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Joana Azeredo
Sanna Sillankorva *Editors*

Bacteriophage Therapy

From Lab to Clinical Practice

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Edited by

Joana Azeredo

LIBRO-Laboratory of Research in Biofilms Rosário Oliveira, CEB – Centre of Biological Engineering, University of Minho, Braga, Portugal

Sanna Sillankorva

CEB – Centre of Biological Engineering, University of Minho, Braga, Portugal

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Editors
Joana Azeredo
LIBRO-Laboratory of Research in Biofilms Rosário Oliveira
CEB – Centre of Biological Engineering
University of Minho
Braga, Portugal

Sanna Sillankorva
CEB – Centre of Biological Engineering
University of Minho
Braga, Portugal

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Preface

This year we are celebrating the 100th anniversary of bacteriophage discovery. Bacteriophage therapy has had glorious and unfortunate periods. Since 1917, bacteriophages were extensively and intensively used as antimicrobial agents to control bacterial infections. In the early part of the century many big pharmaceutical companies produced several bacteriophage products for different applications. The discovery of antibiotics allied to the indiscriminate use of bacteriophages to treat every sort of infection, even nonbacterial, is identified as one of the major reasons that led to the abandonment of bacteriophage therapy. Antibiotics have now been used for approximately 70 years and have reduced illnesses and deaths caused by infectious diseases. However, the emergence of antibiotic resistant bacteria is a major health concern. Each year millions of people become infected with multiple-drug resistant bacteria, which consequently leads to numerous deaths. On this account, there is a revival of interest in bacteriophage as therapeutic agents to control infectious diseases. Despite this renewed interest, there is not yet any product broadly available for human application. There are however a few exceptions where bacteriophages can be applied to human patients as a last resort under the guidelines of the Helsinki treaty and as a therapeutic option in a few European countries. Accordingly, it is important to bring to medical application the products that are being developed at the lab. *Bacteriophage Therapy: From Lab to Clinical Practice* focuses on the methodology of product development and application in clinical context to ensure efficacy, safety, and regulatory compliance.

This book has 21 chapters which have been subgrouped by dividing the experimental approaches suitable for isolating and characterizing bacteriophages to formulating bacteriophage medicinal products and clinical application. Regulatory compliance and safety aspects of bacteriophage therapy are also addressed in the book.

We would like 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 in a simple and comprehensive way to guide bacteriophage researchers throughout the development of a product for medical application. This book also targets a broader audience ranging from clinicians, pharmaceutics, regulatory authorities, and stakeholders.

Braga, Portugal

Joana Azeredo
Sanna Sillankorva

Contents

Contributors

Maryam Agboluaje • *Chemical and Materials Engineering, University of Alberta, Edmonton, AB, Canada*

Joana Azeredo • *LIBRO - Laboratory of Research in Biofilms Rosário Oliveira, CEB – Centre of Biological Engineering, University of Minho, Braga, Portugal*

Bob Blasdel • *Laboratory of Gene Technology, KU Leuven, Kasteelpark Arenberg, Belgium*

J. Rodney Brister • *National Center for Biotechnology Information, National Library of Medicine, National Institutes of Health, Bethesda, MD, USA*

Mary Cano-Sarabia • *Catalan Institute of Nanoscience and Nanotechnology (ICN2), CSIC and The Barcelona Institute of Science and Technology, Barcelona, Spain*

José L. Carrascosa • *Department of Structure of Macromolecules, Centro Nacional de Biotecnología, Madrid, Spain*

Joan Colom • *School of Veterinary Medicine and Science, University of Nottingham, Nottingham, UK*

Pilar Cortés • *Departament de Genètica i Microbiologia, Universitat Autònoma de Barcelona, Cerdanyola del Vallès, Spain*

Ana Rita Costa • *LIBRO - Laboratory of Research in Biofilms Rosário Oliveira, CEB – Centre of Biological Engineering, University of Minho, Braga, Portugal*

Ana Cuervo • *Department of Structure of Macromolecules, Centro Nacional de Biotecnología, Madrid, Spain*

Krystyna Dąbrowska • *Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wrocław, Poland*

Masanori Daibata • *Department of Microbiology and Infection, Kochi Medical School, Kochi University, Nankoku, Kochi, Japan*

Laurent Debarbieux • *Department of Microbiology, Molecular Biology of Gene in Extremophiles, Institut Pasteur, Paris, France*

Raphaëlle Delattre • *Department of Microbiology, Molecular Biology of Gene in Extremophiles, Institut Pasteur, Paris, France; INSERM, IAME, UMR 1137, Paris, France; Service d'Anesthésie-Réanimation, AP-HP, Hôpital Beaujon, Clichy, France*

Nicolas Dufour • *Department of Microbiology, Molecular Biology of Gene in Extremophiles, Institut Pasteur, Paris, France; Service de Réanimation Médico-Chirurgicale, Centre Hospitalier René Dubos, Pontoise, France; INSERM, IAME, UMR 1137, Paris, France*

Alan Fauconnier • *Federal Agency for Medicines and Health Products, Brussels, Belgium; Culture in vivo ASBL, Nivelles, Belgium*

Randolph Fish • *PhageBiotics Research Foundation, Gray's Harbor Community Hospital, Aberdeen, WA, USA; Wound Centers at St Joseph's Medical Center, Tacoma, WA, USA*

Denis A. Ganichev • *Railway Clinical Hospital, Novosibirsk, Russian Federation*

Cristina García-Aljaro • *Department of Microbiology, University of Barcelona, Barcelona, Spain*

Aleksandra Głowacka • *Department of Microbial Biochemistry, Institute of Biochemistry and Biophysics of the Polish Academy of Sciences, Warsaw, Poland*

Piotr Golec • *Laboratory of Molecular Biology (affiliated with the University of Gdańsk), Institute of Biochemistry and Biophysics of the Polish Academy of Sciences, Gdańsk, Poland*

Sally L. Gras • *The Bio21 Molecular Science and Biotechnology Institute, The University of Melbourne, Parkville, VIC, Australia; The Department of Chemical Engineering, The University of Melbourne, Parkville, VIC, Australia*

Juan Jofre • *Department of Microbiology, University of Barcelona, Barcelona, Spain*

Zuzanna Kaźmierczak • *Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wrocław, Poland*

Yulia N. Kozlova • *Laboratory of Molecular Microbiology, Institute of Chemical Biology and Fundamental Medicine SB RAS, Novosibirsk, Russian Federation*

Andrew M. Kropinski • *Department of Food Science, University of Guelph, Guelph, ON, Canada; Department of Molecular & Cellular Biology, University of Guelph, Guelph, ON, Canada; Department of Pathobiology, University of Guelph, Guelph, ON, Canada*

Konrad Krysiak-Baltyn • *The Bio21 Molecular Science and Biotechnology Institute, The University of Melbourne, Parkville, VIC, Australia; The Department of Chemical Engineering, The University of Melbourne, Parkville, VIC, Australia*

Sarah Kuhl • *VA Northern California, Martinez, CA, USA; University of California, San Francisco, CA, USA*

Eugene E. Kulikov • *Winogradsky Institute of Microbiology RC Biotechnology RAS, Moscow, Russia; Moscow Institute of Physics and Technology, Dolgoprudny, Moscow Region, Russia*

Mzia Kutateladze • *George Eliava Institute of Bacteriophages, Microbiology and Virology – 3, Tbilisi, GA, USA*

Elizabeth Kutter • *PhageBiotics Research Foundation, Gray's Harbor Community Hospital, Aberdeen, WA, USA; Bacteriophage Lab, The Evergreen State College, Olympia, WA, USA*

Libera Latino • *Institute for Integrative Biology of the Cell (I2BC), CEA, CNRS, Univ. Paris-Sud, Université Paris-Saclay, Gif-sur-Yvette Cedex, France*

Andrey V. Letarov • *Winogradsky Institute of Microbiology RC Biotechnology RAS, Moscow, Russia; Lomonosov Moscow State University, Moscow, Russia; Moscow Institute of Physics and Technology, Dolgoprudny, Moscow Region, Russia*

Montserrat Llagostera • *Departament de Genètica i Microbiologia, Universitat Autònoma de Barcelona, Cerdanyola del Vallès, Spain*

Małgorzata B. Łobocka • *Department of Microbial Biochemistry, Institute of Biochemistry and Biophysics of the Polish Academy of Sciences, Warsaw, Poland; Faculty of Agriculture and Biology, Autonomous Department of Microbial Biology, Warsaw University of Life Sciences – SGGW, Warsaw, Poland*

Gregory J.O. Martin • *The Department of Chemical Engineering, The University of Melbourne, Parkville, VIC, Australia*

Daniel Maspoch • *Catalan Institute of Nanoscience and Nanotechnology (ICN2), CSIC and The Barcelona Institute of Science and Technology, Barcelona, Spain; ICREA, Barcelona, Spain*

Shigenobu Matsuzaki • *Department of Microbiology and Infection, Kochi Medical School, Kochi University, Nankoku, Kochi, Japan*

Luís D.R. Melo • *LIBRO – Laboratory of Research in Biofilms Rosário Oliveira, CEB – Centre of Biological Engineering, University of Minho, Braga, Portugal*

Maia Merabishvili • *Laboratory for Molecular and Cellular Technology, Queen Astrid Military Hospital, Brussels, Belgium*

Catarina Milho • *LIBRO - Laboratory of Research in Biofilms Rosário Oliveira, CEB – Centre of Biological Engineering, University of Minho, Braga, Portugal*

Vera V. Morozova • *Laboratory of Molecular Microbiology, Institute of Chemical Biology and Fundamental Medicine SB RAS, Novosibirsk, Russian Federation*
Maite Muniesa • *Department of Microbiology, University of Barcelona, Barcelona, Spain*
Jennifer Otero • *Departament de Genètica i Microbiologia, Universitat Autònoma de Barcelona, Cerdanyola del Vallès, Spain*
Diana P. Pires • *LIBRO – Laboratory of Research in Biofilms Rosário Oliveira, CEB – Centre of Biological Engineering, University of Minho, Braga, Portugal*
Jean-Paul Pirnay • *Laboratory for Molecular and Cellular Technology, Queen Astrid Military Hospital, Brussels, Belgium*
Christine Pourcel • *Institute for Integrative Biology of the Cell (I2BC), CEA, CNRS, Univ. Paris-Sud, Université Paris-Saclay, Gif-sur-Yvette Cedex, France*
Hilde Van Raemdonck • *Laboratory for Molecular and Cellular Technology, Queen Astrid Military Hospital, Brussels, Belgium*
Dominic Sauvageau • *Chemical and Materials Engineering, University of Alberta, Edmonton, AB, Canada*
Sanna Sillankorva • *CEB – Centre of Biological Engineering, University of Minho, Braga, Portugal*
Iyo Takemura-Uchiyama • *School of Veterinary Medicine, Azabu University, Sagamihara, Kanagawa, Japan*
Nina V. Tikunova • *Laboratory of Molecular Microbiology, Institute of Chemical Biology and Fundamental Medicine SB RAS, Novosibirsk, Russian Federation*
Igor Tolstoy • *National Center for Biotechnology Information, National Library of Medicine, National Institutes of Health, Bethesda, MD, USA*
Jumpei Uchiyama • *School of Veterinary Medicine, Azabu University, Sagamihara, Kanagawa, Japan*
Takako Ujihara • *Science Research Center, Kochi University, Kohasu, Oko-cho, Nankoku, Kochi, Japan*
Gilbert Verbeken • *Laboratory for Molecular and Cellular Technology, Queen Astrid Military Hospital, Brussels, Belgium*
Daniel De Vos • *Laboratory for Molecular and Cellular Technology, Queen Astrid Military Hospital, Brussels, Belgium*
Gordon Wheat • *PhageBiotics Research Foundation, Gray's Harbor Community Hospital, Aberdeen, WA, USA; Kaiser Permanente Northwest, Saint Peter Hospital Family Medicine Residency, Olympia, WA, USA*

Part I

Isolation of Bacteriophages

Chapter 1

Isolation of Bacteriophages for Fastidious Bacteria

Shigenobu Matsuzaki, Jumpei Uchiyama, Iyo Takemura-Uchiyama, Takako Ujihara, and Masanori Daibata

Abstract

One of the most important factors for successful bacteriophage therapy is, undoubtedly, the isolation of excellent therapeutic candidate bacteriophages. There are only a few reports about active bacteriophages in the fastidious bacteria *Helicobacter pylori*. In this chapter, we describe a method for isolating and purifying KHP30-like bacteriophages in *H. pylori*, which have lytic and pseudolysogenic life cycles.

Key words *Helicobacter pylori*, KHP30-like bacteriophage, Lytic and pseudolysogenic life cycles, Isolation, Purification

1 Introduction

Helicobacter pylori is a Gram-negative, spiral, and microaerophilic bacterium. It is well known as a causative agent in chronic and acute gastritis, gastric ulcers, and gastric cancer [1, 2]. If the bacterium is detected in the stomach by bacterial examination, its eradication will be recommended using antimicrobial agents such as clarithromycin, metronidazole, and amoxicillin. However, as antimicrobial resistance (AMR) is also progressing in the case of *H. pylori* like other bacterial cases, its eradication will become more difficult in the future [2]. Therefore, the isolation of therapeutic candidate bacteriophages for the eradication of *H. pylori* from the human stomach is thought to be meaningful.

Despite the scarcity of reports about active *H. pylori* bacteriophages [3–9], KHP30 is one of the best characterized of these bacteriophages [7, 8]. Bacteriophages that are genetically related to KHP30 are called KHP30-like bacteriophages in this article. Both KHP30 and KHP40, another KHP30-like bacteriophage, have lytic and pseudolysogenic life cycles [7, 8, 10]. Although lytic bacteriophages are thought to be suitable as therapeutic bacteriophages, mutant bacteriophages derived from them that have

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lost pseudolysogenic ability via spontaneous mutations may also be useful for bacteriophage therapy.

In this chapter, we describe isolation and purification methods of KHP30-like bacteriophages in *H. pylori*.

2 Materials

2.1 Bacteriophage Isolation

1. *H. pylori* strains.
2. Brucella broth.
3. Equine serum (inactivated by heating for 30 min at 56 °C).
4. Vancomycin (filtrated using a filter with a pore size of 0.2 μm, 10 mg/mL of water).
5. BEV: Brucella broth including 10% (vol/vol) equine serum and 10 μg/mL of vancomycin (*see* **Note 1**).
6. BEV plate: BEV including 1.5% (wt/vol) agar (ca. 20 mL in a plastic Petri dish with a diameter of 9–10 cm).
7. SBA (soft Brucella agar): Brucella broth including 0.5% (wt/vol) agar and kept at 55 °C in a long glass screw vial (diameter, 1.8 cm; length, 15–18 cm) on a dry hot bath.
8. Sterile plastic tube (10, 50 mL) for culture.
9. Sterile syringe filter with a pore size of 0.45 μm and sterile plastic syringe.
10. Sterile Conradi stick.
11. Micropipette (10 μL and sterile plastic tip for it).
12. Sterile plastic loop (10 μL).
13. Sterile plastic needle.
14. Dry hot bath: equipment for standing SBA-containing grass vial at 55 °C.
15. CO_2 incubator.
16. Shaker in CO_2 incubator.
17. Cooling centrifuge.

2.2 Purification of Bacteriophage Particles

1. BCV: Brucella broth including 0.5% (wt/vol) β-cyclodextrin and 10 μg/mL of vancomycin (*see* **Note 2**).
2. Polyethylene glycol 6000 and Tween 20.
3. TM buffer (10 mM Tris–HCl, 5 mM $MgCl_2$, pH 7.2).
4. DNase I: Deoxyribonuclease I (10 mg/mL).
5. RNaseA: Ribonuclease A (10 mg/mL).
6. AAS solution (100 mM ammonium acetate, 10 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, pH 7.2).

7. CsCl ($\rho = 1.7$, 1.5, and $\rho = 1.3$) in AAS solution.
8. Plastic syringe with a needle or dropper.
9. Dialyzing tube (cut-off molecular weight, 10,000).
10. Cooling centrifuge.
11. Ultracentrifuge.
12. Ultracentrifugation tube (6 mL).

3 Methods

This protocol shows a detailed description of the method previously described [8]. In this protocol, *H. pylori* strains are cultured in air containing 10% CO_2 at 37 °C.

3.1 Preparation of the Supernatant of an H. pylori *Culture*

1. *H. pylori* strains are shaken vigorously in 2 or 5 mL BEV in 10 or 50 mL plastic tubes for 2 days (*see* **Notes 3** and **4**).
2. The strains are centrifuged at 7000 × *g* (or lower) for 5 min at 4 °C, to remove the residual host cells.
3. The supernatants are filtrated using a filter with a pore size of 0.45 μm with a plastic syringe (*see* **Note 5**).

3.2 Easy Screening of Active Bacteriophages: Spot Test and Streak Test

1. 0.5 mL of the host culture fully grown in BEV is placed on the BEV plate, and 5 mL of SBA is poured on it, followed by solidification (i.e., bilayer method) (*see* **Note 6**).
2. For the spot test, 3 μL of the filtrate from Subheading 3.1, **step 3** is spotted onto the surface of the upper layer, which includes the host cells, using a micropipette (Fig. 1a). For the streak test, several lines are gently streaked on the surface of the upper layer using a plastic loop carrying 10 μL of the filtrate (Fig. 1b) (*see* **Note 7**). Subsequently, the plate is incubated for 3 days.
3. Results of the spot test: the appearance of a spot shows that the filtrate possibly includes bacteriophages. However, it does not necessarily indicate that the bacteriophages have propagated in the host. Therefore, after the spot test, plaque-forming activity needs to be examined, to detect active bacteriophages.
4. Results of the streak test: the observation of lysis lines to the end, or lysis lines and plaques following them, implies that the filtrate includes active bacteriophage(s) (Fig. 1b). Conversely, the presence of lysis lines without plaques following them may indicate lysis from without, in which bacteriophages adsorb to the host but do not propagate in it. The absence of lysis lines indicates that there may not be any bacteriophage infection in the host.

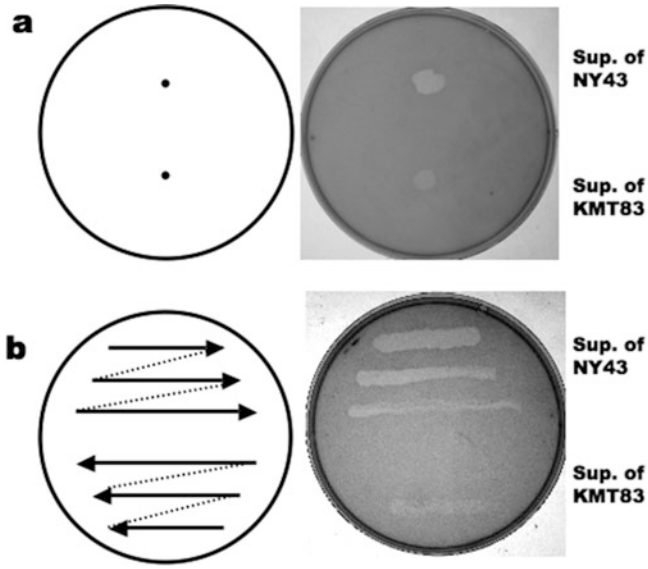

Fig. 1 Spot test and streak test using the supernatants of cultures of the *H. pylori* strains NY43 and KMT83, in which KHP30-like bacteriophages, KHP30 and KHP40, are released, respectively. Upper panel: spot test (3 μL). *Lower panel*: streak test (10 μL). *Left panel*: schematic representation of the procedures. *Right panel*: results obtained after 3 days

3.3 Isolation of H. pylori Bacteriophages by Single-Plaque Isolation Procedure

1. Filtrates in which the presence of bacteriophages has been shown are serially diluted in 0.5 mL BEV (e.g., 1, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}).
2. 0.1 mL of the diluted samples and 0.5 mL of the host culture fully grown in BEV, which showed sensitivity to the active bacteriophage, are placed on the BEV plate, and 5 mL SBA is then poured onto them and mixed evenly. After solidification, the plates are incubated for 3 days.
3. A well-separated single plaque is picked up using a needle, suspended in 0.5 mL of BEV medium, and diluted to 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4} in BEV (*see* **Note 8**).
4. 0.1 mL of each bacteriophage dilute and 0.5 mL of the host culture fully grown in BEV are placed on the BEV plate, and SBA is poured onto it. After solidification, the plates are incubated again for 3 days.
5. Another single plaque is picked up using a needle, diluted, and cultured with the host as described above. The single plaque isolation procedure is repeated at least three times, to avoid contamination by other bacteriophages.

3.4 Preparation of an H. pylori Bacteriophage Stock by Confluent Lysis Procedure

1. A plaque that appears in the third cycle after the procedure described above is picked up using a needle and transferred into 0.5–1 mL of BEV medium.
2. 0.1 mL of the bacteriophage suspension and 0.5 mL of the host culture are placed on five BEV plates, and SBA is poured onto each plate and mixed. After solidification, the plates are incubated for 3 days.
3. 10 mL of liquid BEV are poured onto one of the plates in which the host has been lysed completely. After the upper layer is crushed with a sterile Conradi stick, it is transferred onto the next plate. This procedure is repeated. Finally, all of the crushed upper layers with the medium are transferred into a 50 mL plastic tube and are vortexed vigorously.
4. The tube is centrifuged at 7000 × *g* for 5 min at 4 °C, and the supernatant is filtrated using a filter with a pore size of 0.45 μm.
5. The bacteriophages are probably stable at 4 °C for at least 2 years. For long-term storage, 0.5 mL of the filtrate is mixed with 0.5 mL of 60% (vol/vol) glycerol and is placed at −80 °C.

