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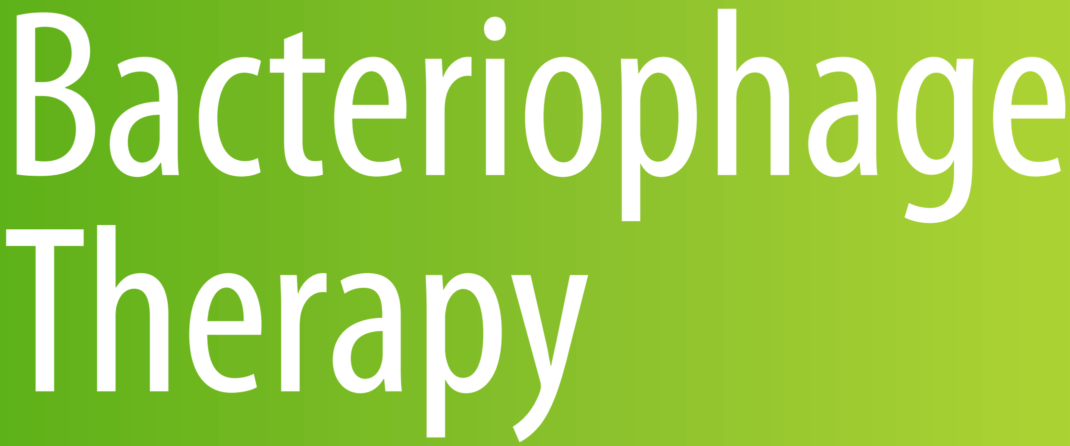

Bacteriophage Therapy

From Lab to Clinical Practice

Second Edition

Methods in Molecular Biology

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Bacteriophage Therapy

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Second Edition

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Preface

Bacteriophage Therapy: From Lab to Clinical Practice was first edited in 2017, during the 100th anniversary of bacteriophage discovery, as a tribute to this important discovery and aiming to contribute to fostering the clinical application of phages in treating infectious diseases. At that time, phage therapy was not broadly recognized in most countries, and the few clinical cases reported resulted from the compassionate use of phage therapy. Six years after the first edition, phage therapy has undergone significant advances from a regulatory, scientific, and technological perspective. This second edition has updated content with expanded coverage of the topics focusing on recent advances.

We have collected 19 chapters which have been subgrouped by dividing the experimental approaches suitable for isolating and characterizing bacteriophages to selecting, producing, and formulating bacteriophage medicinal products and testing in different in vitro and in vivo models. We have also included protocols and guidelines to use bacteriophages therapeutically for different clinical applications, and finally, we introduced a section of protocols describing the latest technologies for bacteriophage engineering.

We want to express our gratitude to all contributing authors whose expertise in the field is highly recognized for their commitment to this book by sharing their knowledge simply and comprehensively to guide bacteriophage researchers throughout the development of a product for medical application. This book is written for undergraduate and graduate medical, pharmaceutical sciences, microbiology, and biotechnology students. It also intends to target a broader audience of industry and healthcare professionals working in phage therapy interested in expanding their knowledge in the field.

Braga, Portugal

Joana Azeredo
Sanna Sillankorva

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Part I

Isolation, and Characterization of Bacteriophages

Check for updates

Chapter 1

Isolation of Bacteriophages for Clinically Relevant Bacteria

Sanna Sillankorva and Paul Hyman

Abstract

The isolation of bacteriophages targeting most clinically relevant bacteria is reasonably straightforward as long as its targeted host does not have complex chemical, physical, and environmental requirements. Often, sewage, soil, feces, and different body fluids are used for bacteriophage isolation procedures, and following enrichment, it is common to obtain more than a single phage in a sample. This chapter describes a simple method for the enrichment and isolation of bacteriophages from liquid and solid samples that can be adapted for different clinically important aerobic bacteria.

Key words Clinically relevant bacteria, Bacteriophage isolation, Purification

1 Introduction

Many of the pathogens responsible for a substantial percentage of nosocomial infections in intensive care units present a high rate of multiple-drug resistance, rendering entire classes of antibiotics redundant [1, 2]. Most of these pathogens, including ESKAPE [*Enterococcus faecium* (E), *Staphylococcus aureus* (S), *Klebsiella pneumoniae* (K), *Acinetobacter baumannii* (A), *Pseudomonas aeruginosa* (P), *Enterobacter* sp. (E)], do not require complex and specific requirements for their growth, thriving on standard enriched culture media under aerobic conditions [3–5]. Antimicrobials are inefficient against ESKAPE due to resistance mechanisms, including drug inactivation, modification of the drug binding sites, changes in cell permeability, or mutation [6–8]. The therapeutic challenges imposed by multidrug-resistant infections involving ESKAPE pathogens have prompted scientists' interest in bacteriophage therapy for years. Several researchers have isolated fully characterized different bacteriophages that target this group of pathogens, and nowadays, hundreds of complete bacteriophage genomes are deposited in public sequence repositories [9, 10].

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There are different bacteriophage isolation methods, including direct plating, but the vast majority continue to rely on the enrichment procedure already detailed by Felix d'Herelle [11]. Direct plating can be used when the samples are already known to be rich in bacteriophages. For instance, direct plating has been successfully used to isolate bacteriophages specific to *Escherichia coli* from sewage and stool samples (reviewed in [12]). Generally, enrichment procedures allow the phages present to infect and multiply, which allows for easier isolation and purification. The enrichment procedure can also be used for a single strain or multiple non-lysogenic strains of the same species added simultaneously. The latter has been shown to improve the likelihood of isolating bacteriophages for the host of choice. This procedure has also been suggested to increase the isolation of broader host range bacteriophages, although this may not be true for all hosts [13]. When using this procedure, lysogenic strains should not be added since this will produce false positive results [14].

In addition to avoiding the use of lysogenic strains, several other factors may determine the choice of bacterial hosts for the enrichment culture. If a specific strain of bacteria is being targeted, for example, for treating a single patient, then that strain can be used alone or included with a mixture of hosts. In some cases, the pathogenic strain may be too virulent or grow too poorly under isolation conditions to be useful. In this case, a surrogate host must be used and the isolated phages later tested to ensure that they can infect and kill the pathogenic strain. As long as the isolation and pathogenic strains are somewhat related, finding phages that infect both is reasonably likely as collections of newly isolated phages tend to have at least some whose host range is fairly broad [15–17].

In this chapter, we detail the methodology to enrich and assess the presence of bacteriophages in samples and how to isolate different bacteriophages taking into account plaque diversity. After this procedure, the isolated bacteriophages will be ready for production and characterization (e.g., host range, genome characteristics, and life cycle) and further testing of their antimicrobial efficacy toward the host to provide a comprehensive analysis if they are suited for phage therapy.

2 Materials

2.1 Enrichment of the Sample for Bacteriophage Isolation

1. Clinically relevant strains or surrogates: overnight grown bacteria in 100 mL Erlenmeyer flask containing 25 mL of sterile LB (*see* **Note 1**). Weigh 25 g of commercial LB and pour into a 1 L bottle. Adjust to 1 L with deionized water. Autoclave at 121 °C for 15 min.

2. Bacteriophage isolation source sample: a liquid or solid sample that possibly contains bacteriophages.
3. Lysogeny broth (LB): weigh 25.0 g, place in a 1 L bottle, and add 1 L of deionized water. Autoclave at 121 °C for 15 min (*see* **Note 2**).
4. Sterile double strength LB media (2 × LB): weigh 40.0 g of LB, place in a 1 L bottle, and add 800 mL of distilled water. Autoclave at 121 °C for 15 min.
5. Sterile saline solution: weigh 9.0 g of NaCl, place in a 1 L bottle, and add 1 L of deionized water. Autoclave at 121 °C for 15 min.
6. Sterile 250 mL and 500 mL bottles.
7. Sterile 50 mL, 100 mL, and 500 mL Erlenmeyer flasks.
8. Sterile 50 mL centrifuge tubes.
9. Sterile 1 mL tubes.
10. Filters: 0.2 μm and 0.45 μm (Whatman™, USA).

2.2 Checking for the Presence of Bacteriophages in the Enriched Samples

1. LB agar: LB broth including 1.2% (wt/vol) agar (ca. 20 mL in a disposable Petri dish with a 9 cm diameter).
2. Overnight grown bacteria in 100 mL Erlenmeyer flasks containing 25 mL of sterile LB.
3. Sterile LB Top Agar (TA): Weigh 12.5 g of LB and 3.0 g of agar and pour into a 500 mL bottle. Adjust to 500 mL with deionized water (*see* **Note 3**). Autoclave at 121 °C for 15 min and store accordingly (*see* **Note 4**).

2.3 Isolation of all Different Bacteriophages Based on Plaque Characteristics from the Enriched Samples

1. Bacterial lawn prepared as follows: Add to an LB agar plate 100 μL of overnight grown bacteria (previously grown in 100 mL Erlenmeyer flasks containing 25 mL of sterile LB) in 3 mL of sterile TA (47 °C).
2. Sterile paper strips [1 cm × 5 cm, paper sheet (80 g per m^2), autoclaved at 121 °C for 15 min].
3. Sterile toothpicks.

3 Methods

3.1 Enrichment of the Bacteriophage Isolation Source

3.1.1 Using Liquid Samples

1. Use 100 mL of sample possibly containing bacteriophages and pour the sample into 50 mL centrifuge tubes.
2. Centrifuge (9000 × g, 10 min, 4 °C) and recover the supernatant.
3. Filter (0.45 μm) the supernatant into a 500 mL sterile Erlenmeyer flask (*see* **Note 5**).

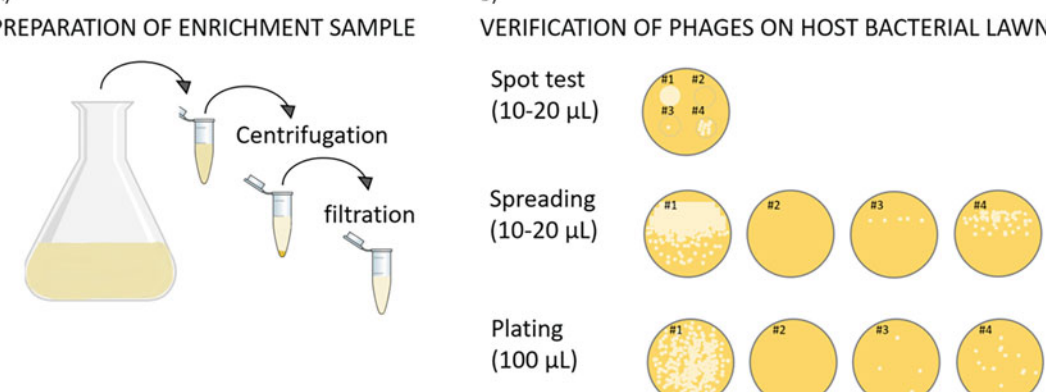

Fig. 1 Assessment of bacteriophages following enrichment of samples: (**a**) steps following enrichment that are performed to remove contaminants; (**b**) three strategies to check for the presence of bacteriophages in four samples (#1 to #4)

4. Add 50 µL of overnight grown bacterial suspension and 100 mL of 2 × LB to the 100 mL of filtered supernatant in the 500 mL Erlenmeyer flask (*see* **Note 6**).
5. Incubate at 37 °C for 24 h, under agitation (120–200 rpm).
6. Add 1 mL of the enriched sample to a 1 mL tube (Fig. 1a) (*see* **Note 7**).
7. Centrifuge (9000 × *g*, 4 °C for 10 min) (*see* **Note 8**).
8. Collect and filter (syringe filter 0.2 µm) the supernatant to a sterile 1 mL tube.

3.1.2 Using Solid Samples

1. Add to a 500 mL bottle 100 mL of saline solution and 10–50 g of solid sample (ex. soil).
2. Mix thoroughly and incubate for at least 1 h at room temperature (*see* **Note 9**).
3. After incubation, pour onto 50 mL centrifuge tubes and centrifuge (9000 × *g*, 10 min, 4 °C).
4. Collect the supernatant and proceed as described above (*see* Subheading 3.1.1, **step 3**).

3.2 Checking for the Presence of Bacteriophages in the Enriched Samples

3.2.1 Spot Test Verification of the Enriched Samples

1. Prepare bacterial lawns of each strain by pouring 100 µL of overnight culture and 3 mL of TA onto an LB agar plate.
2. Let the overlay agar layer solidify.
3. Add 1–4 drop(s) of 10–20 µL of each filtered sample obtained (last steps from Subheadings 3.1.1 and 3.1.2) on the bacterial lawn.
4. Let the plate stand until the drop(s) have completely dried (*see* **Note 10**).

5. Incubate the plate overnight at 37 °C.
6. Check for the presence of bacteriophages indicated by plaques or a zone of lysis (Fig. 1b).

3.2.2 Paper Strip Spreading Method

1. Prepare bacterial lawns as described in Subheading 3.2.1 **steps 1** and **2**.
2. Add a 10–20 μL drop of enriched sample on the left upper corner.
3. Use a paper strip to spread it horizontally throughout the agar, changing the paper after using it every two times to spread the sample throughout the plate (*see* **Note 11**).
4. Incubate the plate overnight at 37 °C.
5. Check for the presence of plaques (Fig. 1b).

3.2.3 Plating the Enriched Sample

1. Prepare different bacterial lawns on LB agar plates containing: overnight culture of one strain (100 μL) and enriched sample (or diluted enriched sample) (100 μL) in 3 mL of TA (*see* **Note 12**).
2. Immediately after adding the TA, swirl the LB agar plate so that the mixture is evenly distributed.
3. Let the overlay agar layer solidify.
4. Incubate the plate overnight at 37 °C.
5. Check for the presence of bacteriophages as indicated by plaques (Fig. 1b).

3.3 Isolation of All Different Bacteriophages Based on Plaque Characteristics from the Enriched Samples

An enriched sample may contain more than one bacteriophage for a specific host. The presence of more than one bacteriophage is verified by visual inspection of the bacteriophage plaques on the bacterial lawns (Fig. 2), which may vary in morphology and size. All different plaques are observed (in the plates obtained after 3.2.2 and 3.2.3) and may then be isolated following the procedure detailed below.

1. Add a drop (10–20 μL) of the enriched sample (obtained in the final steps of Subheadings 3.1.1 and 3.1.2) in the upper left corner (*see* **Note 13**).
2. Streak on a Petri dish containing a previously prepared bacterial lawn of the strain used in the enrichment step downward using paper strips (*see* Fig. 3, **step 1**).
3. Make sure to change the paper strips to transfer always less and less concentrated bacteriophage so that, in the end, the Petri dish will have a higher number of isolated bacteriophage plaques until reaching the end of the plate (Fig. 3a).
4. Incubate the plate overnight at 37 °C.

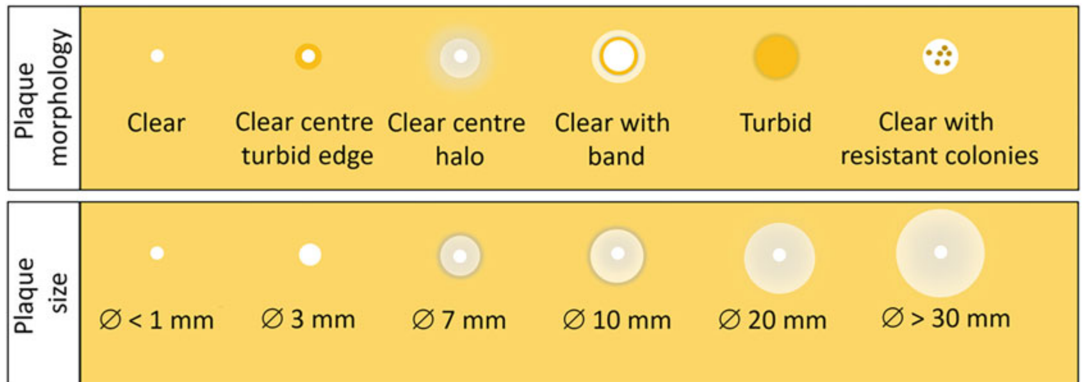

Fig. 2 Examples of bacteriophage plaques that can be formed on bacterial lawns. Morphological differences include plaques varying in turbidity and the presence of halo. Although resistant colonies are not a morphological feature of the plaque itself, this is observed for some bacteriophages that produce very large plaques that increase in halo diameter over time [17]; therefore, these were included. Plaque size also varies in diameter, particularly when the halo increases over time

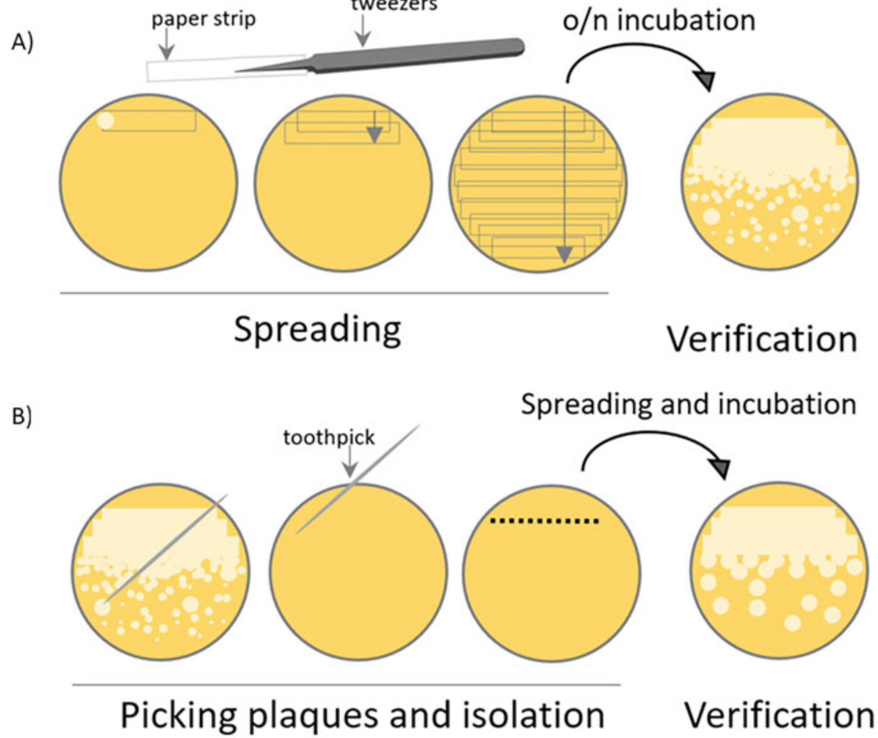

Fig. 3 Using the paper strip procedure to isolate bacteriophages with characteristic plaques: (**a**) spreading the enriched sample with a sterile paper strip to isolate all different plaques; (**b**) use of a sterile toothpick to collect and spread a plaque with specific morphology and size

5. Analyze the bacteriophage plaque morphologies to check for differences (Figs. 2 and 3a).
6. Pick a single bacteriophage plaque using a toothpick and perforate (in a line) the agar plate prepared with a bacterial lawn of the specific host (Fig. 3b).
7. Use sterile paper strips to streak the bacteriophages as described above (*see* Subheading 3.3, **steps 2–3**).
8. Incubate the plates overnight at 37 °C.
9. Repeat **steps 6**–**8** at least thrice or until all bacteriophage plaques are uniform (*see* **Note 14**).

4 Notes

1. In all these methods, there is no need to have a known concentration of bacteria, but the concentration should be enough to form a confluent lawn on the agar plates ($>1 \times 10^9$ colony forming units per mL). If the concentration is too low, the plates might end up with colonies spread throughout the plate and will not allow clear visualization of the bacteriophage plaques.
2. LB is commercially available, but it may be prepared (10 $g.L^{-1}$ of tryptone, 10 $g.L^{-1}$ of sodium chloride, and 5 $g.L^{-1}$ of yeast extract). Adjust the pH to 7.0 with 5 N NaOH. This media was selected since most ESKAPE bacteria grow well in LB. However, other media can be used instead. For instance, Trypticase soy broth can be used to grow *Enterococcus faecium*, *Escherichia coli*, and *Staphylococcus aureus*.
3. For phages presenting tiny plaques, adding less agar to have a final concentration in the top agar lower than 0.6% (wt/vol) agar is frequently helpful. This helps bacteriophages diffuse and will give rise to plaques with a slightly larger diameter. Also, agarose has been successfully used with bacteriophages presenting diffusional limitations due to their large size or formation of aggregates [18]. The addition of certain antibiotics can also aid in the diffusion to allow better visualization of bacteriophage plaques [19].
4. If the intent is to use the TA, after its preparation, let it cool down to 47 °C. If this was prepared in advance and allowed to solidify, it could be melted using a microwave or a boiling water bath.
5. **Steps 2** and **3** are used to discard the vast majority of particles that are usually recovered when collecting raw sewage.

6. The purpose of using 2× strength LB media is to provide the ideal concentration for bacterial growth. Therefore, use a separate flask for each clinically relevant bacteria even though the isolation source is the same. Furthermore, if a mixed culture of hosts is to be used, lysogeny experiments should be performed before mixing any two given bacteria to test for the presence of lysogenic strains of bacteria mixed with non-lysogenic ones. To detect lysogeny, each isolate needs to be tested against the others. If a lysogenic strain is present, plaques will be visible due to the induction of prophages.
7. There is no need to centrifuge higher volumes since 1 mL is sufficient to check for the presence of bacteriophages in enriched samples.
8. In this step, chloroform can be added before the centrifugation to lyse host cells (≈50 μL per 1 mL of sample). However, caution is recommended since some bacteriophages are sensitive to chloroform. For instance, chloroform inactivates filamentous and lipid-containing bacteriophages as well as some tailed bacteriophages [20, 21].
9. Ideally, the sample containing a solid isolation source should be left for a longer time (at least 12 h) in contact with a buffer (e.g., saline, SM buffer, or PBS) so that all bacteriophages can elute from the solid sample to the liquid phase.
10. If using a single LB agar plate for one sample with potential bacteriophages, it is not so crucial that the drop or drops have completely dried out before incubating the plate. However, when samples of different sources are applied, the drops need to dry completely to avoid them from combining, which can potentially alter the result.
11. See Subheading 3.3 for a more detailed explanation of how to proceed with the spreading using the paper strips, and see the procedure graphically depicted in Fig. 3.
12. If the enriched sample is highly concentrated in phages (for instance, checked initially using the spot test), prepare dilutions 1:10 up to 1:10000 dilution and plate. If there is still not a good separation of bacteriophage plaques at this last dilution, consider diluting the sample further.
13. Alternatively, if the plates from verifying bacteriophages' presence present individual plaques, you may consider starting the procedure in Subheading 3.3, **step 6**. Nonetheless, there is a higher probability of missing some phages that may appear in lower numbers.
14. Bacteriophage plaques can be removed with a cut micropipette or Pasteur pipet tip together with the agar. Insert these in a 2 mL tube. Store at +4 °C until there is a need to start

producing the phage. Alternatively, add 1 mL of SM buffer to the 2 mL tube, allow phage elution to the buffer, and remove the agar particles. Then filter (0.2 μm) to remove any possible bacteria collected together with the agar.

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Chapter 2

Observation of Bacteriophage Ultrastructure by Cryo-Electron Microscopy

Ana Cuervo, Patricia Losana, and José L. Carrascosa

Abstract

Transmission electron microscopy (TEM) is an ideal method to observe and determine the structure of bacteriophages. From early studies by negative staining to the present atomic structure models derived from cryo-TEM, bacteriophage detection, classification, and structure determination have been mostly done by electron microscopy. Although embedding in metal salts has been a routine method for virus observation for many years, the preservation of bacteriophages in a thin layer of fast frozen buffer has proven to be the most convenient preparation method for obtaining images using cryo-electron microscopy (cryo-EM). In this technique, frozen samples are observed at liquid nitrogen temperature, and the images are acquired using different recording media. The incorporation of direct electron detectors has been a fundamental step in achieving atomic resolution images of a number of viruses. These projection images can be numerically combined using different approaches to render a three-dimensional model of the virus. For those viral components exhibiting any symmetry, averaging can nowadays achieve atomic structures in most cases. Image processing methods have also evolved to improve the resolution in asymmetric viral components or regions showing different types of symmetries (symmetry mismatch).

Key words Bacteriophage structure, Cryo-electron microscopy, Fast freezing, Data acquisition, Image processing, Three-dimensional reconstruction

1 Introduction

Virus visualization has been strongly related to electron microscopy development. Early after the design and implementation of the first electron microscope, one of the first samples to be visualized was the tobacco mosaic virus [1]. Since then, electron microscopy has become the main technique to detect, classify, and describe the morphology of many different viruses (reviewed in [2]). Besides these applications, structure determination of viruses using transmission electron microscopy (TEM) started in the 1960s, after the pioneering work by Klug and Finch using negatively stained virus

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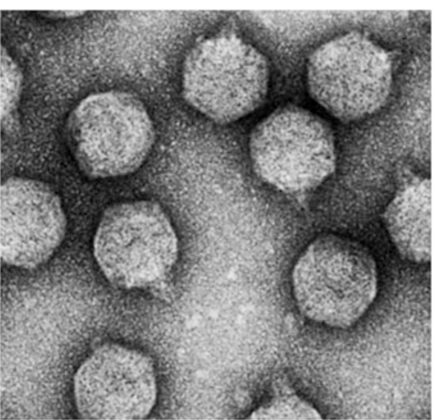
images [3], soon followed by the first attempts to produce three-dimensional reconstructions of viral structures by electron microscopy [4].

The incorporation of cryoprotection in sample preparation for TEM [5] was a fundamental step for extending the use of TEM toward high-resolution structural determination of viruses, allowing data acquisition at a sufficient resolution so as to produce the first near-atomic model of a rotavirus-related particle [6]. The first near-atomic resolution structure of a bacteriophage (ϵ15) was obtained soon after [7].

The two basic procedures for observation of bacteriophage ultrastructure are negative staining and cryo-electron microscopy (cryo-EM). Negative staining allows a rapid overview of the sample by mixing the virus preparation with a heavy metal salt (uranyl acetate, sodium phosphotungstate). After drying, the metal salt produces an accurate cast of the virus particles that can be introduced in the vacuum of the microscope column to be visualized (Fig. 1, left). The metal cast resists electron interaction without much radiation damage and renders projection images that reproduce the ultrastructure of particles up to several nm resolution. This method is simple and fast, thus ideal to check for sample concentration, homogeneity, presence of contaminants, etc.

Nevertheless, the use of a replica or cast from the real virus particles prevents getting high-resolution data. Direct observation of bacteriophage without any chemical fixation or contrasting reagent is only possible by cryo-EM (Fig. 1, right). This method is based on the fast freezing of the virus preparation using cryogenic agents (liquid ethane). High-speed freezing (better than 10^4 degrees per second) produces vitrified water, which is a structureless form of ice that preserves virus structure in a near native environment even under the vacuum [8]. After freezing, samples are kept under liquid nitrogen and introduced into the microscope



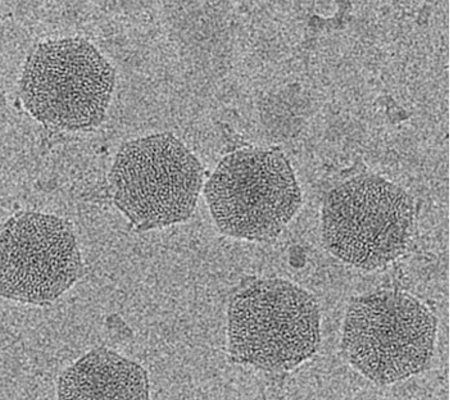


Fig. 1 Left, negative stained T7 bacteriophage. Right, a cryo-EM image of T7 bacteriophage

using specialized sample holders, which maintain the sample at a controlled temperature (−180 °C) and allow to obtain TEM images at this temperature (Fig. 1, right).

The fact that no contrasting reagent is used in cryo-EM implies that images have to be taken using a very low electron dosage to prevent the destruction of the sample due to radiation damage. This results in cryo-EM images having a very low signal-to-noise ratio (Fig. 1, right), and consequently, the observation of viral particles demands certain skills from the operator to detect and select those areas with better image quality. Recording of cryo-EM images has improved greatly in the last few years: While films and charge-coupled devices (CCDs) were traditionally used for data acquisition, the incorporation of direct electron detectors (DEDs) has greatly improved the quality, sensibility, and contrast of the images. Furthermore, DEDs offer the possibility of reducing the limitations derived from sample movement and distortion during the acquisition by using fast frame acquisition [9]. These advantages together have opened the possibility for TEM-based structure determination up to atomic resolution.

In any case, image processing is required for averaging data to enhance viral particles signal, as well as for classification and/or data combination for three-dimensional reconstruction. Different packages for TEM image processing are available to process, classify, average, and reconstruct volumes from TEM projection data. The final results in these procedures are volumes at a defined resolution that have to be validated using standard tests to render the final viral structure. Current developments in microscope acquisition technologies and image processing methods allow for obtaining high-resolution structures in most cases. In cases where the resolution is still too low to solve the structure, hybrid methods including data from other sources (mainly structures of certain viral components solved by x-ray crystallography or alphafold [10]) are very helpful to validate, interpret, and extend the resolution of the TEM-derived three-dimensional models.

2 Materials

2.1 Support Preparation for Negative Staining

1. Negative stained standard grids: metal (copper, gold, titanium, nickel, etc.) perforated circular plates 3 mM in diameter (Fig. 2a). Metal bars are covered by a layer of plastic (as formvar) followed by a thin layer of carbon (Edwards E306 evaporator and Leica ACE 200 are examples of convenient coating devices). Grids for cryo-EM are made of Holey Carbon Film (like QUANTIFOIL®), which is a perforated support foil with circular and square holes (Fig. 2b).

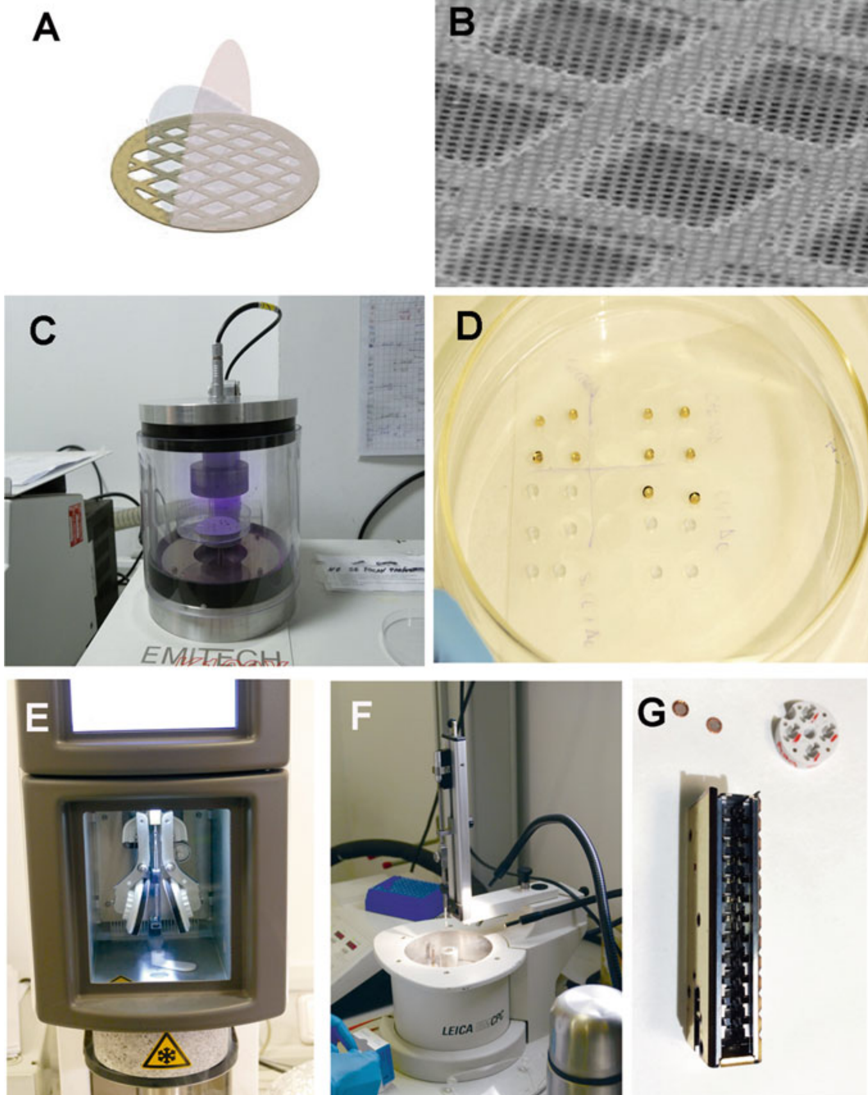


Fig. 2 Gallery of images showing different steps of grid preparation process: (**a**) copper grid covered with plastic and carbon; (**b**) image showing a QUANTIFOIL® grid, where squares and holes can be appreciated; (**c**) Emitech Glow discharge machine; (**d**) glass plate showing grid incubation during negative staining grid preparation; (**e**) Fei vitrobot; (**f**) Leica CPC vitrification devices; and (**g**) grids, grid-boxes, and cartridge needed to introduce grids with autoloader

2. Glow discharge apparatus: moderate cost apparatus such as Emitech K100X, Ted Pella easy Glow, and Edwards 306 can be used to generate low-energy plasma (Fig. 2c).
3. Hydrophobic surfaces (such as Parafilm) (Fig. 2d) can be used for sample adhesion to the EM grid by drop deposition.

4. Buffer: Usually, the same buffer as that containing the sample is used but lowering the salt content (to a maximum of 100 mM) and removing other components, such as sugars or glycerol.
5. Distilled water.
6. EM grid box.

2.2 Fast Freezing

1. Cryogen (usually liquid ethane) kept at −180 °C by liquid nitrogen.
2. EM grid vitrification stations such as FEI Vitrobot and Leica EM GP automatic plunge freezer (Fig. 2e, f).
3. Blotting paper (usually Whatman).
4. Forceps.
5. Cryo-transfer holders (*see* **Note 1**).

2.3 Data Acquisition

1. Most modern microscopes acquire data by fiber-optically coupled CCD, such as the Gatan Orius series.
2. Recent advances in CMOS detectors, such as the Gatan One View, TVIPS TemCam XF-series, and FEI ceta 16 M, offer 4 k × 4 k images well suited for image processing.
3. Since 2012, new types of electron direct detectors are used. Examples of these new detectors are Direct EL DE16 and FEI Falcon II (4 k × 4 k images at frame rates of around 30 Hz), both using direct electron integration, and Gatan K2 Summit and FEI Falcon 3EC, which operates in counting mode and provides frame rates of 400 Hz.

3 Methods

3.1 Negative Staining

1. Carbon-coated grids (*see* **Note 2**) are treated for 10–30 s with low energy plasma in the chamber of a glow discharge apparatus (*see* Subheading 2.2).
2. Then, a small volume (5–10 μL) of the sample is adhered to the carbon surface of the grid and incubated for 1–3 min.
3. Grids are washed into several drops of buffer and then distilled water (Fig. 2d). Finally, the grid is air-dried and kept in an EM grid box (*see* **Note 3**).

3.2 Fast Freezing

1. A small amount (3–5 μL) of purified sample is layered on the surface of a grid and allowed to interact for a couple of minutes.
2. The grid is then taken by forceps and brought into the freezing chamber of a fast freezing equipment (Fig. 2e, f) under controlled humidity and temperature conditions.
3. The grid is blotted using filter paper under controlled force (*see* **Note 4**).

4. After blotting, the grid is immersed in a cryogenic media (usually liquid ethane), which is cooled by liquid nitrogen. After immersion, the grid must be kept under liquid nitrogen until released into the microscope vacuum.
5. Grids are transferred into the EM microscope (*see* **Note 5**) using cryo-transfer holders. The grid is transferred under a controlled temperature (−180 °C) and finally inserted into the microscope column.
6. Care must be taken to prevent exposure of the frozen grid to any atmospheric contaminant, as ice crystals would immediately form on the sample surface.
7. Robotized transfer cartridges (Fig. 2g) facilitate the procedures and lower contamination risks.

3.3 Data Acquisition

1. The first step is taking several images at low magnification to build a grid atlas to identify regions of interest in the grid.
2. Then, grid squares and holes are imaged at higher magnification to select the acquisition areas.
3. The illumination conditions and focusing are set at the final magnification for data acquisition (around 30–70,000×) in areas nearby the actual site to collect the image frames.
4. Electron beam is moved toward the acquisition area, and the data is taken (in about 1 or 2 s of exposition) using either CCDs or direct detectors (DDs) (*see* **Note 6**).

3.4 High-Resolution Structure Determination

3.4.1 Workflow

1. First, single frames are aligned to correct beam-induced movements.
2. The average image is then used to manually or automatically select individual particles.
3. Undesirable particles are eliminated after two-dimensional (2D) classification.
4. A final particle set is selected to be used to build the three-dimensional reconstruction (3D) (*see* **Note 7**).

3.4.2 Processing Software

1. The selected particle set is then processed using RELION [11], EMAN [12], Cryosparc [13], FREALIGN [14], or Xmipp [15], either with no symmetry (Fig. 3a and e) or imposing icosahedral symmetry (Fig. 3b).
2. For regions that do not follow the icosahedral symmetry, besides standard asymmetric reconstruction methods, other software using symmetric relaxation or localized reconstruction methods can be used in order to improve the resolution [16]. (*see* **Note 8**).
3. If the map allows it, the polypeptide chain might be built using Coot [17].

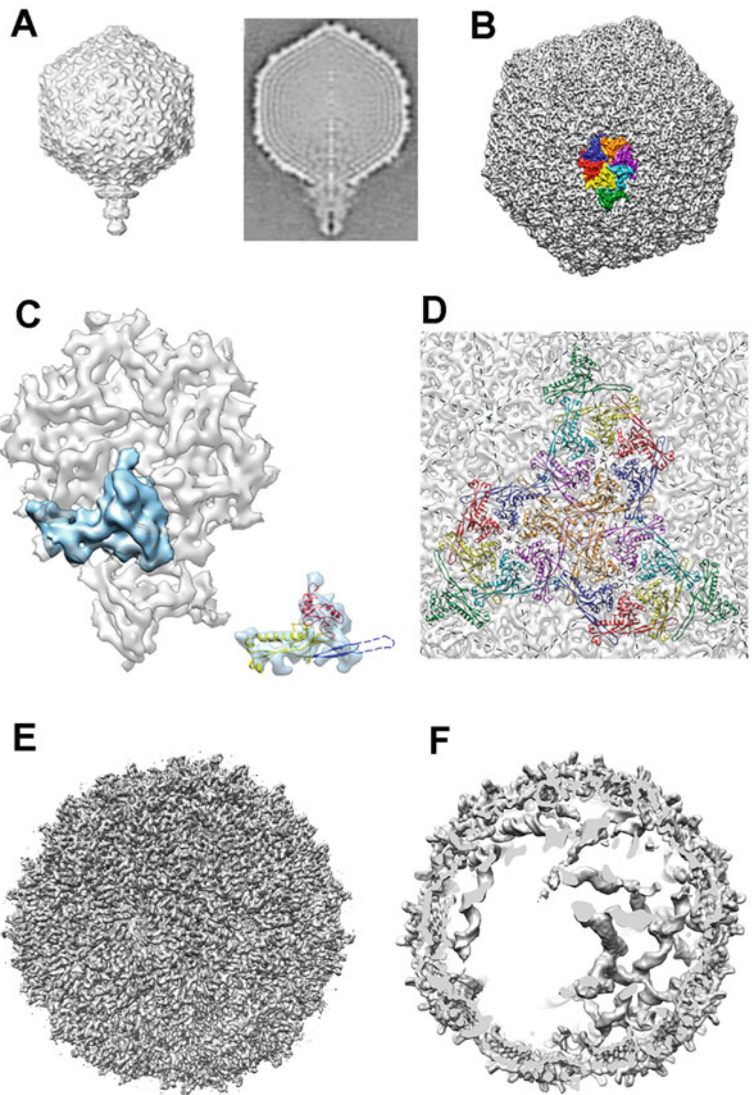


Fig. 3 (**a**) Left, three-dimensional reconstruction of T7 without imposing any symmetry and right, a slide of the same volume [43]; (**b**) icosahedral reconstruction of T7 bacteriophage at 10 Å resolution, the asymmetric subunit composed by a hexon and a subunit of the penton is colored [37]; (**c**) upper panel, T7 asymmetric unit where a protein monomer is colored in blue; bottom panel, docking of the atomic model inside the segmented volume of the monomer [37]; (**d**) close view of the T7 bacteriophage capsid pseudo-atomic model [37]; (**e**) asymmetric reconstruction of MS2 bacteriophage at ~3.6 Å resolution; and (**f**) section of the volume shown in E filtered at ~6 Å [31]

3.5 Structure Determination and the Use of Hybrid Methods

In some cases, cryo-EM maps of viruses are obtained at resolutions ranging from 10 up to 4 Å resolution (Fig. 3b), and the polypeptide chain cannot be built directly. For these cases, hybrid methods can be used to build pseudo-atomic models.

1. Segment the asymmetric unit cell and the monomer (Fig. 3c) using Segger [18] in Chimera [19] at different σ levels to define boundaries.
2. Load the known atomic structures of the structural components (or fragments) into the EM volume using Chimera [19].
3. Move the atomic models as rigid bodies inside the volume to adapt to the EM volume Chimera [19] (Fig. 3c, bottom). The fitting might be refined by maximizing the cross-correlation using Chimera [19].
4. If the fitting is not satisfactory, use flexible fitting methods using imodFit [20], FlexEM, or Coot software [17, 21] (Fig. 3d) (*see* **Note 9**).

4 Notes

1. Transferring frozen grids from the freezing station into the microscope is done at −180° using specialized microscope holders. Each main electron microscope company (FEI, JEOL, and Hitachi) has specific cryo-transfers adapted to the requirements of their respective microscopes. Also, Gatan has cryo-transfers designed especially for the different microscopes from the main companies. The recent use of robot sample holders in the latest electron microscope models has prompted the use of special cartridges that are adapted for transfer under cryo-conditions (Fig. 2g).
2. Grid preparation considerations. A very thin formvar layer is formed from a solution [1% (vol/vol) solution, dissolved in ethylene dichloride]. Clean glass microscope slides are dipped in the formvar solution, allowed to drain and dry, and then released from the glass by slow immersion in a water bath. The floating plastic can then be transferred onto the metal grid surface by lifting away the water. On top of the plastic layer, a thin carbon layer (2–6 nM) can be produced either by carbon deposition or by carbon layer transfer from a pre-loaded support. Thermal carbon deposition is made using specific equipment which heats carbon rods of high purity (less than 5 ppm contaminants) using high-current electrical terminals. Deposition onto the metal/plastic grid is made under vacuum (approx. 10–7 Torr), and the thickness of the carbon deposit is controlled using a quartz monitor. Carbon transfer is performed by transferring a thin carbon layer previously deposited

over a clean mica surface onto a water surface. Then, the carbon layer is released from the mica by slow immersion into a water bath, and the floating carbon can then be transferred onto the metal grid surface by lifting away the water.

3. Sample preparation considerations: The purity and buffer conditions are critical to obtain suitable cryo-EM grids. Usually, negative staining is used for testing different concentrations and spreading conditions. Small contaminants (< 60 kDa) almost invisible in negative staining can hinder the observation of the sample in vitreous ice. Also, buffers containing sucrose, glycerol, or some detergents might produce the same problem. A size exclusion chromatography step can be crucial to eliminate small contaminants and buffer exchange to form nice and thin vitreous ice. The ideal sample should be homogeneous and stable; samples containing flexible domains or multiprotein complexes can be stabilized by cross-linking using techniques such as GraFix [22]. Care must be taken to eliminate from the sample buffer sugars or glycerol, as they interfere badly with the electron irradiation under cryo-EM conditions. High salt concentrations (above 0.5 M) might also induce undesirable side effects for image acquisition. To assure sample suitability, a final dialysis step against a buffer with minimum requirements to sustain virus stability is convenient. It is important to find a proper virus concentration for cryo-EM. Protein concentrations ranging from 0.1 mg/mL to 1 mg/mL are good starting conditions, but every virus preparation has to be individually tested to ensure an even distribution of particles in the EM grid, as well as an optimum number of particles per microscope image to facilitate ulterior image processing. Too crowded fields would prevent obtaining individual virus images required for processing. Too diluted fields make processing lengthy and impractical. In this context, the use of a fast-checking procedure is required. Negative staining has proven to be the easiest and fastest way to obtain information about sample purity, virus homogeneity, and spreading characteristic for viral samples (Fig. 1, left).

4. For cryo-EM preparations, ideally, the samples should have a homogeneous distribution inside the hole during imaging. Nevertheless, certain samples tend to avoid holes or cannot be purified in a sufficient amount to manage to make a good-looking grid. This problem can be solved by depositing the samples on QUANTIFOIL® grids with a thin layer of carbon on top of it. On the other hand, carbon surfaces can produce drift and make the acquisition of high-resolution data difficult. Recently, graphene and gold grids have been shown to avoid drift and to help with particle even distribution inside the hole [23]. For the Vitrobot blotting time, force, temperature, and

humidity have to be determined for every sample, but the standard values range from 1 to 3 s of blotting time, −10 to +5 force, 4 °C to 22 °C, and around 95% of humidity. This is a critical step, as excessive blotting will result in a dry sample, while leaving too much liquid in the grid will result in a too thick ice layer.

5. Transmission electron microscopes: For moderate resolution and screening applications, a microscope equipped with tungsten or LaB6 gun will suffice. For more detailed studies and proper three-dimensional reconstruction at nanometric resolutions, a field emission gun is a must. All major EM companies (FEI, JEOL, and Hitachi) have equipment in both range types.

6. The key step in cryo-EM is the actual imaging of the sample to collect data for visualization and processing. Currently, the achievement of high-resolution 3D structures requires collecting a large amount of images to process. Modern microscopes allow full automation of the procedure, facilitating the collection of thousands of images in one day. Automatic control procedures such as EPU (FEI company), Serial EM [24], Leginon [25], or TOM [26] can be implemented in different microscopes to perform medium throughput data acquisition by EM. In cryo-EM, as the frozen sample is not protected by any staining or fixative, it is extremely sensitive to radiation damage. Exposures higher than 30 e/A^2 usually induce irreversible residue damage and loss of high resolution [27]. Another important incorporation in current methods is the use of direct detectors (DDs). In this case, due to their great sensitivity and data transfer rate, several frames (around 15–40 depending on the DD) can be taken in one-second exposure. These frames are taken as a movie (instead of a single photograph), and individual frames can be analyzed during image processing, allowing playing with the image dose ratio. The last frames presenting higher doses are useful to enhance the contrast during particle picking and are usually removed or down-weighted during the movie average [9, 28]. Usually first frames are also removed as they can be blurred due to beam-induced movement. This high-resolution frame selection and weighting avoids beam-induced movement and radiation damage, resulting in an improvement in the data quality and in a tremendous boost in the resolution potentially attainable by cryo-EM.

7. In data processing, there is not one unique approach for the 3D reconstruction of viral particles from cryo-EM data. Several methods are available to process the two-dimensional projections obtained by cryo-EM from the viral particles to merge in a three-dimensional volume, but they can be divided into two main sets: those which are based on the fact that most

bacteriophages are built following icosahedral symmetry, and make extensive use of this assumption for the whole processing process, and others that consider the viral particles as single particles for processing following general procedures that, in a certain step, do apply the proper symmetries (icosahedral, six-fold, 12-fold, etc.), if any. In the past, processing data with icosahedral symmetry used specific software packages such as Auto3DEM and AutoRTM [29] or Rico [30]. Currently, general software for single particle processing, such as RELION [11], EMAN [12], Cryosparc [13], FREALIGN [14], or Xmipp [15], successfully manage to build high-resolution viral capsid reconstructions imposing icosahedral symmetry or not imposing any symmetry at all (Fig. 3e and f) [31]. The improvement in microscopes, detectors, and image processing software in the last years has helped to increase the number of atomic structures published (Fig. 3e and f). Image processing without imposing icosahedral symmetry (Fig. 3a, e, and f) has the drawback that more individual images are required for the reconstruction but, on the other hand, they can deal with the existence of non-icosahedral structural features in the viral particle: although capsid shells in most bacteriophages are icosahedral (or icosahedrally derived), neither internal components (scaffolds, core, portals, and nucleic acids) nor tails exhibit such symmetry (Fig. 3a and f).

8. Structure determination: The existence of symmetry mismatches is one of the problems that can be found during image processing of bacterial viruses. While most of the capsids present icosahedral symmetry, connectors have 12-fold, some terminases 5-fold, fibers 3-fold, etc. Developments in software methods allowing to relax the symmetry or locally refine regions with different symmetries [16] have evolved to increase the resolution of viral components that do not follow the icosahedral symmetry, such as the tail or other portal vertex components, or asymmetric components such as the nucleic acid.
9. Hybrid methods: The first procedures used the x-ray structures as solid bodies searching for the best orientation to fit the EM volume [32]. These approaches were limited to a first approximation, and it was soon realized that a certain degree of flexibility was required to deal with relative motions and structural transitions present in macromolecular complexes. A number of methods were then developed, allowing certain secondary structure elements of the x-ray structures to move as rigid bodies to adapt better to the EM envelope. These methods use a wide variety of different approaches, from optimizing the fitting of the atomic model to a density map, also taking into account the stereo-chemical properties of the model by

minimizing an energy function [33], or using molecular dynamics flexible fitting by incorporating the EM data as an external potential in conventional MD simulations [21, 34]. Other common procedures for flexible fitting are those included in UCSF Chimera [19], Rosetta [35], or imod-Fit [20]. Flexible docking opens the possibility to extend the resolution of the cryo-EM volumes up to a quasi-atomic resolution map (Fig. 3d), thus gaining insights into dynamic processes involved, for example, in bacteriophage shell maturation [36, 37], DNA packaging [38–40], and ejection [41, 42].

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Chapter 3

Bacteriophage Taxonomy: A Continually Evolving Discipline

Dann Turner, Evelien M. Adriaenssens, Susan M. Lehman, Cristina Moraru, and Andrew M. Kropinski

Abstract

While taxonomy is an often underappreciated branch of science, it serves very important roles. Bacteriophage taxonomy has evolved from a discipline based mainly on morphology, characterized by the work of David Bradley and Hans-Wolfgang Ackermann, to the sequence-based approach that is taken today. The Bacterial Viruses Subcommittee of the International Committee on Taxonomy of Viruses (ICTV) takes a holistic approach to classifying prokaryote viruses by measuring overall DNA and protein similarity and phylogeny before making decisions about the taxonomic position of a new virus. The huge number of complete genomes being deposited with the National Center for Biotechnology Information (NCBI) and other public databases has resulted in a reassessment of the taxonomy of many viruses, and the future will see the introduction of new viral families and higher orders.

Key words ICTV, NCBI, Taxonomy, Morphology, DNA sequence homology

1 Why Is Taxonomy Important?

Humans like to put things into boxes and then give those boxes names. This process provides both a context, this thing is like these others, and a language—together these things are called something. Unsurprisingly, the art and science of grouping things is particularly important in biology, as it provides a basis for the identification and inference of relationships. In this context, taxonomy is the process of establishing criteria for the contents of individual boxes and a consistent framework that unites them.

The defining characteristic of virus taxonomy is a group of concepts that can be assembled into a hierarchy. Each layer of this hierarchy must be defined so that a species is defined as one thing and a genus is defined as a higher-order thing that encompasses all of the species beneath it. One can imagine a variety of criteria that could be used to define a taxonomic hierarchy, and the preferred set

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of taxonomic metrics is dependent on the nature of relationships under scrutiny and the availability of data that can be used in evaluations.

Setting criteria for these taxonomic definitions is really where art meets science. Taxonomic hierarchies often attempt to address longer-range evolutionary relationships that stretch back into the far-distant past, yet the process of assignment is restricted to observations gleaned from the extant data. So, even under the best conditions, when there is plentiful data to evaluate, taxonomic constructs often require inferences beyond available data. Making matters worse, in the context of bacteriophage taxonomy, there has been a shortage of data to evaluate, making it difficult to both establish unifying taxonomic criteria and to extrapolate a taxonomic framework from these criteria. The rise in the number of high-throughput sequence-based studies, including metagenomics, has added a wealth of data which is being used to create more robust phylogenies and taxa [1, 2].

2 Brief History of Phage Taxonomy Prior to 2008

The formal taxonomy of the tailed bacteriophages originated in the pioneering phage classification work of David Bradley (Memorial University, Canada), who used electron microscopy and acridine orange staining to classify these viruses into three morphotypes: A (contractile tail), B (long noncontractile tail), and C (short noncontractile tail) [3, 4]. This system was adopted and extended in 1971 by the then International Committee on Nomenclature of Viruses (ICNV), with the names *Myoviridae, Styloviridae*, and *Pedoviridae* proposed for the three morphotypes by Hans-Wolfgang Ackermann and Abraham Eisenstark of the Bacterial Virus Subcommittee in 1975 [5, 6]. The names of these families were accepted by the International Committee on Taxonomy of Viruses (ICTV) in 1981 (as *Myoviridae* and *Podoviridae*; https://ictv.global/taxonomy/history) and in 1984 (*Siphoviridae*; https://ictv.global/taxonomy/history). In 1998, Ackermann proposed an order, the *Caudovirales* [7] to encompass all the tailed phages, which was approved by a postal vote that year. The classification system at the family level remained largely unchanged for 37 years, and it provided an invaluable framework for the classification of bacterial viruses in the absence of sequence data.

The advent of the “omics era” coupled with renewed interest in bacterial viruses has had a profound effect on phage classification. A seminal paper on phage evolution was published in 1999 by Roger W. Hendrix and colleagues [8]. In their paper, subtitled “All the World’s a Phage,” the authors argue cogently that “all dsDNA phage genomes are mosaics with access, by horizontal exchange, to a large common genetic pool but in which access to the gene

pool is not uniform for all phage." This led to a period in which little advancement was made in official phage taxonomy because it was considered that rampant recombination would blur taxa boundaries.

The next major advance in phage grouping occurred with the publication of the highly controversial "The Phage Proteomic Tree: A Genome-Based Taxonomy for Phage" in which Forest Rohwer and Rob Edwards employed BLASTp to compare the proteomes of 105 fully sequenced phage genomes [9]. It was controversial because of some of the illustrated relationships: The "P2 Myophage" cluster contained coliphages P2 and Mu; the "PZA Podophage" group included a member of the *Tectiviridae* (coliphage PRD1) and *Bacillus* podoviruses PZA and GA-1; and, lastly the "λ-like Siphophage" cluster harbored *Escherichia coli* phage λ, HK97 and 933 W, *Pseudomonas* phage D3, and *Salmonella* phage P22. There is no doubt that the latter phages share important features with λ [10], but the merging of members of the *Siphoviridae* and *Podoviridae* created an intellectual stir.

2.1 Extension of Proteomics to Phage Taxonomy from 2008

From 2002 to 2008, the number of fully sequenced members of the *Caudovirales* in GenBank had increased from 19 to 276. However, the number of ICTV-classified species remained at 36. In 2008, when Rob Lavigne (KU Leuven, Belgium) assumed the Chair of ICTV's Bacterial and Archaeal Viruses Subcommittee, it was time to take a new look at the classification of phages. Using two protein analysis tools, CoreExtractor and CoreGenes, Lavigne and co-workers realized that the T7-like phages actually fell into three distinct clades, which were termed "T7-like virus," "SP6-like virus," and "φKMV-like virus" each containing multiple species [11]. Since these three groups shared a similar genomic organization and the presence of a large, single subunit RNA polymerase, they were grouped into the first phage subfamily, *Autographivirinae*. What linked the species within a clade was that the members shared 40% of their proteins in common, as shown using CoreGenes [12–14]. This threshold was defined based on a comparison of T7 and *Pseudomonas* phage gh-1 [15], during which detailed molecular analysis revealed that this phage was closely related to *Escherichia coli* phage T7. This approach was subsequently used with the *Myoviridae* [16] and *Siphoviridae* [17]. The problem with this total proteome approach is that the genomes being compared have to be fully and correctly annotated, which is not always the case. In addition, CoreGenes is relatively slow and tedious to apply to multiple genomes. The latest version, CoreGenes5.0, allows the determination of the common proteins encoded by a maximum of 21 phages [14].

In 2008, another seminal paper was published recognizing the reticulate nature of phage genomic relationships using a network-based representation of phage populations [18, 19]. The findings

and progress made in this paper were largely ignored for a decade until other researchers used similar approaches to analyze a larger dataset of the dsDNA virosphere, including archaeal viruses [20, 21]. Not until the development of vConTACT and its successor vConTACT2, which use similar gene-sharing networks as first used by Gipsi Lima-Mendez and colleagues, did network approaches to represent phage diversity become really popular [22, 23]. The downside of these approaches is that they need to be converted into a hierarchical classification before they can be used in phage taxonomy.

2.2 DNA Sequence Comparisons Enter the Picture

DNA-DNA sequence relatedness has always been the gold standard for the classification of bacterial strains [24, 25], and while DNA-DNA hybridizations have been used to study phage relationships [26–28], in silico analyses of the sequence relationships between biological entities did not influence phage taxonomy until fairly recently. The most commonly used genome comparison tools were progressiveMauve [29], EMBOSS Stretcher [30, 31], FastANI [32], and dot matrix analysis programs [33–35]. The problem with EMBOSS Stretcher and FastANI is that they are inaccurate below approximately 50% sequence identity, they require collinear genomes, and can only handle pairs of viruses. Other lesser used graphical comparison tools that provide metrics of sequence similarity include BRIG (BLAST Ring Image Generator; [36]), Easyfig [37], Circos [38], CGView [39], and CGView Comparison Tools [40]. Dotplots have been used extensively by scientists associated with the Actinobacteriophage Database projects [41–43], and an example Gepard plot ("**GE**nome **PA**ir – **R**apid **D**otter"; [35]; http://cube.univie.ac.at/gepard) comparing two *Leuconostoc* phage genomes is shown in Fig. 1.

Though progressiveMauve, EasyFig, BRIG, etc., and all of the dot matrix analysis tools produce good figures for manuscripts, they do not express relationships between genomes in quantitative terms such as "percent identity." This is one of the advantages of EMBOSS Stretcher, PASC [PAirwise Sequence Comparison; [44]; (https://www.ncbi.nlm.nih.gov/sutils/pasc/viridty.cgi)], SDT [Sequence Demarcation Tool; [45]; (http://web.cbio.uct.ac.za/~brejnev/)], JSpecies ([46]; https://imedea.uib-csic.es/jspecies/), ANI (Average Nucleotide Identity; [47]; http://enve-omics.ce.gatech.edu/ani/), GGDC (Genome-To-Genome Distance Calculator; [48]), and VICTOR ([49]; https://ggdc.dsmz.de/). However, with the possible exception of ANI and VICTOR, most of these tools have not been used for phage research. To be more precise, VICTOR calculates pairwise identity scores and uses them to calculate the tree and the different taxon levels, but does not output the "percentage identity."

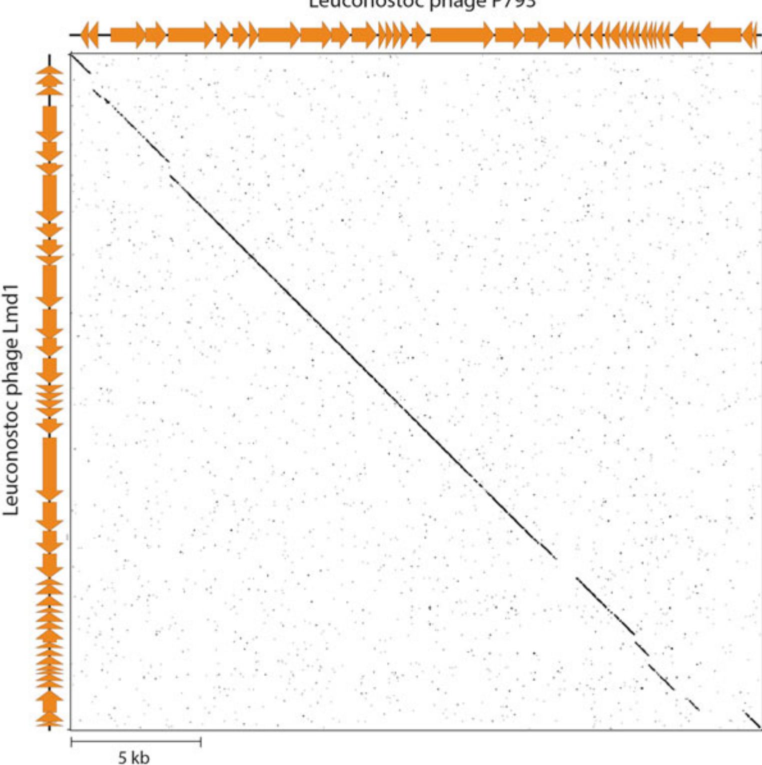

Fig. 1 Gepard dotplot comparing the similarity between the genomes of *Leuconostoc* siphoviruses Lmd1 [93] versus P793 [94] modified to include genome maps generated using EasyFig. Protein-coding genes are depicted as arrows with the orientation indicating the location on the forward or reverse strand. The dark diagonal lines represent syntenic regions

Two groups have made extensive use of DNA sequence homology to group bacteriophages. The first is the Actinobacteriophage Database (https://phagesdb.org/), which currently includes 20,918 phages [50]. The original clustering method [51] has now been modified to include a measure of the Gene Content Similarity (GCS; [52]). Second, in 2014 Grose and Casjens [53, 54] used BLASTn, BLASTp, and quantitative DotPlot data to group 337 sequenced Enterobacterial phages into 56 clusters, many of which now correspond to ICTV-ratified genera.

3 How ICTV Currently Groups Phages into Taxa

It is imperative before continuing this discussion to distinguish between phage isolates and taxa. To quote from the ICTV website, "Viruses are real physical entities produced by biological evolution and genetics, whereas virus species and higher taxa are abstract

concepts produced by rational thought and logic. The virus/species relationship thus represents the front line of the interface between biology and logic"" [55–57]. In a recent opinion piece, this is further clarified as "virus species are human-made taxonomic categories to which viruses are assigned when they satisfy a particular set of properties, known as "species demarcation criteria"" [55, 58]. In practical terms, phage isolates are physical entities that can form plaques on an appropriate host while taxa cannot. Once a new phage is isolated, it can be added to an existing taxon, or in cases where the new isolate is sufficiently distinct from extant isolates, a new taxon can be proposed with the new isolate the exemplar of the new species. The tool for proposing the creation of a new viral order, family, subfamily, genus, and species, as well as for modifying existing taxa, is known as a Taxonomy Proposal (abbreviated as TaxoProp). The template for this can be downloaded from the appropriate ICTV website (https://ictv.global/taxonomy/templates). Anyone can fill in and submit a TaxoProp, but it is generally advisable to work with an appropriate member of the Bacterial Viruses Subcommittee (BVS; https://ictv.global/sc/bacterial), who can offer advice and proofread the intended submission.

The process of placing a new phage isolate within a taxon begins with identifying all related isolates. This can be done by comparing the new isolate sequence to the "nucleotide collection (nt/nr)" database using the BLASTn algorithm [59]. Searches should be restricted to Organism "Viruses (taxid:10239)" or "Caudoviricetes (taxid:2731619)," if the morphology is known. The increase in SARS-CoV-2 sequences means BLASTn often returns a CPU overload message now. To prevent identifying prophages that are part of bacterial genomes, the search is limited to Viruses, unless prophages are what you are interested in. It is also helpful to search against the reference genomic sequences (refseq_genomic) database, as new viral and phage reference genomes are created for each species exemplar [60–62]. Alternatively, one can search against a specific phage species, genus, subfamily, or family. A crude estimate of the overall nucleotide sequence similarity between pairs of genomes can be obtained from the BLASTn search results by multiplying the "Query Cover" by "Per. Ident."

Turner et al. [63] have created "A Roadmap for Genome-Based Phage Taxonomy" in which the demarcation criteria necessary to create the following taxa are enunciated. In the following taxonomic sections, we have quoted liberally from this manuscript.

Species: "The biological species concept [. . .] defines species as interbreeding individuals that remain reproductively isolated from other such groups" (cited from [64]). In the absence of sexual reproduction, gene flow, for example through homologous recombination, has been proposed to result in "interbreeding" [65]. Therefore, viral species can be thought of as a group of strains

with high rates of gene exchange. In nature, this would result in the existence of sequence-discrete populations. A large-scale metagenomic study in the surface oceans found that dsDNA phage populations form discrete genotypic clusters at ~95% average nucleotide identity (ANI) cut-off [64].

This is in line with the current ICTV species threshold. "Two phages are assigned to the same species if their genomes are more than 95% identical over their genome length for isolates." These values can be calculated by a number of tools, such as BLASTn, or using the intergenomic distance calculator VIRIDIC [66]. VIRIDIC calculates nucleotide-based intergenomic identities that are normalized to the viral genome lengths. Therefore, VIRIDIC values are less prone to overestimation of the intergenomic identity as compared with ANI methods, which normalize identity to alignment length. It is worth noting, however, that large direct terminal repeats can still distort VIRIDIC results unless the user ensures that all of the input genomes contain only one copy of the terminal repeat.

In Fig. 2, VIRIDIC has been applied in a quantitative genome comparison of a group of *Leuconostoc* siphoviruses. ICTV does not classify strains, but BVS keeps a list of these and transmits this information to NCBI.

Genus: Turner et al. state: "In search for criteria that create cohesive and distinct genera that are reproducible and monophyletic, the Bacterial Viruses Subcommittee has established 70% nucleotide identity of the genome length as the cut-off for genera." In the case of temperate phages, some leeway to this value is permitted. Genus-level groupings should always be monophyletic in the signature genes, as tested by phylogenetic analysis. In Fig. 2, phage P793 and its homologs are classified into the genus *Limdunavirus*, while phage CHB is a member of the *Unaquatrovirus*. Based upon the above statement concerning strains, phages Diderot and phiLN03 (exemplar) are both strains within the species *Limdunavirus LN03*; phages phiLN6B and Lmd1 (exemplar) within *Limdunavirus Lmd1*; phiLN34 (exemplar), phiLNTR2, P974, CHA, and CHB are strains within *Unaquatrovirus LN34*; and, lastly, Ln-8 and phiLN25 (exemplar) are strains within the species *Unaquatrovirus LN25*.

Subfamily: Subfamilies are to be created when two or more genera are related below the family level. In practical terms, this usually means that they share a low degree of DNA sequence similarity (usually about 40–50%) and that the genera form a clade in a marker tree phylogeny. In the above example, we considered that there was sufficient evidence for a taxonomic relationship between the *Limdunavirus* and the *Unaquatrovirus* to create a subfamily which was named *Mccleskeyvirinae*.

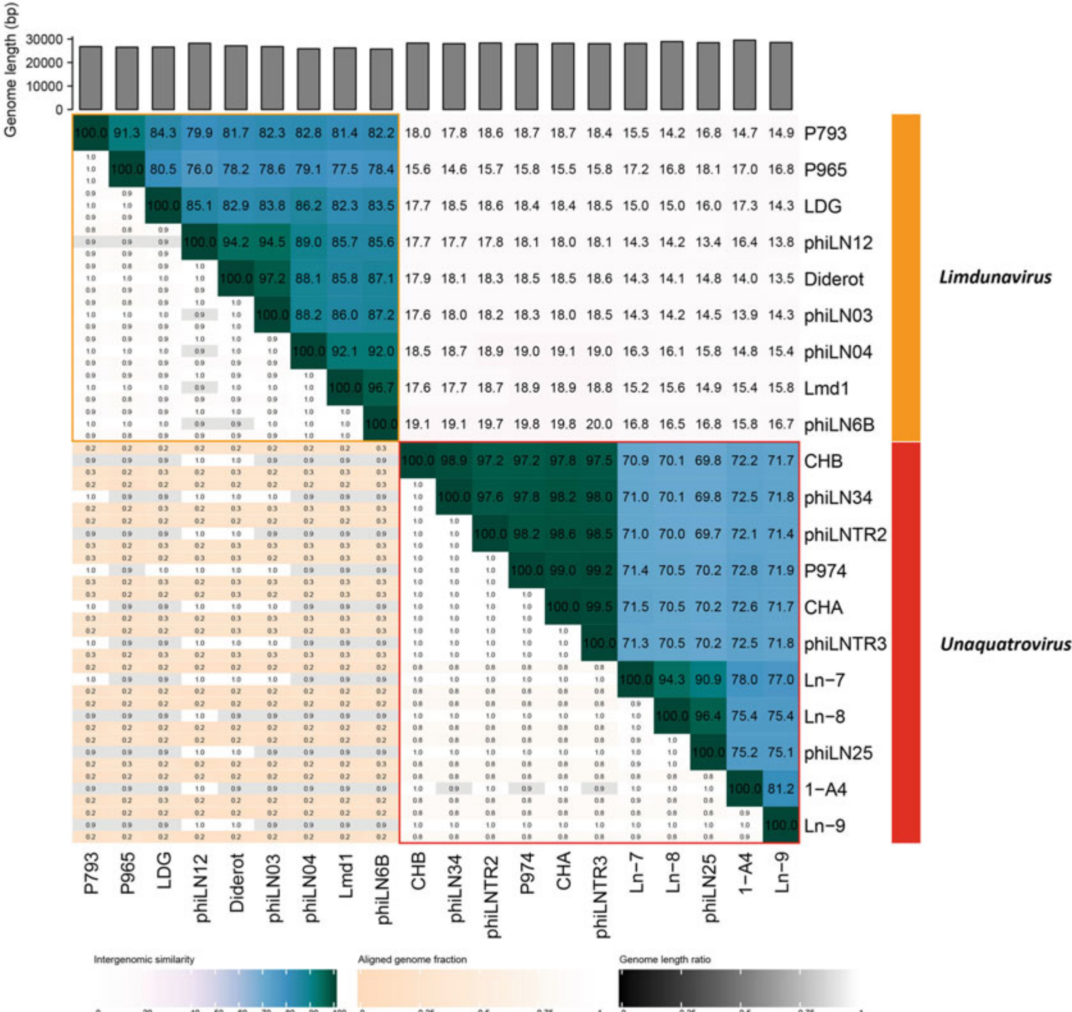

Fig. 2 VIRIDIC analysis of 20 *Leuconostoc* siphoviruses in which two distinct clades (*red square* for *Unaquatrovirus* genus and *yellow square* for *Limdunavirus* genus) are recognizable. The upper-right half shows the nucleic acid intergenomic identities for each genome pair, color coded in a range from white (0% identity) to dark green (100% identity). The lower-left half shows three different indicator values. For each genome pair, these values are given at the intersection of the corresponding row with the corresponding column, as follows (from the top to bottom of the intersection square): (i) aligned fraction genome 1, representing the proportion of the genome found on the row that has been aligned to its pair (the genome on the column); (ii) genome length ratio between the two viruses in a pair; and (iii) aligned fraction genome 2, representing the proportion of the genome found on the column that has been aligned to its pair (the genome on the row). All three values are color coded as well (see legend). Lighter, closer to white colors, indicate well-aligned genome fractions (close to 1) and/or good genome length ratios (close to 1). For example, the pair phiLNTR2 (on the row) and CHB (on the column) has all three indicators equal to 1, meaning that their genome lengths are very similar and that they align along their full genome. On the contrary, the pair phiLNTR2 (row) and phiLN6b (column) have two aligned genome fractions of 0.3 and their genome length ratio of 0.9. This means that even though the lengths of their genomes are similar, they have regions of homology (and therefore align) only on 30% of their respective genome lengths. Their intergenomic identity is also low—19.7

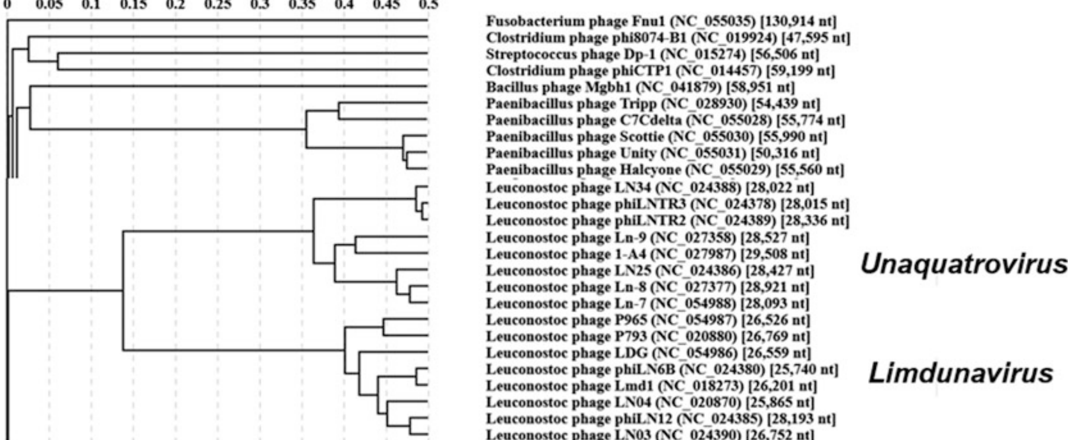

Fig. 3 ViPTree analysis of *Leuconostoc* siphoviruses. ViPTree analysis (https:/www.genome.jp/viptree/) is based upon Rohwer and Edwards's [9] Phage Proteomic Tree and utilizes tBLASTx comparisons between pairs of phage genomes

Family: "The family is represented by a cohesive and monophyletic group in the main predicted proteome-based clustering tools," which can include ViPTree [67], GRAViTy dendrogram [68, 69], vConTACT2 network [22, 23], and VirClust [70, 71]. To add more functional information to these proteome-based clustering tools, we can recommend the PHROGs database (Prokaryotic Virus Remote Homologous Groups; [72]) and Phamerator [73, 74]. PHROGs represents a comprehensive database of viral protein superclusters, which allows HMM comparisons and, thus, enables the detection of more distant homologues and enhances viral genome annotations and the discovery of viral hallmark genes. Members of a viral family share a significant number of orthologous genes (the number will depend on the genome sizes and the number of coding sequences of members of the family). In the case of these *Leuconostoc* phages (Fig. 3), there is insufficient data to support creating a new family.