3.5 Preparation of Purified Bacteriophage Particles Using CsCl Density Gradient Ultracentrifugation

1. 5–10 mL of *H. pylori* culture that has been grown fully in BEV and 0.1–0.2 mL of the bacteriophage stock (usually ca. 1–5 × 10^9 pfu/mL) are transferred to 250–500 mL of BCV medium in a flask and the culture is vigorously shaken for 2–3 days (*see* **Note 9**).
2. The culture is centrifuged at 10,000 × *g* for 10 min at 4 °C, to precipitate the host cell debris.
3. 10% (wt/vol) polyethylene glycol 6000, 3 M NaCl, and 1% (vol/vol) Tween 20 are added to the supernatant and are solubilized well (*see* **Note 10**).
4. The mix is centrifuged at 10,000 × *g* for 20–40 min at 4 °C.
5. The precipitate is resuspended in 2–3 mL of TM buffer including DNaseI (final concentration, 100 μg/mL) and RNaseI (final concentration, 100 μg/mL), followed by incubation at 37 °C for 60 min.
6. 1 mL of the sample is overlaid on top of a stepwise CsCl density gradient consisting of 0.5 mL (ρ = 1.7), 1.5 mL (ρ = 1.5), and 2.5 mL (ρ = 1.3) from the bottom upwards (Fig. 2b) (*see* **Note 11**).
7. The tube is centrifuged at 100,000 × *g* for 60 min at 4 °C.
8. The purified bacteriophage (white band) is obtained using a syringe and needle or a plastic dropper.
9. The purified bacteriophage band is transferred to a dialyzing tube (cut-off molecular weight, 10,000) and is dialyzed against 1000 mL of AAS at 4 °C for 60 min.

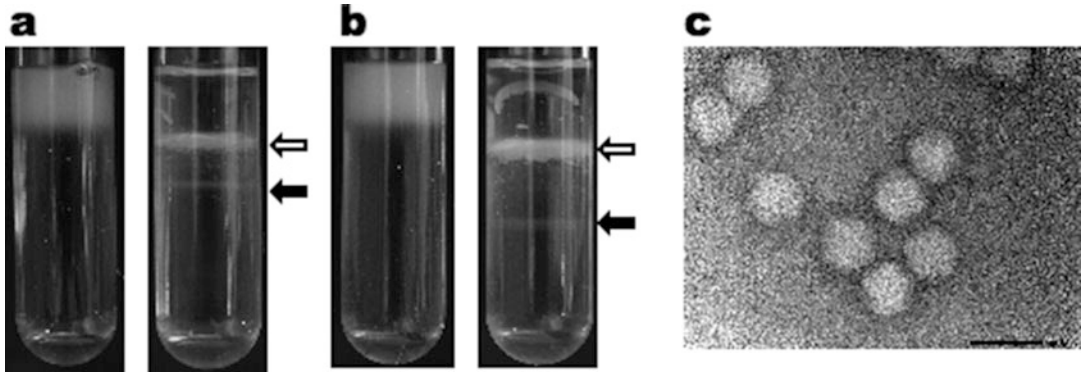

Fig. 2. KHP30 bacteriophage band in two types of stepwise CsCl density gradient ultracentrifugation. (**a**) Usual method: 2.5 mL ($\rho = 1.7$), 2.5 mL ($\rho = 1.5$), and 2.5 mL ($\rho = 1.3$) from the bottom. (**b**) Modified method for the preparation of KHP30-like bacteriophages: 0.5 mL ($\rho = 1.7$), 1.5 mL ($\rho = 1.5$), and 2.5 mL ($\rho = 1.3$) from the bottom. *Left* and *right* in **a** and **b** depict the experiment before and after ultracentrifugation, respectively. *Black arrow*, bacteriophage band. *White arrow*, debris. (**c**) Electron micrograph of KHP30 purified using the unusual method

10. The purified bacteriophage sample can be used for electronic microscope observation, DNA preparation, or virion protein preparation (e.g., Fig. 2c).

4 Notes

1. In the preparation of 1000 mL, 900 mL of water including 28 g of Brucella broth powder (and 15 g agar) are autoclaved and, after cooling to a temperature that allows hand touching, 100 mL of equine serum and vancomycin (final concentration, 10 μg/mL) are added.
2. 1000 mL of water including 28 g of Brucella broth powder and 5 g of β-cyclodextrin are autoclaved and, after cooling, vancomycin (final concentration, 10 μg/mL) is added.
3. Aeration is very important for *H. pylori* culture. A 10 or 50 mL plastic tube is used for 2 or 5 mL culture, respectively, and a shaking speed >200 rpm is recommended.
4. As active KHP30-like *H. pylori* bacteriophages are found rarely (about 1%), the examination of as many as possible *H. pylori* strains is recommended. KHP30-like bacteriophages do not seem to be induced by mitomycin C addition.
5. Usage of a filter with a pore size of 0.2 μm is not recommended because large bacteriophages (such as T4-related bacteriophages) cannot pass through the filter perfectly. Therefore, in general, the use of a 0.45 μm filter is recommended for the examination of unknown bacteriophages.

6. *H. pylori* strains seem commonly to have strong restriction-modification systems. Therefore, as many as possible host strains should be examined during the screening for KHP30-like bacteriophages.
7. The spot test is suitable to examine many filtrates, but the plaque-forming activity needs to be checked. Conversely, in the streak test, plaque-forming activity can be checked in one step.
8. If several types of plaques showing a different morphology or diameter appear, all types of plaques should be picked up independently because they may be taxonomically different from each other.
9. BCV medium is more suitable than BEV medium for large-scale bacteriophage preparation because the presence of equine serum in the medium seems to produce a lot of debris.
10. The addition of 3 M NaCl (usually 0.5 M) and 1% Tween 20 promotes the separation of the bacteriophages from the debris.
11. This is a modified stepwise CsCl density gradient ultracentrifugation. As the density of KHP30-like bacteriophages is low, the bacteriophage band is hardly separated from the upper debris layer in the usual method, which uses an equal volume of CsCl solution with $\rho = 1.7$, 1.5, and 1.3 from the bottom (Fig. 2a and b).

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Chapter 2

Isolation of Bacteriophages of the Anaerobic Bacteria *Bacteroides*

Cristina García-Aljaro, Maite Muniesa, and Juan Jofre

Abstract

Here we describe the detection, enumeration, and isolation of bacteriophages infecting *Bacteroides*. The method is based on the infection of *Bacteroides* host strains and the production of visible plaques in a confluent lawn of the host strain using the double-layer agar method. This is a straightforward methodology that can be applied for the detection, enumeration and isolation of bacteriophages for other anaerobic bacteria, using an appropriate host strain and culture conditions. In the case of bacteriophages of *Bacteroides* the results can be obtained in less than 24 h, although the time could vary depending on the growth rate of the host strain.

Key words Bacteriophage, Double-layer agar method, Anaerobic, Lytic plaques, *Bacteroides*

1 Introduction

A number of bacteriophages have been incorporated in several regulations as indicators of microbial water quality to validate and verify water treatment processes [1–6]. In this respect, the presence of bacteriophages infecting *Bacteroides*, one of the most abundant species in the gut, in an environmental sample indicates the presence of fecal pollution of human or animal origin. Different studies have pointed out that bacteriophages infecting different *Bacteroides* species can be used for microbial source tracking purposes [7–14] although certain geographic variation has also been reported [8, 9, 13, 14]. Despite different molecular detection methods have been reported, detection of bacteriophages using the double-layer agar method with an appropriate host strain is a straightforward and nonexpensive methodology which, besides detection of infecting bacteriophages and evaluation of the microbiological water quality, allows their isolation for different purposes. For example, bacteriophages of *Bacteroides* can be isolated and used to study the bacteriophage receptors, which are different cell surface components that may be involved in the virulence of different *Bacteroides*.

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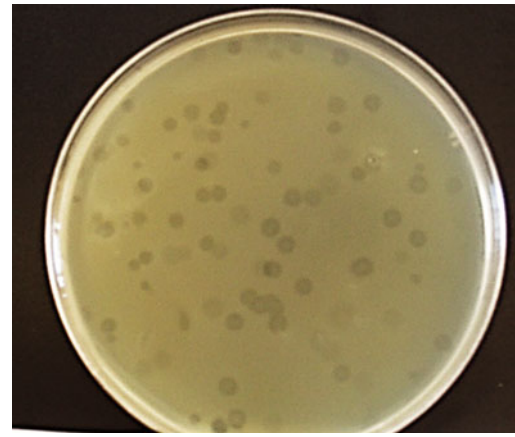

Fig. 1 Typical lytic plaques of phages infecting *Bacteroides fragilis* grown on semisolid BPRMA

In this chapter, we report a methodology for the detection and enumeration of bacteriophages infecting *Bacteroides* based on the double-layer agar method, as an example of bacteriophages for anaerobic bacteria. The most abundant bacteriophages infecting *Bacteroides* belong to the family *Siphoviridae* with flexible tail (dsDNA, long noncontractile tails, capsids up to 60 nm) (Fig. 1). The method relies on the infection of selected bacterial host and the production of visible plaques (clearance zones) in a confluent host bacteria lawn grown under appropriate culture conditions. Bacteriophages detected are those virulent infecting *Bacteroides* by attaching to molecules present in the bacteria cell wall. Virulent bacteriophages may lyse the host cell in 30–40 min under optimal conditions. They produce clear plaques which do not differ very much in size and morphology (Fig. 2). This method can be applied to all types of water, sediments and sludge extracts, as well as shellfish extracts and has been standardized by the International Standard Organization [15].

2 Materials

2.1 Handling and Culturing the Host Strain

1. *Bacteroides fragilis* RYC2056 [10], although other *Bacteroides* species or strains can be used as long as they can grow using the same medium and culture conditions. For example, *B. fragilis* HSP40 (ATCC 51477) [16], *B. thetaiotaomicron* GA17 and others have been successfully used following the same method with minor modifications [17].
2. Basal Bacteroides Bacteriophage Recovery Medium Broth (BPRMB) [18]: weight 10 g meat peptone, 10 g casein peptone, 2 g yeast extract, 5 g NaCl, 0.5 g monohydrated

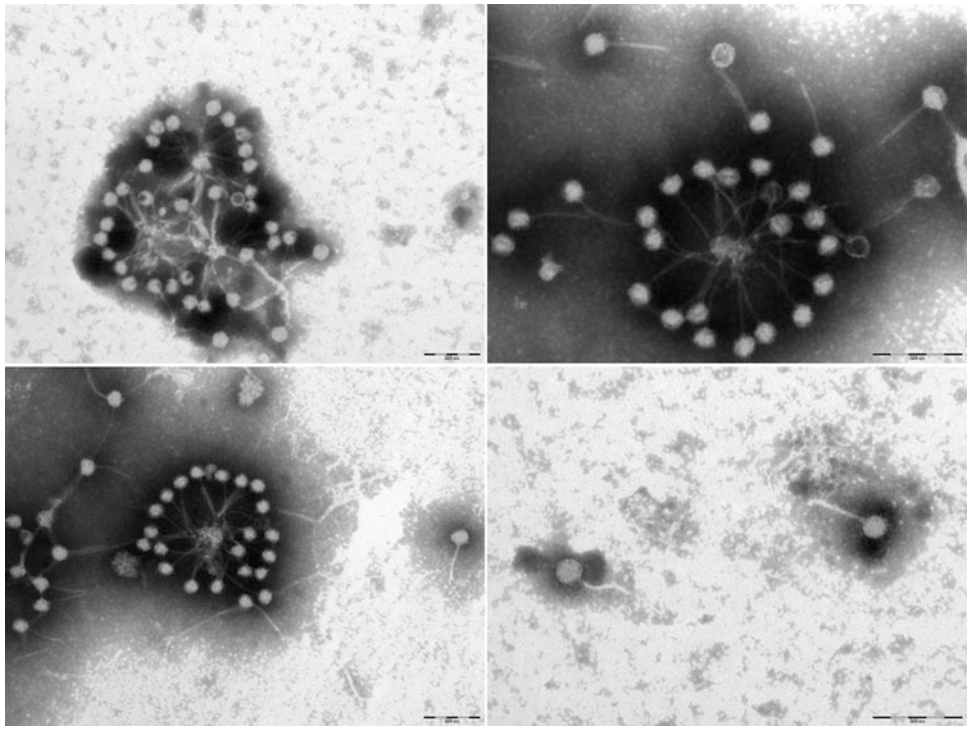

Fig. 2 Electron micrographs of phages infecting *Bacteroides fragilis* and *Bacteroides thetaiotaomicron*. Pictures show phages alone or attached to particles. Negative staining with 2% (wt/vol) KOH phosphotungstic acid (pH 7.2). Bar 200 nm

L-cystein, 1.8 g glucose, 0.12 g $MgSO_4 \cdot 7H_2O$, and add 1000 mL of distilled water. Dissolve the ingredients and add 1 mL of $CaCl_2$ stock solution containing 0.05 g/mL prepared as described below. Sterilize in the autoclave at 121 °C for 15 min. Store in the dark at 4 °C for no longer than 1 month (*see* **Note 1**).

The complete broth (Table 1) should be prepared immediately before use by adding aseptically 10 mL of hemin solution and 25 mL of disodium carbonate solution to 1000 mL of basal broth (*see* **Note 2**). The pH should be adjusted to 6.8 by adding HCl (e.g. 2.5 mL of HCl 35% (vol/vol)).

3. $CaCl_2$ stock solution: weight 5 g of $CaCl_2 \cdot 2H_2O$ in 100 mL of distilled water while heating gently. Cool to room temperature and filter sterilize through a 0.2 μm pore size membrane filter (high-binding protein cellulose ester membrane) [19]. Store in the dark at 4 °C for no longer than 6 months (*see* **Note 1**).
4. Hemin solution: weight 0.1 g of hemin, add 0.5 mL of NaOH 1 M and 99.5 mL of distilled water. Dissolve by magnetic stirring (*see* **Note 3**). The solution can be filter-sterilized

Table 1
Composition of the *Bacteroides* phage recovery medium broth.

Meat peptone	10 g
Casein peptone	10 g
Yeast extract	2 g
NaCl	5 g
Monohydrated L-cystein	0.5 g
Glucose	1.8 g
$MgSO_4$.7H2O	0.12 g
$CaCl_2$ solution (0,05 g/mL)	1 mL
Hemin solution[a]	10 mL
Na_2CO3 solution[a]	25 mL
Distilled water	1000 mL

[a]Add aseptically immediately before use.

through a 0.2 μm pore size high protein binding membrane filter, or sterilized in the autoclave at 121 °C for 15 min. Store at room temperature for no longer than 6 months.

5. Disodium carbonate solution (1 M) (Na_2CO_3): weight 10.6 g of Na_2CO_3 in 100 mL of distilled water and filter-sterilize through a 0.2 μm pore size high protein binding membrane filter. Store at room temperature for no longer than 6 months.
6. Nalidixic acid solution: weight 250 mg Nalidixic acid in 2 mL of NaOH (1 M), then add 8 mL of distilled water until completely dissolved. Filter-sterilize through a 0.2 μm pore size high protein binding membrane filter. Store at 4 °C for no longer than 8 h or at −20 °C for no longer than 6 months.
7. Kanamycin monosulfate: weight 1.25 g of kanamycin monosulfate in 10 mL of distilled water and filter-sterilize through a 0.2 μm pore size high protein binding membrane filter (*see* **Note 4**). Store at 4 °C for no longer than 8 h or at −20 °C for no longer than 6 months.
8. Bacteroides Bacteriophage Recovery Medium Agar (BPRMA): Add 12–20 g of agar (depending on gel strength of agar) to 1000 mL of basal broth (not autoclaved). Sterilize by autoclaving at 121 °C for 15 min. Cool down to between 45 and 50 °C and add aseptically 10 mL of hemin solution and 25 mL of Na_2CO_3 solution to prepare the complete BPRMA (*see* **Note 2**). The pH should be adjusted to 6.8 by adding HCl (e.g. 2.5 mL of HCl 35%

(vol/vol)). Pour into Petri dishes (20 mL in dishes of 9 cm diameter). Allow to solidify and store in the dark at 4 °C for no longer than two months (*see* **Note 1**). Place the plates at room temperature 1–2 h before use to dry them.

2.2 Host Strain Stock Culture Preparation

1. *Bacteroides* host strain.
2. BPRMB tubes (*see* Subheading 2.1).
3. BPRMA plates (*see* Subheading 2.1).
4. Cryoprotector: 10% (wt/vol) bovine serum albumin, 20% (wt/vol) sucrose in water. Sterilize using high protein binding 0.2 μm pore-size cellulose ester membrane filters.
5. Sterile swabs.
6. Anaerobic jar or chamber.
7. Sterile screw-capped vials.
8. Sterile tips.
9. Incubator at 37 °C.
10. −70 °C freezer.

2.3 Preparation of Host Strain Inoculum Culture for Bacteriophage Analysis

1. *Bacteroides* host strain stock culture.
2. BPRMB tubes (*see* Subheading 2.1).
3. BPRMA plates (*see* Subheading 2.1).
4. Sterile screw-capped vials.
5. Sterile tips.
6. Anaerobic jar or chamber.
7. Incubator at 37 °C.
8. Spectrophotometer.

2.4 Bacteriophage Presence/Absence Test

1. *Bacteroides* host strain inoculum culture.
2. BPRMA plates (*see* Subheading 2.1).
3. Semisolid Bacteroides Bacteriophage Recovery Medium Agar (ssBPRMA) tubes: Basal agar medium, agar (6–10 g), depending on the gel strength (*see* **Note 5**). Sterilize by autoclaving at 121 °C for 15 min and distribute in volumes of 50 mL. Allow to solidify and store at 4 °C for no longer than two months. Immediately before use, melt the basal medium, allow to cool down to 45–50 °C and add aseptically 10 mL of hemin solution and 25 mL of Na_2CO_3 solution to prepare the complete ssBPRMA (*see* **Note 2**). The pH should be adjusted to 6.8 by adding HCl (e.g. 2.5 mL of HCl 35% (vol/vol)).
4. Bacteriophage suspension/samples.
5. Sterile tips.

6. Anaerobic jar or chamber.
7. Incubator at 37 °C.
8. Incubator at 45–50 °C.

2.5 Bacteriophage Enumeration

1. *Bacteroides* host strain inoculum culture.
2. BPRMA plates (*see* Subheading 2.1).
3. ssBPRMA tubes (*see* Subheading 2.4).
4. Sterile tips.
5. Anaerobic jar or chamber.
6. Incubator at 37 °C.

2.6 Isolation of Bacteriophages from Plaques

1. Bacteriophage plaques.
2. SM buffer: weight 5.8 g of NaCl, 2 g of $MgSO_4 \cdot 7H_2O$, 0.1 g of gelatin, and add 50 mL of Tris–HCl 1 M at pH 7.5. Adjust to 1000 mL with double distilled water. Sterilize in autoclave at 121 °C for 15 min.
3. BPRMB tubes (*see* Subheading 2.1).
4. BPRMA plates (*see* Subheading 2.1).
5. ssBPRMA tubes (*see* Subheading 2.4).
6. Chloroform.
7. Sterile tubes.
8. Sterile needle.
9. Sterile tips.
10. 0.2 μm pore-size low protein binding membrane filter (PVDF or PES).
11. Anaerobic jar or chamber.
12. Bench centrifuge.

2.7 Preparation of a High-Titer Bacteriophage Suspension in Solid Agar Media

1. Bacteriophage suspension.
2. Bacteroides host strain inoculum culture.
3. BPRMA plates (*see* Subheading 2.1).
4. ssBPRMA tubes (*see* Subheading 2.4).
5. SM buffer.
6. Chloroform.
7. Sterile tubes.
8. Sterile needle.
9. Sterile pipette.
10. Sterile tips.
11. 0.2 μm pore-size low protein binding membrane filter (PVDF or PES).

12. Anaerobic jar or chamber.
13. Incubator at 37 °C.
14. Bench centrifuge.

2.8 Preparation of Reference Bacteriophage Stock Culture

1. Reference bacteriophage: Bacteriophage B56-3 (ATCC 700786-B1) can be used as reference bacteriophage for *B. fragilis* RYC2056, and bacteriophage B40-8 (ATCC 51477-B1) for *B. fragilis* HSP40 (ATCC 51477). Reference bacteriophages have also been isolated for other hosts but to the best of our knowledge they have not been deposited yet in culture collections.
2. Peptone saline: weight 1 g of peptone and 8.5 g of NaCl, and add 1000 mL of distilled water, adjust pH at 7.2. Sterilize in autoclave at 121 °C for 15 min.
3. Sterile glycerol.
4. Sterile tubes.
5. Sterile tips.
6. 70 °C freezer.

3 Methods

3.1 Handling and Culturing the Host Strain

Bacteroides do not require very strict anaerobic conditions for handling or for growing: the bacterium can be handled shortly in the presence of air but agar plates (BPRMA) should be incubated in anaerobic cabinets, jars, or bags containing anaerobiosis generators and anaerobic indicators. Liquid cultures in BPRMB can be performed in glass tubes, flaks or bottles by filling them completely with medium and closing them with screwing caps, without the need of incubating them under anaerobic conditions. Of course, they can be handled and incubated under anaerobic conditions. Other anaerobic bacteria may require more strict anaerobic conditions and different growth media and culture conditions.

3.2 Host Strain Stock Culture Preparation

1. Inoculate a BPRMA plate with the corresponding host strain (cover all the plate twice with a sterile swab to ensure the maximal growth) and incubate 24–48 h at 37 °C in anaerobic jar or chamber.
2. Inoculate 10 mL of BPRMB with at least 1/4 of the cells previously grown the BPRMA plate and incubate overnight at 37 °C (*see* **Notes 6** and **7**).
3. Mix culture and cryoprotector in a 1:1 ratio (vol/vol), avoiding bubble formation. Distribute into sterile screw-capped vials in aliquots of *ca.* 0.5 mL and store at −70 °C.

3.3 Preparation of Host Strain Inoculum Culture for Bacteriophage Analysis

1. Thaw one vial of stock culture at room temperature and streak on a plate of BPRMA covering all the plate. Incubate 24–48 h at 37 °C. After 24 h of incubation check if a dense bacterial growth is visible. Otherwise, incubate for extra 24 h at 37 °C. The plate should be completely covered of cell material before inoculating the strain in **step 2**.
2. Inoculate at least 1/4 of the cell material grown on the plate in 10 mL of prewarmed BPRMB and incubate overnight at 37 °C (*see* **Note 8**).
3. Transfer this culture to fresh prewarmed BPRMB screw capped tube in a ratio 1:10 (vol/vol). Fill completely the tube with BPRMB and incubate at 37 °C. After 2 h measure absorbance every 30 min until reaching approximately 5×10^8 cfu/mL (OD_{620} 0.3–0.5 based on data obtained in our laboratory) (*see* **Note 9**).
4. Take the inoculum culture from the incubator and quickly cool the culture by placing it in ice. Use within 6 h.

3.4 Bacteriophage Presence/Absence Test

1. Add 1 mL of the inoculum culture to 2.5 mL of melted ssBPRMA, previously cooled to 45–50 °C (*see* **Note 10**).
2. Mix well and pour onto a BPRMA plate.
3. Allow to solidify and add a drop (up to 20 μL) of the bacteriophage suspension or filtered samples to be tested (*see* **Note 11**). Allow the drop to adsorb on the agar for at least 15 min.
4. Incubate the plates upside down overnight at 37 °C in anaerobic conditions (*see* Subheading 3.1).
5. Detection of a clearance zone (plaque formation) (*see* **Note 12**).

3.5 Bacteriophage Enumeration

1. Add 1 mL of the inoculum culture grown at OD_{620} 0.3–0.5, and 1 mL of the sample to be tested to 2.5 mL of melted ssBPRMA (*see* **Notes 10** and **13**).
2. Mix well avoiding bubble formation and pour onto a BPRMA plate.
3. Allow to solidify and incubate plates upside down overnight at 37 °C in anaerobic conditions (*see* Subheading 3.1).
4. Count the number of lytic plaques and calculate the number of plaque forming units (pfu) per mL depending on the sample dilution analyzed.

3.6 Isolation of Bacteriophages from Plaques

1. Carefully excise the desired plaque from the over layer of semisolid agar using a sterile needle.
2. Resuspend the bacteriophages in the plaque in SM buffer.
3. Add chloroform at 1:10 (vol:vol). Mix vigorously for 5 min. Centrifuge at 16,000 × *g* for 5 min.

4. Recover supernatant.
5. Inoculate 1 mL of supernatant with 9 mL of host strain inoculum, fill the tube with BPRMB to the top, and incubate overnight at 37 °C.
6. Add chloroform at 1:10 (vol:vol). Mix vigorously for 5 min. Centrifuge at 16,000 × *g* for 5 min. Carefully transfer the aqueous supernatant to a sterile empty agar plate avoiding solvent phase.
7. Filter the supernatant through a 0.2 μm pore-size low protein binding membrane filter (PVDF or PES membranes) [19] (*see* **Note 14**).
8. Enumerate the bacteriophages in the suspension. If higher bacteriophage titer is desired, repeat **steps 5–8** using 1 mL of the suspension.

3.7 Preparation of a High-Titer Bacteriophage Suspension in Solid Agar Media

1. Use 1 mL of bacteriophage suspension obtained in Subheading 3.6 (**step 4**) and proceed as for bacteriophage enumeration. Prepare one control plate without bacteriophage suspension (containing only the host strain and ssBPRMA.
2. After incubation, confluent lysis should be expected in comparison with the bacterial control plate. Add 5 mL of SM buffer and drop it onto the surface of the plate with confluent lysis. Incubate 15 min at 4 °C.
3. Carefully recover the liquid from the surface with a sterile pipette.
4. Add chloroform at 1:10 (vol:vol). Mix vigorously for 5 min. Centrifuge at 16,000 × *g* for 5 min.
5. Recover supernatant and filter it through 0.2 μm diameter pore-size filter.

3.8 Preparation of Reference Bacteriophage Stock Culture (See Note 13)

1. Dilute bacteriophage suspension in peptone saline solution to contain between 40 and 100 pfu/mL.
2. Add sterile glycerol to the bacteriophage suspension (final glycerol concentration should be 10% (vol/vol)).
3. Distribute into aliquots of 1 mL and store at −70 °C.
4. Check the intra- and inter-vial homogeneity (*see* **Note 15**).

4 Notes

1. For long-term storage, ISO method recommends storage at 4 °C in the dark to prevent contamination or undesirable changes in the solution.

2. *Bacteroides* growth is slower as other possible contaminants. Therefore to avoid competition with other microorganisms present in the samples the use of antibiotics is recommended. One of the most common contaminants are Gram-positive cocci. To prevent contamination it is recommended to add Kanamycin monosulfate (final concentration of 100 μg/mL) and Nalidixic acid (final concentration of 100 μg/mL) to the complete broth. Since not all strains of *Bacteroides* display the same resistance range to both antibiotics, how these can affect the growth of the strain should be tested previously.
3. Complete dissolution of hemin is necessary and may last between 30 and 60 min.
4. Many suppliers of kanamycin sulfate contain less than 100% active Kanamycin. Make the necessary corrections to reach 1.25 g of active kanamycin monosulfate to 10 mL of water.
5. The gel strength of ssBPRMA is critical to obtain good results and if possible different concentrations should be tested. Choose the agar concentration that produces the highest plaque counts but also control plaque-size to either reduce confluence or very small plaques. The most commonly used in our lab is 7 g/L.
6. Depending on the growth of the host strain on BPRMA, inoculate 1/8 or more of the cell material grown on the surface of BPRMA, using a sterile cotton swab (e.g. in case of dense growth use 1/8 to inoculate 10 mL BPRMB, in case of poor growth use 1/2 of the full slant).
7. To ensure anaerobic conditions, completely fill the tube with BPRM broth and close the screw-capped tube. Otherwise a tube not completely filled and not completed sealed could be incubated using an anaerobic jar.
8. The overnight liquid culture can be prepared directly from the stock (without intermedium stage in an agar plate) in a ratio 1:10 (vol:vol), into to a screw-capped tube.
9. We strongly recommend performing a growth curve of the host strain in order to calculate the correspondence between CFU/mL and OD_{620} nm, due to possible variations in spectrophotometers. If the cell density of approximately 5×10^8 CFU/mL is not reached within three hours, it is possible to increase the amount of working culture transferred into the BPRMB to a ratio 1.5:10 (vol:vol) or the incubation time.
10. Immediately before use, hemin and Na_2CO_3 solutions, as well as antibiotics (if desired) should be added to ssBPRM. The accuracy of counting bacteriophages depends on the stability of the host strain. In order to check the stability of the strain a

positive control should be performed using the reference bacteriophage B56-3 (ATCC 700786-B1) for RYC2056, B40-8 (ATCC 51477-B1) for HSP40 strain, GA17-1B for GA17 strain, etc.

11. When low numbers are expected, larger volumes of sample can be analyzed and/or a bacteriophage culture enrichment can be performed. For example, for a 100 mL sample volume, prewarm the sample (when a large sample volume is used it is recommended to treat the sample to remove oxygen, which can be done through the bubbling of nitrogen for 5 min at a rate of 5 L/min or through the addition of a reducing solution, such as Na_2S (final concentration 0.04% (wt/vol) or resazurin solution (0.5 mL/100 mL of a solution of 0.025 g/100 mL)) and add to a 250 mL screw capped sterile glass bottle containing 100 mL of prewarmed double-strength complete BPRMB. Add 30 mL of the host inoculum culture, fill the bottle completely with medium, tighten the cap to create anaerobic conditions, and incubate overnight at 37 °C with gentle magnetic stirring. Take 1 mL of the enrichment culture and add 0.4 mL of chloroform, mix well and centrifuge at 3000 × *g* for 5 min. Use a drop of the supernatant to perform the absence/presence test.
12. The drop can result in a clearance zone if the bacteriophage is in a high concentration and in plaque visualization in this zone in case the sample spotted has bacteriophage in lower concentrations.
13. If a high concentration of bacteriophages in the sample is suspected, a tenfold serial dilution of the sample in Peptone saline should be performed.
14. Bacteriophages can be purified using a $CsCl_2$ gradient (optional) to get rid of cell DNA and proteins for bacteriophage genomic and proteomic studies.
15. The reference bacteriophage stock vials prepared should be assessed for intra- and inter-vial homogeneity. Intra-vial homogeneity, understood as homogeneous distribution of bacteriophages within a vial and inter-vial homogeneity, understood as homogeneous bacteriophage counts in all vials as well, are required. The first is achieved by homogenizing properly the vial before bacteriophage enumeration. The second by the performance of control charts of reference bacteriophage stocks, prepared as described elsewhere [20, 21].

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Chapter 3

Isolation of Bacteriophages for Clinically Relevant Bacteria

Sanna Sillankorva

Abstract

A number of bacteriophages deposited in different culture collections target clinically relevant bacterial hosts. In this chapter, we describe a method for isolating bacteriophage plaques for the most common bacteria involved in nosocomial infections.

Key words Clinically relevant bacteria, Bacteriophage isolation, Purification

1 Introduction

There are several lists of clinically important bacteria providing a description of bacteria according to their: family, morphology, oxygen preference, catalase and oxidase reaction, among other characteristics [1–4]. Many of the clinically relevant bacteria are fastidious and anaerobic and bacteriophage isolation for these bacteria is already detailed in Chapters 1 and 2 of this book. Nonetheless, many microorganisms recovered from patients do not have particular requirements for their growth, such as the ESKAPE bacteria: *Enterococcus faecium* (E), *Staphylococcus aureus* (S), *Klebsiella pneumoniae* (K), *Acinetobacter baumannii* (A), *Pseudomonas aeruginosa* (P), and Enterobacter species (E) [5]. These pathogens present a high rate of antibiotic resistance and are responsible for a substantial percentage of nosocomial infections in intensive care units [6–8]. Besides their incidence in infections, these pathogens are also widely known for their multiple-drug resistance rendering entire classes of antibiotics redundant. *E. faecium* and *S. aureus* are Gram-positive bacteria and strains of both species are frequently resistant to vancomycin [9]. *S. aureus* strains resistant to methicillin (MRSA) are a major problem in clinical medicine [10, 11]. The other four ESKAPE pathogens are Gram-negative bacteria. *Klebsiella pneumoniae* strains are intrinsically resistant to penicillins and can acquire resistance to third- and fourth-generation cephalosporins [12]. *A. baumannii* strains show an increase in carbapenem

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resistance, decreasing treatment options to drugs of last resort such as colistin. *P. aeruginosa* strains show widespread resistance to many common first-line antibiotics, and therefore carbapenems, polymyxins, and tigecycline are the drugs of choice; however, resistance to these drugs has also been reported [13, 14]. Enterobacter species are intrinsically resistant to ampicillin, amoxicillin, amoxicillin-clavulanate, first- to fourth-generation cephalosporins, and cefoxitin, and ureido- and carboxypenicillins [15, 16]. This intrinsic resistance to antibiotics is the main reason that has prompted scientists' interest in isolating bacteriophages for these pathogens.

2 Materials

2.1 Enrichment of the Bacteriophage Isolation Source

1. Clinically relevant strains: Overnight grown bacteria in 100 mL Erlenmeyers containing 25 mL of sterile LB prepared as follows: Add 20.0 g of commercially available LB and dissolve in 800 mL of deionized water. Autoclave at 121 °C during 15 min.
2. Bacteriophage isolation source sample: liquid or solid sample that possibly contains bacteriophages.
3. Lysogeny broth (LB): weigh 25.0 g and place in a 1 L bottle and add 1 L of deionized water. Autoclave at 121 °C for 15 min (*see* **Note 1**).
4. Sterile double-strength LB media (2× LB): weigh 40.0 g of LB and 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 and place in a 1 L bottle and add 1 L of deionized water. Autoclave at 121 °C for 15 min.
6. Sterile 500 mL, 250 mL bottles.
7. Sterile 500 mL, and 100 mL Erlenmeyers.
8. Sterile 50 mL Falcon tubes.
9. Filters: 0.2 μm and 0.45 μm (Whatman™, USA).

2.2 Checking for the Presence of Bacteriophages in 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 Erlenmeyers containing 25 mL of sterile LB.
3. Sterile LB Top-Agar (TA): Weigh 17.5 g of LB, 3.0 g of agar, and pour in a 500 mL bottle. Adjust to 500 mL with deionized water (*see* **Note 2**). Autoclave at 121 °C for 15 min and store accordingly (*see* **Note 3**).

2.3 Bacteriophage Plaque Isolation

1. Bacterial lawn prepared as follows: Add to a LB agar plate, 100 mL of overnight grown bacteria in 100 mL Erlenmeyers containing 25 mL of sterile LB, and 3 mL of sterile TA (47 °C).
2. Sterile swabs or sterile paper strips (1 cm × 5 cm, paper sheet (80 g/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 Falcon tubes.
2. Centrifuge (9000 × *g*, 10 min, 4 °C) and recover the supernatant.
3. Filter (0.45 μm) the supernatant to 500 mL sterile Erlenmeyers (*see* **Note 4**).
4. Add 50 μL of overnight grown bacterial suspension, 100 mL of 2× LB, and 100 mL of filtered supernatant to a 500 mL Erlenmeyer (*see* **Notes 5** and **6**).
5. Incubate at 37 °C during 24 h, under agitation (120–200 rpm).
6. Pour the enriched sample onto 50 mL Falcon tubes.
7. Centrifuge (9000 × *g*, 4 °C for 10 min).
8. Collect and filter (syringe filter 0.2 μm) the supernatant to sterile 100 mL bottles.

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 7**).
3. After incubation, pour onto 50 mL Falcon tubes and centrifuge (9000 × *g*, 10 min, 4 °C).
4. Collect and filter (0.45 μm) the supernatant to 500 mL sterile Erlenmeyers.
5. After filtering the supernatant the procedure follows the protocol described above (Subheading 3.1.1, **step 4**).

3.2 Checking for the Presence of Bacteriophages in Samples

3.2.1 Spot Test Verification of the Enriched Samples

1. Prepare bacterial lawns of each strains by pouring to an LB agar plate: 100 μL of overnight culture and 3 mL of TA.
2. Let the overlay agar layer solidify.
3. Add 1–4 drop(s) of 10–20 μL of the 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 8**).

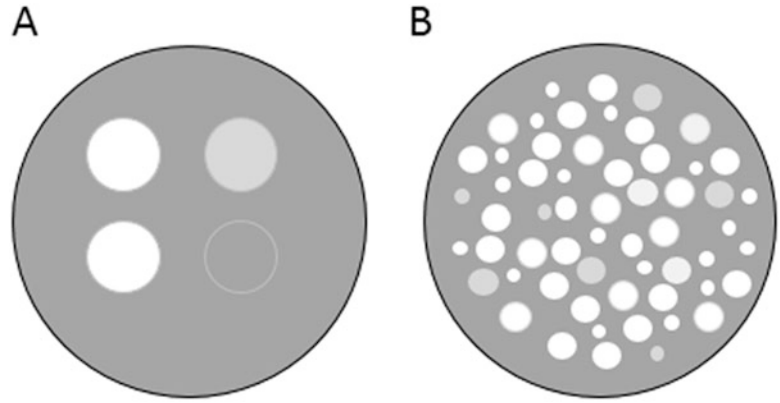

Fig. 1 Verification of bacteriophage presence using (**a**) spot test on a bacterial lawn, and (**b**) plating a diluted enriched sample using the agar-overlay method described by Sambrook and Russell [17]

5. Incubate the plate overnight at 37 °C.
6. Check for clear and turbid lysis zones indicative of the presence of bacteriophages (Fig. 1a).

3.2.2 Enriched Sample Dilution and Plating

1. Prepare different bacterial lawns on LB agar plates containing: overnight culture of one strain (100 μL), 1:10,000 diluted enriched sample possibly containing phages (100 μL), and 3 mL of TA (*see* **Note 9**).
2. Immediately after adding TA, swirl the LB agar plate so that the mixture is evenly distributed in the whole.
3. Let the overlay agar layer solidify.
4. Incubate the plate overnight at 37 °C.
5. Check for clear and turbid lysis zones indicative of the presence of bacteriophages (Fig. 1b).

3.3 Bacteriophage Plaque Isolation

An enriched sample can contain more than one bacteriophage for a specific host. Presence of more than one bacteriophage is done by visual inspection of the bacteriophage plaques on the bacterial lawns. If different size plaques, plaques containing halo, or plaques with differences in turbidity are present (in the plates of Subheading 3.2), each needs to be isolated following the procedure detailed below.

1. Wet the tip of a sterile swab or a sterile paper strip in the bacteriophage suspension obtained in the final steps of Subheadings 3.1.1 and 3.1.2.
2. Streak on a Petri dish containing a bacterial lawn of the strain used in the enrichment step with the swab as you would do when streaking a bacterial colony (Fig. 2a) or downward in case you use paper strips (Fig. 2b).

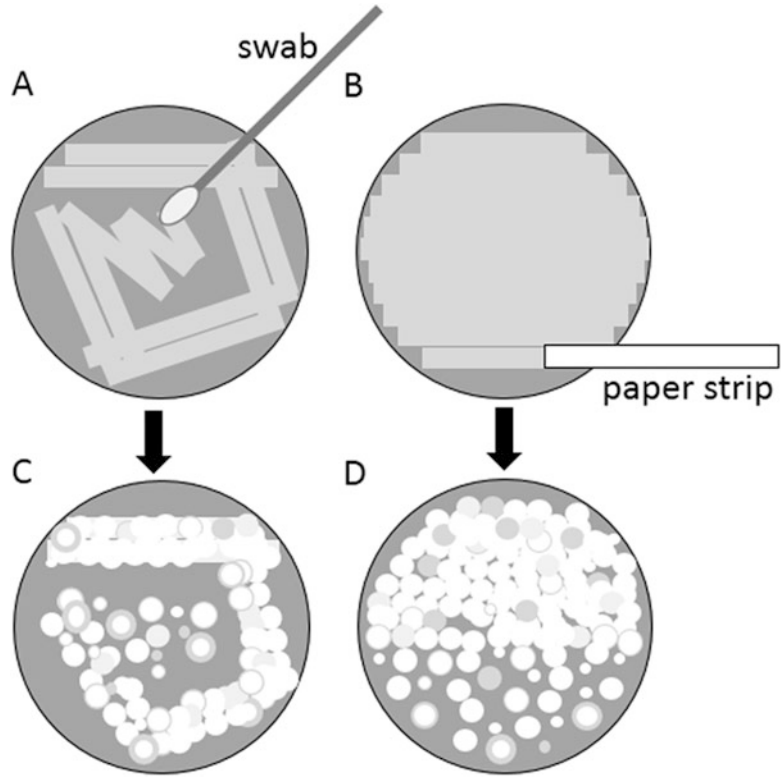

Fig. 2 Spreading an enriched bacteriophage sample on a LB plate with a bacterial lawn. (**a**) Using a sterile swab, (**b**) a sterile paper strip, (**c**) and (**d**) are the respective results after overnight incubation

3. Make sure you change swabs or paper strips to transfer always less and less concentrated bacteriophage so that, in the end, you will have isolated plaques.
4. Incubate the plate overnight at 37 °C.
5. Analyze the bacteriophage plaque morphologies to check for differences (Fig. 2c, d).
6. Pick a single bacteriophage plaque using a toothpick and stick the toothpick several times (in a line), in an agar plate with a bacterial lawn prepared of the specific host.
7. Use sterile swabs to streak the bacteriophages as described right above.
8. Incubate the plates overnight at 37 °C.
9. Repeat **steps 6–8** until all bacteriophage plaques are uniform (*see* **Note 10**).

4 Notes

1. ESKAPE bacteria grow well in LB; however, other media can be used instead. For instance, Tryticase soy broth can be used to grow *Enterococcus faecium* and *Staphylococcus aureus*.
2. For phages presenting very small plaques it is frequently useful to add less agar in order to have lower than 0.6% (wt/vol) agar in the top-agar so that the phages can diffuse better and give rise to plaques with slightly larger diameter.
3. If you intend to use the TA after preparation let it cool down to 47 °C. If you prepare in advance, you can let TA solidify and then use microwave or a water bath to melt the TA.
4. **Steps 2** and **3** are used to discard the vast majority of particles that are usually recovered when collecting raw sewage.
5. The purpose of using 2× strength LB media is to provide the ideal concentration for bacterial growth.
6. Use an Erlenmeyer for each of the clinically relevant bacteria even though the isolation source is the same. Furthermore, lysogeny experiments should be performed prior to mixing any two given bacteria to avoid false-positive results due to the presence of lysogenic strains mixed with nonlysogenic cells. To detect lysogeny, each bacterium, of a specific species, needs to be tested against each other. If a lysogenic strain is present, plaques will be visible due to spontaneous lysis of a small number of lysogenic cells.
7. Ideally, the sample containing solid isolation source should left for longer time (at least 12 h) in contact with any liquid solution so that all phages can elute from the solid sample to the liquid phase.
8. If you use a single LB agar plate for only one possible source of bacteriophage isolation, it is not so important that the drops have completely dried out before incubating the plate. However, if you apply several drops with enriched samples with different source, it is recommended to allow drops to dry completely to avoid that the liquid run to other drops which can potentially alter the result.
9. If using 1:10,000 dilution there is still not a good separation of bacteriophage plaques, it is highly recommended to dilute even more your enriched sample. If the contrary occurs, no visible plaques, use less diluted enriched sample.
10. You can remove with a cut micropipette tip a few bacteriophage plaques together with the agar, and insert this in a 2 mL tube. Store at +4 °C until you need to start producing this phage. Alternatively, add 1 mL of SM buffer to the 2 mL tube, allow

phage elution to the buffer and remove the agar particles. You can filter (0.2 μm to remove any possible bacteria that might been collected together with the agar.