Since the ViPTree analysis is based upon tBLASTx and not BLASTp, CoreGenes3.5 [75] or CoreGenes5.0 (https://coregenes.ngrok.io/) are frequently employed to compare proteomes. Other command line tools such as Roary [76], PIRATE [77], and PanOCT [78] are also available to facilitate pan-genome analysis. It is important to note that ViPTree does not consider gene order and should be interpreted with caution (ideally by supplementing with another tool), particularly if there is a biologically surprising result. For example, when trying to resolve the classification of lambda-like phages above the subfamily level, we have seen clades of different morphotypes group more closely together than related clades of the same morphotype. When the

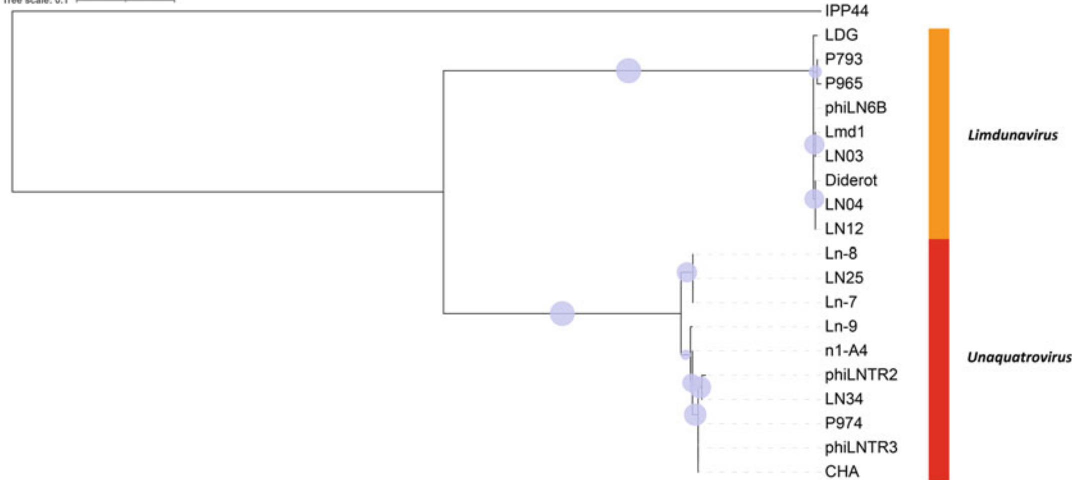

Fig. 4 Large subunit terminase (TerL) phylogenetic tree of the *Leuconostoc* phages with the homologous protein from *Streptococcus* phage IPP44 as the outlier. This was generated using "one click" at http:/phylogeny.lirmm.fr/ [95], exported in Newick format, and visualized using iTOL v6 [96]; the bootstrap support >50% is shown as filled circles, with the size proportional to the value. *Leuconostoc* phage CHB is not included since it is represented, in GenBank, as two pieces. "The 'One Click mode' targets users that do not wish to deal with program and parameter selection. By default, the pipeline is already set up to run and connect programs recognized for their accuracy and speed (MUSCLE for multiple alignment and PhyML for phylogeny) to reconstruct a robust phylogenetic tree from a set of sequences." It also includes the use of Gblocks to eliminate poorly aligned positions and divergent regions. "The usual bootstrapping procedure is replaced by a new confidence index that is much faster to compute". (see [97] for further details)

same genomes are run through GRAViTy, which does take genome organization into account, the same-morphotype clades all cluster together.

As the last step in collecting data to support new taxa, phylogenetic trees are created. These are usually based upon virus hallmark genes, for example, the large subunit terminase, DNA polymerase, nucleotide metabolism, major capsid, or major tail proteins. For the family level, the recommendation is to generate a phylogenomic tree, that is, a tree that is based on an alignment of all the core genes in the family. They need to include maximum likelihood, bootstrapping, and rooting using an outgroup to enable an accurate assessment of monophyly here. The results (Fig. 4) of our analysis of TerL proteins from *Leuconostoc* siphophages are completely in accord with the total proteome and total genome analyses. In summary, BVS now employs a more holistic approach to classifying phages, relying on genomic identity along with a common proteome and phylogeny. It should be pointed out that members of the *Autographiviridae*, *Chaseviridae* [79], and *Xipdecavirus* [80] all encode a large single-subunit RNA polymerase gene, yet morphologically represent podo-, myo-, and siphoviruses, respectively.

Table 1
Change in the numbers of various ICTV-recognized taxa belonging to the order *Caudovirales* or class *Caudoviricetes*

Year				
	2005	**2011**	**2016**	**2021**
Class	0	0	0	1
Order	1	1	1	4
Family	3	3	3	47
Subfamily	0	5	15	98
Genus	18	37	215	1197
Species	36	135	956	3601

3.1 Progress

As more and more phage sequences were deposited in databases, it became apparent that the order *Caudovirales* and its three families was not monophyletic and that its existence inhibited the development of a true phage taxonomy. The BVS initially dealt with this problem by creating parallel families, including the *Herelleviridae*, which was used as a case study [81, 82]. However, this proved ultimately unsatisfactory. Therefore, in 2020, ICTV expanded the number of taxonomic ranks from five to fifteen [83] and in 2022 removed the order *Caudovirales*, and the families *Myoviridae, Siphoviridae,* and *Podoviridae* [84]. Please note that the terms “myovirus,” “podovirus,” and “siphovirus” will continue to be acceptable in describing morphotypes.

The BVS of ICTV has made huge advances in method development and in the classification of bacteriophages (Table 1). This is probably best illustrated by the classification of the T7-like phages: In 2008, these were classified as three species in the family *Podoviridae*; as of 2021, they had become the *Autographiviridae* family, with three subfamilies, 133 genera, and 373 species. In the latest taxonomic update, 1197 of the 2606 viral genera are members of what was known as the *Caudovirales*. In the latest taxonomic update, the order *Caudovirales* was abolished and replaced by the class Caudoviricetes to encompass all bacterial and archael viruses with a dsDNA genome. Concerted efforts are now required by both the BVS and wider bacterial virus research community to establish genomically coherent taxa at the levels of family and order. For classification at the family level, ideally, at least two genera comprised of multiple species are required to determine the presence of several hallmark gene products conserved across all species to be used as demarcation criteria.

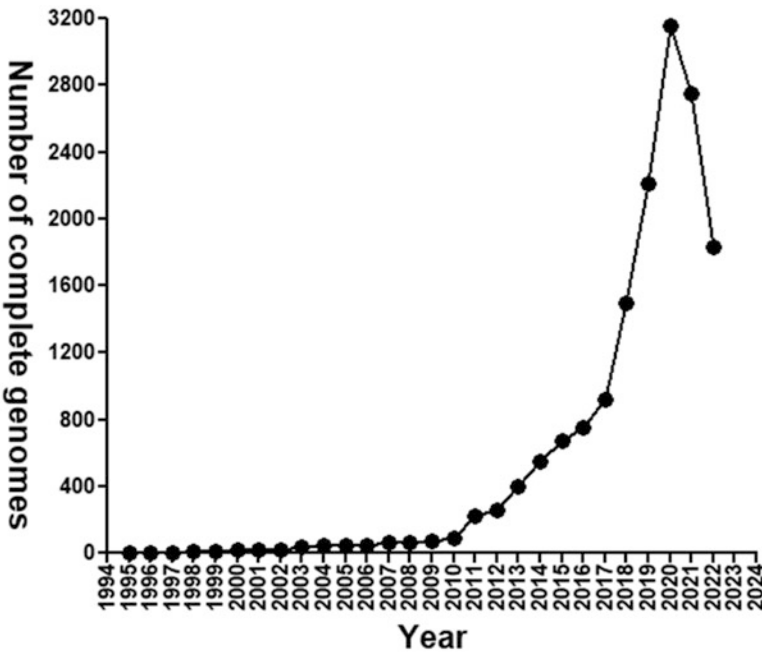

Fig. 5 Annual growth of fully sequenced members of the class *Caudoviricetes* in NCBI. The data is the number of complete *Caudoviricetes* (newer data) or *Caudovirales* (older data) as of September first of each year and was generated using "(((Caudoviricetes) AND complete genome) AND ("2022/08/31" [Publication Date] : "2023/09/01" [Publication Date])) NOT RefSeq"

3.2 Taxonomic Challenges of Modern Sequence Databases

The number of complete phage genomes deposited in the International Nucleotide Sequence Database Collaboration (INSDC) resources annually is staggering (Fig. 5). Since ICTV only reviews TaxoProps once a year and the documentation requires considerable attention to detail, the creation of new taxa lags significantly that of generating complete phage sequences.

Phage sequences in the databases are derived from three sources—direct isolates (phages), bacterial genomes (prophages), and environmental samples (viromes). While there is growing evidence that viromic samples represent complete phages, the same does not extend to prophages, many of which are demonstrably cryptic. In many cases, it is difficult to determine from the online record whether the "phage" was isolated in a laboratory or discovered using bioinformatics approaches. In the case of prophage submissions, the DEFINITION line could be "host genus + prophage + complete genome," or the GenBank sequence record could include the qualifier "/proviral" within the "source" feature key. We would very much like the aforementioned databases to introduce classifications for viral genomes derived from metagenomes and for prophages, to enable the application of filters within BLAST searches. In addition. BLAST searches can reveal "hits" to

metagenome-derived phage-like sequences. A search for "Siphoviridae sp. (human metagenome)" revealed 23,445 sequences that are called "MAG TPA_asm: Siphoviridae sp.," 23,635 deposited under "Siphoviridae sp. (seawater metagenome)," and 24 under "Siphoviridae sp. (animal metagenome)." There are also huge numbers of metagenomically classified *Myoviridae* and *Podoviridae*. Some of those with complete genomes have been classified recently to a new Order, the *Crassvirales*. The presence of those which have not been classified by ICTV can complicate the interpretation of the BLASTn results. If one includes NOT ENV[Division] in the Entrez Query box, all classified environmental (ENV) "hits" will be removed/hidden. Alternatively, we recommend running BLAST searches against the manually curated **IN**frastructure for a **PHA**ge **RE**ference **D**atabase (INPHARED; [85]). Comparisons against the INPHARED database require expertise to create custom BLAST databases for command-line searches.

Incomplete/inaccurate taxonomies and errors in the deposited phage sequences and their annotation contribute to the propagation of errors and can confound classification efforts. In an ideal scenario, submitters should view their sequence record as a living document and take responsibility for updating these records to address errors of annotation or classification as they become apparent.

3.3 Creation of Higher Taxa

It has been proposed that the defining characteristic of the "T4 superfamily" of phages, which infect a wide range of host bacteria in the phyla Proteobacteria and Cyanobacteria, is the presence of approximately 30 conserved proteins [86]. Huge advances have been made in the formalized classification of these viruses by Andrew Millard and his study group, who have created two new families, *Straboviridae* (2 subfamilies, 35 genera) and *Kyanoviridae* (45 genera). These include phages infecting *Aeromonas, Cronobacter, Enterobacter, Escherichia, Erwinia, Klebsiella, Morganella, Pectinobacterium, Proteus, Pseudomonas, Salmonella, Serratia, Shigella, Vibrio*, and *Yersinia*; and *Prochlorococcus* and *Synechococcus*, respectively. *Campylobacter* phages, which by Petrov's definition are T4-like, are to be found in the subfamily *Eucampyvirinae*.

Another age-old taxonomy problem is the relationship between *Escherichia coli* phage λ (*Lambdavirus*) and *Salmonella* phage P22 (*Lederbergvirus*) since these two temperate phages have syntenic genomes and the same type of repressor-anti-repressor regulatory circuitry, but different morphologies. In addition, similarities have been observed with the T1-like phages (*Drexlerviridae*) and N15-like phages (*Ravinvirus*), which share tail morphogenesis genes [87] (http://www.biologyaspoetry.org/PDFs/bgnws018.pdf). While analysis is ongoing, it appears likely that the lower taxonomic groups containing λ, P22, and T1 will remain distinct, with the differences among these groups outweighing their

similarities, but the best approach to structuring these taxa has yet to be determined. At the genus level, in 2020, a large number of unclassified phages that had been described as lambda-like by the historical report, by the sequence submitter, or by NCBI algorithms were collected and placed into 10 genera and 20 species (ICTV TaxoProp 2020.013B). Only one phage presented as a challenging "edge case," having 70–71% nucleotide similarity to all of the phages in each of the two candidate genera. Automated tools slightly favored one genus assignment and manual inspection of the genome alignment slightly favored the other. With no clear "best" result, the phage was ultimately placed within the genus to which it was assigned by automated tools (VIRIDIC, a nucleotide method, and ViPTree, a proteomic method) on the grounds that this is most likely to result in consistent classification by future users who might not inspect every alignment. At the Family, Subfamily, and Genus levels, genome mosaicism has presented some challenges, but does not seem to be the insurmountable challenge that had sometimes been predicted for the historically lambdoid phages.

The last two examples point to the need for the BVS to develop criteria for the identification of higher-order taxons, particularly at this time for families, orders, and classes. Toward this end, one of the authors (CM) is currently developing a web-based tool, VirClust ([72] http://virclust.icbm.de/), aimed to assist researchers with virus classification. VirClust represents a protein-based tool, which calculates protein clusters (based on BLASTp similarity) and protein superclusters (based on HMM similarity) for the input viral genomes. Further, VirClust is using these protein (super)-clusters to (i) cluster hierarchically the input viral genomes, (ii) delineate viral genome clusters based on different intergenomic distance thresholds, (iii) calculate core proteins (signature genes) for the respective viral clusters, and (iv) annotate viral proteins against several databases. To identify higher-order taxons (e.g., Order), it may be necessary to define HMMs of signature genes—see the RdRP of RNA viruses [88].

4 Concluding Statement

Over the last decade, interest in bacteriophages has blossomed. This is a result of three things: (i) the isolation and characterization of phages as alternatives to antibiotics (phage therapy), (ii) the use of these viruses to teach genomics (phage hunting courses; [89–92]), and (iii) the realization that all ecosystems are teaming with phages (metagenomics). Productivity in these areas of phage research has been enhanced by the availability of inexpensive DNA sequencing. Collectively we have seen a massive increase in complete viral genomes in the INSDC databases. At the taxonomic

level, the introduction of an expanded taxonomic field and the removal of the order *Caudovirales* and its three families have and will allow natural evolutionary relationships to be recognized. We have also seen the development of valuable tools for the characterization of metagenomes, along with those which group viruses based on DNA and protein sequence homology.

5 Practical Considerations for Phage Scientists

Before deciding that a newly sequenced phage genome fits within an existing or new genus one should review all the evidence: host; morphotype; lifestyle; genome characteristics (kb, mol% G + C, number of CDS, tRNAs); DNA-DNA relatedness; % protein homologs; and phylogeny. After reviewing all the data, you may decide to lump or split. Do not try and "force" a phage genome into an existing genus, there are plenty of orphan species and genera for which homologs are subsequently isolated. In the case where you experience difficulty, we would recommend that you contact the appropriate member of the Bacterial Viruses Subcommittee (https://ictv.global/sc/bacterial/sc_bacterial).

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Part II

Selection, Production, and Encapsulation of Bacteriophages

Check for updates

Chapter 4

Guidelines to Compose an Ideal Bacteriophage Cocktail

Maia Merabishvili, Jean-Paul Pirnay, and Daniel De Vos

Abstract

Properly designed bacteriophage therapeutics are the cornerstone for a successful outcome of bacteriophage therapy. Here we present an overview of the different strategies and steps that can be taken to develop a bacteriophage cocktail that complies with relevant quality and safety requirements. It is based on empirical bacteriophage therapy knowledge from over a century of experience, more recently performed studies, and emerging technologies. We emphasize the selection of adequate bacteriophages and describe a modified Appelmans' method to improve the overall performance of therapeutic bacteriophages individually and collectively in the cocktail. We present two versions of the method, which differ from each other by the employed techniques to evaluate phage activity and synergy: photometric assessment of bacterial growth versus measurement of bacterial respiration via the Omnilog® system.

Key words Bacteriophage, Cocktail, Composition, Therapeutics, Bacteriophage selection, Bacterial host, Host range, Virulence, Appelmans, Preadaptation, Compatibility, Synergy

1 Introduction

On their way to possibly reclaim their niche in Western medicine, bacteriophage (phage) therapy products are required to achieve a certain quality and to demonstrate safety and efficacy (e.g., using randomized controlled trials) to a certain extent, which is mainly determined by the regulatory framework at hand [1–3]. The statement released by the European Medicines Agency (EMA) after the workshop dedicated to the reacceptance of phage therapy, held on June 8, 2015, emphasizes the urgency for phage therapy clinical trials [4, 5]. However, the pressure caused by the global antibiotic crisis with increasing numbers of newly emerging antibiotic-resistant bacterial strains creates a challenging situation with high expectations from phage therapy [6, 7]. According to recent microbiome and microbiota studies, antibiotics have a larger negative impact on the commensal bacterial flora than previously supposed [8, 9]. More such studies are urgently needed, and alternative approaches in the fight against bacterial infections need to be

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developed. At the same time, high expectations are placed on phage therapy as an addition to the current antibacterial repertoire [10–12].

More and more successful phage therapy cases are being published in the scientific literature, some in high-impact journals, putting increasing pressure on medical doctors to turn to phage therapy as an alternative or supplement to antibiotics in the treatment of antibiotic-resistant or difficult-to-treat infections. Some countries have therefore implemented exceptional measures, under the compassionate use program (France), the magistral preparation framework (Belgium), expanded access (US), or the Declaration of Helsinki (Poland) [13–16], so that phage therapy can be used today to help desperate patients, and pending the marketing of licensed phage products. However, it is anticipated by some that these temporal or other adapted frameworks will have to remain alongside the conventional medicinal product pathway. Indeed, it has been suggested that the licensed defined phage products in the pipeline, which target commercially viable markets, are bound to be inefficient in certain cases, which will have to be met by more flexible, personalized approaches, requiring ditto production and licensing solutions. This should, however, not affect the scrutiny of the whole process of therapeutic phage production and subsequent validation procedures. The development of effective therapeutic phage cocktails and their application in thoroughly designed standardized clinical trials are of utmost importance for the acceptance, and the avoidance of a second (irreversible) rejection, of the phage therapy concept.

Felix d'Hérelle, one of the discoverers of phages and the initiator of the idea of applying phages for therapeutic use, was also the first person to warn that phage cocktails should be designed and developed by laboratories that harbor a vast knowledge in therapeutic phage research, and not by companies focusing primarily on quick profit gain [17].

Current commercialization of phage therapy should exploit the advances in molecular biology (e.g., synthetic biology), biotechnology, and modern biomedical sciences as well as the specific knowledge of phage biology and its co-evolutionary aspects to avoid the failures of the past [18]. Factually, it seems strange that most actors in the field are still pointing at certain perceived negative aspects of phage therapy, such as the specificity of phages and the time needed to select phages that target the patient's infecting bacterial pathogen. At the same time, today, technologies exist to overcome these hurdles, which are mainly related to classical microbiological techniques that have not changed since the time of Pasteur [19].

In this chapter, we provide recommendations on the selection of candidate phages and bacterial strains, their adaptation and production, and their combination in therapeutic phage cocktails.

Our recommendations are based on a century of experience in phage therapeutic production processes and on current scientific research evidence confirming the eligibility of each procedure. We also describe a modification of Appelmans' method [20] to evaluate the stability of phage activity in liquid media cultures, to be applied to enhance the activity and broaden the host range of therapeutic phages, and to define their complementary activity in the cocktail.

1.1 Selection of Bacterial Host Strains

1.1.1 Bacterial Strains Used for the Selection and Initial Propagation of Therapeutic Bacteriophages in the Preproduction Process

The principle of phage therapy is based on the application of the Darwinian ecological-evolutionary theory to control pathogenic microbes at the site of infection. Interactions between bacteria and phages can be described as antagonistic coevolution [21], which prompts the development of phage therapy within the "one health" approach and leads us to consider the "couplet phage/bacterium" as one entity. These incentives should be considered the main advantages of phage therapy and should not be compromised in view of quick commercial success.

Phage cocktails should not be considered medications with a fixed composition, such as classic static chemical drugs (e.g., aspirin and antibiotics). Quite the opposite, they should be produced using a continuous renewal and adaptation process. In this regard, the choice of bacterial strains to be used in the development and production of phage cocktails is crucial. Laboratories aiming at developing phage cocktails should have large, continuously updated collections of relevant pathogenic bacterial strains. At least one-third of such bacterial collections need to be renewed annually with the addition of new isolates originating from the clinical environments and geographical areas where the phage therapeutic product will be used.

The preproduction bacterial strains should be virulent strains, and the strains used in the phage selection process should include:

(i) Species-typical physiological, biochemical, serological, and genetic features.

(ii) Representatives of the clinically relevant bacterial strains/clones that are circulating in the considered clinical settings.

(iii) As such, clinically important non-typical strains of the targeted species can also be added.

Strains should be characterized by state-of-the-art microbiology and molecular biology methods.

Therefore, laboratories developing phage products should be in perpetual interaction with hospitals and routine clinical microbiology laboratories.

Bacterial strains selected for de novo isolation of therapeutic phages (e.g., from sewage water) should be reevaluated yearly to ensure that they maintain the main biological features, including

virulence properties, and to prolong their use in the preproduction process. Strains should be maintained in such a way as to limit the loss of their virulence traits and of other relevant biological features.

As a rule, for the isolation and selection of new phages, at least 10–15 bacterial strains, which exhibit as many different relevant features as possible, should be used [22–25].

1.1.2 Bacterial Strains Used for the Propagation of Bacteriophages in the Production Process

Different requirements are applied to the bacterial strains used directly in the process of phage production, in particular for the large-scale phage propagation process. These strains should be chosen to guarantee the maximal quality and safety of the final phage product. Therefore, the following requirements should be met:

(i) The industrial production strains should have a well-documented pedigree history, identified at the species and strain level based on the state-of-the-art methodology of microbiology and molecular biology (genotyping methods, such as AFLP, PFGE, and MLST) alongside phenotypic characterization. Preferably, genome sequences of the industrial production strains should be available and analyzed to avoid different kinds of unexpected outcomes, such as undesirable horizontal genetic exchange(s) or toxins in the final phage product.

(ii) The production strains should be as benign (e.g., non-toxin producing) as possible to limit purification efforts of the phage product and minimize the risk of toxicity of the final product. Therefore, for producing phage products active against highly virulent bacteria, it is recommended to use so-called surrogate phage-sensitive strains with low pathogenicity [18, 26].

(iii) Production strains should preferably be non-lysogenic. In case such strains are unavailable, they should exhibit a low frequency of spontaneous and induced phage induction to obtain negligible amounts of temperate phages in the final product and avoid incurred horizontal genetic exchanges. The required features can be confirmed by complete genome sequencing and prophage induction methods [27].

(iv) Production strains should not be "mutator strains" to exclude unpredictable mutations during the production process. This can be confirmed by state-of-the-art methods (e.g., disk diffusion tests) [28].

(v) Production strains should preferentially be rapidly growing and easy to culture to ensure the low cost of the commercial end product.

1.2 Selection of Candidate Bacteriophages and Design of a Therapeutic Cocktail

Based on current European regulatory requirements, candidate therapeutic phages should be naturally occurring phages, not genetically manipulated. Although engineered phages have been used in therapy and synthetically produced phages are being developed [19, 29, 30] and might become commonplace in the future, here, we focus on natural phages.

1.2.1 Recommended Isolation Sources for Therapeutic Bacteriophages

Phages can be isolated from various environments, such as river water, sewage, soil, and rhizospheres, as well as from clinical samples, such as pus, urine, feces, body fluids, and others. Using sewage water from multi-profile clinics and hospitals increases the chances of isolating therapeutic phages, as these untreated samples contain high titers of clinically relevant bacteria.

Some newly emerging pathogenic bacterial species or strains are not inhabitants of common environments [31], which challenges the phage isolation process. In this case, phages should be searched in the specialized environments typical for that particular pathogen species or strain [32].

Candidate phages are recommended to be isolated by the enrichment method [33, 34] using a mixture of several preproduction bacterial strains. The enrichment method creates certain biases by selecting fast-replicating phages, which is considered an advantageous feature for therapeutic phages. Using mixtures of bacterial strains in the process also guarantees a broad host range of newly isolated phages [18, 35].

Laboratories involved in developing phage therapeutics should possess large collections of well-characterized candidate phages that are continuously updated.

1.2.2 Requirements for Single Therapeutic Bacteriophage Candidates

There are several requirements for therapeutic phages, which can be considered essential, important, or desirable [36, 37]. However, each requirement should be considered in light of the application aim of the future phage product, such as sense of urgency, target infection site, and duration of treatment. The main principles concerning the choice of candidate phages and the design of therapeutic cocktails are presented in Fig. 1.

Candidate therapeutic phages to be included in phage cocktails should ideally have the broadest host range possible against the target bacterial species/strains. Variable thresholds of host range are considered, depending on the population structure of the bacterial species. However, for most bacterial species, the host range threshold for each individual phage will not exceed 40–50% of the clinical strains, and therefore a mixture of different phages (cocktail) should be assembled for application.

Therapeutic phages should act as "active" killers toward most target strains. They should be able not only to adsorb onto the surface of the bacterial cells but also to overcome bacteria-mediated defense mechanisms, such as restriction-modification, CRISPR/Cas, and abortive infection [38]. Phages that lack these abilities but can adsorb on the bacterial cell wall can still kill bacteria by

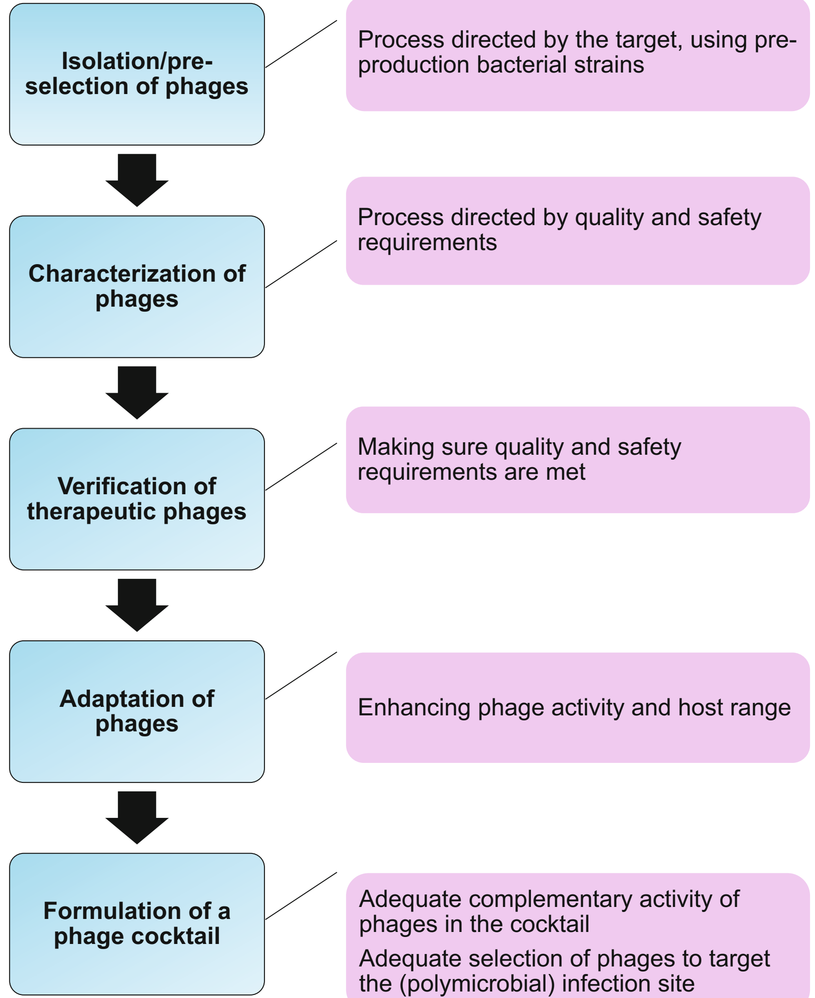

Fig. 1 Main principles of therapeutic phage cocktail design

"lysis/killing from without" when present in high numbers [39]. However, for successful phage therapy, it is essential that the increase of the phage population surpasses that of the bacterial population at the infection site. The ability of phages to propagate on target bacterial strains can be evaluated by determination of the efficiency of plating (EOP) [40], that is, the titer of the phage on a given bacterial cell line compared to the titer observed on the host strain. The threshold EOP should be defined for each bacterial species.

The host range of candidate phages can be broadened by serial passaging (at least three to four times per strain) on 10–15 pre-production bacterial strains, in parallel, in broth cultures, using a modified Appelmans' method [20]. Pre-adapting phages on bacterial strains was already widely applied by d'Hérelle in his first therapeutic experiments [41]. The phage preadaptation process is

based on the coevolution of the phage-bacterium tandem and allows not only to expand the host range of the candidate phages but also to reduce resistance evolution in intermittent and chronic bacterial strains [42].

Therapeutic phages should also have high rates of clearance of target strains. It is considered that phages with a high adsorption rate, short latent period, and large burst size are good candidates to be applied in therapeutic treatment [36, 37]. The above physiological parameters can be defined by the one-step growth test as described by Adams [27].

Therapeutic phages should maintain their killing ability during treatment for as long as possible. However, the emergence of phage-resistant mutants is unavoidable, and therefore another criterion to be taken into account is the frequency of emerging phage-resistant bacterial mutants. As a rule, the frequency of resistant mutants' appearance in phage-sensitive strains should be in the threshold range of $\leq 10^{-7}$–10^{-8} mutants per generation [22–25]. This frequency can be determined using the classical method described by Adams [27]. The ability to delay the selection of resistant mutants can be checked by co-culturing bacteria and phages in broth by the same Appelmans' method.

To preclude any kind of horizontal genetic exchange, therapeutic phages should be exclusively lytic, that is, so-called strictly virulent and non-transducing phages. Their genomes should not encode for any lysogeny-conferring genes, such as integrases, transposases, or repressors. The capacity for generalized transduction, the process by which phages can package any bacterial DNA and transfer it to another bacterium in lytic phages, depends on DNA packaging organization and can be tested by PCR-based methods [43], or by a classic transduction assay [44].

Therapeutic phages should not contain other potentially damaging genetic determinants, such as those encoding for various virulence factors (e.g., toxins) and antibiotic resistance. Genome sequencing of candidate phages is an essential strategy for assessing their safety. It is recommended that each natural phage used for therapeutic purposes has an identified pedigree/history to confirm its naturally evolved origin. Well-defined morphology and classification of the therapeutic phages also include their matching identity verification [37].

Adequate therapeutic phages should have the ability to propagate easily in the appropriate production strains, reaching high titers in a short time. They should also have an acceptable shelf life. This means that one must be able to store the phages in their application form (galenic formulation) while retaining an acceptable activity during a storage period of at least 1 year. To prolong their shelf life, one can refrigerate, freeze, dry, or freeze-dry the phages. The stability of phages in different formulations, such as liquids, spray systems, creams, gels, tablets, and powders, as well as their compatibility with other anti-infectious agents, can be

considered essential characteristics which need to be, partly empirically, investigated. Those characteristics depend on the type of phage, as well as on the formulation itself and its specific application. Phages with excellent stability and compatibility characteristics can be applied using various administration forms and routes.

There are also some (non-essential) desirable features for therapeutic phages, such as exhibiting an immuno-modulatory effect in patients [45, 46] or eliciting phage-resistant bacterial mutants with high fitness costs, including virulence reduction [47, 48]. A good example of the latter feature are lipopolysaccharide (LPS)-specific phages that provoke the selection of resistant bacterial mutants with modified receptors, resulting in reduced bacterial virulence [49, 50].

1.2.3 Phages in Cocktails

Correctly chosen individual therapeutic phages with a sufficiently broad spectrum of activity can be applied as mono-phage therapeutics. Phages, representatives of the genus *Kayvirus* active against *Staphylococcus aureus* and *Staphylococcus epidermidis* strains, are good candidates for such kind of therapeutic products [18, 51]. The host range of these phages usually covers more than 90% of *S. aureus* and around 80% of *S. epidermidis* clinical isolates [23, 52–54]. Preparations containing *Kayvirus* phages proved to be highly effective against various *Staphylococcus* infections [51, 55].

However, for most bacterial species, it is necessary to design the so-called phage cocktails comprising several phages to achieve a collective broader host range of the final product. The efficacy of the final cocktail is considered sufficient if it lyses at least 70–80% of the target clinical strains. The range of this activity threshold mainly depends on the bacterial species. This is a consequence of the specific population structure of a bacterial species, which can be very clonal (e.g., *Salmonella*), panmictic (e.g., *Neisseria gonorrhoeae*), or epidemic (e.g., *Pseudomonas aeruginosa*). A panel of clinical control strains to evaluate ready-to-use cocktails should comprise at least 50 relevant and currently circulating clinical strains per targeted species.

It is important to understand that phage cocktails should not be random mixtures of phages, as many well-documented studies show that specific phages, when applied concomitantly, can interfere with each other upon co-infection [56]. This interference can have synergistic, additive [57, 58], antagonistic, or neutral effects [59, 60]. Therefore, the compatibility of phages in mixtures needs to be determined by challenging them against preproduction strains in broth cultures using Appelmans' method. The resulting phage activity can be monitored by determining bacterial growth inhibition photometrically, fluorometrically, or turbidometrically or by using other more recently developed technologies to follow bacterial metabolisms, such as isothermal calorimetry or the Omnilog® system [61–64]. If a number of therapeutic phages collectively demonstrate higher efficacy compared to individual phages, the positive interference between phages of the same cocktail is guaranteed.

Phage cocktails must be designed as combinations of phages prompting various kinds of resistance mechanisms in bacterial mutants. For instance, it is recommended to choose phages with different bacterial cell wall receptor recognition sites [57]. Individual phages with multiple bacterial recognition sites are also considered advantageous, for example, T-even dual-receptor phages [65, 66].

When targeting infection sites with polymicrobial communities, complex cocktails containing mixtures of mono and/or complementary phages active against each bacterial genus/species should be applied. The Pyobacteriophage and Intestibacteriophage cocktails, which were initially designed by d'Hérelle himself [67] and are still produced by several phage companies in different countries (e.g., *Eliava BioPreparations LTD*, Tbilisi, Georgia), contain phages active against five and seven different bacterial genera, respectively [68].

Pyobacteriophage targets infections in different parts of the human body, such as skin and soft tissue (wounds) and respiratory and urinary tracts. On the other hand, Intestiphage is widely applied in intestinal infections such as dysentery, salmonellosis, and dysbiosis. While designing such cocktails, the microbial composition of target infection sites needs to be determined through epidemiological surveys (incidence and prevalence data), considering the (frequency of) simultaneous occurrence of different bacterial species at the infection site. Antagonistic and synergistic activity between different bacterial species should also be considered. For example, during early phage trials, it was observed that phage cocktails applied to prevent or treat gas gangrene caused by the anaerobic bacterium *Clostridium perfringens* should also contain phages active against staphylococci and streptococci but not against *Escherichia coli* strains. The observation was based on the fact that staphylococci and streptococci create favorable conditions for anaerobic bacteria, while *E. coli* exhibits a natural antagonism against them [69].

2 Materials

2.1 Modified Appelmans Method for the Preadaptation of Phages Against Bacterial Strains and the Evaluation of Phage Complementary Activity in a Therapeutic Cocktail

1. OD_{600} spectrophotometer.
2. Centrifuge (6000 × *g*).
3. Sterile centrifuge tubes.
4. 15 mL culture tubes resistant to chloroform.
5. Serological pipettes.
6. Variable micropipettes (200 μL and 1000 μL).
7. Sterile tips for the micropipettes.
8. 5 mL syringes and needles.

9. 0.45 or 0.22 μm syringe filters.
10. Broth medium specific for the growth of the targeted bacterial species/strains (including supplements).
11. Chloroform (optional).
12. Suspensions of the individual phages or phage mixtures that need to be tested. It is recommended to use high phage titers ($\geq 10^9$ PFU/mL), as determined by their propagating strains, as EOPs on clinical strains can be low.
13. Broth culture of the preproduction bacterial strain(s), in a log growth phase, at a minimal concentration of 10^8 CFU/mL defined based on OD_{600} (0.2–0.4).

2.2 Modified Appelmans' Method for the Preadaptation of Phages on Bacterial Strains Using the Omnilog® System

1. Omnilog® (Biolog) system.
2. Spectrophotometer.
3. Centrifuge (6000 × *g*).
4. 96-well culture plates.
5. Serological pipets.
6. One- and multi-channel pipets and tips.
7. Reagent reservoirs.
8. 15 mL test tubes.
9. Syringes for small volumes and needles.
10. 0.45 or 0.22 μm syringe filters.
11. Broth media specific for the growth of the targeted bacterial species/strains (including supplements).
12. Omnilog® tetrazolium dye, 100 times concentrated, provided by the manufacturer.
13. Suspensions of the individual phages or phage mixtures that need to be tested. It is recommended to use high phage titers ($\geq 10^9$ PFU/mL) determined on their propagating strains, as EOPs on clinical strains can be low.
14. Fresh liquid cultures of the considered bacteria, grown to 10^8 CFU/mL determined based on OD_{600} (0.2–0.4) and subsequently diluted to ~10^7 CFU/mL.

2.3 Evaluation of Complementary Activity of Phages in the Cocktail with the Omnilog® System

1. Omnilog® (Biolog) system.
2. Spectrophotometer.
3. 96-well culture plates.
4. Serological pipets.
5. One- and multi-channel pipets and tips.
6. Reagent reservoirs.
7. Broth medium specific for the growth of the targeted bacterial species/strains (including supplements).

8. Omnilog® tetrazolium dye, 100 times concentrated, provided by the manufacturer.
9. Two or more phages with a titer of 10^9 PFU/mL and supplemented with Omnilog® tetrazolium dye. The titers are defined on the phages' propagating strains.
10. Fresh liquid cultures of the considered bacteria, grown to 10^8 CFU/mL determined based on OD_{600} (0.2–0.4) and subsequently diluted to ~10^7 CFU/mL.

3 Methods

We describe two modifications of Appelmans' method, used to preadapt phages on clinical strains and to determine the complementary activity of phages in a therapeutic cocktail. The two versions differ in how bacterial growth inhibition is monitored, measuring optical density (OD_{600}) in the first version while measuring bacterial respiration using the Omnilog® system in the second version. In the Omnilog® system, the change of color of a tetrazolium redox dye is measured (in an automated way) in short intervals (typically every 15 min) to determine a bacterial respiration curve as a reporter for bacterial cell growth [63]. The main advantage of the latter system is that it allows the real-time determination of living, respirating bacterial cells instead of an overall cumulative optical density.

3.1 Modified Appelmans Method for the Preadaptation of Phages Against Bacterial Strains and the Evaluation of Phage Complementary Activity in a Therapeutic Cocktail

1. Add 4.5 mL of broth medium into 15 mL culture tubes.
2. Prepare three extra tubes as positive and negative controls. The positive control is a tube with only bacterial culture. Negative controls are two tubes, one with individual phage/mixture of phages and another with only broth media in it.
3. Prepare serial tenfold dilutions of each individual phage or phage mixture up to the concentration of 10^0 PFU/mL in the prepared culture tubes by transferring 0.5 mL from the previous tube to the next tube containing 4.5 mL broth. Remove and discard 0.5 mL from the last dilution tube.
4. A negative control tube with individual phage or phage mixture only should be present as the first tenfold dilution of the phage suspension(s).
5. Add bacterial culture, resulting in a final concentration of 10^6 CFU/mL, to each phage dilution tube and the positive control tube.
6. Incubate the tubes at bacterial species/strains' specific conditions (temperature, aerobic/anaerobic environment) for 48 h or longer in case of slow-growing species.

7. Bacterial growth and phage activity can be monitored by measuring OD_{600} after 24/48/72 h of incubation.
8. Results should be compared to positive and negative controls.
9. In case the method is used for phage preadaptation, the tube with the highest phage dilution that shows an OD_{600} value close to the negative control is selected, and chloroform is added up to a final volume of 2.0% (vol/vol) (optional), after which the tube is vortexed shortly and incubated at 4 °C for at least 1 h.
10. After incubation, the lysate can be used directly for the next rounds, or the upper phase without chloroform can either be centrifuged at 6000 × *g* for 15 min or aspirated through a needle into a syringe, and filtrated through 0.45 or 0.22 μm filters, or both procedures can be applied (*see* **Note 1**).
11. The obtained lysate is put through the entire above-described process for at least three to four rounds, each time on another bacterial strain from the preproduction panel (*see* **Note 2**) to achieve stable lysis at dilution 10^{-7}, as recommended in the protocols of the Eliava Institute [22–25, 70].
12. If the method is used to evaluate the complementary activity of phages, after **step 8**: OD_{600} measurement values for the phage combinations should be compared to those for the individual phages. Values should be lower in the case of a mixture because the phages demonstrate higher activity than individual phages. The test can be performed on each bacterial strain or a mixture of strains representing the preproduction strain panel.

3.2 Modified Appelmans' Method for the Preadaptation of Phages on Bacterial Strains Using the Omnilog® System

1. Transfer the culture broth medium (the total volume depends on the number of wells used in the experiment) into a reagent reservoir and dilute the Omnilog tetrazolium dye 100 times in it. Mix well by pipetting up and down to ensure complete dissolution of the dye in the solution.
2. Distribute the broth medium supplemented with tetrazolium dye into the experimental and control wells of the 96-well plate, at 180 μL per well, using a multi-channel pipet. Distribute 200 μL of medium to the control wells (Fig. 2).
3. Add 20 μL of phage solution to the wells of the first column of the 96-well plate. Make tenfold serial dilutions by transferring 20 μL of the phage solution from the wells of the previous column (starting from the first one) into the wells of the next column using a multi-channel pipette. Make dilution up to 10^0 PFU/well, changing tips whenever making a new dilution. Remove and discard 20 μL of the solution from the wells of the last column of dilution.

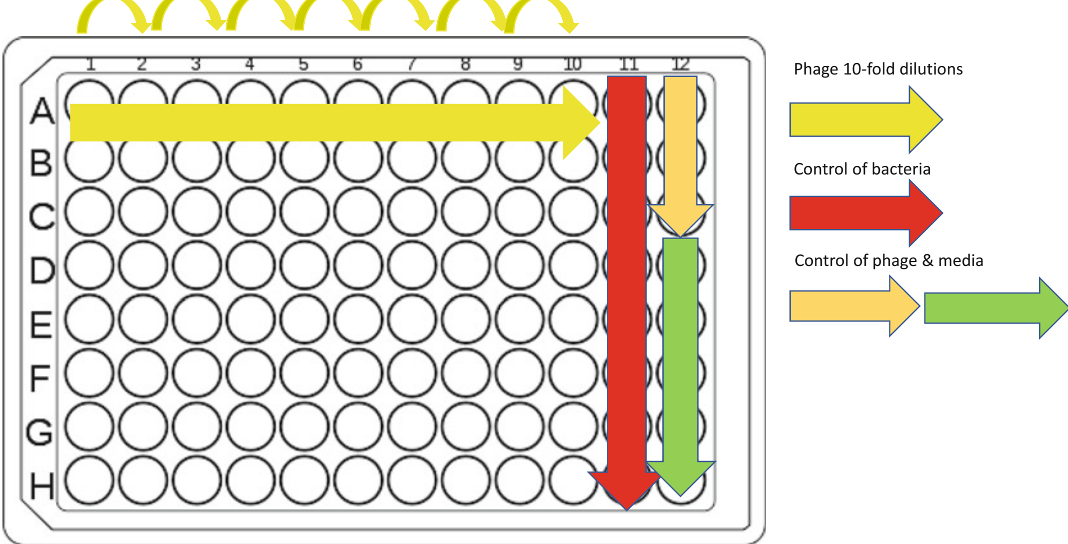

Fig. 2 Schematic representation of a 96-well plate with the distribution of experimental and control wells for the Appelmans' method adapted for the Omnilog® system

4. Distribute 20 μL of bacterial suspension to each experimental well. As such, each well contains 10^5 CFU of bacteria.
5. Designate all wells of one column as positive controls by adding only bacterial suspension.
6. Prepare two types of negative control wells in the last column, one with only phage and one with only media.
7. Incubate the 96-well plate into the Omnilog® device at 37 °C and monitor the results within the 24–48 h incubation period.
8. After incubation, the wells with the highest dilution and the lowest initial concentration of phages where bacterial strains did not grow, or their growth was inhibited significantly (within hours) compared to the positive control wells, should be chosen.
9. The contents of the chosen wells are pooled into a 15 mL centrifuge tube. The tube is then centrifuged at 6000 × g for 10 min, and the supernatant is filtered through a 0.45 or 0.22 μm membrane filter.
10. Use the received phage lysate to prepare the new experiment as described above (*see* **Note 3**).

3.3 Evaluation of Complementary Activity of Phages in the Cocktail with the Omnilog® System

Volumes are given for two phages and one plate.

It is recommended to plan the experiments in advance in Excel file format.

1. Transfer the culture broth medium (the total volume depends on the number of wells used in the experiment) into a reagent

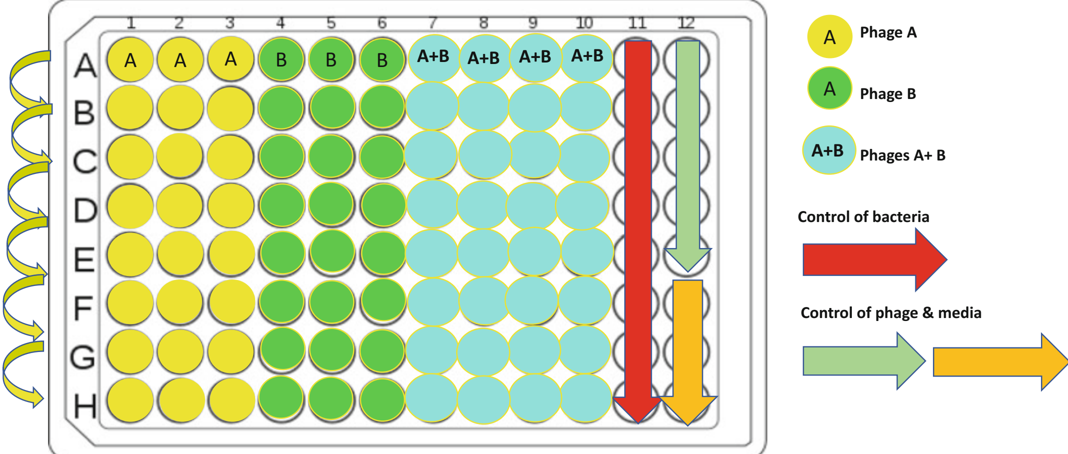

Fig. 3 Schematic representation of a 96-well plate with the distribution of experimental and control wells for the Appelmans' method adapted for the determination of the complementary activity of phages in a mixture, using the Omnilog® system

reservoir and dilute the Omnilog® tetrazolium dye 100 times in it. Mix well by pipetting up and down to ensure complete dissolution of the dye in the solution.

2. Distribute the broth medium supplemented with tetrazolium dye into the experimental and control wells of the 96-well plate using the multi-channel pipet at a volume of 160/180/200 μL per well, depending on the well designation (Fig. 3).
3. Add 20 μL of each phage solution with a titer of 10^9 PFU/mL to the wells of the first row, taking into account the phage distribution (Fig. 3). As such, the wells of row A will contain 10^7 PFU/well. Make tenfold serial dilutions by transferring 20 μL of the solution from the wells of the previous row (starting from the first one) into the wells of the next row using a multi-channel pipet and changing tips whenever making a new dilution.
4. Remove and discard 20 μL of the phage solution from the last dilution wells (row H in Fig. 3). Add phages into the negative control wells of column 12.
5. Distribute the bacterial suspensions (10^7 CFU/mL) to each experimental well at a volume of 20 μL. As such, each well contains 10^5 CFU of bacteria. The range of multiplicity of infection (MOI) in the wells from row A to row h will be 100–0.00001 (Fig. 3).
6. Designate all wells of column 11 (Fig. 3) as positive control wells by adding only bacterial suspension at a volume of 20 μL.

7. Prepare negative control wells by not adding bacterial culture to individual phage, phage mixtures, and broth media in the wells of column 12 (Fig. 3). Adjust all volumes to a final 200 μL.
8. Incubate the 96-well plate in the Omnilog® device at 37 °C and monitor the results within 24/48/72 h.
9. After completing the incubation process analyze the raw data using the Omnilog® Data Analysis software. Bacterial growth curves should be compared to negative and positive controls.
10. Phage mixtures (cocktails) can be considered fit for therapeutic use if they demonstrate at least a neutral activity, which is defined as such when the bacterial strain growth curve obtained for the mixture mimics the curve of the most active individual phage at the same MOI. In the case of additive and synergistic effects, the total growth inhibition time of the tested bacterial strain is longer compared to the ones received for each individual phage at the same MOI.

4 Notes

1. When processing without chloroform, the tubes of interest can be directly centrifuged and/or filtered, as explained above.
2. These rounds are performed to obtain adapted individual phages ready to be included in the future phage cocktail.
3. The procedure can be repeated as many times until stable lysis is achieved at the preferred dilution of 10^{-7}, as recommended in the protocols of the Eliava Institute [11–14, 56].

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Chapter 5

Rapid Bench to Bedside Therapeutic Bacteriophage Production

Tiffany Luong, Andrew D. Sue, and Dwayne R. Roach

Abstract

It has been over 100 years since bacteriophages (phages) were used as a human therapeutic. Since then, phage production has dramatically evolved. Current phage preparations have fewer adverse effects due to their low bacterial toxin content. As a result, therapeutic phages have become a predominant class of new antimicrobials and are being widely used for compassionate treatment of multidrug-resistant (MDR) infections. We describe herein a protocol for the production and ultrapurification of phages. By this technique, it is possible for a lab experienced with the process to produce $>10^9$ plaque-forming units (PFU) per mL of Gram-negative phages that meet FDA endotoxins limits for intravenous infusions in as little as 48 hours. We provide illustrations of the process and tips on how to safely remove bacterial toxins from phage lysates. Although dependent on the phage strain, the approach described can rapidly generate and purify phages for a variety of applications.

Key words Bacteriophage, Amplification, Purification, Ultrapurification, Diafiltration, Concentration, TFF, Phage therapy

1 Introduction

Bacteriophage, also known as phage, is a virus that attacks and destroys bacteria. Phages are found anywhere and everywhere bacteria can be found: water, air, soil, and in the body. Their activities control the levels of all bacteria in nature. In 1919, Félix d'Hérelle, a French-Canadian microbiologist, who at the time was at the Pasteur Institute in Paris, used phages to treat dysentery bacillus. d'Hérelle's work set the basis for phage therapy. Currently, only the Eliava Institute in Tbilisi, Georgia, has maintained a mainstream use of phage therapy since its discovery. For the past several years, the practice of using phages for patients has been under redevelopment and modernization [1–3]. Antibiotic resistance has become a leading public health threat, and the discovery of new classes of

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antibiotics has stagnated [4]. Phages are not affected by antibiotic resistance mechanisms in general [1, 2], and virions themselves are generally considered safe for human and animal use [5–7].

Virulent phages exclusively use the lytic cycle for replication in bacterial host cells [8]. A phage seeks a host and, when found, binds to the cell surface and injects its DNA genome inside the host. In a matter of seconds, the host cell is hijacked for intensive virion replication that results in the bacterium bursting open, releasing newly produced phages to repeat the process. In this manner, the phage lysate can expand to $>10^{10}$ plaque-forming units per milliliter (PFU/mL) in growth medium under optimal host conditions. Many phage strains are easy to produce with standard microbiology equipment.

However, the production bottleneck arises during phage purification. Phage replication accumulates a high amount of toxins in lysates, such as endotoxins, exotoxins, soluble bacterial proteins, peptidoglycans, nucleic acids, and induced prophages [9, 10]. Their presence in the bloodstream may cause septic reactions with a variety of symptoms such as fever, hypotension, nausea, shivering, and in extreme cases, fatal shock. Endotoxins are lipopolysaccharide (LPS) molecules associated with the outer membranes of Gram-negative bacteria and make up the most common toxins that must be removed from pharmaceutical phage products. When these bacterial cells are lysed by phages, their cell membranes rupture and endotoxins are released into the solution. Endotoxins in solution range from molecular weights of 10,000 to 1,000,000 Daltons (Da) [11]. However, since phages are in the same molecular weight range as endotoxins, filtration cannot be used to adequately separate the endotoxins from the target phages. A harmonized standard for recommended maximum endotoxin exposure is 5.0 endotoxin units (EU) per kg of body weight (interpreted as within 1 hour) for most drugs based on an average patient weight of 70 kg [12]. Troubleshooting endotoxin-contaminated phage suspension may become necessary during phage production. Moreover, cold-stored phage suspensions may see the number of viable phages decrease over time, while the concentration of endotoxins detected may not [13].

Double polyethylene glycol (PEG) precipitation is a traditional approach for purifying phages [14, 15]. As phage particles are made up mostly of coat proteins around their genetic material, solutions containing PEG and high salt can force phage particles to aggregate into clusters. This low-cost method needs minimal equipment, and the precipitation is performed by general centrifugation. PEG precipitation, however, faces several disadvantages. The method requires the addition of large amounts of PEG and NaCl, some of which remain as a residue in the phage pellet and are difficult to eliminate. Certain other contaminants, such as LPS and exotoxins, are generally co-precipitated with phages [16, 17]. For these

reasons, depending on the purpose, PEG-precipitated phages usually require further methods of purification, such as density gradient ultracentrifugation (DGU), to remove co-precipitated impurities [14, 15]. DGU is a highly versatile method that has been widely used for the concentration and purification of phages [14, 18, 19]. This approach allows the separation of phages and contaminants based on buoyant density differences independent of size and shape. In this method, the sample is passed through a steep density gradient that contains a very high concentration of cesium chloride (CsCl) and ultracentrifugation. Multiple bands may be visible after ultracentrifugation; the top band(s) corresponds to debris, LPS, and unassembled phages (e.g., genome-free capsids), while the lower band contains the target phage that can be collected by puncturing specialized tubes [18, 20]. Drawbacks associated with CsCl DGU are that some phages cannot withstand long periods of ultracentrifugation at more than 100,000 × g force, and osmotic shock or interaction with CsCl and/or extensive dialysis may lead to loss of phage infectivity [21]. Last, not all laboratories can access the required equipment for this method.

Diverse chromatography-based methods enable the separation and purification of phages. The target phage can be purified based on its interaction types as well as its characteristics. Accordingly, the separation functionality is broadly categorized into [1] size, [2] charge, [3] hydrophobicity, and [4] affinity. Size exclusion chromatography (SEC) separates molecules based on their size by filtration through a gel. Given the large size of phages relative to most bacterial components and other small compounds such as LPS, exotoxins, ions, and salts, SEC can be applied as a complementary step for phage purification [16]. The main advantages of SEC are the low costs of resins and simplicity in operation as samples are eluted isocratically, so there is no need to use different buffers during the separation. However, this technique lacks selectivity, suffers from low productivity, and scaling up is restricted because the column can get saturated with host cell debris, consequently preventing the separation of large phages from endotoxins. Another nonspecific technique is ion exchange chromatography (IEC), which is one of the most efficient methods for separating charged particles. Phages are separated according to the strength of their overall ionic interaction with a solid phase material [14, 17, 22]. There are two types of IEC: anion- and cation-exchange chromatography (AEC and CEC, respectively). A disadvantage of this method is the risk of eluting contaminant proteins and endotoxins with similar electrostatic interactions as the target phage. Phages have a pH-dependent negative surface charge in most solutions, as do LPS [23]. The other drawback is the need to connect the chromatography column to a costly FPLC or HPLC instrument. By contrast, affinity chromatography, also called affinity purification, makes use of specific binding interactions between

molecules [14, 17, 18, 22]. This method uses synthetic ligands for specific elution and involves the impregnation of poly(ε-lysine) into cellulose beads, which provide high endotoxin selectivity [24]. A combination of electrostatic, hydrophobic, and hydrogen bond interactions are present in the affinity chromatography. The current drawbacks of affinity chromatography are its low yield and high salt concentration requirement for substance elution.

More recent methods have been applied to phage purification. Extraction with organic compounds can efficiently remove endotoxins from phage samples, such as 1-octanol [25, 26]. The removal of octanol is achieved by multiple centrifugation steps and the use of a speed vacuum to remove the residual organic solvent [17, 22, 25]. The main drawback is inconsistency in sample phage recovery [17, 25, 26], sometimes being as low as 15% [17].

With several options at each step of the production process, this protocol selects appropriate methods for the production and purification of phages using different criteria, including cost, equipment, ease, time, yield, and purity of the product. The steps intrinsic to our process to rapidly generate highly pure small-batch phage stocks that meet regulatory guidelines for intravenous (IV) administration are timed process execution to match phage-bacteria kinetics and modifications that have been used to optimize ultrafiltration (UF) and diafiltration (DF) by both us [18] and others [14, 17, 20]. Our process first minimizes the quantity of bacterial toxins that accumulate during phage replication by using several techniques to limit bacterial overgrowth and reduce bacterial debris in phage lysates. Next, we use a semi-permeable membrane with active force applied to drive UF and DF to get target phages ready for the next processing step by enabling sample cleanup, purification, concentration, buffer exchange, and desalinization. Last, we use affinity chromatography resin to selectively capture the conserved region of the inner core of LPS molecules and thereby remove all kinds of endotoxins from Gram-negative bacteria. We also include our aseptic/sterile fill-finish methods, which are ideal for small-batch biomedical applications. Together, this streamlined process efficiently produces many phages in as little as 48 hours and economically.

2 Materials

2.1 Equipment

1. Biological safety cabinet (BSC).
2. Microbiological incubators: static and refrigerated incubator shaker (*see* **Note 1**).
3. Single and multichannel pipettes: various volumes.
4. Serological pipet controller.

5. Spectrophotometer.
6. High-speed centrifuge with fixed angle rotors (e.g., Beckman Coulter J-Lite JLA-8.1000 fixed angle rotor).
7. Thermocycler.
8. Electrophoresis chamber and gel imager.
9. Double-pan balance scale.
10. Peristaltic pump and size 16 pump head (*see* **Note 2**).
11. Tube rotator.
12. Multimodal microplate reader.

2.2 Bacteriophage and Bacterial Host Strains

We recommend before phage production that both phage and host bacterial genomes have been sequenced and annotated. Both phage and host bacteria genomes require screening for virulence factors, antibiotic resistance and toxin genes, and prophages [27–29]. Prophages integrated into the host genome may not only harbor additional virulence factors but also introduce an added risk of horizontal gene transfer (transduction) or may interfere with the infectivity of the target phage [27, 30]. If any undesirable elements are identified in either the phage or host genome, we recommend using alternative strains.

1. Bacteriophage stock (recommend sequenced and 0.2 μm filtered).
2. Bacterial strain used to propagate bacteriophage strain (recommend sequenced).

2.3 Reagents

Reagents should be prepared using sterile Milli-Q water, autoclaved (45 min at 121 °C), and stored at room temperature unless otherwise indicated.

1. Bacterial broth and solid (agar) media.
2. Phosphate buffered saline (PBS) (10X): weigh 17.8 g of Na_2HPO_4, 2.4 g of KH_2PO_4, 80 g of NaCl, 2 g of KCl, and dissolve in 800 mL of sterile water. Add water to 1 L. Dilute 10:1 in sterile water to make a 1X working solution (10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCI).
3. PCR primers (10 μM concentration).
4. Tris-acetate-EDTA (TAE) buffer (10X): weigh 48.5 g Tris and dissolve in 800 mL of sterile water. Add 11.4 mL glacial acetic acid and 20 mL 0.5 M EDTA (pH 8.0). Add water to 1 L. Dilute 10:1 in sterile water to make 1X working solution (40 mM Tris, 20 mM acetic acid, 1 mM EDTA).
5. 2% agarose gel: 0.8 g agarose, 40 mL 1X TAE.
6. 0.5 M NaOH.
7. Endotoxin-free water.

8. 95% Ethanol and 0.2 M NaOH solution.
9. 2 M NaCl.
10. 10 mM sodium phosphate buffer (PBS): Dilute 10X PBS 10:1 in endotoxin-free water and filter sterilize.
11. DNase I.
12. RNase A.
13. 100% Ethanol.

2.4 Consumables

When appropriate (i.e., if plastics are not sterilely packaged), non-sterile glassware and plastics should be autoclave-sterilized prior to use.

1. Disposable inoculating loops: 1 μL.
2. Disposable plastic cuvettes.
3. Test tubes and caps.
4. Petri plates: 100 x 15 mm.
5. Pipette tips: various volumes.
6. Serological pipets: various volumes.
7. Polypropylene conical centrifuge tubes: 15 mL and 50 mL.
8. DNase/RNase-free strip PCR tubes and caps.
9. Microcentrifuge tubes: 1.5 and 1.7 mL.
10. Graduated glass media bottles: various volumes.
11. Glass Erlenmeyer flasks with GL45 screw cap: 250 mL and 2 L.
12. GL45 screw cap with 0.22 μm polytetrafluoroethylene (PTFE) hydrophobic membrane (Corning).
13. Polycarbonate centrifuge bottles with cap assemblies (recommend 1 L).
14. Luer Lock tip syringes: 10 mL and 50 mL.
15. Syringe filters: 0.2 μm.
16. Syringe needles: 23G x 1–1/2″.
17. Rx-Vent™ Filtered Venting Needles.
18. 0.8 | 0.45 μm polyethersulfone (PES) double-layer dead-end filter capsule.
19. 0.45 | 0.2 μm PES double layer dead-end filter capsule.
20. Vivaflow® 200 Crossflow Cassettes: 100 KDa MWCO PES membrane.
21. ¼” flexible rubber tubing and clamps.
22. Depyrogenated glass vials (pre-assembled with stopper and aluminum crimp seal).
23. 96-well microplates: clear U-bottom and black flat-bottom.

2.5 Recommended Kits

1. Endotoxin Removal: EndoTrap® HD (Lionex) *or* Pierce™ High-Capacity Endotoxin Removal Resin assay (Thermo Scientific).
2. Endotoxin Quantification: ENDONEXT™ EndoZyme® II – Recombinant Factor C (rFC) Endotoxin Detection assay (Bio-Vendor) *or* Pierce™ Chromogenic Endotoxin Quant assay (Thermo Scientific).

3 Methods

All procedures must be performed aseptically/sterilely in a biological safety cabinet.

3.1 Preprocessing

3.1.1 Bacterial Host Standard Curve

1. Streak bacteria on an agar plate from a glycerol stock using a sterile loop. Incubate overnight at the temperature that is optimal for bacterial growth.
2. Pick a colony-forming unit (CFU) and culture it in the appropriate nutrient-rich broth, overnight at the optimal temperature for growth.
3. In a BSC, air-dry five agar plates for 1 h.
4. Subculture 50 μL in 5 mL of fresh liquid broth in a sterile test tube. Incubate until an OD_{600} ~ 0.8 is reached. Place the test tube on ice.
5. Aliquot culture into a sterile cuvette and measure absorbance using a spectrophotometer. Dilute the culture to OD_{600} 0.8 with sterile bacterial broth in a sterile cuvette. Continue dilutions in twofold increments to obtain cuvettes with OD_{600} 0.8, 0.4, 0.2, 0.1, and 0.05.
6. Prepare two identical 8-well columns of PBS diluent in a 96-well microtiter plate. Add 198 μL of PBS to the top 2 wells and 90 μL in the remaining 14 wells.
7. Sample 2 μL from the OD_{600} 0.8 bacterial culture and add to the first microwell containing 198 μL of PBS and mix by repetitive pipetting (repeatedly dispensing and withdrawing solution from the microwell). Sample 2 μL of bacterial culture again and add to the second microwell containing 198 μL.
8. Use a multichannel pipette to serially dilute in tenfold increments by aliquoting 10 μL from the first row of wells to the second and mix by repetitive pipetting. Change pipette tips and repeat for the remaining six wells.
9. Withdraw 4 μL from the first column of 8 microwells using a multichannel pipette. Spot 8 × 4 μL aliquots to the leftmost edge of the agar plate. Repeat 8 × 4 μL sampling from the first column and spot on the agar plate next to the first column of

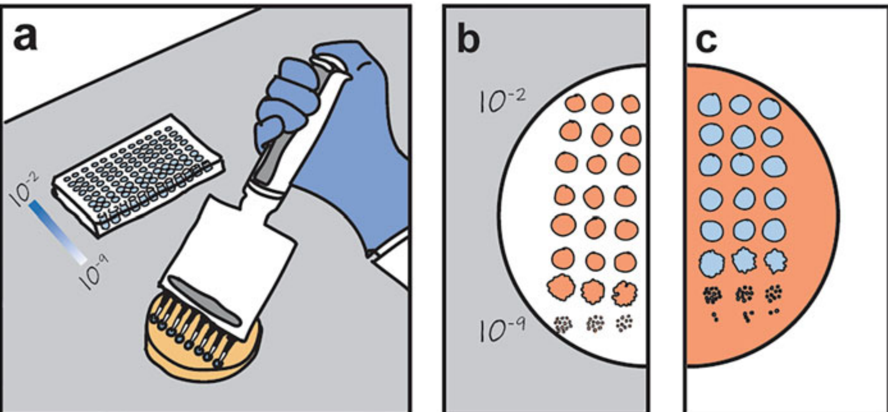


Fig. 1 Illustration of bacteria titration or Spot+ phage titration. (**a**) Example setup using a multichannel pipette and 96-well plate for serial dilutions. (**b**) Desired 48-spot plating for bacterial quantification. Counting should proceed from the row containing ten or more distinct CFUs. (**c**) Desired 48-spot plating for Spot+ phage titration. Counting should proceed from the row containing ten or more distinct PFUs

spots. Repeat 8 × 4 μL sampling from the first column a third time, spotting on the agar plate next to the second column of spots (Fig. 1a).

10. Repeat 4 μL spotting in triplicate, withdrawing from the second column of 8 microwells. Dry spots completely before closing the Petri plate lid and moving the plate (*see* **Note 3**).
11. Repeat dilution and titration steps 6–10 for the remaining diluted cuvettes.
12. Incubate all agar plates at the appropriate bacterial growth conditions until colonies are countable (Fig. 1b).
13. Calculate bacterial CFU/mL by averaging the number of CFUs visible in all six spots from both dilution series. Selected spots should contain 10–40 countable CFUs. Divide the average CFUs by 4 (volume spotted), times the dilution factor (column position), and times by 1000 (convert μL to mL).
14. Plot the CFU/mL (y-axis) against OD_{600} reading (x-axis). Generate a trendline and check that the R^2 of the line is >0.9. The equation of the trendline will allow you to approximate the host OD_{600} value necessary to achieve the desired concentration CFU/mL.

3.1.2 Spot+ Phage Titration

Traditionally, phages are quantified using the top agar overlay method [8]. We recommend a double aliquot 48-spot serial titration per sample of interest. This quantification method is quick and easily performed with general laboratory equipment. The Spot+ method increases sample coverage, depth, and technical repetition to improve accuracy over a typical spot titration method.

1. In a BSC, air-dry agar plates for 1 h. Dry one plate for each sample to be titered.
2. Pour 2 mL of bacterial culture at OD_{600} 0.2 onto a dried agar plate to seed. Gently swirl to spread the liquid evenly over the entire surface. Tilt the Petri plate and immediately collect excess liquid for disposal. Repeat seeding for each dried agar plate needed.
3. Air-dry seeded plates for 30 min.
4. Prepare two identical 8-well columns of PBS diluent in a 96-well microtiter plate. Add 198 μL of PBS to the top 2 wells and 90 μL in the remaining 14 wells.
5. Sample 2 μL from the phage preparation and add to the first microwell containing 198 μL of PBS and mix by repetitive pipetting (repeatedly dispensing and withdrawing solution from the microwell). Sample 2 μL of phage again and add to the second microwell containing 198 μL.
6. Use a multichannel pipette to serially dilute in tenfold increments by aliquoting 10 μL from the first row of wells to the second and mix by repetitive pipetting. Change tips and repeat for the remaining six wells.
7. Withdraw 4 μL from the first column of 8 microwells using a multichannel pipette. Spot the 8 aliquots in the leftmost area of the seeded agar plate. Repeat 8 × 4 μL sampling from the first microwell column and spot onto the agar plate next to the first column of spots. Repeat 8 × 4 μL sampling from the first microwell column a third time, spotting on the agar plate next to the second column of spots (Fig. 1a).
8. Repeat step 7 and perform 4 μL spotting in triplicate, withdrawing from the second column of 8 microwells.
9. Dry spots completely before closing the Petri plate lid and moving. Incubate at the appropriate bacterial growth conditions until plaques are countable (Fig. 1c).
10. Calculate phage PFU/mL by averaging the number of PFUs visible in all six spots from both dilution series. Selected rows should contain 10–40 PFUs. Divide the average PFUs by 4 (volume spotted), times the dilution factor (column position), and times by 1000 (convert μL to mL).

3.1.3 Pilot Phage Amplification

1. Pick a fresh CFU from the host strain plate and grow to ~10^8 CFU in 5 mL of broth medium.
2. Add phages at ~10^7 PFU to achieve a multiplicity of infection (MOI) of 0.1.
3. Incubate the culture for 10–12 h, then harvest or cool to 10 °C for the remainder of the time (*see* **Note 4**).

4. Determine the window for cooling the phage preparation by titering and checking the OD_{600} of the lysate the following day (*see* **Note 5**).
5. Harvest the phage lysate in a sterile 15 mL conical and centrifuge for 10 min at 8000 × g.
6. Decant the supernatant into a sterile syringe with an attached 0.22 μm filter unit. Filter into a fresh 15 mL conical.
7. Aliquot 100 μL of the filtered lysate into a PCR tube.
8. Store at 4 °C.

3.1.4 Lysate Prophage Detection

Even if a putative prophage is detected bioinformatically, it may not necessarily be liberated during lysate production. Use the pilot phage lysate as a PCR template for verification.

1. Identify prophages from a fully assembled host strain genome using PHASTER or other software of choice (*see* **Note 6**).
2. Obtain the FASTA file for the putative prophage(s) from the tool of choice.
3. Design primers for the prophage(s) using NCBI Primer-BLAST or other tools of choice.
4. Make a master mix for each potential prophage or phage strain tested (*see* **Note** 7).
5. Vortex briefly to mix. Centrifuge briefly to settle.
6. Distribute 22.5 μL of master mix into freshly labeled PCR tubes.
7. Add 2.5 μL of the pilot phage lysate as template into PCR tubes.
8. Set the PCR temperatures according to the polymerase/kit chemistry. Use an annealing temperature specific for the phage primers designed in step 3.
9. While the PCR reaction is running, prepare a 2% agarose gel and add DNA gel stain.
10. Load the PCR reaction after the gel has set for 20 min. Run gel electrophoresis using an appropriately sized DNA ladder and samples.
11. Analyze and image gel using a gel documentation system (*see* **Note 8**).

3.2 Batch Phage Amplification

Phage production typically begins with long culture incubations (e.g., overnight/18–24 h) followed by lysate centrifugation and 0.2 μm filter sterilization. As mentioned, bacterial toxins accumulate during phage amplification, and a large quantity of free endotoxin results from violent cell lysis. It is also common for target bacterial cells to evolve resistance to phage infection during these long incubations. These evolved mutants thrive in optimal culture

conditions and overgrow in the lysate. We recommend switching to cold growth incubation, 1–2 h prior to the visible outgrowth of phage-resistant bacteria. Time-kill kinetics assays help understand interactions that exist between host bacteria and phages, including time to resistance. For example, we recommend when using *Pseudomonas aeruginosa* phages to reduce the incubation temperature after ten hours to suppress the outgrowth of evolved phage-resistant mutant cells while maintaining phage production. Similarly, we recommend for *Staphylococcus aureus* phages to reduce the incubation temperature after 22 hours to achieve the same suppression. This temperature drop prevents resistant bacterial growth, which causes a clearer lysate while maintaining long incubation times for higher phage yields. The addition of two centrifugation steps significantly enhances the removal of intact bacterial cells and bacterial debris, including large endotoxin aggregates. Figure 2 shows a flow diagram briefly describing this process.

1. Pick a CFU isolated using the streak plate method on solid growth medium and culture in the appropriate nutrient-rich broth medium overnight to bulk up a pure culture (*see* **Note 9**).
2. Subculture 50 μL in fresh liquid broth to get the bacterial population growing exponentially (i.e., OD_{600} 0.1–0.8).
3. Add 1 L of broth medium to each of the three sterile autoclaved glass Erlenmeyer flasks with GL45 screw cap and pre-warm to bacterial growth temperature.
4. Add exponentially growing bacteria at an optical density that corresponds to a count of ~10^{10} CFU per flask and exchange the flask's cap for an autoclaved GL45 vented screw cap with 0.22 μm PTFE hydrophobic membrane. Incubate the flasks for one generation at growth temperature with gentle agitation (Fig. 2a).
5. After bacteria have acclimated and grown for one generation, add phages at ~10^9 PFU to achieve a multiplicity of infection (MOI) of 0.1 (Fig. 2a).
6. Incubate the flasks for 10–12 h, then cool to 10 °C for the remainder of the time until 18 h (*see* **Note 4**).
7. Harvest amplified phages by decanting lysates into sterile centrifuge bottles (e.g., 1 L bottles) and balance the three bottles against one another using a double pan balance scale (Fig. 2b).
8. Centrifuge at 8000 × *g* for 30 min at 4 °C.
9. Decant supernatant containing phage particles without disturbing the solid pellet into new sterile centrifuge bottles (Fig. 2c). Repeat centrifugation at 8000 × *g* for 30 min at 4 °C (*see* **Note 10**).
10. Collect phage particles without disturbing the tiny pellet into sterile glass storage bottles.

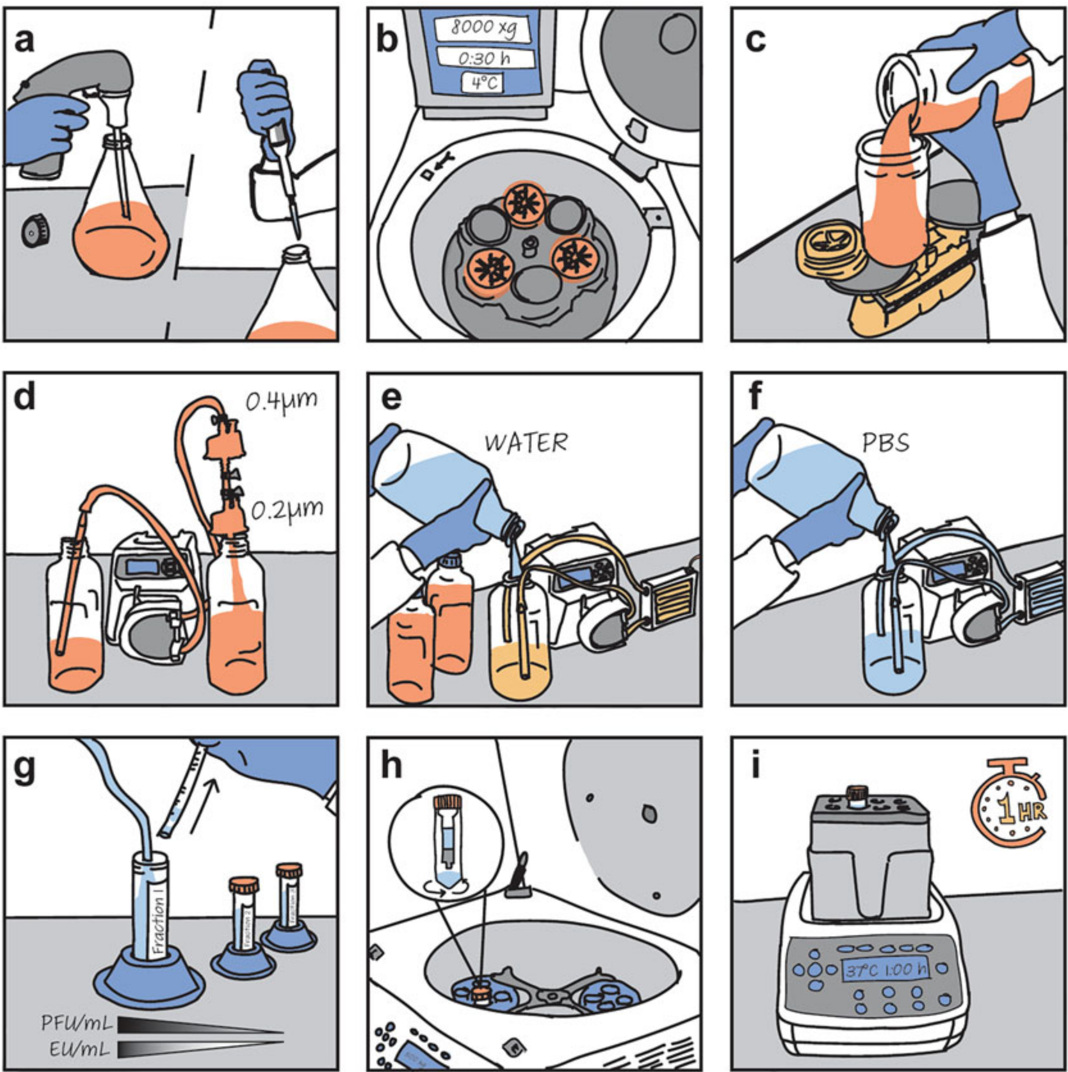


Fig. 2 Illustrated schematic of phage production and purification process. (**a**) Phage amplification. Bacteria should be added to the sterile growth medium and grown for one generation with a vented screw cap to allow gas exchange (*left*). Phages should be added to achieve a MOI of 0.1 (*right*). (**b**) First centrifugation. Centrifuge produced phage lysates at 8000 × g for 30 min at 4 °C. (**c**) Second centrifugation. Avoid the bacterial pellet and carefully decant the supernatant into new centrifuge bottles. Re-balance bottles and repeat centrifugation at 8000 × g for 30 min at 4 °C. (**d**) Dead-end filtration sterilization. Sterilize the lysate by connecting in tandem a 0.8 | 0.45 μm capsule filter to a 0.45 | 0.2 μm filter capsule. (**e**) Diafiltration. Remove storage buffer from the CFF cassette, flush with water, and set up the CFF cassette as pictured. The intake and retentate lines should recirculate from the bottle containing lysate. Flow all phage lysate through the CFF. Concentrate to 50% volume and dilute with cold ultrapure water. (**f**) Washing. Exchange spent lysate for cold ultrapure storage buffer. Stepwise concentrate and dilute phage with storage buffer. (**g**) Concentrating. When the phage concentrate appears visibly clear, purge the CFF and collect phage particles in three or more fractions. (**h**) Endotoxin removal. Sterilely prepare reagents for endotoxin removal. Perform endotoxin removal per manufacturer's instructions with suggested modifications to prevent phage loss. (**i**) Removal of nucleic acids. Remove free DNA/RNA by 1 h enzyme treatment

3.3 Phage Purification

3.3.1 Dead-End Filtration Sterilization

1. In a BSC, connect a 0.8 | 0.45 μm heterogeneous PES double layer filter capsule to a 0.45 | 0.2 μm heterogeneous PES double layer filter capsule with autoclaved ¼″ flexible tubing and clamps as shown in Fig. 2d.
2. Prepare a disposable 10 mL serological pipet by removing the cotton or filter from its end. Feed the tubing through the peristaltic pump head, insert the serological pipet into the end of the tubing, and place it into sterile water.
3. Precondition the filter membrane by pumping 500 mL of sterile water through the capsule filters. Discard the flow through.
4. Switch the serological pipet and tubing to the bottle containing phage lysate and pump it into sterile glass bottles (*see* **Note 11**).
5. Store all filtered lysates at 4 °C. This is a potential pause step; depending on the phage strain, the lysate can be stored at 4 °C for up to several months without significant loss in titer.

3.3.2 Ultrafiltration and Diafiltration

This process uses crossflow filtration (CFF) for combined UF and DF of up to 5 L batch volumes. UF and DF are commonly used for the development and manufacturing of biological therapeutics, such as proteins and antibodies, as well as therapies that rely on viral nanoparticle delivery [31, 32]. The Vivaflow 200 CFF cassette uses a polyethersulfone membrane (PES), which is popular for phage applications given its hydrophilic properties and low protein binding. The semi-permeable membrane retains larger target phages while allowing smaller molecules to filter out. A 100 kDa MWCO is adequate to retain most phages. DF enables medium to buffer exchange by adding a new cold buffer to the phage retentate. By returning the sample to the original volume, UF/DF can be repeated until the sample reaches a targeted level of clarity with the new buffer. The Vivaflow's unique switchback channels parallel to the filtration membrane improve phage dissociation from endotoxins and require less pressure to drive filtration. The cassette design is easy to set up, gentle on phage particles, and its transparency allows for monitoring flow.

1. In a BSC, connect the CFF cassette with autoclaved 6.4 mm outside diameter (O.D.) tubing as shown in Fig. 2e. Connect the filtrate line to the port on top of the cassette, the retentate line to the upper side port, and the intake line to the lower side port.
2. Feed the intake line through the peristaltic pump head, insert a disposable 10 mL serological pipet into the end of the tubing, and place the pipette into a bottle with sterilized chilled (4 °C) ultrapure water. Keep the cassette in an open circuit by placing both the retentate and filtrate lines in a waste container.

3. Pump 500 mL of autoclaved cold ultrapure water through the cassette to remove storage EtOH and to condition the membrane with pure water.
4. Transition the cassette into a continuous circuit. Insert the intake and retentate lines into the glass bottle containing sterilized cold phage lysate, as shown in Fig. 2e. Only the filtrate line should remain in the waste container.
5. Recirculate the phage lysate through the cassette until 50% volume remains in the bottle and add cold ultrapure water to double volume (*see* **Note 12**).
6. Concentrate to 50% again and repeat the ultrapure water dilution by doubling the volume (*see* **Note 13**).
7. Concentrate to 50% again and dilute with cold sterile PBS to double the volume (Fig. 2f).
8. Concentrate to 30% and dilute with cold sterile PBS to double the volume.
9. Concentrate to 20% and dilute with cold sterile PBS to double the volume.
10. Concentrate to 10% and dilute with cold sterile PBS to double the volume, repeat until the lysate is visibly clear (*see* **Note 14**).
11. To collect phage particles, set up and label three sterile conical centrifuge tubes as "1st, 2nd, 3rd Fractions." Add 30 mL of sterile cold PBS to tubes 2 and 3.
12. Concentrate to ~30 mL and pause the peristaltic pump. Pour the ~30 mL of phage solution into the empty conical tube and carefully insert the intake and retentate lines into the conical. Restart pumping until ~5 mL remains in the conical tube. Pause the pump. See Fig. 2g.
13. Collect the first fraction by draining the cassette; this will allow you to collect the remaining ~30 mL of dead volume of the cassette and tubing. Remove the intake line from the conical and restart the pump to completely flush the cassette into the 50 mL conical. Pause the pump (*see* **Note 15**).
14. Immediately insert the intake and retentate lines into the second conical tube containing 30 mL sterile cold PBS and start the pump to recirculate the buffer to recover the phage remaining in the cassette. When the volume reaches ~5 mL, pause the pump, remove the intake line, and restart the pump to completely flush the cassette again. Pause the pump after all the liquid is collected.
15. Repeat the recirculation and draining from step 14 using the third conical tube containing sterile cold PBS. Pause the pump after all liquid is collected. Immediately refill the cassette with buffer for short-term storage until cleaning using manufacturer's instructions (*see* **Note 16**).
16. Store fractions at 4 °C (*see* **Note 17**).

3.3.3 Chromatographic Removal of Endotoxin

Although all prior processing steps reduce and remove endotoxins from phage samples, CFF fractions generally contain endotoxins above regulatory limits for intravenous applications. For example, up to 10^5–10^6 EU/mL. Therefore, chromatographic endotoxin removal may be required depending on phage concentration and intended application. For instance, a 10^{11} PFU/mL phage sample with an endotoxin level of 10^4 EU/mL would not need additional endotoxin removal steps if dilution in pyrogen-free saline was done to achieve a therapeutic dose of 10^9 PFU/mL. The commercially available Pierce High-Capacity Endotoxin Removal Resin and EndoTrap HD assays have a binding capacity of 2×10^6 and 5×10^6 EU/mL, respectively. Below is described the specific use of a 1 mL Pierce Chromogenic Endotoxin Removal Spin Column with a sample capacity of 10 mL with modifications to the manufacturer's instructions. Samples should be prepared using endotoxin-free water, and the column should only be uncapped in a BSC. Resin columns can be regenerated for reuse, but each column should be dedicated to only a single phage strain.

1. Prepare the 1 mL column according to the manufacturer's instructions. Each time you spin out a solution, loosen the column cap and remove the column's bottom plug. When adding a solution to the resin slurry, secure the column cap and replace the bottom plug (*see* **Note 18**).
2. Prepare buffers for use according to the manufacturer's instructions. Kit modification: Spike the endotoxin-free equilibration buffer with sterile-filtered NaCl solution to achieve a final solution of 10 mM PBS 0.4 M NaCl.
3. Following the removal of the ethanol storage solution, NaOH regeneration buffer, salting with NaCl buffer, and equilibration with water and sodium phosphate buffer, the column is ready for sample addition. Kit modification: Increase the phage concentrate's NaCl concentration to 0.4 M NaCl by spiking in sterile NaCl solution (Fig. 2h) (*see* **Note 19**).
4. Mix end-over-end using a tube rotator at 4 °C for 1 h.
5. Collect the sample in a sterile conical tube (*see* **Note 20**).
6. Once endotoxin column removal is completed, filter (0.2 μm syringe filter) the final phage preparation into a sterile conical tube (*see* **Note 21**).
7. Store at 4 °C and maintain sterility for qualification/quantification.

3.3.4 Free Nucleic Acids Digestion

Phage products can contain residual DNA from host cell substrates. It is, therefore, possible that such residual DNA could encode or harbor harmful molecules or elicit an innate immune response in the patient. It is not clear what health risk the DNA can pose to the product recipients, but often manufacturing can be designed to

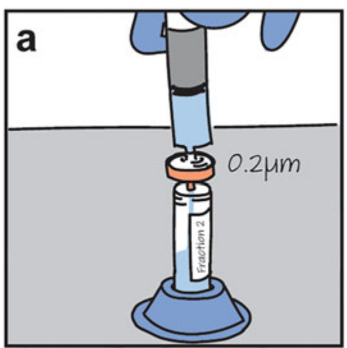
minimize the risk by reducing the levels of DNA. The World Health Organization and U.S. Food and Drug Administration guidelines recommend that 10 ng/dose and 200 base pairs be the limits of content and size of residual DNA in the final product dose [33]. Enzymatic digestion of phage samples removes free DNA and RNA while intact virions are unharmed and protected by their protein capsid.

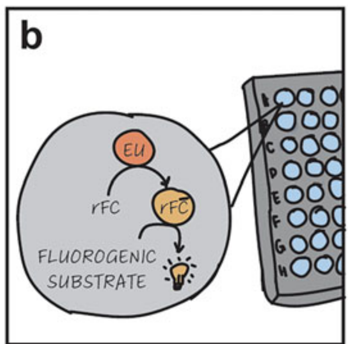
1. Preheat a heat block to 37 °C.
2. Dilute DNase I stock to a concentration of 100 U/mL. Place on ice.
3. Dilute RNase A stock to a concentration of 100 U/mL. Place on ice.
4. Determine how many mLs of phage preparation were produced. If you are not using the entire stock, sterilely aliquot the fraction that will be treated.
5. Add 2.5 μL DNase I and 2.5 μL RNase A per 100 μL of phage preparation being treated.
6. Treat phage preparation for 1 h at 37 °C, with the lid heating off (Fig. 2i).
7. Store the phage preparation at 4 °C.

3.4 Final Preparation Validation

3.4.1 Sterilization and Titration

1. Prepare a fresh exponentially growing culture of host bacteria.
2. Air-dry an agar plate for 1 h in a BSC.
3. Pre-wet a 0.22 μm PES syringe filter using sterile phage storage buffer (i.e., PBS).
4. Decant the nucleic acid-free phage preparation into the syringe. Filter sterilize into a fresh sterile 15 mL conical tube (Fig. 3a).





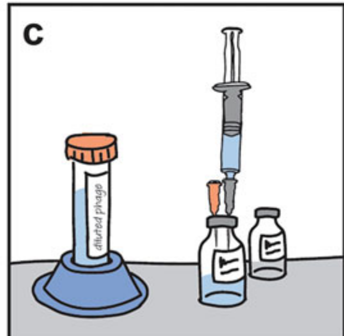


Fig. 3 Illustrated schematic of phage sterilization, validation, and fill-finish. (**a**) Syringe sterilization. Syringe filter the final phage preparation before obtaining a final phage titer, validation, and/or long-term storage. (**b**) Endotoxin quantification. Perform endotoxin quantification using a kit with an appropriate detection range. (**c**) Vial filling. UV sterilize all supplies and vials before use. Filter sterilize the diluted phage preparation before sterile distribution into vials

5. Aliquot 100 μL of the phage preparation into a microcentrifuge tube.
6. Store 0.22 μm filtered phage preparation at 4 °C and protect from UV for longer-term storage.
7. Perform spot titration using the phage aliquot as outlined in Subheading 3.1.2.

3.4.2 Endotoxin Quantification

We recommend choosing either the EndoZyme recombinant factor C or Pierce chromogenic LAL-based assays. Traditional endotoxin quantification uses the Limulus amebocyte lysate (LAL) test, which contains specialized blue blood cells from the wild Atlantic horseshoe crab, *Limulus polyphemus*, as a component because they react to the presence of endotoxins in a way that can be measured and quantified. The EndoZyme assay is a sustainable test, using a recombinant version of the first enzyme in the LAL clotting cascade. The EndoZyme assay also has a larger detection range from 0.005 to 50 EU/mL.

1. Select an assay, prepare the assay reagent mixture, and set the microplate reader to the settings as per the manufacturer's instructions.
2. Dilute the phage sample in endotoxin-free water to achieve a dilution series of 10^{-4}, 10^{-6}, and 10^{-8} PFU/mL to ensure at least one measurement is within the linear dynamic range of the assay.
3. Add reagents and run the microplate reader protocol as per the manufacturer's instructions (Fig. 3b).

3.5 Sterile Fill-Finish

The term "sterile fill-finish" is used for drug products that are sterile injectables (e.g., liquids filled in vials or syringes). Because there is no process to sterilize the phage product in its final container, it is critical that containers be filled and sealed in an extremely controlled environment [34]. Ensuring sterility is not a trivial task, and failure can have catastrophic—even life-threatening—consequences for a patient. The success of aseptic processing and sterile fill-finish operations relies on mitigating contamination from each of these sources: (i) personnel, (ii) drug product components and containers, (iii) cleanroom facilities, and (iv) equipment and processes.

1. Clean a BSC with HEPA filtration using 70% EtOH and UV sterilize for 20 min.
2. Place all containers and consumables in the cabinet and UV treat for 20 min (*see* **Note 22**).
3. Dilute the phage stock with the desired sterile and endotoxin-free chilled solution in a sterile 50 mL conical tube. Gently vortex (*see* **Note 23**).

4. Pre-wet a 0.22 μM PES syringe filter with phage diluent (i.e., PBS), and filter sterilize the diluted phage preparation into a fresh sterile 50 mL conical tube.
5. Insert a filtered sterile vented needle into the vial's rubber stopper (*see* **Note 24**).
6. Draw 4.5 mL in a 5 mL Luer-Lock tip syringe with a 23G x 1–1/2″ needle. Pierce the rubber stopper and aliquot 1 mL into the vial (Fig. 3c).
7. Carefully remove both the syringe needle and the vented needle (*see* **Note 25**).
8. Repeat until all vials are filled.
9. When finished, protect vials from UV light and store at 4 °C.

4 Notes

1. If your incubator is not equipped with refrigeration, phage production will need to be manually paused during pilot production (*see* Subheading 3.1.3).
2. A pump with a convex-roller head design is gentler on phages compared to a flip-type head design.
3. A 115 mm Petri plate will support a 48-spot grid of six columns of eight spots each (*see* Fig. 1b–1c). We recommended using this layout to titer CFU/PFU in two sets of triplicate spots.
4. Modern microbiological shaking incubators can be refrigerated and programmed to support temperature cycles. Alternatively, cultures can be moved to a 4–14 °C environment. We find that harvesting phage production after 10 hours achieves titers upwards of 10^8 PFU/mL while minimizing bacterial overgrowth during additional incubation. This timing may change depending on the growth rate of the host bacteria, so perform pilot phage production (*see* Subheading 3.1.3).
5. Once bacterial density reaches OD_{600} 0.1–0.2, it should be immediately harvested or transferred for cooling. Additional bacterial growth will result in difficulty during dead-end filtration.
6. At the time of this writing, PHASTER is accessible using a web browser and accepts FASTA files or GenBank accession numbers [35]. Alternate tools include PhiSpy and Prophage Hunter [36, 37].
7. A standard PCR protocol (such as [38]) should be used. Individual reagent volumes will depend on the polymerase/PCR kit being used. Each reaction will require 22.5 μL of the master mix.

8. Bacterial strains can also be cured of prophages using mitomycin C induction or UV light treatment [39]. However, prophage curing can cause changes in phage susceptibility/bacterial fitness. We recommend repeating pilot phage lysate production if prophage curing is performed.
9. Culture in liquid broth medium gives bacteria easy access to the available nutrients compared to static bacterial growth on solid medium, also known as an agar culture. Agitation to keep the bacteria dispersed through the medium during incubation can aid this access further. Liquid media will also dilute out waste products as they are formed, distributing them through the culture. Consequently, a greater mass of bacteria may be obtained for an equivalent volume of liquid as opposed to solid media.
10. When pouring into a new container, the pellet may not be easily observed. Nonetheless, carefully handle the centrifuge bottles to avoid disturbing the pellet. The supernatant may appear clear; however, it is critical to perform a second spin to enhance the removal of gross bacterial debris and prevent clogging of capsule filtration units.
11. By centrifuging lysates twice, 3 L can generally be filtered with the capsule filters without the need for cleaning in between. If the 0.45 | 0.2 μm filter becomes clogged, follow the manufacturer's instructions to clean the filter (e.g., using 0.5 M NaOH), rinse with sterile water, and resume filtration.
12. If phage lysates are in multiple storage bottles, continue to add all phage lysate to the CFF before switching to the ultrapure water dilution step.
13. The CFF will wash proteins and salts through the membrane while leaving phage particles in the retentate and exchange spent growth media for the desired storage buffer. This buffer exchange will occur simultaneously with concentration, where liters of lysate are concentrated up to 100-fold.
14. Repeat this step until the phage solution has visible clarity, denoting the remaining broth and small particles have been washed away (i.e., diafiltration).
15. This will briefly dry the CFF membrane and proceed through fraction collection quickly to ensure the membrane does not stay dry for long.
16. Phage may be collected with further repeats. However, as you collect fractions, phage concentration decreases.
17. Typically, CFF concentrates phage lysates by 1–2 orders of magnitude and concentrates endotoxin to ~10^4–10^5 EU/mL of buffer. We recommend proceeding with endotoxin removal

using Fraction 2, which has lower endotoxin content than Fraction 1, while the phage concentration typically stays within the same order of magnitude. If time permits, titer the phage concentrate before proceeding.

18. For improved endotoxin removal, we recommend column regeneration using 0.2 M NaOH overnight (16–18 h) incubation at room temperature. Regenerate the column during phage overnight production if you plan to remove endotoxin immediately after CFF concentration.
19. When using the Pierce endotoxin removal resin, an insufficient salt concentration significantly decreases final phage sample recovery. Spiking the equilibration buffer and phage concentration to ~0.4 M NaCl, as recommended in the manufacturer's troubleshooting guide, has significantly improved the recovery of *P. aeruginosa* phage particles.
20. If desired, the column can be immediately regenerated, and the endotoxin removal process can be repeated. A second removal step may further reduce endotoxin concentration by up to twofold. However, we do not recommend going beyond two column passages as the final phage recovery is reduced in each passage.
21. Pre-wet the syringe membrane with endotoxin-free buffer to prevent loss of phages during final sterile filtration.
22. UV does not penetrate into materials, particularly plastics, very well. Although UV can disinfect an empty BSC, it will only disinfect the outer surface of any material in a BSC.
23. Filtration typically causes a loss in phage concentration. Calculate and add at least 10% more phage stock to compensate for diluted phage losses.
24. For sterile glass vials, it is necessary to vent the gas from the vial and allow the solution in.
25. At minimum, replace the needles each time a new syringe is used.

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Chapter 6

Bacteriophage Production in Compliance with Regulatory Requirements

Jean-Paul Pirnay, Maia Merabishvili, Daniel De Vos, and Gilbert Verbeken

Abstract

In this chapter, we discuss production requirements for therapeutic bacteriophage preparations. We review the current regulatory expectancies and focus on pragmatic production processes, implementing relevant controls to ensure the quality, safety, and efficacy of the final products. The information disclosed in this chapter can also serve as a basis for discussions with competent authorities regarding the implementation of expedited bacteriophage product development and licensing pathways, taking into account some peculiarities of bacteriophages (as compared to conventional medicines), such as their specificity for, and co-evolution with, their bacterial hosts. To maximize the potential of bacteriophages as natural controllers of bacterial populations, the implemented regulatory frameworks and manufacturing processes should not only cater to defined bacteriophage products. But, they should also facilitate personalized approaches in which bacteriophages are selected ad hoc and even trained to target the patient's infecting bacterial strain (s), whether or not in combination with other antimicrobials such as antibiotics.

Key words Bacteriophage therapy, Antibiotic resistance, Quality and safety, Production, Manufacturing, Medicinal product, Drug

1 Introduction

Bacteriophage preparations for human use were classified as medicinal products in the European Union (EU) and as drugs in the United States (US) based on the literal implementation of definitions. Basically, any substance presented as having properties for treating or preventing disease in human beings is considered to be a medicinal product (EU) or a drug (US). As a result, bacteriophage products are subjected to the same manufacturing requirements, clinical trials, and marketing authorizations as conventional medicines such as antibiotics.

This means that potential bacteriophage candidates, which produce suitable levels of bacterial reduction, should be selected using preclinical in vitro studies. These bacteriophages should then

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be subjected to preclinical in vivo testing to determine the toxicity, immunogenicity, and dosing of the treatment. Bacteriophage products exhibit complex and poorly understood pharmacodynamics and pharmacokinetics (PD/PK). In the human body, bacteriophages exhibit short half-lives (15–30 min) as they are efficiently eliminated by the patient's immune system, and especially by the reticuloendothelial system, but simultaneously they can multiply exponentially in the presence of host bacteria. It is, therefore, very challenging to identify appropriate measures for PD/PK and pharmacological endpoints [1, 2]. Next, selected bacteriophages should be produced according to Good Manufacturing Practices (GMP), a challenging task. Defined bacteriophage products will often consist of several bacteriophages. There are two main purposes for using cocktails of bacteriophages in bacteriophage therapy: (i) to try to overcome bacterial resistance, as resistance to bacteriophage cocktail was shown to readily emerge in vitro [3] as well as in vivo [4, 5], and (ii) to streamline bacteriophage therapy to become compatible with "real-world" clinical practice [6].

For certain clinical indications, bacteriophage cocktails that contain bacteriophages able to lyse a wide variety of pathogenic bacteria—so-called broad spectrum "poly-phage" cocktails—have been (and are still) used in Eastern European practice (e.g., the *IntestiPhage* and *PyoPhage* cocktails produced by the Eliava Institute in Tbilisi, Georgia). The availability of such pre-made cocktails could indeed streamline certain treatments and could be important in acute multidrug-resistant (MDR) infection situations (empirical treatments). However, these cocktails are historically produced using an approach that resembles the back-slopping method used for the production of yogurt. In this method, a small amount of the previous batch of bacteriophage cocktail is added to a culture medium containing an inoculum of a selection of targeted bacterial pathogens, updated with problematic—bacteriophage-resistant—strains that emerged during the previous usage period of the cocktail. These bacteriophages and bacteria are dosed to obtain a balanced growth rate of bacteriophages and bacteria, creating the optimal conditions for their productive interactions upon incubation [7]. The result of this process is a stable bacteriophage community containing bacteriophages which are present at different concentrations and evolved to work in concert or even in synergy.

However, according to current customary regulations, for example, those enforced by the European Medicines Agency (EMA) and the US Food and Drug Administration (FDA), all bacteriophages that make up a bacteriophage cocktail should have been shown to be individually acceptable and then mixed into a stable product. In addition, the dynamics between the individual components of a bacteriophage cocktail should also be assessed.

Bacteriophage products deemed acceptable by the competent authorities for medicines should then be submitted to costly Phase I to IV clinical trials for clinical safety and efficacy measurement. Finally, an elaborate application dossier for marketing authorization should be compiled and submitted for assessment.

Regulators believe that the current medicinal product regulation is adequate for bacteriophage therapy products and that no dedicated regulatory framework is needed [8]. They might be right concerning industrially produced single bacteriophages or predefined bacteriophage cocktails, providing the necessary modifications and logical exemptions to the regulatory framework.

However, some researchers feel that these defined and "stationary" bacteriophage products will encounter difficulties in dealing with geographical diversities and time-bound shifts in bacterial populations or with the emergence of evolved (mutated) epidemic strains that are no longer susceptible to the "immutable" bacteriophage products. The long-term use of predefined cocktails is bound to elicit considerable bacterial bacteriophage resistance. Although not much is known about the rate at which this resistance would occur in clinical settings (e.g., in an individual during the course of bacteriophage therapy), there are indications that resistance to bacteriophages will arise with a similar or higher frequency to that of antibiotic resistance [4, 5, 9, 10]. Because the efficacy of predefined bacteriophage cocktails is likely to decrease over time, they would need to be regularly adapted and re-approved for use. Today, individual approvals should be obtained for each cocktail variant due to their unique composition [1].

According to many bacteriophage researchers, the current medicinal product (EU) or drug (US) development and approval pathways are not suitable (too rigid and unnecessarily costly) for customized or sustainable bacteriophage therapy approaches [11]. Bacteriophages coevolve with their host bacteria and ensure the Earth's ecological equilibrium in several environmental or ecological niches. In fact, we need to consider the coevolving bacteriophage/bacterium couplet, which is continually coevolving and engaged in an arms race and, as such, provides a long-term sustainable antibacterial approach. Indeed, for each existing or emerging pathogen, there is a bacteriophage to be found, which makes bacteriophage therapy sustainable.

Unfortunately, the more flexible and sustainable the bacteriophage therapy concept, the more difficult it is to comply with the current medicinal product (drug) development and approval system (Fig. 1), which was developed to cater to widely used and industrially prepared chemical molecules such as aspirin.

In addition, the established pharmaceutical industry ("big pharma"), with which and for which the current medicines framework was developed, is not ready to invest in bacteriophage therapy products. Weak intellectual property protection (IPP) of natural

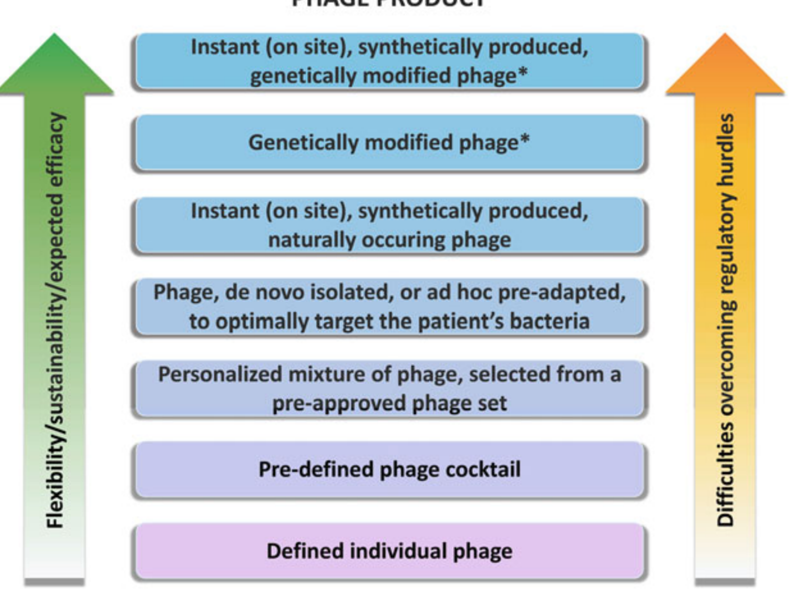

Fig. 1 The more flexible and sustainable the bacteriophage product, the more regulatory hurdles the product needs to overcome

entities such as bacteriophages, and bacteriophage specificity and resistance issues, which are bound to prevent widespread and long-term uses of single bacteriophages or predefined bacteriophage cocktails, and a lack of randomized clinical trials showing bacteriophage therapy efficacy, compromise their substantial investments [12].

One would assume that naturally occurring bacteriophages are relatively cheap and easy to produce, but GMP manufacturing requirements, regulatory issues, and the empirical evidence suggesting that predefined bacteriophage cocktails will need to be regularly updated and approved to adapt to bacterial population shifts and to counteract bacterial resistance will probably mean that comparable costs to conventional antibiotics should be anticipated [13]. It was estimated that the median and mean capitalized R&D costs to bring a drug to the market were $985 m and $1336 m, respectively [14]. The median and mean costs for five new antibiotics included in this analysis were $1260 m and $1297 m, respectively [14].

A few small and medium-sized enterprises (SMEs) did pick up the gauntlet and are slowly moving along the elaborate and expensive conventional medicinal product (drug) licensing pathway. However, some issues are starting to materialize, for instance, during the European Commission–sponsored *PhagoBurn* study, the first major bacteriophage therapy trial under modern medicinal

product regulatory standards in the EU [15]. In this randomized, controlled, double-blind phase 1/2 trial, the efficacy and tolerability of a cocktail of bacteriophages to treat burn wounds infected by *Pseudomonas aeruginosa* was evaluated. Manufacturing of the study's investigational products conforming to the guidelines recommended by the competent authorities took 20 months and spent the largest part of the study budget [16]. In addition, the complex cocktail of no less than 12 *Pseudomonas aeruginosa* bacteriophages, which was developed to ensure an acceptable host range, was retrospectively shown to be inactive (in vitro) against *P. aeruginosa* strains found in three out of ten patients [15], thus confirming the predicted bacteriophage specificity issues. Because the study product targeted only one of the many bacterial species that are known to cause burn wound infections, physicians were reluctant to include patients [16]. Irrespective of the results of the clinical trial, which were relatively disappointing [15], the preliminary phase of the *PhagoBurn* study unveiled some of the constraints of the conventional medicines framework, which would never be able to cater to personalized bacteriophage therapy approaches without considerable adaptation of production and documentation requirements. For some, the study had underpinned the need for a dedicated regulatory framework for personalized bacteriophage therapy approaches to enable a timely supply of efficient bacteriophage preparations to conduct the desperately needed safety and efficacy studies and to respond to urgent local infection issues or public health threats, such as the *Escherichia coli* STEC O104:H4 outbreak in Germany in 2011 [17].

Competent authorities for medicines seem to be increasingly aware of these issues but tend to believe that they will be able to find a solution within the existing framework, in consultation with developers and manufacturers. Although they seem to be more or less on the right course as far as predefined cocktails are concerned, it is highly improbable that they will be able to squeeze variable or custom-made (personalized) bacteriophage preparations through the conventional medicinal product funnel in timelines that are compatible with bacteriophage therapy in critically infected patients.

The setup of bacteriophage libraries (bacteriophage banks) of preapproved bacteriophages could allow for the timely assembly and approval of bacteriophage cocktails [1]. In that context, individual bacteriophages would be characterized in vitro using structural, genomic, and efficacy analyses. Pre-characterization could also establish safety and efficacy, including suitable in vivo testing in animal models [1].

A regulatory framework that places emphasis on process controls instead of characterizing each single bacteriophage strain could also make it easier and cheaper to update bacteriophage cocktails [18]. Such a regulatory framework could be based on

the Quality by Design (QbD) concept, which is increasingly applied to the development and production of biopharmaceutical molecules [19]. The QbD approach entails designing quality into the process and the product and this in a science- and risk-based manner. Understanding patients' needs and determining the specific science and quality characteristics of the product that are linked to safety and efficacy are crucial components of QbD.

It was also suggested to use the Biological Master File (BMF) approach that is currently applied to certain chemical drugs [20], that is, the compilation and submission, for regulatory approval, of a stand-alone package covering only part of a dossier. The BMF could, for instance, cover only the active ingredient (the bacteriophage) of a bacteriophage preparation. One BMF would be submitted for each individual bacteriophage. The BMF concept mainly focuses on the quality of the manufacturing process (GMP) and safety issues. Unfortunately, European legislation does not allow the application of this concept to biologically active substances.

In this chapter, we review the relevant (for the Western world) and current regulatory requirements and some important topics with regard to the development and production of therapeutic bacteriophage preparations, such as bacteriophage identification and characterization; bacteriophage cultivation; relevant quality, safety, and efficacy controls; and bacteriophage product storage. This chapter is meant to help institutions that wish to manufacture bacteriophage products for human use to define production processes; implement relevant controls to ensure quality, safety, and efficacy of the final products; and meet the applicable regulatory expectancies. It can also serve as a basis for discussions with competent authorities regarding the development of expedited bacteriophage product manufacturing and licensing pathways with relevant and pragmatic requirements, allowing for the full exploitation of bacteriophages as natural controllers of bacterial populations.

Bacteriophage-derived products, such as endolysins, are static chemicals similar to antibiotics and should not face difficulties obtaining approval via conventional medicinal product or drug pathways. Therefore, we will only focus on products that contain natural whole (intact) bacteriophages.

2 Regulatory Requirements

As stated earlier, the EMA and FDA classified therapeutic bacteriophage products as medicinal products (more specifically "biological medicinal products") and drugs [bacteriophage applications are handled by the Division of Vaccines and Related Product Applications part of the Center for Biologics Evaluation and Research

(CBER)], which makes it simple in terms of the requirements for the manufacturing processes of bacteriophages: They will need to be carried out according to GMP.

However, to date, two decades after the so-called phage therapy renaissance, no bacteriophage products have been approved for human use in the EU or the US.

In the absence of commercially available bacteriophage preparations and in the face of an uncurbed antimicrobial resistance crisis, bacteriophage therapy is sporadically applied in the Western world, often under the umbrella of Article 37 (Unproven Interventions in Clinical Practice) of the Declaration of Helsinki [21]. This framework places the main responsibility on the treating physician, who needs to obtain expert advice and the patient's informed consent, but does not impose specific requirements for the medicines that will be used in the "unproven intervention."

In addition, several EU and US patients suffering from difficult-to-treat bacterial infections are known to have traveled abroad for bacteriophage therapy.

National health services and academia have a role in carrying out clinical research for the benefit of public health and without necessarily making a profit [18]. In some Western countries, policymakers entered into discussions with these stakeholders on setting a dedicated bacteriophage therapy framework with adapted manufacturing requirements to exploit and further explore the specific nature of bacteriophages as antibacterials. As a result, some countries allow non-EMA/FDA-approved (for market placement) bacteriophage preparations to be used in consultation with competent authorities.

- In the US, the FDA is committed to facilitating the testing of bacteriophage therapy in clinical trials. Clinical trials are performed under FDA oversight in the investigational new drug (IND) program. In the absence of FDA-approved bacteriophage therapy products for human applications, the FDA can allow the compassionate use (expanded access) of bacteriophage therapy. Where non-emergency expanded use authorization can take a while to obtain, the CBER may authorize emergency-use expanded access for single patients within hours of the request. Case studies include successful bacteriophage therapy treatment against multidrug-resistant *Acinetobacter baumannii* and *P. aeruginosa*. Recently, the FDA gave *Adaptive Phage Therapeutics* an Expanded Access IND authorization to use bacteriophage therapy for COVID-19 patients with a critical secondary bacterial infection. Bacteriophages can be sourced from companies (products in development) or academic laboratories, and their manufacturing (GMP compliance is not mandatory), purification, and formulation are variable. They must, however, meet certain quality standards set by the competent authorities.

- In Australia, bacteriophage products have been applied in compassionate treatments under a comparable scheme [22].
- In Poland, bacteriophage therapy is an experimental treatment that may be performed at the Ludwik Hirszfeld Institute of Immunology and Experimental Therapy and is covered by the Declaration of Helsinki and a national Act. Bacteriophages can be applied under similar conditions to Article 37 of the Declaration of Helsinki. More than 150 patient cases performed between 2008 and 2010 were reported with positive clinical results [23].
- In Belgium, since 2018, therapeutic bacteriophages can be delivered to individual patients as components of magistral preparations (known as compounding pharmacies in the US). These bacteriophage preparations are formulated by (or under the supervision of) hospital pharmacists upon prescription by a medical doctor [24]. Magistral preparations are regulated by Article 3 of the "medicinal product Directive" (Directive 2001/83). The active pharmaceutical ingredients (APIs) of magistral preparations, the bacteriophages in this case, must meet the requirements described in a monograph (a "recipe" describing their manufacturing and quality control testing processes) in an official Pharmacopoeia. However, in the absence of such an official monograph, "non-authorized" ingredients can be used as long as they are accompanied by a certificate of analysis, which in Belgium must be issued by a Belgian Approved Laboratory (BAL). The BAL is a quality control laboratory that has been granted accreditation by the appropriate Belgian regulatory authorities to perform batch release testing of medicinal products. Such a laboratory exists in each Member State. On January 10, 2018, the "phage API monograph" was granted a formal affirmation by the Belgian Federal Agency for Medicines and Health Products (FAMHP) [24]. Well-characterized bacteriophages (and their production hosts) that pass the quality control performed by a BAL receive a "green" genomic passport and can be stored in a bank for future use as seed lots for bacteriophage API productions. Phage APIs are manufactured (by private companies and academic or public institutions) according to the API monograph, and each batch is accompanied by a batch record. A BAL performs an external quality assessment of each produced bacteriophage API batch. The phage APIs, together with the batch record and the external (BAL) quality assessment, are provided to the hospital pharmacies for further formulation into magistral preparations, according to the prescriptions of medical doctors. There are initiatives to extend the Belgian "magistral preparation" bacteriophage therapy framework to the European level (via the European Pharmacopoeia), enabling an expeditious reintroduction of personalized bacteriophage therapy into Europe [25].

- In France, bacteriophage therapy is applied in a compassionate use setting as unauthorized medicines that are being evaluated in clinical trials or have entered the marketing authorization application process, and for which early study results have demonstrated safety and efficacy [2]. Compassionate use falls under the responsibility of the Member States, with EMA providing recommendations. The Agence Nationale de Sécurité du Médicaments et les Produits de Santé (ANSM) may approve [*autorisation temporaire d'usage* (ATU)] the bacteriophage therapy requests after evaluation by a committee of experts. Fifteen patients were treated between 2006 and 2018 [2]. Recently, two GMP-certified *Staphylococcus aureus* bacteriophage products from the French biotech company *Pherecydes* were authorized for the treatment of bone infections via the *autorisations d'accès compassionnels* (AAC) program, which is less complex than the ATU framework. In addition, ANSM has approved the use of Belgian non-GMP-certified phage APIs in a handful of patients, meaning that they do not strictly adhere to the "medicine that are being tested or have entered the marketing authorization application process after early study results have demonstrated safety and efficacy" condition, after thorough analysis, and in the interest of the patients.

Irrespective of the shape of (future) bacteriophage therapy regulatory frameworks, requirements for the production of therapeutic bacteriophage products should always give precedence to patient safety and preferably be determined in consultation with the competent authorities for medicines.

3 Production Processes

Not much information regarding bacteriophage product manufacturing is publicly available. Companies, and even some public institutes, are reluctant to publish technical information with regard to bacteriophage production processes and quality controls [6]. The information listed below is based on the scientific literature and on our personal experiences.

3.1 Bacteriophage Propagation

Bacteriophage cultivation requires validated aseptic conditions. Developing processes based on closed (automated) systems or bioreactors, as opposed to open systems, increases safety and is an important step toward GMP compliance (where GMP is required) [26]. In addition, producing bacteriophage products in closed systems will prevent the dissemination of (pathogenic) bacteria and bacteriophages in the production facility, reducing the chances of contamination problems. Potent bacteriophages that have spread in the production environment might induce unwanted lysis of bacteria for months to years after their initial use

[27]. Materials may be introduced to a closed system, or air may be introduced or exhausted, but this must be done in such a way as to avoid exposure of the product or the production environment to contaminants such as bacteria and bacteriophages. This can be done using filters, for example, high-efficiency particulate air (HEPA) filters capturing nanoparticles, including bacteria and bacteriophages. Where the production process is not fully closed or opened (a functionally closed system), for example, to install a filter or connect tubing, a GMP grade A environment is ideally required for manipulation. If no GMP grade A environment is used, a validated sanitization or sterilization step is necessary after closing, and prior to process use. The use of closed production systems minimizes the risks of contamination and could facilitate bacteriophage productions in less controlled environments (e.g., a GMP grade D environment). This can be important with regard to the production of bacteriophage therapy products in the developing world [28].

Bourdin et al. investigated the pilot scale amplification of *E. coli* bacteriophages for bacteriophage therapy [29]. Using various production systems such as wave bags, stirred-tank reactors, and even simple Erlenmeyer flasks, acceptable peak titers of 10^9 to 10^{10} pfu/mL were obtained. The bacteriophage strain and the time between bacterial and bacteriophage inoculation were shown to have the greatest impacts on final bacteriophage titers [29]. The choice of broth had only a weak effect on the final bacteriophage titer. The inoculation concentrations and timings of bacteriophages and bacteria are important to obtain a balanced growth rate of bacteriophages and bacteria, creating the optimal conditions for their productive interactions upon incubation, and (ideally) need to be optimized for each production [7]. The selection and possible preadaptation of adequate therapeutic bacteriophages, combinations of bacteriophages, or combinations of bacteriophages and antibiotics (in search of synergistic effects), and the selection of proper bacterial production hosts are explained in another chapter of this book ("Guidelines to Compose an Ideal Bacteriophage Cocktail"). Using well-defined or completely defined media will increase safety and reproducibility [26]. It is essential that the media used for bacteriophage propagation are free of potentially dangerous contaminants such as prions, viruses, and allergens [30]. Vieu (reviewed in [31]) showed that for IV (intravenous) use, the quality of media used to produce bacteriophages was important and that the media that were best tolerated by patients were those containing the least amount of large protein molecules [32]. Montclos (reviewed in [31]) recommended against the cultivation of bacteriophages in media containing animal extracts [33]. Traditional growth media for bacteria often do contain animal extracts (implying a risk of transmission of infectious agents such as BSE) [33]. Therefore, Merabishvili et al. decided to use a bacterial growth medium certified to be free of animal proteins for the

production of their bacteriophage therapy product [34]. The most important medium remnants in the bacteriophage product were soy hydrolysate and yeast extract, inherent components of the broth used for bacterial growth and bacteriophage production. The final theoretical concentrations of these remnants, before endotoxin removal, were 25 and 125 mg/mL, respectively. Sauve (reviewed in [31]) described the cultivation of bacteriophages in broth without peptone to avoid the shock induced by peptone [35]. It should be noted that some defined media are *for research use only* and require upgrading *for further manufacturing use* or *clinical use*.

To represent a potential practical solution for developing countries, large-scale bacteriophage amplification should be achieved in a relatively inexpensive way while ensuring the production requirements (e.g., GMP compliance) [29]. Relatively inexpensive closed cultivation systems, in combination with inexpensive microbiological media containing only food-grade supplements, can be used for growing bacteria and bacteriophages.

In the (near) future, therapeutic bacteriophage genomes, whether designed or not, might be predicted using artificial intelligence and subsequently synthesized, transcribed, and translated into functional bacteriophages using an onsite, instant, bacterial cell-free production system [36]. A device producing such synthetic bacteriophages would have considerable advantages over classically produced (in bacterial hosts) bacteriophages:

1. There is no need for physical therapeutic bacteriophage banks.
2. There is no need to dispatch the patient's bacterial isolates and the selected and produced therapeutic bacteriophages all over the world.
3. Synthetic bacteriophages against bacteria causing eminent public health threats [17] or against bacteria (suspected to be) used for bioterrorism [37] can be timely produced on site.
4. Bacteriophages targeting bacteria causing lethal diseases, for which no non-pathogenic production host strains are available, and whose propagation requires biosafety level-3 (BSL-3) bio-containment precautions can be synthesized in BSL-1 conditions.
5. When bacteriophages cannot readily be isolated from sampling sites, bacteriophage genomic sequences extracted from metagenomic data [38] can be used to produce synthetic bacteriophages.
6. Synthetic bacteriophage preparations contain no (or smaller amounts of) molecules that could have a negative impact on patients (e.g., endotoxins).
7. Devices can be adapted to produce synthetic bacteriophages during extended space travel and space colonization [39].

3.2 Bacteriophage Purification

Bacteriophages need to be separated from other culture components prior to human (or animal) administration. In the early days of bacteriophage therapy, host bacterial remnants were suggested to have contributed to treatment failures. Today, competent authorities for medicines, such as the US FDA and the EMA, mandate that any modern bacteriophage therapy product be made to GMP standards. These standards include a very high level of purification and sterilization.

Basic bacteriophage purification protocols involve relatively simple methods, such as clarification of lysed cultures via either centrifugation or filtration [40]. Gill and Hyman discussed the small-scale purification of bacteriophage therapy products [6]. Lysis of bacteria and subsequent release of bacteriophages in the culture medium may be accomplished via incubation with small quantities of chloroform. Note that for therapeutic bacteriophages to be used in humans, organic solvent lysis should be avoided during the purification process (but not necessarily from the initial isolation procedure or stock storage), in part due to regulatory concerns. Lysozyme or bacteriophage lysins could be used instead. After lysis, remaining bacterial cells or cell debris are usually removed by low-speed centrifugation. This centrifugation step may be followed by filtration over 0.45 and/or 0.22 μm membrane filters.

In the case of Gram-negative bacteria, crude lysates also contain bacterial endotoxin, which is strongly pro-inflammatory. Therefore, most Western bacteriophage therapy studies have opted to purify bacteriophage preparations more thoroughly. The degree of further purification is largely a function of the type of application the bacteriophages will be used for [6]. A more thorough purification is especially warranted in systemic or invasive rather than topical or oral applications. For invasive bacteriophage product applications such as IV injections, more stringent purifications, which usually involve ultracentrifugation, a series of filtration and washing/buffer-exchange steps, and/or various forms of chromatography, are required. The most common laboratory-scale purification methods involve the concentration of bacteriophages using cesium chloride or sucrose gradient ultracentrifugation. However, this technique is time-consuming, labor-intensive, and requires expensive specialized equipment and skilled operators, and especially the requirement for ultracentrifugation limits the scalability of the procedure [6]. Therefore, different procedures have been developed to precipitate bacteriophages, which eliminates the need for ultracentrifugation. Often, *low-speed* centrifugation is still needed to pellet the bacteriophage-containing precipitant [6]. For large volumes, continuous flow centrifugation, a filtration step, or hydroxyapatite column chromatography might be substituted for centrifugation. Polyethylene glycol (PEG) is the most commonly used precipitating agent. Flocculation has also been used to concentrate bacteriophages from large volumes [6]. The resulting flocs

can be collected by filtration, sedimentation, or low-speed centrifugation. Chromatographic procedures have also been reported to reduce endotoxin levels. Uhr et al. (discussed in [31]) reported a purification method consisting of precipitation with ammonium sulfate and passage through a diethylaminoethyl (DEAE) cellulose anion exchange column with 0.1 M ammonium acetate [41]. Bourdin et al. investigated the pilot-scale purification of *E. coli* bacteriophages for therapeutic use [29]. Bacteriophage lysates could be sterilized using 0.22 μm membrane filters without titer loss. Concentration by differential centrifugation eliminated cellular debris. Ultracentrifugation and PEG precipitation led to 1-log and 0.5-log bacteriophage losses, respectively. Medium-speed centrifugation, ultrafiltration, and chromatography methods were associated with only minimal titer losses. On the other hand, ultracentrifugation led to 90% endotoxin removal. Merabishvili et al. used a commercially available chromatographic column that specifically binds endotoxins [34].

Endotoxin limits have been established by the FDA [42] for injectable and parenteral drugs and for medical devices (discussed in [29]). Endotoxin levels in bacteriophage preparations for IV use should not exceed 5 endotoxin units (EU) per kilogram of body weight per hour [30]. There is no defined oral endotoxin limit dose, but oral administration to mice of 10^6 EU of *E. coli* endotoxin per mouse elicited no toxicity [29].

4 Quality, Safety, and Efficacy Requirements for Bacteriophage Products

In 2015, an international panel consisting of 29 "bacteriophage experts" from ten countries elaborated on quality, safety, and efficacy requirements for sustainable bacteriophage therapy products [43]. These requirements were tailored to the production of therapy products, starting from banked bacteriophages (Master Seed lots), possibly over intermediate bacteriophage products (Working Seed lots or Active Substances), to finished products. The design was roughly based on the specifications of the EU Tissue and Cell Directives (EUTCD). The EUTCD framework introduced dedicated safety and quality standards for human tissues and cells, for which strict pharmaceutical requirements are too challenging and which are not classified as medicinal products (drugs). Table 1 contains an updated version of these requirements, including an exhaustive list of possible control tests. As for all biological products, bacteriophage product release testing should address purity, concentration, consistency, identity, and biosafety.

The exact processes, tests, and limits that will actually be applied will depend on the route of administration (e.g., topical or systemic), on the regulatory framework the product will need to comply with, and on discussions with relevant competent authorities.

Table 1
Quality, safety, and efficacy requirements for bacteriophage (phage) therapy products

A. Production environment
When production activities include the processing of intermediate, bulk, or finished phage products exposed to the environment, this must take place in an environment with specified air quality and cleanliness to minimize the risk of contamination. The effectiveness of these measures must be validated and monitored. Where intermediate, bulk, or finished products are exposed to the environment during processing, without a subsequent microbial inactivation process, an *air quality* with particle counts and microbial colony counts equivalent to those of Grade A as defined in the current European Guide to Good Manufacturing Practice (GMP), Annex 1 and Directive 2003/94/EC is required with a background environment at least equivalent to GMP Grade D in terms of particles and microbial counts.
Because phages are dynamic entities that interact with their bacterial host and their environment, some of them can possess properties that may negatively impact human health. Therefore, in the EU, phage manipulation is covered by European Directive 2000/54/EC related to the exposure of workers to biological agents. When genetically modified phages are involved, risk assessment should be made in agreement with the specifications of European Directive 2009/41/EC on the contained use of genetically modified microorganisms [27]. Most natural therapeutic phages can be classified in risk class 1 since they do not **a priori** represent any direct risk to human health or the environment (unless they are carrying undesired genes). Often, biosafety containment levels (BSL) will be determined by the bacterial host strain. A well-characterized lytic *Escherichia coli* phage that contains no undesired genes would require only BSL-1, while propagation of this phage in *E. coli* O157:H7 would require BSL-2. It is recommended to avoid unwanted release of phages and bacteria in the production facility as well as in the environment. Large phage concentrations could possibly perturb environmental bacterial populations. The main precautions consist of properly inactivating all biological wastes.
B. Production processes, equipment, and materials
All equipment and materials must be designed and maintained to suit their intended purposes, and any hazard to recipients and staff must be minimized. All critical equipment and technical devices must be identified and validated, regularly inspected, and preventively maintained in accordance with the manufacturers' instructions. Where equipment or materials affect critical processing or storage parameters (e.g., temperature, pressure, particle counts, and microbial contamination levels), they must be identified and must be the subject of appropriate monitoring, alerts, alarms, and corrective action, as required, to detect malfunctions and defects and to ensure that the critical parameters are maintained within acceptable limits at all times. All equipment with a critical measuring function must be calibrated against a traceable standard if available. Maintenance, servicing, cleaning, disinfection, and sanitation of all critical equipment must be performed regularly and recorded accordingly.
Production processes must be described in detail (equipment, materials, culture media, additives, culture conditions, purification steps, etc.) in standard operating procedures (SOPs) and must be validated (procedures published in relevant peer-reviewed journals could be considered "validated").
SOPs must detail the specifications for all critical materials and reagents. In particular, specifications for culture media, additives (e.g., solutions), and packaging materials must be defined. Critical reagents and materials must meet documented requirements and specifications and when applicable the requirements of Council Directive 93/42/EEC of June 14, 1993, concerning medical devices and Directive 98/79/EC of the European Parliament and of the Council of October 27, 1998, on **in vitro** diagnostic medical devices. If possible, animal-component-free culture media and additives should be used [the Note for Guidance on Minimizing the Risk of Transmitting Animal Spongiform Encephalopathy Agents via Human and Veterinary Medicinal

Products (EMEA/410/01) in its current version is to be applied]. If animal-product-free media are not used, transmitting animal spongiform encephalopathy (TSE)–free certification should be obtained for all components containing products of animal origin. Analytical methods can be validated according to (a) EMEA/CHMP/EWP/192217/2009 "Guideline on bioanalytical method validation" or (b) CPMP/ICH/381/95 "ICH Topic Q 2 (R1) Validation of Analytical Procedures: Text and Methodology." Bacteria and phage bank systems need to be set up. These bank systems typically consist of ***Master Seed*** lots and ***Working Seed*** lots. The generation and characterization of the banks should be performed in accordance with the principles of CPMP/ICH guideline Q5D. The banked phages and bacteria should be characterized for relevant phenotypic and genotypic markers so that the identity, viability (activity for phages), and purity of organisms used for the production are ensured. Biological Resource Centers [44] could function as repositories for phage Master Seeds and host bacteria.			
C. Quality assurance and quality control (QA/QC) specifications			
Products/characteristics	**Control test**	**Limits of acceptance**	**Recommended test procedures**
C.1. Host bacteria used in production (stock suspensions)			
The bacterial hosts used in the production process—with the exception of selection, adaptation, and efficiency of plating (EOP) and host range determination—should be as safe (or least pathogenic) as feasible.			
Origin	Document pedigree/history/pathogenicity level	Known origin	Screening of scientific literature, lab books, consignment letters, etc.
Identification	Identification of the species and strain levels	Matching species and strain identification	State-of-the-art clinical microbiology techniques Highly discriminating (molecular/genomic) typing techniques (e.g., MLST, AFLP, PFGE, and Rep-PCR) State-of-the-art DNA sequencing and analysis (bioinformatics) procedures
Most often it will not be possible to find or quickly generate a suitable ***host*** bacterium that is free of prophages or phage-like elements, but one should nevertheless strive to use non-lysogenic strains, containing as few phages or other	Induction of phages Host genome screening for phage or phage-like elements	As few spontaneously produced (or by induction) temperate phages, complete prophage sequences, or phage-like elements as possible[1]	In vitro induction methods (e.g., Mitomycine C [47] or UV induction) State-of-the-art DNA sequencing and analysis (bioinformatics) procedures

(continued)

Table 1 (continued)

phage-like elements of genetic exchange [45, 46] as possible			
Avoid mutator strains as host bacteria	Screen for mutator strains in case of doubt	No mutator strain	State-of-the-art tests (e.g., fosfomycin and rifampicin Disk Diffusion Tests) [48]
Validated preservation/storage (cryopreservation, freeze-drying, etc.)	Monitor storage conditions (e.g., temperature)	Variable, depending on the preservation method	Variable (e.g., temperature probes and temperature indicator labels)
C.2. Phages (Master Seed lots)			
Origin	Document phage pedigree/history (e.g., isolation source)	Known origin Natural or naturally evolved phages	Screening of scientific literature, lab books, consignment letters, etc.
Identification	Identification of the family (subfamily), genus and species, and strain level Morphology and biology	Matching identification, morphology, and biology	State-of-the-art DNA or RNA sequencing and analysis procedures[2] Highly discriminating genotyping techniques (e.g., AFLP and fRFLP [49])[3] Matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometer [29] State-of-the-art classification according to the International Committee on Taxonomy of Viruses (ICTV) State-of-the-art electron microscopy (optional)[4]

Not containing potentially damaging genetic determinants (e.g., conferring toxicity, virulence, lysogeny, or antibiotic resistance)	Genome analysis for known potentially damaging genetic determinants	Absence of potentially damaging genetic determinants[5]	State-of-the-art DNA or RNA sequencing and genome analysis (bioinformatics) procedures[2]
Non-transducing (optional) [50][6]	Screen for "general transduction"	Does not pack random host DNA in a portion of progeny phage particles	Transduction assay [51]
In vitro efficacy	Determination of host range on a panel of target species (reference) strains	Broad host range (if possible) Variable threshold according to species (e.g., >75% for *S. aureus*)	Spot test [52] Titration of phages against target bacteria according to the soft-agar overlay method and determination of the efficiency of plating (EOP) [53]
	Stability of lysis (optional)[7]	Stable lysis in broth culture for 24–48h	Appelmans method [54], possibly modified using modern technologies (e.g., using isothermal colorimetry or redox chemistry)
	EOP under conditions similar to eventual clinical application (optional)	Threshold EOP value	EOP determination [53]
	Determination of the frequency of emergence of phage-resistant bacteria	Low frequency of emergence of resistance	Method described by Adams [53]
	Determination of growth parameters such as optimal replication temperature and pH, duration of the latent period, average burst size, and binding rate (optional) [6]	Threshold values	One-step growth curve [52]
	Determination of bacterial cell wall binding domains (optional)	Choose phages with cell wall binding domains that are less prone to modification and avoid combining phages with similar cell wall binding domains to reduce the chances of overlapping cross-resistance [6]	State-of-the-art molecular biology methods

(continued)

Table 1 (continued)

Improvement/adaptation/ "training" (optional)	Optimization of host range	Broadened and stable host range	Appelmans method [54], possibly modified using modern technologies (e.g., using isothermal colorimetry or redox chemistry) Host range expansion (HRE) on agar medium [55]
	Shifting optimal growth parameters (temperature, pH, etc.) toward the physiological conditions of the clinical application site	Threshold values	Sequential titrations of phages against target bacteria under gradually changing conditions [52]
	Optimization of phage cocktails toward reducing phages hampering each other upon co-infection [52]	Reduced hampering	Cocultivation of phages [52]
Validated preservation/storage (cryopreservation, freeze-drying, etc.)	Monitor storage conditions (e.g., temperature)	Variable, depending on the preservation method	Variable (e.g., temperature probes, and temperature indicator labels)
C.3. Phages (working seed lots/active substances)			
Quantitative determination of active substance (phages)	Phage titration	Variable. Typically log(8)–log(10) plaque-forming units (PFU)/ml	Soft-agar overlay method [53]
Identification of the active substance	Genomic fingerprinting	Matching genomic fingerprint (max. deviation depends on the method)	State-of-the-art DNA or RNA sequencing and genome analysis (bioinformatics) procedures[2] State-of-the-art genotyping techniques (e.g., AFLP and fRFLP [49]) MALDI-TOF mass spectrometer for phage identification [29]

Microbial contamination	Sterility (when there is no sense of urgency)[8]	Sterile (absence of microorganisms)	Membrane filtration method based on the European Pharmacopoeia (EP)
	Absence of pathogens (when there is a sense of urgency)	Aseptic (absence of pathogens)	State-of-the-art clinical microbiology methods
Toxicity	Bacterial endotoxin or lipopolysaccharides (LPS) quantification [56]	Depends on posology and method and route of administration. The maximum level for intravenous applications for pharmaceutical and biological products is set to 5 endotoxin units per kg of body weight per hour (EP)	Limulus amebocyte lysate (LAL) assay according to the EP (e.g., kinetic-QCL method) Recombinant Factor C (rFC) test (EP method) Monocyte activation test (MAT) (EP method) Rabbit pyrogen test (EP method)
Bacterial DNA contamination[9]	Screen for (potentially damaging) host bacterial DNA	Absence of potentially damaging genetic determinants that are known to be present in the host bacterium	Methods for the quantification of bacterial DNA in general (e.g., PicoGreen) or for the quantification of known DNA sequences (e.g., qPCR)[10]
Acidity or basicity of aqueous solution	pH measurement	Variable (typically 6.5–7.5)	pH test (EP method)
Purity	Clarity of phage solution	Absence of visible particles	EP method, CPMP-ICH guideline
Validated preservation/storage (cooling, cryopreservation, freeze-drying, etc.)	Monitor/record/demonstrate storage conditions (temperature)	Variable (e.g., 2–8 °C)	Variable (e.g., temperature probes, temperature indicator labels, etc.)
C.4. Finished products			
Bulk products may be diluted [typically to log(5)-log(7) pfu/ml], combined, or added to a carrier (hydrogel, ointment, cream, bandage, etc.) prior to clinical use. Dilution solutions, carriers, and packaging materials must meet documented requirements and specifications and when applicable the requirements of Council Directive 93/42/EEC of June 14, 1993, concerning medical devices. Carriers must be chosen that allow the required phage activity during the intended application period (stability). The following information must be provided either on the label or in accompanying documentation: (a) description (definition) and, if relevant, dimensions of the phage product; (b) date of production of the phage product; (c) storage recommendations; (d) instructions for opening the container, package, and any required manipulation/reconstitution; (e) expiration dates (incl. after opening/manipulation); (f) instructions for reporting serious adverse reactions and/or events; (g) presence of potentially harmful residues (e.g., antibiotics, ethylene oxide); (h) contraindications; and (i) how to dispose of unused (expired) phage products.			

(continued)

Table 1 (continued)

Validated storage (cold storage)	Monitor/record/demonstrate storage conditions (temperature)	Variable (e.g., 2–8 °C)	Variable (e.g., temperature probes, temperature indicator labels, etc.)
D. Shelf life of phage stock suspensions, working solutions, and finished products (at recommended storage conditions)			
Stability	Periodic quantitative determination of the active substances (phages) or breakdown products Periodic determination of sterility Periodic pH measurements	The shelf life is the time period during which the product remains sterile and the activity and pH remain within specified limit thresholds	Soft-agar overlay method [53] CPMP-ICH guideline, Q5C, Q1A Membrane filtration method (EP method) pH test (EP method)
E. Surveillance			
The clinical use of phage therapy products must be surveyed and reported, including possible adverse events and reactions associated with the use of phage therapy products. A centralized (publicly available) reporting system is warranted.			

[1] *E. coli* phages for human phage therapy can be propagated on *E. coli* strain K803, a derivative of laboratory strain K-12 lacking prophage lambda [29]. In some cases, however, it may be impossible to successfully cure some host strains that are indispensable for the production of some therapeutically interesting phages. In addition, in some cases, it might be necessary to use phages that were isolated from the patient's bacteria and that are not able to replicate in known host strains devoid of prophages. However, since that kind of phage preparations are only designed to be used in that given patient, any remaining traces of DNA from that host bacterium would be orders of magnitude less than the amount already present in the patient from whom that bacterium was isolated for this purpose

[2] To note, metagenomic analysis was recently used to identify distinct phage types and to screen for undesired genes and bacterial DNA in an undescribed phage preparation [57]

[3] This genetic fingerprint can be used to timely identify bacteriophages and confirm their presence in Working Seed lots and in finished products, without having to re-perform full genome sequencing. It is, however, expected that fast, low-cost, and accurate full genome sequencing and analysis (of bacteriophages) will replace routine microbial genotyping techniques in the near future

[4] In some cases (e.g., novel bacteriophages with no homology in databases), electron microscopy could provide important information and could thus be warranted

[5] In general, it is recommended to only use lytic phages (and no temperate phages) in phage therapy. Lytic phages are more potent killers of host bacteria, making them more effective in therapy than temperate phages. Following lysogenic induction, temperate phages may transfer fragments of host bacterial DNA into non-targeted bacteria (possibly belonging to other species). This phenomenon is called transduction or phage-mediated horizontal gene transfer (HGT). If these DNA fragments contain toxin-encoding or antibiotic resistance-mediating genes, temperate phages could thus produce new pathogenic strains. According to some researchers, lytic variants of transposable phages and phages that produce pseudolysogenic conditions (bacteria are infected, but there is no intracellular development of phage) should also be excluded [58]. Only a direct comparative study of a new phage in relation to other phages' interactions on the surface of infected bacterial biofilm could provide a reliable indication regarding its safety in therapeutic applications [58]. However, in the future, the dogma that the use, in treatment, of temperate phages is impossible or undesirable because of the danger of HGT might be abandoned in certain circumstances (science- and risk-based decision, taking into consideration the patients' needs). In certain bacterial species, the number of strictly virulent phages is small and it might not be possible to isolate adequate new virulent phages in due time. Phage-mediated HGT is abundant and virtually ubiquitous in bacterial populations and the additional and immediate danger to the patient related to the use of temperate phages in the course of phage therapy (days) is bound to be limited. Moreover, if a temperate phage acts as a lytic phage in relation to a particular pathogen, the probability of HGT might not be higher than for inherent genetic virulent phages [59]. In the future, temperate

phages might specifically be used in therapy, for example, to introduce, by lysogenization, genes conferring sensitivity to antimicrobials [60] or to inhibit virulence traits [61]. Finally, antibiotic stress was also shown to induce genetic transformability in human pathogens [62]

[6]Today, it is not feasible to exclude the possibility of low levels of generalized transduction by therapeutic phages into any of the infecting and commensal bacteria present in or on the patient. The use in phage therapy of phages that mediate some random general transduction might be considered in certain circumstances (science- and risk-based decision, taking into consideration the patients' needs)

[7]In some cases, phages that produce stable lysis will not be found in a timely fashion. Phages that induce relatively fast in vitro bacterial resistance might then be considered

[8]In some cases, sterility may not be required (e.g., "non-sterile for topical application")

[9]Working Seed lots can be contaminated with low levels of DNA derived from the host bacteria used in production. Potentially damaging genetic determinants (e.g., conferring toxicity, virulence, or antibiotic resistance) might then be transferred (through transformation) to bacteria present in or on the patient, which could potentially make them (more) pathogenic. While this would be expected to occur at a level well below exchanges already going on within the patient's body involving their own pathogenic bacteria and phages already resident, it makes sense to select hosts that are as devoid of pathogenicity factors as reasonably possible for growing therapeutic phage and treating the phage with DNase in the course of its purification to destroy such contaminants. If no non-pathogenic bacterial strain is available for growing the phage, constructing a "defanged" host strain, with all pathogenicity determinants deleted, could be envisaged as the best main step in avoiding this issue. Note that the use of non-pathogenic host bacterial strains also reduces the potential hazard to the personnel involved in the production of therapeutic phages

[10]A threshold level should be determined. Note that some DNA quantification methods might also pick up phage DNA

The clinical efficacy of bacteriophage therapy products should be evaluated using clinical trials, which will not be addressed in this chapter. For a bacteriophage product release, relevant in vitro potency assays, which can give a prediction of the expected clinical efficacy, should be implemented. Similar to other custom-made medicines, such as mesenchymal stem cells (MSCs) [26], efficacy could largely be based on previous in vitro and possibly on in vivo experiments carried out in animals (or in equivalent organ or tissue in vitro models). The immunosuppressive capacity of MSCs is tested in vitro. Likewise, the anticipated antibacterial clinical efficacy of bacteriophage products could also be predicted in vitro, for instance, using validated virulence and host range determination assays (Table 1). Standard efficacy criteria are often used to claim activity for chemical antimicrobials against particular pathogens (e.g., ATCC 27853, a reference strain of *P. aeruginosa*). Many valid tests were developed [7], mainly in the early years of bacteriophage therapy, but these are all based on ancient laborious microbiology methods (and should thus be automated and modernized), and there is no consensus on which tests and acceptance criteria should best be applied [6]. Suitable criteria, for instance, based upon a defined lower kill level, should be established [6].

For bacteriophage products that will be used in sustainable and flexible bacteriophage therapy concepts, it is crucial that safety controls, which will be applied to the release of bacteriophage products, allow for a fast determination of the identity and purity of the products. However, we would like to stress that less stringent requirements should be considered for bacteriophages intended to deal with urgent public health issues or medical emergencies, of course pending compliance (as quick as possible) to the applicable regulatory expectancies.

In 1986, the Ministry of Health of the USSR prescribed the following requirements for injectable staphylococcal bacteriophage preparations [55]:

1. Transparent, colorless, or light-yellow appearance.
2. Activity (in broth for 48 h) on ten different strains that were not used during production.
3. Activity determined using the double-agar method.
4. pH 7.3 ± 0.1.
5. Sterility.
6. Absence of pyrogens (tested in rabbits).
7. No toxic response in mice.
8. No unfavorable reaction in guinea pigs.

Conformity of the product to the specifications needed to be determined using methods that were approved by the Ministry of Health [63]. Bacteriophage products that conform to the above-mentioned specifications have been used in numerous patients.

In 2009, Merabishvili et al. described the small-scale, laboratory-based, production and quality control of a bacteriophage cocktail, consisting of exclusively lytic bacteriophages, designed for bacteriophage therapy of *P. aeruginosa* and *S. aureus* infections in burn wound patients [34]. This cocktail, consisting of three carefully selected bacteriophages, was "sterilized" by filtration and purified of endotoxins using a chromatographic column that specifically binds endotoxins. Quality controls included stability (shelf life), determination of pyrogenicity (in rabbits), sterility and cytotoxicity (toward keratinocytes), confirmation of the absence of temperate bacteriophages, and transmission electron microscopy (TEM)-based confirmation of the presence of the expected virion morphologic particles as well as of their specific interaction with the target bacteria. Bacteriophage genome and proteome analysis confirmed the lytic nature of the bacteriophages and the absence of toxin-coding genes and showed that the selected bacteriophages were close relatives of known bacteriophages. To date, this bacteriophage cocktail has been applied topically, but also intravenously, in several patients, without adverse reactions.

Recently, Fish et al. used a commercial preparation of staphylococcal bacteriophage sb-1 to treat intransigent diabetic toe ulcers [64]. The bacteriophage was completely sequenced, and bacteriophage lysates were sterile filtered and column-purified. Aliquots were independently tested and approved for sale by the Georgian Ministry of Health [63].

5 Conservation, Storage, and Stability

Bacteriophages consist of a DNA or RNA genome encapsulated by protein structures. As such, they are susceptible to many factors known to affect proteins, including exposure to organic solvents, high temperatures, pH, and ionic strength. For instance, low pH and digestive enzyme activities are known to affect bacteriophage activity in the gastrointestinal tract. Bacteriophage preparations need to be formulated for storage but also for subsequent clinical application.

The addition of Mg^{2+} led to an *E. coli* bacteriophage preparation that maintained an acceptable titer after storage for more than a month at 30 °C [29]. Some bacteriophages are relatively heat resistant and can even survive pasteurization temperatures. Other bacteriophages, like T4-like bacteriophages, however, showed rapid titer losses at 70 °C [29]. Bacteriophages can also be preserved by freeze-drying, spray-drying, or encapsulation [30].

According to most bacteriophage researchers, the best way to store bacteriophages is by cooling [30]. Sometimes substances that enhance bacteriophage stability in water (e.g., albumins, salts, or gelatin) are added. Refrigerated *E. coli* bacteriophages in

bacteriophage buffer were shown to maintain titers (<0.5-log titer decrease) for up to 2 years [29], and *P. aeruginosa* and *S. aureus* bacteriophages for at least 1 year at 2–8 °C [34].

Bacteriophage stock solutions were also successfully (without significant loss of titer) frozen at temperatures varying from −18 °C to −196 °C in appropriate media, with or without protective agents such as gum acacia [65]. Bacteriophages can also be frozen inside freshly infected susceptible bacteria at the early stages of bacteriophage development [66]. The infected bacteria release mature bacteriophages upon thawing, enabling an instant recovery of bacteriophages with high efficiency. The only limitation of this method is the sensitivity of the bacteriophage host to freezing.

Many strategies have been used for stabilizing, immobilizing, and encapsulating bacteriophages, including spray-drying, freeze-drying, spray freeze drying, extrusion dripping methods, and emulsion and polymerization techniques [67–70]. Spray drying was used to produce very stable bacteriophage formulations for the treatment of *P. aeruginosa* lung infection [68, 71, 72]. Liang et al. lyophilized *Campylobacter* bacteriophages, using tryptone as a stabilizer, with a limited titer reduction [73]. Transmission electron microscopy revealed that the titer reduction was associated with capsid collapse. The freeze-dried bacteriophages were stable under refrigerated vacuum conditions and showed no significant titer changes over 3 months of incubation at 4 °C [73]. *S. aureus* bacteriophage ISP was lyophilized with stabilizers sucrose and trehalose, resulting in a decrease of only 1 log immediately after lyophilization [74] and maintaining stability during a 7-year storage period [75].

Encapsulating bacteriophages in a protective barrier can confer acid stability before the functional release of the encapsulated bacteriophages in the gastrointestinal tract. Bacteriophages have been nano-encapsulated into liposomes, with the encapsulation yield being affected by the aggregation of bacteriophages or by the binding of large numbers of bacteriophages to the outside of the formed liposomes [76]. Microfluidic-assisted encapsulation into liposomes resulted in high encapsulation efficiency for *P. aeruginosa* bacteriophages, translating into minimal titer reduction [77]. Bacteriophages have also successfully been microencapsulated in alginate gel particles [78] and chitosan-alginate beads with a honey and gelatin matrix [79].

The materials from which the storage containers are made are also of importance. When comparing glass, polyethylene, polypropylene, and polystyrene storage containers, Richer and colleagues observed that bacteriophages tend to adsorb on the surface of more hydrophobic containers, while bacteriophage suspensions are relatively stable in more hydrophilic vials [80]. The polypropylene surface of the Eppendorf-type and Falcon-type containers were shown to accommodate from 10^8 to 10^{10} pfu/mL of

bacteriophages from the suspension, which in some cases might result in complete scavenging of bacteriophages by the container wall, from its content. The addition of the surfactant Tween20 and/or plasma treatment was shown to modulate surface wettability, thus inhibiting bacteriophage adsorption to the container walls [80].

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Chapter 7

Nano/microformulations for Bacteriophage Delivery

Pilar Cortés, Mary Cano-Sarabia, Joan Colom, Jennifer Otero, Daniel Maspoch, and Montserrat Llagostera

Abstract

Encapsulation methodologies allow the protection of bacteriophages for overcoming critical environmental conditions. Moreover, they improve the stability and the controlled delivery of bacteriophages which is of great innovative value in bacteriophage therapy. Here, two different encapsulation methodologies of bacteriophages are described using two biocompatible materials: a lipid cationic mixture and a combination of alginate with the antacid $CaCO_3$. To perform bacteriophage encapsulation is necessary to dispose of a purified and highly concentrated lysate (around 10^{10} to 10^{11} pfu/mL) and a specific equipment. Both methodologies have been successfully applied for encapsulating *Salmonella* bacteriophages with different morphologies. Also, the material employed does not modify the antibacterial action of bacteriophages. Moreover, both technologies can be adapted to any bacteriophage and possibly to any delivery route for bacteriophage therapy.