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Part II

Characterization of Bacteriophages

Chapter 4

In Vitro Activity of Bacteriophages Against Planktonic and Biofilm Populations Assessed by Flow Cytometry

Diana P. Pires and Luís D.R. Melo

Abstract

The in vitro activity of bacteriophages against planktonic cultures and biofilms is commonly evaluated by culture methods. However, these methods can lead to an underestimation of total bacterial cells when they undergo different physiological states.

This chapter describes the methodology used to assess the in vitro activity of bacteriophages against planktonic cultures of bacteria in different metabolic states and biofilm populations by flow cytometry.

Key words Phage infection, Planktonic cultures, Biofilm, Flow cytometry

1 Introduction

Biofilms can be briefly defined as communities of microorganisms attached to surfaces and surrounded by a self-produced exopolymeric matrix. It has been described that the majority of the human bacterial infections are biofilm-related [1]. Biofilm formation constitutes one of the major hurdles on the treatment of nosocomial infections, as the antibiotic concentration required to eradicate biofilms is usually difficult to obtain in vivo [2]. Therefore, after the establishment of a bacterial biofilm infection, it becomes very difficult to treat using antibiotic therapy.

Comparatively to planktonic cultures, biofilms are composed of bacterial cells that are in a wide range of physiological states [3]. Although some cells are active, most of the biofilm cells display a genome-wide adaptation to that lifestyle, including downregulation of basic cell processes [4]. These cells with low metabolic rates, termed dormant cells, are defined as bacteria that survive for long periods of time without dividing and might not form CFUs on regular culture media [5–7]. Consequently, antibiotic therapy designed to target cells under replication is not effective against biofilms [8, 9].

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Bacteriophages are known to control exponentially growing planktonic cells and even biofilms. However, limited studies have evaluated the interaction of bacteriophages and cells in stationary-phase [10–12]. One of the issues associated with the study of mature biofilms is their increased proportion of dormant bacteria [13], some of which are viable but nonculturable, leading to an underestimated number of total viable bacteria when a CFU analysis is performed [6, 14].

Flow cytometry is a technique initially developed for studying mammalian cells and later adapted for use with bacteria [15]. This technique allows rapid, accurate and highly reproducible analysis of individual cells within a population [15]. It has been routinely used for monitoring the in vitro activity of antimicrobial compounds by assessing the cell number and viability [15, 16]. Besides cell viability, flow cytometry further allows to monitor active but nonculturable bacterial cells as well as to study parameters such as size and the physiological diversity of populations [15, 17–19].

Herein we describe the methodology to analyze the bacterial response to bacteriophage, assessing cell viability of bacteria under different metabolic states by flow cytometry.

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 as well, according to the requirements of the host bacterium.

2.1 Bacteriophage Infection of Planktonic Cultures

1. Purified bacteriophage suspension (*see* **Note 1**).
2. LB broth prepared according to the manufacturer's instructions (*see* **Note 2**).
3. Bacterial culture: Place one colony of the bacterial host into a glass flask with 25 mL of LB and incubate at appropriate temperature for 16 hours at 120–150 rpm (*see* **Note 3**).
4. Stationary phase culture: Dilute the overnight grown culture 1:100 (vol/vol) with LB to a final volume of 50 mL (*see* **Note 4**) and incubate at appropriate temperature with agitation (120–150 rpm) for 48 h (*see* **Note 5**).
5. LB deprived of nutrients (LB_{N-}). Centrifuge the stationary phase culture in sterile 50 mL centrifuge tubes, collect and filter (0.22 μm) the supernatant (*see* **Note 6**).
6. Saline Magnesium buffer (SM buffer): 100 mM NaCl, 8 mM $MgSO_4 \cdot 7H_2O$, and 50 mM Tris–HCl, pH 7.5.

7. Sterile 100 mL glass flasks.
8. Sterile 50 mL tubes.
9. Syringes.
10. Syringe filters (0.22 μm).

2.2 Biofilm Formation and Bacteriophage Infection of Biofilms

1. Purified bacteriophage suspension.
2. Bacterial culture: *see* Subheading 2.1, **item 3** for preparation.
3. LB broth.
4. SM Buffer.
5. 96-well microtiter plates (*see* **Note 7**).
6. Wash solution: NaCl 0.9% (wt/vol) (*see* **Note 8**).
7. Sterile 1.5 mL tubes.
8. Sterile 50 mL flasks.
9. Ultrasonic bath.

2.3 Flow Cytometry Analysis

1. Purified bacteriophage suspension.
2. Bacterial cells from biofilms and planktonic cultures.
3. Phosphate-buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4, pH 7.4.
4. Vortex.
5. SYBR Green (SYBR): 1:40,000 of SYBR Green I (Invitrogen, Carlsbad, CA, USA).
6. Propidium Iodide (PI): 20 μg/mL propidium iodide (Sigma-Aldrich, St. Louis, MO, USA) (*see* **Note 9**).
7. Sterile 3 mL PP tubes: 75 × 10 mm.
8. Flow cytometer.

3 Methods

Carry out all procedures at room temperature unless specified otherwise.

3.1 Bacteriophage Infection of Planktonic Cultures

3.1.1 Bacteriophage Activity Against Exponentially Growing Cells

1. Dilute 1:100 (vol/vol) the bacterial culture grown for 16 h with LB to a final volume of 50 mL and incubate in a 100 mL flask at appropriate temperature with agitation (120–150 rpm) until an OD_{600nm} of approximately 0.4–0.5.
2. Add the bacteriophage suspension to the bacterial culture in order to obtain the multiplicity of infection (MOI) required. In control experiments, use SM Buffer instead of bacteriophage suspension.

3. Incubate the suspension at appropriate temperature with agitation (120–150 rpm) and take samples at different time points for flow cytometry analysis.

3.1.2 Bacteriophage Activity Against Stationary Phase Cells

Adopt exactly the same protocol described in Subheading 3.1.1; however, replace **step 1** (exponentially growing cells) with the following step:

1. Adjust the OD_{600nm} of the stationary phase culture to 0.4–0.5 using LB_{N-}.

3.2 Bacteriophage Infection of Biofilms

1. Dilute overnight grown bacterial culture 1:100 (vol/vol) with LB.
2. Add 200 μL of the bacterial suspension to each well of a 96-well microtiter plate.
3. Incubate the microtiter plates at appropriate temperature under agitation (120–150 rpm) for the appropriate time.
4. After incubation, discard all media and wash the wells with 200 μL of wash solution to remove planktonic bacteria.
5. Add 200 μL of LB with the bacteriophage suspension to each well in order to obtain the MOI required. In control experiments use SM Buffer instead of bacteriophage suspension.
6. Incubate the microtiter plates at appropriate temperature under agitation (120–150 rpm) for the appropriate time.
7. Prepare biofilm samples for flow cytometry according to the following steps:
   (a) Discard all the medium from the wells and wash the wells with 200 μL of wash solution to remove planktonic bacteria and bacteriophages.
   (b) Add 200 μL of wash solution to each well and sonicate using an ultrasonic bath (*see* **Note 10**).
   (c) Homogenize the suspension in each well by pipetting and transfer to sterile 1.5 mL tubes.

3.3 Flow Cytometry Analysis

1. Open a new protocol in the flow cytometer software.
2. Set the plots listed below, on logarithmic scale, for bacteria visualization:
   (a) Forward Scatter (FSC) vs. Side Scatter (SSC)—relative size vs. granularity.
   (b) SYBR vs. PI.
3. Set the volume of sample to be analyzed to ¼ of the total sample volume (e.g. 50 μL of a 200 μL sample).
4. Set the flow rate in order of this to be low (e.g. 10 μL/min as used in DNA analysis).

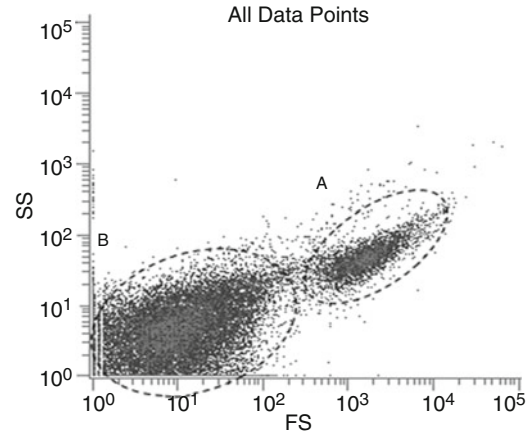

Fig. 1 Representative FSC vs SSC dot plot with the bacterial population on-scale, with region A set around the target bacterial population and region B around the background

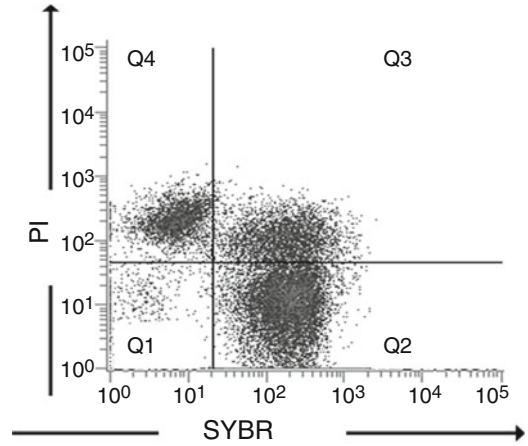

Fig. 2 Representative SYBR vs PI dot plot of double stained bacteria. Q1 – SYBR-/PI- (cellular debris); Q2 – SYBR+/PI- (live cells); Q3 – SYBR+/PI+ (compromised cells) and Q4 – SYBR-/PI+ (dead cells)

5. Acquire 200 µL of PBS suspension to define the background in the FSC vs. SSC dot plot.
6. Add 20 µL of planktonic suspension (1:10 diluted), into a PP tube with 180 µL of PBS and acquire on the flow cytometer (*see* **Note 11**).
7. Gate all dot plots in the bacterial population (*see* Fig. 1).
8. Adjust adequately the voltage and the gains so the unstained bacteria are on the Q1 region (*see* Fig. 2).
9. Add 20 µL of planktonic suspension (1:10 diluted), into a PP tube with 180 µL of a solution containing 1:40,000 of SYBR (*see* **Note 12**).

10. In a new PP tube add 20 μL of planktonic suspension (1:10 diluted) and 180 μL of a solution containing 20 μg/mL of PI (*see* **Note 12**).
11. Vortex and incubate all samples for 5–20 min at room temperature and protected from the light (*see* **Note 13**).
12. Acquire single-stained samples and set the compensations if necessary.
13. Add 20 μL of each bacterial suspension (1:10 diluted) into a PP tube with 180 μL of a solution containing 1:40,000 of SYBR and 20 μg/mL of PI.
14. Vortex and incubate for 5–20 min all samples at room temperature and protected from the light.
15. Acquire double-stained samples. The compensation values optimized with unstained and single-stained cells should be adequate, but fine-tune alterations may be necessary.
16. Analyze the obtained data regarding:
    (a) SYBR Median fluorescence intensity and if an increase in intensity is observed this is indicative of increased metabolic state.
    (b) Cell counts/μL (*see* **Note 14**).
    (c) Number of intact, compromised, and dead cells (*see* Fig. 2).

4 Notes

1. To purify the bacteriophages use the method described by Sambrook and Russell [20].
2. 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.
3. The procedure is described for fast growing bacteria. However, if experiments are performed with slow growing bacteria, the incubation time needs to be adjusted.
4. Alternatively to LB, minimal media can also be used when performing experiments with stationary-phase cells [10].
5. Some bacterial species need a longer incubation period to enter in stationary phase. Bacterial growth kinetics should be performed prior to these experiments.
6. This medium corresponds to the culture medium deprived of nutrients, so the cells will not reactivate as would possibly happen using fresh nutrients.

7. 96-well polystyrene plates are commonly used for high-throughput biofilm formation experiments. Alternatively, 6, 12, 24 or 48-well polystyrene plates can be used. The biofilm formation can occur in the microplate or in coupons (i.e. acrylic, silicone, etc.) that mimic better the real surface where biofilms are formed. Biofilms can also be formed in MBEC™ assay plates in which biofilms are formed on the 96 pegs of the plastic lid.
8. The wash solution can also be LB medium or PBS.
9. Bacterial cells stained with dual-color LIVE/DEAD® can be ran in a flow cytometer for analysis of their viability. This dual-stain system reports on cell viability via membrane integrity, and can been used to measure the viability of bacteria growing in biofilm communities. The LIVE/DEAD® kit makes use of the different permeability of the green SYTO9 and red propidium iodide (PI) dyes [21]. While SYTO9 penetrates the membrane of all cells and bind their DNA, PI can only penetrate damaged membranes [21]. Since PI exhibits a stronger affinity for nucleic acids, SYTO9 is displaced by PI and consequently, live cells will be stained with green and dead cells with a red fluorescence [21, 22]. In alternative to SYTO, SYBR green can also been used as a component of the LIVE/DEAD staining to assess the cell viability by flow cytometry [21, 23]. Furthermore, it was reported that this fluorochrome can be used to assess the physiological state of bacterial cells [24].
10. Prior to these experiments, the sonication conditions (time and power) should be optimized. There are some ultrasonic baths on the market that heat beyond the set temperature and cause an undesirable heating of the sample therefore this should be taken into account in the selection process before acquiring this equipment.
11. Using unstained bacteria will define the bacterial population on FSC vs. SSC dot plot drawing a gate around bacterial cells. This gating will allow to eliminate electronic background and/or debris interference. Individual FSC and SSC histograms should be analyzed to guarantee that the bell-shaped 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.
12. When the emission spectra of different fluorochromes overlap, the fluorescence of derived from more than one fluorochrome may be detected. To correct for 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 that is being measured (e.g. SYBR green-stained bacteria should be FL-1 (SYBR) positive/FL-4 (PI) negative and PI-stained bacteria FL-1 (SYBR) negative/FL-4 (PI) positive).

13. The time of incubation vary with bacterial morphology and sample matrix (e.g. 5–10 min for *Staphylococcus epidermidis*; 20 min for *Pseudomonas aeruginosa*).
14. Most flow cytometers cannot 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 5

Observation of Bacteriophage Ultrastructure by Cryo-electron Microscopy

Ana Cuervo 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 has been mostly done by electron microscopy. Although embedding in metal salts has been a routine method for virus observation for many years, preservation of bacteriophages in a thin layer of fast frozen buffer has proven to be a 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 to achieve 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 procedures help to render near-atomic resolution structures.

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 design and implementation of the first electron microscope, one of the first samples to be visualized was tobacco mosaic virus [1]. Since then, electron microscopy became a main technique to detect, classify, and describe the morphology of many different viruses (reviewed in [2]). Besides these applications, structure determination of viruses using TEM started in the 60's, after the pioneering work by Klug and Finch using negatively stained virus images [3], soon followed by the first attempts to produce three-dimensional reconstructions of viral structures by electron microscopy [4].

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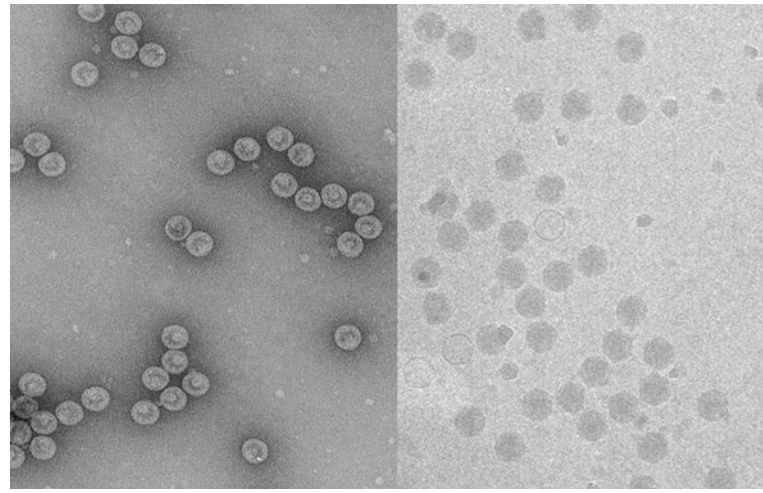

Fig. 1 *Left*, negative-stained T7 bacteriophage. *Right*, a cryo-EM image of T7 bacteriophage, where full and empty viruses can be appreciated

The incorporation of cryoprotection in sample preparation for TEM [5] was a fundamental step for extending the use of TEM towards high-resolution structural determination of viruses, allowing data acquisition at 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 ($\epsilon 15$) was obtained soon after [7].

The two basic procedures for observation of bacteriophage ultrastructure are negative staining and cryo-electron microscopy (cyo-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 resist electron interaction without much radiation damage and renders projection images which 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 to get 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, introduced into the microscope using specialized sample

holders which maintain the sample at 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 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 CCDs were traditionally used for data acquisition, the incorporation of direct electron detectors (DDs) has improved greatly the quality, sensibility and contrast of the images. Furthermore, DDs offer the possibility of reducing the limitations derived from sample movement and distortion during acquisition by using fast frame acquisition. 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. In this context, hybrid methods, including data from other sources (mainly structures of certain viral components solved by x-ray crystallography) 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 (cupper, 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 CyoEM are made of Holey Carbon Film (like QUANTIFOIL®), which is a perforated support foil with circular and square holes (Fig. 2b).
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.

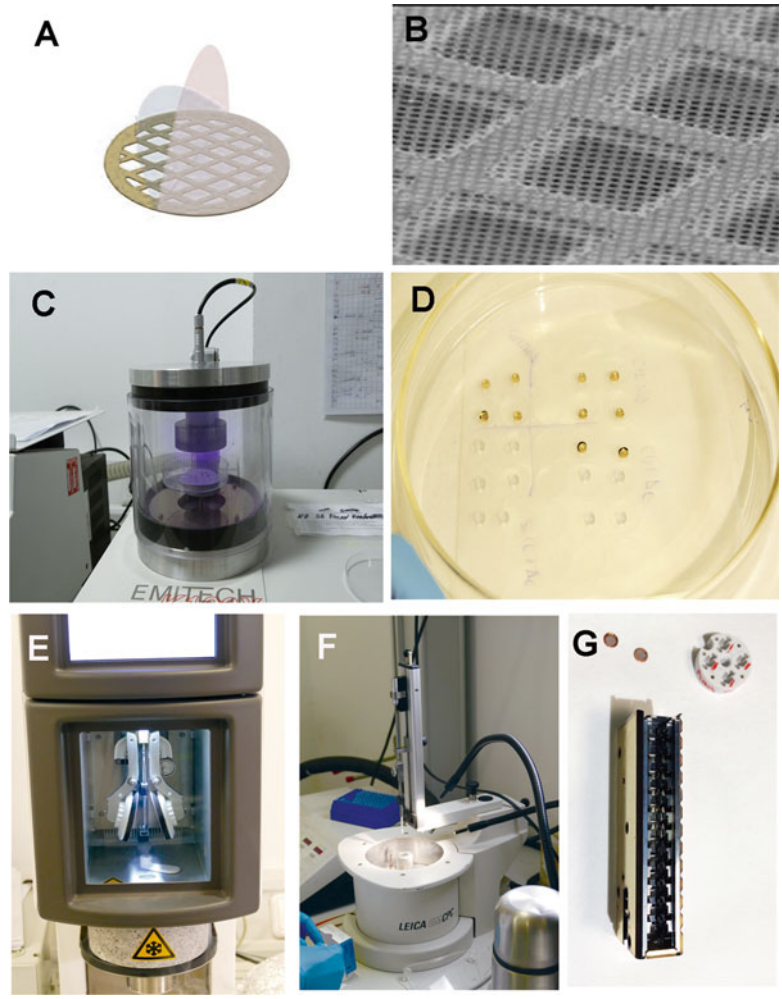

Fig. 2 Gallery of images showing different steps of grid preparation process. (**a**) Cupper grid covered with plastic and carbon. (**b**) Image showing a QUANTIFOIL® grid, where squares and holes can be appreciated. (**c**) Emitech Glow discharge machine. (**d**) Glass plate showing grid incubation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

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, 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 Whatmann).
4. Forceps.
5. Cryo-transfer holders (see **Note 1**).

2.3 Data Acquisition

1. Most modern microscopes acquire data by fiber-optically coupled CCD, as the Gatan Orius series.
2. Recent advances in CMOS detectors, as the Gatan One View, TVIPS TemCam XF-series, and FEI ceta 16 M, offer 4k × 4k images well suited for image processing.
3. Since 2012, a new type of electron direct detector is used. Examples of these new detectors are Direct EL DE16 and FEI Falcon II (4k × 4k 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 provide 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 (Subheading 2.2).
2. Then, a small volume (5–10 μL) of the sample are adhered to the carbon surface of the grid and let incubate 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 into 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 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 of 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 near by the actual site to collect the image frames.
4. Electron beam is moved towards the acquisition area and the data is taken (in about 1 or 2 s of exposition) using either CCDs or 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. For a general type of particles, without assuming any symmetry, the selected particle set is processed using RELION [9], EMAN [10], FREALIGN [11] or Xmipp [12] (Fig. 3a).
2. For icosahedral particles specific processing packages such as Auto3DEM and AutoRTM [13] or Rico [14] might be used, (Fig. 3b) (*see* **Note 8**).
3. If the map allows it, the polypeptide chain might be built using Coot [15].