Key words Bacteriophages, Liposomes, Alginate, Nanoparticles, Microparticles

1 Introduction

Bacteriophages have gained increasing attention as a global measure to tackle the spread of antibiotic resistance under a One Health concept involving human and veterinary medicine and the agri-food sector [1–3]. However, despite the numerous advancements in their preparation, bacteriophages meet some empirical challenges posed by the host system depending on the route of administration. These include loss of stability, low bioavailability, non-targeted delivery, rapid clearance by the reticuloendothelial system, and antibody-mediated inactivation [4, 5]. The oral route has been evidenced as the most convenient and often applied for the delivery of bacteriophages [4]. However, bacteriophages are sensitive to different environmental factors encountered during their intestinal tract transit as extreme pH values or temperature, which causes their breakdown and subsequent loss of stability and

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infectivity and a reduced therapeutic efficacy [6]. Additionally, the residence time of bacteriophages in the intestinal tract is very short [7].

Encapsulation methodologies protect the bacteriophages from overcoming critical environmental conditions [8–12]. For bacteriophage encapsulation, the following aspects should be addressed: (i) the use of biocompatible materials for allowing their administration to living beings or foods, (ii) the choice of encapsulation technologies that do not inactivate bacteriophages, and (iii) the appropriate size of encapsulated bacteriophages according to their application.

Among different biomaterials, alginate is one of the most used for bacteriophage encapsulation. This is due to its high viscosity, which confers mucoadhesive properties [13, 14]. Moreover, microcapsules of alginate in combination with other materials [chitosan [15–17], $CaCO_3$ [18, 19], pectin [20, 21], and whey protein [22, 23]] can be obtained in order to improve their mucoadhesive properties and also to modify the release kinetics of bacteriophages. Alginate is a polymer that allows encapsulation by ionotropic gelation technique. Alginate hydrogel is gradually formed through the reaction between divalent cations (i.e., Ca^{2+}) and guluronate blocks of the alginate chains, giving a structure termed "egg-box" suitable to entrap molecules higher than 10,000 Daltons as lower mass molecules would diffuse through the capsule wall. Current strategies for ionotropic gelation encapsulation are dripping, spraying, or atomization drying, each of which results in capsules with different micrometric sizes. Nevertheless, all are based on the use of two solutions. The first is the solution containing the alginate and the material to be encapsulated, while the second is the gelation solution, which is formed by calcium chloride and allows the polymerization of the alginate [24].

Cationic lipids are another biocompatible material for bacteriophage encapsulation as they readily allow the encapsulation of such biological entities which are negatively charged *(studies reviewed in 8–11)*. The liposomes provide the following properties: (i) barrier to protons, thus protecting bacteriophages against gastric acidic pH [25]; (ii) promoters of mucoadhesivity, owing to their positively charged surfaces [26, 27], which would prolong the intestinal residence time of bacteriophages; (iii) in oral therapy against intestinal pathogens, they are degraded upon contact with intestinal bile salts [28], allowing the delivery of bacteriophages in the desired site; (iv) their nanometric size facilitates their administration; (v) their capacity of diffusion through the mucosa allows their interaction and possible absorption [29]; and (vi) liposomes protect bacteriophages from the neutralizing antibodies [30].

Different methodologies have been developed for lipid encapsulation [31], the thin-film hydration method being the most widely used [32] since hydrophobic interactions allow obtaining

multilamellar, unilamellar vesicles, or liposomes. For vesicle formation, a lipid mixture dissolved in an organic solvent is evaporated on a glass surface, forming a thin lipid layer that is later hydrated with water, sucrose, or other electroless solution and stirred gently. This causes the separation of lipid sheets from the surface and eventually forms large multilamellar vesicles. As bacteriophages are complex entities with hundreds of proteins and nucleic acid, multilamellar or unilamellar vesicles of around 1 μm are required to increase encapsulation effectiveness. However, the reduction of the vesicles' size by extrusion confers higher stability to the capsules [33]. Other modifications that can be used include electroforming [34] and gel-assisted hydration [35]. Recently, microfluidic approaches have been attempted to overcome the potential drawbacks (i.e., control of size and efficiency of encapsulation) of the other techniques [36].

Two methodologies of bacteriophage encapsulation are described in detail below and include all the encapsulation steps, the determination of the encapsulation efficiency and the size of the capsules, and their microscopic observation. One of them uses a lipid cationic mixture and rends capsules of nanometer size (from 309 to 326 nm) with an encapsulation efficiency of around 50% [33]. The other one uses a combination of alginate and the antacid $CaCO_3$, gives capsules of micrometric size (from 123.7 to 149.3 μm), lower than described by other authors, and the encapsulation efficiency is nearly 100% [19]. To perform both methodologies, it is necessary a purified and highly concentrated lysate (around 10^{10} to 10^{11} pfu/mL) and a specific equipment. *Salmonella* bacteriophages with short and long tails have been encapsulated with both methodologies, and their efficiency in reducing *Salmonella* in a model of oral therapy in poultry was significantly higher along the time than non-encapsulated bacteriophages [19, 33]. Both methodologies can also be adapted to any bacteriophage and possibly to any delivery route for bacteriophage therapy.

2 Materials

2.1 Lipidic Encapsulation

1. 2 mL of the bacteriophage suspension in 10 mM $MgSO_4$ at a concentration of 10^{11} pfu/mL (*see* **Note 1**).
2. Lipid mixture: 1,2-dilauroyl-rac-glycero-3-phosphocholine (DLPC), cholesteryl polyethylene glycol 600 sebacate (Chol-PEG600), cholesterol (Chol), and cholesteryl 3β-N (dimethylaminoethyl) carbamate hydrochloride (cholesteryl) in a molar ratio of 1:0.1:0.2:0.7. Dissolve each lipid in chloroform (100 mg/mL). Make a solution containing 106 μL of DLPC, 17 μL of cholesterol-PEG600, 13 μL of Chol, 64 μL of

cholesteryl, and 1 mL of pure chloroform in a round-bottom flask under sterile conditions. Mix the solution in a vortex for 1 min (*see* **Note 2**) and remove the organic solvent under vacuum (10 min) and nitrogen (30 min) to afford a dry lipid film (*see* **Note 3**).

3. Chloroform.
4. 10 mL round-bottom flasks (Fisher Scientific International Inc., Pittsburgh, USA).
5. Polycarbonate membrane (pore size: 400 nM, Whatman, Maidstone, UK).
6. Extruder (Lipex Biomembranes Inc., Vancouver BC, CA).
7. Rotavapor R-210 (Büchi Labortechnik AG, Flawil, SW).

2.2 Alginate/$CaCO_3$ Encapsulation

1. 50 mL of the bacteriophage suspension in 10 mM $MgSO_4$ at a concentration of 10^{11} pfu/mL.
2. Sodium alginate 1.8% (wt/vol).
3. $CaCO_3$ 1% (wt/vol).
4. 300 mL coagulation round tank with magnetic stirring at 500 rpm.
5. Calcium chloride coagulation solution by dissolving 3 g of $CaCl_2$ into 150 mL of deionized water [final concentration of 2% (wt/vol)] (*see* **Note 4**).
6. 10 mM $MgSO_4$.
7. 10 mL round-bottom flasks (Fisher Scientific International Inc., Pittsburgh, USA).
8. 50 mL Falcon tubes (Fisher Scientific International Inc., Pittsburgh, USA).
9. Magnetic stirrer (IKA Works GmbH & Co, Staufen, GE).
10. Peristaltic pump (Ismatec ISM830, Wertheim, GE).
11. ViscoMist™ Spray Nozzle with 381 mm of inner diameter (Lechler Inc., Metzingen, GE).
12. Nitrogen.
13. Centrifuge (Beckman Coulter, Pasadena, USA).

2.3 Characterization of Nano/microparticles

1. Size and zeta potential disposables cuvettes (DTS1070, Malvern Instruments, Malvern, UK).
2. ZetaSizer Nano ZS (Malvern Instruments, Malvern, UK).
3. MasterSizer 2000 (Malvern Instruments, Malvern, UK).

2.4 Encapsulation Efficiency

1. Polystyrene sterile tubes (5 mL).
2. 1.5 mL microcentrifuge tubes.
3. 50 mM bile salts.

4. 10 mM $MgSO_4$.
5. 0.22 μm syringe PES filter (Merck-Millipore, Darmstadt, GE).
6. 10 mL sterile syringes.
7. Agar plates.
8. Bacterial culture at exponential growth rate.
9. Soft agar [0.7% (wt/vol)].
10. Dry thermo Block (JP Selecta, Abrera, SP).
11. Incubator (37 °C) (Memmert GmbH + Co.KG, Schwabach, GE).

2.5 Microscopy of Nano/microparticles

1. SYBR gold (Molecular Probes, Oregon, USA).
2. Vybrant™ DiI cell-labeling solution (Molecular Probes, Oregon, USA).
3. Amicon Ultra-15 Centrifugal Filters 50 K (Merck-Millipore, Darmstadt, GE).
4. Carbon-coated film meshes.
5. Standard copper grids.
6. Coated glass slides.
7. 4′,6-diamidino-2′-phenylindole dihydrochloride (DAPI; Molecular Probes, Oregon, USA).
8. Liquid ethane.
9. Liquid nitrogen.
10. Jeol JEM-1400 transmission electron microscope (Jeol Ltd., Tokyo, JP).
11. Leica TCS SP5 laser confocal microscope (Leica Microsystems, Mannheim, GE).
12. Zeiss Axio observer.Z1 inverted optical microscope (Carl Zeiss, Oberkochen, GE).

3 Methods

3.1 Lipidic Encapsulation

This method of encapsulation of bacteriophages is based on the thin-film hydration method [32].

1. Hydrate the dry lipid mixture film with 2 mL of the bacteriophage suspension under stirring for 1 h at 200 rpm. Under these conditions, the stacks of liquid crystalline lipid bilayers become fluid and swell, resulting in their detachment during agitation and their self-closure to form large multilamellar vesicles (LMVs) (*see* **Note 5**).

2. Homogenize the LMVs suspension using a manual extruder with a polycarbonate membrane (pore size: 400 nm) for obtaining unilamellar vesicles. The LMV suspension is passed through the extruder ten times using a 1 mL syringe.
3. The capsules obtained are stable at 4 °C for at least six months.

3.2 Alginate/$CaCO_3$ Encapsulation

The methodology employed for the encapsulation of bacteriophages using alginate-$CaCO_3$ follows a previously described protocol with some variations [15].

1. Add 0.5 g of $CaCO_3$ and 0.9 g of alginate to 50 mL of the bacteriophage suspension to obtain a final concentration of 1% (wt/vol) and 1.8% (wt/vol), respectively.
2. Stir the mixture in round-bottom flasks using a magnetic stirrer at 700 rpm overnight at room temperature.
3. Pump the "bacteriophage/alginate/$CaCO_3$" mixture with the peristaltic pump at a feed flow rate of 1.5 mL/min into the $CaCl_2$ bath under stirring (500 rpm), using a spray nozzle coupled to a nitrogen gas flow at 3 bars.
4. Harden the gelatinized capsules by maintaining them in the coagulation bath for 90 min at 500 rpm.
5. Clean the formed capsules placed in 50 mL Falcon tubes by centrifugation at 469 × *g* for 5 min and remove the supernatant.
6. Add 30 mL of 10 mM $MgSO_4$ and resuspend the pellet by vortexing.
7. Repeat the centrifugation step thrice in the 50 mL Falcon tubes. The final cleaned pellet is diluted with 10 mM $MgSO_4$ until a 50 mL final volume.
8. The capsules obtained are stable at 4 °C for at least six months.

3.3 Characterization of Nano/microparticles

3.3.1 Lipidic Nanoparticles

The particle-size distributions and the zeta potential values of the capsules are determined in the ZetaSizer Nano ZS apparatus by measuring the electrophoretic mobility and using a dynamic light scattering (DLS) analyzer combined with non-invasive backscatter technology.

1. Dilute the sample 1:10 with distilled water and place 1 mL of the diluted sample into the size and zeta potential disposables cuvette.
2. Measure each sample three times for 10 runs at 25 °C in the ZetaSizer apparatus.
3. Take the measures of three different experiments to determine the mean particle diameter (*see* Fig. 1), the mean zeta potential of the dispersed system, and the standard deviations (*see* **Note 6**).

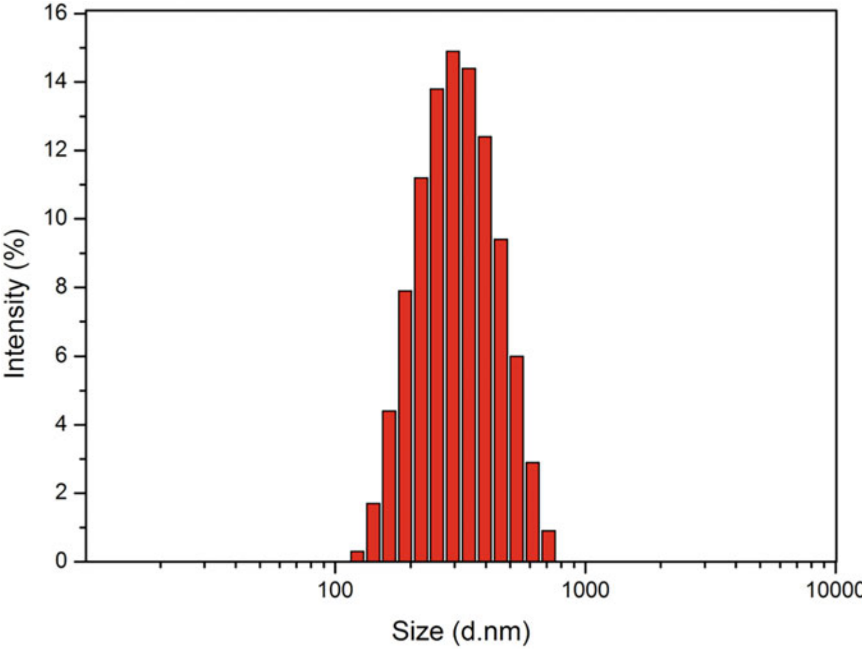


Fig. 1 Example of the particle size distribution of liposome-encapsulated bacteriophage of the *Lederbergvirus* genus, as measured by dynamic light scattering

3.3.2 Alginate/$CaCO_3$ Microparticles

Particle size distribution is determined by a granulometric assay using the Mastersizer 2000 based on a laser diffraction analyzer.

1. Put 1 mL of the sample into the Hydro SM dispersion unit of the Mastersizer 2000 apparatus, which contains water as dispersant media, stirring at 1500 rpm.
2. Measure each sample three times at 25 °C for 10 s and with an obscuration limit above 3%.
3. Take the measures of three different experiments and determine the mean for determination of size (mean diameter) and the standard deviation (*see* Fig. 2).

3.4 Calculation of Encapsulation Efficiency

3.4.1 Lipidic Nanoparticles

1. Prepare a sterile polystyrene tube (5 mL) with 2 mL of soft agar [0.7% (wt/vol)] and maintain the tubes at 50 °C in a Dry Thermo Block.
2. Make the appropriate dilutions of the result of encapsulation with 10 mM $MgSO_4$ in 1.5 mL microcentrifuge tubes and plate them onto adequate agar plates by the double agar layer method [37], using the appropriate bacterial tester strain.
3. Put the plates in an incubator at 37 °C for 18 h.
4. The bacteriophage concentration obtained corresponds to the concentration of free bacteriophages (C_{free}).

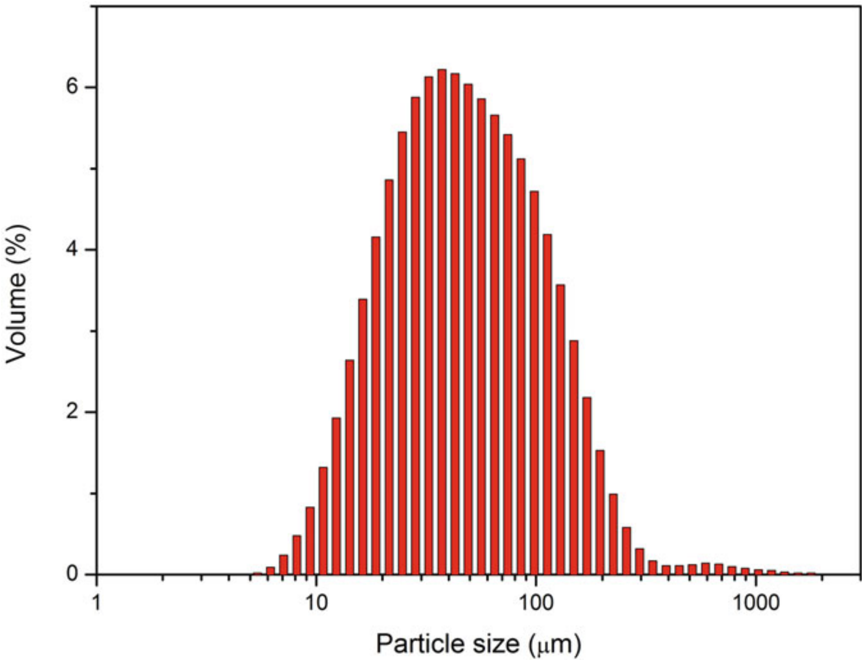


Fig. 2 Example of the particle size distribution of alginate/$CaCO_3$ encapsulated bacteriophage of the *Lederbergvirus* genus, as measured by laser diffraction

5. Treat 0.5 mL of the result of encapsulation with 0.5 mL of 50 mM bile salts to disrupt the liposomes (*see* **Note 7**). Put the mixture in 1.5 mL microcentrifuge tubes.
6. Make the appropriate dilutions after treatment with bile salts with 10 mM $MgSO_4$ and plate them onto adequate agar plates by the double agar layer method [37], as described before. The bacteriophage concentration obtained corresponds to the total bacteriophage concentration (C_{total}).
7. Repeat the experiment at least three times and calculate the mean of C_{free} and C_{total}.
8. Apply the following formula:

$$\text{Encapsulation efficiency }(\%) = [(C_{total} - C_{free})/C_{total}] \times 100$$

3.4.2 Alginate/$CaCO_3$ Microparticles

1. Prepare a sterile polystyrene tube (5 mL) with 2 mL of soft agar [0.7% (wt/vol)] and maintain the tubes at 50 °C in a Dry Thermo Block.
2. Make the appropriate dilutions of the result of encapsulation with 10 mM $MgSO_4$ in 1.5 mL microcentrifuge tubes and plate them onto adequate agar plates by the double agar layer method [37], using the appropriate bacterial tester strain.
3. Put the plates in an incubator at 37 °C for 18 h.

4. The bacteriophage concentration obtained corresponds to the total concentration (C_{total}) (*see* **Note 8**).
5. Take a sample of the result of encapsulation and filter with a syringe of 10 mL through a 0.22 μm PES filter to retain the encapsulated bacteriophages.
6. Make appropriate dilutions of the filtered and plate them onto agar plates, as described before. The bacteriophage concentration obtained corresponds to the concentration of free bacteriophages (C_{free}).
7. Repeat the experiment at least three times and calculate the mean of C_{free} and C_{total}.
8. Apply the following formula:

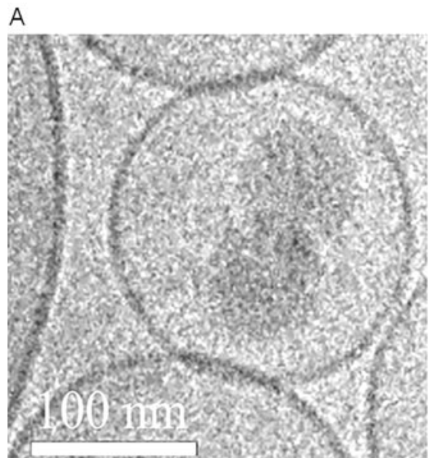
$$\text{Encapsulation efficiency } (\%) = [(C_{total} - C_{free})/C_{total}] \times 100$$

3.5 Microscopy Observation

Liposome integrity (morphology and lamellarity) is examined by cryo-TEM using a JEOL-JEM 1400 microscope.

3.5.1 Lipidic Nanoparticles Cryo-TEM Microscopy

1. Place 5 μL of the result of encapsulation onto carbon-coated film meshes supported by standard copper TEM grids.
2. Leave the grid to dry for 30 s and blot it with a double layer of filter paper to obtain a thin film (*see* **Note 9**).
3. Plunge the grid into liquid ethane at −180 °C and then transfer it into liquid nitrogen (−196 °C) for storage until use.
4. Transfer the vitrified specimens into the microscope and observe the morphology and lamellarity of the capsules containing bacteriophages (*see* Fig. 3a).



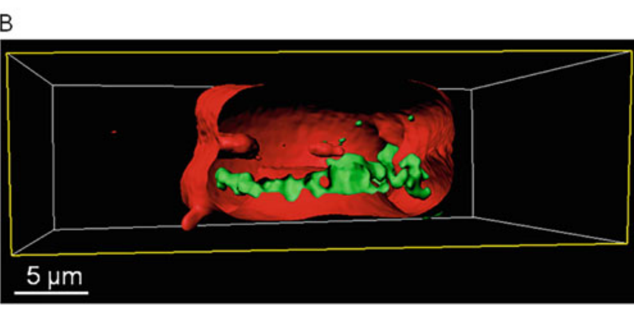


Fig. 3 Example of Cryo-TEM image of a liposome-encapsulate bacteriophage of the *Lederbergvirus* genus (scale bar, 100 nM) (**a**), and 3D confocal microscopy cross-sectional image of this bacteriophage, labeled with SYBR gold (*green*) and encapsulated into fluorescent DiI-labeled liposomes (*red*), (scale bar, 5 μm) (**b**)

Confocal Microscopy

To confirm that the bacteriophages have indeed been encapsulated within the liposomes, fluorescently labeled samples are observed by Leica TCS SP5 confocal microscope.

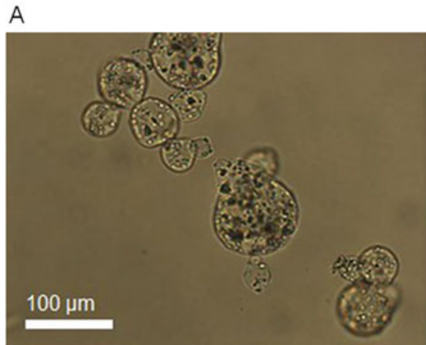
1. Stain the bacteriophage with 100× SYBR-gold by adding 0.02 mL of SYBR-gold to 10 mL of bacteriophages (10^{11} pfu/mL) suspended in 10 mM $MgSO_4$.
2. Incubate overnight at dark [38] and purify using Amicon Ultra-15 Centrifugal Filters 50 K three times (*see* **Note 10**).
3. Prepare fluorescent-labeled lipid thin film (*see* **Note 11**) by adding the Vybrant DiI cell-labeling solution to the DLPC/Chol-PEG/Chol/cholesteryl lipid mixture.
4. Prepare liposomes using the Vybrant Dil fluorescent lipid film and the fluorescent SYBR-gold-labeled bacteriophages, following the protocol described in Subheading 3.1.
5. Add 30 μL of labeled nanocapsules onto a coated glass slide and observe in Leica TCS SP5 confocal microscope (*see* **Note 12)** (*see* Fig. 3b).

3.5.2 Alginate/$CaCO_3$ Microparticles

Optical Microscopy

The morphology of alginate microcapsules is examined in the Zeiss Axio observer.Z1 microscope.

1. Add a drop of microparticles onto a glass slide and observe in the microscope at environmental conditions (*see* Fig. 4a).



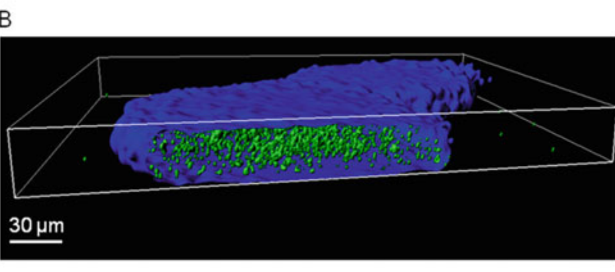


Fig. 4 Example of optical microscopy image of an alginate/$CaCO_3$ encapsulated bacteriophage of the *Lederbergvirus* genus (scale bar, 50 μm) (**a**), and 3D confocal microscopy cross-sectional image of this bacteriophage, labeled with SYBR gold (*green*) and encapsulated into DAPI-labeled alginate/$CaCO_3$ (*blue*) (scale bar, 30 μm) (**b**)

Confocal Microscopy

To confirm that the bacteriophages have indeed been encapsulated within alginate capsules, fluorescently labeled samples are to be observed by Leica TCS SP5 confocal microscope.

1. Label the bacteriophage with SYBR-gold as described in Subheading 3.5.1.
2. Fluorescent label the alginate polymer with 4′,6-diamidino-2′-phenylindole dihydrochloride (DAPI) following a previously described protocol [39].
3. Encapsulate SYBR-gold labeled bacteriophages into a DAPI-alginate matrix, following the protocol described above (*see* Subheading 3.2).
4. Add 30 μL of sample onto a coated glass slide and observe in the Leica TCS SP5 confocal microscope (*see* **Note 13**) (*see* Fig. 4b).

4 Notes

1. Bacteriophages must be used after purification by ultracentrifugation, applying the appropriate speed according to the size of the bacteriophage. After ultracentrifugation, ultrafiltration of the bacteriophage lysate using the Amicon Ultra-15 Centrifugal Filters de 100 K is recommended (100MWCO; Merck Millipore), following the manufacturer's instructions.
2. The total solution volume is 1.2 mL, and the total lipid concentration is 17 mM. This protocol was validated for a maximum lipid solution volume of 30 mL.
3. The times indicated correspond to the Rotavapor R-210 connected to a vacuum pump which allows the total evaporation of the solvent.
4. The volume of the calcium chloride bath is always three times the volume of the phage to be encapsulated.
5. These conditions were validated for a maximum vesicle volume of 60 mL per batch.
6. Typical values of zeta potential range between +31 mV and + 35 mV.
7. This concentration was appropriated for breaking the capsules, allowing the delivery of the bacteriophages. It was confirmed that 50 mM of bile salts had no significant effect on the infectivity of the bacteriophages encapsulated by us with this methodology.

8. In this case, the total concentration of bacteriophages is obtained without additional treatments due to divalent ions (Ca^{2+}), which are essential for the stability of the alginate capsules. Therefore, their degradation when plating might be a consequence of the sequestration of divalent ions during the gelation of the double agar layer.
9. Appropriate film thickness would be between 20 and 400 nm.
10. It is important to adjust the volume with 10 mM $MgSO_4$ between each purification step.
11. The mixture is made by adding 10 μL of Vybrant DiI solution per 20 mg of lipid mixture.
12. The resonance scanning mode of the Leica TCS SP5 confocal microscope is applied due to the fast Brownian movement of the particles due to their small size.
13. In this case, the resonance scanning mode is not necessary as the size of this type of capsule is big enough to reduce their Brownian movement.

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Part III

From In Vitro to Animal Efficacy Studies

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Chapter 8

Phage-Host Interaction Analysis Using Flow Cytometry

Maria Daniela Silva and Luís D. R. Melo

Abstract

Phage-host interactions are commonly evaluated by culture-based methods. However, these techniques are very laborious and time-consuming. Therefore, other time-efficient, not labor-intensive, and cost-effective methods have been developed.

This chapter describes the methodology used to assess the susceptibility of planktonic cultures of bacteria to phage infection and to study their interactions over time by flow cytometry.

Key words Flow cytometry, Phage, Synchronized infection

1 Introduction

Bacteriophages, like all viruses, depend on the host cell for their reproduction. A successful lytic infection cycle involves three distinct programmed steps. Firstly, the phage adsorbs to the susceptible bacterial host cell, injects its genome, and overtakes the host cell machinery to synthesize phage proteins. Subsequently, phage DNA is replicated, and structural proteins are formed. The last step involves the assembling and packaging of phage particles and the lysis of the host cell to release the newly formed phages [1, 2].

An increasing number of bacterial infections are recalcitrant to antibiotics, being difficult to treat by conventional treatment. This has been accompanied by a growing interest in phage therapy. A specific phage can be used to treat an infection caused by a specific bacterial strain, in a tailor-made approach. The selection of the most appropriate phage is, therefore, of utmost importance. Understanding which phage can infect a specific species or even strain and the dynamics of their interaction is very important. This has been commonly evaluated by culture-based techniques, which take a considerable amount of time [3].

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Flow cytometry is a reliable and rapid method that can be used to evaluate the susceptibility of a specific bacterial strain to a specific phage, an alternative to time-consuming plating procedures. By measuring the light scattered and the fluorescence emitted by cells, a bacterial population can be studied at a single-cell level [4]. Flow cytometry has been used to evaluate the antibacterial activity of different compounds by quantifying cell numbers and assessing their viability, allowing the discrimination of live, dead, and damaged cells [5, 6]. The physiological state of the cells and their size can also be evaluated [7]. Importantly, both planktonic and biofilm cells can be analyzed, and their response to phage infection can be investigated by flow cytometry [8–11].

Herein we describe a flow cytometry method to follow the progression of phage infection through bacterial cell staining with viability fluorophores. In addition, to evaluate bacterial viability, SYTO BC can be used as a metabolic probe to evaluate the bacterial physiological state [8]. This is helpful to inform on how the bacteria respond to phage infection through the assessment of the nucleic acid content inside the cell, which can also mean phage replication [9]. This way, the proposed methodology allows the enumeration of bacterial cells and the evaluation of their metabolic activity and viability in real time, being a promising approach to study phage-host interactions and quickly evaluate host susceptibility for phage screening.

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 be used, depending on the requirements of the host bacterium.

2.1 Synchronized Bacteriophage Infection of Planktonic Cultures

1. Purified bacteriophage suspension at minimum 1×10^9 PFU. mL^{-1} (*see* **Note 1**).
2. Lysogeny broth prepared according to the manufacturer's instructions (*see* **Note 2**).
3. Bacterial culture: Place one colony of the bacterial host into a 25 mL glass flask with 5 mL of broth medium (LB or other) and incubate at the appropriate temperature (37 °C or other) for approximately 16 h at 120–150 rpm (*see* **Note 3**).
4. Saline Magnesium buffer (SM buffer): 100 mM NaCl, 8 mM $MgSO_4 \cdot 7H_2O$, and 50 mM Tris–HCl, pH 7.5.
5. Sterile 50 mL and 100 mL glass flasks.

2.2 Flow Cytometry Analysis

1. Purified bacteriophage suspension at minimum 1×10^9 PFU. ml^{-1} diluted in SM Buffer (*see* **Note 1**).
2. Planktonic bacterial cultures at exponential phase.
3. Phosphate-buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4.
4. Vortex.
5. SYTO BC: 250 nm of SYTO BC Green Fluorescent Nucleic Acid Stain (Thermo Fisher Scientific, Waltham, MA, USA) (*see* **Note 4**).
6. Propidium Iodide (PI): 20 $\mu g.mL^{-1}$ propidium iodide (Sigma-Aldrich, St. Louis, MO, USA) (see **Note 4**).
7. Sterile 3 mL Polypropylene (PP) tubes: 75 × 10 mm (*see* **Note 5**).
8. Flow cytometer equipped with a 488 nm laser and FITC and PC5.5 bandpass filters.

3 Methods

Carry out all procedures at room temperature unless specified otherwise.

3.1 Synchronized Bacteriophage Infection of Planktonic Cultures

1. Dilute in PBS 1:100 (vol/vol) the bacterial culture grown for 16 h to a final volume of 20 mL and incubate in a 100 mL flask at an appropriate temperature with agitation (120–150 rpm) until an optical density (OD_{600nm}) that corresponds to approximately 2×10^8 CFU/mL (*see* **Note 6**).
2. Transfer 10 mL of the culture to a new flask and add the bacteriophage suspension in order to obtain a multiplicity of infection (MOI) that ensures a synchronous infection (>95% reduction of bacterial cells in 5 min [12]) (see **Note 7)**.
3. Take one sample immediately (time 0), incubate the suspension at an appropriate temperature with agitation (120–150 rpm), and take samples at different time points (corresponding to early, middle, and late phases of phage infection) for flow cytometry analysis (*see* **Notes 8** and **9**).

3.2 Flow Cytometry Analysis

1. Open a new protocol in the flow cytometer software using a 488 nm laser.
2. Set the plots listed below, on a logarithmic scale, for bacteria visualization:
   (a) Forward Scatter (FSC) vs. Side Scatter (SSC)—relative size vs. granularity.
   (b) SYTO BC vs. PI (*see* **Note 10**).

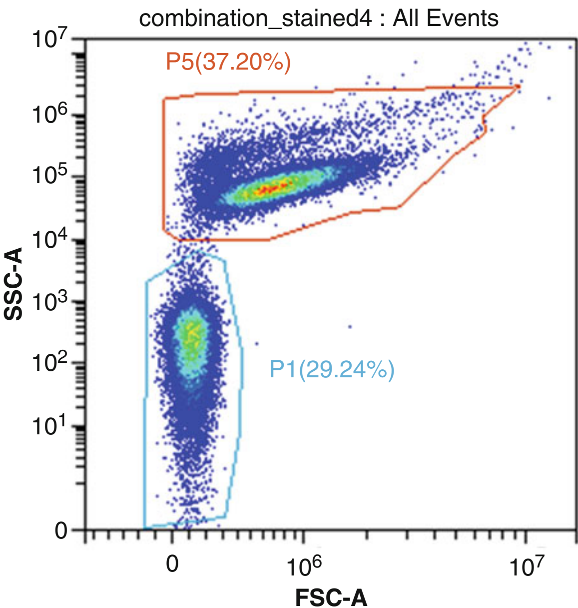

Fig. 1 Representative FSC vs. SSC dot plot with the bacterial population on-scale, with region P5 set around the target bacterial population and region P1 around the background

3. Set the histograms listed below, on a logarithmic scale, for bacterial fluorescence visualization:
   (a) SYTO BC (choose the appropriate channel).
   (b) PI (choose the appropriate channel).
4. Set the sample volume to be analyzed to 0.25 volumes of the total sample volume (e.g., 75 μL of a 300 μL sample).
5. Set the flow rate to 10 μL/min (*see* **Note 11**).
6. Acquire 300 μL of PBS suspension to define the background in the FSC vs. SSC dot plot.
7. Add 30 μL of bacterial suspension (1:10 diluted) into a PP tube with 270 μL of PBS and acquire the events on the flow cytometer (*see* **Note 12**).
8. Create a gate to include all dot plots in the bacterial population (*see* Fig. 1).
9. Adjust the gains adequately, so the unstained bacteria are 100% on the unstained region for both colors.
10. Add 30 μL of bacterial suspension (1:10 diluted) into a PP tube with 270 μL of a solution containing 250 nM of SYTO BC (*see* **Note 13**).
11. In a new PP tube, add 30 μL of planktonic suspension (1:10 diluted) and 270 μL of a solution containing 20 μg.mL^{-1} of PI (*see* **Note 13**).

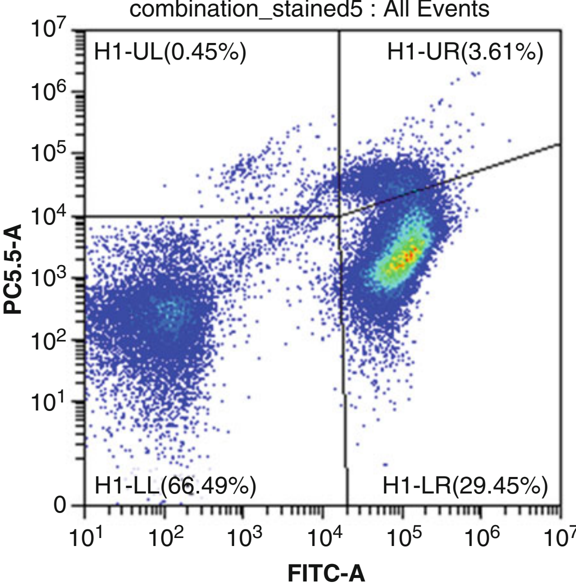

Fig. 2 Representative SYTO BC vs. PI dot plot of double-stained bacteria. H1-LL – SYBR-/PI- (cellular debris), H1-LR – SYBR+/PI– (live cells), H1-UR – SYBR+/PI+ (compromised cells), and H1-UL – SYBR-/PI+ (dead cells)

12. Homogenize the samples by vortexing for 5 s.
13. Incubate all samples for 5–20 min at room temperature and protect from the light (*see* **Note 14**).
14. Acquire single-stained samples and set the compensations if necessary (*see* **Note 15**).
15. Add 30 μL of each bacterial suspension (1:10 diluted) into a PP tube with 270 μL of a solution containing 250 nM of SYTO BC and 20 $\mu g.L^{-1}$ of PI.
16. Vortex as above, incubate for 5–20 min all samples at room temperature, and protect from the light.
17. Acquire the events of double-stained samples (*see* Fig. 2). The compensation values might need slight modifications due to the interaction of both stains (*see* **Note 16**).
18. Analyze the obtained data regarding:
    - (a) SYTO BC Median fluorescence intensity and the number of peaks. If differences in this parameter are detected, they indicate an increase in the nucleic acid content inside the cell, which may be a consequence of phage replication.
    - (b) Cell counts per μL (*see* **Note 17**).
    - (c) Number of intact, compromised, and dead cells.

4 Notes

1. Use the method described by Sambrook and Russell for bacteriophage purification [13].
2. Lysogeny broth is commercially available, but it may also be prepared using the following components: 10 $g.L^{-1}$ of tryptone, 10 $g.L^{-1}$ of sodium chloride, and 5 $g.L^{-1}$ of yeast extract. Adjust the pH to 7.0 with 5 N NaOH.
3. The procedure is described for fast-growing bacterial species. For instance, *Staphylococcus epidermidis* overnight cultures reach approximately 8×10^9 $CFU.mL^{-1}$. However, if experiments are performed with slow-growing bacterial species, the incubation time might need adjustments.
4. Analysis of the viability of bacterial cells by flow cytometry can be performed by LIVE/DEAD® staining. The membrane integrity of the cells allows the assessment of their viability by using this dual-stain system, which contains the green SYTO BC and red propidium iodide (PI) dyes [14]. SYTO BC penetrates the membrane of all cells and binds their DNA, while PI can only penetrate damaged membranes [14]. Since PI displays a stronger affinity for nucleic acids, SYTO BC is displaced by PI and, therefore, live cells will be stained with green and dead cells with red fluorescence [14, 15]. SYBR green can also be used as an alternative to SYTO BC as a component of the LIVE/DEAD staining in order to evaluate cell viability by flow cytometry [14, 16].
5. Although typically 3 mL PP tubes are used for flow cytometry analysis, depending on the equipment, other recipients can be used (e.g., polypropylene 2 mL tubes and 96-well microtiter plates).
6. A concentration of 10^8 $CFU.mL^{-1}$ is optimal to have a good resolution in the majority of flow cytometers. Samples can be saturated and have abortive events if they are more concentrated ($>10^9$ $CFU.mL^{-1}$). In opposition, more diluted samples (10^7 $CFU.mL^{-1}$) can be near the limit of detection of some flow cytometers and consequently not well detected.
7. A multiplicity of infection of 50 guarantees a synchronous infection for *Pseudomonas aeruginosa* phages [10].
8. Not all phages have a lytic cycle corresponding to early, middle, and late phage infection. Nevertheless, this method can monitor the progress of phage infection [17].
9. Samples should be immediately analyzed.
10. To detect SYTO BC and PI fluorescence, 525/40 and 610/20 nm bandpass filters are typically used.

11. A low flow rate is suggested to be used in DNA analysis; however, this should be optimized for every flow cytometer.
12. Using unstained bacteria will define the bacterial population on FSC vs. SSC dot plot drawing a gate region rounding bacterial cell events. This gating will eliminate the electronic background and/or debris interference. Individual FSC and SSC histograms should be analyzed to guarantee that the populations are not cut off on the display. Peak shapes and resolution from noise will vary with bacterial morphology and sample matrix. The gate will vary with bacterial morphology and sample matrix.
13. When the emission spectra of different fluorochromes overlap the fluorescence derived from more than one fluorochrome may be detected. To correct this phenomenon, fluorescence compensation might be used. It is important to analyze single-stained samples to guarantee that the fluorescence detected in a particular detector is derived from the fluorochrome being measured (e.g., SYTO BC-stained bacteria should be SYTO BC positive and PI negative, while PI-stained bacteria should be SYTO BC negative and PI positive).
14. The time of incubation varies with bacterial morphology and sample matrix (e.g., 5–10 min for *Staphylococcus epidermidis* and 20 min for *Pseudomonas aeruginosa*).
15. Compensation is a correction of a possible signal overlap between the channels of the emission spectra of different fluorochromes. This is particularly important for multi-channel experiments.
16. If the protocol was well optimized for both unstained and single-stained cells, it should be adequate for double-staining.
17. Most flow cytometers are not able to directly provide the cell concentration or absolute count of cells in a sample. In those cases, cell counting beads should be used.

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Chapter 9

Bacteriophage Control of Infectious Biofilms

Luciana Meneses, Sanna Sillankorva, and Joana Azeredo

Abstract

Biofilm formation, a strategy of bacterial survival, is a significant concern in different areas, including health, where infectious biofilms are very difficult to combat with conventional antimicrobial therapies. Bacteriophages, the viruses that infect bacteria, are promising agents to prevent and control biofilm-related infections. This chapter describes a series of standard procedures that can be used to study the potential of bacteriophages for biofilm control, from biofilm formation to bacteriophage treatment and evaluation of its efficacy.

Key words Bacteriophage, Biofilm, Control, Quantification

1 Introduction

The biofilm mode of growth is the principal bacterial and archaeal lifestyle, representing 40–80% of all bacterial biomass on Earth [1]. Biofilms are characterized by aggregates of microbial communities surrounded by a matrix of extracellular polymeric substances (EPS) [2]. Over the years, in vivo knowledge has made it clear that biofilm formation and growth are not dependent on a surface, as biofilms can exist as non-surface-attached or surface-attached aggregates. Therefore, the biofilm life cycle process can now be defined by three major steps: (a) the bacteria start forming the suspended or attached aggregate, (b) the bacteria produce EPS and accumulate more bacteria for aggregate expansion, and (c) the disaggregation of parts of the biofilm or detachment of single cells ready to colonize other niches [3]. Biofilm formation constitutes a protective mode of growth in which bacteria are more tolerant to external pressures, such as changing environmental conditions and antimicrobial agents [4, 5].

The high tolerance of biofilms to antibiotics is a critical concern in health care, making the treatment of bacterial infections extremely challenging [6, 7]. Therefore, developing alternative or

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complementary strategies to conventional antibiotic therapy is crucial for preventing and treating biofilm-related infections. Bacteriophages, the viruses of bacteria, are ubiquitous in the environment and are considered very promising for controlling bacterial biofilms [8]. Besides the natural ability of bacteriophages to specifically infect bacteria by injecting the DNA and replicating inside of the host to release new phage particles through cell lysis, bacteriophages may also have other features that make them attractive for biofilm control. These include the production of polysaccharide-degrading enzymes able to disrupt the biofilm matrix and enhance bacteriophage penetration and replication inside of the biofilm or the ability of bacteriophages to infect stationary-phase cells [9].

In human patients, the use of bacteriophages to treat bacterial infections—Phage Therapy—can be particularly beneficial for infections caused by multidrug-resistant bacteria and biofilm-related infections, where antibiotic treatment frequently fails. Therefore, in recent years, many case reports with positive outcomes of using Phage Therapy for biofilm control have been reported [10]. However, further research is still needed to assess the best treatment conditions (e.g., dosing, duration, antibiotic combinations) to ensure a good treatment outcome. For that, it is essential to develop standardized methods to evaluate bacteriophage efficacy against biofilms formed in vitro, taking into account the specific features of each biofilm prior to clinical use [11, 12].

Although other methods can be used to evaluate bacteriophage/biofilm interactions in vitro, such as three-dimensional infection models, microtiter plate-based methods are still the most widely used. These methods enable the high throughput testing of multiple variables simultaneously and can be easily adapted to simulate different growth conditions, including temperature and shaking [13].

In this chapter, we detail a comprehensive protocol to evaluate the efficacy of bacteriophages against in vitro biofilms, which can be easily adaptable for different bacterial strains and culture conditions. The protocol contains details on how to handle surface-attached biofilms, from biofilm formation to treatment with bacteriophages and evaluation of their efficacy through two different biofilm assessment methods—viable cells enumeration by colony-forming unit (CFU) counts and total biomass quantification by crystal violet (Fig. 1). To better mimic the host environment, for a better translation of bacteriophage efficacy from in vitro studies to clinical use, clinically relevant growth media simulating the body fluids can be used for biofilm growth, and combination with other antimicrobials can also be tested following the same protocol.

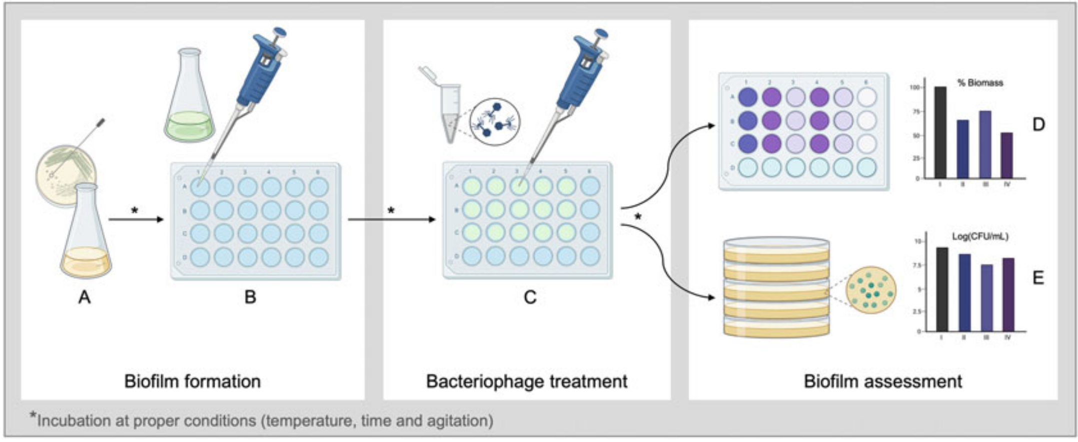

Fig. 1 Schematic of in vitro evaluation of bacteriophage treatment of biofilms. A bacterial inoculum is prepared from a fresh agar plate of the desired strain and incubated at proper conditions (**a**). The microplate is inoculated with overnight grown bacteria diluted with the desired culture medium (**b**). The spent culture medium is replaced with fresh medium and the desired bacteriophages are added to the desired concentration or multiplicity of infection (MOI) (**c**). Biofilm control efficacy by bacteriophages is evaluated at least based on reduction of biomass (crystal violet assay) (**d**) and reduction of viable cell counts (CFU/mL) (**e**), preferably together with bacteriophage titration. [To study the prevention of biofilm formation, bacteriophages can be added before or during microplate inoculation with bacteria (**b**)]

2 Materials

Pseudomonas aeruginosa strain PAO1 is used in this protocol as an example strain. Lysogeny broth (LB) is the culture medium used in the procedures described, but other alternative media can be used (*see* **Note 1**). All the solutions are prepared using distilled water and stored at room temperature unless indicated otherwise.

2.1 Biofilm Formation

1. *P. aeruginosa* strain PAO1 as −80 °C frozen stock [20% (v/v) glycerol].
2. LB agar (LBA) plates: prepare LB according to the manufacturer's instructions and add 1.2–1.5% (wt/vol) of agar (*see* **Note 2**). Autoclave at 121 °C for 15 min and pour 20 mL onto standard 90 mm × 15 mm Petri dishes.
3. Sterile LB: prepare LB according to the manufacturer's instructions. Autoclave at 121 °C for 15 min.
4. Sterile 50 mL Erlenmeyer flask with an aluminum lid.
5. 24-well sterile microplates (*see* **Note 3**).
6. Sterile inoculation loop (10 μL).

2.2 Biofilm Control with Bacteriophages

1. Liquid stocks of bacteriophages stored at 4 °C.
2. Sterile LB (*see* Subheading 2.1).
3. Sterile Saline Magnesium buffer (SM buffer): prepare 1 M Tris-HCl buffer (pH 7.5)—add 6.06 g of Tris-Base into a 250 mL bottle to a final volume of 50 mL in water, adjust the pH to 7.5 with HCl, and sterilize by autoclaving for 15 min at 121 °C. Prepare SM buffer—add 5.8 g of NaCl, 2.0 g of $MgSO_47H_2O$, 50 mL of 1 M Tris-HCl (pH 7.5), and water to a final volume of 1 L in a 1 L bottle and sterilize by autoclaving for 15 min at 121 °C.
4. Sterile 15 mL Falcon tubes.
5. 24-well microplate (*see* **Note 3**) with pre-formed PAO1 biofilms (*see* **Note 4**).

2.3 Evaluation of Biofilm Treatment with Bacteriophages

2.3.1 Quantification of Biofilm Culturable Cells

1. Sterile LB (*see* Subheading 2.1).
2. LBA plates (*see* Subheading 2.1).
3. Sterile saline: prepare 0.9% NaCl in water—weigh 4.5 g of NaCl into a 500 mL bottle and add 500 mL of distilled water. Autoclave at 121 °C for 15 min.
4. Sterile cell scrapers.
5. Sterile 96-well microplates.

2.3.2 Quantification of the Biofilm Biomass

1. Sterile saline (*see* Subheading 2.3).
2. Methanol 100% (vol/vol).
3. Crystal violet 1% (vol/vol).
4. Distilled water.
5. Acetic acid 33% (vol/vol).
6. Sterile 96-well microplates.

2.3.3 Bacteriophage Titration by Drop Plaque Assay

1. Overnight grown bacterial culture.
2. Sterile LB soft agar: prepare LB according to the manufacturer's instructions with 0.4–0.7% (wt/vol) of agar. Autoclave at 121 °C for 15 min and store at 50–60 °C. For over 2 days, store the soft agar at 4–21 °C and melt it before use.
3. LBA plates (*see* Subheading 2.1).
4. Sterile SM buffer (*see* Subheading 2.2).
5. Sterile 96-well microplates.

3 Methods

Carry out all procedures at room temperature unless stated otherwise.

3.1 Biofilm Formation

1. Streak the bacterial strain PAO1 from −80 °C stock into an LBA plate using an inoculation loop.
2. Incubate the plate overnight (~16 h) at 37 °C.
3. Use a loop to inoculate a single colony of PAO1 from the fresh agar plate into a 50 mL Erlenmeyer flask filled with 15 mL LB.
4. Incubate the flask overnight at 37 °C with constant shaking (*see* **Note 5**).
5. Add 990 μL of LB per well to a 24-well microplate (*see* **Note 6**).
6. Add to each well 10 μL of PAO1 overnight culture adjusted to an OD_{600} of 1.0 (*see* **Note 7**).
7. Let the biofilm grow for 24 h (*see* **Note 8**) at 37 °C with a constant agitation of 120 rpm (*see* **Note 9**).

3.2 Biofilm Control with Bacteriophages

1. Prepare the bacteriophage working solutions. Start by calculating the volume of bacteriophage needed for the biofilm control experiment according to the desired final bacteriophage concentration [plaque-forming units (PFU) per mL] using Eq. 1 or according to the desired multiplicity of infection (MOI) using Eq. 2 (*see* **Note 10**). Once the final concentration is known, perform dilutions of the bacteriophage stocks of known titer in SM buffer using 15 mL Falcon tubes.

$$\text{Phage stock titer (PFU/mL)} \times \text{Volume of phage (mL)} = \text{Phage final concentration (PFU/mL)} \times \text{Volume of the well (mL)} \quad (1)$$

$$\text{MOI} = \frac{\text{Phage stock titer (PFU/mL)} \times \text{Volume of phage (mL)}}{\text{Biofilm culturable cells (CFU/mL)} \times \text{Volume of the well (mL)}} \quad (2)$$

2. Gently remove all spent medium from each well of the 24-well microplates resulting from Subheading 3.1, without touching the bottom and sides of the well. This contains non-adhered cells (*see* **Note 11**).
3. Wash with 1 mL of fresh LB (*see* **Note 12**).
4. Add 990 μL of fresh LB (*see* **Note 13**).
5. Add the bacteriophage at the final desired concentration (PFU/mL) or MOI to the biofilm formed, using at least two replicate wells for each condition tested. For instance, for a final

bacteriophage concentration of 1×10^8 PFU/mL or for an MOI of 1, starting from a bacteriophage stock with a titer of 1×10^{10} PFU/mL and with a biofilm with 1×10^8 CFU/mL, add 10 μL of bacteriophage in the 24-well microplate.

6. Leave two well for the untreated controls, where 990 μL of LB is added instead.
7. Incubate for 24 h (*see* **Note 8**) at 37 °C with a constant agitation of 120 rpm (*see* **Note 9**).

3.3 Evaluation of Biofilm Treatment with Bacteriophages

This protocol describes the two most commonly used methods to evaluate the effect of bacteriophages on biofilms: quantification of biofilm culturable cells by the CFU assay and quantification of biofilm biomass by crystal violet assay. However, additional methods can be used (*see* **Note 14**). It is also important to quantify the bacteriophages during biofilm treatment to understand the population dynamics.

3.3.1 Quantification of Biofilm Culturable Cells

1. Gently remove all the spent medium from each well (*see* **Note 15**) without touching the bottom and sides of the well.
2. Wash with 1 mL of sterile saline.
3. Add 1 mL of fresh sterile saline.
4. Use a cell scraper to scrap the biofilm (*see* **Note 16**).
5. Transfer 200 μL of each well, by pipetting up and down to mix the samples, to the first row of a 96-well microplate.
6. Add 180 μL of saline to the remaining wells.
7. Perform successive serial dilutions (1:10) (*see* **Note 17**): Add 20 μL of each sample to 180 μL of saline, starting from the first row to the last row of the microplate, to obtain serial dilutions from 0 to 10^{-7}.
8. Plate a 5 μL drop of each dilution (10^{-2} to 10^{-7}) in triplicate on an agar plate with LB.
9. Let the agar plates stand still until the drops have completely dried.
10. Incubate the plates overnight at 37 °C.
11. Count the colonies formed in the drop of the dilution with 5–50 bacterial colonies.
12. Calculate the number of CFUs per mL using Eq. 3.

$$\text{Biofilm culturable cells (CFU per mL)} = \frac{\text{Nr. of colonies} \times \text{Dilution factor}}{\text{Volume of sample plated (mL)}} \tag{3}$$

13. Convert the amount of biofilm culturable cells to log density per mL ($\log_{10}$ CFU/mL) and compare the log reduction

between all the conditions tested. The reduction obtained is calculated taking into account the $\log_{10}$ CFU/mL of the control samples and the $\log_{10}$ CFU/mL of the phage-treated samples.

3.3.2 Quantification of Biofilm Biomass

1. For biofilms formed in a 24-well microplate, gently remove all the spent medium from each well (1 mL) (*see* **Note 18**).
2. Wash with 1 mL of saline without disturbing the biofilm (*see* **Note 6**) and remove the added volume.
3. Wash again carefully with 1 mL of saline and remove the added volume.
4. Add 1 mL of methanol to fix the biofilm.
5. Leave the plates on the hood, taking care not to disturb the biofilm. Leave the plates without the lid for 15 min or until the wells have completely dried.
6. Add 1 mL of 1% crystal violet to stain the biofilm and leave for 15 min without disturbing the biofilm.
7. Remove 1 mL of crystal violet and wash the excess with 1 mL of distilled water without disturbing the biofilm.
8. Add 1 mL of 33% acetic acid and pipette up and down to dissolve the stain.
9. Transfer 200 μL of the resulting solution to a 96-well microplate and read the absorbance at 595 nm.
10. Convert the absorbance values to percentages based on the untreated control wells and compare the percentage of biofilm biomass reduction between all the conditions tested.

3.3.3 Bacteriophage Titration by Drop Plaque Assay

1. To calculate the bacteriophage titer on the planktonic phase use a 50 μL sample of spent medium (containing non-adhered cells). For bacteriophage counts in the biofilm phase use a 50 μL sample of the homogenate after biofilm dispersion (*see* Subheading 3.3.1).
2. Transfer 200 μL of the bacteriophage samples to the first row of a 96-well microplate.
3. Fill the remaining wells with 180 μL of SM buffer and prepare successive serial dilutions (1:10) (*see* **Note 17**): Add 20 μL of each sample to 180 μL of SM buffer, starting from the first row to the last row of the microplate, to obtain serial dilutions from 0 to 10^{-7}.
4. Add 100 μL of overnight grown bacterial culture and 3–5 mL of LB soft agar to a 90 mm × 15 mm Petri dish containing LBA, gently swirl to spread the volume to the whole plate, and let dry for 10 min.

5. Plate a 10 μL drop of each dilution on the upper part of an agar plate from the previous step, tilt the plates at 75°, and stop before the drops touch the other side of the plate.
6. Let the agar plates stand still, with the lids open, until the drops have completely dried.
7. Incubate the agar plates overnight at 37 °C.
8. Count the phage plaques formed in the drop of the dilution with 5–50 phage plaques.
9. Calculate the bacteriophage titer in PFUs per mL using Eq. 4.

$$\text{Bacteriophage titer (PFU per mL)} = \frac{\text{Nr. of phage plaques} \times \text{Dilution factor}}{\text{Volume of sample plated (mL)}} \quad (4)$$

4 Notes

1. Lysogeny broth is commonly used for *P. aeruginosa* growth, but other media can be used instead, such as Tryptic Soy Broth (TSB), nutrient broth (NB), or media mimicking in vivo conditions. LB is commercially available or can be prepared as follows: add 10 g of tryptone, 10 g of NaCl, and 5 g of yeast extract to a final volume of 1 L in water; adjust the pH to 7.0 with NaOH; and sterilize by autoclaving for 25 min at 120 °C.
2. Alternatively, commercially available LBA can be used according to the manufacturer's instructions.
3. Other sizes of well plates can be used instead, such as 48-well or 96-well plates.
4. This protocol focuses on the use of bacteriophages to control established biofilms, but it can be adapted to study the ability of bacteriophages to inhibit biofilm formation by adding the bacteriophages before or at the same time as the bacterial culture for biofilm formation (*see* Subheading 3.1, **step 6**).
5. Different shaking speeds can be used for bacterial culture growth (100–250 rpm).
6. According to the type of microplate, the volumes should be adjusted to a maximum final volume of 200 μL for 96-well plates, 400 μL for 48-well plates, and 1 mL for 24-well microplates.
7. A PAO1 culture in LB with an OD_{600} of 1.0 corresponds to approximately 10^9 CFU/mL. For other strains or other bacterial species, the correspondence between OD_{600} and CFU/mL should be previously checked and used in the experiment to obtain 10^6–10^7 CFU/mL of bacteria on each well to start biofilm formation.

8. Incubation time can be changed to better mimic the in vivo conditions, according to the type of biofilm being studied. The culture medium should be renewed every 24 h to promote biofilm growth and not the growth of planktonic cells.
9. Biofilms can also be grown with different shaking speeds or without shaking to better mimic the in vivo conditions, according to the type of biofilm being studied.
10. The number of biofilm culturable cells (CFU/mL) should be previously calculated after the desired time of biofilm growth in three independent experiments performed at least in duplicate, following the steps described for the quantification of biofilm culturable cells (see Subheading 3.3.1).
11. *P. aeruginosa* PAO1 forms surface-attached biofilms in LB. However, the use of other strains or culture conditions can lead to the formation of non-surface-attached biofilms. For suspended biofilms: remove half of the spent medium from each well (500 μL) without disturbing the bacterial aggregates and gently add 500 μL of fresh LB.
12. The washing step can also be performed with sterile saline instead of LB.
13. For other well sizes of microplates, the volume of medium to be replaced should also be half of the total volume used on each well for suspended biofilms and all the volume used on each well for attached biofilms (*see* **Note 6**).
14. The evaluation of bacteriophage effect on biofilm prevention or control should not be based on a single method. Results from biofilm biomass characterization should be complemented with CFUs quantification. Also, additional methods can be used in combination, including biofilm imaging and metabolic activity assays.
15. In the case of suspended biofilms: replace the well plate lid with a sealing tape and begin biofilm dispersal by placing the plate first in a shaker for 5 min at 900 rpm and then in an ultrasonic bath for 5 min at 40 kHz. Shaking and sonication conditions should be optimized for a good biofilm dispersion. Alternatively, suspended biofilms can be homogenized using a vortex, homogenizer, or bead ruptor, after transferring the biofilm to an appropriate tube.
16. Alternative biofilm dispersion methods, such as sonication, can also be implemented for surface-attached biofilms.
17. Alternatively, 1.5 mL Eppendorf tubes can be used to perform serial dilutions (100 μL of each sample to 900 μL of solvent).
18. For the crystal violet assay, the biofilms need to be attached to the wells for the bacterial aggregates not to be removed during the washing steps. Therefore, this method does not apply to suspended biofilms.

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Chapter 10

Studying Bacteriophage Efficacy Using a Zebrafish Model

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Abstract

The rise of bacteria resistant to the antibiotics currently in use (multiple drug-resistant, MDR) is a serious problem for patients affected by infections. This situation is even more worrying in the case of chronic bacterial infections, such as those caused by *Pseudomonas aeruginosa* (*Pa*), in patients with cystic fibrosis (CF). As an alternative to antibiotic treatments, the use of bacteriophages (phages) to fight bacterial infections has gained increasing interest in the last few years. Phages are viruses that specifically infect and multiply within the bacteria without infecting eukaryotic cells. It is well assumed that phage therapy has a high bacterial specificity, which, unlike antibiotics, should limit the damage to the endogenous microbiome. In addition, phages can kill antibiotic-resistant bacteria and perform self-amplification at the site of the infection.

The protocol detailed in this chapter describes how the antimicrobial effect of phages can be studied in vivo in the zebrafish (*Danio rerio*) model infected with *Pa*. The same procedure can be applied to test the effectiveness of several different phages killing other bacterial species and for the rapid preclinical testing of phages to be used as personalized medicine.

Key words Zebrafish, *Pseudomonas aeruginosa*, Phages, Fluorescence imaging, Bacterial burden, Infection model

1 Introduction

The use of bacteriophages (phages) to fight bacterial infections has gained increasing interest in the last years as an alternative to antibiotic treatments. Phages are viruses that specifically infect and multiply within bacteria, without infecting eukaryotic cells [1]. In Eastern Europe, phages have been used for decades to treat a variety of bacterial infections, and thus, they are considered generally safe. On the contrary, in the Western world, phage therapy was mostly eclipsed by the advent of antibiotics and only recently has regained interest in overcoming the problem of multiple drug-resistance (MDR) insurgence [2]. Besides several cases of compassionate treatments [3–10], the idea of phage therapy as a

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standard therapy protocol is getting closer, as witnessed by the starting of various clinical trials aiming to assess the safety and effectiveness of phage mixtures against *Pseudomonas aeruginosa* (*Pa)* in patients with cystic fibrosis (CF) (ClinicalTrials.gov Identifier: NCT04596319).

At present, few animal models are used to test the phage efficacy as antimicrobials. Among the invertebrates, there are the insects *Drosophila melanogaster* and *Galleria mellonella*, and the worm *Caenorhabditis elegans*, whereas among vertebrates, mouse, rabbit, chicken, hamster, and zebrafish models are used [11]. Zebrafish (*Danio rerio*) is for sure the cheapest and easiest vertebrate model to manipulate and the response in terms of antimicrobial activity of phages is comparable to that obtained with other animals [12, 13]. Zebrafish embryos are particularly suitable for the study of host-bacterial interactions, and the innate immune system is evolutionarily conserved between humans and fish. The embryos are transparent and genetically tractable, making them more useful than mammalian embryos for studying aspects of infectious diseases. For instance, it is possible to perform both a systemic infection, by injecting the pathogens into the circulation, and a local infection by injecting subcutaneously, intramuscular or intraventricular (eventually followed by phage administration via the same route).

Moreover, it is to note that until 5 days post fertilization, zebrafish embryos are not considered an animal model for the Italian and European laws (Italian D.lgs March 26, 2014 and 2010/63/EU and 89/609/EEC), thus allowing to follow the 3R rules (Reduction, Refinement, and Replace) for animal use in research and to cut the costs for the tests.

Following bacterial infection, the success of phage therapy treatments can be easily demonstrated by increased survival of the embryos and decreased bacterial burden after plating homogenized embryos, offering the possibility to validate its efficiency in a quick (5 days) and cheap way. In this chapter, we describe our screening platform for phage efficacy, which includes in vitro and in vivo analyses. We applied our platform for the screen of phages able to kill *Pa* strains, but this system could be applied to test phage efficacy against other bacteria isolated directly from patients in the frame of a personalized infection treatment.

2 Materials

This protocol is described with *Pseudomonas aeruginosa* (*Pa*) PAO1 strain [14] carrying a GFP plasmid (GFP^+-PAO1) conferring resistance to carbenicillin [12], as a model strain. Two Schitoviridae (formerly classified as Podoviridae), vB_PaeP_PYO2 (GenBank accession: MF490236) and vB_PaeP_DEV (GenBank accession: MF490238), and two Myoviridae, vB_PaeM_E215 (GenBank

accession: MF490241) and vB_PaeM_E217 (GenBank accession: MF490240), are used (individually or assembled in a phage cocktail). Phages were isolated using PAO1 strain following standard procedures [15] (not described in this protocol), by identifying clear plaques to produce raw phage stocks. To develop zebrafish models of infection and phage therapy, the wild-type AB strain (European Zebrafish Resource Center, EZRC) is used. All buffers and media used are sterilized by autoclaving (121 °C, 15 min) unless otherwise stated.

2.1 GFP$^+$-PAO1 Culture Preparation

1. Lennox lysogeny (LB) broth: weigh 10 g of NaCl, 5 g of yeast extract, and 10 g of tryptone and add to 1 L of distilled water (pH = 7–7.2). For LB supplemented with carbenicillin, add 1 mL of carbenicillin (300 mg/mL) to 1 L of LB.
2. LB agar plates (for bacterial plating): add 10 g of agar to 1 L of LB broth. Pour 25 mL per standard 90 mm × 15 mm Petri dish. For plating phages: use 5.7 g/L of agar in LB broth (*see* **Note 1**).
3. Incubator for bacterial plates.
4. Benchtop centrifuge (1.5–2 mL tubes).

2.2 High-Titer Bacteriophage Stocks Preparation and Purification

1. LB broth and LB agar plates prepared as described in Subheading 2.1.
2. LB soft agar: Add 6 g of agar to 1 L of LB broth.
3. RNase stock solution (1 mg/mL).
4. DNase stock solution (1 mg/mL).
5. NaCl powder.
6. PEG 6000 powder.
7. TN buffer: Add 1.21 g of Tris HCl pH 8.0 and 8.77 g of NaCl to 1 L of distilled water.
8. CsCl solutions for density gradients: $d = 1.3$, add 20.49 g to 50 mL of TN; $d = 1.4$, add 20.28 g to 50 mL of TN; $d = 1.5$, add 34.13 g to 50 mL of TN; $d = 1.6$, add 41.2 g to 50 mL of TN.
9. Syringe filters: pore diameter size 1.2 μm and 0.22 μm.
10. Dialysis tubes with membrane cut-off = 6000 Dalton.
11. Bacterial endotoxin removal column, with specific affinity for lipopolysaccharide (LPS).
12. Transparent and thinwall polyallomer ultracentrifuge tubes.
13. Incubator for bacterial plates.
14. Shaker with rotatory function.
15. Benchtop centrifuge (1.5–2 mL tubes).
16. Ultracentrifuge.

2.3 Zebrafish (Danio rerio) Breeding and Embryos Collection

Adult zebrafish must be bred according to international (EU Directive 2010/63/EU) and national guidelines (Italian decree March 4, 2014, n. 26) on the protection of animals used for scientific purposes. Briefly, adult zebrafish are maintained in a circulating water system according to general guidelines [16, 17], following a 14/10 h light/dark cycle, a constant temperature of 28 °C, optimal pH of 7.2, and conductivity values between 400 and 600 μS, respectively.

1. Egg collection: common kitchen fine-mesh strainer or 3 mL plastic Pasteur pipettes are used.
2. 1X E3 embryo medium: prepare a 50X E3 stock solution by adding 73.0 g of NaCl, 3.15 g of KCl, 9.15 g of $CaCl_2$, and 9.95 g of $MgSO_4$ in 5 L of distilled H_2O. The 50X E3 doesn't need to be autoclaved and is stored at room temperature (RT). Dilute the 50X E3 solution 1:50 in distilled water and add 200 μL of 0.05% methylene blue.
3. 1X 1-Phenyl-2-Thiourea embryo medium (1X PTU): Prepare a 10X concentrated PTU stock solution by adding 0.3 g of PTU in 1 L of 1X E3. Dilute (1:10) the 10X PTU solution in 1X E3 medium. PTU solutions don't need to be autoclaved and are stored at 4 °C.
4. Incubator for embryo growth (temperature 22–34 °C).

2.4 Bacterial Infection and Phage Treatment

1. GFP^+-PAO1 culture.
2. Bacteriophage suspension at different concentrations, diluted in TN buffer and prepared immediately before use.
3. Physiological solution: Add 9 g to 1 L of distilled H_2O.
4. Phenol red vital tracer, stock solution (Sigma Aldrich).
5. 1X E3 and 1X PTU embryo media (*see* Subheading 2.3).
6. Pronase working solution (5 mg/mL): Prepare the 5X pronase stock solution by dissolving 50 mg of pronase powder into 10 mL of 1X E3; prepare aliquots and store them at −20 °C. To prepare the pronase working solution (1 mg/mL), add 1 vol of the working solution to 4 vol of 1X PTU.
7. 1X tricaine working solution: first prepare a 25X tricaine stock solution by adding 0.08 g of tricaine powder in 20 mL of deionized H_2O. Prepare aliquots and store at −20 °C. To prepare the 1X tricaine working solution, dilute 1:25 the 25X tricaine stock solution in 1X PTU.
8. Petri dishes: 90 mm × 15 mm and 35 mm × 15 mm.
9. Stereomicroscope: M205FA, Leica (Wetzlar, Germany) equipped with a fluorescent lamp and a GFP filter (excitation 488 nm).

10. Borosilicate-glass micro-injection needle. The needles are obtained from borosilicate capillaries (1 mm O.D. × 0.78 mm I.D.; Harvard Apparatus, Holliston, Massachusetts, USA). The capillaries are mounted on a puller, where they are heated in the center while being pulled at both ends. In this way, the heated region extends and gets thinner until it breaks down into two micro-needles. Adjust puller parameters to get the most appropriate shape and dimension of the needles (*see* **Note 2**).
11. 1.5% Agarose gel tracks for microinjection: Agarose gel supports with tracks for positioning embryos during injection must be prepared in advance as follows: dissolve 1.5 g of agarose powder (molecular biology grade) in 100 mL of distilled H_2O and pour in a standard 90 mm × 15 mm Petri dish to create a thin layer; two standard microscope slides are positioned obliquely at an angle of 50–70° in the liquid agarose gel layer to create tracks; once the agarose gel has solidified, the slides are carefully removed from the agarose gel leaving track shapes. Agarose gel supports can be stored at 4 °C for up to 2 weeks.
12. Injection system with micro-loader: microinjection is performed through a micromanipulator (Micromanipulator 5171; Eppendorf) and a microinjector (Femtojet; Eppendorf).
13. Micro-loaders: Femtotip® capillary micro-loader, Eppendorf.
14. Common laboratory tweezers with thin tips.
15. 3 mL Pasteur plastic pipette.
16. Confocal 35-mm coverglass-bottom Petri dish (coverglass size: 22 × 22 mm; coverglass thickness: 0.13–0.16 mm).
17. Insulin syringe.
18. 1.5% low-melting-point (LMP) agarose solution: dissolve 1.5 g of LMP agarose powder in 100 mL of 1X PTU.
19. Fluorescent confocal microscope: SP2 confocal inverted microscope (Leica, Wetzlar, Germany), equipped with a GFP filter (excitation 488 nm) and 10X objective (HC PL APO 10X).

2.5 Outcomes

1. 1X tricaine solution prepared as described in Subheading 2.4.
2. Physiological solution prepared as described in Subheading 2.4.
3. Phosphate buffer saline (PBS)/1% Triton X-100: add 100 μL of Triton X-100 to 900 μL of PBS.
4. Insulin syringe with a 25-G needle.
5. LB agar plates prepared as described in Subheading 2.1.
6. Stereomicroscope, described in Subheading 2.4.
7. Micro-loaders: Femtotip® capillary microloader, Eppendorf.
8. Petri dishes: 90 mm × 15 mm and 35 mm × 15 mm.

3 Methods

3.1 GFP+-PAO1 Culture Preparation

1. GFP+-PAO1 glycerol stock is freshly streaked out on LB agar and incubated at 37 °C o/n.
2. A single colony of GFP+-PAO1 is inoculated in 5 mL of LB broth supplemented with carbenicillin (300 μg/mL), and the culture is incubated o/n at 37 °C with 200 rpm rotatory shaking.
3. The day after, the bacterial culture is diluted in the same medium to $OD_{600} = 0.1$ and incubated as before up to $OD_{600} = 0.5$ [corresponding to about 5×10^8 colony forming units (CFU)/mL] (*see* **Note 3**).
4. One-fifth of the bacterial culture (e.g., 2 mL) is centrifuged at 16,000*g* for 2 min and the bacterial pellet is resuspended in 1 mL of sterile physiological solution, for example, at $OD_{600} = 1$, equivalent to ~10^9 CFU/mL.
5. GFP+-PAO1 culture is titered on LB agar and stored at 4 °C until use (*see* **Note 4**).

3.1.1 Preparation of the Bacterial Inoculum for In Vivo Experiments

1. 500 μL of GFP+-PAO1 culture (previously prepared and titered as described in Subheading 3.1) is vortexed for 15 s and then passed through a syringe with a 25G needle (*see* **Note 5**).
2. Different titers (from 5×10^7 CFU/mL to 5×10^8 CFU/mL) of homogenized bacterial suspension are prepared by dilution in physiological solution with 10% phenol red (*see* **Note 6**).

   *This step is necessary only when the bacterium is first studied, in order to set a proper dose to infect the embryos. For *Pa* infections, we routinely use a culture of PAO at ~1.5×10^8 CFU/mL.

3.2 High-Titer Bacteriophage Stocks Preparation and Purification

High-titer phage preparations are produced by infection of a liquid PAO1 culture with raw phage stocks, as described by Henry and colleagues [18], with some modifications to improve purity for the needs of in vivo experiments [12]. High-titer stocks are prepared individually for each phage used in the experiments.

1. A single colony of PAO1 is inoculated in 5 mL of LB broth and incubated at 37 °C with 200 rpm rotatory shaking. The resulting bacterial culture is diluted in 500 mL of LB broth to $OD_{600} = 0.01$ and incubated at 37 °C with 200 rpm shaking to $OD_{600} = 0.05$ (about ~5×10^7 CFU/mL).
2. An aliquot of a raw phage preparation containing ca. 2.5×10^7 phages (*see* **Note 7**) is added to the PAO1 culture to reach a multiplicity of infection (MOI) of 10^{-3}. The infected bacterial culture is incubated at 37 °C with 100 rpm shaking until the OD_{600} falls to ~0.1–0.3 (*see* **Note 8**).

3. The bacterial lysate is incubated with 1 μg/mL of RNase and 1 μg/mL of DNase at 37 °C for 30 min to remove extracellular bacterial nucleic acids, then centrifuged at low speed (5000 × *g*) at 4 °C for 30 min (*see* **Note 9**).
4. The supernatant is carefully recovered and filtrated with a 1.2 μm pore diameter filter.
5. 58 g/L of NaCl and 105 g/L of PEG6000 are added to the supernatant. The suspension is dissolved under stirring for 20 min at RT and kept o/n at 4 °C. Then, it is centrifuged at a high speed (20,000 × *g*) for 30 min to allow phage precipitation. The supernatant is discarded, and the phage-enriched pellets are resuspended in 15 mL of TN buffer.
6. The concentrated phage suspension is purified by the Cesium chloride (CsCl) density gradient method prepared by stratifying equal volumes of CsCl solutions of decreasing concentrations (*see* Materials) in polyallomer ultracentrifuge tubes (e.g., for SW41 rotor) (*see* **Notes 10** and **11**). 3.5 mL of PEG-concentrated phage suspension is layered on top of the CsCl gradient in each tube. Four tubes are usually prepared.
7. The four tubes are inserted into the proper ultracentrifuge rotor (*see* **Note 12**) and centrifuged at 100,000 × *g* for 2 h at 4 °C.
8. The tubes are slowly removed and cautiously locked on a support clamp. The white opalescent band that forms inside the CsCl gradient is gently sucked up from the tube using a 5 mL syringe with a 19G needle (*see* **Note 13**).
9. The aspired phage suspension is transferred into a new polyallomer ultracentrifuge tube and centrifuged at 150,000 × *g* at 4 °C for at least 16 h (*see* **Note 14**).
10. The visible white opaque band is collected as described in the previous step (*see* **step 8**).
11. The opaque band is dialyzed two times for 20 min into dialysis tubes with membrane cut-off = 6000 Dalton against 500 mL of water and then against 500 mL of TN buffer o/n at 4 °C.
12. The dialyzed phage preparation is filtered with a 0.22 μm pore diameter filter.
13. Phage preparation is flowed through a bacterial endotoxin removal column (e.g., EndoTrap HD, Hyglos, Germany) to remove the LPS (*see* **Note 15**).
14. The endotoxin level of the phage preparation is measured with a quantification assay (e.g., Pierce LAL Chromogenic Endotoxin Quantification assay, Thermo Scientific) (*see* **Note 16**).
15. Phage stock is stored at 4 °C until use.

3.2.1 Preparation of the Phage Suspension for In Vivo Experiments

1. High-titer phage stocks (prepared as described in Subheading 3.2) are titered by agar overlay method and diluted in TN buffer to 1×10^9 PFU/ml.
2. The phage cocktail is prepared immediately before use, to ensure accurate phage titers. Single high-titer phage stocks are equally combined to obtain an overall titer of 1×10^9 PFU/mL. Both single phages and cocktails are added with 10% phenol red before use.
3. Phage preparation is diluted in TN buffer to reach the MOI of interest as determined in preliminary experiments (*see* **Note 17**).

3.3 Zebrafish (Danio rerio) Breeding and Embryos Collection

1. For egg collection, 1:1 male:female ratio breeding pairs are transferred to specific smaller breeding tanks in the afternoon, where females and males are separated by a net [19].
2. The following morning, as the light cycle starts, the dividing net is removed, and mating occurs, leading to spawning and egg fertilization. Fertilized eggs are collected from the bottom of the tanks using a fine-mesh strainer or a plastic pipette and gently transferred to a 90 mm × 15 mm Petri dish containing fresh 1X E3 embryo medium (*see* **Note 18**). Any impurity collected together with the eggs is carefully removed from the Petri dish with a plastic pipette.
3. Embryos are grown in the 1X E3 embryo medium at 28 °C in a dedicated incubator (*see* **Note 19**). Starting from 24 h post fertilization (hpf), embryos are grown in 1X PTU to inhibit pigmentation. Embryos are monitored daily, removing unfertilized, necrotic or undeveloped embryos and providing fresh 1X PTU medium (*see* **Notes 20** and **21**).

3.4 Bacterial Infection and Phage Treatment

A protocol is described here to study the efficacy of single/cocktail bacteriophages in counteracting *Pa* acute bacterial infection in vivo.

3.4.1 Injection of Bacterial Suspensions in Embryos

1. At 48 hpf, embryos are mechanically or chemically dechorionated with sterile thin needles or tweezers or by pronase digestion (*see* **Note 22**). For pronase digestion, the embryos are gently pipetted in the pronase working solution until the chorions break; then they are immediately transferred to fresh 1X E3 medium and rinsed with 1X E3 medium several times to remove pronase residues (*see* **Note 23**).
2. Approximately 5 min before starting the experimental procedures, embryos are anesthetized in a Petri dish containing 1X tricaine.
3. Embryos at the same developmental stage [19] and without any morphological issues (e.g., pericardial or yolk sac edema) are selected under a stereomicroscope for the experiments.

4. A borosilicate-glass needle for microinjection is loaded with 5 μL of the bacterial suspension with a 20 μL pipette and a thin micro-loader and is assembled on the injection system (*see* **Note 24**).
5. The injection needle is opened by cutting off its tip with sterile thin tweezers and the dimension of the drop is set based on the scalebar in the ocular of the stereomicroscope and by adjusting pressure and injection-time values of the microinjector. Usually, a drop of 2 nL is optimal for both systemic and local injections (*see* **Note 25**).
6. Five to ten anesthetized embryos are transferred to the agarose gel tracks and gently lined up and positioned laterally with a micro-loader tip (i.e., with the duct of Cuvier pointing toward the tip of the needle) (*see* **Note 26**). Excess of 1X tricaine working solution is removed from the tracks with a thin plastic pipette, to limit embryo movements.
7. Embryos are microinjected:
   - For systemic intravascular injection, the needle is delicately inserted in the dorsal side of the duct of Cuvier, where it starts spreading over the yolk sac, and a volume of 2 nL of bacterial suspension is injected. If embryos are correctly injected into the vascular system, a brief, rapid wave appears in the yolk stream as the drop is injected (*see* **Note 27**).
   - For local injections in the hind-brain ventricle, the needle is delicately inserted laterally in the dorsal region of the head, right in the ventricle, and 1–2 nL of bacterial suspension is injected, paying attention not to perforate the basal layer (*see* **Note 28**).
   - As a control for successful bacterial infection, approximately every 20 injections, a volume of the bacterial suspension is injected directly in drops of 30 μL of sterile physiological solution. The drops are plated on LB agar plates and grown o/n at 37 °C to assess the actual number of injected CFU per embryo (*see* **Note 29**).
8. Repeat from step 6 until all the embryos are inoculated with the bacterial suspension.
9. The inoculated embryos are transferred in a Petri dish with fresh 1X PTU and incubated at 32 °C (*see* **Note 30**).

3.4.2 Selection of Locally Injected GFP$^+$ PAO1 Infected Embryos

The use of the GFP$^+$ PAO1 strain allows live tracking of the progression of bacterial infection in the embryos. For local infection experiments, GFP$^+$ PAO1-positive embryos can be selected, discarding those uninfected, at 3 h post-injection (hpi), before phage treatment, through live monitoring of the infection (*see* **Note 31**).

1. At 3 hpi, embryos are anesthetized with tricaine working solution in a 35 mm Petri dish previously filled with a slight layer of 1.5% agarose.
2. Embryos are placed under a fluorescence stereomicroscope.
3. Injected embryos are dorsally oriented to assess the presence of the GFP^{+} signal in the brain ventricle (*see* **Note 32**).
4. Following induction of PAO1 bacterial infection, 2 dpf (days post fertilization) wild-type (wt) zebrafish embryos are ready to be treated with phages and to monitor the progression of bacterial infection.

3.4.3 Injection of Bacteriophage Suspensions in Embryos

1. Three hours after the injection of the bacterial suspension, the inoculated embryos are prepared and injected with the phage suspension (single or cocktail) or TN buffer (control group) following the procedure described in Subheading 3.4.1 (from **step 2**).
2. As a control, approximately every 20 injections, the actual number of injected PFU/embryo is assessed as done for bacteria (*see* Subheading 3.4.1, **step** 7) but employing the agar overlay method to titer phages.
3. The treated embryos are transferred to new Petri dishes and incubated at 32 °C in 1X PTU.

3.4.4 Time-Lapse Confocal Microscope Imaging of Phage Therapy in Locally/Systemically Infected Embryos

For both systemic and local infection, time-lapse experiments can be exploited to evaluate the efficacy of phage treatment.

1. Immediately after phage administration (or TN buffer for control groups), locally/systemically infected embryos are anesthetized with a tricaine working solution (*see* **Note 33**).
2. One phage-treated embryo and one control are pipetted in a glass bottom Petri dish (35 mm diameter) and then the anesthetic is dried out with an insulin syringe.
3. A few drops of warm 1.5% LMP agarose solution are carefully added to cover and embed the embryos (*see* **Note 34**).
4. Using a stereomicroscope, the two embryos are immediately positioned in the desired orientation and side-by-side, with a fine tip: dorsally for the local infection assay or laterally for the systemic one.
5. The LMP agarose layer is cooled for 5–6 min until solidification (*see* **Note 35**).
6. The glass bottom Petri dish is gently filled with a thin layer of tricaine working solution, to keep the embryos moist during microscopic acquisition.
7. Embryos are placed under a fluorescent confocal microscope, at a constant temperature of 32 °C if possible (*see* **Note 36**).

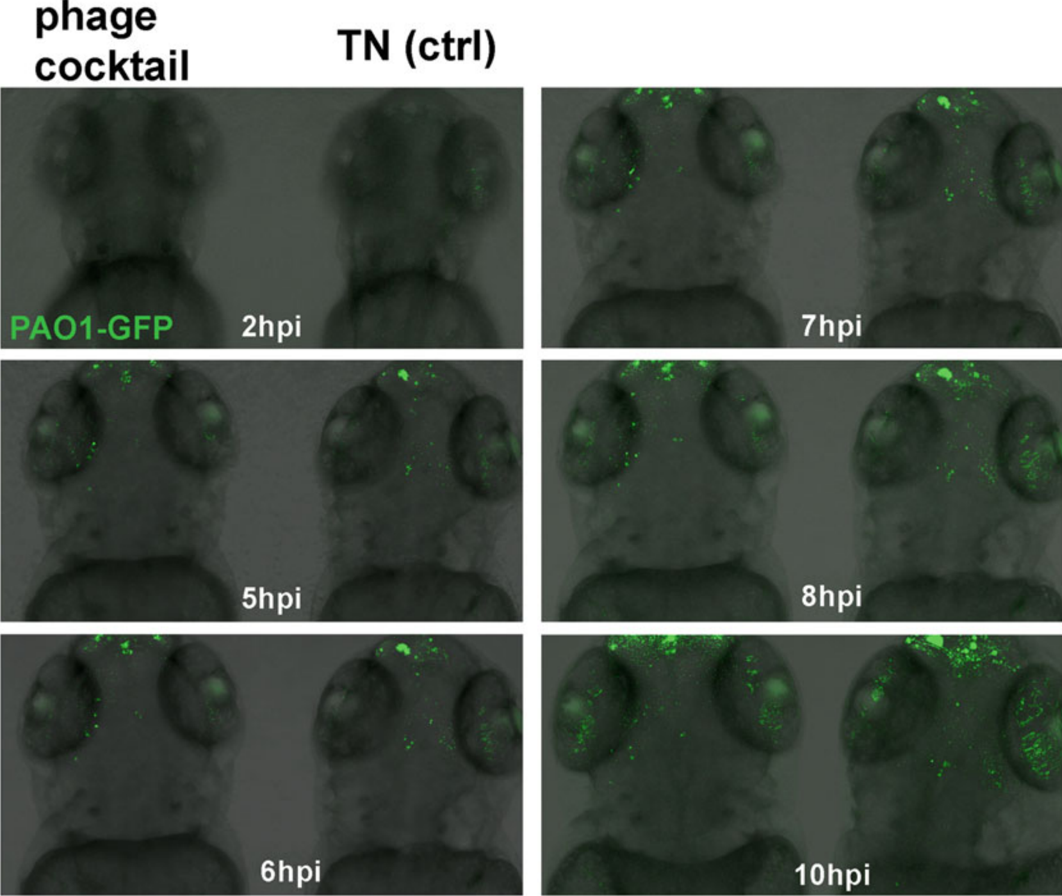

Fig. 1 Time-lapse of infected embryos. Representative time-lapse experiment of locally GFP$^+$ PAO1 infected embryos, treated (phage cocktail, MOI = 5) or not (TN) with phages and acquired for 10 hpi. Different time-points of the progression of the local infection following the microinjection of ~200 CFU/embryo of GFP$^+$ PAO1 culture in the brain ventricle are shown

8. Images of the two adjacent embryos are acquired for 24 h in bright field and fluorescence time-lapse acquisition using the following parameters:

   10X objective (HC PL APO 10X), series of 150–170 μm z-stacks at 1 μm intervals every 10 min (for local infection experiment) or 250 μm z-stacks at 1.5 μm intervals every 15 min (for systemic infection experiment) (*see* **Note 37**).

9. The frames resulting from the acquisition are assembled and converted to an ".mpeg" movie using ImageJ software (Fig. 1).

3.5 Outcomes

In this chapter, some key outcomes are described to evaluate and score the effectiveness of phage treatment on infected embryos by fluorescence imaging and plating techniques.

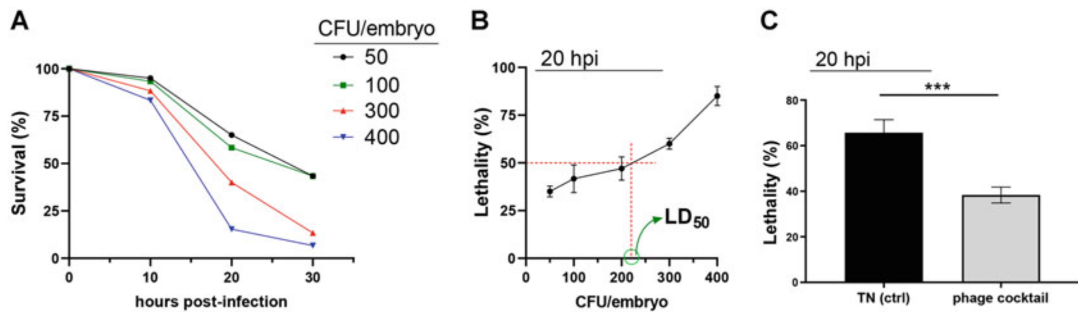

Fig. 2 Setting of the bacterial inoculum for infection experiment. In order to set a proper dose to infect the embryos, it is necessary to individuate the LD_{50} of the bacterium when it is first studied. (a) Survival rate of 48 hpf embryos infected with different doses (CFU/embryo) of a PAO1 culture, at 10, 20, and 30 hpi. (b) Determination of LD_{50} in 48 hpf zebrafish embryos infected with different doses of PAO1. In the representative graph, the lethality of the embryos is observed at 20 hpi. (c) Lethality at 20 hpi of embryos infected with ~250–300 CFU/embryo of PAO1 at 48 hpf and treated with the phage cocktail. Mean and SEM of three independent experiments are reported, *student's t*-test

3.5.1 Evaluation of the Severity of Bacterial Infection

The extent of a systemic acute bacterial infection under different experimental conditions (systemic/local administration, treated/ not treated with phages) is evaluated at 10, 20, and 30 hpi considering:

Morphology: embryos with altered morphology are scored considering the overall state of the embryo, the presence of cardiac edema, curved spine, absence of circulation, or necrotic tissue spots.

Lethality: embryos in necrosis or completely lacking heartbeat are scored as dead (*see* **Note 38**).

The half maximal lethal concentration 50 (LD_{50}) dose of PAO1 (dose that kills 50% of infected embryos) at 20 hpi (*see* **Note 39**) is calculated by infecting embryos with different doses (e.g., 50, 100, 300, 400 CFU/embryo, as described in Subheading 3.4.1 preparation of bacterial *inoculum*). The dose is chosen to properly assess the efficacy of phage treatment, usually close to the LD50 value. Consequently PFU/embryo are determined to reach MOI = 1 (or beyond) (Fig. 2).

3.5.2 Determination of Bacterial Burden by CFU Counts

This section describes a method to quantify the bacterial load of systemic/local infections in embryos at two different time points post-infection based on CFU counts in homogenized embryos.

1. At 8 or 20 hpi, groups of 5/10 embryos from each experimental condition are anesthetized as described in Subheading 3.4.1 and transferred in a 1.5 mL centrifuge tube (*see* **Note 40**).
2. The anesthetic solution is replaced with 200 μL of sterile physiological solution to wash the embryos and immediately replaced with 200 μL of fresh sterile tricaine working solution

(*see* **Note 41**). Then, the anesthetic is replaced with PBS/1% Triton X-100, and embryos are completely disaggregated by pipetting with a 200 μL micro-pipette and by passing them into a sterile insulin syringe with a 25-G needle (*see* **Note 42**).

3. Serial dilutions in sterile physiological solution (1:10, 1:100, 1: 1000) are prepared from the homogenates and 10 μL of each dilution are plated on LB agar plates added with ampicillin (100 μg/mL) (*see* **Note 43**) and incubated o/n at 37 °C (*see* **Note 44**).
4. After 12–14 h, GFP+ colonies are counted under a fluorescence stereomicroscope considering, for each experimental condition, the dilution(s) where colonies are easily distinguishable from each other (*see* **Note 45**) (Fig. 3). To estimate the score of bacterial burden (BB) (CFU/embryo), the following calculation is performed:

$$\mathrm{BB(CFU/embryo)} = \frac{\text{Number of colonies} \times \text{Dilution factor}}{\text{Number of embryos}}$$

3.5.3 Determination of Bacterial Load by Fluorescent Pixel Count (FPC)

This section describes a method to quantify the bacterial load of systemic/local infection in embryos at two different time points post-infection based on the quantification of the GFP+ signal from infecting bacteria.

1. At the proper time post-infection (8 or 20 hpi), phage-treated and control-infected embryos are prepared for fluorescence imaging as described in Subheading 3.4.2 (*see* **Note 46**).
2. Embryos are carefully positioned, dorsally or laterally, using a micro-loader tip (*see* **Note 47**).
3. Bright-field and fluorescence images of the brain ventricle region or the whole embryo are sequentially acquired using a fluorescence stereomicroscope equipped with a fluorescence lamp and digital camera and mounting a GFP-filter (excitation of 488 nm), maintaining the same acquisition parameters for all embryos (*see* **Note 48**).
4. To quantify the bacterial load, the GFP signal is estimated as the amount of fluorescent pixels in each embryo (FPC, area) with Fiji (ImageJ software, developer: Wayne Rasband) as follows (as described also by Phan and colleagues [20]):

   (1) manually select as region of interest (ROI), that is, the head area or the whole body of the embryo, with "polygon selection" function; (2) subtract the background of the image; (3) run "make binary" function to convert the image to B&W and set a common threshold level; (4) run "measure area" function to determine the area of fluorescent pixels within the selected ROIs, avoiding autofluorescence of the yolk sac (*see* **Note 49**).

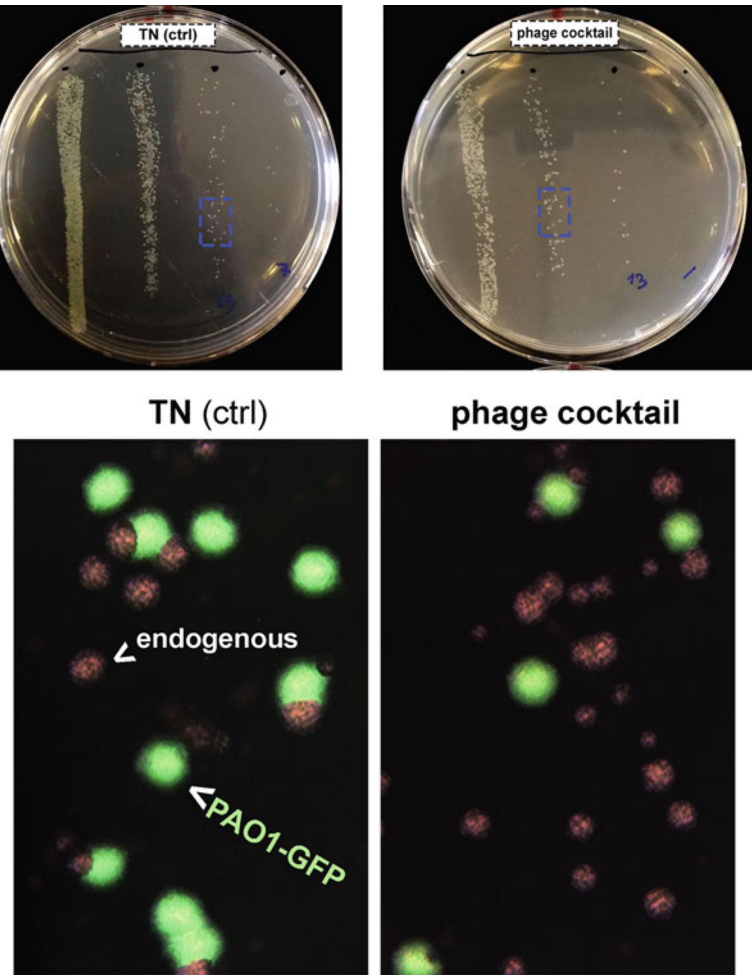

Fig. 3 Estimation of the bacterial burden. Colonies derived from the plating of homogenized infected embryos treated or not (ctrl) with phage cocktail are analyzed under fluorescence stereomicroscope to detect GFP^+ bacteria (PAO1-GFP), avoiding counting colonies of endogenous zebrafish bacteria (endogenous). Merged bright-field and fluorescence images are shown (bottom panel)

4 Notes

1. This type of LB agar layer serves as a base for LB soft agar, to favor in bacterial lysis by phages.
2. Set the puller with the following settings: heat 550, pull 90, pressure 40, time 70, and velocity 80.
3. Mid-exponential phase *Pa* cultures are used for in vivo experiments. The test of other bacterial genera might require stationary-phase cultures.

4. Use freshly prepared bacterial cultures for greater reliability. If not possible, bacterial cultures can be stored at 4 °C up to 24 h to avoid drops in bacterial titer.
5. Homogenization of the bacterial suspension avoiding the formation of clumping that could alter the injection dose is a critical step.
6. Phenol red tracing dye is useful to aid visualization during the injection procedure. The toxicity of the tracing dye on the bacterium used for the experiment should be tested by titrating the bacterial suspension before and after phenol red addition.
7. To determine the title of raw phage preparations, measure the PFU number following the established agar overlay method [15], using PAO1 as the bacterial indicator.
8. The time required depends on the phage growth cycle. It usually takes about 3–5 h to get massive culture lysis.
9. This crucial step allows debris and non-lysed bacterial cells to settle without damaging the phage particles.
10. CsCl is toxic, thus, proper safety procedures must be adopted to handle and dispose of it.
11. To avoid the formation of inaccurate gradients, fill the tube gradually and slowly with CsCl solutions (e.g., drop by drop along the wall of the tube). After preparation, use CsCl gradient immediately to avoid the shuffle effect.
12. To avoid dangerous centrifuge malfunction, ensure that the four tubes are balanced, with a maximum weight difference of 0.01 g. CsCL $d = 1.4$ solution can be used to accurately balance the tubes.
13. Usually, the white band is positioned between the density regions $d = 1.5$ and $d = 1.4$, according to the density of the phage particles. To ease the individuation of the band, place a black background behind the tube.
14. To avoid erroneous shuffling of suspension and to preserve the formation of the phage-enriched band in the correct position in the tubes, let the centrifuge run stop without braking.
15. For this purpose, the "Pierce high-capacity endotoxin removal resin" kit (Thermo Scientific), following the protocol "Endotoxin removal procedure using the batch method with spin columns," can be used.
16. The level of residual endotoxins in phage preparation should be below the maximum quantity recommended for intravenous administration of 5 international units/kg/hour.
17. Generally, a MOI = 1 is used as the starting dose for the evaluation of phage therapy.

18. To avoid contaminations that could affect embryo development remove the debris from the Petri dish.
19. To prevent defects in the development of the embryos, limit to 60/70 the number per Petri dish during growth.
20. Dead or unfertilized eggs usually appear opaque and can be distinguished under a stereomicroscope. To determine embryo stage, dechorionate a few embryos and observe them under a stereomicroscope.
21. Regular changes of fresh 1X E3 medium and frequent elimination of dying embryos allow for achieving a relatively sterile growth condition.
22. To facilitate the subsequent microinjection, remove the chorion before starting the experimental procedure to be sure that the embryos are completely straight and stretched out.
23. To accelerate the decohorionation process, incubate pronase-exposed embryos at 37 °C for 4 min and progressively dilute the pronase solution by adding 1X E3 medium while gently pipetting the embryos.
24. The microinjection system requires a stereomicroscope, a micromanipulator (Micromanipulator 5171; Eppendorf), and a microinjector (Femtojet; Eppendorf).
25. Reference values to be considered: compensation pressure ~15 hPa; injection pressure ~300–600 hPa; and injection time ~0.4–0.5 s. Higher injection time and pressure values may damage the anatomical structures of the embryos or their physiology while injecting.
26. Alternatively, use a hair loop tool to orient the embryos on the track, reducing potential damaging events [21].
27. To successfully perform a systemic intravascular injection, blood circulation must be well established in the embryos. Therefore, consider injecting embryos from 26 hpf onward.
28. For intravascular injection, set the micromanipulator arm at about 45° to the surface of the injection track; for local injection, set it at about 20°.
29. It is necessary to monitor this to ensure the correct evaluation of the bacterial *inoculum* by considering the average value of CFU/embryo derived from titration of the inoculated drops.
30. The incubation of infected embryos at 32 °C is necessary to allow sustained bacterial proliferation within the embryos. This incubation temperature can be adjusted according to the optimal growth conditions of the studied bacterium.
31. At 3 hpi, the GFP^+ PAO1 signal is still not appreciable in systemically infected embryos because the bacteria are spread

throughout the entire embryo rather than concentrated. Therefore, embryos are considered infected based on the dye tracker visible during the injection procedure.

32. To facilitate dorsal visualization of the embryos, pierce the agarose with a fine plastic tip (e.g., tips for P10 micropipette) and place the yolk sac of the embryos inside.
33. Expose the embryos to anesthetic for at least 5–6 min before the imaging experiment to achieve a more significant anesthetizing effect.
34. Add 40 μL of 25X tricaine solution to 960 μL of 1.5% LMP-agarose solution to achieve a greater anesthetizing effect. Avoid using LMP-agarose solution at a temperature higher than 40 °C.
35. To accelerate the solidification, carefully place the dish on an ice cooler (or cold keeper).
36. Some confocal microscopes are equipped with a temperature chamber. If present, set the temperature as close as possible to the optimal one for bacterial growth, promoting bacterial colonization and infection of the embryo, but no higher than 34 ° C to avoid damage in embryo growth.
37. To enable an adequate definition of bacterial cells, do not set the z-stack parameter below 1.5 μm, given the dimension of the bacterial cells themselves.
38. Necrotic embryos appear white and not transparent.
39. 20 hpi is the appropriate time to determine LD50 of PAO1 infection. Other bacteria may require different timing to effectively determine LD50, depending on the rate of infection.
40. It is essential to start from a known number of embryos (better if precisely the same number or very similar) in each experimental category to have comparable conditions and precisely estimate the bacterial burden (*see* **step 4**). As a control, groups of uninfected embryos are used.
41. These steps are essential to wash the surface of the embryos from the non-sterile growth medium, achieving an approximate degree of sterility and eliminating most of the bacteria contained in the growth medium for subsequent analysis.
42. If necessary, vortex the tubes for 30″ to improve homogenization.
43. The PAO1 strain is ampicillin-resistant.
44. Plating serial dilutions of the same homogenate is fundamental to ensure to obtain well-defined and countable bacterial colonies for each experimental condition.
45. Only GFP+ colonies derived from the PAO1-GFP infection are counted. Colonies derived from endogenous antibiotic-resistant bacteria are not considered [22].

46. It is also possible to fix the embryos in PBS/4% PFA o/n at 4 °C (or for 2 h at RT). Keep the fixed embryos at 4 °C and photograph them within a week (the timeframe in which the GFP signal remains stable). However, it is still advisable to acquire them in vivo to avoid the interference of fixation-dependent autofluorescence.
47. To facilitate image acquisition, transfer small groups of 4–5 embryos at a time, keeping them moist in a little amount of anesthetic solution (e.g., 1 mL of volume).
48. The three-dimensional size of the yolk sac can affect image quality, making it difficult to acquire focus. To obtain a proper 2D image, use the tip of a micropipette to drill a hole in the agarose layer and arrange the yolk sac of the embryo inside the hole.
49. To standardize the quantification of the GFP+ signal, use the same ROI selection mask for all embryos under analysis, moving it to the proper position for each embryo.

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Chapter 11

Use of Galleria mellonella as an Animal Model for Studying the Antimicrobial Activity of Bacteriophages with Potential Use in Phage Therapy

Lucía Blasco and María Tomás

Abstract

Interest in phage therapy has increased in the last decade, and animal models have become essential in this field. The larval stage of the wax moth, *Galleria mellonella*, represents an easy-to-handle model. The larvae have an innate immune response and survive at 37 °C, which is ideal for infection and antimicrobial studies with bacteriophages. In this chapter, we describe the procedures used to study the antimicrobial activity of bacteriophages in a *G. mellonella* infection model.

Key words *Galleria mellonella*, Animal model, Bacteriophage, Infection, Antimicrobial

1 Introduction

Galleria mellonella (the greater wax moth) is an invertebrate arthropod belonging to Lepidoptera. The larval stage of the wax moth can be used as an animal model in infection studies. Mammalian species have been widely used as infection models and in antimicrobial testing because of their similarities to humans. However, insect models are preferred (when possible) because they are not subject to ethical limitations, as invertebrates are excluded from the Animals Act 1986 [1]. The use of insects also has other advantages, such as ease of maintenance, low cost, absence of need for anesthesia, and the possibility of pretreatment with chemical inhibitors [2]. Among the insect models currently used, *G. mellonella* larvae are notable for their short life span, easy-to-handle size, incubation temperature of 37 °C, and the possibility of precise dosing [1, 3]. In addition, results obtained using the wax moth model are positively correlated with those obtained using mammalian models to study the virulence of *Pseudomonas aeruginosa* [4].

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The use of *G. mellonella* as an infection model has significantly increased in the last decade, and the value of the larval model for studying the virulence of infections with pathogens, including bacteria, viruses, and fungi, and for testing new antimicrobial agents has been established. Another important factor supporting the use of *G. mellonella* larvae in such studies is that although they do not have an adaptive immune system like mammals, they have an innate immune system that includes cellular and humoral defense responses. The cellular response involves hemocytes, which phagocytose and encapsulate pathogens. The encapsulation stage depends on the production of antimicrobial peptides, melanization, and coagulation of the hemolymph [2, 5].

G. mellonella larvae have also been used in many studies to test the efficacy of bacteriophages (or phages, i.e., viruses that infect bacteria) in treating bacterial infections. Phage therapy uses bacteriophages to treat bacterial infections. Animal models have been widely used to test the efficacy of phages in treating bacterial infections, and the *G. mellonella* model has become popular in recent years [6]. It was established for the first time in 2009 that a bacteriophage increased the survival of *G. mellonella* larvae infected with *Burkholderia cepacia* [7]. The value of this model for studying phage therapy to treat different pathogens in the ESKAPE group (*Enterococcus faecium*, *Staphylococcus* aureus, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp.) has also been confirmed in various studies [8–15]. Bacteriophages or phage-derived enzymes can be combined with antibiotics, and in such cases, *G. mellonella* larvae have proven to be valuable for in vivo testing of the efficacy of the combined treatment; sublethal doses of antibiotics together with bacteriophages or phage-derived enzymes have been found to improve the survival of infected larvae [11, 16, 17].

In summary, the larval stage of *G. mellonella* can be used as an in vivo model for testing the bacteriophages with potential use in phage therapy, alone or in combination with antibiotics. This model can also be used in prescreening prior to mammalian models (such as mice), thus reducing the number of animals needed in infection studies. This chapter presents a protocol for treating infected *G. mellonella* larvae with bacteriophages that can be used in studies evaluating the efficacy of different bacteriophages as therapeutic agents.

2 Materials

2.1 Strains of Bacteria and Types of Bacteriophages

As this is a generic protocol for use with different bacteria and bacteriophages, specific strains or species are not cited.

1. Bacterial strain used to propagate the bacteriophage. The strain should not contain prophages or virulence or resistance genes.
2. Bacterial strain used as the host strain causing infection.
3. Bacteriophage strain, selected following the bacterial strain used.
4. *G. mellonella* larvae: Between 10 and 20 *G. mellonella* larvae per test group: final-instar stage, of size 2–3 cm and weight 180–250 mg (*see* **Note 1**).

2.2 Culture Media and Buffers

1. Lysogeny Broth (LB): 10 g tryptone, 5 g yeast extract, 5 g NaCl, made up to 1000 mL with distilled water. Autoclave at 120 °C for 15 min.
2. LB agar plates: 10 g tryptone, 5 g yeast extract, 5 g NaCl, 20 g agar, made up to 1000 mL with distilled water. Autoclave at 120 °C for 15 min.
3. Tryptone Agar (TA): 10 g tryptone, 5 g NaCl, 15 g agar, made up to 1000 mL with distilled water. Autoclave at 120 °C for 15 min.
4. Tryptone Agar soft (TA soft): 10 g tryptone, 5 g NaCl, 4 g agar, distilled water to 1000 mL. Autoclave at 120 °C for 15 min.
5. Phosphate buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, and 1.8 mM KH_2PO_4, made up to 1000 mL with distilled water. Autoclave at 120 °C for 15 min.
6. Saline-magnesium buffer (SM): 100 mM NaCl, 8 mM MgSO4. $7H_2O$, 50 mM Tris–HCl (pH 7.5), made up to 1000 mL with distilled water. Autoclave at 120 °C for 15 min.

2.3 Equipment

1. Petri dishes (90 mm diameter).
2. Filter paper.
3. Sterile 250 mL Erlenmeyer flasks for bacterial culture.
4. Micropipettes of volume 10 μL, 100 μL, and 1000 μL.
5. Pipettes of volume 5 mL to 25 mL.
6. Sterile syringe microfilter 0.45 μm.
7. Amicon®Ultra-15 centrifugal filter units (100 KDa).
8. Sterile syringe of 20 mL.
9. Hamilton microliter™ 0.1 mL syringe.
10. Sterile scalpel.
11. Sterile cotton swabs.
12. Ethanol 70% (v/v).
13. Chloroform.
14. Sterile 1.5 mL microtubes.

15. 15 mL and 50 mL sterile polypropylene tubes
16. Microcentrifuge.
17. Centrifuge.
18. Spectrophotometer.
19. Water bath.
20. Balance.
21. Incubator.
22. Autoclave.

3 Methods

3.1 Preparation of Bacteria

The medium and culture conditions can be adapted to optimal conditions for each bacterium.

1. Remove one loop full of the bacterial strain from a frozen vial and streak it onto an LB agar plate. Incubate at 37 °C overnight.
2. Select colonies and inoculate individually in 5 mL LB. Incubate overnight at 37 °C and 180 rpm.
3. Harvest the cultures by centrifugation at 4000 × *g* for 15 min. Discard the supernatant and wash the precipitated culture twice with PBS by centrifuging at 4000 × *g* for 15 min. Finally, suspend the cultures to obtain 1×10^{10} colony-forming units (CFU) per mL (*see* **Note 2**).
4. Make serial dilutions (0- to 12-fold), plate them on LB agar, and incubate overnight at 37 °C. Count the number of colonies and calculate the number per mL (CFU/mL = number of colonies × 10 × reciprocal of dilution).

3.2 Bacteriophage Preparation

1. Dilute 1:100 a propagation strain overnight culture in 20 mL LB by stirring at 180 rpm at 37 °C until the culture reaches the optical density corresponding to the optimal growth phase for infection (*see* **Note 3**).
2. Infect the culture with the selected bacteriophage at the appropriate multiplicity of infection (MOI) and maintain agitation at the appropriate temperature for the host bacterial strain until the culture is cleared (*see* **Note 4**).
3. Add chloroform to a final concentration of 1% and incubate at room temperature for 30 min. Centrifuge at 4000 × *g* for 15 min and sterile-filter the supernatant through a 0.45 μm microfilter to recover the cell-free bacteriophage lysate (*see* **Note 5**).
4. Store the bacteriophages at 4 °C until required.

5. To concentrate and purify the bacteriophage, add 15 mL of bacteriophage suspension to an Amicon filtration device (*see* **Note 6**).
6. Centrifuge the Amicon tube at 4000 × *g* for 5 min until the suspension is concentrated to <10 mL. Repeat this step as many times as necessary by adding another 15 mL of bacteriophage lysate.
7. Wash the bacteriophage lysate by adding 15 mL of SM buffer and centrifuge at 4000 × *g* for 5 min until the suspension is <10 mL. Repeat this **step 3** times and finally reduce the volume to <10 mL to concentrate the bacteriophage by at least ten-fold.
8. Collect the bacteriophage lysate from the upper case of the Amicon filter device with a pipette and store at 4 °C until use.
9. Determine the bacteriophage concentration using the double agar overlay titration method [18].

3.3 Preparation of G. mellonella Larvae

1. Immediately after receiving the larvae, remove all dead and dark-colored larvae and larvae entering the pupal stage. Dark coloration is a sign of unhealthy specimens; healthy larvae should be creamy white in color (*see* **Note 7**).
2. The larvae should be stored in wood shavings for 7 days in darkness at 15 °C.
3. One day before the start of the assay, select larvae of similar size and weight, distribute them in Petri dishes lined with a filter paper with a 10 cm diameter, and maintain them in darkness at 4 °C without food for 24 h (*see* **Note 8**).

3.4 G. mellonella Infection: Testing the Lethal Dose (LD_{50})

Prior to infection and/or treatment studies with the *G. mellonella* larvae, it is important to establish the lethal dose of each bacterial host strain tested, as all strains (even those of the same species) have different fitness and infectious capacities. For testing the activity of the antimicrobial agents, the 50% lethal dose (LD_{50}), i.e., the amount of inoculum that causes the death of 50% of the larvae 24 h post-infection, is determined. Lower or higher LDs will hamper testing of the antimicrobial activity.

1. Select 10–20 larvae of the wax moth *G. mellonella* of similar weight and size for each infection group. The groups will correspond to each concentration of inoculum and the PBS control group (Fig. 1a).
2. Prepare the inoculum with the host bacteria diluted in PBS at concentrations ranging from 10^3 CFU/mL to 10^{10} CFU/mL (*see* **Note 9**). Load 10 μL of the bacteria into a Hamilton syringe (*see* **Note 10**), which has been previously sterilized with 70% ethanol.

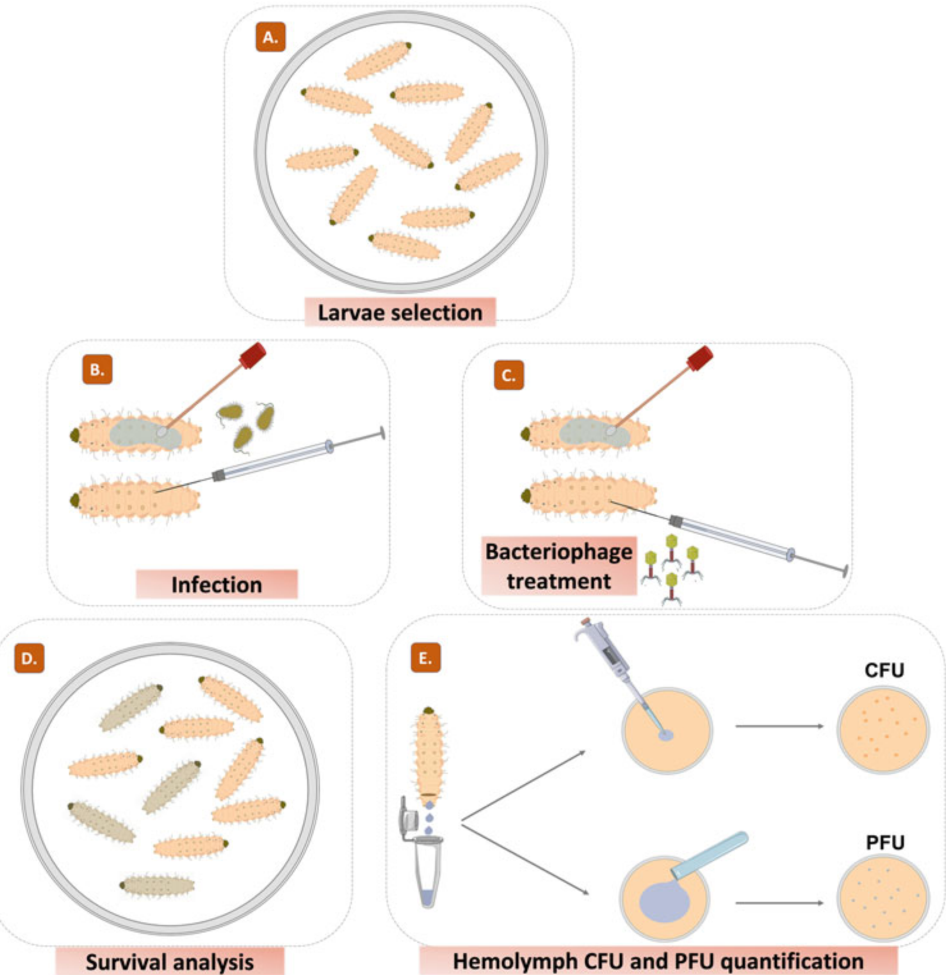

Fig. 1 Use of *G. mellonella* larvae in bacteriophage studies. (**a**) Selection of healthy larvae of similar weight and size. (**b**) Infection of the larvae by injecting the bacteria in the last left proleg. (**c**) Treatment of the infected larvae with the bacteriophage by injection in the last right proleg. (**d**) Analysis of survival by quantification of dead (dark-colored) larvae. (**e**) Quantification of the CFUs and PFUs in the hemolymph extracted from the larvae

3. Disinfect the larvae with a cotton swab soaked in 70% ethanol (Fig. 1b).
4. Pick up one larva and hold it by its head, between the thumb and index finger of one hand. Support the body of the larvae with the middle finger of the same hand, exposing the prolegs. Inject 10 μL of the bacterial solution in the last left proleg (*see* **Note 11**) (Fig. 1b). Transfer the larva to a clean Petri dish.
5. Incubate injected larvae at 37 °C for 24 h. Count the surviving specimens. The concentration of inoculum that results in 50% survival after 24 h can be used in the following assays (Fig. 1d).

3.5 *G. mellonella* Infection and Treatment with Bacteriophages

1. The experimental groups should consist of 10–20 *G. mellonella* larvae (Fig. 1a). The groups will correspond to treatment with bacteriophages, infection control, bacteriophage control, and SM buffer control.
2. Prepare the inoculum with the host bacteria diluted in SM buffer at the previously established LD_{50}. Load 10 μL of the bacteria into a previously sterilized Hamilton syringe.
3. Disinfect larvae with a cotton swab soaked in 70% ethanol (Fig. 1b).
4. Pick up and hold the larvae as previously described and inject 10 μL of the bacterial solution in the last left proleg (Fig. 1b). Larvae in the treatment group and in the infection control group will be inoculated with bacteria. Transfer the infected larvae to the corresponding Petri dish.
5. Inoculate a group of larvae similarly but with SM buffer.
6. Incubate all groups of larvae in darkness at 37 °C.
7. After 1 h, remove the larvae from the bacteriophage treatment group and inoculate them with the bacteriophage as previously done for bacteria, but in the last right proleg (Fig. 1c). The bacteriophage inoculum (10 μL) is previously prepared at the concentration of interest, usually 10–100 MOI. Place the larvae in the corresponding Petri dish (*see* **Note 12**).
8. Incubate the plates in darkness at 37 °C.

3.6 Survival Analysis

1. Count and record the dead larvae every 24 h for a maximum of 96 h (*see* **Note 13**) (Fig. 1d). To verify the death of the larvae, pinch with forceps or prod with a pipette tip: no movement should be observed. Discard any larvae showing signs of metamorphosis.
2. Calculate the larval survival rate using a statistical program such as GraphPad Prism.

3.7 Extraction of Larval Hemolymph for Quantification of Bacteria and Bacteriophages

1. Select 3 larvae from each group every 24 h from 0 h until the end of the assay. Place in a 15 mL tube.
2. To anesthetize the larvae, place each tube in ice for 10–15 min until the larvae no longer move.
3. Place the larvae in a Petri dish and make an incision between the last two segments (beside the tail) using a scalpel.
4. Before the extraction of hemolymph, weigh the 1.5 mL tubes that will be used.
5. Collect (by squeezing) the hemolymph from three larvae and pool in a 1.5 mL tube (*see* **Note 14**) (Fig. 1e). Carry out within 10 min to prevent coagulation of the hemolymph.
6. Weigh the 1.5 mL tubes after extraction of the hemolymph.

7. Dilute the hemolymph tenfold in SM buffer.
8. To count CFUs, spread 100 μL from each hemolymph dilution in LB plates and incubate overnight at 37 °C. Count the number of colonies and calculate the CFU per mL, as described in Subheading 3.1. (Fig. 1e).
9. To count the number of plaque-forming units (PFU), infect a culture of the host or the propagation strain at the ideal growth stage with 10 μL of each hemolymph dilution and spread in TA using the double agar overlay method. Incubate the plates overnight at 37 °C and determine the PFU; calculate the concentration of PFU per mL as described for the CFU in Subheading 3.1. (Fig.1e).
10. Place the bodies of the larvae in 15 mL tubes for disposal (*see* **Note** 7).

4 Notes

1. Wax moth larvae can be purchased from commercial suppliers. If possible, research-grade larvae should be used to ensure accurate and repeatable results. Alternatively, larvae can be purchased from pet stores.
2. Prior to this step, the number of CFUs at each incubation time or the optical density measured at a wavelength of 600 nm (OD_{600nm}) should be determined. The correlation between the OD_{600nm} and the CFU depends on the bacterial strain, age of the culture, culture medium, and spectrophotometer used.
3. The bacteriophage should be selected prior to the treatment of *G. mellonella*. Host range and bacteriophage infectivity should be characterized [19, 20] prior to testing. Some cultures must be supplemented with divalent ions by adding $CaCl_2$ or $MgCl_2$ to facilitate bacteriophage-host binding.
4. MOI is the number of bacteriophage particles that infect one cell, i.e., MOI = 10 indicates that a cell is infected by 10 bacteriophage particles.
5. The use of chloroform increases the number of bacteriophages recovered as it releases the fully assembled virions remaining inside the bacterial cytoplasm.
6. Ultracentrifugation is recommended for large volumes of bacteriophage lysate.
7. Discarded larvae should be frozen for 24 h to ensure death and autoclaved before disposal.
8. Food deprivation reduces the larval immune response making larvae more susceptible to infection.

9. The concentrations tested to establish the LD_{50} can be varied according to the bacterial strain and species.
10. Optionally, an infusion pump can be used, with an injection volume of 10 μL and a flow rate of 3.6 mL/h.
11. If resistance to the entry of the needle is noted, the position should be slightly changed until the needle penetrates the larvae easily. When the injection is carried out correctly, leakage of small drops of hemolymph may occur but should stop immediately.
12. For testing the synergic effects of antibiotics and bacteriophages, prepare a mixture of bacteriophage and antibiotic at the desired concentrations and inoculate 10 μL of the suspension in the larva proleg.
13. Melanization can be monitored to determine the progress of infection in the larvae [6].
14. Each larva will yield 15–50 μL of hemolymph, depending on its size.

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Part IV

Host Interaction and Clinical Application

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Chapter 12

Interaction of Bacteriophages with the Immune System: Induction of Bacteriophage-Specific Antibodies

Katarzyna Gembara and Krystyna Dąbrowska

Abstract

In all cases when a bacteriophage makes a direct contact with a mammalian organism, it may challenge the mammalian immunological system. Its major consequence is the production of antibodies specific to the bacteriophage, particularly IgM, IgG, and IgA as the typical response. Here we present protocols applicable in studies of the ability of bacteriophage to induce specific antibodies; immunization to whole virions or to isolated phage proteins has been included. The protocols have been divided into three parts: purification, immunization, and detection (enzyme-linked immunosorbent assay, ELISA).

Key words Antibody, Immunogenicity, ELISA, Immune response, Immunization, Bacteriophage, IgM, IgG, IgA

1 Introduction

Antibodies (Ab), also called immunoglobulins (Ig), are immunological proteins produced mainly by B cells, which are specialized white blood cells. They are capable to differentiate to plasma B cells, the major reservoir of immunological memory. The function of antibodies is to identify and neutralize pathogens and foreign elements that invade the system and to target pathogens, improving the efficiency of other elements of the immune system that, without cooperation with antibodies, have a nonspecific mode of action. An antibody specifically recognizes a unique molecule that is called an antigen. The precision of their match is often compared with the key-lock match. Antibodies are commonly considered as the most efficient immunological tools. Antibodies are produced by B cells, but antigen-specific helper T cells are able to provide stimulating signals to induce the B cell to differentiate into antibody-secreting cells. This is the T-dependent type of immune response, the key pathway for developing immunological memory. Bacteriophages are nonpathogenic viruses, but human and animal immune systems

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effectively respond to phages with specific antibodies. Phages induce an immune response, at least in the majority by the T-dependent pathway [1–3].

Antibodies may represent different classes (isotypes) and subclasses that determine their biological role. The most frequent and considered regular mode of response is the first induction of IgM followed by IgG in blood serum as well as IgA secretion on mucosal surfaces. IgMs are characteristics of the primary response, while IgGs result from response maturation. This isotype demonstrates improved specificity and is most important for systemic neutralization. IgAs primarily present an immune barrier on mucosal surfaces, for instance, in the gastrointestinal tract. Of note, the secretion of specific IgA on mucosal surfaces is typically paralleled by its rise in circulation. In all cases when a bacteriophage makes a direct contact with a mammalian organism, it may challenge the mammalian immunological system [1, 4], and its major consequence is the production of antibodies specific to the bacteriophage, typically (as observed in tailed phages), starting with IgM, and followed by IgG and IgA [5, 6]. Antibody production, however, appears to depend on the route of bacteriophage administration and on the individual features of a bacteriophage. It further depends on the application schedule and dose [1]. As a consequence, it is not easy to draw a general conclusion on bacteriophage immunogenicity or to propose a universal model for investigation of its impact on therapeutic approaches. These range from insignificant or undetectable [7–10] to devastating [11–13].

Phages are complex structures consisting of many proteins. Further, each protein displays many potential epitopes. For this reason, antibodies called "phage-specific," when resulting from human or animal body exposure to a complete phage virion, are, in fact, a sum of responses to different phage proteins (polyclonal). Antibodies that specifically recognize one phage epitope (monoclonal) can be developed using biotechnological methods [14]. Probably the major biological significance relates to phage-neutralizing antibodies [13]. Neutralization of phage by antibodies occurs when these antibodies bind proteins crucial for the ability of phage to infect bacteria, for instance, proteins engaged in binding to the bacterial surface [15, 16]. Antibodies targeting other sites of virions may, however, induce indirect neutralization of phage, executed by nonspecific elements of immunity that cooperate with antibodies.

Here we present protocols applicable in studies of the ability of bacteriophage to induce specific antibodies. Antibacteriophage antibodies can be studied in human sera, as they result from natural contact with these viruses or from bacteriophage treatment. Antibacteriophage antibodies can also be studied in animal models, conveniently in murine models, and mouse immunization has been included in the protocols presented herein. Because bacteriophage virions are typically complex structures containing many

different proteins with a multitude of potential antigenic epitopes, it may be useful to investigate separate bacteriophage proteins. Thus, bacteriophage proteins are included in the protocols presented here as potential antigens. All proposed protocols can be applied in studies where commercial monoclonal antibodies specific to a bacteriophage or bacteriophage protein are unavailable.

Protocols have been divided into three parts: purification, immunization, and detection (enzyme-linked immunosorbent assay, ELISA). Purification is necessary to remove bacterial remains, which are often highly reactive to the immune system and may cause a false-positive detection or other interference at further steps. Therefore this protocol is recommended in addition to standard procedures routinely conducted in many laboratories [17–19].

Immunization protocols are designed to study and compare the ability of bacteriophage to induce antibodies. They can be useful for investigating the consequences of antibody induction in therapeutic trials or just for preparing highly reactive polyclonal antibodies able to detect and/or neutralize bacteriophage. However, natural antibodies are also investigated in animals and humans. In such cases, only purification and detection protocols will be applicable [20, 21]. In any type of study, serum from a studied individual must be prepared.

The detection of specific antibodies is based on ELISA, but it can be replaced with any alternative method for antibody detection. Here, a variant of indirect ELISA has been applied (Fig. 1); an investigated antigen (a bacteriophage or a protein) is immobilized, then investigated sera are allowed to react with the antigen, and to bind specific antibodies from serum to the immobilized antigen. These serum antibodies are further detected using a detection antibody (commercial) that selectively recognizes the investigated class of antibodies from serum. The detection antibody is conjugated with an enzyme that turns a chemical substrate into a readable signal that allows for the quantification of the reaction.

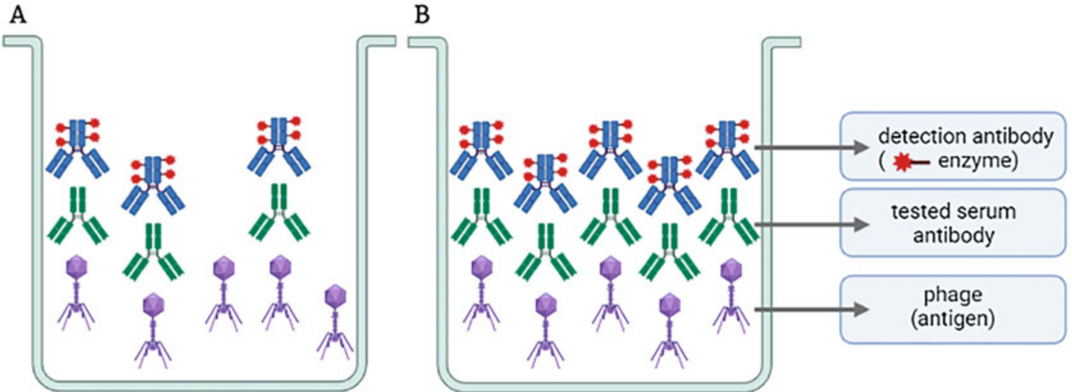

Fig. 1 Detection of bacteriophage-specific antibodies in human or animal serum by indirect ELISA. (**a**) Low serum concentration of phage-specific antibody, (**b**) high serum concentration of phage-specific antibody

2 Materials

Total volumes of necessary compounds must be calculated individually, according to the experimental design and the resulting number of samples.

2.1 Preparation of Bacteriophages or Bacteriophage Proteins

1. Samples for testing: (i) bacteriophages purified in any standard procedures such as chromatography, ultracentrifugation, etc., or (ii) bacteriophage proteins purified using two independent chromatography methods, e.g., by affinity chromatography and size exclusion chromatography or size exclusion and ion exchange chromatography.
2. LPS-affinity kit: EndoTrap Blue or EndoTrap HD (Lionex, Germany).
3. 50-mM $CaCl_2$, apyrogenic.
4. Syringe PVDF filter 0.22 μm, sterile.
5. Phosphate-buffered saline (PBS): 10-mM Na_2HPO_4, 2.7-mM KCl, 1.8-mM KH_2PO_4, 137-mM NaCl, apyrogenic, sterile, in the amount calculated from the sample volume as follows: (i) 300 × protein preparation volume or (ii) 600 × bacteriophage preparation volume (*see* **Note 1**).
6. Dialysis tubes 3–50 kDa (for proteins, according to a protein MW) or 300–1000 kDa (for bacteriophage).
7. 1.5 mL Eppendorf tubes.
8. EndoZyme (bioMerieux, France) (or other quantitative LPS activity assay, including Limulus Amebocyte Lysate assay).

2.2 Immunization of Mice

1. Mice (6–10-week-old) should be bred in specific pathogen-free (SPF) or germ-free conditions (*see* **Notes 2** and **3**).
2. Highly purified preparation of bacteriophage or protein (prepared according to Procedure 3.1). For calculation of the amount, see doses and schedule in Table 1.
3. Vehicle (buffer or solvent identical to that used for tested bacteriophage or protein) for the treatment of control mice. Preferentially phosphate-buffered saline (PBS): 10 mM Na_2HPO_4, 2.7 mM KCl, 1.8 mM KH_2PO_4, 137 mM NaCl, apyrogenic, sterile.
4. Syringes or other medical equipment for application of tested preparations.

2.3 Testing Specific Antibody Levels in the Blood

1. Serum separated from animal or human blood (using a standard procedure).
2. Nunc MaxiSorp flat-bottom 96-well plates (Thermo Fisher Scientific) or other plates designed for preferential adsorption of proteins.

Table 1
Examples of schedules and doses for immunization with bacteriophage according to its relative immunogenicity (doses in pfu per mouse)

Day	0	5–10	14	21	28	35	42	49	56	63	70
High immune reactivity	10^9	**Test for IgM** (*see* **Note 12**)	10^9		10^9	**Test for IgG**					
Medium immune reactivity	10^9–10^{10}	**Test for IgM** (*see* **Note 12**)		10^9–10^{10}			10^9–10^{10}	**Test for IgG**			
Low immune reactivity	10^{10} or more	**Test for IgM** (*see* **Note 12**)		10^{10} or more			10^{10} or more			10^{10} or more	**Test for IgG**

3. Highly purified preparation of bacteriophage or protein (prepared according to Procedure 3.1).
4. A microtiter plate sealing film or other accessories for covering the plates during incubation.
5. 5. Albumin (1% [wt/vol]) (alternatively to albumin any commercial ELISA plate blocking reagent may be used).
6. Phosphate-buffered saline (PBS): 10 mM Na_2HPO_4, 2.7 mM KCl, 1.8 mM KH_2PO_4, 137 mM NaCl.
7. PBS with 0.05% (wt/vol) Tween 20.
8. Detection antibody (*see* **Note 4**). The detection antibody must be specific for the serum antibody whose induction will be investigated, and it needs to be conjugated to the substrate-specific enzyme. Examples of detection antibodies: peroxidase-conjugated mouse anti-human IgG for the detection of human IgG; peroxidase-conjugated goat anti-mouse IgM for the detection of murine IgM.
9. Substrate/substrates for peroxidase: tetramethylbenzidine (TMB), e.g., TMB X-Treme (ImmunO4, Westminster, MD, USA) or equivalent.
10. 2N H_2SO_4.
11. Plate reader (450 nm and 550 nm).

3 Methods

3.1 Preparation of Bacteriophage or Bacteriophage Proteins

Induction of a specific humoral response and its detection generally require highly purified preparations, in the case of both bacteriophages and bacteriophage proteins. Purification is performed to limit any bacterial remains, which are often highly reactive to the immune system and may cause a false-positive detection or boost the immune reaction with no relevance to bacteriophage activity (*see* **Note 5**). Highly purified bacteriophage or proteins can be used either for specific immunization as the challenging agent or for the detection of antibodies in animals and humans.

1. Prepare three Eppendorf tubes (tubes 1, 2, and 3), each containing 200 μL of LPS-affinity resin EndoTrap into an Eppendorf tube and centrifuge the tubes 1200 × *g*, 5 min. Carefully remove the supernatant, minimizing the loss of slurry (*see* **Notes 6** and 7).
2. Gently resolve the slurry in tubes 1, 2, and 3 in 400 μL of Regeneration Buffer (delivered with LPS-affinity kit) and centrifuge the tubes at 1200 × *g* for 5 min. Carefully remove the supernatant, minimizing the loss of slurry.
3. Repeat **step 2** three times.

4. Gently resolve the slurry in tubes 1, 2, and 3 in 400 μL of Equilibration Buffer (delivered with LPS-affinity kit) and centrifuge at 1200 × *g* for 5 min. Carefully remove the supernatant, minimizing the loss of slurry.
5. Repeat **step 4**.
6. Mix the slurry in tube 1 with your sample (bacteriophage or protein). The sample volume may range from 0.2 mL to 50 mL.
7. Add Ca^{2+} up to 100 μM.
8. Shake gently for 1 h at room temperature (RT).
9. Centrifuge at 1200 × *g* for 5 min. Carefully collect supernatant (your sample), minimizing its loss. Preserve the slurry for its later recycling or throw it away.
10. Repeat **steps 6–9** using tube 2 (*see* **Note 8**).
11. Repeat **steps 6–9** using tube 3 (*see* **Note 8**).
12. Filter your sample using a 0.22 μm syringe PVDF filter.
13. Dialyze your sample against high-purity sterile PBS (or another solvent, if required) at 4 °C:
    (a) Proteins should be dialyzed at least overnight, including three changes of PBS, using a dialysis tube with a cutoff pore size appropriate for the protein (usually 3–50 kDa).
    (b) Bacteriophage should be dialyzed for 2 days, including 5–6 changes of PBS, using dialysis tubes with a cutoff pore size of 300–1000 kDa (unless a lower cutoff has been experimentally determined) (*see* **Note 9**).
14. Filter your sample using a 0.22 μm syringe PVDF filter.
15. Test endotoxin content in the sample via EndoLISA or Limulus Amebocyte Lysate assay (*see* **Note 10**).

3.2 Immunization of Mice

Please note that all animal experiments must be conducted according to appropriate ethical guidelines and regulations (e.g., EU Directive 2010/63/EU for animal experimentations, ARRIVE: Animal Research: Reporting of In Vivo Experiments guidelines or equivalent), and they must be approved by appropriate Ethical Committees for Experiments with the Use of Laboratory Animals.

Immunization of mice allows for the production of specific sera containing polyclonal antibodies, suitable, e.g., for the detection of the investigated bacteriophage or protein in biological and environmental samples, for testing their ability to induce specific antibodies, or for further studies of effects that immunization may have on bacteriophage activity in vivo. Different administration routes can be used to challenge animals, and here we present subcutaneous injection and oral treatment (*see* **Note 11**).

Table 2
Examples of schedules and doses for immunization with a purified bacteriophage protein according to its relative immunogenicity (doses in micrograms per mouse)

Day	0	5–10	14	21	28	35	42	49	56	63	70
High immune reactivity	20–50	**Test for IgM** (*see* **Note 12**)	30–50		30	**Test for IgG**					
Medium immune reactivity	30–60	**Test for IgM** (*see* **Note 12**)		50–100			50–100	**Test for IgG**			
Low immune reactivity	100–200	**Test for IgM** (see **Note 12**)		200–300			200–300			200	**Test for IgG**

1. Challenge

   Inject mice subcutaneously with 200 μL of bacteriophage or protein preparation (*see* **Note 12**). Control mice need to be injected with vehicle. For recommended schedules, see Tables 1 and 2.

   Alternatively

   Alternatively, if not choosing parenteral challenge (for instance, due to ethical reasons), add bacteriophages to drinking water to a final concentration of 4×10^9 pfu/mL (a daily dose of approximately 2×10^{10} pfu per mouse) or higher; when necessary for phage stability, use a nontoxic buffer in concentrations tolerable for animals.

2. Sample murine blood from the lateral tail vein and terminate the experiment when ready (*see* **Note 13**).

Typical patterns of specific IgM, IgG, and IgA induction in mice challenged with bacteriophage (tailed phages) are presented in Figs. 2 and 3.

3.3 Testing Specific Antibody Levels in Blood

This procedure is designed for the use of peroxidase-conjugated antibody. **Steps 1**–**7** are universal, while **steps 8**–**12** can be freely modified for other kinds of detection.

1. Cover MaxiSorp flat-bottom 96-well plate sterilely, overnight at 4 °C with bacteriophage (5×10^9 pfu/mL) or bacteriophage proteins (10 μg/mL), 100 μL per well (*see* **Note 14**).
2. Remove bacteriophage or protein preparations and wash the plate five times with PBS (*see* **Note 15**).
3. Block the plate for 1 h with 1% (wt/vol) albumin, 150 μL per well, RT.
4. Remove albumin and wash the plate five times with PBS.

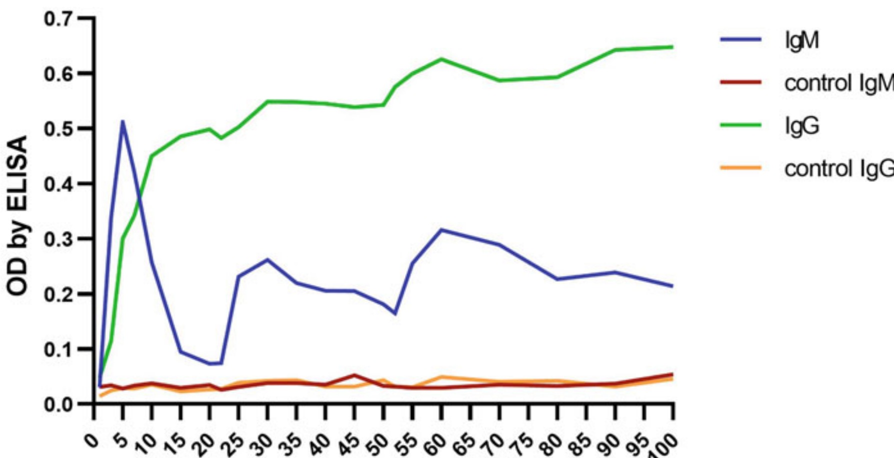

Fig. 2 Induction of specific IgM and IgG in mice challenged with bacteriophage parenterally on days 0, 20, and 50, example pattern. IgM – serum level of phage-specific IgM in bacteriophage-challenged mice; control IgM – serum level of phage-specific IgM in control mice; IgG – serum level of phage-specific IgG in bacteriophage-challenged mice; control IgG – serum level of phage-specific IgG in control mice; intensity – relative intensity according to OD ELISA

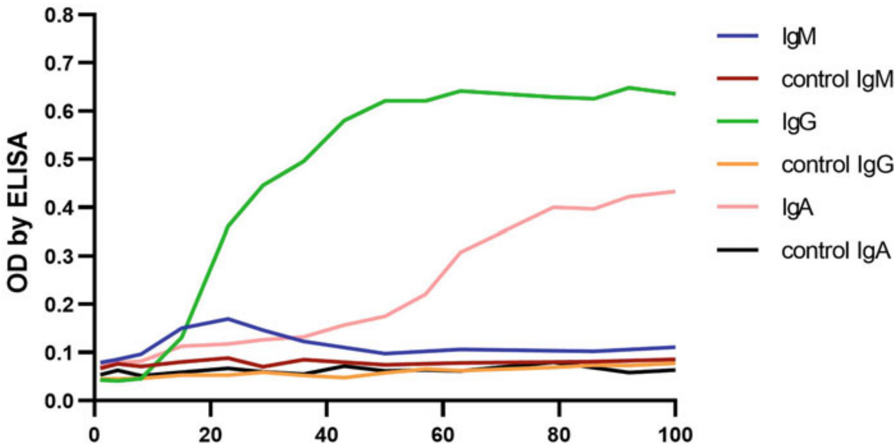

Fig. 3 Induction of specific IgM, IgG, and IgA in mice challenged with bacteriophage per os in drinking water, continuously, example pattern. IgM – serum level of phage-specific IgM in bacteriophage-challenged mice; control IgM – serum level of phage-specific IgM in control mice; IgG – serum level of phage-specific IgG in bacteriophage-challenged mice; control IgG – serum level of phage-specific IgG in control mice; IgA – serum level of phage-specific IgA in bacteriophage-challenged mice; control IgA – serum level of phage-specific IgA in control mice; intensity – relative intensity according to OD ELISA

5. Add 100 μL of diluted serum (*see* **Note 16**) to each well and incubate at 37 °C for 2 h.
6. Remove serum and wash the plate five times with PBS with 0.05% (wt/vol) Tween 20.
7. Prepare a fresh dilution of a detection antibody according to the manufacturer's instructions.

8. Add diluted detection antibody (100 μL per well) and incubate the plate for 1 h in the dark, RT.
9. Remove the detection antibody and wash the plate five times with PBS with 0.05% (wt/vol) Tween 20.
10. Add detection substrate: TMB X-Treme reagent (100 μL per well) and incubate the plate for 20–40 min. In positive wells, a blue color will gradually appear.
11. Add H_2SO_4 (50 μL per well); the color will turn yellow.
12. Read the plate without further incubation: absorbance at 450 nm (read 1) and 550 nm (read 2). Subtract the read 2 value from the read 1 value to calculate OD by ELISA (read 1 – read 2 = OD by ELISA) (*see* **Note 17**).

4 Notes

1. Alternatively, when PBS cannot be used, e.g., due to unstable protein or bacteriophage, other nontoxic buffers can be used.
2. All mice should be tested for specific antibodies of interest before they are used for an experiment. Preexistence of bacteriophage antibodies in properly bred mice under SPF conditions is unusual but not impossible. Animals of lower standard inbreeding, e.g., MD (minimal disease standard), are not recommended for immunological studies.
3. Any wild-type strain should be appropriate (e.g., BALB/c, C57BL/6) unless the study requires a knockout or other modified strain to investigate particular biological mechanisms. Also, there is no important reason to choose male or female animals unless an experimental design requires that. Typically, mice need to be young.
4. This procedure is designed for the use of peroxidase-conjugated antibody, but it can be freely modified for other kinds of detection.
5. In some types of experiments, specifically those that are NOT designed to compare or assess the intensity of the immune response, it might be less important to remove nonspecific boosters of the immune system, such as LPS. In cases where the only purpose of immunization is to obtain a highly reactive serum (with a high level of specific antibodies), LPS in a bacteriophage or protein preparation can be tolerable or even helpful. This is because LPS massively induces cytokines and other positive regulators of the immune response, thus making it stronger.
6. LPS (lipopolysaccharide, endotoxins) is a typical compound present in bacteriophage lysates of Gram-negative bacteria due to the natural structure of these bacteria. However, in

many cases, bacteriophage lysates of Gram-positive bacteria also contain endotoxins. This is because of the contamination of the culture media and/or contamination of the bacteriophage preparation at various stages of production. Endotoxins are very stable and extremely immunoreactive; therefore, for practical reasons, it may also be necessary to purify bacteriophage lysates of Gram-positive bacteria by LPS affinity. In case of any doubts, a preparation needs to be tested for its endotoxin content, e.g., by EndoLISA (Hyglos GmbH).

7. The amount of the EndoTrap resin of any type can be optimized according to (i) the amount of endotoxin content in a particular sample and (ii) individual efficiency of endotoxin removal from this sample, which largely depends on individual characteristics of a particular bacteriophage or protein (and can only be tested experimentally). The theoretical calculation can be done according to the manufacturer's information on the resin capacity. In this case, it is necessary to test a sample for purification for its endotoxin content before purification.
8. Every time, add Ca^{2+} as in the first round.
9. Dialysis with high cutoff membranes (300–1000-kDa pores) is crucial for the preparation of bacteriophages for immunological testing. It allows for the removal of residual peptidoglycan, bacterial DNA, LP (boosters), and bacterial proteins (nonbacteriophage antigens). Please note that in many cases, no assays for assessing their contaminations in bacteriophage samples are available.

   Some bacteriophages become unstable when purified with EndoTrap resins (e.g., some *Pseudomonas* bacteriophages). In such cases, only dialysis with 300–1000-kDa membranes can be applied for bacteriophage purification without all preceding **steps** (**1**–**12**), but it usually takes more time and more PBS changes to achieve a satisfactory result. This needs to be optimized individually for a bacteriophage.
10. We typically use a purified sample for ELISA or mice injection only if the LPS content is less than 1 unit per mL or per mouse, respectively.
11. Other routes of delivery are also possible, although s.c. is widely considered the most efficient and safe. Other common and convenient routes that can be used are: intraperitoneal (i.p.), intravenous (i.v.), intranasal, *per rectum* [22], intravesical [9], or other. Please use anesthetic drugs for animals when applicable. Please note that bacteriophage administered per os may be much less immunogenic than when administered parenterally [1], and induction of serum IgG by isolated bacteriophage proteins administered per os is very improbable. In addition, a researcher must be cautious when delivering

bacteriophage per os, because when using a stomach probe, microinjuries may result in artificial delivery of bacteriophage to blood. When possible, bacteriophage delivery in food or water is recommended for per os studies. Subcutaneous injection is typically expected to result in the most efficient induction of antibodies, while oral administration typically requires substantially more phage (total) and long exposition [1].

12. Adjuvants are not recommended unless necessary.

13. Please note that the typical peak of specific IgM in blood is temporary, usually within the fifth to tenth day after challenge; then a decrease in IgM level and a significant increase in IgG may be expected (*see* also Fig. 2).

14. Please note that all necessary controls must be included. The proper set of controls depends on the experimental design, but usually empty wells are used as the assay background control, wells with an antigen but without serum are included to control cross-reactivity of the detection antibody and the antigen, a positive control should be used for the optimization of detection antibody reactivity, and control (nonreactive) proteins are recommended as negative controls for protein immunogenicity testing (other controls should be considered if applicable). Control mice must be tested in the same way. Each sample on each cover must be tested in duplicate. In some experiments, serial dilutions of serum (*see* **Note 15**) or a standard curve (*see* **Note 16**) are needed.

15. For the whole procedure, you may use a multichannel pipette and/or an ELISA plate washer.

16. Following standard procedures of ELISA assays, serum dilutions should be individually optimized by testing serial dilutions (1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128, 1/256, 1/512, ...) to ensure the reliable signal and quality of serum. Many experiments apply serum dilutions between 1/100 and 1/10000.

17. ELISA units can also be calculated according to Miura et al. [23, 24]. This needs to be accommodated into the experimental design. Specifically, you need a standard serum (high antibody level, the best quality) that needs to be developed before your ELISA test, e.g., by Procedure 3.2. Standard serum dilution series must be included on each of your ELISA plates to obtain a standard curve for the calculation of ELISA units for each sample. This allows for direct comparison between plates and experiments. Simple presentation of immunization in the time course can be presented as relative intensity of immunization, i.e., OD by ELISA, but this is not appropriate for direct comparisons.