3.5 Structure Determination and the Use of Hybrid Methods

In many cases, cryo-EM maps of viruses are obtained at resolutions ranging from 12 up to 8 Å resolution (Fig. 3b) and the polypeptide chain cannot be built directly, in 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 [19] in Chimera [20] 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 [20].

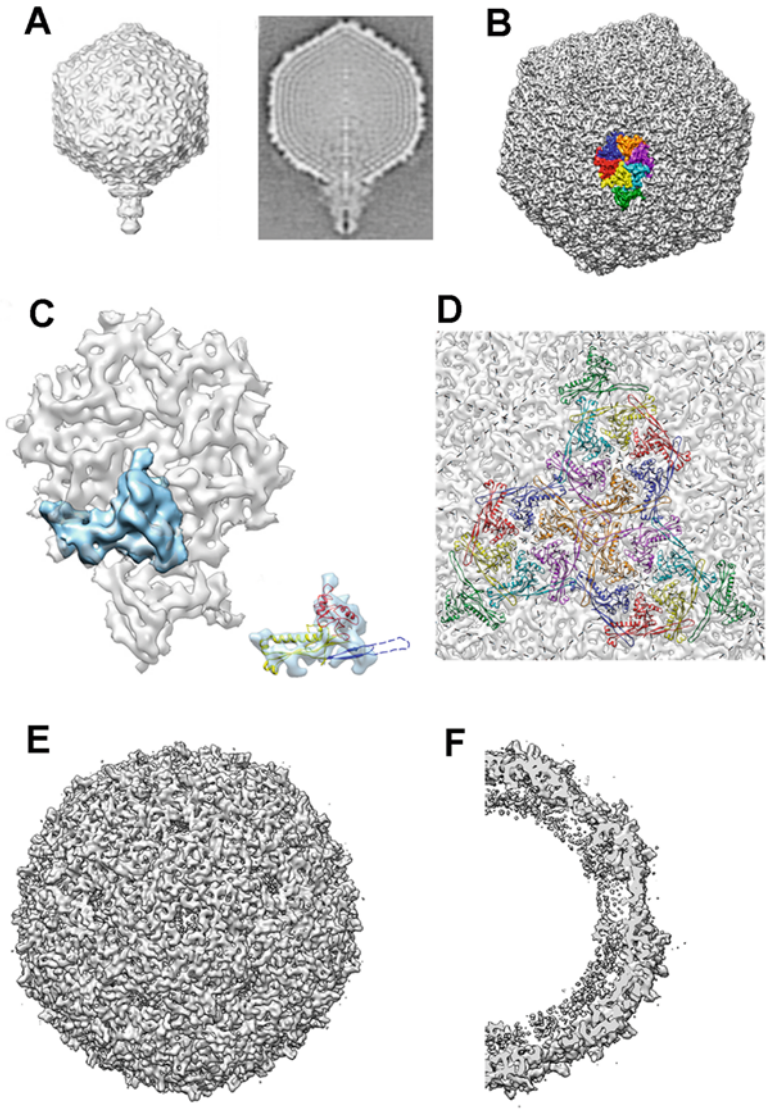

Fig. 3 (**a**) *Left*, three-dimensional reconstruction of T7 without imposing any symmetry; and right, a slide of the same volume [16]. (**b**) Icosahedral reconstruction of T7 bacteriophage at 10 Å resolution, the asymmetric subunit composed by an hexon and a subunit of the penton is colored [17]. (**c**) *Upper panel*, T7 asymmetric unit where a protein monomer colored in *blue*; *bottom panel*, docking of the atomic model inside the segmented volume of the monomer [17]. (**d**) Close view of the T7 bacteriophage capsid pseudo-atomic model [17]. (**e**) Icosahedral reconstruction of MS2 bacteriophage at ~5 Å resolution; and (**f**) section of the volume shown in **e** [18]

3. Move the atomic models as rigid bodies inside the volume to adapt to the EM volume Chimera [20] (Fig. 3c, bottom). The fitting might be refined by maximizing the cross-correlation using Chimera [20].
4. If fitting is not satisfactory use flexible fitting methods using FlexEM or Coot software [21, 15] (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, 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. Recent use of robot sample holders in 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 be then 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 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 on 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 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. 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 to 1 mg/mL are good starting conditions, but every virus preparation has to be individually tested to assure 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 dilute fields make processing lengthy and impractical. In this context, the use 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 the top of it. On the other hand, carbon surfaces can produce drift and make it difficult the acquisition of high-resolution data. Recently, graphene and gold grids have 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 1–3 s of blotting time, −10 to +5 force, 4–22 °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 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, 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 achievement of high-resolution 3D structures requires collecting a big amount of images to process. Modern microscopes allow a full automation of the procedure, facilitating the collection of a thousand of images in one day. Automatic control procedures

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. Last frames presenting higher dose are useful to enhance the contrast during particle picking and are usually removed or down-weighted during movie average [28, 29]. Usually first frames are also removed as they are 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 of the data quality and in a tremendous boost in the resolution potentially attainable by cryo-EM.

7. In data processing, there is not a unique approach for 3D reconstruction of viral particles from cryo-electron microscopy 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 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 those other that consider the viral particles as single particles for processing following general procedures that, in a certain step, do apply the proper symmetries (icosahedral, 6-, 12-fold, etc.), if any. Some specific software to work applying icosahedral symmetry are packages as Auto3DEM and AutoRTM [13] or Rico [14]. General software for single particle processing are packages as RELION [9], EMAN [10], FREALIGN [11] or Xmipp [12] which have been successfully applied to build high-resolution viral capsid reconstructions imposing icosahedral symmetry. The improvement in microscopes, detectors and image processing software in the lasts years has helped to increase the number of atomic structures published (Fig. 3e, f). Image processing without imposing icosahedral symmetry (Fig. 3a), 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, nucleic acids) nor tails exhibit such symmetry (Fig. 3a, right).

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. Fibers present also the problem of the flexibility that highly complicates the alignment of the images. Both problems can be solved by carrying masked 3D refinement. In order to apply local symmetries to the different parts or to avoid aligning flexible undesirable domains, the area of interest is selected in the images by creating different masks, thus allowing to specifically align the parts of the model suitable to get high-resolution [29].
9. Hybrid methods: First procedures used the x-ray structures as solid bodies searching for best orientation to fit the EM volume [30]. 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 optimized fitting the atomic model to a density map also taking into account the stereo-chemical properties of the model by minimizing an energy function [31], or using molecular dynamics flexible fitting by incorporating the EM data as an external potential in conventional MD simulations [21, 32]. Other common procedures for flexible fitting are those included in UCSF Chimera [20] or Rosetta [33]. 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 [17, 34], DNA packaging [35, 36] and ejection [37, 38].

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Chapter 6

Bacteriophage Taxonomy: An Evolving Discipline

Igor Tolstoy, Andrew M. Kropinski, and J. Rodney Brister

Abstract

While taxonomy is an often-unappreciated branch of science it serves very important roles. Bacteriophage taxonomy has evolved from a mainly morphology-based discipline, characterized by the work of David Bradley and Hans-Wolfgang Ackermann, to the holistic approach that is taken today. The Bacterial and Archaeal Viruses Subcommittee of the International Committee on Taxonomy of Viruses (ICTV) takes a comprehensive approach to classifying prokaryote viruses measuring overall DNA and protein identity and phylogeny before making decisions about the taxonomic position of a new virus. The huge number of complete genomes being deposited with 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. Not surprising the art and science of grouping things is particularly important in biology, as it provides a basis for identification and inference. In this context, taxonomy is the process of establishing criteria for the contents of individual boxes and a framework that unites them.

The defining characteristic of 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 genus is defined as a higher-order thing that captures one or more 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 of taxonomic metrics is dependent on the nature of relationships under scrutiny and the availability of data used in evaluations.

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Setting criteria for these taxonomic definitions is really where art meets science. Taxonomic hierarchies often attempt to address longer range relationships that stretch back into the 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.

2 Bacteriophage Taxonomy

2.1 Brief History of Bacteriophage Taxonomy to 2008

The formal taxonomy of the tailed bacteriophages derived from the pioneering bacteriophage 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) [1, 2]. 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 & Abraham Eisenstark of the Bacterial Virus Subcommittee in 1975. The names of the families in their present state was accepted by International Committee on Taxonomy of Viruses (ICTV) in 1981 (as *Myoviridae* and *Podoviridae*; http://www.ictvonline.org/virusTaxonomy.asp?msl_id=7) and in 1984 (*Siphoviridae*; http://www.ictvonline.org/virusTaxonomy.asp?msl_id=9). In 1998, Ackermann proposed an order, the Caudovirales [3], to encompass the tailed bacteriophages, which was approved by a postal vote that year. Therefore the classification system at the family level has been in its present state for over 30 years; and has proved invaluable in the classification of bacterial viruses.

The advent of the 'omics era coupled with renewed interest in bacterial viruses has had a profound effect on bacteriophage classification. The seminal paper of bacteriophage evolution was published in 1999 by Roger W. Hendrix and colleagues [4]. In their paper subtitled "All the world's a phage" the authors argue cogently that "all dsDNA bacteriophage 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 bacteriophage taxonomy, since rampant recombination would blur and taxa boundaries.

The next major advance in bacteriophage grouping occurred with the highly controversial "Phage Proteomic Tree" in which Rohwer and Edwards employed BLASTp to compare the proteomes of 105 fully sequenced bacteriophage genomes [5]. 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 bacteriophages are lambdoid [6] but the lumping of members of the *Siphoviridae* and *Podoviridae* created an intellectual stir.

2.2 Extension of Proteomics to Bacteriophage Taxonomy from 2008

By 2008 the number of fully sequenced members of the Caudovirales had increased from 75 to 349 and with Rob Lavigne (KU Leuven, Belgium) in the chair of the Bacterial and Archaeal Viruses Subcommittee of ICTV it was time to take a new look at the classification of bacteriophages. Using two BLASTP-related tools, CoreExtractor.vbs and CoreGenes, Lavigne, and coworkers realized that the T7-like bacteriophages actually fell into three distinct clades "T7-like virus," "SP6-like virus," and "φKMV-like virus" each containing numerous species [7]. Since these three groups shared a similar genomic organization and the presence of a large a single subunit RNA polymerase, they were grouped into the first bacteriophage subfamily, *Autographivirinae*. What linked the species within a clade was that the members shared 40% of their proteins in common as shown using CoreGenes [8, 9]. This number was based upon a comparison of *Pseudomonas* phage gh-1 [10] which detailed molecular analysis revealed was closely related to *E. coli* phage T7. This approach was reiterated with the *Myoviridae* [11] and *Siphoviridae* [12]. The problems 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.

2.3 DNA Sequence Comparisons Enter the Picture

DNA–DNA sequence relatedness has always been the gold standard for the classification of bacterial strains [13] (but *see* [14], and while DNA–DNA hybridizations have been used to study bacteriophage relationships [15–17], in silico analyses of the sequence relationship between biological entities did not influence bacteriophage taxonomy until fairly recently. The most commonly used genome comparison tools are progressiveMauve [18], EMBOSS Stretcher [19, 20] and dot matrix analysis tools [21–23]. The problem with EMBOSS Stretcher is that it is inaccurate below approximately 50% sequence identity, it requires collinear genomes; and, it can only handle pairs of viruses. Other lesser used tools

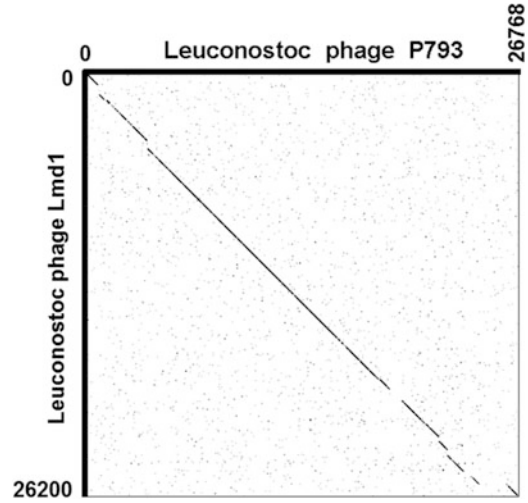

Fig. 1 Gepard dotplot comparing the similarity between the genomes of *Leuconostoc* siphoviruses Lmd1 (Kleppen et al. 2012) versus P793 (Kot et al. 2013). The ordinate and abscissa are visually enhanced in this diagram. The *dark line* represents syntenic regions

include BRIG (BLAST Ring Image Generator) [24], Easyfig [25], Circos [26], CGView [27] and CGView Comparison Tools [28]. Dotplots have been used extensively by scientists associated with the Actinobacteriophage Database projects [29–31], and an example Gepard plot ("*GE*nome *PA*ir—*R*apid *D*otter"; [23]; http://cube.univie.ac. at/gepard) comparing two *Leuconostoc* bacteriophage genomes is shown in Fig. 1. Though all of these 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; [32]; http://www.ncbi.nlm. nih.gov/sutils/pasc/), and SDT (Sequence Demarcation Tool; [33]; http://web.Cbio.uct.ac.za/~brejnev/; JSpecies; [34]; (http://imedea.uib-csic.es/jspecies/), ANI (Average Nucleotide Identity; [35]; http://enve-omics.ce.gatech.edu/ani/), GGDC (Genome-To-Genome Distance Calculator; [36]; http://ggdc.dsmz.de/) though none of the latter resources have been used for bacteriophage research.

Two groups have made extensive use of DNA sequence homology to group bacteriophages. The first of these is the Actinobacteriophage Database (http://phagesdb.org/) which includes almost 9000 bacteriophages [31]. In 2014 Grose and Casjens [37] used BLASTn, BLASTp, and quantitative DotPlot data to group 337 sequenced Enterobacterial bacteriophages into 56 clusters many of which corresponded to ICTV-ratified genera.

3 Bacteriophage Grouping into Taxa

3.1 How ICTV Currently Groups Bacteriophages into Taxa

It is imperative before continuing this discussion to distinguish between bacteriophage 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." (http://www.ictvonline.org/virusTaxInfo.asp). Following this logic, a taxa is a human construct that contains some number of physical bacteriophage isolates. Once a new bacteriophage is isolated it can be added to an existing taxa, or in cases where the new isolate is sufficiently distinct from extant isolates, a new taxa can be proposed. The tool for proposing the creation of a new viral order, family, subfamily, genus, and species as well as modifying existing taxa is known as a Taxonomy Proposal Submission Template (abbreviated as TaxoProp) which can be downloaded from the ICTV website. Anyone can fill-in and submit a TaxoProp, but it is generally a good idea to work with an appropriate member of the Bacterial and Archaeal Viruses Subcommittee (BAVS; http://www.ictvonline.org/subcommittee.asp?se=5&committee=56) who can offer advice and proofread your intended submission.

For logistical reasons BAVS is currently only producing TaxoProps where the complete sequences of two or more related bacteriophages have been deposited in one of the public databases belonging to the International Nucleotide Sequence Database Consortium (INSDC [38]), which include GenBank, DNA Data Bank of Japan or European Nucleotide Archive.

The process of placing a new bacteriophage isolate within a taxa 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 [39]. Searches should be restricted to Organism "Viruses (taxid:10239)" to prevent identifying prophages that are part of bacterial genomes. It is also helpful to search against the reference genomic sequences (refseq_genomic) database, as new viral and bacteriophage reference genomes are created for each species [40, 41]. For simple quantitative DNA comparisons one can use EMBOSS Stretcher (see proviso above) or by multiplying the BLASTn "Query cover" by "Ident" one can get a crude estimate on the overall and nucleotide identity. For larger comparative groups we recommend Gegenees [42]. In the following figure (Fig. 2) this resource, available from http://www.gegenees.org/, which is written in Java and can be run on Linux, MacOS, or Windows platforms has been applied in quantitative analysis of a group of currently unclassified *Leuconostoc* bacteriophages. One can readily see that these fall into three

Phage Name	1-A4	Ln-9	Ln-8	phiLN25	phiLN34	phiLNTR3	phiLNTR2	phiMH1	P793	phiLN12	phiLN03	phiLN04	phiLN6B	Lmd1
1-A4	100.0	55.2	54.6	55.4	46.9	46.5	46.0	0.8	0.0	1.1	0.1	0.0	0.1	0.0
Ln-9	56.2	100.0	55.0	53.7	44.0	44.0	44.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Ln-8	56.4	53.9	100.0	88.4	47.2	48.5	48.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0
phiLN25	57.1	53.5	89.9	100.0	48.2	48.6	48.3	0.0	0.1	0.0	0.0	0.0	0.0	0.0
phiLN34	48.7	45.5	50.1	48.5	100.0	93.6	94.6	0.0	1.5	1.5	1.5	1.6	1.6	1.5
phiLNTR3	47.6	45.3	50.4	48.7	93.6	100.0	97.2	0.0	1.4	1.4	1.3	1.3	1.3	1.2
phiLNTR2	47.5	45.7	49.7	47.7	93.7	96.3	100.0	0.1	1.4	1.4	1.3	1.1	1.2	1.2
phiMH1	0.7	0.0	0.0	0.0	0.0	0.0	0.1	100.0	0.1	1.2	0.4	0.1	0.0	0.0
P793	0.0	0.0	0.0	0.1	1.6	1.6	1.6	0.1	100.0	62.6	64.5	59.7	55.6	56.3
phiLN12	1.2	0.0	0.0	0.0	1.3	1.3	1.3	1.6	60.0	100.0	86.8	73.8	65.1	63.8
phiLN03	0.1	0.0	0.0	0.0	1.5	1.5	1.5	0.5	64.1	91.5	100.0	72.5	68.9	66.5
phiLN04	0.0	0.0	0.0	0.0	1.4	1.4	1.3	0.2	61.3	80.4	75.4	100.0	75.3	75.3
phiLN6B	0.1	0.0	0.0	0.0	1.7	1.7	1.6	0.1	57.9	72.8	71.8	75.8	100.0	91.6
Lmd1	0.0	0.0	0.0	0.0	1.3	1.3	1.3	0.0	57.0	69.4	68.1	74.1	89.8	100.0

Fig. 2 Gegenees BLASTn analysis, using the "Accurate parameters" (fragment size, 200 bp; step size, 100 bp), of the genomic sequences of 14 *Leuconostoc* siphoviruses

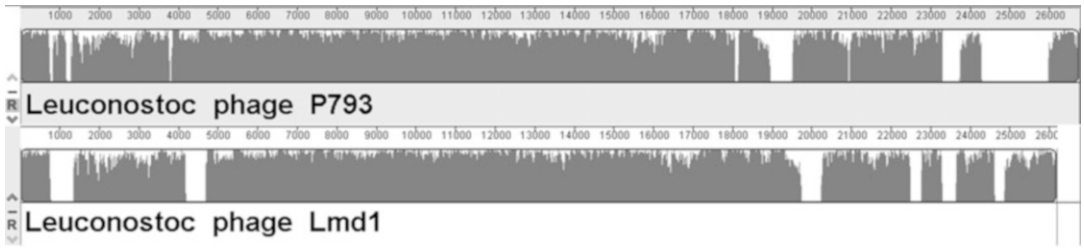

Fig. 3 progressiveMauve alignment of the genomes, in fasta format, of *Leuconostoc* bacteriophages Lmd1 and P793. The *grey*-colored *blocks* indicate the regions of 1 to 1 best alignment with rearrangement breakpoints in a different random color. The *white* regions are significantly different. The degree of sequence similarity between regions is given by a similarity plot within the colored blocks with the height of the plot proportional to the average nucleotide identity (Aaron Darling, personal communication)

genomogroups (boxed in Fig. 2), represented by bacteriophages 1-A4, phiMH1 and P793. If taxa were established from these, they would be tentatively called "Una4virus," "Mh1virus," and "P793virus" after the first genome to be deposited in a public database. Please note that names of taxa cannot begin with a numeral or Phi [43] nor can it include a hyphen.

We now employ a more holistic approach to classifying bacteriophages relying on common morphology, genomic identity, and synteny (*see* Fig. 3), along with a common proteome and phylogeny.