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Chapter 13

Bacteriophage Treatment of Infected Diabetic Foot Ulcers

Vera V. Morozova, Yulia N. Kozlova, Denis A. Ganichev, and Nina V. Tikunova

Abstract

Diabetic foot ulcers occur as a common complication of diabetes. The concomitant infection significantly delays the healing of the ulcers. Antibiotic treatment of infected ulcers is complicated by the formation of microbial biofilms, which are often heterogeneous and resistant to antibiotics. Bacteriophage therapy is considered an additional approach to the treatment of infected wounds. Here, we describe the basic method of application of bacteriophages for the treatment of infected diabetic foot ulcers, including very large ones.

Key words Bacteriophage therapy, Lytic bacteriophage, Diabetic foot ulcer, Antibiotic resistance, Diabetes, Polymicrobial infection

1 Introduction

Diabetic foot ulcers occur as a result of several factors, including peripheral neuropathy, atherosclerotic peripheral arterial disease, and mechanical changes in the bony architecture of the foot [1–3]. Approximately 5% of patients with diabetes develop foot ulcers each year, and 1% of these cases need amputation, making diabetes the leading cause of nontraumatic lower-extremity amputations in many countries [4, 5]. The comprehensive management of diabetic foot ulcers requires offloading the wound using appropriate therapeutic footwear, daily saline, or similar dressings to provide a moist wound environment, debridement when necessary, antibiotic therapy if osteomyelitis or cellulitis is present, control of blood glucose, and correction of peripheral arterial insufficiency [2, 6]. In the last few years, amputation preventions has become a key objective of clinicians providing care to patients with diabetic foot problems, and a multidisciplinary team approach for the treatment of foot ulcers was introduced in several hospitals [6–8].

Bacterial infections are common in diabetic foot ulcers, which are very susceptible to pathogens. There are local infections or

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critical colonization, infections with regional signs (cellulitis), and systemic infections (often causing fever). Infection must be diagnosed and treated promptly and adequately, as healing of the infected ulcers is significantly delayed or even prevented by the concomitant infection [9]. Aerobic gram-positive cocci (staphylococci and enterococci) are the main causative agents of diabetic foot infections and usually cause mild-to-moderate infections in patients. Other most common pathogens are gram-negative rods, such as *Pseudomonas aeruginosa* and members of the *Enterobacteriaceae family.* Gram-negative bacteria infect patients with chronic wounds, especially those previously treated with several courses of antibiotics. This type of ulcers often contains polymicrobial communities, where Gram-negative bacteria are predominant, and obligate anaerobic pathogens may be present [9–11].

Systemic antibiotic treatment of infected chronic wounds is often complicated due to (a) low bioavailability of antibacterial drugs caused by reduced microcirculation, (b) formation of microbial biofilms preventing penetration, and (c) bacterial antibiotic resistance [12, 13]. Therefore, the development of new approaches, as alternative or additional to antibiotic therapy, is required. One of the approaches is the implementation of lytic bacteriophages for treatment. In general, phages may be administered intravenously or topically; the latter option gives direct access to a chronic wound bacterial infection that is hidden from the immune system. In addition, it is the most cost-effective, safe, and convenient route for phage delivery. Topical application is possible by direct instillation, encapsulation in hydrogels, or impregnation of dressings [14–16]. Here we describe a basic method of topical bacteriophage treatment of diabetic foot ulcers.

Several commercial therapeutic phage cocktails have been developed in the Republic of Georgia, USA, Ukraine, and Russia [17]. In Russia, commercial phage preparations from NPO "Microgen" have been approved for therapy and are available in pharmacies. We test the activity of these preparations against the clinical strains, and if any of these phage preparations are active against the tested strain and the phage titer is high enough, we recommend using them. Otherwise, we use a customer preparation based on well-studied bacteriophages from our own collection (Figs. 1 and 2 present examples of such a bacteriophage treatment).

2 Materials

1. Collection of sterile bacteriophage preparations: Each bacteriophage is stored at a concentration of at least 10^7 pfu/mL and should be pre-characterized, including determination and analysis of genome sequence and lytic properties (*see* **Note 1**).

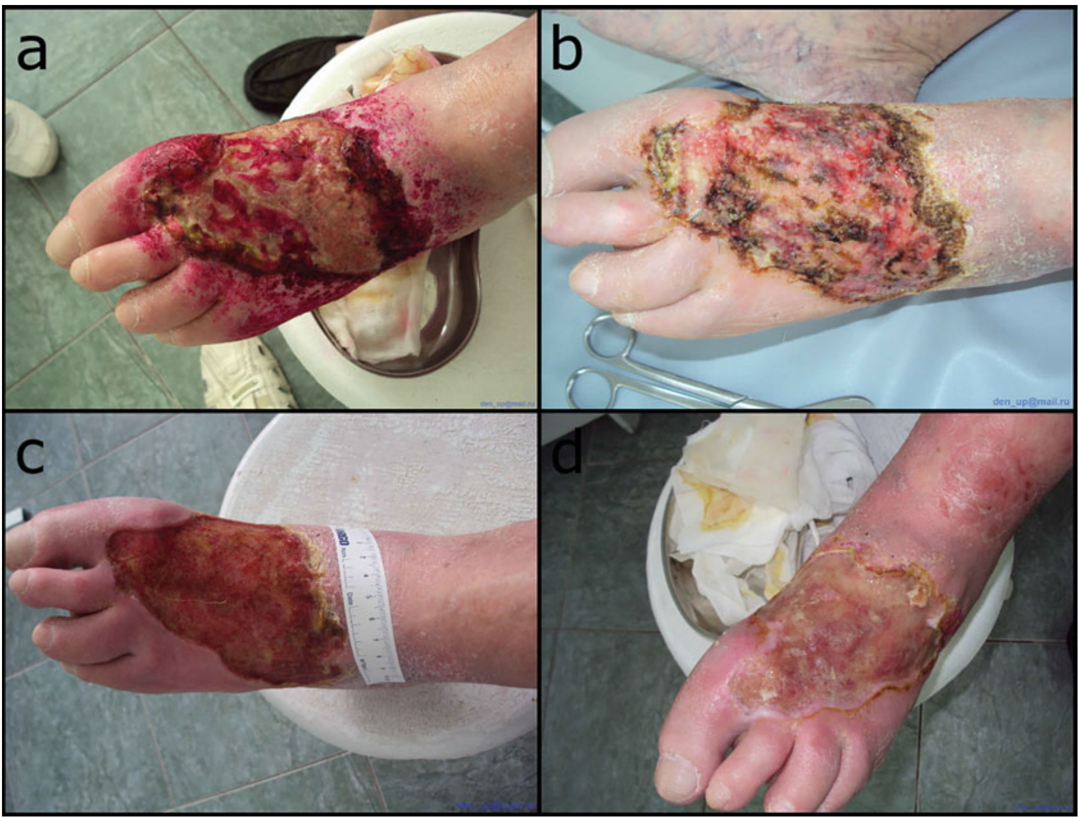

Fig. 1 Clinical case of eradication of MRSA infection with the help of bacteriophage therapy. Patient Sh., 60 years old, history of diabetes with multiple ulcers. (**a**) Beginning of bacteriophage therapy by Piobacteriophage (NPO "Microgen"), 4-12-2013. (**b**) Treatment continues, 14-12-2013. (**c**) The end of the bacteriophage treatment, 25-12-2013. (**d**) Wound continues to improve, MRSA infection is not detected, 01-01-2014

2. Indicator host strains.
3. Lysogeny Broth (LB): weigh 25.0 g of commercially available LB and dissolve in 1 L of deionized water. Autoclave at 121 °C for 15 min.
4. Trypticase soy agar (TSA) or LB agar plates: weigh 25.0 g of commercially available LB or 30 g of TSB and add 12–15 g of bacto-agar and dissolve in 1 L of deionized water. Autoclave at 121 °C for 15 min. Dispense 20–30 mL into Petri plates and allow it to solidify at room temperature (*see* **Note 2**).
5. TSB or LB top agar: prepare broth as described above and add 8% (wt/vol) of bacto-agar.
6. SM-buffer: add 50 mL of 1 M Tris-HCl (pH 7.5), 5.8 g of NaCl, and 2 g of MgSO4 × $6H_2O$ to a 1 L bottle and 1 L deionized water. Filter-sterilize or autoclave at 121 °C for 15 min.

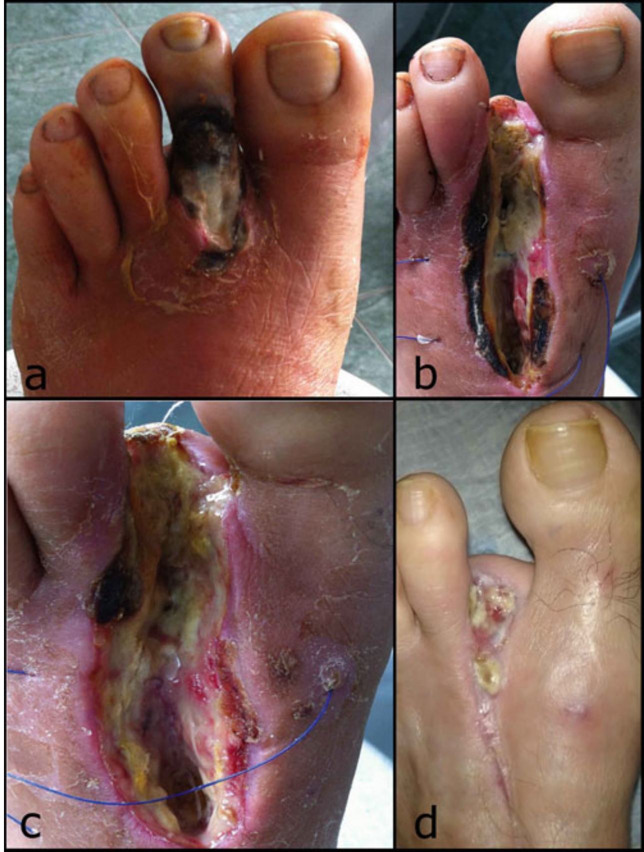

Fig. 2 In this particular case, wound was infected by MRSA resistant to other tested antibiotics. Lytic Staphylococcus bacteriophage preparation was used to prevent further growth of infection and possible subsequent amputations. (**a**) Before elective amputation, 30-05-2013. (**b**) Beginning of bacteriophage therapy after amputation, 4-06-2013. (**c**) Treatment continues, 10-06-2013. (**d**) Healing of the wound, 4 weeks later

7. Sterile 0.9% (wt/vol) saline solution: weigh 9.0 g of NaCl and place in a 1 L bottle and add 1 L deionized water. Autoclave at 121 °C for 15 min.
8. Transport swabs with liquid Amies medium.
9. Filters (0.22 μM) (Millipore, Sartorius or other manufacturers).
10. Appropriate sterile dilution tubes (such as 1.5 mL capped microcentrifuge tubes).
11. Large sterile 15 mL tubes.
12. Glass bottles (1 L).

3 Methods

3.1 Microbial Strain Isolation and Identification

1. Diagnosis of infection in diabetic foot ulcers is determined by a podiatrist on the base of clinical signs such as redness, temperature, pain, tenderness, edema, suppuration, and the presence of suspected discharge.
2. Tissue specimens or swabs from the ulcer for isolation of bacteria. Specimens obtained by aspiration or ulcer curettage are preferable to wound swabs.
3. Bacterial isolation and identification are carried out using a routine microbiological protocols, according to the lab opportunities. Isolated bacteria are grown on selective agar media with subsequent use of Biochemical Analyzer GENIII OmniLog (BioLog, USA) for identification. In difficult identification cases, 16S rRNA sequencing is carried out.

3.2 Selection of Specific Bacteriophage/Bacteriophages

1. Inoculate a colony of each bacterial strain isolated from the patient in 5-mL broth (usually LB medium) and incubate overnight with shaking at 37 °C (*see* **Note 3**).
2. Mix 200 μL of the resulting bacterial suspension with 3–4 mL of top agar that has been brought to a boil and cooled to 45 °C in a sterile tube and pour it on the LB agar in a Petri dish. Allow it to solidify.
3. Screen appropriate bacteriophage preparations from available commercial bacteriophage cocktails and/or the laboratory collection. The appropriate bacteriophage preparations are preparations that include bacteriophages specific to the same bacterial genus as the examined microbial strain (*see* **Note 4**).
4. Apply a drop of each tested bacteriophage preparation on the bacterial lawn, dry plates, and incubate overnight at 37 °C. Examine plates after some hours of incubation and the next morning. Bacteriophages are considered active if they cause clear confluent plaques on the tested bacterial lawn.
5. Determine the titer of active bacteriophages on bacterial lawns of tested strains in top agar. To do this, make tenfold dilutions of bacteriophage preparations in sterile SM buffer and apply drops of each dilution on the bacterial lawn. To accurately determine the titer, this should be done in replicates.
6. Dry plates and incubate them at 37 °C. Examine plates after some hours of incubation and the next morning. Count plaques and determine the titer. We use the bacteriophage preparation for bacteriophage treatment if its titer is 10^7 pfu/mL or higher on the patient strain (*see* **Note 5**).

3.3 Making Bacteriophage Preparation for Treatment

According to our experience, bacteriophage treatment success depends on the species of infectious agent/agents in the diabetic ulcer, so a bacteriophage preparation or a cocktail of bacteriophage preparations is best prepared individually.

1. In the case of *Staphylococcus* or *Enterococcus* mono-infection, choose the bacteriophage showing the best lysis of the examined strain and the highest titer. Use a laminar flow cabinet to seal the chosen bacteriophage preparation into sterile vials of 10 mL aliquots. One vial is intended for single use in the treatment to avoid contamination. Incubate vials at 37 °C for 24 h to control the sterility of the preparation. Do not use the preparation if it becomes cloudy!
2. In the case of mono-infection caused by *Pseudomonas aeruginosa* or some other gram-negative bacterium, try to make a bacteriophage cocktail consisting of 2–3 active bacteriophages. Use a laminar flow cabinet to prepare the bacteriophage cocktail. Equalize the titers of the bacteriophage preparations using sterile SM buffer and mix bacteriophages. Seal the mixture into sterile vials of 10 mL aliquots. One vial is intended for single use in the treatment to avoid contamination. Incubate aliquots at 37 °C for 24 h to control sterility of preparation. Do not use the preparation if it becomes cloudy!
3. In the case of mixed infection, prepare a bacteriophage cocktail consisting of active bacteriophages as described in **items 1** and **2** of this section.

3.4 Bacteriophage Treatment

It is necessary to clarify that bacteriophage therapy is used to eliminate the infection from the ulcer as part of a comprehensive management, as mentioned in the Introduction section. Our current main criteria for bacteriophage treatment are the presence of microbial infection, a clear indication for elective amputation, and poor response to previous antibacterial therapy, including strain-directed antibiotics. Written informed consent must be provided by the patient before beginning bacteriophage therapy.

The procedure of bacteriophage treatment includes the following:

1. Debride the wound. One possible way is to use a water-jet dissector (e.g., HELIX HYDRO-JET, ERBE Elektromedizin, Germany) to remove necrotic tissue and clean the wound. It is essential to use an antiseptic solution, for example, Chlorhexidine or Myramistin, which do not affect the viability of bacteriophages, or apply a sterile 0.9% (wt/vol) saline solution.
2. Dilute the bacteriophage preparation with sterile 0.9% (wt/vol) saline solution (1:2–1:10) to a minimal volume depending on the size of the wound cavity.

3. Rinse the wound cavity with the bacteriophage preparation, cover it with gauze soaked in bacteriophage preparation, wait for 10–15 min, and wrap it with dressing. When it becomes dry, replace the gauze with a new one soaked in bacteriophage preparation. Repeat replacement up to four times per day.
4. After 5–6 days of treatment, take a swab for control microbiological analysis. The titer of the infectious agent should be lower by at least 3–4 orders of magnitude or even absent (*see* **Note 7**). Repeat microbiological analysis every 5–6 days of treatment.
5. Continue bacteriophage treatment for 2–3 weeks.
6. Protect the wound surface using a nonadhesive dressing, such as Urgotul (Urgo Medical, Great Britain), during the period of granulation.

4 Notes

1. The genomes of the bacteriophage used for treatment should not contain genes encoding toxins or providing lysogenic bacteriophage infection. Lytic properties may be characterized by several microbiological methods, for example, lytic activity assay [18].
2. Some bacterial strains need a specific agar medium for bacterial lawn formation. *Enterococcus* strains often grow slowly on LB plates and need a rich medium. Many strains from the *Proteus* genus demonstrate swarming motility on LB plates that complicate visualization and correct counting of plaques. Use CLED agar for growing these strains. One can store plates in closed polyethylene packet at 4 °C for up to 2 months.
3. For rapidly growing cultures, it is possible to use a single large colony to prepare the bacterial lawn, skipping **item 2** of Subheading 2.2. Take a large colony, thoroughly re-suspend it in 200-μL broth, add 3–4 mL of top agar brought to a boil and cooled to 45 °C in a sterile tube, and pour the mixture on the LB agar in a Petri dish.
4. As mentioned above, in Russia, there are therapeutic bacteriophage preparations (NPO "Microgen"), which are approved for therapy and available in pharmacies. We test the activity of these preparations against the clinical strains as well, and if the bacteriophage preparation is active against the tested strain and the bacteriophage titer is high, we recommend using it.
5. If the bacteriophage is active against examined clinical strain but demonstrates low titer ($<10^7$ pfu/mL), it is necessary to re-develop the bacteriophage preparation using the indicative host strain or the clinical (tested) strain as host. For this

purpose, grow the bacterial culture in LB broth to OD_{600} 0.3–0.4, add bacteriophage with MOI 0.001–0.01, and incubate it until complete lysis of bacterial culture. Then, remove cell debris using a centrifuge (8000 × *g*, 10 min) and sterilize lysate filtering through a 0.22 μM filter. Determine the titer of bacteriophage preparation using the tested strain. Check preparation for sterility. Do not use the preparation if it becomes cloudy.

6. Polymicrobial infections in diabetic foot ulcers may be represented by two or more pathogens, and often it is not possible to pick up active bacteriophages for all of them. In this case, while the use of bacteriophages specific to one or two suspected agents leads to their elimination, other pathogens can persist and even increase their titer. Even in this case, bacteriophage therapy can be used, but it must be combined with other antimicrobial preparations, including appropriate antibiotics. Bacteriophages can destroy suspected bacteria and disrupt biofilms, which increases the bioavailability of other antimicrobials.
7. The bacteriophage therapy of infected diabetic foot ulcers may be accompanied by changes in the spectrum of bacterial agents. If repeated microbiological analysis demonstrates a new infective agent (or agents), the selection of a new bacteriophage preparation is required.

Acknowledgments

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Chapter 14

A Review of Phage Therapy for Bone and Joint Infections

Tristan Ferry

Abstract

There is a strong rationale for using phages in patients with bone and joint infections (BJIs). Indeed, specific phages can infect and replicate in bacterial pathogens and have also demonstrated their activity in vitro against biofilm produced by different bacteria. However, there is a high variability of the different clinical forms of BJI, and their management is complex and frequently includes surgery followed by the administration of antibiotics. Regardless of the availability of active phages, optimal ways of phage administration in patients with BJIs are unknown. Otherwise, all BJIs are not relevant for phage therapy. Except for diabetic foot infection, a BJI with bone exposure is potentially not a relevant indication for phage therapy. On the counterpart, prosthetic joint infections in patients for whom a multidisciplinary expert team judges a conservative approach as the best option to keep the patient's function seem to be a relevant indication with the hypothesis that phage therapy could increase the rate of infection control. The ESCMID Study Group for Non-traditional Antibacterial Therapy (ESGNTA) was created in 2022. One century after the first use of phages as a therapy, the phage therapy 2.0 era, with the possibility to evaluate personalized phage therapy in modern medicine and orthopedic surgery, is just open.

Key words Phage therapy, Bacteriophages, Biofilm, Bone and joint infections, Prosthetic joint infections, Osteomyelitis

1 Introduction

Bacteriophages (phages for short) are natural viruses that specifically target replicative bacteria [1, 2]. Each phage is highly specific to a bacterial species [2, 3]. Virulent phages induce bacterial lysis by hacking the intracellular bacterial machinery. Phage multiplication in a bacterial population could lead, in theory, to the total eradication of the bacterial community.

Bone and joint infections (BJIs) are considered one of the most difficult-to-treat bacterial infections, mainly because bacteria can modify their phenotype by producing biofilm, a common process for bacteria to survive in a different hostile environment [4]. As phages also demonstrated their ability to target the biofilm matrix and to synergistically impact it with antibiotics, there is a strong

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rationale for using phages in patients with BJI, especially in the context of disseminating increased antimicrobial resistance [5]. However, BJIs are a mix of different bacterial infections that are very heterogeneous, with different pathophysiologies and diagnoses, and not all of them are probably relevant for phage therapy [6, 7]. In 2017, the multidisciplinary team at Hospices Civils de Lyon started to implement a phage therapy center hosted by our French health ministry-labeled referral center to manage complex BJIs (http://www.crioac-lyon.fr) [4, 8]. One of the objectives of PHAGE*in*LYON *Clinic* is to promote and evaluate phage therapy in patients with complex BJI and dead-end situations [9]. The first challenge is to identify relevant clinical situations where phages could be an added value. In this chapter, the rationale for phage therapy in BJI, the type of infections that are relevant for phage therapy, and the guidelines of treatment based on our clinical experience are described.

2 Relevance of Phage Therapy to Treat BJI

2.1 Pathophysiology of BJI

During the pathophysiology of BJI, several players are involved. The host is infected by a pathogen that has reached a joint or the bone from different potential ways. It could reach the joint or the bone: (i) during a trauma with an open fracture that leads to direct bone tissue exposure [10]; (ii) during any invasive procedure such as surgery (arthroplasty aimed at replacing a degenerative joint by a prosthesis is the most common one) [11]; (iii) by a step-by-step contamination of the tissue following skin and soft-tissue impairment [12]; and finally (iv) by a hematogenous seeding from a separate infectious site, which could be clinically obvious or occult [13]. The host opposes this invasion with its immune system, producing acute inflammation by recruiting macrophages and polymorphonuclear cells, the first line of defense against bacterial infectious agents. Different pathogens could be involved, and each of them has different replicative and virulence properties and also different capacities to produce toxins, invade the tissue, escape to the immune response, and finally produce biofilm, especially into a dead bone or at the surface of an implant [7]. Indeed, orthopedic implants such as prostheses, plates, screws, and cement used to replace a joint or stabilize a bone are highly susceptible to bacterial colonization, representing a particular microenvironment that facilitates biofilm production [14]. Biofilm is a common process for bacteria to survive in a different hostile environment, inside or outside a host. It corresponds to a community of bacteria that forms a complex multicellular structure that aims to persist over time. The bacteria first adhere to an inert surface and then modifies their phenotype by (i) reducing replication and virulence properties and (ii) producing a dedicated extracellular matrix acting as a slime

that can have various compositions [15]. Once the biofilm is produced, it stays inseparable from the colonized surface. Of note, the biofilm is tolerant to the immune system, as it is quite impossible for macrophages and polymorphonuclear cells to eradicate the biofilm and other components of the immune system cannot penetrate the biofilm. Maturation of the biofilm could take days or weeks, depending on the pathogen, surface composition, and environment. During and after maturation, the bacteria survive embedded into the slime in a stationary or dormant growth phase and constitute with the biofilm a complex structure that exhibits extreme resistance to environmental stresses, including mechanical and chemical stresses [15]. It is well known in clinical practice that bacteria can persist for decades in biofilm, as there are many clinical examples of patients with latent or progressive infections with symptoms that could appear so far from the bacterial inoculation [14, 16]. Biofilm-associated infections are very complex to manage as most of the time, the implant where the biofilm has been formed has to be removed to cure the patient [17]. Unfortunately, implant removal is frequently associated with significant morbidity, with a need for iterative surgical procedures associated with a significant loss of function and/or life-threatening clinical conditions [17, 18]. Currently, there are no antibiotics and interventions except implant removal that could cure a patient with a matured biofilm at the surface of an implant. Patients with BJIs that need to be treated are symptomatic patients, with, for instance, postoperative infection following an arthroplasty where, after inoculation, the two distinct bacterial phenotypes of pathogenic bacteria cohabit: (i) the biofilm phenotype with dormant bacteria and local mild inflammation at the implant surface that could lead to prosthesis loosening and (ii) the planktonic phenotype with the same pathogen, in a different state, with high capacities of replication and virulence that could be associated with inflammatory pain, fever, and purulent discharge [17]. Finally, during the process of BJI, some particular bacteria, especially *Staphylococcus aureus,* could be responsible for penetration into the host's cells such as endothelial of bone cells, and may have the ability to survive and to persist, also constitute a subsequent reservoir of bacteria [19].

2.2 The Double Action of Phages

Based on these pathophysiology processes, phage therapy is, in theory, quite relevant in treating patients with BJI for many reasons. First, many studies have shown that active phages can target bacteria embedded in biofilm [20–22]. This antibiofilm activity of phages should be variable and dependent on the chosen phage bacteria. It may be due to the action of phage-derived enzymes, such as depolymerases, holins, and/or lysins [23–25], that target particular key elements involved in the biofilm biomass. Acting directly on the biofilm structure, these enzymes may significantly impact an infected patient with matured biofilm (Fig. 1, panel **a**).

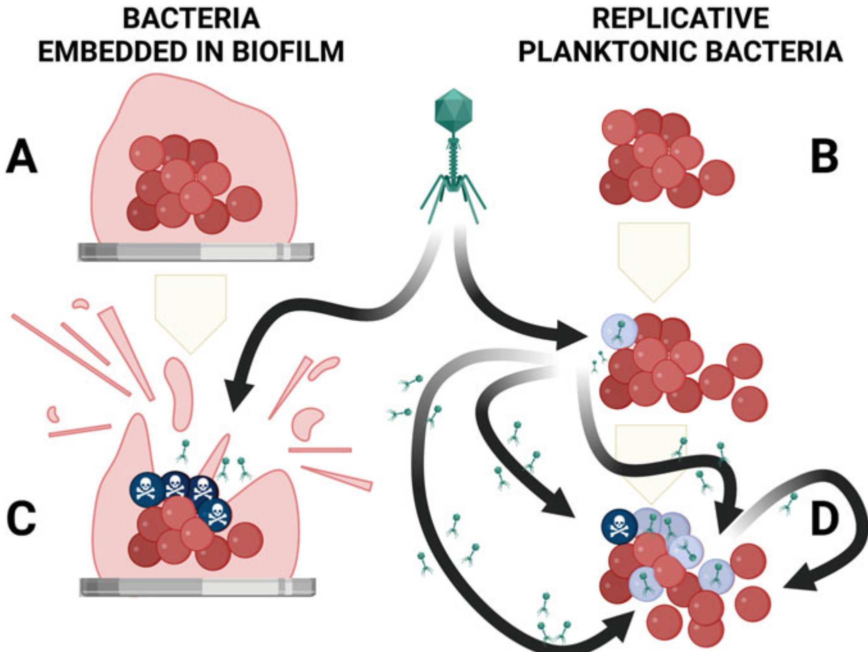

Fig. 1 Two distinct bacterial phenotypes of pathogenic bacteria responsible for BJI (**a** and **b**) and activities of phage against both of them (**c** and **d**). During the pathogenesis of BJI, pathogenic bacteria frequently produce biofilm, especially when in contact with an implant, and the biofilm matures with time (**a**). Bacteriophages exhibit dose-dependent antibiofilm activity, with an impact on the structure of the biofilm and on the bacterial biomass incorporated into the biofilm (**c**). In the process of BJI, the involved pathogen is also present in the state of replicative planktonic bacteria (**b**). Active phages have the ability to infect replicative bacteria (bacteria in blue color), hack the intrabacterial machinery, and finally produce a large amount of new viral particules that, by destroying their host (bacteria in black color), can also infect the replicative bacteria that is located in a close environment. Note that it is a dynamic process, with competition between the pathogenic bacteria that replicate themselves, with the capacity of the phages to infect and replicate among the whole bacterial population (**d**)

Indeed, these phage enzymes may directly reduce biofilm thickness and its biomass by facilitating the killing of dormant bacteria embedded in the biofilm matrix (Fig. 1, panel **c**). The other phage antibacterial activity is its well-known capacity to infect and kill planktonic bacteria (Fig. 1, panel **b**). Phages use the bacterial intracellular machinery to replicate, and upon cell lysis, hundreds of new viral particles become available to infect the other bacteria present in the immediate environment [1, 4]. This process is self-sustaining and could amplify itself by competing with the capacities of the bacteria to escape this infection and replicate (Fig. 1, panel **d**). In theory, if the process of phage infection of planktonic bacteria is effective, it leads to the total disappearance of the bacterial population.

By combining actions on the biofilm and actions on replicative planktonic bacteria, active phages would have a great place in the

available arsenal to treat patients with complex BJIs, especially as it has been demonstrated that the activities of such phages could be synergistic with antibiotics. However, BJIs are very diverse, and phage therapy may not be relevant to treat all of them.

3 High Variability of the Different Clinical Forms and Complexity of the Management of the Disease

3.1 All BJIs Are Not the Same Diseases

BJI is a generic term that means that the bone and/or joint tissue are infected [7]. However, BJI can encompass distinct infections based on how bacteria reach the infected tissue, infection site, clinical presentation, host's general conditions, local anatomy, and existence of an implant. This considerable disease heterogeneity is crucial because phage therapy may not be adequate for all BJI. Figure 2 presents different BJIs with different ways of contamination at different locations, illustrating the aforementioned point.

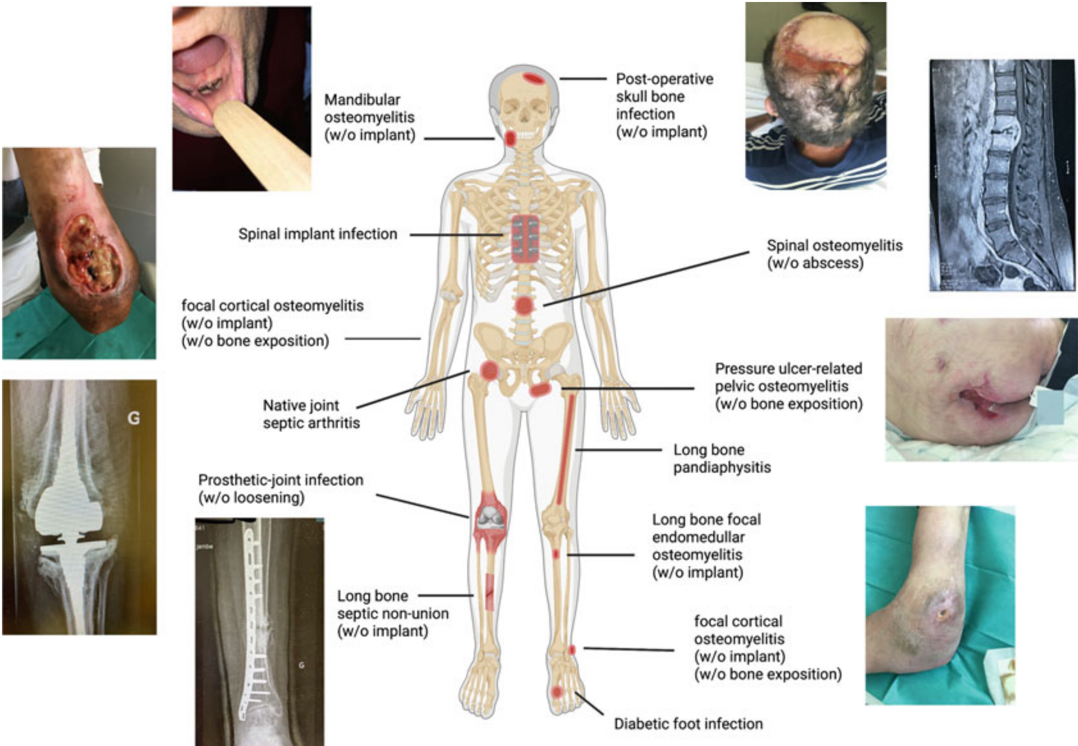

Fig. 2 Large heterogeneity of BJIs, which can be located at the surface of any bone of the skeleton and could be associated or not with an implant, with an abscess or a bone exposition. All bones and/or joints of the body could be infected, and clinical management of each of them significantly differs depending on several parameters such as the host comorbidity, pathophysiology of the infection, and its clinical presentation

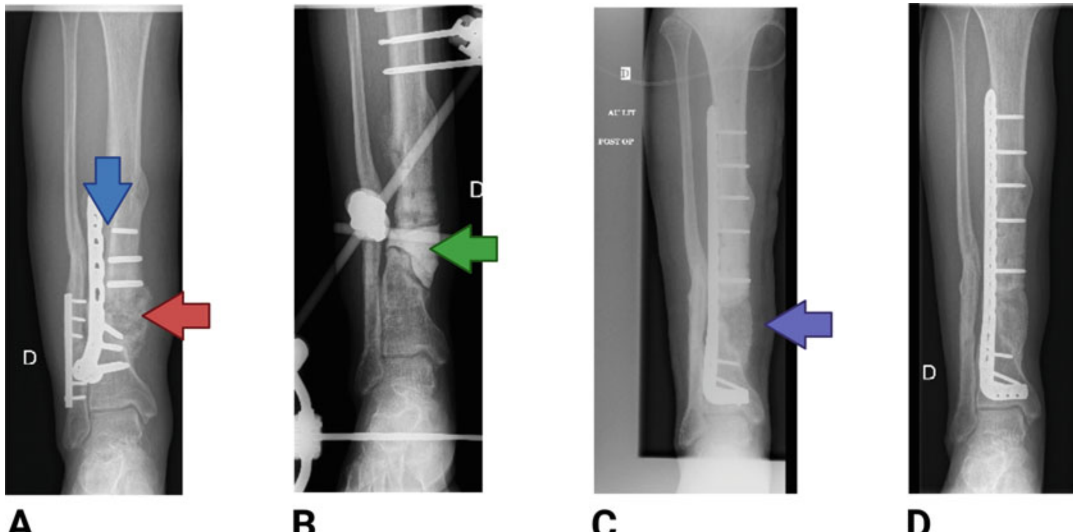

Fig. 3 Patient with a complex right tibial septic nonunion following a trauma that needed set up of a plate few years before, with bone destruction and necrosis due to the infection at the site of the facture (red arrow), with mechanical instability and broken screws (blue arrow) (**a**). Surgical treatment with a two-stage approach as described by Masquelet et al. (**b**) Resection of the infected bone, external fixation, setup of a temporary cement to fill the dead space and induce a vascularized membrane around the cement (**c**) and, 6–8 weeks later, incision of the membrane to remove the cement and bone grafting (purple arrow) with setting up of a new plate (osteosynthesis). During the follow-up, consolidation was observed, and amputation was avoided (**d**)

3.2 Management of Posttraumatic Infections

Posttraumatic infections (also called fracture-related infections) frequently involve the lower limbs, long bones, and the tibia. In this clinical situation, it is usual to perform osteosynthesis with plates to facilitate the consolidation of the bone. A bacterial inoculation at the time of the trauma could impair the consolidation process and lead to septic nonunion (Fig. 2), a complex-to-manage clinical situation at risk for amputation [10]. Indeed, subsequent surgeries are frequently required to rule out the infected bone and the infected implant, with the performance of a bone resection (Fig. 3). The standard of care at this stage to manage the bone defect is to perform an induced membrane technique (initially accidentally discovered in 1986 by Pr. Masquelet) that significantly improves the future potential for consolidation [26]. In a few words, the bone gap is filled during bone resection and bone sampling for microbiology by a polymethyl methacrylate cement spacer, and a new transient stabilization is performed. Impregnated cement with antibiotics for a local delivery could be used, but only heat-stable antibiotics are appropriate, as the cement rises in temperature during its reconstitution (≈100°C) a few minutes before its use. Empirical systemic antibiotics are immediately prescribed after surgery and then adapted to the bacteriological results to target pathogens responsible for the infection. The spacer induces

a richly vascularized pseudosynovial membrane around itself that takes several weeks to become biologically active [27]. Then, a new surgery is performed to open the induced membrane, rule out the cement, and perform definite stabilization and bone grafting in this closed biological chamber where biofilm and bacteria have been previously eradicated [26].

Another form of fracture-related infection is chronic osteomyelitis. After the fracture and despite the bacterial inoculation, a consolidation still occurs. However, a focal intramedullary infection could be responsible for the occurrence of clinical signs of infection that could appear many months or years after the trauma, with pain and discharge (Fig. 2). The prognosis and challenge of long bone chronic osteomyelitis are totally different in comparison with septic nonunion, as the problem is only infection and not the absence of consolidation. The standard of care includes surgical debridement of the medullary canal, microbiological sampling to identify the pathogens responsible for the infection, management of the intramedullary dead space that could be filled by a bone substitute (composed of a calcium sulfate and hydroxyapatite matrix and also an antibiotic; gentamicin is the most common antibiotic used in such devices), and systemic antibiotics that have to be adapted to the bacteriological results [28]. Long-bone pandiaphysitis is a particular posttraumatic chronic osteomyelitis with extensive intramedullary infection of the bone that could be particularly difficult to treat, with a high risk of relapse and functional impairment, especially as it could lead to amputation, total femur replacement, and/or limb disarticulation (Fig. 2) [29].

3.3 Prosthetic Joint Infections

Another common form of BJI is prosthetic joint infection (Fig. 2). A bacterial contamination could occur during prosthesis implantation or exchange of a prosthesis despite the maximal preventive measures currently set up in operating rooms [14]. The infection could be associated with acute symptoms such as fever and inflammatory joint effusion with purulence or can show up several years after inoculation with prosthesis loosening, that is, loss of the fixation of the prosthesis at the prosthesis-bone interface due to an inflammatory process generated by the infection. In acute prosthetic joint infection with clinical symptoms that recently appeared, the standard of care is to perform arthrotomy (surgical opening of the joint), bacteriological sampling, synovial debridement, pulsed lavage of the joint part of the prosthesis to limit the adherence of bacteria and prevent biofilm formation, and prescription of systemic antibiotics that have to be adapted to the final microbiological results. In patients with chronic prosthetic joint infection, the biofilm is considered to be completely matured, and prosthesis explantation, with reimplantation during the same or subsequent procedure, is recommended to eradicate the biofilm. Prosthesis exchange is totally relevant in patients with mechanical problems such as prosthesis loosening [14, 30].

3.4 BJIs Related to Step-by-Step Contamination of the Bone

Other forms of BJI are infections related to step-by-step contamination of the bone tissue following skin and soft-tissue impairment. This normally occurs in patients with paraplegia who are at risk of pressure ulcer-related pelvic osteomyelitis, intravenous drug users with focal cortical osteomyelitis, or diabetic patients with neuropathy who are at risk of diabetic foot osteomyelitis (Fig. 2). The standard of care for such infections frequently consists of surgical debridement with or without bone resection, microbiological sampling, skin and soft-tissue closure (to avoid iterative superinfection), and antimicrobial therapy that has to be adapted based on the microbiological results. Notably, most of these BJIs are polymicrobial [12, 31, 32].

3.5 Hematogenous Seeding BJIs

The last group of BJI is hematogenous seeding BJI from a separate infectious site that could be clinically obvious or occult. Native joint septic arthritis and spinal osteomyelitis (spondylodiscitis) are the most common hematogenous BJIs (Fig. 2). Bacterial identification is frequently performed using blood cultures and by performing punctures that must be done at the site of infection. These infections are most of the time acute, and the standard of care is frequently the use of adapted antibiotics. Indeed, surgery is only considered for infections that do not respond to antibiotics, for relapsing infections, or if large abscesses and/or bone destruction are associated [33, 34].

4 Guidelines for the Administration of Phages in Patients with BJIs

4.1 Historical Ways of Phage Administration

In patients with BJIs, we can imagine different ways of administration for phages. The historical way of administration is the local application of phages, which was common for trauma- and war-related infections before the antibiotic era [35]. André Raiga-Clémenceau, a French orthopedic surgeon who learned about phages with Felix d'Herelle, underlined the complexity of treating these infections and the failure of phage therapy in patients with BJIs [36]. He wrote: “At the bone necrosis stage, it [the bacteriophage] can only succeed in stopping the progression of the infection but can do nothing for the bone that death has deprived of vascularization; this bone will be sequestered and the lesion is now a matter only for surgery. To do anything else is, in my opinion, to commit an error of therapeutic indication”. Since the development of antibiotics that were stable chemically and had a broad spectrum of action against most of the bacteria responsible for infections, the use of phage therapy totally collapsed in the West. With the development of orthopedic surgery and implementation of different surgical technics that can promote bone healing, bone consolidation, and reconstruction of joints with arthroplasty, the standard of care for BJIs in modern medicine is mainly based on surgery.

Indeed, the objectives of surgery is to solve mechanical problems, to eradicate the biofilm, and to identify the pathogens involved in the process of infection. Following the surgery, empirical antibiotics are prescribed, and then could be replaced by targeted antibiotics active on the pathogens that grew in culture. In the East of Europe, especially in Georgia, Poland, and Russia, phage therapy remained a common practice in patients with BJI, but not in combination with orthopedic surgery [37, 38]. The approach in these countries remained linked to the historical (and respectable) use of phages, mainly in the outpatient setting, with phages that are not of pharmaceutical grade for injection use. In patients with long bone septic nonunion, focal cortical osteomyelitis, or endomedullar osteomyelitis, phage therapy is proposed as a daily topical treatment if the patient experiences a discharge, combined with daily oral intake of phages during several weeks. The concept is as follows: (i) iterative topical administration of phages on a discharge may allow phages to reach the bacteria in the underlying bone foci of infection, thanks to the replicative properties of the phages, and (ii) iterative daily oral intake of phages may allow phage absorption in the bloodstream with recognition of the targeted bacteria in the bone foci of infection and self-sustained amplification at the site of infection. These approaches could not be considered the most appropriate for the administration of phages for BJI at that time as (i) it does not take into account the surgery and (ii) the systemic pharmacokinetics of phages after oral intake is not well known.

4.2 Ways of Administration in Contemporary Orthopedic Surgery

In modern medicine, based on the recent literature that is now available about the use of pharmaceutical-grade phages in patients with BJIs, the two ways of administrations that have to be considered as "conventional" are the intravenous route as systemic exposition, and administration directly during surgery, and the use of direct injections, as the local exposition (Fig. 4) [4, 39, 40]. For intravenous use, pharmaceutical-grade phages are mandatory to limit any safety concerns. However, some alternative administrations also need to be considered. Systemic alternative administrations to treat patients with BJI could be done using nebulization, especially a vibrating mesh nebulizer that can increase systemic exposition throughout the lung (Fig. 4, panel **b**) [41]. The other alternatives are oral phages and rectal suppositories, but pharmacokinetic studies are required for all these potential ways of administration (Fig. 4, panels **c** and **d**) [42]. Local administration during surgery is, in theory, the best time for local treatment, but the administration of phages could be complex depending on the different anatomies in each kind of BJI (Fig. 4, panel **e**). Local administration of phages as topical use in patients with a BJI discharge has to be considered an alternative way of administration (Fig. 4, panel **g**). Moreover, this way of administration is probably challenging to evaluate and does not solve the mechanical

Fig. 4 Different ways of administration that are used or could be used in patients with BJIs. Systemic administrations: panel **a**: The intravenous way of administration has to be considered as the conventional way for systemic administration. Panel **b**: Nebulization, especially using vibrating mesh nebulizer with the ability to increase systemic exposition throughout the lung, is an emerging and interesting way of systemic administration that needs to be explored. Panels **c** and **d**: Gut administration with the use of oral or rectal route is historically considered as a potential way of systemic administration for patients with a BJI, considering that phages could reach the bloodstream throughout the gut, and then the bone focus of infection. Local administrations. Panel **e**: Direct local application of phages in a liquid form directly at the site of infection has to be considered as the conventional local way of administration. Panel **f**: The use of innovative formulation such as hydrogel that may facilitate the application during surgery on an infected implant and that may facilitate a prolonged local release of phages is of great interest, has to be developed and evaluated, and is considered as an alternative way of administration

orthopedic problem that could be associated. A potential combination with a dedicated hydrogel that could be used locally during surgery and that can deliver phages during the time at the surface of an implant is of huge interest and also has to be considered as an alternative local way of administration that needs to be developed (Fig. 4, panel **f**) [43].

4.3 Immunization and Clearance of Phages Depending on the Way of Administration

Depending on the different ways of administration (local or intravenous) and the type of phage used to treat the patient, it would be required to evaluate the risk of phage clearance, immunization, and neutralization by the immune system [44, 45]. Indeed, as phages are viral particles, they could be immediately filtered and cleared by the mononuclear phagocyte system (previously called the

reticuloendothelial system), especially in the liver, and the spleen, with also potential clearance by the kidney, especially after intravenous injection [46, 47]. Moreover, phages are recognized by the immune system and can then induce an immune response, especially the production of antibodies [47, 48]. As phages are highly prevalent in the environment, low titers of phage antibodies would be expected in part of human beings, and titers may increase after phage therapy. On the other hand, a human being could also be utterly naïve to phages. The pharmacokinetics of this immunization and its clinical impact on phage activity and the clinical outcome are not well known and might depend on several factors, such as the type of phages used, way of administration, and host's response [49]. We could hypothesize that intravenous administrations would be more associated with phage clearance and immunization than local injections. However, intravenous administrations may facilitate access to particular foci of infection that are not reachable with local administrations, especially if there is no anatomical space where phages could be locally injected.

4.4 The Use of Phages Is More or Less Relevant and Easy in the Field of BJI

The optimal way of administration of phages to treat a patient with a particular BJI should be local administration, systemic, or both. However, taking all of the elements discussed above into account and facing the heterogeneity of BJIs, the use of phages is more or less relevant and accessible, especially if there is a bone exposition or none, if there is an anatomical space where phages could be injected or not, and if the pathogen responsible for the infection could be identified before surgery or not [4]. Moreover, the phage clearance and consequences of a potential immunization should be diverse depending on the way of administration, and the clinical impact of the reactions of the host is unknown.

4.5 Potential Nonrelevant and Relevant Indications of Phage Therapy to Treat BJIs

After (i) describing the heterogeneity of BJIs and (ii) defining the conventional and alternative administrations of phages based on the recent literature and expertise, we can classify the different BJIs as relevant and nonrelevant for the development of phage therapy. Of course, this classification could change depending on the future clinical data that will be collected and on the potential impact of intravenous use of phages in terms of phage clearance and immunization.

4.5.1 Nonrelevant Indications for Phage Therapy

Culture-negative BJI: Phage therapy is a highly specific treatment, and demonstrating phage activity in vitro on the bacteria involved in the infection process is mandatory. Culture-negative BJIs are not rare, as depending on the clinical presentation, up to 7–25% of BJIs are associated with negative cultures [50–52]. Culture-negative BJI may be caused by slow-growing bacteria or by common bacteria, but for which culture is inhibited by the intake of antibiotics. In this particular setting, treating these patients with phage therapy is obviously inappropriate.

Absence of active phages (phage resistance): Phage resistance is a standard process that environmental and pathogenic bacteria have primarily developed, and several mechanisms have been described: resistance to phage adsorption, prevention of the phage DNA entry into the bacteria, intracellular phage DNA cleavage, but also different systems that can avoid phage replication into the bacteria [2]. Determining the activity of phages on the patient's strain seems to be required, and the lack of activity led to the avoidance of phage therapy.

BJIs with bone exposition: Currently, phage therapy seems nonrelevant for BJIs with bone exposition. First, the current management of BJI with bone exposition, such as pressure ulcer-related pelvic osteomyelitis and focal cortical osteomyelitis, involves performing a bone debridement to remove the dead bone and to identify the pathogen(s). Then, performance of skin and soft-tissue coverage (sometimes including muscles) is most-of time required, and this surgical step is crucial anatomically to avoid iterative superinfections of the bone, to facilitate its vascularization and also the exposition to antibiotics, and finally to protect the bone mechanically from the outside [10, 12]. Finally, as there is no anatomical space where phages could be injected as a liquid formulation during surgery, local phage therapy seems impossible at present in such BJI. In theory, a potential gel formulation with active phages should be possible. However, complete identification of the pathogens involved in the infection is available only after surgery. This is a strong limitation for perioperative phage intervention. Finally, intravenous phage therapy is technically feasible during the postoperative time, targeting the bacteria that have been identified, but as these BJIs are mostly plurimicrobial, this approach seems complicated. Of note, diabetic foot infections (see the chapter dedicated to diabetic foot infection) seem to be an exception [53].

4.5.2 Potential Indications for Phage Therapy

Long bone focal intramedullary osteomyelitis: As the microbiological diagnosis of long bone focal intramedullary osteomyelitis is frequently available only after bone debridement and skin and soft-tissue closure, local phage therapy is not a feasible option for such patients. Intravenous phage therapy with pharmaceutical-grade phages is, in theory, feasible after surgery, depending on the identification of the involved bacteria and its susceptibility to phages. The current development of calcium sulfate bone substitutes loaded with large-spectrum antibiotics such as gentamicin seems to be a more relevant innovative approach than phage therapy. Indeed, bone substitutes can fill the dead space after surgery, limit intramedullary bleeding and hematoma formation, deliver gentamicin locally for weeks, and promote bone healing as their matrix is constituted by calcium sulfate and hydroxyapatite [28, 54].

Fracture-related infections: Local phage therapy is difficult to set up in fracture-related infections. First, the microbiological diagnosis of fracture-related infections is also available only after surgery, and second, there is no anatomical space where phages can be easily applied during surgery. Some preclinical data show that daily postoperative local phage therapy in a liquid form (saline) could be administered at the infected site through a subcutaneous tube, but such a tube may greatly facilitate the occurrence of a new infection in clinical practice [55]. Developing a hydrogel loaded with phages is an option for the potential future perioperative application of phages. However, not knowing the microbiological diagnosis before surgery is a considerable barrier to developing perioperative phage therapy in this clinical setting [43]. Finally, intravenous phage therapy is one potential option, easily feasible in clinical practice after surgery and phage susceptibility testing, provided that it does not take too long to perform. In 2019, Nir-Paz et al. published the case of a patient with a trauma-related left tibial infection associated with extensively drug-resistant *Acinetobacter baumannii* and multidrug-resistant *Klebsiella pneumoniae* who has been treated intravenously with adapted phages and antibiotics [56].

4.5.3 Relevant Indications and Strategies Currently Developed in the Clinic

Long-bone pandiaphysitis: Long-bone pandiaphysitis is a putative relevant indication for phage therapy. Local and/or systemic treatments must be considered and discussed as in other clinical settings. Intravenous treatment would always be feasible with pharmaceutical-grade phages provided the bacteria could be identified and that phages are shown to be active in vitro on the patient's strain. Sometimes, abscesses are associated with long-bone pandiaphysitis and would be puncturable before surgery, giving the microbiological diagnosis before surgery. During surgery, extensive debridement of all abscesses is generally performed, and reaming of the intramedullary space has to be performed. The concept of reaming is to perform intramedullary debridement of the infected long bone using a guide, facilitating the excision of sequestrum and infected dead bone. Reaming is associated with intramedullary bleeding from the bone cortex, which limits the use of local phages that would be mechanically flushed by intramedullary bleeding [57, 58]. However, phages could be locally administered using intramedullar tubes, but these patients are at risk of new infection throughout the tube. Intravenous phage therapy is, in theory, feasible in patients with long-bone pandiaphysitis after the identification of the pathogen after the surgery.

5 Phage Therapy Experiences for BJI

In 2007, the Queen Astrid military hospital (Brussels, Belgium) was the first Western European hospital that reinitiated a phage therapy program [59]. Since then, a process of phage production has been set up to treat patients fulfilling the criteria of article §37 of the Declaration of Helsinki (World Medical Association), which mentions the following: "In the treatment of an individual patient, where proven interventions do not exist or other known interventions have been ineffective, the physician [. . .] may use an unproven intervention if [. . .] it offers hope of saving life, re-establishing health, or alleviating suffering" [60]. Among external requests that the Queen Astrid military hospital receives, some are complex BJIs. In the Leuven university hospital (located 30 km from Brussels), a coordination group for phage therapy was created to structure the care path of patients presenting different kinds of complex infections, such as BJIs. A standardized multidisciplinary treatment protocol has been set up, and some patients with femoral pandiaphysitis (but not exclusively) received such treatment. A first surgical debridement is performed in order to identify the pathogens from deep and bone tissue specimens. Then, active phages from the Queen Astrid military hospital or other libraries, such as the Eliava Institute in Georgia, are selected. During a new surgery, a draining system is placed into the intramedullar space for the injection of phages (Fig. 5) during surgery and then three times per day for 7–10 days. All patients have to receive also active systemic antibiotics. The disadvantages of this approach are the need for several surgeries and the risk of occurrence of a healthcare-associated new infection throughout the tubes [45, 47].

Based on our experience at PHAGE*in*LYON *Clinic*, long-bone pandiaphysistis would be one relevant indication. However, to avoid iterative surgeries, our approach is to first detect before surgery if there is a soft-tissue abscess associated with the long-bone pandiaphysistis that could be puncturable before surgery. If a pathogen such as *S. aureus* is found before surgery, we perform the phage susceptibility testing before surgery, and in case of susceptibility, we treat the patient with active phages intravenously administered exclusively (case reports will be published soon). The limitation is the need to achieve microbiological diagnosis before surgery and to have at our disposal pharmaceutical-grade active phages that could be injected intravenously.

5.1 BJIs Associated with Accessible Abscesses

Based on the PHAGEinLYON *Clinic* experience, in patients with accessible abscesses, the management would be adapted to and personalized depending on the clinical situation. In patients with implant-associated complex BJIs with abscesses, the added value of phage therapy in patients for whom surgical abscess debridement

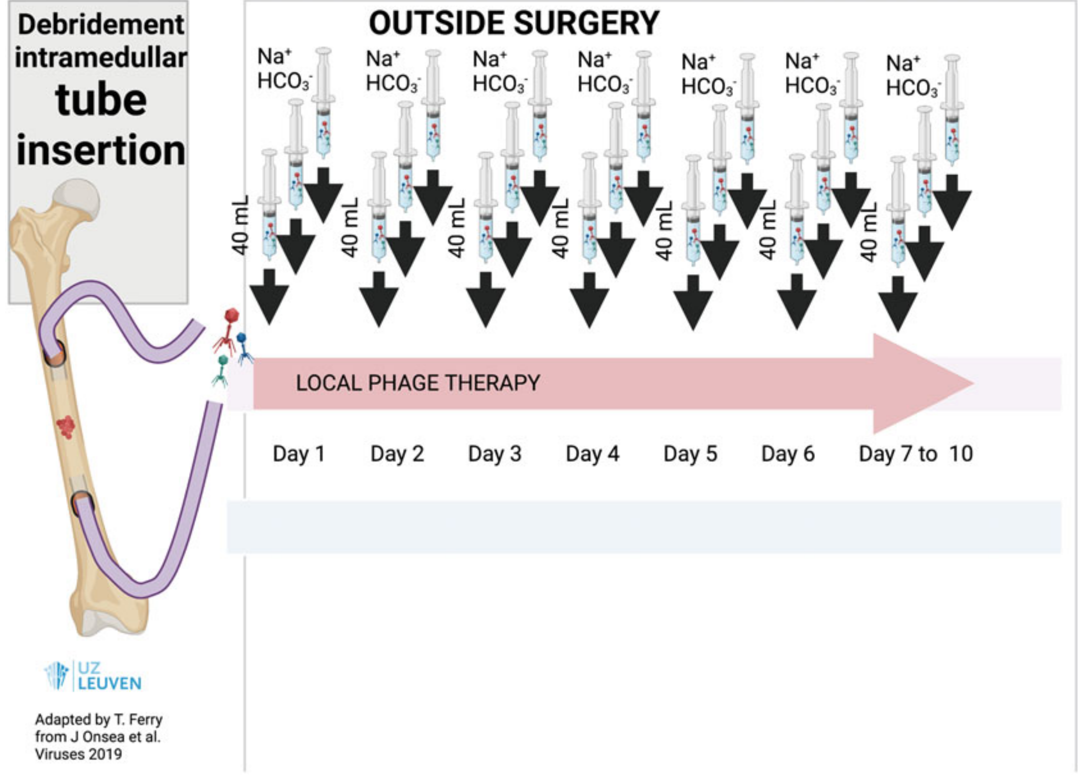

Fig. 5 Leuven standardized phage therapy protocol for patients with complex BJIs such as pandiaphysitis. First tubes were surgically set up into the intramedullar space to rinse the intramedullar space with sodium bicarbonate (1.4%) (to create an alkaline environment). Second, 40 mL of the phage solution was injected, and before wound closure, a gentamicin-impregnated sponge soaked in phage solution is placed on the infected bone. Just after surgery, 40 mL of the phage solution is injected three times per day for 7–10 days, and the tubes have to be closed for 10 min after each administration

and implant removal are performed would be difficult to demonstrate. From our point of view, the limitation of the therapeutic options (antimicrobial resistance) in patients with residual abscess cavity after surgical debridement and/or the inability to remove an infected implant is the determinant elements that we have to take into account in the indication for phage therapy and for its ways of administration. We recently published a case of *Pseudomonas aeruginosa* spinal infection with an abscess that was treated with surgical debridement and perioperative administration of active phages in the space of the abscess (Fig. 6) [61]. A second local administration was performed during a subsequent surgery. Intravenous phages were also prescribed as a companion, and we observed a favorable outcome. Furthermore, we recently encountered patients with relapsing abscesses associated with implant-associated infections for whom debridement and implant removal were unsuitable for functional reasons (Fig. 7). As the control of the infectious process

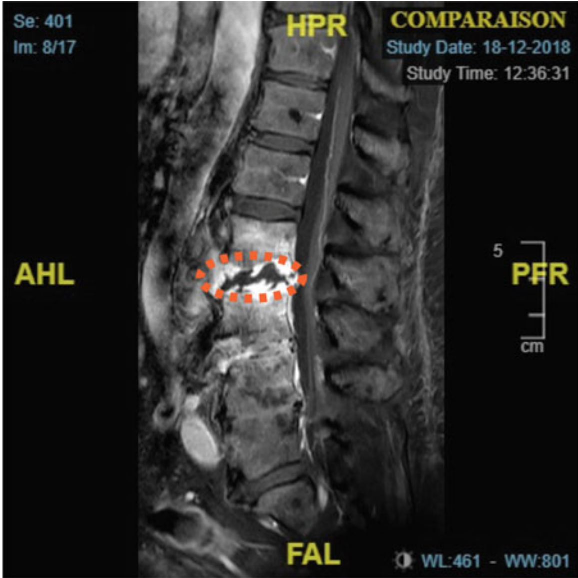

Fig. 6 Mid-sagittal magnetic resonance imaging view of a patient with a spinal abscess due to pandrug-resistant *P. aeruginosa* treated with local administration of phages into the abscess space and intravenously. The abscess has been delimited by the red circle (additional picture to that previously published)

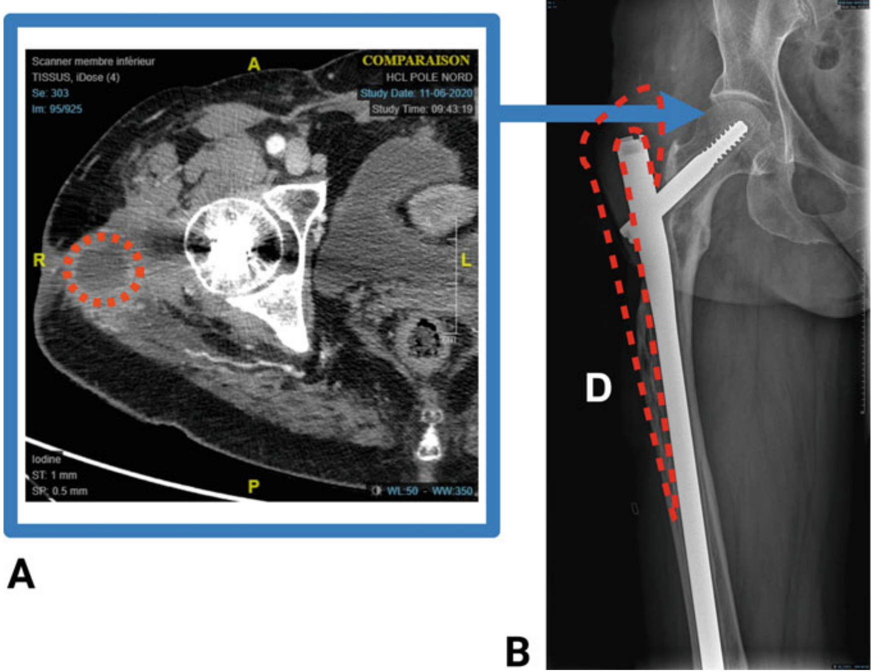

Fig. 7 Transversal CT scan view (panel **a**) of a patient with a complex relapsing *S. aureus* implant-associated infection for whom explantation of the implant was not desirable. Indeed, explantation or exchange of the nail visible on X-ray (panel **b**) is associated with definitive femoral fracture with risk of amputation

is usually not possible in this clinical situation without surgery, we qualified that as a relevant indication for phage therapy in combination with antibiotics. A one-shot phage injection under sonography was performed, and under suppressive antimicrobial treatment, the outcome was favorable (case report not yet published). Finally, we believe that BJIs with abscesses are potentially relevant indications of phages, especially if there is still a cavity after surgical abscess debridement, if a total surgical debridement of the abscess is not technically feasible, or if the abscess is associated with an orthopedic implant that cannot be ruled out for mechanical reasons.

Onsea et al. reported the Leuven experience with phages in patients with complex BJI. Some of them had purulent osteomyelitis with abscesses. In these clinical situations, surgical debridement with the administration of phages, as described above, was applied [45].

5.2 Prosthetic Joint Infection

Prosthetic joint infection is potentially the most relevant indication for phage therapy. Currently, a conservative approach is proposed for patients with acute prosthetic joint infection. In this approach, it is considered that during acute infection, the time to the inoculation of the bacteria is <3 months, and the biofilm produced by the pathogen is not yet matured [30]. The conservative surgery is based on arthrotomy (surgically opening the articulation), exchange of the polyethylene part of the prosthesis (polyethylene is a small plastic part of the prosthesis that facilitates the sliding of the two metal parts of the prosthesis), abundant pulsed lavage with saline to reduce the bacterial inocula, closure of the joint and closure of the skin and soft tissue. Then antibiotics could be started after the surgery. This procedure is called "DAIR," which stands for "Debridement Antibiotics and Implant Retention." Except in the acute infection setting, there is a clear indication of a one- or two-stage exchange of the complete prosthesis to eradicate the biofilm. However, these procedures are associated with significant morbidity, loss of bone stock, iterative surgeries, and several postoperative complications. Based on our experience, we believe that phage therapy would be relevant in patients with nonacute prosthetic joint infections for whom explantation is undesirable to avoid loss of function and/or amputation [18].

In 2021, Cano et al. from the Mayo Clinic published the case of a patient with a relapsing *Klebsiella pneumoniae* prosthetic knee infection in dead-end clinical situation for whom personalized pharmaceutical phages were produced and then used exclusively intravenously. Of note, the patient failed previous surgical procedures, and salvage combination of intravenous antibiotics (meropenem) for 8 weeks with a total of 40 weekday injections of active phages controlled the diseases, with antimicrobial suppression with minocycline [62]. This procedure is called "Haverty Protocol," with Haverty being the name of the patient (Fig. 8). Since the

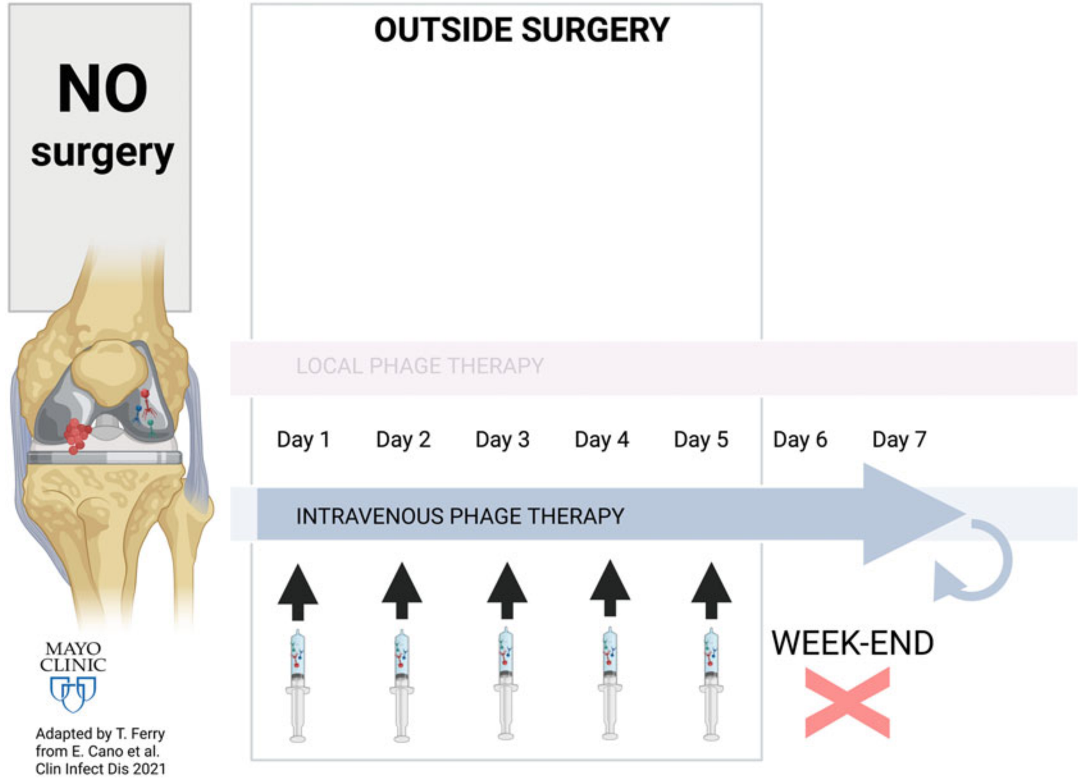

Fig. 8 "Haverty Protocol" proposed by Mayo Clinic based on experience from a patient for whom the infection was successfully controlled with exclusive intravenous injections of phages (total of 10 weekday injections) followed by suppressive antimicrobial therapy

experience, the Mayo Clinic started a collaborative program to develop phages to treat patients with prosthetic joint infections as salvage therapy (ClinicalTrials.gov Identifier: NCT05314426).

In the paper published in 2022 by Doub et al., the authors reported the experience of phage therapy for treating 10 recalcitrant periprosthetic joint infections [63]. The location (hip, knee, or elbow), clinical presentation (w/o sinus tract), pathogen (s) involved (*S. aureus, S. lugdunensis, S. epidermidis, Enterococcus faecalis,* or *K. pneumoniae*), surgery that has been performed (DAIR or revision), and phages used (from Yale Center for Phage Biology and Therapy or Adaptive phage therapeutics) in this case series were heterogeneous. The authors proposed two different protocols for bacteriophage therapy in patients with prosthetic joint infection, and they selected for each patient the one that seemed to be the most appropriate, based on the individual risk/benefit ratio. Protocol 1 (Fig. 9) includes one local injection and daily intravenous injections for 5 days; Protocol 2 (Fig. 10) includes five daily local and intravenous injections. Of note, the authors discussed that using a catheter is controversial in patients with a

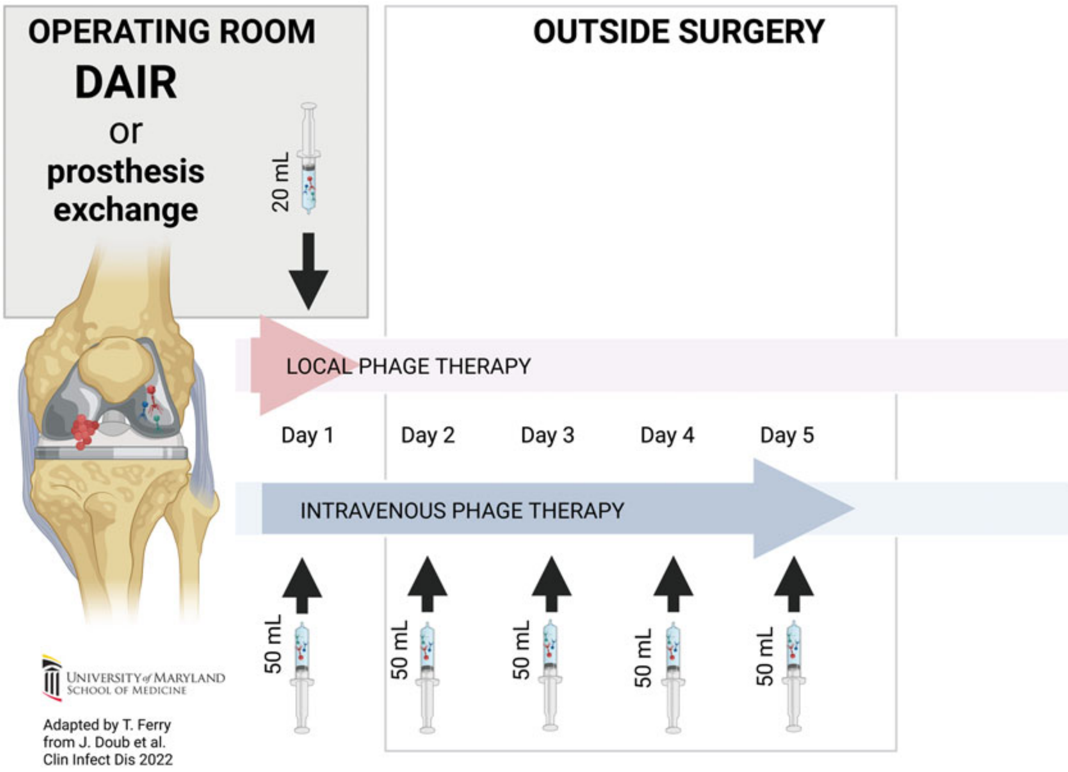

Fig. 9 "Protocol 1" for bacteriophage therapy in prosthetic joint infection in Baltimore, Maryland, with one perioperative local administration and one intravenous injection the day of the surgery, followed by one intravenous injection per day for 4 days

prosthetic joint infection, as it could be associated with nosocomial superinfection throughout the catheter [63]. Even if this risk may be limited, it unfortunately exists and limits the extensive use of this protocol. Moreover, there is another limitation of the use of catheters in patients with arthroplasty treated with DAIR that the authors did not discuss: the catheter can get stuck by the two parts of the prosthesis (especially for knee arthroplasty), which may lead to catheter rupture and to subsequent complications and risk for superinfection. Wire-reinforced catheters, such as an epidural catheter, could be used for that purpose, but crushing and catheter rupture remain potential complications (Fig. 11).