When we apply CoreGenes3.5 analysis [44] to the *Leuconostoc* bacteriophage proteomes (Fig. 4) we note that while phage phiMH1 is still an outlier, the proteomes of the *Una4virus* and

Phage Name	1-A4	Ln-9	Ln-8	phiLN25	phiLN34	phiLNTR3	phiLNTR2	phiMH1	P793	phiLN12	phiLN03	phiLN04	phiLN6B	Lmd1
1-A4	100.0	87.3	80.2	77.7	84.4	84.4	83.3	8.9	64.9	59.9	59.4	59.4	59.4	58.5
Ln-9		100.0	86.1	81.4	83.7	83.7	82.6	7.8	68.4	61.1	62.8	62.8	62.8	61.9
Ln-8			100.0	95.5	90.9	90.9	89.3	5.7	68.0	63.0	64.6	64.6	64.6	63.8
phiLN25				100.0	90.2	90.2	89.2	6.0	68.5	63.4	65.1	65.1	65.1	64.2
phiLN34					100.0	100.0	98.8	6.0	71.0	63.4	65.1	65.1	65.1	64.2
phiLNTR3						100.0	98.8	6.0	71.0	63.4	65.1	65.1	65.1	64.2
phiLNTR2							100.0	7.9	70.2	62.7	64.3	64.3	64.3	64.0
phiMH1								100.0	8.4	10.0	8.3	6.7	6.7	5.7
P793									100.0	93.9	96.2	96.2	96.2	95.0
phiLN12										100.0	97.6	95.1	95.1	93.9
phiLN03											100.0	94.9	94.9	93.7
phiLN04												100.0	97.4	98.8
phiLN6B													100.0	98.8
Lmd1														100.0

Fig. 4 CoreGenes3.5 analysis, using default BLASTp threshold score of 75 (http:/binf.gmu.edu:8080/CoreGenes3.5/). The average of the reciprocal values is recorded

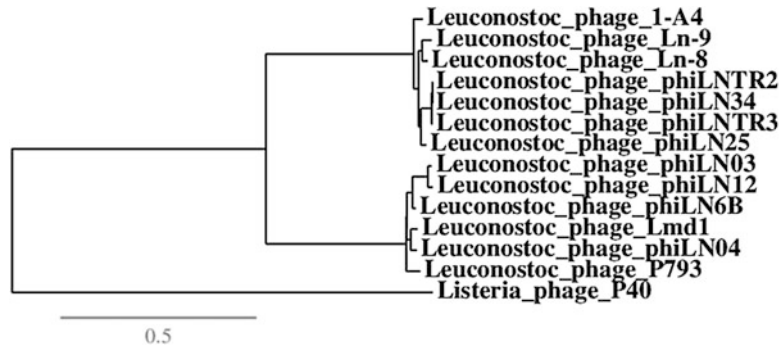

Fig. 5 DNA polymerase phylogenetic tree with the Listeria phage P70 homolog as an outlier, rather than the Leuconostoc phage phiMH1 protein, since this virus lacks a DNA polymerase constructed using "one click" at http://phylogeny. Lirmm.fr/ [3]. "The "One Click mode" targets users that do not wish to 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* (Anisimova and Gascuel 2006) for details"

the *P793virus* are related. This strongly suggests that these two genera are part of a new subfamily.

As the last step in collecting data to support new taxa are phylogenetic trees. These are usually based upon the large subunit terminase, major capsid, and major tail proteins, but in this case we have used we have used the DNA polymerases. The results (Fig. 5) are completely in accord with the total proteome and total genome analyses.

3.2 Problems with the Current Taxonomy

It has been proposed that defining characteristic of the "T4 superfamily" of bacteriophages, which infect a wide range of host bacteria in the phyla Proteobacteria and Cyanobacteria, is the presence of approximately 30 conserved proteins [45]. Currently only a fraction of these viruses have been classified into a subfamily *Tevenvirinae*. A more inclusive molecular analysis reveals that these bacteriophages display as much diversity as is seen among the viruses which make up the order *Herpesvirales* (M. Sullivan, personal communication). The fact that none of the cyanomyophages are currently classified by ICTV requires immediate attention.

Another age-old taxonomy problem is the relationship between *E. coli* phage λ and *Salmonella* phage P22, since these two temperate lambdoid phages have syntenic genomes and the same type of repressor-anti-repressor regulatory circuitry. Furthermore, at the protein level they share 21.5% homologous proteins, yet they belong to different genera in different viral families. This also needs to be resolved.

Lastly, *Escherichia* phage phiEcoM-GJ1 [46] and *Erwinia* phage vB_EamM-Y2 [47] are two members of the *Myoviridae*; while *Xanthomonas* phage Xp10 "an odd T-odd phage" [48], is a member of the *Siphoviridae* yet they possess the defining feature of the *Podoviridae* subfamily *Autographivirinae*—a single subunit RNA polymerase. Moreover, if one compares the proteomes of Xp10 and *Xanthomonas* phage Paz [49], a member of the *Phikmvvirus*, they share 14 (27.45% homologs) including DNA polymerase, primase, helicase, spannin, internal protein, and endolysin.

In each of these cases there is compelling evidence that the phages are related, and this relationship should be recognized in a logical taxonomy. In two of the three cases presented the problem is caused by a top heavy taxonomy which relies on morphology. The BAVS is currently investigating whether it is time to do away with the Caudovirales and its three families, and introduce new families and orders such as the "*Lambdaviridae*," "*Saltoviridae*" [50], and "*Tevenvirales.*"

4 The Age of Genomics and Beyond

Often the urge to classify precedes the actual isolation of viruses in numbers sufficient to delineate classification strategies. If one considers the various possibilities of genome arrangements and sequences as a universe of sorts, our knowledge of this space is akin to star gazing. From the vantage of a major city, only a few objects can be seen in the night sky, and there is little sense of either expanse or pattern. Yet, the same night sky observed far away from city lights reveals clusters of stars and patches of darkness that together create patterns from one horizon to the next.

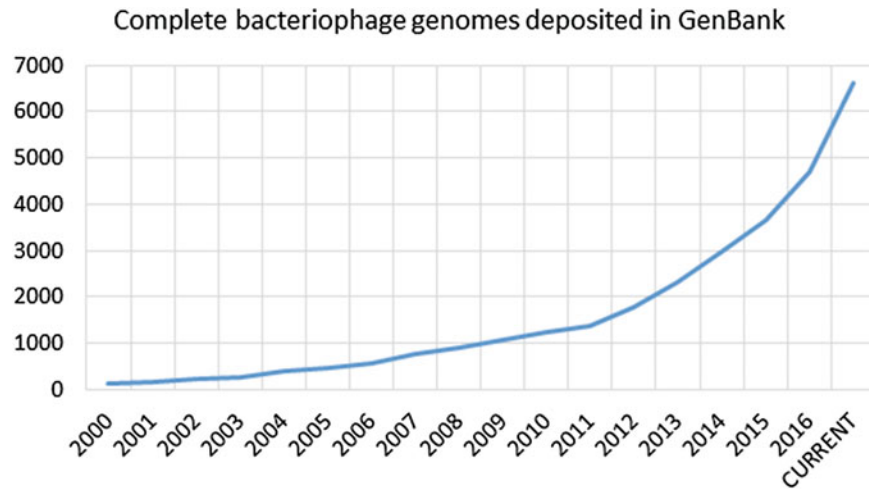

Fig. 6 Cumulative number of bacteriophage genomes marked as "complete" available from GenBank on January 1 of each indicated year

Like stargazing, it is difficult to discern relationships among genome sequences without the benefit of large numbers of representative genomes. Put simply, the would-be genome based taxonomist is dependent on extant sequence space on which to base their analysis. If the goal of taxonomy is to provide phylogenetic representations of sequence space—that is to say a taxonomic structure built upon the backbone of evolutionary inference—then there must be enough sequence representation to effectively draw models of relatedness. Devising taxonomic structures would be much simpler if one knew the entirety of extant sequence space as groupings would presumably be obvious, and the focus would change to modeling implied evolutionary relationships between groups.

While early attempts at genome based taxonomy suffered from the general lack of available genomes, the good news is that recent sequencing efforts have given rise to more than 30,000 bacteriophage nucleotide records and 6300 complete genomes (Fig. 6 [51]). Unfortunately, despite these efforts, today we still stand far from a complete representation of bacteriophage sequence space. Indeed, the extent of genomes not yet sequenced remains unclear. If prophage and other viral representations in microbial sequence sets are any indication, then there are many groups of unique viruses not yet captured as complete genome sequences in public databases [52, 53]. So when one speaks of developing approaches to bacteriophage taxonomy, they must mean just that—developing approaches based on a foggy view of extant sequence space with the expectation that the view will clear in coming years as increasing numbers of bacteriophage genomes are sequenced.

So where does that leave us? Well, all does not appear lost. Though the entirety of extant sequence space is unknown, patterns

are emerging among those sequenced genomes that are available, and there is now enough sequence data to begin framing a conceptual view of bacteriophage sequences. Among dsDNA bacteriophages there are clearly clusters of highly similar genomes. For example, there are at least 68 bacteriophage genomes that share 94% coverage with the Mycobacteriophage PG1 genome (AF547430) as determined by BLASTn analysis (J.R. Brister, personal observation). There is very little nucleotide variation within these colinear regions, and tight nucleotide sequence relationships are accompanied by extensively shared predicted protein content easily revealed by tBLASTx analysis. The small gaps in coverage seen in these analyses highlight short regions that are unique to subsets of genomes within the highly similar group, so in this cluster of genomes we can gain insight into both allelic variation within colinear genome regions and structural variation associated with a small number of variable components [54, 55].

A second set of higher-order genomic relationships is also evident among currently sequenced genomes, and beyond near identical relationships, genomes can be grouped based on clear nucleotide co-identity, restricted to interspersed, subgenomic regions. For example, Blastn analysis demonstrates a hierarchal set of Enterobacteria phage T4 clusters—one defined by near identical nucleotide sequences, a second defined by greater than 85% coverage and increased variation within shared regions, and a third defined by greater than 40% coverage and much greater variation within shared regions.

At least three groups of T4-like genomes are also seen with tBLASTx analysis (Altschul et al. 1997), roughly corresponding to the groups seen with BLASTn analysis (Fig. 7). The most similar group is characterized by 98% or greater coverage and corresponds to the nearly identical genomes seen in the BLASTn analysis. The second group shows extensive, syntenic amino acid identity to T4 interspersed with islands of unique sequence. The least similar group shows less than 80% T4 coverage and a punctate pattern of amino acid similarity, most notably around T4 nucleotide positions 30,000 where a number of replication genes are encoded and 75,000–105,000 where a number of structural genes are encoded. This group includes many genomes with fairly weak nucleotide similarity to T4, such as RB49 which shares only 20% nucleotide coverage with T4 as determined by BLASTn analysis.

Comparing the BLASTn and tBLASTx analysis of T4-like genomes, several key concepts emerge. First, there exist a group of genomes that can be defined by global nucleotide and protein similarities. Second, there exists a group that can be defined by nucleotide and protein homologies across a syntenic subset of T4 genes. This second group could be thought of as a core gene content group and will likely be an important bridge between

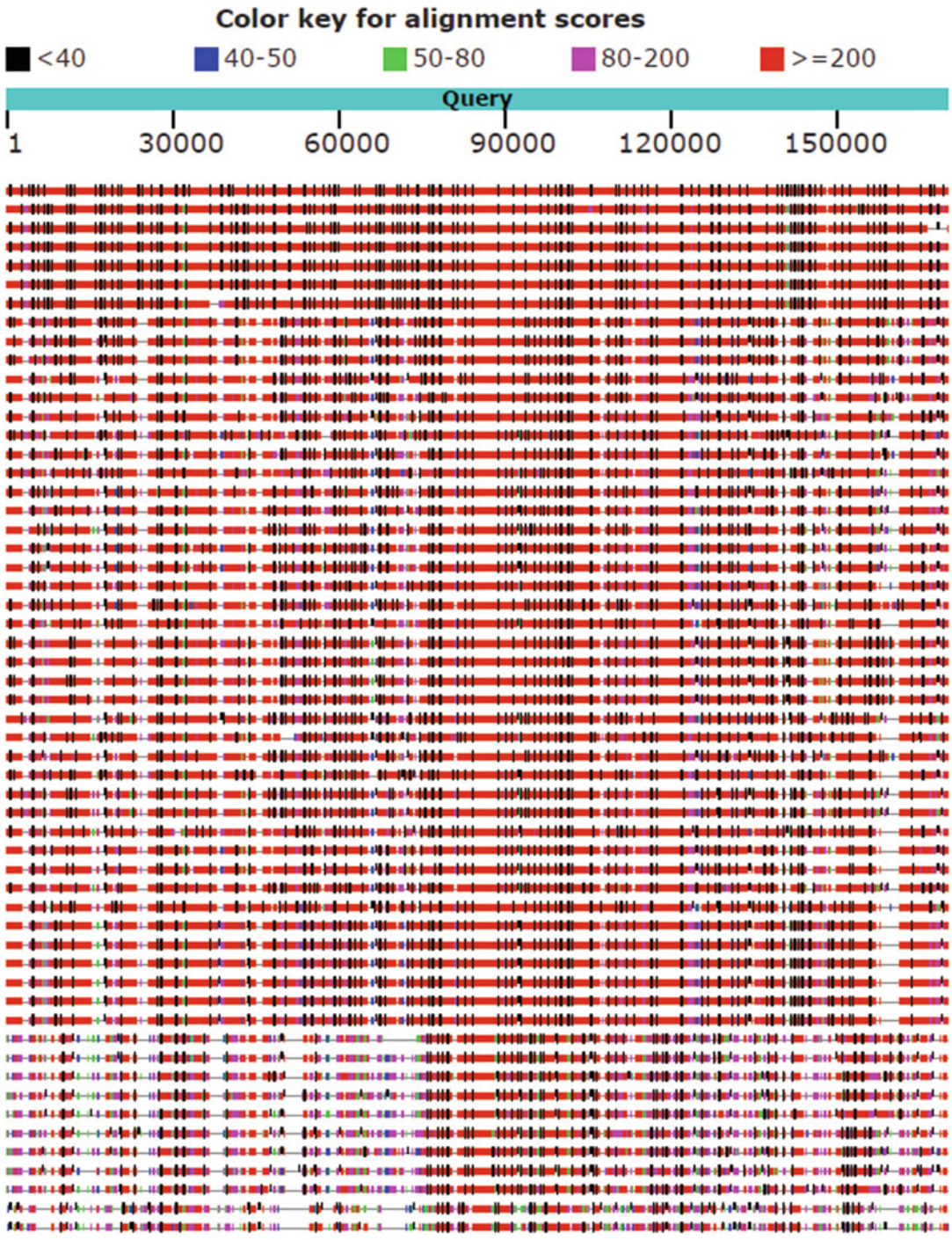

Fig. 7 Comparative Enterobacteria phage T4 (AF158101.6) tBLASTx analysis against all GenBank *Myoviridae* sequences marked as "complete" in GenBank. Graphic representation of the 100 most similar genomes are shown

nearly identical genomes and more distant evolutionary relationships defined by core protein domains or gene models [56–58].

While our current view of bacteriophage sequence space remains murky, emerging clusters of near identical genomes and gene content groups offer a peek at possible conceptual frameworks going forward. These examples portend a taxonomic approach where natural genome clusters of nearly identical genomes define the tips of a taxonomic hierarchy, syntenic gene content groupings define larger branches, and longer range conserved domains and/or gene models define the trunk. As new genomes are sequenced and new clusters emerge, the relationships within bacteriophage sequence space should become clearer.

5 Practical Considerations for Bacteriophage Scientists

Before deciding that a newly sequenced bacteriophage genome fits within an existing or new genus one should review all the evidence: host, morphotype, life style, 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 bacteriophage genome into an existing genus, there are plenty of orphan species for which homologs are subsequently isolated. Expect major changes in the classification of phages from ICTV and NCBI.

Acknowledgement

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Part III

Bacteriophage Selection and Cocktail Formulation

Chapter 7

Determination of the Bacteriophage Host Range: Culture-Based Approach

Andrey V. Letarov and Eugene E. Kulikov

Abstract

The bacteriophage host range is one of the most practically important characteristics of each bacterial virus. Here the classical plate-culture-based approach for bacteriophage host range determination is described. The important considerations related to interpretation of the data and limitations of the methods are discussed.

Key words Host range, Titer, Efficiency of plating, Resistance, Infectivity

1 Introduction

Bacteriophage host range is by definition a repertoire of bacterial species and strains that are able to support multiplication of the given virus. It is therefore impossible to determine a complete host range practically, since in no experiment can all of the existing bacterial strains be tested for susceptibility to the chosen bacteriophage. In laboratory practice, the bacteriophage's host range is normally determined against a pre-selected panel of the bacterial test cultures (for example, a set of clinical isolates of a given pathogenic species obtained in some region or during some period of time, or some other strain set made on purpose). We shall also note that assessing the bacteriophage growth directly is difficult, so the researcher generally has to resort to a number of proxies of this parameter, based mostly on the bactericidal action of the bacteriophage;

- The ability of the bacteriophage to cause clearance of growing liquid bacterial cultures.
- The ability of the bacteriophage stock to form spots of reduced or inhibited growth of the bacterial lawn on solid media.

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- The ability of the bacteriophage to form individual plaques on the bacterial lawn
- The ability of the bacteriophage to inhibit the growth of the bacterial strains plated on agar containing a high dose of the bacteriophage (bacteriophage agar).

The way of determining the bacteriophage bactericidal activity influences the determined host range quite significantly, even if the same bacterial cultures are tested against the same bacteriophages. We thus have to keep in mind some preliminary considerations.

If the bacteriophage does not clear the growing bacterial culture rapidly, this bacterial strain should not automatically be excluded from the potential bacteriophage hosts. The initial bacteriophage-induced drop in optical density of the liquid culture could be rapidly compensated for by the growth of bacteriophage-resistant mutants selected by bacteriophage action or even by re-growth of genetically bacteriophage-sensitive bacteria [1, 2] that presumably gain transitional physiological resistance. Sometimes when the culture reaches a stationary or pre-stationary growth phase it becomes temporarily immune to lysis by the specific bacteriophage [3] before visible signs of this effect appear. So, the liquid-culture-based bacteriophage assay protocols like Appelmans' titer assay [4] should be left out from the bacteriophage host range determination practice unless they are applied rationally.

Bacteriophage spot testing assays give much more information on the biology of bacteriophage–host interaction, allowing the researcher not only to observe the effects, but also to easily isolate the biological entities of interest. The signs of bacteriophage lysis such as the plaques, sterile spots or locally reduced lawn growth on solid media are generally reproducible, allow the bacteriophage titer counting in the case of plaques, and are frequently used for quick bacteriophage host range determination. The main parameter influencing this assay is the efficiency of plating (EOP) of the bacteriophage, which may differ by several orders of magnitude on different host strains [5, 6]. The bacteriophage's EOP on the strain yielding the highest value of the titer (often the strain that was used to grow the bacteriophage stock, however in some cases the plating on other strains may give higher titer values) is arbitrarily taken as 1.0. The EOP on any other strain X is defined as a ratio of the bacteriophage titer determined on this strain to the titer determined on that chosen host strain with presumed EOP = 1.0. The decreased EOP may be due to the action of host bacteriophage-resistance systems blocking the intracellular virus development [7, 8], or in some cases due to poor bacteriophage adsorption to the cells [9]. If the EOP on some strain is dramatically reduced to 10^{-6}–10^{-5}, a possible explanation (along with above mentioned action of the host anti-bacteriophage systems) is that a point mutation (e. g. in genes encoding virion proteins recognizing the

bacteriophage receptor) could be required for bacteriophage adaptation to the particular host strain. Also, normally the bacteriophage needs to be able to produce about 5–10 bacteriophage per cell in the conditions on the plate to be able to produce a visible plaque. The threshold level of the EOP above which the strain can be considered as bacteriophage sensitive should be established explicitly as a function of the practical purposes of the host range determination. For example, if the experiment is set up to predict a potential therapeutic efficacy of the given bacteriophage preparation, the host strains yielding low EOP of this bacteriophage stock may be considered as resistant. To the contrary, when one is looking for the alternative bacteriophage hosts, the initially low EOP may be not a problem, since it can often be improved by adaptation of the bacteriophage *via* 1–2 rounds of growth on the new host strain, caused by an inherent bacteriophage genome plasticity. In any case, the spot test should not be limited to application of only a single drop of the concentrated bacteriophage stock on the bacterial lawn; multiple dilutions should always be tested, preferably made up to observe the single plaques. It is important that the lawn growth inhibition upon the concentrated bacteriophage stock application may not always indicate efficient bacteriophage infection. The clear spot may also be due to the killing of the cells by lysis from without [10] or even due to the presence of the traces of bacteriostatic/bactericidal substances such as chloroform or antibiotic compounds in the concentrated bacteriophage stock.

It is also important to keep in mind that sometimes the bacteriophage that is able to replicate on a particular host strain may not form the well-defined plaques on it. This may happen due to a variety of reasons including, for example, very high frequency of lysogenization (only in temperate bacteriophages), the small burst size on that host (must be at least about 6–8 bacteriophage per cell to make a plaque), the existing temporary physiological resistance of the majority of the cells in the culture to the bacteriophage (thus focusing the bacteriophage on the minor fraction of bacteriophage-sensitive cells) (*see* for example [11]). The giant bacteriophages sometimes do not form the usual plaques because of the natural limitation of virion diffusion imposed by top agar layer [12, 13]. In the latter case, the replacement of the agar in the top layer of the plates by 0.2% (wt/vol) low melting point agarose may improve the plaque formation [14, 15].

Alternative methods of the bacteriophage growth detection should be considered if it is important to detect host strains supporting the bacteriophage growth without forming plaques. Among such methods, potentially adaptable to high throughput, we may mention the epifluorescence microscopy visualization [16], the detection of the bacteriophage particles by whole virion agarose electrophoresis [15], quantitative PCR, bioluminescent measurement of ATP or ATP-producing enzymes released from host cells

upon bacteriophage lysis [17] and other physicochemical approaches. In each case the accommodation of the procedure to the conditions of the experimental design used is necessary to avoid errors (most frequently false—positive results). It is also possible to monitor the increase of the bacteriophage titer upon incubation with such strains using biological titration on a sensitive test strain. However in this case one should be aware of possible EOP decrease of the bacteriophage progeny obtained on the new host when it is plated on the standard strain lawn, for example due to bacteriophage modification and restriction or other mechanisms. It is worth mentioning that the strains in which only on a fraction of the cells are susceptible to the bacteriophage may feature some phase variations of the surface receptor and/or bacteriophage resistance mechanisms [7, 18]). Some temperate bacteriophages are able to chemically modify the host surface receptor moieties required for other bacteriophages, e.g. LPS O-antigen, using specific transferases encoded by the bacteriophage genome [19], thus rendering the host strain unavailable for infection by bacteriophages using the native O-antigen as a primary receptor. Therefore in some cases the derivative suitable for bacteriophage plaque growth may be isolated from the culture of such a semi-susceptible strain [20].