Since the setup of the PHAGE*in*LYON *Clinic* program, we considered that phage therapy was relevant in patients with prosthetic joint infection for whom it was essential to keep the function (and so for whom explantation or revision was not possible or not desirable) [4, 18, 64]. Indeed, performing a joint puncture and injecting phages in a liquid form during or after surgery is easy, especially for the knee location. We implemented the "Phago-DAIR" procedure, i.e., the use of phages during a DAIR procedure

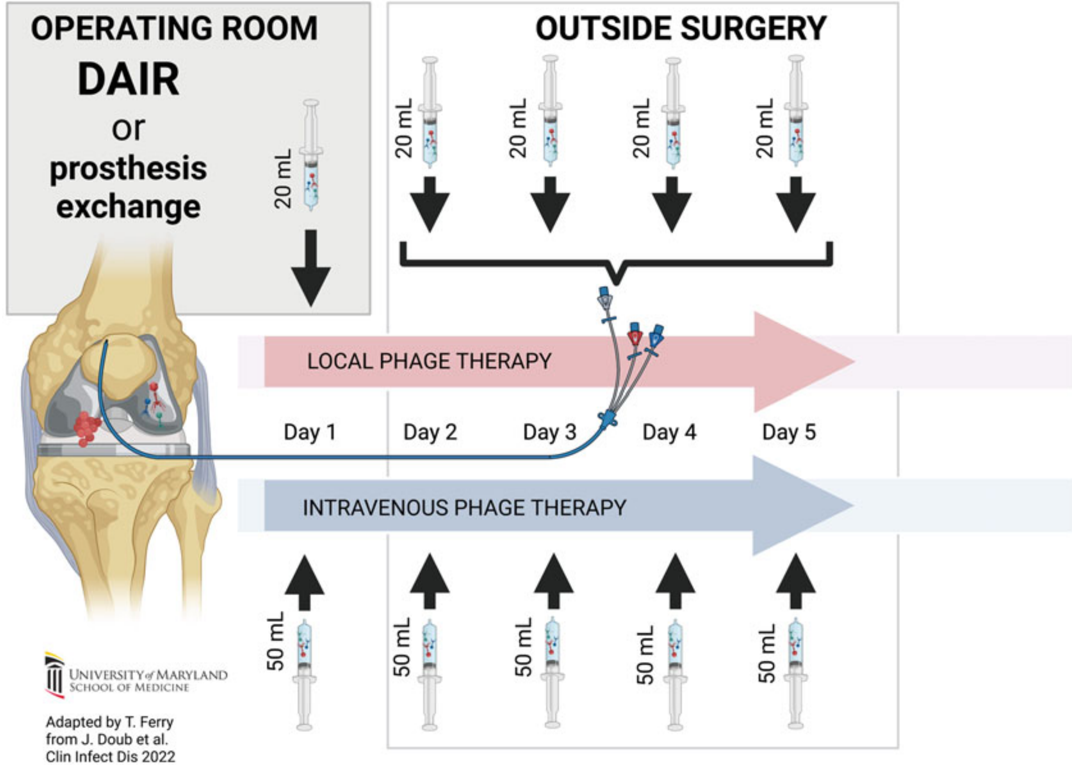

Fig. 10 "Protocol 2" for bacteriophage therapy in prosthetic joint infection in Baltimore, Maryland, with one perioperative local administration and one intravenous injection the day of the surgery, followed by four additional local daily injections using a Hickman catheter and one intravenous injection per day for 4 days

that was published in Frontiers in Medicine in 2020 [65]. The concept is to select patients with chronic and relapsing infection for whom a DAIR, followed by primary antimicrobial therapy for 3 months and then by suppressive antimicrobial therapy, was indicated as a salvage procedure [9]. As the probability of controlling the infectious process in this clinical setting is low, we hypothesized that the use of phages might increase it in such patients. We performed traditional open DAIR. Then the joint was closed in a watertight manner without using a drainage tube, and a single injection at the end of the procedure in the operating room of a significant volume of a cocktail of active phages freshly prepared by the hospital pharmacist was performed to replace the joint spacer with a saline solution containing phages (Fig. 12). We also applied this procedure for arthroscopic DAIR with success in one patient [66]. During the long-term follow-up, under suppressive therapy, we observed some patients with clinical relapse for whom subsequent injections of phages were exclusively performed under sonography. As repeated subsequent expositions of phages might be associated with a more potent anti-biofilm effect, we proposed

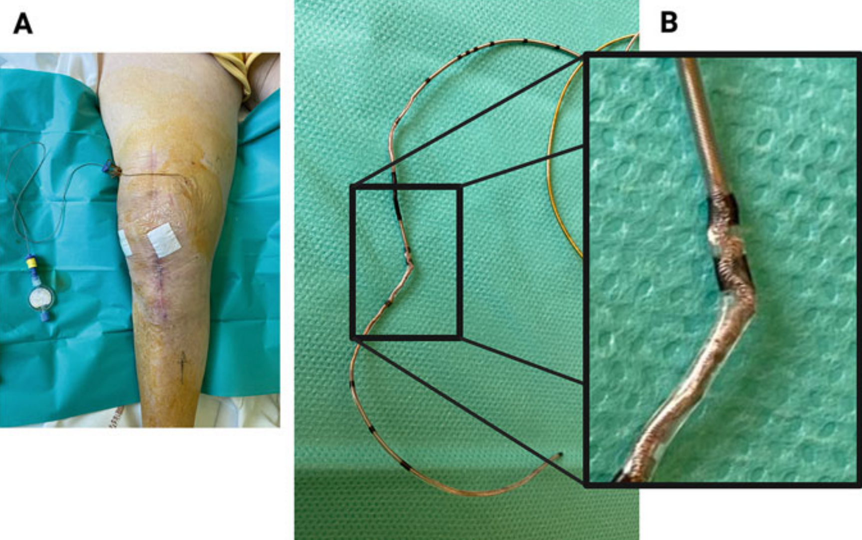

Fig. 11 Wire-reinforced epidural catheter (FlexTip Plus® Epidural catheter) inserted during arthroscopic DAIR surgery to try to inject phages postoperatively in a patient with knee arthroplasty (panel **a**). Unfortunately, the catheter has been compressed by the different component of the knee prosthesis, and was almost broken (wire-reinforced catheter was preferred rightly to limit the risk of rupture) (panel **b**). The catheter was removed following bending maneuvers of the knee prosthesis, and a surgical revision was not necessary. Acknowledgement to Mikhail Dziadzko and Constant Foissey for the helpful discussions and management of the catheter in this patient

three injections under sonography with a 1-week interval between each injection empirically. The positive points about this approach are as follows: (i) there is no need for a catheter, so there are no catheter complications; (ii) it allows evaluation during the bacterial culture of the joint fluid as inflammatory markers; and (iii) it is a minimally invasive procedure that is entirely acceptable for the patient (Fig. 13). As we recorded a positive outcome in patients for whom we performed such salvage procedure, this concept was integrated into the "PhagoDAIR I" clinical trial (ClinicalTrials.gov Identifier: NCT05369104). We also concluded that three injections of phages with a 1-week interval would probably be more appropriate than a single injection during DAIR. Then we modified the PhagoDAIR procedure by completing the preoperative injection with another injection under sonography 1 week later and again 1 week later (Fig. 14), totaling three injections with a 1-week interval. In clinical practice, it is not easy to select active phages before surgery, receive them before surgery, and prepare them on the day of the surgery. Consequently, we proposed, as a final evolution of the procedure, open or arthroscopic DAIR first, followed by reception of the final microbiological diagnosis and, after selecting the active phages, three injections under sonography with a 1-week interval during the primary antimicrobial therapy

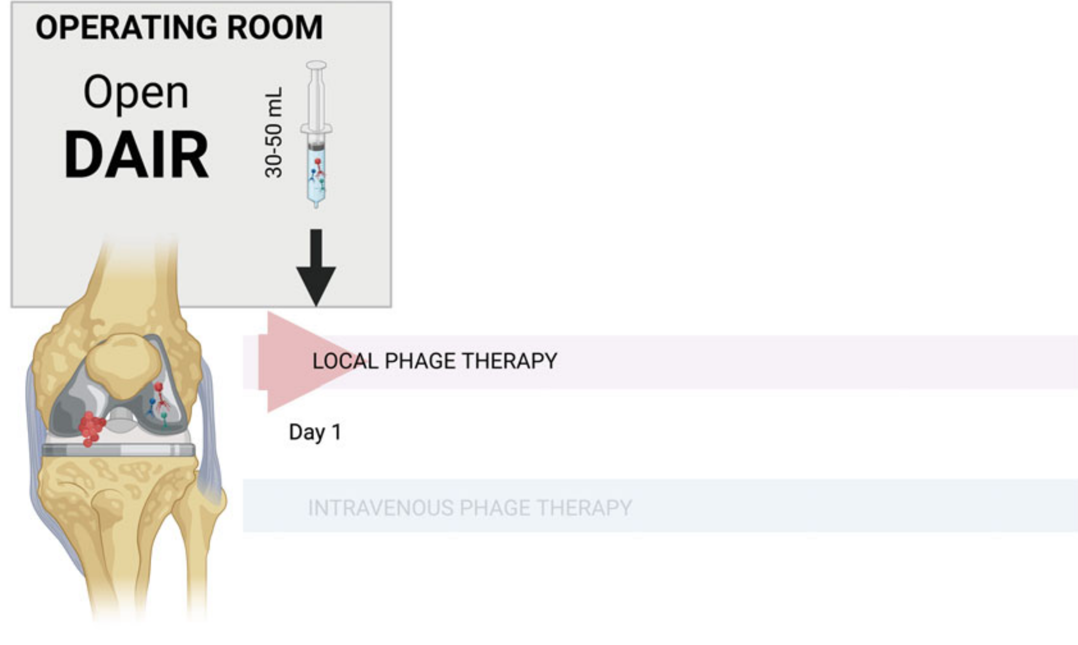

Fig. 12 The initial "PhagoDAIR" procedure (one-shot injection of phages at the end of a DAIR procedure) for patients with prosthetic joint infection that has been set up at the CRIOAc Lyon during the implementation of the PHAGE*in*LYON *Clinic* program

(Fig. 15). Finally, in patients for whom surgery was not feasible at all and some active phages and antibiotics were available, we proposed an exclusive medical approach with an intensive scheme of phage administration by combining daily intravenous and iterative local administrations of phages (every 2 days) for several weeks (Fig. 16). Evaluation of the safety and long-term success rate of each strategy is ongoing.

6 Conclusion and Perspective

Even if there is a rationale for using phages in patients with BJI, and regardless of the potential availability of active phages, phage therapy is complex in such patients. Due to a large pathophysiology and clinical presentation heterogeneity, all BJIs are not relevant indications for phage therapy. To demonstrate an added value of phage therapy as an adjuvant in patients with selected BJIs, several points need to be achieved: (i) concentrate the expertise in referral BJI centers, as has been done in France and Europe with the establishment of CRIOAc centers in France, and follow the initiative of the

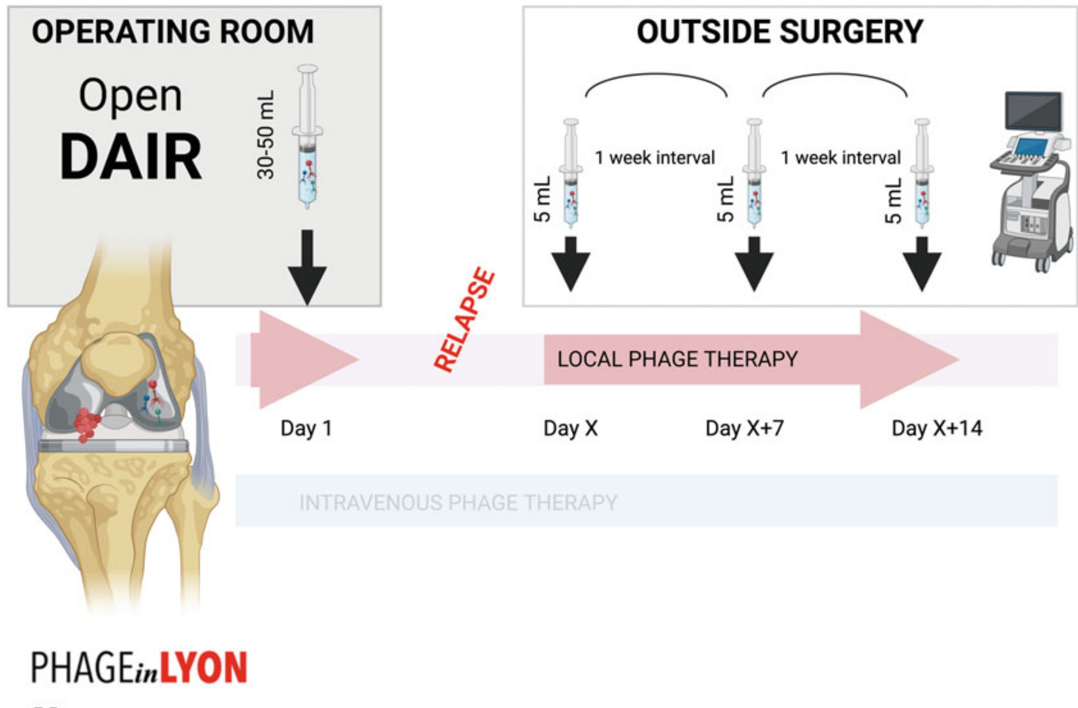

Fig. 13 Management of patients with prosthetic joint infection who experienced a relapse after the initial "PhagoDAIR" procedure for whom exclusive active-phage injections have be performed under sonography (three injections with an interval of 1 week between each injection); PHAGE*in*LYON *Clinic* program

European Bone and Joint Infection Society, the ESCMID Study Group for Implant-Associated Infections (ESGAI), and the ESCMID Study Group for Non-traditional Antibacterial Therapy (ESGNTA); (ii) select patients for whom phage therapy could be relevant as salvage therapy; (iii) establish compassionate use in expert center in close relationship with the healthcare authority; (iv) determine the optimal exposition to phages (local or systemic exposition, way of administration, number of injections) to achieve clinical response; and (v) develop phase II and then phase III clinical trials that could be set up in centers that interact between each other and where phages from academic of private origin could be evaluated.

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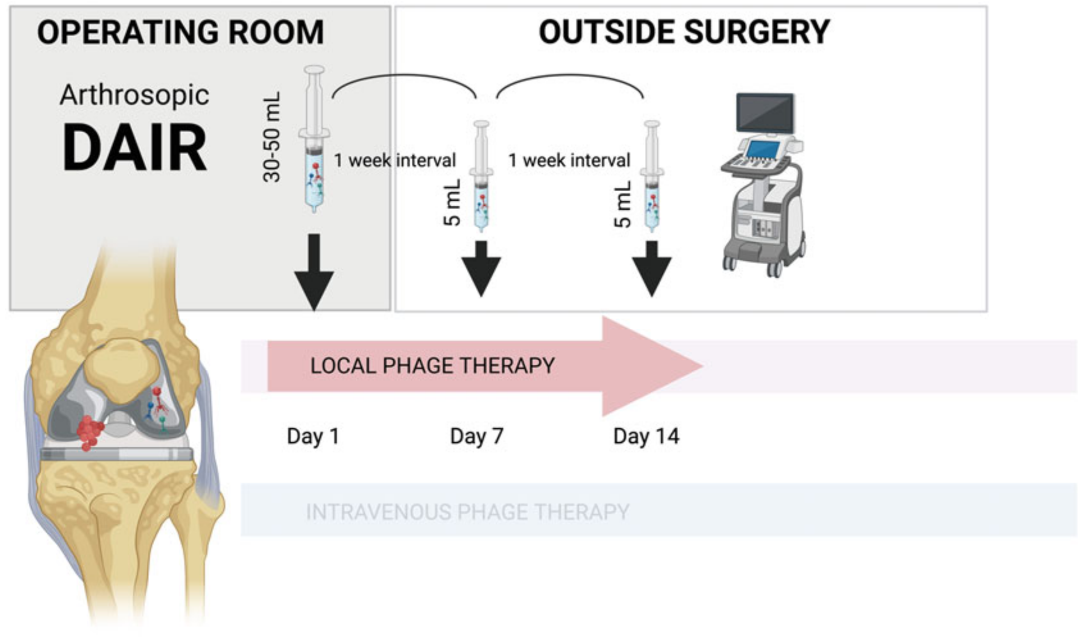

Fig. 14 Evolution of the "PhagoDAIR" procedure in patients with prosthetic joint infection with phage administration during DAIR surgery, followed by another injection performed under sonography 1 week later and again followed by another injection performed under sonography 1 week later, totaling three phage injections; PHAGE*in*LYON *Clinic* program

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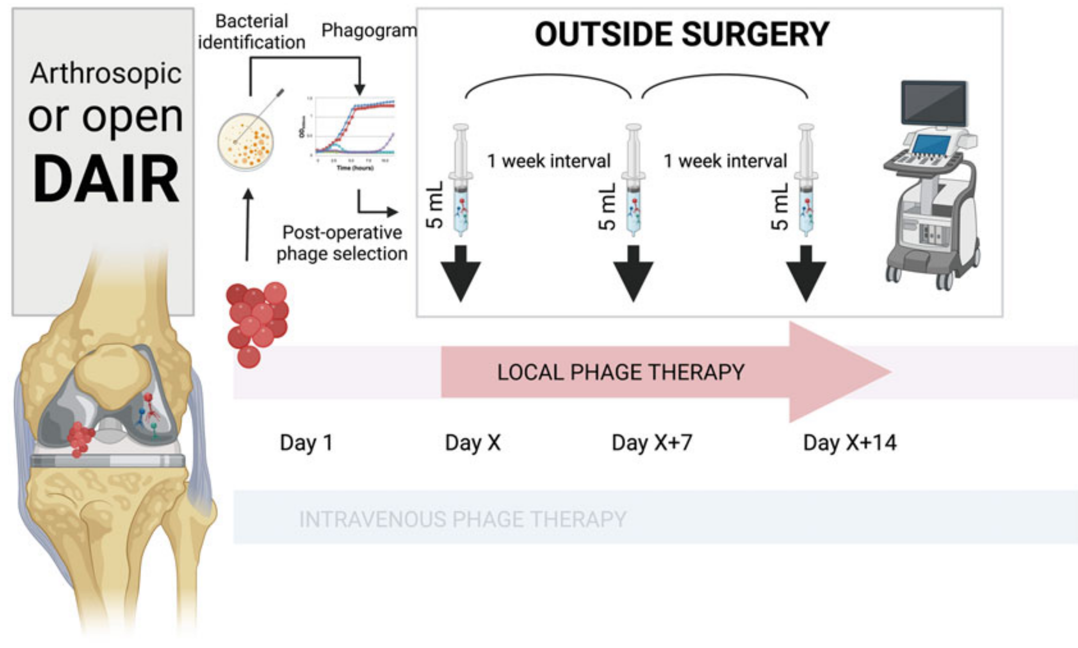

Fig. 15 Evolution of the "PhagoDAIR" procedure in patients with prosthetic joint infection with phage administration after DAIR surgery, one injection per week performed under sonography for 3 weeks, totaling three phage injections; PHAGE*in*LYON *Clinic* program

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Tristan Ferry, Myrtille Le Bouar, Thomas Briot, Gilles Leboucher, Camille Kolenda, Tiphaine Roussel-Gaillard.

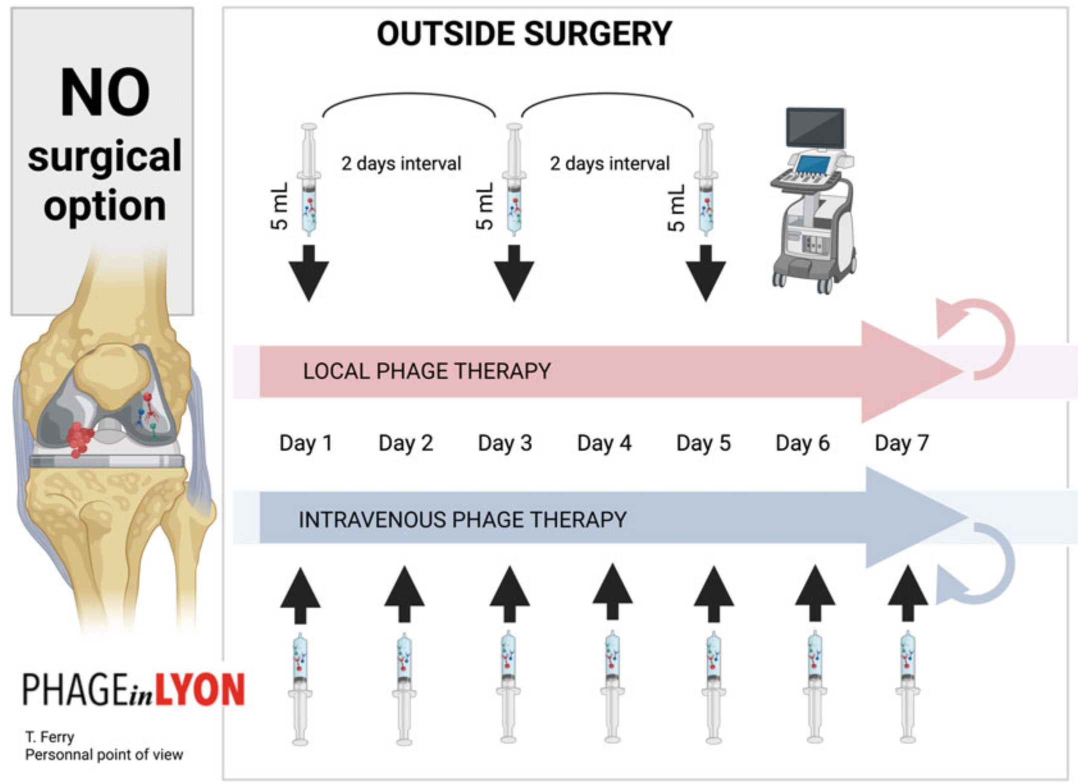

Fig. 16 Proposal for extensive phage exposition in patients with extremely severe prosthetic joint infection (potentially with abscesses) for whom surgery is not considered as a possible option; PHAGE*in*LYON *Clinic* program

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Chapter 15

Successful Use of Phage and Antibiotics Therapy for the Eradication of Two Bacterial Pathogens from the Respiratory Tract of an Infant

Vera V. Morozova, Yulia N. Kozlova, and Nina V. Tikunova

Abstract

Phage therapy can be a useful approach in a number of clinical cases associated with multidrug-resistant (MDR) bacterial infections. In this study, we describe a successful consecutive phage and antibiotic application to cure a 3-month-old girl suffering from severe bronchitis after tracheostomy. Bronchitis was associated with two bacterial agents, MDR *Pseudomonas aeruginosa* and a rare opportunistic pathogen *Dolosigranulum pigrum*. The phage cocktail "Pyobacteriophage" containing at least two different phages against isolated MDR *P. aeruginosa* strain was used via inhalation and nasal drops. Topical application of the phage cocktail removed most of *P. aeruginosa* cells and contributed to a change in the antimicrobial resistance profile of surviving *P. aeruginosa* cells. As a result, it became possible to choose and administer an appropriate antibiotic that was effective against both infectious agents. Complete recovery of the infant was recorded.

Key words Respiratory tract infection, Multidrug resistance, *Pseudomonas aeruginosa*, Phage therapy, *Dolosigranulum pigrum*

1 Introduction

The growing level of antibiotic resistance of nosocomial agents worldwide leads to the search for alternative methods of therapy. One possible method is the application of lytic bacteriophages – bacterial viruses that are able to destroy bacterial cells. Two main approaches to phage therapy are intravenous and topical phage administration. One of the most accessible places for the use of bacteriophages is the respiratory tract, where phages spread easily and can be administered by inhalation, rinsing solution, or nasal drops.

Many reports on phage treatment have been published over the last few years. Among them, 20 cases of phage treatment of low respiratory tract infections have been reported and analyzed in a

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comprehensive review [1]. These reports describe phage treatment of patients with multidrug-resistant (MDR) bacterial infections, and the applied protocols vary in the doses of phages, methods of administration, and duration of treatment. Three recent investigations have described the successful use of phages for the treatment of MDR *Pseudomonas aeruginosa* low respiratory tract infection [2–4]. For all these patients, phage therapy was proposed after administration of antibiotics therapy to control infection failed, and phages were used in a complex therapy with antibiotics. It seems to be a preferable way to administer therapy, because patients often have mixed infection with divergent antibiotic sensitivity. In addition, complex phage–antibiotic therapy substantially reduces the probability of selecting phage- and antibiotic-resistant clones of infectious agents. Moreover, it is not always possible to rapidly obtain therapeutic phages for all bacterial strains isolated from the sample from a patient; therefore, the addition of an appropriate antibiotic in the course of therapy leads to better results in pathogen elimination and recovery. Here, we describe a successful case of phage and antibiotic treatment of a 3-month-old girl suffering from bronchitis associated with MDR *P. aeruginosa* infection.

2 Patient

The patient was a 3-month-old girl with congenital pathology of the laryngeal muscles, which caused serious breathing problems. At the age of 2 weeks, the treatment included tracheostomy. A month later, the infant became ill with severe bronchitis. Three consecutive courses of different antibiotics were carried out, each of which led to a temporary improvement for several days only. As a result, the child's condition did not improve but worsened: fever, weakness, and anxiety were noted, and the infant's weight did not increase.

3 Bacterial Isolates

Two bacterial isolates were revealed by washing saline from the tracheostomy tube. The first pathogen was *P. aeruginosa*, a dominant microorganism with a titer of ~10^6 cfu/mL (colony-forming units per ml). The susceptibility of the isolate to antibiotics was determined using a disk diffusion test according to the guidelines of EUCAST 10.0 (https://eucast.org). Disks with antibiotics (OXOID) were applied to the lawn of the investigated culture on Mueller–Hinton agar (OXOID). The isolate was resistant to 30 tested antibiotics, including carbapenems, cephalosporins, and aminoglycosides. This bacterial strain was sensitive only to fluoroquinolones, which are toxic and not recommended for the treatment of infants.

The second strain was *Dolosigranulum pigrum*, which was detected by washing saline with a titer of ~10^3 cfu/mL. The species was identified by sequencing a fragment of the 16S rRNA gene (GenBank OQ355445). This bacterial species belongs to the *Carnobacteriaceae* family and has been reported to be a rare opportunistic pathogen causing respiratory tract infections, keratitis, and cholecystitis [5–7]. The identified *D. pigrum* isolate was sensitive to all tested antibiotics, including beta-lactams.

P. aeruginosa and *D. pigrum* strains were deposited in the Collection of Extremophilic Microorganisms and Type Cultures (CEMTC) of ICBFM SB RAS as *P. aeruginosa* CEMTC 2052 and *D. pigrum* CEMTC 2053.

4 Bacteriophages

Several commercial preparations of therapeutic phage cocktails produced by Microgen in Russia were screened against *P. aeruginosa* CEMTC 2052 using a spot test [8]. It was revealed that "Pyobacteriophage" cocktail was active. This phage preparation contains a set of different lytic phages specific to *Pseudomonas aeruginosa, Staphylococcus aureus*, *Enterococcus* spp., *Proteus* spp., *Klebsiella pneumonia,* and *Escherichia coli*.

To estimate the titer of "Pyobacteriophage", tenfold dilutions of phage preparation were dropped on the lawn of tested bacterial isolate, and plates were incubated at 37 °C overnight. The next day, plates were examined for the presence of phage plaques (negative colonies). "Pyobacteriophage" produced plaques of different sizes and morphologies, and some of them were surrounded by a "halo" associated with the exopolysaccharide depolymerizing activity of phage proteins. Therefore, it was supposed that the *P. aeruginosa* isolate was sensitive to at least two phages from the cocktail. The total titer of phages on a lawn of the isolate was calculated as ~10^7 pfu/mL (plaque-forming units per ml), and according to the manufacturer's instructions, this was sufficient for the administration of the cocktail.

Currently, therapeutic phages specific to *D. pigrum* are not developed. Whereas the initial titer of *D. pigrum* was low, phage treatment was focused on the eradication of *P. aeruginosa* infection.

5 Results and Discussion

"Pyobacteriophage" was administered by inhalation (2 mL with a titer of 10^7 pfu/mL) using a compressor nebulizer-inhaler. In addition, it was applied in nasal drops (1–2 drops into each nasal passage). Phage preparation was applied twice a day. Washing saline from the tracheostomy tube was tested for the presence of bacteria

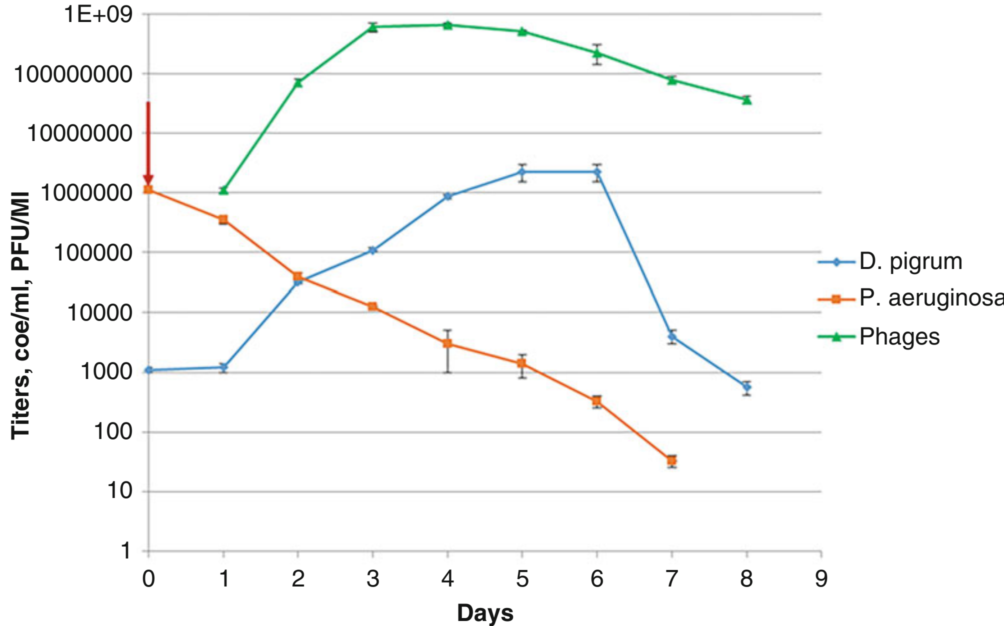

Fig. 1 Dynamics of bacterial and phage populations in the course of phage treatment. Titers were measured in cfu/ml for bacteria and pfu/ml for phages. Red arrow marks the start of phage administration. No phages specific to *P. aeruginosa* CEMTC 2052 were detected in saline before treatment

and phages once a day before phage administration. Phages were administered as monotherapy (without antibiotics) for 6 days. Substantial clinical improvement was observed; fever and anxiety reduced on the third day after the start of phage treatment. Side effects were not detected.

To evaluate the effectiveness of phage administration, the presence of both bacteria and phages was monitored by washing saline collected from a tracheostomy tube during the course of the treatment (Fig. 1). The amount of *P. aeruginosa* was found to be reduced by four orders of magnitude within 6 days of phage administration. On the contrary, the total titer of phages rapidly grew in the first 3 days, was high (~10^9 pfu/mL) for the next 3 days, and later began to decrease.

Each dose of administered preparation contained ~2×10^7 viable phages; therefore, a significant increase in the phage titer was associated with their reproduction in *P. aeruginosa* cells. A further decline of the phage titer was caused mainly by a lack of susceptible cells for reproduction.

The amount of the second microbial isolate *D. pigrum* CEMTC 2053 was initially low; however, on the fifth day of phage administration, its titer became slightly higher than the titer of *P. aeruginosa* CEMTC 2052 before treatment (Fig. 1). As there were no therapeutic phages specific to *D. pigrum*, an

antibiotic should be selected and used. Simultaneously, phage infection might lead to a change in antibiotic sensitivity in the surviving *P. aeruginosa* cells and the emergence of phage resistance.

To choose an appropriate antibiotic, a number of surviving colonies (the so-called survivors) of *P. aeruginosa*, which grew on Nutrient agar plates on the fifth day of phage treatment, were tested for sensitivity to antibiotics (according to the guidelines of EUCAST 10.0 (https://eucast.org)) and "Pyobacteriophage". It was found that the tested survivors showed various profiles of antibiotic resistance, and most of them became resistant to phages (Table 1). All clones were sensitive to cefotaxime, meropenem, and ticarcillin clavulanate.

Starting from the sixth day of treatment, cefotaxime was administered (i.v. in a dose of 50 mg/kg twice a day, 5 days), whereas "Pyobacteriophage" was no longer used. After that, *P. aeruginosa* and *D. pigrum* titers became undetectable in the washing saline. Then, the respiratory tube was removed, and the girl successfully recovered.

6 Conclusion

The combined application of bacteriophages and antibiotics seems a preferable way of therapy for MDR bacterial infections for a number of reasons. First, phage-antibiotic therapy substantially reduces the probability of selecting phage- and antibiotic-resistant clones of infectious agents. Second, there may be mixed infections, and it is not always possible to rapidly obtain therapeutic phages for all bacterial agents. So, phage preparation can be active against one group of infectious agents, whereas antibiotic(s) can effectively eliminate other pathogen(s); therefore, the combined use of phages and antibiotics leads to better treatment and patient recovery. One more reason to use combined therapy is that administration of phages may contribute to a change in the antimicrobial resistance profile of survived cells. As a result, it becomes possible to administer an appropriate antibiotic and completely eliminate pathogens.

Here, complete recovery of the infant with severe bronchitis was recorded after consecutive phage and antibiotic treatment. Even though the local application of the phage cocktail did not completely destroy the primary pathogenic bacterium (*P. aeruginosa*) and was not active against the second agent (*D. pigrum*), this preparation removed most of the leading agents and contributed to a change in the resistance profile of surviving *P. aeruginosa*. As a result, it was possible to choose an appropriate antibiotic and cure the infant.

Table 1
Heterogeneity of *P. aeruginosa* clones after phage treatment

Isolate	Sensitivity to antibiotics								Sensitivity to phages
	Gentamicin	Amikacin	Cefepime	Ceftazidime	Cefoperazone	Cefotaxime	Meropenem	Ticarcillin clavulanate	
Initial strain	I	R	R	R	R	R	I	R	S
1	R	R	R	R	I	S	S	S	S
2	R	I	R	I	I	S	S	S	R
3	R	S	R	R	S	S	S	S	R
4	R	S	S	S	S	S	S	S	R
5	R	S	R	S	S	S	S	S	R
6	I	S	S	S	I	S	S	S	R

1–6, residual clones of *P. aeruginosa* after phage treatment. Abbreviations: *R* resistant, *I* intermediate, *S* sensitive

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Part V

Phage Engineering Approaches

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Chapter 16

Genetic Engineering and Rebooting of Bacteriophages in L-Form Bacteria

Jonas Fernbach, Susanne Meile, Samuel Kilcher, and Martin J. Loessner

Abstract

The rapid increase of circulating, antibiotic-resistant pathogens is a major ongoing global health crisis, and arguably, the end of the "golden age of antibiotics" is looming. This has led to a surge in research and development of alternative antimicrobials, including bacteriophages, to treat such infections (phage therapy). Isolating natural phage variants for the treatment of individual patients is an arduous and time-consuming task. Furthermore, the use of natural phages is frequently hampered by natural limitations, such as moderate in vivo activity, the rapid emergence of resistance, insufficient host range, or the presence of undesirable genetic elements within the phage genome. Targeted genetic editing of wild-type phages (phage engineering) has successfully been employed in the past to mitigate some of these pitfalls and to increase the therapeutic efficacy of the underlying phage variants. Clearly, there is a large potential for the development of novel, marker-less genome-editing methodologies to facilitate the engineering of therapeutic phages. Steady advances in synthetic biology have facilitated the in vitro assembly of modified phage genomes, which can be activated ("rebooted") upon transformation of a suitable host cell. However, this can prove challenging, especially in difficult-to-transform Gram-positive bacteria. In this chapter, we detail the production of cell wall-deficient L-form bacteria and their application to activate synthetic genomes of phages infecting Gram-positive host species.

Key words Bacteriophage engineering, L-form bacteria, Synthetic biology, Phage therapy

1 Introduction

General overuse and prescription of antibiotics in agriculture and healthcare throughout the previous century have led to a steady increase in the amount of circulating and newly arising antimicrobial-resistant pathogens [1, 2]. Bacteriophages (phages) are a natural alternative to conventional antibiotics and have been used successfully in many compassionate use cases to treat bacterial infections where the current standard of care fails [3–5]. The majority of current therapeutic applications utilize natural phages (i.e., genetically wild type), which can be associated with several shortcomings such as narrow host range, emergence of bacterial

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resistance to phage infection, phage-mediated horizontal gene transfer of antibiotic resistance, and prophage integration [6–8]. These limitations are mostly a result of phage-host coevolution, which constitutes a complex interplay between factors, including receptor adaptations, host defense mechanisms, and intricate phage-phage interactions [9]. Bacteriophage engineering has successfully addressed some of these hurdles, e.g., by altering or broadening of host range, by removing genetic determinants of lysogeny or through the delivery of antimicrobial effector genes to enhance therapeutic efficacy. Furthermore, through the engineering of lysis-deficient phage, the release of inflammatory bacterial components (such as endotoxin) can be minimized [5, 10–15]. For an overview of phage engineering applications, we refer the reader to [16].

Several bacteriophage engineering methodologies have been published to date and are based either on homologous recombination (HR) or on the assembly and rebooting of synthetic genomes [13, 14, 17]. The key advantage of using HR-based engineering is the ability to edit large phage genomes, which represent a significant fraction of therapeutic phage candidates. However, these methods are laborious and require functional cloning systems within the host organism. Furthermore, cloning can be limited by gene toxicity. In general, HR produces a mixture of wild-type and engineered phages, necessitating a subsequent selection step to identify and isolate the engineered phage. Many of these challenges can be overcome by assembling modified phage genomes using in vitro methods. Such protocols are fast and simple, enable the simultaneous introduction of multiple modifications in a single step, and do not require laborious screening or cloning of potentially toxic genes. In vitro-assembled genomes must be activated to allow the production of virus progeny, which can either be achieved within a suitable host cell upon transformation [17–19] or using in vitro transcription/translation systems (TXTL) [20–22]. Most rebooting methods are based on *Escherichia coli* and are therefore well established for Gram-negative bacteria, particularly for phages that infect Enterobacteriaceae. Phages that target Gram-positive bacteria are more challenging to modify using synthetic methods (i) because their thick peptidoglycan layer prevents the efficient transformation of assembled genomes [15] and (ii) because the current cell-free phage production systems are optimized for phages of Gram-negative bacteria [23].

The transformation barrier can be overcome by using bacteria that grow in the absence of a functional cell wall [24, 25]. These so-called L-forms can take up large DNA molecules and have been shown to efficiently reboot wild-type and synthetic phage DNA in response to simple polyethylene-glycol (PEG) chemical transformation, even across genus boundaries [19, 26]. For example, the *Listeria monocytogenes* (*L. monocytogenes*)-derived L-form strain Rev2L reboots several phages that infect Gram-positive hosts [19].

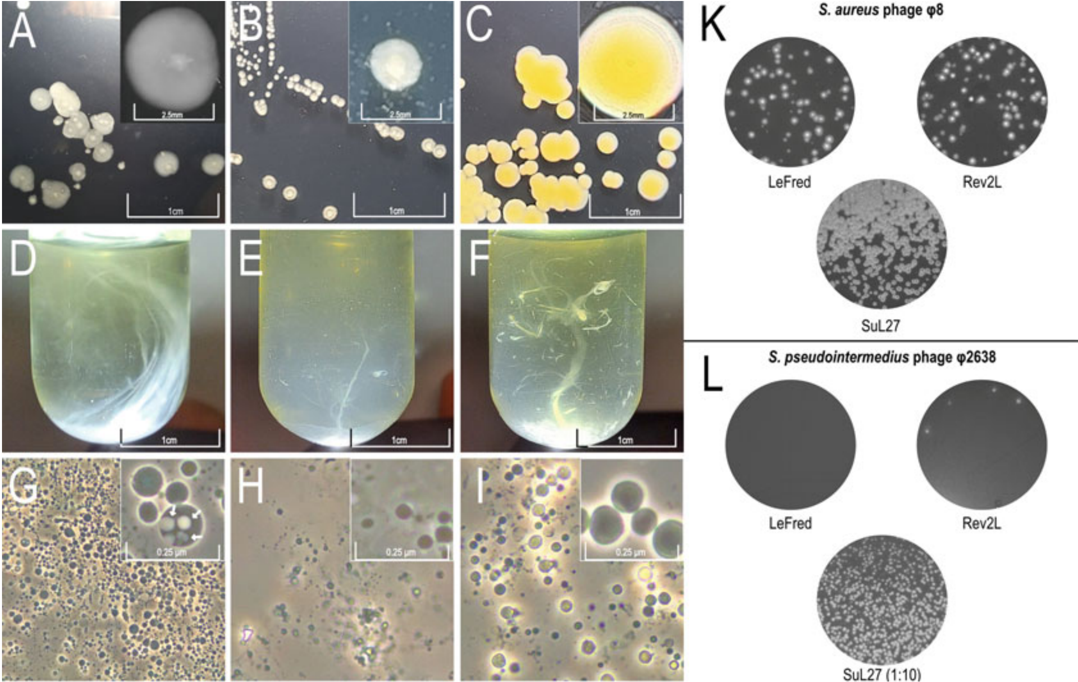

Fig. 1 Cell wall-deficient L-form bacteria. Panels (**a–c**) show the characteristic "fried-egg" morphology of L-form colony growth on antibiotic-supplemented DM3 agar plates for L-form variants derived from *S. aureus* (**a**), *L. monocytogenes* (**b**), and *S. xylosus* (**c**). (**d–f**) show varying, filament-like growth characteristics of L-form variants derived from *S. aureus* (**d**), *L. monocytogenes* (**e**), and *S. xylosus* (**f**) in antibiotic-supplemented DM3 liquid culture. Using microscopic imaging (**g–i**), one can distinguish the morphological differences between L-form variants derived from *S. aureus* (**a**), *L. monocytogenes* (**b**), and *S. xylosus* (**c**). The propagation via intracellular vesicle formation often observed in L-form bacteria [27] is visible (**g,** white arrows). (**k**) shows results of the rebooting of *S. aureus* bacteriophage φ8 genomic DNA in L-form variants derived from *S. aureus* (LeFred), *L. monocytogenes* (Rev2L), and *S. xylosus* (SuL27). For each rebooting reaction, 500-μL supernatant was plated on the corresponding phage propagation host. Each visible plaque in the bacterial lawn is an indication of a successfully rebooted, viable phage particle. (**l**) shows corresponding results for *S. pseudintermedius* phage φ2638 (50-μL supernatant was used for LeFred and Rev2L, 5 μL for SuL27)

L-forms have been observed across a wide range of bacterial species and could therefore offer a simple solution for the rebooting of phages infecting the most important Gram-positive AMR pathogens. L-forms can be readily obtained by culturing bacteria in an osmotically stable milieu in the presence of high concentrations of cell-wall active compounds, such as β-lactam antibiotics and bacteriophage endolysins [27]. Figure 1 presents characteristics of various L-forms grown on solid and liquid medium, as well as images visualizing key microscopical features.

Several caveats are associated with L-form rebooting. First, not all L-forms reboot genomic phage DNA, and not all phage genomes can be rebooted in a given L-form. In Fig. 1, we demonstrate this limitation using three L-form strains derived from *L. monocytogenes*, *Staphylococcus xylosus*, and *Staphylococcus aureus*.

The ability of an L-form to efficiently reboot a given phage genome is likely linked to phage-host compatibility factors, such as codon usage, promoter compatibility, and host-specific DNA modifications. Although cross-genus rebooting of phage DNA has been observed, in our experience, the efficiency of genome activation diminishes with phylogenetic distance. It is, therefore, advisable to isolate new L-form strains for the target species of interest. Here, we present the procedural workflow used for the development of stable, culturable L-form bacteria capable of rebooting synthetic phage DNA to form viable, infectious phage progeny.

2 Materials

The protocol is presented with *S. aureus* strain SH1000ΔmutS as a sample strain. Species-specific media, cultivation conditions, or other variations are indicated where applicable. Solutions and reagents should be handled and stored at room temperature unless indicated otherwise. Analytical-grade reagents and ultrapure water (Milli-Q or any other water meeting purity standards of >=18.2 MΩ cm^{-1} at 25 °C) should be used unless stated otherwise. All buffers and media should be sterilized by autoclave at 121 °C for 15 min unless stated otherwise.

2.1 Antibiotic-Induced L-Form Conversion

1. *S. aureus* strain SH1000ΔmutS (*see* **Note 1**) as −80 °C frozen stock (25% glycerol (v/v)).
2. Brain-heart infusion (BHI) nutrient broth: 37 g/L BHI in water, pH 7.4. Weigh 37 g of BHI (powder form) into a graduated cylinder and fill up to a volume of 1 L (*see* **Note 2**). Mix using a magnetic stir bar and adjust the pH to 7.4 (*see* **Note 3**).
3. BHI agar plates: Prepare BHI nutrient broth as described above and add 14 g/L agar to the solution prior to autoclaving (*see* **Note 4**). Pour 25 mL of autoclaved BHI agar per standard 10 cm petri dish.
4. Appropriate bacterial culture container (*see* **Note 5**).
5. DM3 supplement solution: 91.6 g/L succinic acid, 7.7 g/L glucose, 5.4 g/L K_2HPO_4, 2.3 g/L KH_2PO_4, 6.3 g/L $MgCl_2.6H_2O$ in water (pH 7.3). For 1 L of medium, begin by adding ~400 mL water to a > 1 L graduated cylinder. Add 91.6 g succinic acid and adjust the pH to 7.3 using NaOH (10 M) (*see* **Note 6**). Add the remaining ingredients, fill up to 1 L with water, and stir until all components have fully dissolved. Sterilize by sterile filtration (0.22 μm micropore filter) (*see* **Note 7**).

6. DM3 nutrient broth: 14.29 g/L casein peptone (tryptone), 14.29 g/L yeast extract in water. Dissolve 5 g casein peptone (tryptone) and 5 g yeast extract in 350 mL water (*see* **Note 8**).
7. Osmotically stable DM3 medium: 350 mL DM3 nutrient broth (from **item 6**), 645 mL DM3 supplement solution (from **item 5**), 5 mL bovine serum albumin (BSA, 2% (w/v)). Mix 645 mL DM3 supplement solution with 350 mL DM3 nutrient broth. Add 5 mL of sterile filtered BSA (2% (w/v)) (*see* **Note 9**).
8. DM3 agar plates: Prepare DM3 nutrient broth and supplement solution as described above, except that 8 g/L agar is added to the nutrient broth prior to autoclaving (*see* **Note 10**). Furthermore, the supplement solution must be warmed to ~50–60 °C before mixing with the autoclaved DM3 nutrient broth agar to prevent the agar from congealing before plates can be poured. Add antibiotics (if specified) once the agar has cooled to ~60 °C, mix gently by inverting the flask, and proceed immediately to pouring 25 mL per standard 10 cm petri dish.

2.2 DNA Synthesis and Assembly

1. Designed primer pairs (*see* Subheading 3.2)
2. DNA polymerase/PCR-kit compatible with the specific phage/bacterium genome characteristics such as G/C content
3. Gel electrophoresis setup
4. PCR purification kit of choice
5. Gibson isothermal DNA assembly reagent (e.g., GeneArt™ Gibson Assembly HiFi Master Mix)

2.3 L-Form Rebooting and Phage Recovery

1. Assembled phage DNA (*see* Subheading 3.2)
2. Solubilized penicillin at appropriate stock concentrations (e.g., 200 mg/mL)
3. Spectrophotometer for OD600 turbidity measurement
4. Falcon™ 50 mL Conical Centrifuge Tubes or equivalent
5. Polyethylene glycol (PEG) 20,000 (40% (w/v)) in water
6. Suitable bacterial phage propagation host as a cryostock or plate streak

3 Methods

All procedures should be carried out at room temperature unless stated otherwise. Cultivation conditions of 37 °C and BHI nutrient media pertain to *S. aureus* specifically and should be adapted for other bacterial species accordingly.

3.1 Antibiotic-Induced L-Form Conversion

1. Use an inoculation loop to spread SH1000ΔmutS onto a BHI agar plate. Place the plate at 37 °C and let grow overnight (*see* **Note 11**).
2. Fill an appropriate aerated bacterial culture container (*see* **Note 5**) with 5 mL BHI broth and inoculate with a single colony from the incubated agar plate. Let grow overnight at 37 °C with constant agitation (180 rpm culture shaker).
3. Dip a fresh inoculation loop into the overnight culture and spread the residual liquid onto a DM3 plate (supplemented with 200 μg/mL penicillin) (*see* **Notes 12** and **13**).
4. Wrap the plate in sealing film to reduce moisture loss and store at 37 °C.
5. Inspect the plate every few days until colonies displaying the characteristic "fried-egg" morphology of L-form cultures are visible (Fig. 1a–c). The cells should also be inspected under the microscope to verify L-form conversion (Fig. 1g–i).
6. Use an inoculation loop to scrape cell material from an L-form colony and spread it onto a fresh DM3 (+200 μg/mL penicillin) plate.
7. Repeat **steps 4–6** two more times (*see* **Note 14**).
8. Beginning with passage #3, in addition to transferring picked L-form colonies onto a new plate, also inoculate 1 mL DM3 (+200 μg/mL penicillin) liquid culture (*see* **Note 15**) with scraped cell material (*see* **Note 16,** Fig. 2).
9. Let the culture grow at 37 °C without agitation until L-form growth is visually evident (Fig. 1d–f).
10. Homogenize the cell suspension by vortexing and transfer 10 μL into a fresh 1 mL DM3 (+200 μg/mL penicillin) liquid culture. Repeat **steps 9** and **10** several times (at least thrice or more, Fig. 2, *see* **Note 17**).
11. Once reliable liquid growth has been achieved in iterative passages, the homogenized cell suspension should be frozen as a cryostock at −80 °C in 25% (v/v) glycerol.
12. Inoculate 5× 1 mL DM3 liquid medium (+200 μg/mL penicillin) from the cryostock (*see* **Note 18**). If L-form growth (OD600>= 0.15) can be achieved reliably from the cryostock, the L-form strain is deemed viable and tested for the ability to reboot phage DNA. This can be done by extracting genomic phage DNA and using it during the rebooting reaction as template DNA, as described in Subheading 3.3. Note that this correlates with but does not guarantee the success of rebooting synthetically assembled DNA of the same sequence.

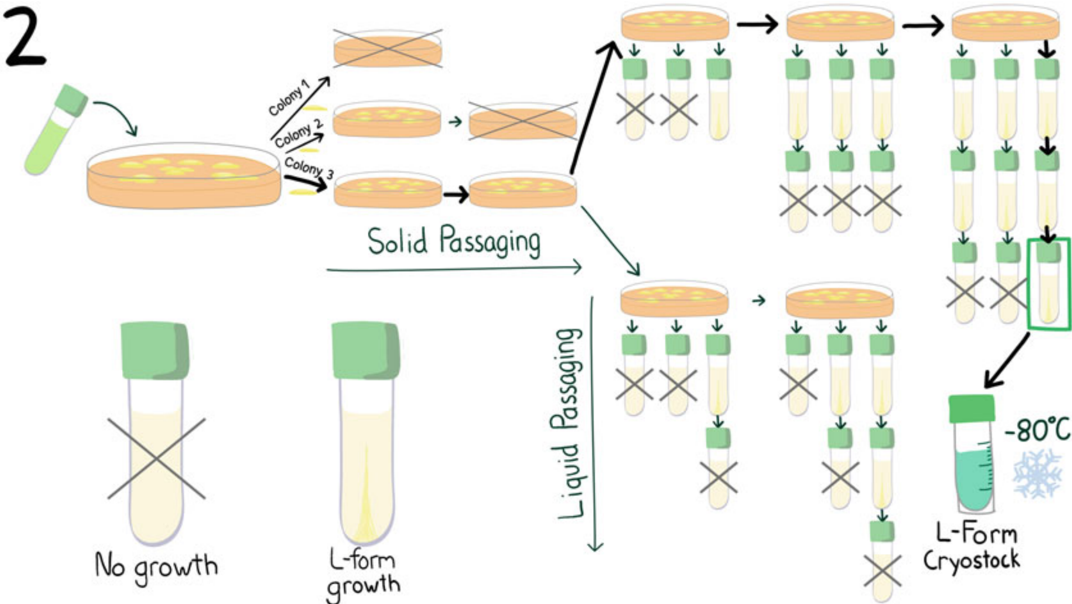

Fig. 2 Schematic of one possible variant of the L-form cultivation workflow. Briefly, DM3 (+Pen200) agar plates are streaked with *S. aureus* wild-type cells and then serially passaged, first on solid and then on solid and liquid medium until reliable L-form growth in liquid culture is achieved. Thicker arrows trace the path of a successful series of passages

3.2 DNA Synthesis and Assembly

1. Design primers to split the phage genome into equally sized fragments with ~40-bp overlaps at each end (Fig. 3, *see* **Notes 19** and **20**). Primers should be designed in concordance with the provider's specifications for the polymerase used to amplify the fragments (*see* **Note 21**).
2. Perform polymerase chain reaction to obtain each genome fragment using the previously designed primers and provider-specified PCR conditions. Phage lysate (1 μL) can initially be used as template (*see* **Note 22**).
3. Perform gel electrophoresis with each sample following PCR to determine correct, pure amplification of the intended genome fragments (*see* **Note 23**).
4. Perform PCR purification with the obtained PCR products prior to genome assembly (*see* **Note 24**).
5. Assemble the genome fragments using Gibson isothermal DNA assembly reagent according to the provider's specifications (*see* **Note 25**).

3.3 L-Form Rebooting

1. Pre-warm DM3 liquid medium to 37 °C (*see* **Note 26**).
2. Add 200 μg/mL penicillin to the prewarmed DM3.
3. Distribute 1 mL of supplemented DM3 (+Pen200) into sterile glass culture vials (*see* **Notes 5** and **27**).

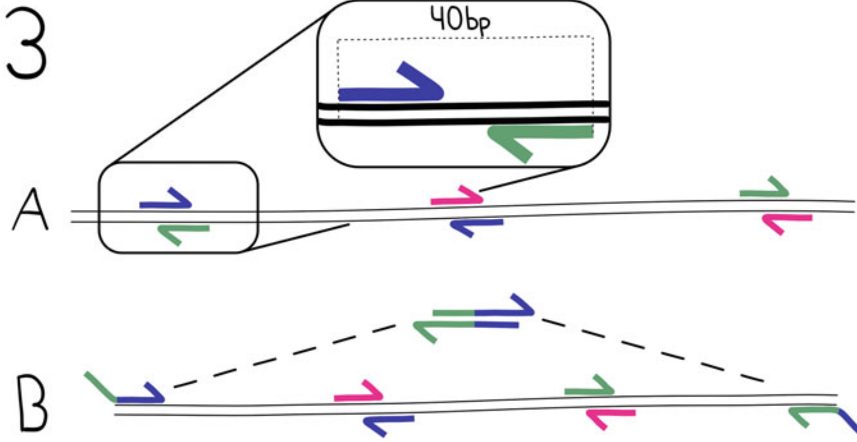

Fig. 3 Schematic of primer pair placement (colored arrows) on the bacteriophage genome (parallel black bars). For circularly permuted, terminally redundant phage genomes (**a**), primer pairs can be positioned arbitrarily on the sequence; however we recommend roughly equidistant primer pairs and overlapping placement at fragment junctions of at least 40 bp. Panel (**b**) shows the appropriate placement of terminal primers for fixed-end bacteriophage genomes and their complementary 5′ synthetic overhangs (colored appendages of the terminal primers)

4. Inoculate each aliquot with L-form from the cryostock using a sterile inoculation loop (*see* **Note 18**).
5. Mix gently and place at 37 °C (without agitation) for 18–32 h (*see* **Note 28**).
6. Disperse the aggregated L-forms by vortexing until the culture appears homogenous (*see* **Note 29**).
7. Determine the OD600 of the culture and adjust to OD600 = 0.15 using prewarmed DM3 medium.
8. Pipette 100 μL L-form culture (OD600 = 0.15) into a 50 mL canonical centrifuge tube or a similar tube.
9. Add the assembled DNA (*see* **Note 30)** and mix thoroughly by pipetting.
10. Add 150 μL 40% PEG (w/v) and mix thoroughly by pipetting (*see* **Note 31**).
11. Incubate at RT for 5 min.
12. Add 10 mL DM3 medium (without antibiotics) prewarmed at 37 °C and mix gently.
13. Incubate at 37 °C for 18–24 h without agitation (*see* **Note 28**).
14. Prepare a culture of a host compatible with the rebooted phage in preparation for phage recovery (*see* **Notes 5** and **32**).

3.4 Phage Recovery

1. Perform soft-agar overlays of the L-form supernatant using the phage propagation host and incubate plates overnight at 37 °C. If rebooting was successful, rebooted phages contained in the supernatant should form plaques on the propagation host. (*see* **Note 33**).
2. Individual plaques formed on the bacterial lawn with high likelihood stem from rebooted phage particles and can be isolated and propagated for downstream application (*see* **Note 34**).

4 Notes

1. We have achieved L-form conversion for a number of different bacterial species, including *S. aureus*, *L. monocytogenes*, and *S. xylosus*.
2. ddH20 can be used instead of MilliQ in this step. To avoid the very fine nutrient powder dispersing into the air while filling the graduated cylinder, add a small amount of water (e.g., 100 mL) to the cylinder before adding the powder.
3. Concentrated HCL and NaOH (10 M, lower concentrations can be used for fine adjustments) are used to adjust the pH.
4. It is advisable to add the agar directly to the flask to be autoclaved to avoid agar clumps adhering to the graduated cylinder when transferring the solution into the flask. Also, adding a magnetic stir bar to the flask prior to sterilization allows for easier dissolution of the agar after autoclaving and prior to pouring.
5. For example, a 20 mL glass vial with a loose aluminum lid for aerobic growth conditions.
6. Succinic acid is a weak acid, and titration with a strong base such as NaOH leads to a very steep sigmoidal curve at pH 7.3. Thus, when nearing pH 7.3 during titration, the more drastic the change in pH is for the same volume of NaOH added. Add smaller volumes or lower concentrations of NaOH to not shoot beyond pH 7.3. Alternatively, titration with a weak base is possible but has not been tested.
7. It is recommended to use a large-volume micropore filter in combination with a vacuum pump for large volumes.
8. Preparing precisely 350 mL DM3 nutrient broth in a 1 L flask will allow us to fill up to 1 L with DM3 supplement solution after autoclaving.
9. The final concentration of BSA in the DM3 medium is 0.01% (w/v).

10. This refers to the agar concentration in the final medium solution (after adding DM3 supplement solution). That is, the concentration during autoclaving is ~23 g/L (8 g/ 350 mL).
11. *Staphylococcus aureus* can also be grown at room temperature with ~three-fold reduction in growth speed (can be advantageous for culturing over the weekend).
12. If L-form growth cannot be achieved using a specific combination and concentration of antibiotics, variations should be attempted (e.g., 500 μg/mL fosfomycin or other cell-wall active antibiotics at variable concentrations and combinations). We advise determining the MIC for each host/antibiotic combination.
13. DM3 agar plates are much softer than conventional 14 g/L plates, and the surface can be easily penetrated, which is to be avoided. Spread the bacterial cells carefully. Alternatively, several microliters of the overnight culture can be pipetted onto the plate and dispersed using a conventional cell spreader.
14. The passage number is from experience and likely arbitrary. The number of passages needed to establish stable L-form growth has not been systematically quantified and may vary significantly between bacterial species.
15. Depending on the aeration of the culture vial and the corresponding amount of medium evaporation, this volume may be scaled up to 5 mL.
16. It is not feasible to conduct the passaging in a systematic manner since the parallel passaging of liquid and solid cultures would lead to an exponential increase in the number of samples. See passaging scheme in Fig. 2.
17. The rationale behind this iterative passaging is the accumulation of mutations and/or metabolic changes, which result in a stable, fast-growing L-form phenotype over time.
18. Depending on the density of the culture prior to freezing, increased amounts of cell material may be needed to inoculate the liquid culture to ensure reliable growth.
19. In our experience, more than 10 fragments and/or fragments longer than 12 kB in length have proven difficult to assemble, showing exceedingly low yields of rebooted genomes and limiting our success to phage genomes up to ~80 kB. These parameters, as well as the optimal length of the fragment overlaps, should be designed in concordance with the specifications of the DNA assembly kit used.
20. For circularly permuted phage genomes, the overall placement of primer pairs on the genome is arbitrary. For phages with a constant genome structure (cohesive ends or nonpermuted

terminal redundancy), the precise physical structure must be known to design primers that allow for artificial circularization of the genome prior to transformation (see Fig. 3b).

21. The polymerase of choice depends on the bacterial species in question and usually depends on factors such as G/C content, potential for secondary structure formation, DNA modifications, etc.
22. The optimal template amount depends on the phage in question and can initially be adjusted simply by diluting the lysate. We recommend purifying genomic phage DNA to quantify the amount of PCR template used.
23. If PCR impurities (i.e., unspecific bands during gel electrophoresis) cannot be resolved by troubleshooting the PCR reaction, preparative gel extraction can be used to purify fragments. In our experience, visualizing DNA bands with a UV transilluminator can severely damage the DNA. We strongly recommend using a blue-light transilluminator and a suitable dye such as GelGreen.
24. This can be done using a conventional PCR purification kit. This step is not required by the GeneArt™ Gibson Assembly HiFi Master Mix specifications but is recommended to increase yield.
25. Genome assembly may also be achieved by restriction-ligation but is more laborious and has not been tested.
26. This temperature is also *S. aureus*-specific. The temperature should be adapted to the bacterial species in question.
27. Multiple cultures can be set up for redundancy in case of failure of growth but is not stringently necessary if L-form inoculation from the cryostock proves consistently reliable.
28. Times can vary greatly between different L-form and phage variants and should be adapted accordingly. In case of exceedingly low rebooting efficiency, one can attempt to optimize by varying incubation times prior to (i.e., OD600 at inoculation) and/or during (time post inoculation) rebooting. Additional variations concerning final PEG concentration, the volume of DM3 addition for PEG dilution, and PEG molecular weight can also be used. The values used in our protocol have previously been optimized for the rebooting of *L. monocytogenes* phages in Rev2L [19]. Toxic payloads may also negatively affect rebooting efficiency, particularly if the payload in question is designed to have an intracellular effect.
29. Multiple samples can be pooled prior to vortexing, as long as growth is visible.
30. To maintain osmolarity, this volume should be kept <20 μL. Larger volumes have not been tested.

31. Wide bore pipette tips make it easier to mix the highly viscous PEG. You can make your own wide bore tips by cutting 1–2 mm off a regular tip using flamed scissors/scalpel.
32. The culture medium should be adapted accordingly if a non-*S. aureus* propagation host is used.
33. Because rebooting efficiency can vary widely between different L-form/phage combinations, in our experience, it has been advantageous to perform the soft-agar overlay with different amounts of L-form solution (e.g., 500 μL, 50 μL, 5 μL) to guarantee distinct plaque formation. In some cases, using a large volume of L-form solution can negatively influence the growth of the propagation host during soft-agar overlays.
34. A suitable control consists of rebooting an incomplete assembly (i.e., leaving out one or more fragments for the synthetic phage assembly). Should plaques form from the control reaction, this is an indication of contamination. This could be in the form of foreign bacteriophage contamination or the rebooting of residual genomic phage DNA used as PCR template. The latter can be avoided by including a *DpnI* digestion of either the PCR or assembly product to remove methylated DNA from the reaction mix.

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Conflict of Interest Statement S.K. is a part-time employee of Micreos Pharmaceuticals, and M.J.L. is a scientific advisor to Micreos Pharmaceuticals. J.F. and S.M. declare no conflict of interest.

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Chapter 17

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

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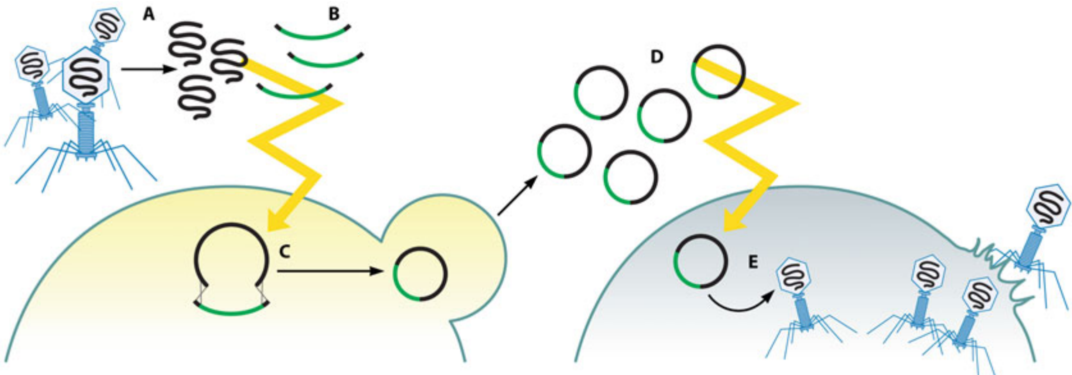

Fig. 1 Bacteriophage recombineering of electroporated DNA. Purified phage DNA (**a**) and dsDNA recombineering substrates (**b**) are co-electroporated into cells (**c**). Recombination between their homologous regions (in orange) (**d**) results in recombinant phage particles (containing DNA fragments 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).

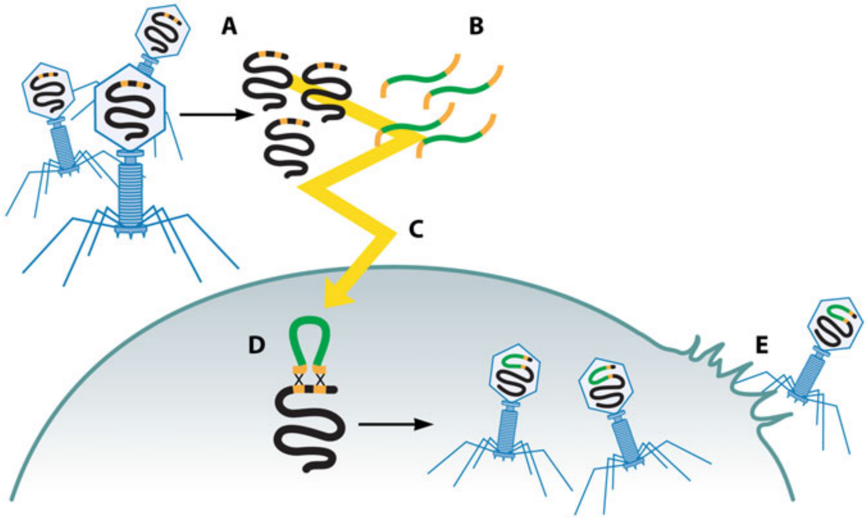

Fig. 2 Yeast-based assembly of phage genomes. Purified phage DNA (**a**) is electroporated into *S. cerevisiae* together with linear YAC molecules with overhangs (in black) homologous to the 5′ and 3′ ends of the linear phage genome (**b**). Recombination in the yeast cell enables genomic subcloning (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× *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.

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Chapter 18

Genetic Engineering of Therapeutic Phages Using Type III CRISPR-Cas Systems

Courtney M. Hill and Asma Hatoum-Aslan

Abstract

The functional characterization of "hypothetical" phage genes is a major bottleneck in basic and applied phage research. To compound this issue, the most suitable phages for therapeutic applications—the strictly lytic variety—are largely recalcitrant to classical genetic techniques due to low recombination rates and lack of selectable markers. Here we describe methods for fast and effective phage engineering that rely upon a Type III-A CRISPR-Cas system. In these methods, the CRISPR-Cas system is used as a powerful counter-selection tool to isolate rare phage recombinants.

Key words Bacteriophage engineering, Type III-A CRISPR-Cas, CRISPR-Cas10, Phage genome editing, Staphylococci

1 Introduction

Phages are the most abundant biological entities in nature and play critical roles in shaping prokaryotic populations [1]. After their discovery by Frederick Twort and Felix d'Herelle over a century ago [2, 3], phages served as early model organisms for studying horizontal gene transfer and bacterial evolution. As such, phages helped lay the foundations of modern molecular biology and, in the process, have generated many of the tools routinely used in contemporary molecular and genetic research, including phage-derived enzymes (e.g., T7 RNA polymerase and T4 ligase), enzymes originating from antiphage immune systems (e.g., restriction modification and CRISPR-Cas), and platform technologies based on whole phages (e.g., phage display). Soon after their discovery, phages were also recognized for their potential to be used as antibacterial therapeutics [4]. Although the discovery and development of antibiotics overshadowed the early use of phage therapy in Western nations, the emergence of multidrug-resistant bacteria has ignited a renewed interest in exploiting phages as antimicrobial agents. With

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the advent of next-generation sequencing technologies, new phages and their genome sequences are being published at an accelerated pace. However, as the sequenced phage collection continues to expand, so does the number of phage genes with unknown functions. Whether to gain deeper insights into phage biology, or to create safe and effective therapeutics and technologies, the development of robust genetic tools is critical to advance phage research.

A variety of approaches for phage genome engineering are currently available and have been extensively reviewed elsewhere [5–7]. One critical aspect that should be considered when planning the best engineering strategy is the lifestyle of the phage of interest. Temperate/lysogenic phages have the ability to integrate their genetic material into the host chromosome and remain dormant for many generations. Thus, temperate phage genomes are best accessed using the tools available for genetic engineering of the host. In contrast, virulent/lytic phages, which are the most suitable for therapeutic applications, reproduce and kill the host within minutes or hours of infection. This rapid and destructive life cycle makes genetic manipulation of virulent phages more challenging. The most commonly used methods for engineering phages rely upon homologous recombination, in which a phage of interest is allowed to recombine with a related phage (via co-infection) or a donor DNA construct introduced into the host. However, the major limitations of these approaches stem from low recombination rates and lack of selectable markers, which together necessitate a labor-intensive screening process to recover the desired mutants. Helping to overcome these limitations, CRISPR-Cas (named after Clustered regularly-interspaced short palindromic repeats – CRISPR-associated) systems have been successfully employed in conjunction with homologous recombination as counterselection tools to facilitate the recovery of phage recombinants [8].

CRISPR-Cas systems are a family of adaptive immune systems within prokaryotes that protect against foreign nucleic acids [9–11]. A CRISPR locus consists of a CRISPR array, containing alternating short, direct repeats and spacers with similar lengths adjacent to a set of *cas* genes. CRISPR-Cas immunity functions in three steps: (i) adaptation, (ii) biogenesis, and (iii) interference (Fig. 1a). During adaptation, Cas proteins capture segments of invading nucleic acids (typically from plasmids and phages) and integrate them as "spacers" in between the repeats in the CRISPR array. During biogenesis, the CRISPR array is transcribed and processed to produce mature CRISPR RNAs (crRNAs) consisting of single spacer sequences, and *cas* genes are transcribed and translated. The crRNAs then form complexes with one or multiple Cas proteins. During interference, effector complexes recognize and degrade foreign nucleic acid "protospacers" that are complementary to the crRNA in the complex. CRISPR-Cas systems are currently divided

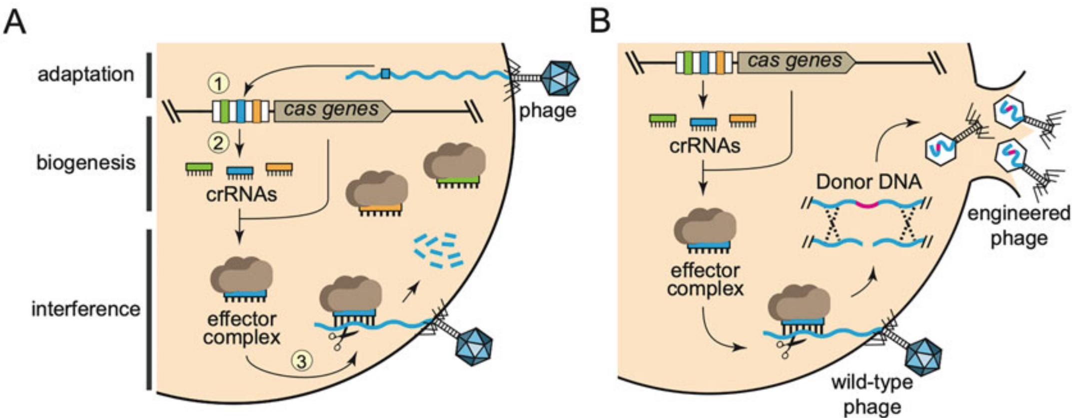

Fig. 1 General mechanism of CRISPR-Cas immunity (**a**) and general approach for phage engineering (**b**)

into two classes and six types (I–VI). Class 1 systems (I, III, and IV) utilize effector complexes composed of multiple Cas proteins, whereas Class 2 systems (II, V, VI) only require a single Cas protein to recognize and degrade nucleic acid invaders [12]. Of these systems, types I–III have been more commonly used to facilitate phage engineering [8]. In the simplest form of this approach (Fig. 1b), the CRISPR-Cas system is programmed to target the region of interest in the phage genome, and a donor DNA construct harboring the targeted region with the desired mutation(s) is supplied *in trans*. During infection, a tiny fraction of phages may recombine with the donor DNA construct and acquire the desired mutations, thus rendering recombinant phages resistant to CRISPR immunity. The CRISPR-Cas system is then used to eliminate wild-type phages from the population and thereby reveal the rare recombinants.

This chapter describes approaches for engineering staphylococcal phages using a type III-A CRISPR-Cas system, CRISPR-Cas10, native to *Staphylococcus epidermidis* RP62a [13]. This system contains three spacers and nine CRISPR-associated *cas* and *csm* genes that have been previously shown to block plasmid conjugation and phage infection [14–16] (Fig. 2a). In this system, the Cas10-Csm effector complex, which is composed of Cas10, Csm2, Csm3, Csm4, Csm5, and a crRNA, recognizes foreign RNA targets [17, 18] (Fig. 2b). One critical feature that enables Cas10-Csm to discriminate between "self" and foreign (i.e., "non-self") RNA is the absence of complementarity between the repeat-derived eight nucleotides on the 5′-end of the crRNA (known as the 5′-tag) and the opposing protospacer flanking sequence (PFS, also known as the anti-tag) on the targeted RNA [19] (Fig. 2 C and D). When Cas10-Csm binds non-self RNA, at least three catalytic activities are triggered in the complex: degradation of RNA by Csm3 [20], cleavage of DNA by Cas10 [20, 21], and production of cyclic

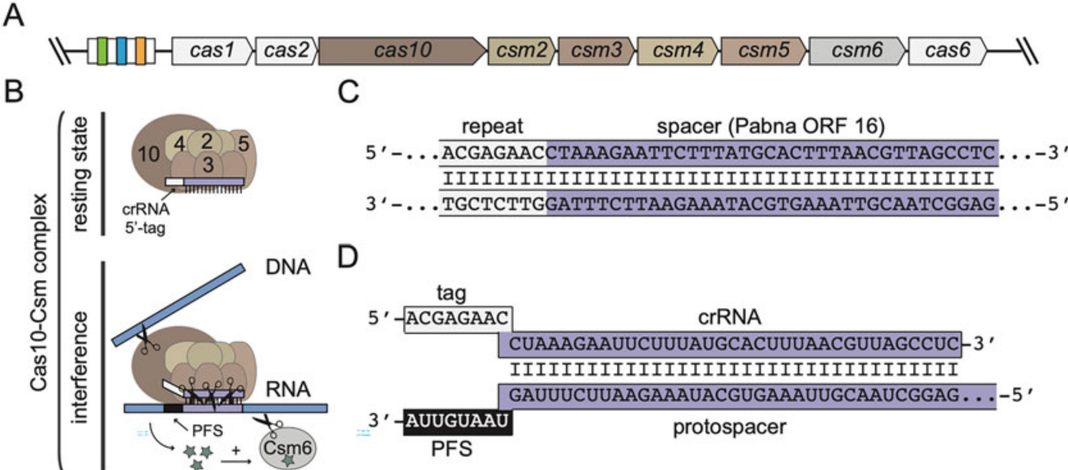

Fig. 2 CRISPR-Cas10 immunity in *S. epidermidis* RP62a. (**a**) The CRISPR locus in *S. epidermidis* RP62a. (**b**) The Cas10-Csm effector complex in resting state (top) and during interference (bottom). (**c**) The sequences of a repeat (gray) and spacer (purple) designed to target *Staphylococcus* phage Pabna open reading frame (ORF) 16. (**d**) Sequences of the corresponding crRNA (top) and protospacer region (bottom) highlighting the absence of complementarity between the crRNA 5′-tag and PFS/anti-tag region

oligoadenylate molecules by Cas10 [22–24]. These second messengers bind and activate the Csm6 ribonuclease, which is not a part of the complex but is essential for immunity, particularly when targeted genes are expressed late in the phage replication cycle [25]. In addition, conserved "housekeeping" nucleases, including PNPase, RNase J, and RNase R, have been shown to participate in interference [26–28]. The combined activities of these Cas and non-Cas nucleases, in conjunction with the high tolerance for mismatches between the crRNA and corresponding protospacer [29], result in a robust immune response that is nearly impossible for targeted phages to escape. This feature makes CRISPR-Cas10 (and perhaps other type III systems) particularly powerful as counterselection tools to facilitate phage engineering [13].