If the host range screening involves the testing of large numbers of the bacterial strains for sensitivity to a limited set of bacteriophages (e. g. if one studies the sensitivity of a commensal *E. coli* community to a therapeutic bacteriophage), a direct plating of bacteria on bacteriophage-containing agar is a very elegant solution, highly increasing the assay throughput. The "raw" set of sensitive strains identified by this method should be submitted for a detailed study of their parameters (EOP, plaque formation etc.) if such information is desired.

It is very important to keep in mind that the observable bacteriophage activity against the bacterial culture applied to the bacteriophage agar (*see* Subheading 3.2 below) is often different from complete arrest of the bacterial growth. It is important to avoid the transfer of copious amounts of bacteria on a bacteriological loop or the toothpicks, since in many cases the strains, perfectly suitable for the bacteriophage multiplication with high EOPs, willingly grow on the bacteriophage agar due to "outgrowing" of the bacterial colonies when a raised part of it is not making any contact with the bacteriophage. Application of bacterial suspension in drops is preferable, supplied with a control plate with the same medium without the bacteriophage. After the accumulation of minimal experience, one can distinguish very reliably the overall traits of positive (significant growth inhibition) and negative results. For the first trials we suggest using several dilutions of the bacterial suspension. This will also help to estimate the approximate frequency of bacteriophage-resistant clone occurrence in sensitive bacterial cultures. We also recommend using positive and negative controls (sensitive and

resistant strain suspensions) to confirm the properties of the bacteriophage agar prepared for the experiments.

2 Materials

1. Bacterial cultures and bacteriophage stock(s) with known titer (s)
2. Sterile liquid medium appropriate for the bacterial strains used.
3. Plates with solid medium containing 15 g of bacto-agar per 1 L. For *E. coli* and many other heterotrophic bacteria, tryptic soy broth or LB medium containing 10 g of Tryptone, 5 g of yeast extract and 5 g of NaCl per 1 L of deionized water is suitable. For bacteria with different nutrient requirements use specialized media (*see* **Note 1**).
4. Sterile soft agar containing 6 g of bacto-agar per 1 L and the same nutrients as the bottom agar.
5. Sterile 13 × 170 mm glass tubes with caps.
6. Sterile Eppendorf tubes of 1.5 and 0.5 mL capacity.
7. Table-top Eppendorf centrifuge with maximum speed 10,000 × *g* or more.
8. Water bath adjusted for 48 °C or thermoblock with 4 × 10 or more holes the right size for the glass tubes.
9. Microwave oven.

3 Methods

3.1 Determination of the Bacteriophage Infectivity by Top Agar Method

This technique is very similar to the drop assay method of bacteriophage titration [21].

1. Remove the required number of plates with bottom agar from +4 °C storage. One needs to use plates poured in advance, and preferably incubated overnight at 37 °C, as they give out much less moisture during cultivation. At least one plate per bacterial strain is required (*see* **Note 2**). If they are too recently poured, it is possible to put them partially open in a laminar flow hood to dry the agar surface and to adjust the medium to room temperature. Label the plates.
2. Melt the top agar in a microwave oven until it comes to a gentle boil and no visible clumps are left, dispense 3 mL aliquots to sterile glass test tubes (e.g. 13 × 170 mm), and place them in water bath or dry block at +48 °C, so the medium is kept molten. Use within a few hours.

3. Prepare the suspensions of log-phase liquid cultures of bacterial strains to be tested. For aerobic bacteria, it is better to grow them with vigorous agitation in the shaker-incubator under their optimal growth temperature in small (50 mL) Erlenmeyer flasks or in test tubes containing 3 mL of the medium and covered by loose caps or aluminum foil. In many cases, cells from a fresh plate resuspended in the liquid medium or in physiological saline work as well. The density of the bacterial suspension should be 10^8–10^9 cfu/mL (normally about $OD_{600} = 0.4$–1.0).
4. Take the tube with top agar, immediately add 50 μL of bacterial suspension, mix by rotating the tube between the palms (the vortexing may produce foam) and pour to the center of plate containing solid medium. Do it quickly enough, so the bacteria will not perish due to a thermal shock. Spread the top agar layer over the whole surface of the bottom agar by gentle tilting the plate, and let solidify on horizontal surface. Do not cover the plate, as the condensing water would cause undesired "swamping", causing the mix-ups of bacterial cultures. The plates with the inoculated lawns prepared in such a way should be used within 3 h. Inoculate one or more (if many bacteriophages have to be tested) plates for each bacterial strain. If more than five bacteriophages are tested it is convenient to use square plates (for 10 × 10 cm square plate, 6–8 mL of the top agar should be used instead of 3 mL).
5. Centrifuge your bacteriophage stocks for 5 min at maximum speed of a table-top centrifuge to remove possible bacterial contamination. Prepare serial dilutions of the stocks. To do this, for each stock prepare six Eppendorf tubes with 990 μL of physiological saline or bacteriophage SM buffer. Label them as "-2", "-4", "-6" and "-8" and indicate also the bacteriophage name. Add 10 μL of the bacteriophage stock to the first tube, mix by brief vortexing. Transfer with new pipette tip 10 μL aliquot of this dilution to the second tube and repeat the same operations until the last tube is used. This will yield the dilutions by 10^2, 10^4, 10^6 and 10^8 times respectively. The tenfold dilutions may be used instead of 100-fold to ensure the countable number of the plaques in at least some of the drops, however it will increase the time and material consumption that may be crucial for a large-scale screening experiment.
6. Draw on each plate the grid and mark the lines according to the bacteriophages. Apply drops of the bacteriophage stocks dilutions on the surface of the top agar in the corresponding line of the grid. One may use a single pipet tip for each bacteriophage if one moves from the highest dilution (10^{-8}) to the lowest. Apply also a drop of nondiluted bacteriophage (10^0). We

suggest to use small drops of 5 μL to avoid smearing of them by the water expelled from the solid medium.

7. Let the drops to be absorbed by the agar; dry the plate 10 min under the laminar hood (no UV irradiation!).
8. Incubate the plates under the optimal conditions for the growth of the bacteria. For rapidly growing cultures an overnight incubation is sufficient, for slower growing a longer incubation may be needed. In the latter case seal the plates with parafilm to avoid the drying of the agar. It is important to keep the plates inverted to avoid water condensation on agar surface.
9. Read your plates. If the bacteriophage produces isolated plaques in any dilution, the bacterial strain used for the lawn can be considered as sensitive. A separate estimate of EOP would be useful for distinguishing of the fully sensitive strains and those to which the bacteriophage may be adapted by further passaging. If the inhibition of the lawn growth is observed at high bacteriophage concentrations but no plaques appear in stronger dilutions, the strain may be considered as basically resistant (or more detailed evaluation of its interactions with a particular virus should be performed).

3.2 Testing of the Bacterial Strains' Susceptibility to Bacteriophage Using Bacteriophage Agar

1. Remove the required number of plates with bottom agar from +4 °C storage. It is better to use plates poured in advance, as they give out much less water during cultivation. At least one plate per bacterial strain is required needed. Put them partially open in a laminar flow hood to dry the agar surface and to adjust the medium to room temperature. Label the plates. Dry them, if necessary, partially uncovered for 10–15 min under the laminar hood or, if the plates are already dry let them heat up to the room temperature for 10–20 min. Label the plates appropriately.
2. Melt the top agar in a microwave oven until it just boils and no visible clumps are left, place sterile glass test tubes (e.g. 13 × 170 mm), in water bath or dry block at +48 °C, dispense 3 mL aliquots into them so the medium is kept molten.
3. Take the tube with molten top agar, add an aliquot of the sterile (*see* **Note 3**) bacteriophage stock containing 10^8–10^9 pfu of the bacteriophage. Mix by rotating the tube between the palms (vortexing may produce foam) and pour to the center of plate. Spread the top layer over the whole surface of the bottom agar by gentle tilting the plate and let solidify on horizontal surface. The bacteriophage agar is thus prepared. Prepare a control plate with sterile top agar overlay without addition of any bacteriophage. Draw identical grids on test and control plates to assure the same pattern of application of the bacterial strains.

4. Prepare the suspensions of your bacterial strains to be tested. Include the positive and negative controls, known sensitive and resistant strains respectively. For preparation of the suspensions, mark one sterile 0.5 mL Eppendorf tube for each strain. Place in it 100 μL of sterile liquid medium or physiological saline. Touch a colony of the strain with a sterile toothpick and resuspend the cells in the liquid by rubbing the toothpick against the bottom of the tube.
5. Apply 3 μL drops of the bacterial suspensions (*see* **Note 4**) in the same pattern on the test (with bacteriophage agar) and control (sterile double-layer agar) plates. Let the drops to be absorbed or dry open plates for 10–15 min under the laminar hood.
6. Incubate the plates at appropriate temperature overnight (or longer if slow growing bacteria were used). Compare the growth on the test and on control plates. It is important that the sensitive strains may undergo complete inhibition on the bacteriophage agar or give more or less significant number of the colonies in the spots. Sometimes a confluent growth or growth in the shape of a ring contouring the spots may appear, but this growth is markedly weaker than on the control plate. To avoid any ambiguity serial dilutions of the cell suspensions should be applied instead of single drops. The action of some bacteriophages that produce weak, tiny and turbid plaques on a given strain, is not easy to observe on a bacteriophage agar. Such bacteriophage–host pairs will usually not be identified by this screening method.

4 Notes

1. Some media used for bacterial cultivation may contain substances inactivating the bacteriophages. Carefully examine the composition of specialized media if using them.
2. In the case when one needs to test a large number of bacteria for their ability to support the plaque growth of one or a few bacteriophages, it is convenient to use multiple minilawns on the same plate. To do so, place 0.6 mL of the molten top agar to the 1.5 mL Eppendorf tube and place them to the dry block at 46 °C. Take a tube out of the block, rapidly add 15–20 μL of the bacterial suspension. Mix by brief pipetting with 100 μL pipet and transfer it on the plate with the bottom agar marked with large grid (for 90 mm plate, 6–8 wells will be appropriate). Spread the top layer over the agar surface within the well of the grid. Let it solidify. The pad of the top agar formed this way is large enough to accommodate 3–4 drops of the bacteriophage

dilutions. Several bacterial cultures may be thus tested on the same plate.

3. The bacteriophage stock might be filtered in advance through 0.22 μm filter, however the centrifugation at maximum speed on the table top centrifuge removes bacterial contamination well enough to allow use of the stock for direct bacteriophage agar preparation. We recommend running such centrifugation twice. After the first round, transfer the supernatant to a new Eppendorf tube leaving some amount of the liquid over the pellet to avoid resuspending it. After the second centrifugation take the aliquot carefully from the top part of the tube. Generally, a moderate bacterial contamination (100–200 contaminant colonies per plate) does not interfere severely with the interpretation of the results.
4. For more reliable results, or in case if the estimate of the resistant clone frequency is desired, we suggest applying serial dilutions of the bacterial suspension instead of a single drop. If the suspensions are prepared as described here, their concentrations are likely to be around 10^7 cfu/mL, so the tenfold dilutions down to 10^{-3} (that would correspond to 10–100 cfu per drop) will be sufficient. To prepare these dilutions, use similar 0.5 mL tubes with 90 μL of the liquid medium. Transfer 10 μL as is described in Subheading 3.1 for bacteriophage dilutions. In this case, the pipetting with the tip used to bring 10 μL aliquot from previous dilution to the tube can be used to mix the suspension instead of vortexing.

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Chapter 8

Recovery and Characterization of Bacteria Resisting Infection by Lytic Bacteriophage

Libera Latino and Christine Pourcel

Abstract

Bacteria and bacteriophages coexist and coevolve, bacteriophages being obligatory predators exerting an evolutionary pressure on their prey. Mechanisms in action vary depending on the bacterial genomic content and on the regulation of the bacteriophage cycle. To assess the multiplicity of bacterial genes involved in resistance as well as the changes in the bacteriophage interactions with the bacteria, it is necessary to isolate and investigate large numbers of independent resistant variants. Here we describe protocols that have been applied to the study of *Pseudomonas aeruginosa* and four of its virulent bacteriophages belonging to the *Podoviridae* and *Myoviridae* bacteriophage families. Mutations are identified using whole genome sequencing of resistant variants. Phenotypic analyses are performed to describe the changes conferred by the mutations.

Key words Bacteriophages, Bacterial phenotype, Genome sequencing, Complementation

1 Introduction

Cocktails are prepared that associate different bacteriophages on the basis of their host range, but often very little is known on their biology, the interactions with their host and their capacity to select for resistant mutants. It is thought that the use of several bacteriophages belonging to different genera will lower the frequency of resistance development [1, 2]. However, many bacteriophages use common structures as receptor, and therefore a mutant selected by one bacteriophage will resist a large number of other bacteriophages [1, 3]. Furthermore, the bacteriophage receptors present at the surface of the bacteria play a role in the bacterial life cycle and virulence. Mutations in genes encoding these elements might reduce the fitness of the bacteria, but it might as well give them new characteristics and make them more aggressive [4].

Therefore it is important to perform experiments aiming at the isolation and analysis of multiple resistant mutants in order to estimate the frequency and diversity of the mechanisms at play.

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The availability of whole genome sequencing at more and more accessible costs makes it possible to identify each mutation and help understanding the dynamics of bacteriophage-driven bacterial evolution.

2 Materials

2.1 Determining the Adequate Multiplicity of Infection (MOI) for the Infection

1. When clinical strains and their bacteriophages are used, manipulations are performed in a biosafety level 2 (BSL2) laboratory containment.
2. Bacteria are grown in appropriate medium depending on those you are using. For *Pseudomonas aeruginosa*, LB (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl), LB agar (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl, 1.5% (wt/vol) agar) are commonly used to grow bacteria in planktonic form or on plate, respectively.
3. Bacteriophages are suspended in SMG buffer (5.8 g/L NaCl, 2 g/L $MgSO_4$, 0.1 g/L gelatin, 1 M Tris–HCl [pH 8.0]).
4. The bacteria and bacteriophages are mixed and the suspension is poured on agar plates using top agar (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl, 0.7% (wt/vol) agar) at 45°C.

2.2 Getting Bacteria Resistant to a Lytic Bacteriophage

All materials and media reported in Subheading 2.1 are used.

2.3 Testing for Phenotypes

1. A stereomicroscope (Motic DM143) equipped with a Moticam 580 camera or equivalent is used to observe and photograph the colonies.
2. For the swarming test the following medium is used: 20 mM NH_4Cl, 12 mM Na_2HPO_4, 8.6 mM NaCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 11 mM dextrose, 0.5% (wt/vol) casaminoacids, 0.5% (wt/vol) agar.
3. For twitching test: LB plus 1.5% (wt/vol) agar.
4. Biofilm formation is quantified using Crystal violet 0.1% (wt/vol).
5. The optical density (OD) is recorded using the microplate reader CLARIOstar, BMG LABTECH's or equivalent.

2.4 Identification of Mutations by Whole Genome Sequencing

1. Before DNA extraction, bacteria are pelleted and resuspended in phosphate-buffered saline (PBS) buffer (8 g/L NaCl, 0.2 g/L KCl, 1.44 g/L Na_2HPO_4, 0.24 g/L KH_2PO_4).
2. Bacterial lysis is achieved by adding 1 volume of 2× lysis buffer (20 mM Tris [pH 7.8], 20 mM EDTA, 20 mM NaCl, 1% (wt/vol) SDS) to the bacterial suspension.

3. Phenol and chloroform solutions are manipulated under the fume hood.

2.5 Confirmation of Mutants

1. For complementation, a shuttle vector, capable of replicating into *E. coli* and into the bacteria of choice should be used.
2. Amplicons are PCR-amplified using Taq polymerase and amplification buffer as recommended by the supplier, and 1 μM oligonucleotides.
3. Preparation of libraries and Illumina sequencing is performed by a specialized in-house service or a company.
4. Sequencing data are analyzed using GeneiousR10 (Biomatters, New Zealand).

3 Methods

There are five main steps in the characterization of bacteriophage-resistant variants: (1) determination of the adequate multiplicity of infection (MOI) for the infection, (2) isolation of bacterial resistant mutants, (3) phenotypic analyses, (4) whole genome sequencing and data analysis, (5) complementation of the mutants. The methods described here can be applied to any bacterial species, using appropriate culture media and growth conditions.

3.1 Determining the Adequate Multiplicity of Infection (MOI) for the Infection

The best conditions for selecting resistant mutants must be determined for each pair of bacteria and bacteriophages, as growth rate and burst size may vary, and also in terms of the purpose of the investigation. The aim of the proposed protocol is to obtain separated colonies surviving infection on solid medium. An overnight culture started from a single colony of the parental wild-type strain should be diluted and titrated in order to know the exact amount of colony forming unit (cfu) corresponding to a fixed optical density at 600 nm (OD_{600}) (*see* **Note 1**). The bacteriophage stock solution must be titrated as well.

1. Prepare seven sterile tubes and label them according to the different MOI (0.01, 0.1, 1, 10, 100, 1000, 10,000).
2. Dilute the overnight bacterial culture and transfer an adequate amount in the different tubes in order to have a tenfold decreasing amount of cfu (10^9, 10^8, 10^7, 10^6, 10^5, 10^4 and 10^3).
3. Add 10^7 bacteriophage plaque-forming units (pfu) in each tube and keep them at room temperature (RT) for 15 min in order to let bacteriophages adsorb on the bacterial surface.
4. Add 4 mL of top agar, pre-equilibrated at 45 °C, in each tube and pour them on the previously prepared agar plates (round 90 mm Petri dishes).
5. Invert and incubate the plates at appropriate temperature for 72 h.

6. Where it is possible, count the bacteriophage-resistant colonies and calculate the frequency of resistance when using 10^7 pfu with the following equation:

$$\mu = \frac{N}{N_0},$$

where μ is the frequency of resistance, N is the number of resistant colonies recovered after incubation for 72 h and N_0 is the cfu used for the infection of the considered plate.

7. For further infections use the MOI for which well-separated colonies are obtained (~50 colonies per plate).

3.2 Getting Bacteria Resistant to a Lytic Bacteriophage

Two important factors must be considered to obtain a large variety of mutants and to be able to identify changes compared to the wild-type susceptible strain. First, it is necessary to start the culture, subsequently used for the infection, from a single colony and to prepare genomic DNA from this culture; this will be used as a reference for the identification of the mutations in the resistant variants. Second, the infection is performed on solid medium, in order to trap the infected bacteria inside the top agar (*see* **Note 2**) and to get separated colonies formed independently.

3.2.1 Obtaining the Resistant Colonies

Steps 1–3 are typically done at the onset of a new project, in order to start from a single parental genome.

1. Streak the bacterial strain in order to get separated colonies.
2. Starting from a single colony, prepare a bacterial culture in the appropriate medium and let bacteria grow overnight.
3. Store 0.5 mL of the culture at −80 °C adding an equal volume of glycerol 60% (vol/vol) (*see* **Note 3**). This reference stock will be used for any additional preparation of fresh bacterial culture at least in the course of a given project.
4. Measure the OD and if necessary, concentrate or dilute the bacterial culture in order to obtain a suspension containing enough cfu to perform infection at the MOI determined in Subheading 3.1.
5. Transfer the determined amount of cfu in a new sterile tube, add 10^7 pfu and keep the mixture for 15 min at RT.
6. Add 4 mL of top agar, pre-equilibrated at 45 °C, and pour the mixture on a fresh agar plate.
7. Incubate the plate for 72 h at a standard growth temperature for the used bacterial strain.
8. Pick up separated colonies from the plate used for the infection (Fig. 1), possibly with different size and morphology, streak them onto fresh agar plates and repeat the purification for a total of three times (*see* **Note 4**).

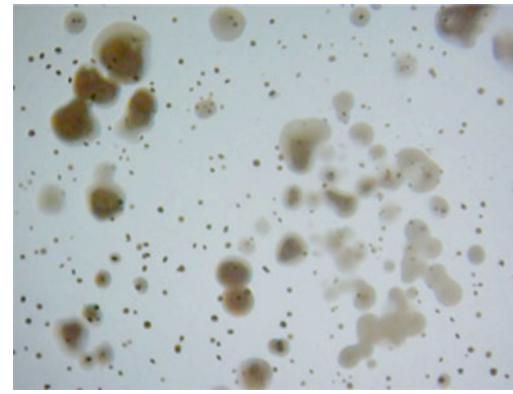

Fig. 1 Section of a plate containing colonies resisting phage infection

3.2.2 Storage of the Bacteriophage-Resistant Bacteria

Once the bacteriophage-resistant colonies are purified, they are stored for further studies, and a lysate is prepared for DNA extraction.

1. A single bacteriophage-resistant colony is picked up from the agar plate and grown overnight in liquid medium at optimal temperature condition.
2. 0.5 mL of the overnight culture is mixed with an equal volume of glycerol 60% (vol/vol) for storage at −80 °C.
3. 1 mL of the culture is transferred to a 2 mL eppendorf tube and centrifuged at 15,000 × *g* for 1 min to pellet the bacteria.
4. The pellet is resuspended in PBS and one volume of 2× lysis buffer is added for purification of whole genome DNA (*see* **Note 5**) as described in Subheading 3.4.1.