Here, a two-step protocol for introducing precise mutations in a staphylococcal phage genome is described (Fig. 3). In the first step, a targeting strain is created that consists of a staphylococcal host harboring a chromosomally encoded CRISPR-Cas10 system and p*crispr-spcφ*, a plasmid with a mini-CRISPR array (i.e., a single repeat and spacer) that targets the region in the phage genome to be mutated. This targeting strain is first challenged with the phage of interest to confirm the effectiveness of the chosen spacer. In the second step, an editing strain is created in which a donor DNA construct is introduced into p*crispr-spcφ* to create p*crispr-spcφ-donor*. The donor DNA construct consists of a phage-derived sequence harboring the desired mutations as well as wild-type (WT) phage sequences flanking both sides. The second step

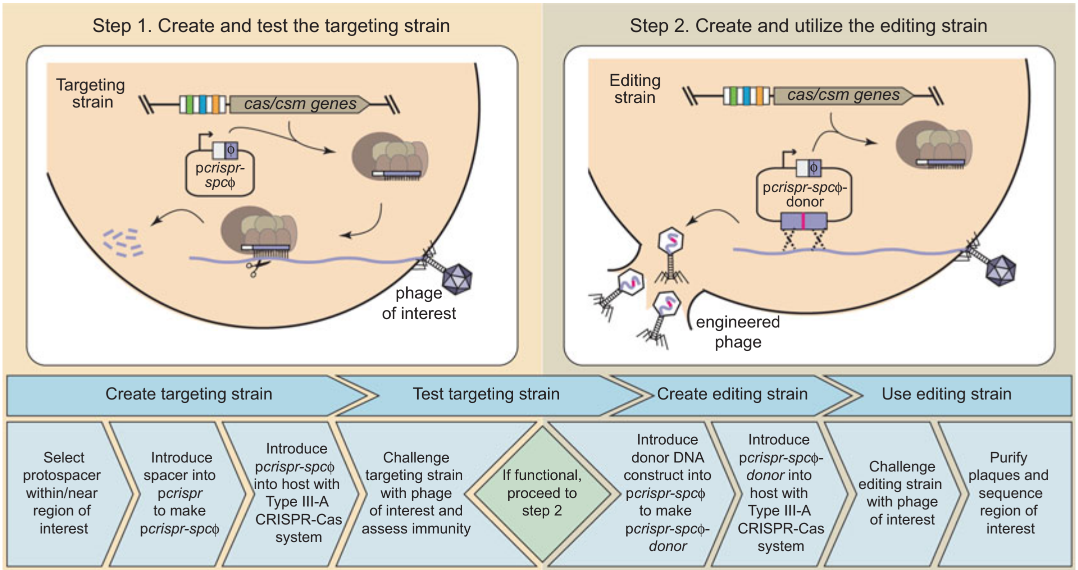

Fig. 3 The two-step approach for phage engineering using a host with native CRISPR-Cas10 system

involves simply propagating phages on the editing strain—during phage challenge, only those phages that have recombined with the donor construct and acquired the desired mutations will escape CRISPR-Cas10 interference and successfully replicate on the editing strain. The resulting plaques (i.e., zones of bacterial growth inhibition) are purified, and the phages within are sequenced to confirm the presence of the mutations. Several variations of this approach are also described, which can be utilized to (i) introduce precise mutations distal to the targeted region, (ii) create deletions in a single step, or (iii) edit phages that infect CRISPR-less hosts. Given that type III systems are relatively widespread among archaea and bacteria, we anticipate that similar strategies may be broadly applied to engineer the genomes of diverse phages.

2 Materials

2.1 Strains

1. Overnight culture of *Staphylococcus epidermidis* RP62a or another host bacterium with a functional chromosomally encoded CRISPR-Cas10 system.
2. Phage of interest (~1×10^9 plaque-forming units (pfus) per milliliter).

2.2 Growth Media and Reagents

1. Tryptic Soy Broth (TSB): 17.0 g/L pancreatic digest of casein, 3.0 g/L papaic digest of soybean, 2.5 g/L dextrose, 5.0 g/L sodium chloride, and 2.5 g/L dipotassium phosphate. Autoclave at 121.1 °C for 45 min. This is used for routine cultivation of staphylococci.

2. TSB supplemented with chloramphenicol (10 μg/mL). This is used to select for plasmids containing p*crispr*, which harbors *cat* (chloramphenicol acetyltransferase), a gene that confers chloramphenicol resistance.
3. TSB supplemented with 500 mM sucrose. TSB is prepared and autoclaved at 121.1 °C for 45 min. Once cooled, sucrose is added to the TSB and the solution is filtered through a 0.45 μm filter. This is used for recovery after electroporation.
4. Tryptic Soy Agar (TSA): 15.0 g/L pancreatic digest of casein, 5.0 g/L papaic digest of soybean, 5.0 g/L sodium chloride, and 15.0 g/L agar. Autoclave at 121.1 °C for 45 min. Supplement with $CaCl_2$ (5 mM final concentration) before pouring plates. This is used for routine cultivation of bacteria and as the bottom layer of agar for plating phage using the double-agar overlay method.
5. Heart Infusion Agar (HIA): 15.0 g/L agar, 10.0 g/L casein peptone, 5.0 g/L meat peptone, 5.0 g/L sodium chloride, 3.0 g/L yeast extract, and 2.0 g/L beef heart infusion. Prepare agar at 0.5× concentration for standard top agar overlays or 0.3× concentration for creating high-titer phage lysates. Autoclave at 121.1 °C for 45 min. Store at 55 °C after autoclaving to maintain molten/liquid form.
6. 5 M $CaCl_2$ solution (filter-sterilized). This is used to supplement the top agar layer to 5 mM final concentration just prior to use.
7. 10% (vol/vol) Glycerol supplemented with 500 mM sucrose (filter sterilized). This is used for preparing electrocompetent staphylococci.

2.3 Plasmid Construction via PCR

1. Appropriate PCR primers (described in sections below).
2. Nuclease-free distilled and deionized water (dH_2O).
3. 5× Phusion HF or GC buffer.
4. dNTPs (10 mM).
5. Phusion DNA Polymerase (2 U/μL).
6. Molecular-grade agarose.
7. TAE buffer: 40 mM Tris, 20 mM acetic acid, and 0.4 mM EDTA. This is used for agarose gel electrophoresis.
8. Rig for agarose gel electrophoresis.
9. PCR clean-up kit.
10. 0.025 μm Mixed cellulose membrane filter.
11. 0.45 μm Syringe filter.
12. 0.45 μm Bottle filter.

13. Gibson Assembly Master Mix: 80 μL 5X ISO buffer, 0.2 μL T5 exonuclease (10 U/μL), 5 μL Phusion polymerase (2 U/μL), 40 μL Taq ligase (40 U/μL), 175 μL dH_2O. Store at −20 °C in 15 μL aliquots.
14. 5× ISO buffer: 3 mL 1 M Tris-HCL (pH 7.5), 300 μL 1 M $MgCl_2$, 240 μL 100 mM dNTP mix (25 mM each: dGTP, dCTP, dATP, dTTP), 300 μL 1 M DTT, 1.5 g PEG-8000, 300 μL 100 mM NAD+, fill to 6 mL with dH_2O. Store at −20 °C in 320 μL aliquots.
15. Thermocycler.

2.4 Phage Genomic DNA Extraction

1. 0.5-M EDTA, pH 8.0
2. 10% (wt/vol) SDS
3. Proteinase K (20 mg/mL).
4. Phenol/chloroform/isoamyl alcohol, 25:24:1 (PCI).
5. 3 M NaOAc
6. 100% (vol/vol) EtOH
7. 75% (vol/vol) EtOH
8. 10% (vol/vol) glycerol.

3 Methods

3.1 Select a Functional Protospacer

For CRISPR-Cas10 to effectively target the phage of interest, a 35-bp spacer sequence must be introduced into the plasmid p*crispr* directly downstream of the repeat sequence. The spacer and corresponding protospacer should abide by the targeting rules for type III systems: (i) The protospacer should fall within a transcribed region, (ii) The crRNA should be complementary to the targeted mRNA, and (iii) there should be little/no complementarity between the 5′-end of the crRNA (5′-tag) and the opposing region (PFS/anti-tag) adjacent to the protospacer (Fig. 2b). The selected protospacer should be located within (ideal) or near the region in the phage genome to be mutated. Examples of a functional spacer sequence and corresponding crRNA-protospacer match in *Staphylococcus* phage Pabna [30] are shown in Fig. 2c, d, respectively. Appropriate protospacers in any gene of interest can be identified using the protospacer selector tool [13], which is designed to locate all potential protospacers exhibiting the characteristics described above. To access and use this tool, follow the steps below:

1. Go to GitHub using the following link: https://github.com/ahatoum/CRISPR-Cas10-Protospacer-Selector
2. Download the two Python files, “MainScript.py” and “GNfunctions.py,” into the same directory.

3. Open the file "MainScript.py" and paste the gene to be targeted in the appropriate field in the 5′-3′ direction.
4. Adjust the 5′-tag sequence if using a different type-III CRISPR-Cas system. The 5′-tag sequence is set to that of CRISPR-Cas10 in *S. epidermidis* RP62a as a default.
5. Run the code. Once the script is finished running, it will create a "Results.txt" output file in the same directory. The file will contain four columns representing the following: protospacer coordinate (5′-3′), protospacer sequence, PFS/anti-tag sequence, and spacer sequence to be cloned into p*crispr*.

3.2 Construct the Targeting Strain

Once a protospacer has been selected, a spacer (the reverse complement of the protospacer) must be introduced into the p*crispr* plasmid immediately downstream of the repeat sequence (Fig. 4a). This plasmid encodes the leader region for the CRISPR locus in *S. epidermidis* RP62a and a single repeat, as well as a gene that confers chloramphenicol resistance. Previous work has shown that a single spacer in this plasmid works together with the chromosomally encoded CRISPR locus in *S. epidermidis* RP62a to efficiently target diverse lytic staphylococcal phages [13]. The targeting spacer is introduced into p*crispr* via inverse PCR, followed by phosphorylation and circularization of the PCR product (Fig. 4b). The specific steps are as follows:

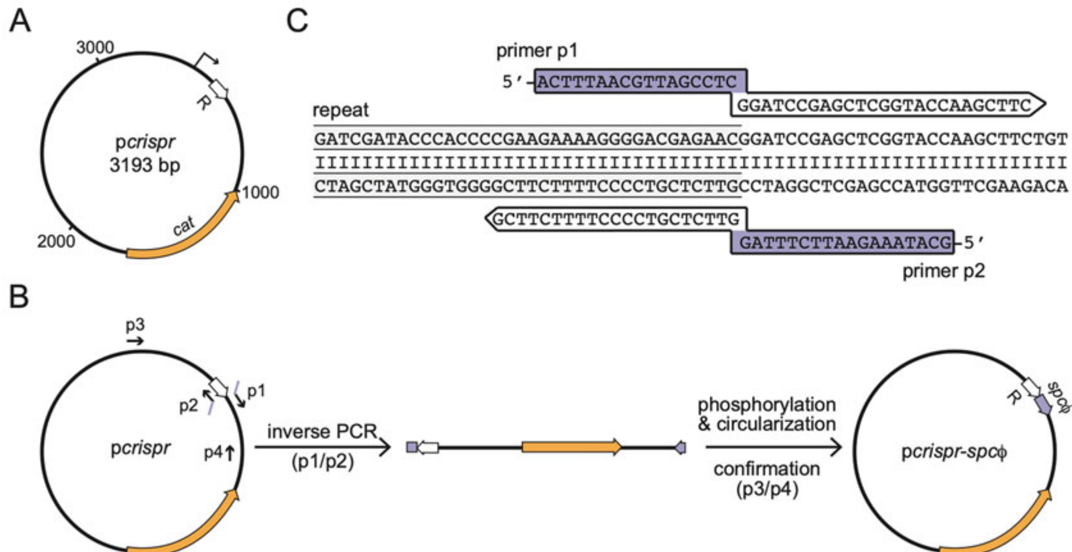

Fig. 4 Constructing p*crispr-spcφ*. (**a**) Illustration of the base plasmid p*crispr*. R, repeat sequence; *cat*, chloramphenicol acetyltransferase. (**b**) The process of using inverse PCR to generate p*crispr-spcφ* with primers p1 and p2. (**c**) The sequences of primers p1 and p2 used for the introduction of the spacer sequence shown in Fig. 2c. The 5′-overhangs containing the spacer sequence are highlighted in purple

1. Design a pair of primers that anneal to the repeat sequence (reverse) and downstream of the spacer sequence (forward) with 5′ overhangs that encode the spacer sequence (Fig. 4c, primers p2 and p1, respectively).
2. Set up a 50 μL PCR reaction with the designed primers, p*crispr* plasmid as a template, and high-fidelity thermostable DNA polymerase such as Phusion. Subject the reaction to the following PCR program: 95 °C for 30 s, 1 cycle; 95 °C for 10 s, 55 °C for 10 s, and 72 °C for 3 min, 30 cycles; and 72 °C for 6 min.
3. Resolve the PCR product on a 1% (wt/vol) agarose gel to check for the expected size. Once confirmed, purify the PCR product using a standard PCR clean-up kit. Elute the PCR product in a final volume of 20 μL of dH_2O.
4. Phosphorylate the 5′-ends of the PCR product in a 20 μL reaction containing 17 μL of the purified PCR product, 2.0 μL of 10× T4 DNA ligase buffer, and 1.0 μL of T4 polynucleotide kinase (10 U/μL). Incubate the reaction at 37 °C for 60 min and heat-inactivate at 80 °C for 20 min.
5. Circularize the PCR product by adding the following to the above reaction: 2.5 μL 10× T4 ligase buffer, 1.0 μL T4 DNA ligase (400 U/μL), and 1.5 μL dH_2O to obtain a 25 μL final volume. Incubate the reaction mixture overnight at room temperature.
6. The next day, add 15 μL dH_2O to dilute the reaction to ~40 μL and dialyze the plasmid against dH_2O on a 0.025-micron (pore-size) mixed cellulose membrane filter for 20 min using the drop dialysis method.
7. Introduce the dialyzed ligation reaction into electrocompetent cells of the *Staphylococcus* host bacterium (*see* Subheadings 3.10 and 3.11 for preparation and electroporation of competent staphylococci, respectively).
8. Inoculate several transformants in TSB supplemented with chloramphenicol (10 μg/mL) and confirm that the targeting spacer has been inserted correctly. Confirmation can be accomplished by PCR-amplifying the repeat-spacer region using primers that anneal upstream and downstream of the spacer (Fig. 4b, p3 and p4) and sequencing the resulting PCR product via Sanger sequencing. Once confirmed, the targeting plasmid is known as p*crispr-spcφ*, and the strain bearing this plasmid is called the "targeting strain" (*see* **Note 1**).

3.3 Confirm Functionality of the Targeting Strain

Once the targeting strain has been constructed, it must be challenged with the phage of interest to confirm that immunity is intact. CRISPR-Cas10 function is indicated by significant reduction/complete elimination of phage plaques when dilutions of the phage lysate are spotted on the targeting strain as compared with a

control strain that is devoid of CRISPR targeting. This can be tested using the double-agar overlay method [31], which is described below:

1. In a sterile 15 mL conical tube, combine 4 mL molten HIA top agar (described in Subheading 2.2) with 100 μL overnight culture of the targeting strain and 4 μL of 5 M $CaCl_2$.
2. Cap the tube and gently invert the mixture several times to get an even suspension.
3. Pour the mixture atop a prewarmed plate containing a solid layer of TSA supplemented with 5 mM $CaCl_2$.
4. Prepare a second overlay plate as above using a strain harboring the p*crispr* plasmid (without the targeting spacer) as a negative control (NC).
5. Allow the top agar overlays to solidify at room temperature for ~15 min.
6. Prepare tenfold serial dilutions (diluted out to 10^{-7}) of high-titer phage lysate.
7. Spot 5 μL of each dilution atop the HIA top agar overlays and allow spots to dry at room temperature for ~20 min.
8. Incubate the plates overnight at 37 °C and enumerate plaques the following day. If CRISPR-Cas10 immunity is present, there should be a significant reduction in plaque numbers when comparing the plate containing the targeting strain and the NC plate. Robust antiphage immunity is indicated by complete or near-complete elimination of phage plaques when plated on the targeting strain. For troubleshooting, see notes as follows: If general clearing of the bacterial lawn occurs under the most concentrated phage spots and plaques fail to appear in more diluted spots (see **Note 2**); If the targeting strain does not provide immunity to the phage of interest (see **Note 3**); and if the targeting strain exhibits weak immunity to phage of interest (see **Note 4**).

3.4 Construct the Editing Strain

Once the targeting strain has been confirmed to have antiphage immunity, the p*crispr-spcφ* plasmid is used as the backbone to construct p*crispr-spcφ-donor* via Gibson assembly [32] (Fig. 5a). The p*crispr/spcφ-donor* plasmid consists of p*crispr-spcφ* plus a donor DNA construct. The donor DNA construct should contain (i) a version of the protospacer (i.e., targeted region in the phage) containing the desired mutation(s) and (ii) between 100 and 1000 nucleotides of perfect homology (i.e., homology arms) flanking both sides of the protospacer region (Fig. 5b). The desired mutation(s) may overlap with the protospacer (ideal) or lie up to 500 nucleotides distal to the protospacer (see Subheading 3.7). Because the protospacer-crRNA duplex can tolerate as many as

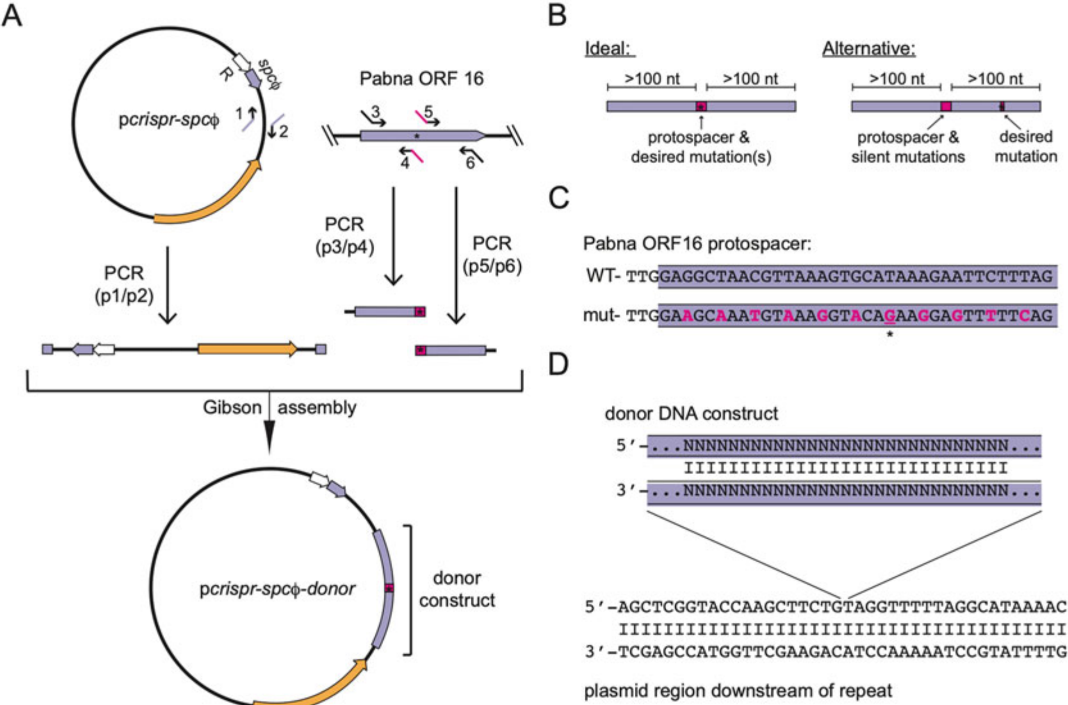

Fig. 5 Constructing the p*crispr-spcφ-donor*. (**a**) A three-piece Gibson assembly is used to generate p*crispr-spcφ-donor* with three pairs of primers. The plasmid p*crispr-spcφ* is used as the backbone, and the donor construct is derived from the phage genome. The final p*crispr-spcφ-donor* construct is shown at the bottom. R, repeat sequence; *spcφ*, a spacer targeting the phage region of interest. (**b**) The donor DNA construct is shown in which desired mutations (marked by asterisks) can be placed on top of the protospacer (left) or up to 500 nucleotides away from the protospacer (right). In both cases, silent mutations should be added to the protospacer such that at least five mutations (total) are present. (**c**) Wild-type (top) and mutated (bottom) sequences of the selected protospacer in phage Pabna. Mutated nucleotides are shown in magenta, and the single nonsynonymous desired mutation is underlined and marked with an asterisk. (**d**) The sequence in p*crispr-spcφ-donor* downstream of the repeat into which the donor DNA construct is inserted

five mismatches [29], additional silent mutations in the protospacer region should be added in the donor construct to reach a minimum of five mutations (Fig. 5c, *see* **Note 5**). The entire donor construct is introduced directly downstream of the repeat-spacer array in p*crispr-spcφ* (Fig. 5d). Once p*crispr-spcφ-donor* is constructed, it is introduced into *S. epidermidis* RP62a (or another host with a functional type III-A CRISPR-Cas system) to create the editing strain. The process for constructing the editing strain is described in detail below.

1. Design and obtain three sets of primers to amplify: (a) the plasmid p*crispr-spcφ* backbone (Fig. 5a, p1/p2), (b) the region in the phage genome directly upstream of the protospacer (Fig. 5a, p3/p4), and (c) the region in the phage genome directly downstream of the protospacer (Fig. 5a, p5/p6). The

forward primer that amplifies the region downstream of the protospacer and the reverse primer that amplifies the region upstream of the protospacer (p5 and p4, respectively, in Fig. 5a) should contain the version of the protospacer sequence with desired mutation(s) plus silent mutations in their 5′-overhangs.

2. Perform PCR with each of the three primer pairs described above to amplify each fragment of the plasmid to be constructed. The PCR reaction with the primer set designed to amplify the plasmid backbone will use p*crispr/spcφ* as a template, and the two primer sets designed to amplify each homology arm will use phage genomic DNA as a template. A high-fidelity thermostable polymerase, such as Phusion, should be used following the protocol described in Subheading 3.2, but with elongation times adjusted for the length of each fragment to be amplified (~1 min of elongation per 1 kilobase pair of DNA, plus a final extension cycle that is twice the elongation time).

3. Resolve the PCR products on a 1% (wt/vol) agarose gel to check for the expected sizes. Once confirmed, purify the PCR products using a standard PCR clean-up kit and measure their concentrations (in ng/μL) using a NanoDrop or another spectrophotometer. These concentrations will be used to determine the volume of each fragment to be added to the Gibson Assembly reaction.

4. For a 2–3 fragment assembly, combine 0.02–0.05 pmol of each PCR product (~5 μL total) with 15 μL of Gibson Assembly Master Mix in a PCR tube. Add dH_2O so that the total volume of the reaction becomes 20 μL. For optimal cloning efficiency, it is recommended to add 50–100 ng of backbone in a 2:1 molar ratio of insert:backbone. This ratio can be adjusted, if necessary, but the volume of PCR products should not exceed 20% (vol/vol) of the total reaction.

5. Incubate the reaction at 50 °C for 60 min. The incubation time can be shortened to 30 min if desired, but utilizing the full 60 min typically results in higher transformation efficiency.

9. Add 20 μL dH_2O to dilute the reaction to ~40 μL and dialyze the reaction against dH_2O on a 0.025-micron (pore-size) mixed cellulose membrane filter for 20 min using the drop dialysis method.

6. Introduce the Gibson-assembled construct into *S. epidermidis* RP62a or another host containing a functional type III-A CRISPR-Cas system using electroporation (see Subheadings 3.10 and 3.11). The entire dialyzed Gibson Assembly reaction should be used for transformation.

7. The following day, pick several transformants and confirm that the donor construct has been inserted correctly within the editing strain using PCR and Sanger sequencing. The primer pair used to confirm the insertion of the targeting spacer (described in Subheading 3.2) can be used to confirm the insertion of the donor DNA insert. Once confirmed, the strain bearing p*crispr-spcφ-donor* is called the "editing strain" (*see* **Note 6**).

3.5 Utilize the Editing Strain to Generate Phage Recombinants

Once the editing strain is confirmed, it is challenged with the phage of interest to produce recombinants that have acquired the desired mutation(s) and thus evade CRISPR-Cas10 immunity. Phage challenge can be performed using the top agar overlay method described in Subheading 3.3 or by co-culturing the phage with the editing strain in liquid culture for up to 12 h prior to plating. Regardless of the method used, the targeting strain should be used as a NC in parallel to confirm that WT phages remain sensitive to CRISPR-Cas10 immunity. The co-culturing method is described below:

1. In a 1.5 mL microtube, combine 500 μL overnight culture of the editing strain with 50 μL phage of interest (~1×10^9 pfu/ml). Supplement the mixture with 5 mM $CaCl_2$.
2. Incubate the mixture at 37 °C for 1–2 h without agitation. As a NC, an identical mixture containing the targeting strain (instead of the editing strain) should be prepared and incubated as above.
3. Combine the entire phage-host mixture with 4 mL HIA top agar supplemented with 5 mM $CaCl_2$ and overlay on TSA plates containing 5 mM $CaCl_2$ as described in Subheading 3.3. Alternatively, the phage-host mixture can be pelleted in a microcentrifuge at 13,000× *g* for 2 min and filtered using a 0.45 μm syringe filter to remove the bacterial cells. Tenfold serial dilutions of the filtered lysate can then be spotted atop an overlay containing the editing strain.
4. Incubate the plates overnight at 37 °C and check for plaques the following day. The presence of plaques on the editing strain and absence of plaques on the targeting strain is a good sign that phage editing was successful and the plaques were created by phages that managed to recombine with the donor DNA construct and take up the desired mutation(s) (*see* **Note 7**).

3.6 Purify and Confirm Phage Recombinants

It is possible that a portion of the putative phage recombinants were unable to acquire the desired mutation(s), especially if the editing strain was constructed in such a way that the desired mutation(s) occur distal to the protospacer/silent mutations (see Subheading 3.7). Therefore, the putative recombinants must be purified and the presence of the desired mutation(s) confirmed as follows:

3.6.1 Purify Putative Phage Recombinants

1. Pick several well-isolated plaques from the editing strain plate using sterile 1 mL micropipette tips and eject each plaque in 500 μL TSB.
2. Vortex the resuspended plaques for 10–30 s to release phage particles from the agar and centrifuge at 13,000× *g* for 2 min to pellet the agar and bacterial cells present in the media. The supernatant is called the "low-titer lysate."
3. Prepare tenfold serial dilutions of the low-titer lysate (from 10^{-1} to 10^{-7}) and spot 5 μL of each dilution on HIA top agar overlays containing the targeting strain as described in Subheading 3.3. Incubate the plates overnight at 37 °C.
4. Repeat steps a-c twice more so that each phage is purified at least 3 times before moving on to the next step.

3.6.2 Prepare High-Titer Phage Lysates

1. In a 15 mL conical tube, combine 200 μL of each low-titer phage lysate (obtained after the third round of purification) with 300 μL overnight bacterial culture in 7 mL of HIA top agar prepared at 0.3× concentration. Supplement with 5 mM $CaCl_2$.
2. Cap the tube and invert it several times to create an even suspension.
3. Pour the phage-host mixture atop a TSA plate containing 5 mM $CaCl_2$ and allow it to solidify at room temperature for ~10 min. Incubate the plates overnight at 37 °C.
4. Harvest the entire top agar layer into a 50 mL conical tube using a sterile cell scraper and add 25 mL fresh TSB atop the agar. Cap the tube securely and vortex for 5 min to release the phages from the agar.
5. Pellet the agar and cell debris at 10,000× *g* for 10 min.
6. Filter the resulting phage supernatant through a 0.45 μm bottle filter.
7. Enumerate phages (in pfu/mL) by plating serial dilutions of the lysate atop a lawn of host cells as described in Subheading 3.3. The phage titer should be at least 1×10^8 pfu/mL before moving on to phage genomic DNA extraction.

3.6.3 Extract Genomic DNA from High-Titer Phage Lysates

1. In a 1.5 mL microtube, combine 300 μL of high-titer phage lysate with 12 μL 0.5 M EDTA, 15 μL 10% (wt/vol) SDS, and 5 μL Proteinase K (20 mg/mL).
2. Incubate the tube at 55 °C for 1 h.
3. Add 300 μL PCI and vortex for 1 min.
4. Centrifuge at 13,000 × *g* for 5 min at room temperature (RT).

5. Transfer the upper aqueous layer into a fresh microtube without disturbing the white layer between the organic and aqueous layers.
6. To the aqueous layer, add 20 μL 3 M NaOAc (pH 5.2) and 900 μL 100% (vol/vol) EtOH. Invert the tube 3–4 times.
7. Chill the mixture on ice for 10 min.
8. Centrifuge at 13,000× *g* for 5 min at room temperature.
9. Carefully pour out the supernatant and add 500 μL 75% (vol/vol) EtOH. Gently invert the tube 2–3 times to wash the pellet.
10. Centrifuge at 13,000× *g* for 5 min at room temperature.
11. Carefully pour out the supernatant and aspirate any remaining EtOH using a pipette tip.
12. Air-dry the pellet for 5–10 min.
13. Resuspend the pellet in 20 μL dH_2O.
14. Measure the concentration of the DNA using a NanoDrop or similar spectrophotometer.
15. Dilute the extracted DNA 1:10 and use 1–2 μL for PCR (or 1–2 μL of the undiluted DNA if necessary).

3.6.4 Sequence Phage DNA

1. Perform PCR using the phage DNA as a template and primers that flank the region of interest in the phage genome.
2. Sequence PCR products using Sanger sequencing to confirm that the putative recombinant phages have indeed acquired the desired mutation(s).

3.7 Introducing Mutations Distal to the Targeted Region

The sections above describe the design and construction of targeting and editing strains in which the targeted region (i.e., protospacer) and the region to be mutated in the phage directly overlap. As an alternative approach, the desired mutation(s) may be introduced in a region distal to the protospacer (Fig. 5b). This approach is beneficial when it is necessary to avoid cloning a gene or phage sequence that is toxic to the cell or otherwise resistant to cloning attempts. If using this approach, the donor DNA construct should harbor the desired mutation(s) up to ~500 nt distal to the protospacer in addition to at least 5 silent mutations across the protospacer region to allow recombinant phages to evade CRISPR-Cas10 immunity.

3.8 Creating Deletions in a Single Step

If deletions in the phage genome are desired, it may be possible to generate such mutants in a single step, provided that the region to be deleted is not essential for phage survival. We have observed that deletion mutants can be selected from the phage population by simply plating a high-titer lysate atop a targeting strain in which p*crispr-spcφ* is programmed with a spacer that targets the region to be deleted (our unpublished results).

3.9 Editing Phages that Infect CRISPR-Less Hosts

If the desired host does not possess its own CRISPR system, an alternative approach can be utilized in which the plasmid p*crispr-cas/Δcas1Δcas2* is used instead of p*crispr*. Unlike p*crispr*, which only contains the leader sequence of the CRISPR locus and a single repeat, p*crispr-cas/Δcas1Δcas2* encodes the seven CRISPR-associated genes (*cas10-cas6*) that are required for interference along with a single repeat sequence transcribed under control of the type III-A native promoter (Fig. 6a). This plasmid has been shown to effectively facilitate editing of phages that infect a CRISPR-less *Staphylococcus* host using the same approach described above [13] (Fig. 6b, c).

3.10 Preparing Electrocompetent Staphylococci

1. Grow the desired strain overnight while shaking at 37 °C in 10 mL TSB and appropriate antibiotics.
2. Pour the entire overnight culture in a sterile 250 mL Erlenmeyer flask and dilute the culture with fresh TSB until the optical density at 600 nm (OD_{600}) reaches ~0.5 (~1×10^8 cfu/mL).
3. Incubate the diluted culture at 37 °C with shaking for 30 min.
4. Chill the flask on ice for 10 min. From this step forward, everything should remain on ice.
5. Divide the culture into two 50 mL conical tubes and centrifuge at 4 °C, 4000× *g* for 10 min.
6. Discard the supernatant and resuspend the pellets in an equal volume of ice-cold dH_2O. Repeat the above wash step.
7. Discard the supernatant and resuspend the pellets in 1/20 volume of ice-cold 10% (vol/vol) glycerol.
8. Combine the pellets into a single tube and centrifuge at 4 °C, 4000× *g* for 10 min.
9. Discard the supernatant and resuspend the pellet in 1/25 volume of ice-cold 10% (vol/vol) glycerol and centrifuge as above.
10. Discard the supernatant and resuspend the pellet in 1/200 volume of ice-cold 10% (vol/vol) glycerol and divide into 50 μL aliquots. Use immediately for transformation or store at −80 °C until needed.

3.11 Electroporating Competent Staphylococci

1. Dialyze the plasmid against dH_2O on a 0.025-micron (pore-size) mixed cellulose membrane in a petri dish for 20 min using the drop dialysis method. Be careful to pipette the sample onto the center of the filter disc.
2. Thaw the desired competent cells on ice for 5 min and then remove from ice and thaw at room temperature for 5 min. Thaw an extra aliquot to be used as a NC.

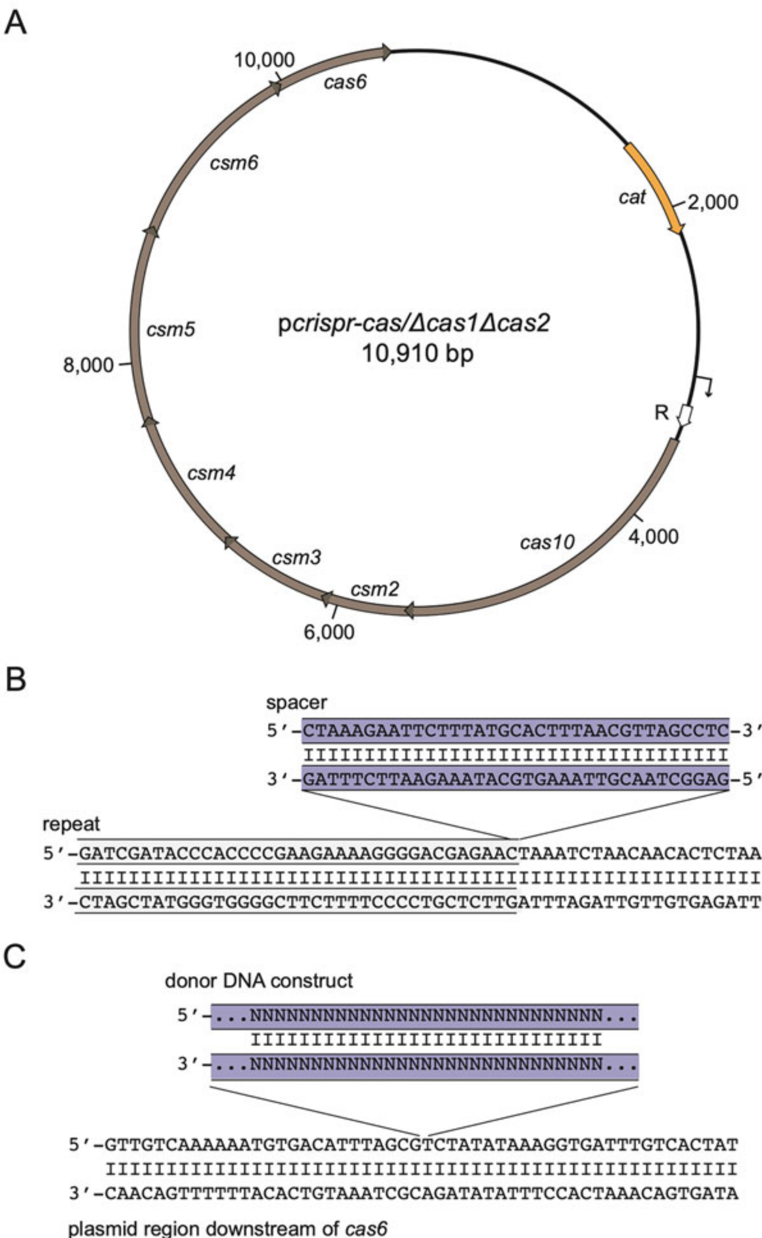

Fig. 6 Plasmid p*crispr-cas*/Δ*cas1*Δ*cas2*. (**a**) Illustration of the full plasmid. R, repeat sequence; *cat*, chloramphenicol acetyltransferase. (**b**) Sequence of the repeat (gray) and spacer (purple) designed to target *Staphylococcus* phage Pabna open reading frame (ORF) 16. (**c**) The sequence in p*crispr-cas*/Δ*cas1*Δ*cas2* downstream of *cas6* into which the donor DNA construct is inserted

3. Centrifuge the competent cells at 5000× *g* for 1 min to pellet the cells and resuspend in 50 μL 10% (vol/vol) glycerol +500 mM sucrose.
4. Combine the dialyzed plasmid/reaction mix with the competent cells and transfer the entire mixture to a 2 mm electroporation cuvette.
5. Electroporate the sample using the following program: voltage, 2100 V; capacitance, 25 μF; resistance, 100 Ω.
6. Immediately following electroporation, add 1 mL recovery media (TSB supplemented with 500 mM sucrose) to the cuvette and transfer to a sterile 1.5 mL microtube.
7. Incubate the cells at 37 °C with shaking for 1–2 h. For *S. epidermidis*, it is recommended to use the full 2 h recovery.
8. Plate 200 μL of the recovered culture onto agar plates containing the appropriate antibiotics and incubate overnight at 37 °C.
9. Check for colonies on the following day. There should be no colonies present on the NC plate.

4 Notes

1. The inability to recover *Staphylococcus* transformants containing a p*crispr*-based plasmid with a new spacer inserted could indicate that the selected spacer might be targeting one or more chromosomal loci in the bacterium. Such self-targeting is toxic to cells and causes cell death. If repeated attempts at creating p*crispr-spcφ* with the initial selected protospacer fail, try again with a different protospacer.
2. If general clearing of the bacterial lawn occurs under the most concentrated phage spots and plaques fail to appear in more diluted spots, this phenomenon is called "lysis from without" [33]. There can be many causes for this phenomenon, including the presence of lysins (i.e., cell wall-degrading enzymes) in the phage lysate, which impedes the growth of the bacterial lawn. If this occurs, it should not be mistaken for plaques.
3. If the targeting strain does not provide immunity to the phage of interest, several potential problems may be indicated. First, the protospacer may be designed incorrectly, so we suggest to first double-check to make sure that the crRNA binds to both the coding strand and the mRNA of the targeted gene. Second, the gene of interest may not be transcribed. If this is the case, it would be advisable to select a protospacer in an adjacent region that is known to be transcribed and employ the distal editing approach (see Subheading 3.7). Third, if a host other than *S. epidermidis* RP62a is used, the native CRISPR-Cas10 system

may not be functional. If the functionality of the native system has not been tested, it would be advisable to use the heterologous p*crispr-cas/Δcas1Δcas2* system from *S. epidermidis* (see Subheading 3.9). Finally, the phage might encode anti-CRISPR protein(s) that subvert type III systems. If this is suspected, it would be advisable to use a different CRISPR-Cas type for phage editing, or a different approach altogether.

4. If the targeting strain exhibits weak immunity to phage of interest (i.e., there is modest (1–2 orders of magnitude) reduction in plaque count after challenging the targeting strain with phage as compared with the NC), this might indicate that the targeted locus is not essential for phage survival, and the plaques may be originating from phage mutants that have randomly lost the targeted locus. To determine if this is the case, purify escaping phages and check for mutations on or near the protospacer. Note that a mutation that inactivates the promoter driving expression of the protospacer region may also lead to phage escape from CRISPR-Cas10 immunity. Even if such mutations occur, it still may be possible to recover the desired mutant by following the editing protocols described and screening through several putative recombinants.
5. When designing silent mutations, it is best to select alternative codons that have been used elsewhere in the same gene to maintain proper codon usage in the phage. A restriction endonuclease site can be added to the region containing the silent/desired mutation(s) to facilitate the screening of putative recombinants. After DNA extraction of the putative recombinants, the region of interest can be amplified and the PCR product digested with the appropriate restriction enzyme to quickly assess the presence of the mutation(s).
6. The inability to recover *Staphylococcus* transformants containing a p*crispr-spcφ*-based plasmid with the donor DNA inserted might indicate that the phage-derived sequence in the donor construct encodes a protein that is toxic to the cells. If this is suspected, try shortening the homology arms (to a length no shorter than 100 nucleotides) or employing a distal editing approach to avoid including a whole toxic gene (see Subheading 3.7).
7. If no plaques are present after culturing phage on editing strain, it is plausible that the intended mutation might impair phage viability. If this is the case, consider introducing a less disruptive mutation.

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Declaration of Interests A. H.-A. is the inventor of a US patent relating to this work (US20180251787A1).

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Chapter 19

Robust and Reproducible Protocol for Phage Genome "Rebooting" Using Transformation-Associated Recombination (TAR) Cloning into Yeast Centromeric Plasmid

Ivan Baykov, Olga Kurchenko, Ekaterina Mikhaylova, Vera V. Morozova, and Nina V. Tikunova

Abstract

Production of infectious bacteriophage based on its genome is one of the necessary steps in the pipeline of editing phage genomes and creating synthetic bacteriophages. This process is called "rebooting" of the phage genome. In this chapter, we describe key steps required for successful genome "rebooting" using a native host or intermediate host. A detailed protocol is given for the "rebooting" of the genome of T7 bacteriophage specific to *Escherichia coli* and bacteriophage KP32_192 that infects *Klebsiella pneumoniae*.

Key words Synthetic bacteriophage, Genome rebooting, Podovirus, Transformation-associated recombination cloning, Gap repair cloning

1 Introduction

In recent years, interest in the development of synthetic bacteriophages has grown rapidly because synthetic phages can be one of the alternatives to antibiotics (reviewed in [1–4]). The reason for the increased interest in bacteriophages is that the proportion of bacteria with multiple antibiotic resistance is steadily growing, and it is becoming more and more challenging to discover new structures of antibiotics. Phages are natural antagonists of bacteria and have a number of advantages over antibiotics: (i) due to their complex structure, they have a huge variety; (ii) unlike antibiotics, phages are able to multiply at the site of infection; (iii) when target pathogenic bacteria are destroyed at the site of infection, phages are eliminated naturally; and (iv) both rational and evolutionary approaches can be used to create new variants of phages. However,

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there are also limitations to the use of phages: many natural phages, especially those with podovirus morphology, have a narrow host specificity, which makes their wide use difficult for now.

The host specificity of phages is mainly determined by the structure of the tail proteins – tail spikes or tail fibers. Therefore, one of the approaches in the development of synthetic phages is to use the framework of a lytic phage and embed suitable tail spikes into it, thereby directing the phage to the desired target [2, 4–8]. Another application of the synthetic approach is to study the functions of certain phage genes via directed modification of these genes. The most effective methods for this are genome assembly and embedding it into a yeast centromeric plasmid using transformation-associated recombination (TAR) cloning (also named "gap repair cloning") [5, 9–12] and genome assembly using the Gibson method [13, 14]. Both of these methods imply that after editing the genome, it is necessary to obtain infectious phage particles based on genomic DNA (the so-called rebooting of phage genomes). To date, several protocols have been published describing methods for rebooting the phage genome [5, 14, 15]. The protocol using an integration of genome into a yeast centromeric plasmid [5, 12] contains two bottlenecks: (i) yeast centromeric plasmid DNA is extremely low copy (1–2 copies per yeast cell [16]), and (ii) *Escherichia coli* is less efficiently transformed by extended DNA (more than 20 kb) than by conventional plasmids (less than 10 kb). So, it is difficult to use this protocol of rebooting phage genomes in many cases.

In this study, we optimized the yeast low-copy plasmid DNA isolation protocol by using an easily scalable and inexpensive method of yeast plasmid DNA purification (mechanical destruction by glass beads and DNA precipitation with alcohol without using the expensive zymolyase/lyticase enzyme). As a result, a protocol for rebooting phage genomes was created, which allowed obtaining a sufficient number of phage plaques (more than 10^3 in the case of phage T7 and ~100 in the case of phage KP32_192) even when using laboratory-made electrocompetent cells with moderate competence (5×10^8–1×10^9 cfu/μg).

2 Materials

2.1 Incorporation of the Phage Genome into Yeast Centromeric Plasmid (TAR Cloning)

1. Vortex mixer.
2. Set of automatic pipettes with sterile tips.
3. Laboratory microbiological laminar flow hood.
4. Benchtop incubator shaker.
5. Spectrophotometer (600 nm).

Table 1
Oligonucleotides

pRS_T7_adaptor_dir	5′ – GTGTTACCTTGAGTGTCTCTCTGTGTCCCTTGTCTCAT GAGCGGATACATA–3′
pRS_T7_adaptor_rev	5′ – GGGGGAACTTTAGGTCCGTACACTGTGAGACCTTGTTCATG TGTGTTCAAAAAC–3′
pRS_KP192_adaptor_dir	5′ – GATACTATATGTTGATGTCTCTGTGTCCCTTGTCTCAT GAGCGGATACATA–3′
pRS_KP192_adaptor_rev	5′ – GGGGGATAACCAAAAGTGTAAACTGTGAGACCTTGTTCATGT GTGTTCAAAAAC–3′
T7_screening_dir	5′ – GGGCCTATGGAGTTCCTATAGGGTCC–3′
KP192_screening_dir	5′ – TGGACTGGAGACTCTCGTGTGCTC–3′
pRS_screening_rev	5′ – GATGAAAAGGACCCAGGTGGC–3′

Regions corresponding to the terminal sequences of the bacteriophage genome are underlined

6. Disposable plastic cuvettes.
7. Benchtop centrifuge for 15 and 50 mL conical tubes.
8. Benchtop microcentrifuge.
9. Sterile 1.5 and 0.2 mL polypropylene tubes.
10. Sterile 15 and 50 mL polypropylene Falcon tubes.
11. *Saccharomyces cerevisiae* strain BY4741 (ATCC 4040002).
12. Phage T7 genomic DNA, 100–500 ng/μL.
13. Phage KP32_192 genomic DNA (GenBank ID MH172261), 100–500 ng/μL.
14. Nuclease-free water.
15. Deoxyribonucleoside triphosphates (dNTPs) mix, 5 mM each.
16. Primers (see Table 1).
17. Pfu DNA polymerase.
18. DreamTaq DNA polymerase.
19. 10× DreamTaq Green buffer.
20. pRSII415 plasmid DNA (Addgene #35454),
21. PCR thermal cycler.
22. Agarose low EEO.
23. Ethidium Bromide Solution, 10 mg/mL.
24. Microwave oven.
25. Horizontal gel electrophoresis camera.
26. Power supply Power Pac HC.

27. DNA gel extraction kit.
28. 1-kb DNA ladder.
29. 6× DNA loading dye.
30. UV transilluminator.
31. Microvolume spectrophotometer NanoDrop.
32. YPD liquid broth. For 300 mL: D-glucose 6 g, yeast extract 3 g, Bacto peptone 6 g, autoclave at 121 °C.
33. YNB-Leu agar (dropout media). For 300 mL: D-glucose 6 g, yeast nitrogen base with ammonium sulfate and without amino acids 2 g, complete supplement mixture without leucine (CSM-leu) 0.21 g, Bacto agar 6 g, adjust pH to 5.6 with 1 M NaOH, autoclave at 121 °C.
34. Plastic Petri dishes, 9 cm.
35. 150 mL Glass Erlenmeyer flasks.
36. 50% (wt/vol) Polyethylene glycol 3350 (PEG-3350) solution in water, filter-sterilized.
37. 100-mM Lithium acetate (LiAc) solution in water, filter-sterilized, 10 mL.
38. 1 M LiAc solution in water, filter-sterilized, 10 mL.
39. 0.22-μm Sterile filters with PES membrane.
40. 10 mL Sterile syringes.
41. Salmon sperm DNA (SS-DNA) solution, 10 mg/mL.
42. Thermo shaker for 0.5 and 1.5 mL tubes.

2.2 Isolation of a Yeast Centromeric Plasmid Containing the Bacteriophage Genome

1. YNB-Leu broth (dropout media). For 300 mL: D-glucose 6 g, yeast nitrogen base with ammonium sulfate and without amino acids 2 g, complete supplement mixture without leucine (CSM-leu) 0.21 g, adjust pH to 5.6 with 1 M NaOH, autoclave for 15 min at 121 °C.
2. Nuclease-free water.
3. Glycerol, Biotechnology grade.
4. Yeast lysis buffer: 2% (wt/vol) Triton X-100, 1% (wt/vol) sodium dodecyl sulfate, 0.1 M NaCl, 1-mM EDTA, 10-mM Tris HCl, pH 7.5.
5. 0.3–0.6 mm Glass beads.
6. Buffer-saturated phenol (equilibrated with 10 mM Tris HCl, pH 8.0).
7. Chloroform.
8. 100% Isopropanol.
9. 70% (vol/vol) Ethanol.

10. RNAse A solution (10 mg/mL).
11. 5 M NaCl solution.
12. 250 mL Glass Erlenmeyer flasks.

2.3 Preparation of Electrocompetent E. Coli Cells

1. Sterile 0.5 mL polypropylene microcentrifuge tubes.
2. 50 mL Polypropylene Falcon tubes.
3. *E. coli* TOP10 (Invitrogen, USA).
4. HEPES powder.
5. Deionized water (mQ-grade).
6. pUC18 or pUC19 plasmid DNA,
7. Petri dishes containing LB-agar supplemented with 50–100 μg/mL ampicillin.
8. Ice bucket.
9. Cold 10% (vol/vol) glycerol solution.
10. 0.5-L Bottle with screw cap.
11. 750 mL Glass Erlenmeyer flasks.
12. YENB liquid broth. For 1000 mL: nutrient broth 8 g (contains 3 g of beef extract and 5 g of peptone), yeast extract 7.5 g, adjust pH to 7.0, autoclave for 15 min at 121 °C.

2.4 "Rebooting" of the T7 Bacteriophage Genome in Its Natural Host (E. coli)

1. Sterile 15 mL polypropylene Falcon tubes.
2. Sterile 1.5 mL microcentrifuge tubes.
3. *E. coli* TOP10 (fresh overnight culture).
4. Frozen electrocompetent *E. coli* TOP10 cells (competency $>5 \times 10^8$ cfu/μg).
5. Electroporation cuvettes with 2 mm gap.
6. SOC medium.
7. Yeast DNA sample containing the genome of T7 phage integrated into yeast centromeric plasmid pRSII415.
8. Soft agar (0.8% (wt/vol) Bacto agar in water, autoclaved).
9. GenePulser Xcell equipped with PC module.
10. Laboratory incubator.

2.5 "Rebooting" the Genome of Bacteriophage KP32_192 Specific to K. pneumoniae Using an Intermediate E. coli Host

1. *K. pneumoniae* strain 2274 (Collection of Extremophile Microorganisms and Type Cultures of ICBFM SB RAS, K26/K30 capsular serotype).
2. Yeast DNA sample containing the genome of KP32_192 phage integrated into yeast centromeric plasmid pRSII415.
3. Chloroform.

3 Methods

3.1 Incorporation of the Phage Genome into a Yeast Centromeric Plasmid (TAR Cloning)

Prior to the insertion of phage genome into the yeast centromeric plasmid pRSII415, the 5′- and 3′-terminal sequences of the phage genome should be determined. These sequences should be added to the adaptor primers, which are used to obtain the vector fragment. Here, we describe the process of incorporating genomes of phages T7 (specific to *E. coli*) and KP32_192 (specific to *K. pneumoniae*) into the yeast centromeric plasmid pRSII415 backbone (*see* **Note 1**).

3.1.1 Obtaining a PCR Copy of Yeast Plasmid pRSII415 Flanked by 5′- and 3′-Terminal Sequences of the Phage T7 Genome

1. Prepare the PCR mixture consisting of the following components (on ice): 5 μL 10× DreamTaq Green buffer, 39 μL DNAse-free water, 2.5 μL dNTP (5 mM each), 1.25 μL of 10 μM pRS_T7_adaptor_dir and 1.25 μL of 10 μM pRS_T7_adaptor_rev primers, 1.25 unit (U) of DreamTaq DNA polymerase, 0.63 U of Pfu DNA polymerase, and 0.1–1 ng of pRSII415 plasmid DNA. The amount of the obtained PCR product should be enough for 20–30 yeast transformations.
2. Perform amplification of the vector DNA fragment according to the parameters given in Table 2 (*see* **Note 2**). PCR products can be frozen (−20 °C) until the next step.
3. Prepare a 1% (wt/vol) agarose gel with 9-mm-wide wells.
4. Load the PCR product into the wells of the gel and run electrophoresis for 20–40 min with a voltage of 5–10 V/cm. Use a suitable DNA ladder to determine the size of the PCR product.
5. Place the gel on the ultraviolet (UV) transilluminator and cut out the gel fragments containing vector DNA with a size of 3000 bp. Try to avoid prolonged exposure of the DNA to UV light. Transfer the gel fragments to a 1.5 mL tube. The fragments can be frozen (−20 °C) until the next step.

Table 2
PCR program for vector fragment amplification

Stage	Duration	Temperature	Cycle count
1. Initial denaturation	3 min	94 °C	1
2.1. Denaturation	15 s	94 °C	35
2.2. Primer annealing 2.3. Elongation	30 s 7.5 min	50 °C 72 °C	
3. Final elongation	5 min	72 °C	1

6. Extract DNA from the gel fragments using any suitable kit. Elute the DNA with two consecutive 30-μL portions of nuclease-free water.
7. Freeze the product for 15–20 min because this promotes the renaturation of DNA, which is partially denatured during purification on silica-spin columns.
8. Thaw the sample and measure the DNA concentration using the microvolume spectrophotometer (e.g., NanoDrop). We typically obtain a concentration of 90–120 ng/μL (approximately 6 μg of total DNA). Additionally, you can analyze the sample by gel electrophoresis in 1% (wt/vol) agarose gel.
9. Store the DNA samples at −20 °C.

3.1.2 Integration of Phage Genomic DNA into Yeast Plasmid Backbone (TAR Cloning)

1. Prepare a starter culture of the BY4741 yeast strain on the morning of the day before performing the transformation. Place 5 mL of YPD broth into a sterile 50 mL conical tube and add a small portion of the frozen yeast suspension (10–20 μL). Incubate the tube at 27–30 °C and 180 rpm agitation for approximately 24 h.
2. On the next day, add 3 mL of yeast culture into 30 mL of YPD broth in a 150 mL flask. Incubate at 30 °C and 180 rpm until the OD_{600} reaches 4–6 (approximately 4 h) (*see* **Note 3**).
3. Centrifuge yeast cells at 3000× *g* for 5 min at room temperature (RT) in a sterile 50 mL tube. Discard the supernatant.
4. Add 25 mL of sterile deionized water under aseptic conditions and resuspend the yeast cell pellet. Centrifuge cells at 3000× *g* for 5 min at RT. Discard the supernatant.
5. Repeat **step 4** (yeast washing). Remove excess supernatant using a pipette.
6. Resuspend the pellet in 1 mL of 100 mM sterile lithium acetate solution, transfer the suspension to a 1.5 mL tube, and precipitate cells at 3000 × g for 5 min at RT using a benchtop microcentrifuge. Remove the supernatant using a pipette.
7. Resuspend the cell pellet in 400 μL of 100 mM lithium acetate. The total volume of the suspension should be 700–850 μL.
8. Mix 2 μL of the cell suspension with 1 mL of deionized water (500-fold dilution) and measure the absorbance OD_{600} against water. We usually obtain OD_{600} ~ 0.5, which means OD_{600} ~ 250 for undiluted yeast suspension. Calculate the volume of suspension to be taken, given that you need approximately 10^8 cells for each transformation (*see* **Notes 4** and **5**).
9. Dilute the required amount of salmon sperm DNA (SS-DNA) in nuclease-free water to a concentration of 2 mg/mL. You need 25 μL of SS-DNA solution per transformation, including the negative control. Heat the sample for 5 min at 98 °C to

Table 3
Yeast transformation mixtures

	Experiment	Negative control
50% (wt/vol) PEG-3350	240 μL	240 μL
1 M Lithium acetate	36 μL	36 μL
Denatured SS-DNA (2 mg/mL)	25 μL	25 μL
pRS_part_for_T7 DNA (from 3.1.1)	200 ng	200 ng
T7 genomic DNA	500–1500 ng	–
Nuclease-free water	To 360 μL	To 360 μL

denature the DNA. Chill the sample immediately by placing the tube in a refrigerator or ice to prevent the DNA from re-annealing (*see* **Note 6**).

10. Prepare yeast transformation mixtures (Table 3) in 0.5 or 1.5 mL tubes. Mix well.
11. Mix the tube with the yeast cell suspension. Place the required volume of the suspension, containing approximately 10^8 yeast cells, into new 0.5- or 1.5 mL tubes (*see* **Note 4**). Mark the tubes, and pellet the yeast using a benchtop microcentrifuge at 12,000× *g* for 30 s. Remove the supernatant using a pipette.
12. Add the transformation mixtures to the yeast pellets, each into an appropriate tube. Resuspend by pipetting or vortexing (*see* **Note 7**).
13. Incubate tubes with yeast suspensions at 42 °C for 30–60 min without shaking (*see* **Notes 8** and **9**).
14. Prepare YNB-Leu agar plates. You will need one plate for each transformation.
15. Pellet the transformed yeast cells in a benchtop centrifuge at 12,000× *g* for 30 s. Remove the supernatant using a pipette.
16. Resuspend the pellets in 100–200 μL of sterile deionized water and spread 10 μL of the suspension per YNB-Leu agar plate using a sterile spreader. Cover the plates and turn them over.
17. Seal the plates with parafilm and place them in a dark place for 3–4 days at RT. When colonies with a diameter of 1.5–2 mm are formed, put the plates into the refrigerator. Experimental plates typically contain 5–10 times more colonies than negative-control plates.

3.1.3 Yeast Colony Screening

When the whole phage genome is inserted into a yeast plasmid backbone, PCR screening is mainly unnecessary, especially if the number of colonies on the experimental plate is 5–10 times higher

Table 4
PCR program for yeast colony screening

Stage	Duration	Temperature	Cycle count
1. Initial denaturation	3 min	94 °C	1
2.1. Denaturation	30 s	94 °C	30
2.2. Primer annealing	30 s	52 °C	
2.3. Elongation	40 s	72 °C	
3. Final elongation	5 min	72 °C	1

than that on the negative-control plate. However, when the phage genome is assembled from several fragments [5, 8], PCR screening of yeast colonies can significantly facilitate further work.

1. Design a pair of primers so that the resulting PCR product includes one of the "phage genome-pRS backbone" junctions and its length is within the 300–600-bp range. You can use primers suggested in this protocol (T7_screening_dir and pRS_screening_rev, Table 1).
2. Transfer 10–15 yeast clones onto a new YNB-Leu agar plate. This helps remove the remnants of the DNA used for yeast transformation (*see* **Note 10**). Leave the plates in a dark place at RT for 2 days. Note that PCR works better with fresh yeast colonies (within 5 days after colony formation).
3. Prepare a PCR master mix to screen several clones plus 1–2 clones from the negative-control plate. For every 20 μL of PCR master mix, you will need the following: 2 μL 10× DreamTaq Green buffer, 16 μL DNAse-free water, 1 μL dNTP (5 mM each), 0.5 μL of each 10 μM forward- and reverse-screening primers, and 0.5 U DreamTaq DNA polymerase. Dispense the master mix in 20 μL aliquots into 0.2 mL tubes. Put a small portion of the fresh yeast colony into each tube.
4. Perform PCR according to the parameters given in Table 4.
5. Analyze 3–5 μL of the PCR mixture by electrophoresis in 1.5% (wt/vol) agarose gel. Use 100-bp or 1-kb DNA ladder. A clone is considered positive if there is a DNA band of the appropriate size (~320 bp in case of T7_screening_dir primer) in the gel.

3.2 Isolation of a Yeast Centromeric Plasmid Containing the Bacteriophage Genome

This protocol is adapted from the previously developed protocols [17–19].

1. Inoculate a positive yeast clone from a plate or frozen glycerol stock into 20 mL of YNB-Leu broth in a 250 mL flask. Incubate for 24–48 h at 27–30 °C with shaking at 180 rpm until the OD_{600} reaches 6–9.

2. To store a yeast sample, mix 0.5 mL of yeast culture with 0.5 mL of 50% (vol/vol) sterile glycerol solution in a 1.5 mL tube. Mix thoroughly and freeze at −70 °C.
3. Centrifuge 10 mL of yeast culture at 3000× g for 5 min in a 15 mL tube. Discard the supernatant. Repeat the precipitation in the same tube with the remaining portion of yeast. Discard the supernatant, and remove the excess with a pipette. The yeast pellet can be frozen at −20 °C until the DNA is isolated.
4. Add 1150 μL of yeast lysis buffer to the yeast pellet. Add approximately 2 g of glass beads (0.3–0.6 mm) (*see* **Note 11**), 500 μL of buffer-saturated phenol (pH 8.0), and 500 μL of chloroform. Mix vigorously on a powerful vortex mixer for 2 min to disrupt yeast cells.
5. Allow the glass beads to settle for 30–60 s and divide the yeast suspension between two 1.5 mL tubes. Centrifuge the tubes at 12,000–14,000× *g* for 5 min to separate the aqueous and organic phases.
6. Transfer the upper aqueous phase containing nucleic acids (600–650 μL) to two new 1.5 mL tubes and add 0.7 volumes (450 μL) of 100% isopropanol to each tube. Mix thoroughly, and then incubate for 5 min at RT.
7. Precipitate nucleic acids by centrifugation at 14,000× *g* for 15 min at RT. Discard the supernatant.
8. Add 500 μL of 70% (vol/vol) ethanol, and wash the pellet and the walls of the tube carefully. Centrifuge at 14,000× *g* for 5 min, and remove the supernatant. Spin down drops of liquid and remove the remaining supernatant using a pipette. Leave the tubes open for 10–15 min to dry the pellet.
9. Dissolve the pellet in 100 μL of DNAse-free water, and add 2 μL of 10 mg/mL RNase A solution and incubate for 10 min at 37 °C. Add 4 μL of 5 M NaCl and 70 μL of 100% isopropanol and mix well. Incubate for 5 min at RT.
10. Precipitate the DNA by centrifugation at 14,000× *g* for 15 min, and remove the supernatant using a pipette. Leave the tubes open for 10–15 min to dry the precipitate. Resuspend the pellet in 30 μL of nuclease-free water or TE buffer by pipetting. Keep the tubes overnight at 4–6 °C to completely dissolve the DNA precipitate (*see* **Note 12**).
11. The next day, centrifuge the tubes with the DNA solution at 12,000× *g* for 3 min to precipitate an insoluble precipitate. Transfer 60 μL of the yeast DNA supernatant containing the pRSII415 plasmid with the inserted phage genome (*see* **Notes 13** and **14**) to a new tube. Store frozen at −20 °C.

3.3 Preparation of Electrocompetent *E. coli* Cells

The use of highly electrocompetent cells and the details of the electroporation process have a great impact on the efficiency of the "rebooting" of the phage genome. Here, we provide a detailed protocol that allows us to obtain cells with a transformation efficiency of 5×10^8–1×10^9 colonies per 1 μg of pUC19 plasmid, which is sufficient for genome rebooting.

1. Prepare overnight culture by inoculating *E. coli* TOP10 or *E. coli* DH10B strain (*see* **Note 15**) into 4 mL of LB in a 50 mL tube. Incubate overnight at 37 °C with shaking at 180 rpm.
2. Prepare 450 mL of 1 mM HEPES solution (pH 7.0–7.2) in deionized water (*see* **Note 16**). Make sure the bottle is clean and does not contain any traces of salts or detergents. After dissolving HEPES, titrate the solution to pH 7.0 with 1 M NaOH. Place the bottle at 4–6 °C.
3. On the next day, inoculate two 2 mL portions of overnight culture into two 750 mL flasks containing 150 mL of YENB broth. Incubate at 37 °C with shaking at 240 rpm (*see* **Note 17**) until the OD_{600} reaches 0.5 (*see* **Note 18**).
4. Place the flasks with cultures and six new 50 mL tubes in an ice-water bath for 20 min. Transfer the bottle of 1 mM HEPES and the 40 mL tube of 10% (vol/vol) glycerol from the refrigerator to an ice-water bath. Cool the centrifuge with the rotor and adapters to 4 °C.
5. Gently mix the contents of the flasks. Transfer 25 mL portions of bacterial culture into 50 mL tubes. Make sure that the flasks and tubes are always cold (0–4 °C) till the end of this procedure (Subheading 3.3).
6. Centrifuge the bacteria at 2000× g for 5 min at 4 °C, and use mild acceleration and deceleration values (e.g., 6–7 out of 9) during this step until the end of this procedure. Discard the supernatant.
7. Repeat **steps 5** and **6**.
8. Place the tubes with pellets in an ice bath. Add approximately 10 mL of ice-cold 1 mM HEPES to each tube. Shake closed tubes vigorously by hand (oscillatory motion) to resuspend the pellets. Do not remove the tubes from the ice while shaking. Do not use pipetting or vortexing, as this will reduce cell competence or viability.
9. After complete resuspension of the pellets, add ice-cold 1 mM HEPES to each tube to a final volume of 45 mL.
10. Repeat **steps 6** and **8**, and skip **step 7**. Add ice-cold 1 mM HEPES to each tube to a final volume of 25 mL.

11. Pellet the cells at 2000× *g* for 5 min at 4 °C. Carefully discard the supernatant – the pellets can easily flake off.
12. Add ice-cold 10% (vol/vol) glycerol to the cell pellets to a final volume of about 5 mL. Resuspend the pellets by shaking the tubes on ice. Pellet the cells at 2000× *g* for 5 min at 4 °C. The resulting pellets are very loose, so carefully but quickly discard the supernatant, trying not to lose cells.
13. Shake the tubes vigorously by hand to resuspend the pellets in the remaining supernatant. Do not remove the tubes from the ice while shaking. Transfer the suspension from six tubes into one. Mix the cell suspension by inverting the tube.
14. Dilute 2 μL of cell suspension 100-fold and measure the OD_{600} value. Multiply by 100 (dilution factor) and calculate the cell concentration in the suspension, considering that 1 optical unit corresponds to 5×10^8 cells/mL. Typically, the cell concentration is $(2-3) \times 10^{10}$ cells/mL. If you get a higher concentration, dilute the cells with ice-cold 10% (wt/vol) glycerol.
15. Place 40–60 of 0.5 mL tubes on ice, and leave the caps open. Gently mix the cell suspension by hand. Transfer 50 μL aliquots of the cell suspension into each tube, gently shaking the tube with cells occasionally. Close the tubes and transfer them to a zip bag placed on ice. Sign the date and the strain, and put the bag at −70 °C (*see* **Notes 19** and **20**).
16. The next day, take one of the tubes and test the transformation efficiency of the cells using 10 pg of pUC18 or pUC19 plasmid (1 μL of 10-pg/μL solution). Dilute plasmid from the stock solution just before use because DNA at low concentrations degrades rapidly even when stored at −20 °C. After electroporation (see Subheading 3.4), spread 50 μL of cell suspension over LB-agar plate containing ampicillin. We usually get 500–1000 colonies, which corresponds to an efficiency of $(1-2) \times 10^9$ cfu/μg.

3.4 "Rebooting" of the T7 Bacteriophage Genome in Its Natural Host (*E. coli*)

1. Use a freshly prepared overnight culture of *E. coli* TOP10 or another strain susceptible to phage T7. You will need 400 μL of culture for each sample, including one negative-control sample.
2. Take 0.2 cm electroporation cuvettes according to the number of samples, including negative control, and put them on ice (*see* **Note 21**). Place 15 mL tube containing 10 mL of SOC medium into the incubator at 37 °C (*see* **Note 22**). Warm up the shaker incubator to 37 °C.
3. Thaw electrocompetent *E. coli* TOP10 cells on ice (*see* **Note 15**). Add 3 μL of the yeast DNA sample containing the plasmid

pRSII415 with the integrated phage T7 genome (*see* **Note 23**). Mix gently by finger flicking and incubate on ice for 5 min. Mark 15 mL tubes according to the number of samples.

4. Turn on the electroporator and select standard settings for 0.2 cm cuvette: 2500 V, 200 ohms, 25 μF. Make sure that the wires of the chamber are connected to the electroporator.
5. Transfer the mixture of cells and DNA to a pre-chilled cuvette, and do not remove the cuvette from the ice. Close the cuvette with a lid. Gently tap the bottom of the cuvette so that the drops of mixture drain down. Quickly clean the cuvette surface using a towel and place it into the electroporation chamber. Be sure to close the chamber lid; otherwise, the pulse will not pass. Press the Pulse button on the electroporator.
6. Immediately add 900 μL of warm SOC medium (37 °C or RT) to the cuvette. Remove the cuvette from the chamber, gently mix cell suspension by pipetting 2–3 times, and transfer cells to the corresponding 15 mL tube. Incubate the tube at 37 °C with shaking at 180 rpm for 1 h. Proceed to electroporation of the next sample.
7. Prepare LB-agar plates. Melt 100 mL of soft agar and place it in a water bath at 45–50 °C. From now, work carefully so as not to contaminate your laboratory with the T7 phage.
8. Make three tenfold dilutions [10^{-1}, 10^{-2}, and 10^{-3}] of transformed cells in LB in 1.5 mL tubes.
9. Put 100-μL aliquot of appropriate dilution into a sterile 15 mL tube, and add 4 mL of melted soft agar (45–50 °C) and 400 μL of *E. coli* TOP10 overnight culture. Mix well and pour the mixture onto the surface of the LB-agar plate. Allow the soft agar to solidify.
10. Repeat **step 9** for the remaining dilutions using a separate 15 mL tube for each dilution. Prepare LB-agar plate with negative-control sample by mixing 4 mL of soft agar with 400 μL of overnight *E. coli* TOP10 culture.
11. After the soft agar solidifies, turn the plates over and incubate them at 37 °C. You should detect the phage plaques in soft agar within 3–5 h. We usually get approximately 500 plaques on a plate with 10^{-2} dilution (*see* **Note 24**).

3.5 "Rebooting" the Genome of Bacteriophage KP32_192 Specific to K. pneumoniae *Using an Intermediate* E. coli *Host*

Because *Klebsiella* cells are surrounded by a polysaccharide capsule, their transformation by exogenous DNA is highly inefficient. Therefore, it is advisable to "reboot" the genome using an intermediate host such as *E. coli*, thus increasing the efficiency of transformation by several orders of magnitude [5]. This method may be suitable for "rebooting" the genomes of various phages that infect Gram-negative bacteria.

1. Prepare a fresh overnight culture of *Klebsiella*. Use a strain susceptible to the bacteriophage.
2. Repeat **steps 2–7** of Subheading 3.4 “Rebooting” of the T7 Bacteriophage Genome in Its Natural Host (*E. coli*). Increase incubation time after electroporation to 2–2.5 h (*see* **Note 25**).
3. After finish of incubation, add 50 μL of chloroform to the cells and mix vigorously using a vortex for 2 min. This promotes cell lysis and the release of phage particles.
4. Transfer the cell suspension to a 1.5 mL tube and centrifuge at 12,000× g for 1 min. Transfer the upper aqueous phase containing the phages to a new 1.5 mL tube.
5. Put phage-containing supernatant into a sterile 15 mL tube and add 4 mL of melted soft agar (45–50 °C) and 500 μL of fresh overnight *Klebsiella* culture. Mix well and pour the mixture onto the surface of the LB-agar plate. Allow the soft agar to solidify. Prepare the negative-control plate, likewise omitting the phage-containing supernatant.
6. When the soft agar solidifies, turn the plates over and incubate them at 37 °C. You should detect the phage plaques on the experimental plates and bacterial lawn on the negative-control plate within 4–6 h (it may take longer).

4 Notes

1. Some bacteriophages with podovirus morphology (e.g., phi29) contain covalently attached proteins at the termini of their genomic DNA. Because these proteins are necessary for the infection process, the protocol described here is unsuitable for such bacteriophages.
2. In our experience, it was necessary to increase the elongation time to 7 min and 30 s (which is just over 2 min/kb) to obtain a sufficient amount of PCR product. By increasing the length of pRS-regions of adaptor primers (Table 1), the duration of the elongation stage can be reduced to the usual 1 min/kb.
3. Dilute a small portion of the culture tenfold to measure the OD_{600} value.
4. We assumed that $OD_{600} = 1$ corresponds to 10^7 cells/mL. Therefore, if the suspension has an $OD_{600} = 250$, then $(10^8$ cells$) / (250 \times 10^7$ cells/mL$) = 0.04$ mL $= 40$ μL of undiluted yeast suspension should be taken per transformation.
5. Yeast settles quickly, so shake the tube occasionally.
6. Successful transformation requires the carrier salmon sperm DNA to be predominantly in denatured single-stranded form.

7. Make sure the yeast is evenly distributed throughout the liquid. If it is not mixed well enough, the viscous PEG and single-stranded DNA solution will settle at the bottom as a clear liquid, hence reducing the transformation efficiency.
8. Increasing the incubation time can increase the transformation efficiency significantly.
9. Usually, the yeast cells form flakes and settle down quickly, but we did not notice any decrease in the transformation efficiency.
10. Negative yeast colonies (containing only pRS-derived fragments without phage genome) grown on master plates may contain residuals of the DNA used for transformation on the cells' surface. So you can obtain false-positive results if you do not re-plate these colonies.
11. It is possible to replace glass beads with washed quartz sand of medium size (~0.5 mm) without loss of disruption effectiveness.
12. It takes time for the precipitated DNA to dissolve.
13. The resulting yeast DNA preparation predominantly contains fragmented genomic DNA and possibly episomal yeast plasmids. Because one yeast cell contains only 1–2 copies of the centromeric plasmid pRSII415, it is impossible to see the corresponding band by gel electrophoresis because it is masked by the rest of the DNA.
14. You can perform PCR using primers that amplify DNA fragments comprising the "genome-pRS" junction or primers that amplify a fragment of the bacteriophage genome to ensure that the resulting preparation contains the target plasmid DNA with the inserted phage genome.
15. The DH10B strain and its derivatives are known for their ability to efficiently transform by extended DNA (more than 10 kb) via electroporation. We prefer to use the TOP10 strain.
16. The use of 1 mM HEPES compared with mQ water allows us to increase the transformation efficiency by 1.5-fold. Do not use 10% (vol/vol) glycerol in water to wash cells. In our experience, this greatly reduces cell viability due to the toxicity of glycerol.
17. This agitation speed is suitable for an Infors Ecotron incubator shaker.
18. Cells grow slightly slower in low-salt YENB medium, having a doubling time of approximately 35 min. Check the OD_{600} value for the first time 1 h after inoculation and then every 15 min. Measure more often when the optical density reaches 0.3–0.4.

19. You can freeze test tubes in a foam bath using liquid nitrogen. We did not notice any deterioration in the competence of cells frozen directly in the freezer.
20. Cells prepared in this way retain their competence for at least several months.
21. You can also use 0.1 cm cuvettes. In this case, use the following electroporation parameters: 1800 V, 200 ohms, 25 μF.
22. The use of SOC medium helps achieve 5–10 times higher electroporation efficiency than LB broth or 2xYT broth. If you store SOC under frozen conditions, ensure that the precipitated salts have completely dissolved after thawing.
23. You can use linear T7 genomic DNA as a positive control. However, cellular exonucleases rapidly degrade the termini of such DNA. Thus, the efficiency of genome rebooting is 2–3 orders of magnitude lower. Therefore, for a successful rebooting, 100–500 ng of linear genomic DNA should be used per transformation.
24. The number of plaques depends on (i) the quality of the yeast DNA sample, (ii) level of competence of the cells, and (iii) time of incubation of transformed *E. coli* (T7 phage is able to amplify quickly, which increases the number of plaques).
25. Increasing the time of incubation of *E. coli* after transformation greatly improves the efficiency of genome "rebooting."

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