3.2.3 Testing for Bacteriophage Susceptibility

1. 0.5 mL of the bacterial culture prepared as in Subheading 3.1 (**steps 1** and **2**) are transferred to a sterile tube.
2. 6 mL of top agar, pre-equilibrated at 45 °C, are added and the mixture is poured onto a squared agar plate (13 × 13 cm).
3. The bacterial-agar layer is let to cool down for few minutes.
4. 10 μL of seven tenfold bacteriophage dilutions (containing approximately 10^7, 10^6, 10^5, 10^6, 10^4, 10^3 and 10^2 pfu) are spotted on the bacterial-agar layer.
5. The bacteriophage drops are left for 15 min under the hood at room temperature before incubating the plate at optimal temperature.
6. For each variant and for the susceptible parental strain, the number of plaques is counted and the efficiency of plating is calculated using the following equation:

$$x = \frac{P_v}{P_s} \times 100,$$

where x is the efficiency of plating expressed in percentage, P_v is the titer of the bacteriophage on the bacteriophage-resistant variant and P_s is the titer of the bacteriophage on the susceptible parental strain.

7. Record the characteristics of plaques (*see* **Note 6**).

3.2.4 Testing for Presence of Free Bacteriophages

Persistence of bacteriophages in pseudolysogens or in carriers has been observed frequently with lytic bacteriophages in different bacterial species, such as *P. aeruginosa* [3] or *Campylobacter jejuni* [5] for example. In order to test for the presence of free bacteriophage particles released in the culture supernatant, a spot-test can be used to screen a large number of bacteriophage-resistant variants followed by polymerase chain reaction (PCR) assay for confirmation.

1. Grow the bacteriophage-resistant variant and the control parental strain overnight in 2 mL of medium.
2. Transfer 1 mL of each culture to a separate sterile eppendorf tube.
3. Centrifuge at 15,000 × *g* for 1 min to pellet the bacteria.
4. Recover the supernatants and transfer them into another eppendorf tube.
5. Plate the susceptible parental strain on a squared Petri dish following the procedure reported in Subheading 3.2.3 (**step 1–3**).
6. Spot 10 μL of each bacteriophage-resistant variant supernatant on the susceptible strain layer.
7. Let the drops dry under the hood for 15 min and then incubate the plate overnight at optimal temperature. If a lysis zone is observed, the selected variant could possibly be a pseudolysogen (*see* **Note 7**).
8. Select bacteriophage primers and perform a standard PCR assay on total DNA extracted as described in Subheading 3.4.1 (*see* **Note 8**).

3.2.5 Stability of the Pseudolysogen/Carrier State

1. Starting with the variants purified by three colony-replatings (Subheading 3.2.1, **step 8**), streak the bacteria on an agar plate in order to obtain about 100 separated colonies.
2. Pick colonies with a sterile toothpick or plastic tip and successively touch a fresh agar plate then the agar plate with a bacteria-agar layer prepared as reported in Subheading 3.2.3

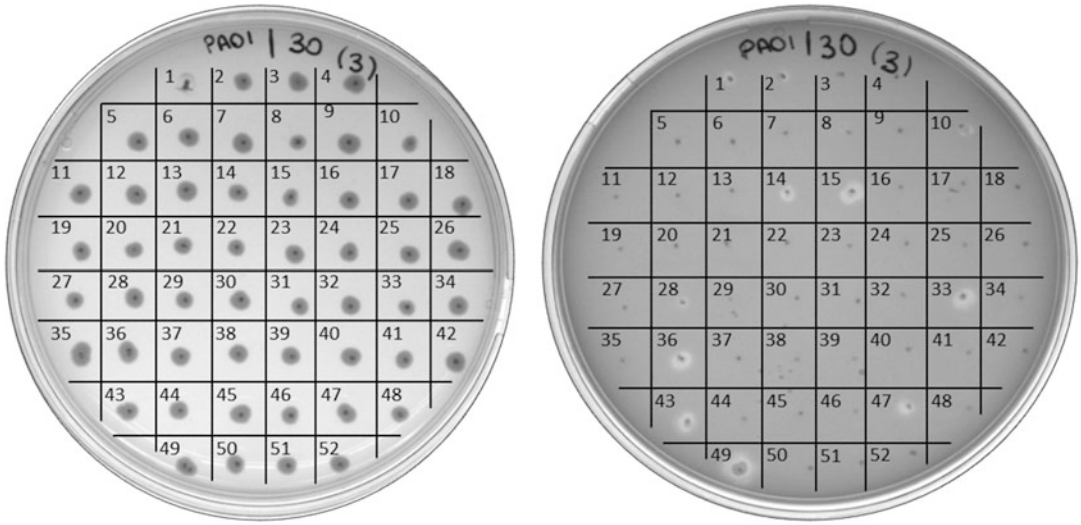

Fig. 2 Test to detect pseudolysogens

(**step 1–3**). Using a numbered grid placed under the agar plate, 52 colonies can be tested on a single round Petri plate (Fig. 2).

3. Incubate the plates overnight at the appropriate temperature.
4. Record the number of colonies surrounded by a lysis zone on the bacteria agar-layer (Fig. 2).
5. From the agar plate, pick a bacteriophage-positive colony and streak it on a fresh agar plate to obtain about 100 separated colonies as described in **step 1**.
6. Incubate overnight at the appropriate temperature.
7. Repeat **steps 2–4** as long as colonies producing bacteriophages are observed.

3.3 Testing for Phenotypes

3.3.1 Observation of the Colonies

The observation of colonies under the stereomicroscope allows the determination of their size and morphological characteristics in comparison with the parental strain. In particular, it is important to record whether the aspect is rough or shiny, if the shape is regular or irregular, the color of the colonies, the diameter and if they have a mucoid appearance. Taking photographs at every step of purification is very important in order to track the evolution of the bacterial population (Fig. 3).

3.3.2 Motility

Motility assays give information on the capacity of the cells to move. Two simple tests can be performed, twitching which necessitates the presence of pili [6], and swarming which proceeds with production of surfactant, pili and flagella [7].

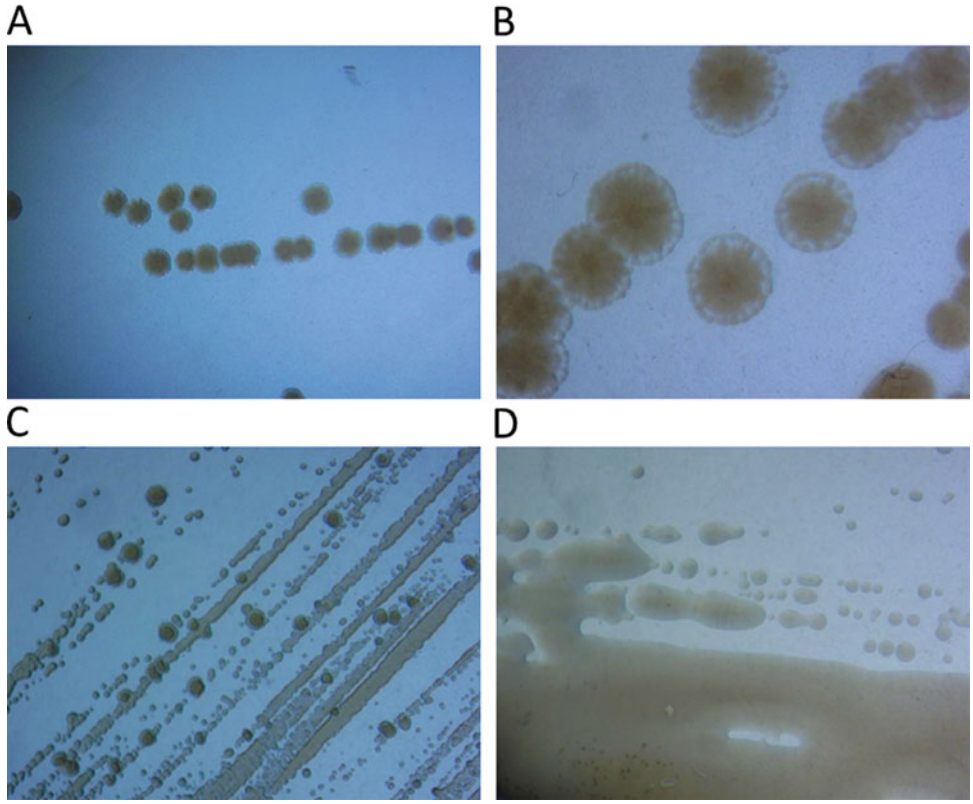

Fig. 3 Phenotypic aspect of *P. aeruginosa* phage-resistant colonies. (**a**) Parental strain; (**b**) colonies with transparent edges; (**c**) pseudolysogen showing colonies with different shapes; (**d**) mucoid colonies

Twitching

1. For twitching motility assessment, 1 μL of an overnight bacterial culture (OD_{600} of 2) is inoculated between the agar and the plastic surface of twitching medium plates.
2. The diameter of the motility zone around the inoculation site is measured after 24 h incubation at appropriate temperature. It can be observed directly on the bottom of the plate or following staining with crystal violet (Subheading 3.3.3, **step 7–9** using an appropriate amount of crystal violet to cover the bottom of the plate).

Swarming

1. For the swarming assay, 2 μL of an overnight bacterial culture are deposited in the center of a freshly prepared plate containing 20 mL of swarming medium, and the plate is incubated for 48–72 h at appropriate temperature.
2. Observe the shape (circular or dendritic) and diameter of the bacterial growth on the surface of the medium.

3.3.3 Biofilm Formation

Several external membrane structures and molecules, including pili, are necessary for the bacteria to adhere to a solid surface and to

form a biofilm, an important virulence factor [8]. This capacity can be tested by measuring adherence to plastic surfaces. To facilitate the analysis of multiple replicates, the test is performed in 96-well microplates into a microplate reader.

1. Set-up well-plates by diluting overnight bacterial culture to an OD_{600} of 0.1 into fresh medium.
2. Fill the well plates by adding 200 µL of diluted culture of each variant. For each variant fill the entire column in order to have replicates (test 12 variants per 96-wells plate).
3. Incubate the plates for at least 48 h at an appropriate temperature.
4. Read the plates at OD_{595}.
5. Dump out excess liquid and planktonic cells by turning the plates over and shaking out the liquid into a biohazard waste sack (biofilms will remain adhered to the wells walls).
6. Rinse the plates twice (each time dumping into a waste sack) with PBS.
7. Add 200 µL of a 1% (wt/vol) crystal violet solution to each well.
8. Leave at room temperature for 30 min.
9. Rinse 3–4 times with water, shake out and blot on a stack of paper towels. Make sure to get rid of all excess cells and dye (there is no need to be gentle).
10. Redissolve the crystal violet attached to the biofilm adding 200 µL absolute ethanol per well and leave the plate for 15 min at RT.
11. Read the OD_{595} and subtract to the initial values obtained at **step 4**.

3.4 Identification of Mutations by Whole Genome Sequencing

For whole genome sequencing using Nextera, the chromosomal DNA must be of high quality, pure, and unsheared. To this end we favor the classical phenol/chloroform extraction. We detail here the preparation of bacterial DNA. Purification of bacteriophage DNA follows the same protocol, starting with purified bacteriophages instead of a bacterial pellet.

3.4.1 Purification of DNA

1. Prepare a 1 mL overnight culture of bacteria.
2. Transfer it to a 2 mL eppendorf tube.
3. Centrifuge for 1 min at 15,000 × *g* to pellet the bacteria, discard the supernatant and resuspend the pellet in 200 µL of PBS.
4. Add 200 µL of 2× lysis buffer and mix gently.
5. Add proteinase K at a final concentration of 50 µg/mL and incubate for 2 h at 50 °C.

6. Add 400 µL of phenol saturated with 100 mM Tris–HCl [pH 7.5], mix thoroughly by shaking the tube (do not vortex).
7. Centrifuge for 5 min at 15,000 × *g*, transfer the supernatant to a clean 2 mL eppendorf tube (*see* **Note 9**).
8. Add one volume of phenol/chloroform 1/1 mixture, mix, and centrifuge. Collect the supernatant and transfer into a new tube.
9. Add one volume of chloroform and repeat the extraction procedure.
10. To the final supernatant, add NaCl to a final concentration of 200 mM, and 2 volumes of ethanol.
11. Centrifuge at 15,000 × *g* for 10 min at 4 °C.
12. Discard the supernatant and add 400 µL of 70% (vol/vol) ethanol (diluted in water) to rinse the pellet.
13. Centrifuge at 15,000 × *g* for 10 min at 4 °C.
14. Discard the supernatant, let the pellet dry on the bench and resuspend in 200 µL Tris–EDTA buffer.

3.4.2 Illumina Sequencing

To identify a new mutation in a bacterial chromosome, with a high confidence level, we found that Miseq Illumina sequencing technology, producing 150–250 bp paired-end reads with a 100-fold coverage is appropriate and convenient. For a 6 Mb genome such as that of *P. aeruginosa*, a minimum of 1.5 million 250 bp reads is recommended, representing an average coverage of 80×. For a bacteriophage genome, 50,000 reads are largely sufficient but it is not always possible to get such a small amount of reads, due to technical constraints. Sequencing can be done by companies or specialized platforms.

We have tested two different approaches to produce libraries, both being satisfactory for circular genomes or bacteriophage genome with circular redundancy:

1. Mechanical shearing of DNA (Truseq library) and selection of fragments with a mean size of 900 bp.
2. Nextera technology.

In a single MiSeq sequencing run, eight to twelve bacterial genomes can be sequenced simultaneously.

Importantly, the Nextera technology is not recommended for sequencing a bacteriophage genome with fixed ends as the "tagmentation" process underlying this approach will not be able to reach the very ends. Libraries for sequencing fixed ends bacteriophages or unknown bacteriophages should be made from physical shearing of DNA (TruSeq) which will on the contrary favor the sequencing of preexisting DNA ends [9].

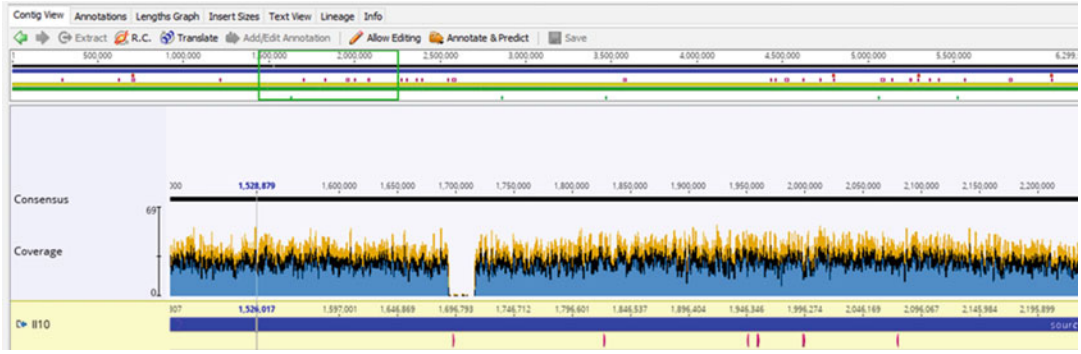

Fig. 4 Screenshot of GeneiousR10 interface. A deletion can be easily observed when the reads of the variant are mapped onto the reference parental strain genome

3.4.3 Data Analysis Using Geneious

To identify a mutation, the sequencing reads of the resistant mutant genome are aligned to the full genome sequence of the parental strain. Single nucleotide changes or insertion/deletions can be visualized using a graphic interface.

Different algorithms are available to compare two genomes by alignment, and to map reads on a reference genome. GeneiousR10 (Biomatters, New Zealand) offers many tools, some developed by Biomatters.

The different steps of the analysis are as follows:

1. Map the reads onto the reference genome, identify positions which correspond to a mutation and calculate the percentage of reads containing the mutation (Fig. 4) (*see* **Note 10**).
2. When a single nucleotide polymorphism (SNP) or an insertion/deletion (indel) is identified in a variant, sequencing reads mapping in the mutated gene plus 1 kb on both sides are recovered, de novo assembled, and the contig is aligned with the parental genome. This allows the precise localization of indels. Mutations are confirmed by PCR amplification of the affected gene and Sanger sequencing (Subheading 3.5.1).
3. Recover the reads that do not map onto the parental genome and perform a de novo assembly. This step will reveal the potential existence of bacteriophage reads.
4. If bacteriophage DNA is present and a genome is assembled, align this genome to the bacteriophage used to derive the resistant variant. This may uncover mutations in the bacteriophage maintained in the pseudolysogen.

3.5 Confirmation of Mutants

It is necessary to confirm the reality of mutations observed from sequencing data and the simplest way is to analyze by PCR an aliquot of the same DNA preparation that was used for whole genome sequencing. Then complementation can be performed if one wishes to prove that the observed mutation is indeed responsible for inducing resistance to bacteriophages.

3.5.1 PCR Amplification and Sanger Sequencing

In order to verify the existence of a SNP or a small deletion, 20 bp oligonucleotides are selected at about 100 bp distance on both sides of the mutation. PCR is performed using purified DNA and Taq polymerase, as recommended by the supplier. PCR products are run on a 2% (wt/vol) agarose gel in 0.5× TBE in order to check their quality and length, and sequenced by Sanger sequencing.

For large deletions, a first primer pair is selected on both sides of the deletion and a second pair inside the deleted region. PCR is performed and the products are visualized on a 2% (wt/vol) agarose gel.

3.5.2 Complementation

A complementation assay allows to formally demonstrate that the observed mutation is responsible for the resistance to bacteriophages. For this, the wild-type gene is cloned into an expression vector and transferred into the variant.

As a vector for *P. aeruginosa*, we have used the pUCP24 plasmid, a generous gift of Dr. Schweizer [10]. This is a shuttle vector which replicates in *E. coli* and in *P. aeruginosa*, and contains a multiple cloning site downstream lacZ.

1. The parental gene is PCR-amplified using oligonucleotides including restriction sites for *BamH*I and *Hind*III.
2. The amplicon is digested with *BamH*I and *Hind*III, ligated into the vector similarly digested and transformed into *E. coli*, in which replication of pUCP24 is optimal [10].
3. A selected recombinant is used to transform *P. aeruginosa* strain by electroporation using the fast protocol described by Choi et al. [11].
4. Transformants are selected using 10 μg/mL Gentamicin.
5. The presence of the plasmid in colonies growing on Gentamicin plates is verified by PCR amplification.
6. The morphology of the verified transformants is observed under the stereomicroscope.
7. The transformants are tested for their susceptibility to the bacteriophages (*see* **Note 11**).

4 Notes

1. For the reference *P. aeruginosa* strain PAO1 we measured that a bacterial culture at an OD_{600} of 1 corresponds to 10^9 cfu/mL.
2. We believe it is important to try and mimic the conditions in which bacteriophage therapy would be applied to patients. Trapping the infectious centers inside agar is reminiscent of communities living in biofilms. Low bacteriophage titers are employed thus preventing the sudden killing of a large

proportion of bacteria and the release of high amount of toxins, such as lipopolysaccharide (LPS) that could induce a severe inflammatory response. The amount of bacteriophages will be sufficient to allow multiplication in the host, release of new bacteriophages and infection of adjacent bacterial cells.

3. New mutations can appear rapidly in bacteria, particularly when working with a strain which has not yet adapted to growth in laboratory conditions. So it is important to be able to go back to the culture used to isolate bacteriophage-resistant mutants, for example when performing phenotypic assays.
4. We consider that three steps of colony isolation by streaking and regrowth are necessary and sufficient to obtain a pure population of bacteria and to eliminate bacteriophages that could be attached to the surface of the bacteria. For a given experiment at least 20 independent colonies must be purified that way and stored.
5. Bacterial crude SDS lysates can be maintained at 4 °C for extended length of time, for future DNA purification.
6. Resistance is not always a clear-cut absence of bacteriophage growth. Plaques might be smaller as compared to those obtained when bacteriophage is spotted on the parental strain, and the titer may be highly reduced.
7. To exclude the presence of bacteriophages absorbed on the surface of the bacteria, it is possible to perform a virucide assay as described by de Siquiera et al. [12].
8. It is necessary to check the nature of the bacteriophage to exclude contamination by other bacteriophages used in the laboratory. Similarly, we recommend to verify the genotype of the bacterial strains and variants when there is a risk of contamination. For *P. aeruginosa* we used a simplified Multiple locus variable number of tandem repeat analysis (MLVA) method derived from Vu-Thien et al. [13]. This consists in using the VNTRs which have previously been identified as informative to distinguish the strains routinely used in the laboratory.
9. The supernatant must not be too viscous, in order to avoid pipetting the phenol phase. If this is the case, dilute by adding PBS to the sample, mix and centrifuge again.
10. At a given mutated position, the percentage of reads possessing the mutation may sometimes be less than 100%. This indicates that a proportion of the bacterial cell has the wild-type phenotype, whereas the rest is mutated. This may be due to reversion of the mutation, for example in the case of phase variation mutation. It can also result from a secondary mutation event.

11. Transformation with a wild-type gene does not always complement a mutation, particularly when a single amino acid is modified. This is due to competition between the mutated and wild-type protein.

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Chapter 9

Guidelines to Compose an Ideal Bacteriophage Cocktail

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

Abstract

Correctly designed bacteriophage therapeutics are the cornerstone for a successful outcome of bacteriophage therapy. Here we overview strategies on how to choose bacteriophages and their bacterial hosts at different steps of a bacteriophage cocktail development in order to comply with all quality and safety requirements based on the already existing essentially empirical experience in bacteriophage therapy and current accomplishments in modern biomedical sciences. A modification of the classic Appelmans' method (1922) to assess stability of bacteriophage activity in liquid media is presented in order to improve the overall performance of therapeutic bacteriophages individually and collectively in the cocktail.

Key words Bacteriophage therapeutics, Bacteria and bacteriophage of choice, Host range, Virulent bacteriophages, Adaptation of bacteriophages, Bacteriophage compatibility

1 Introduction

On the way to reclaim its niche in modern medicine, bacteriophage therapy needs to meet the challenging demands of the regulatory pathways of modern medicinal products and prove safety and efficacy based on well-documented clinical trials performed according to the current medical standards [1–3]. The statement released by the European Medicines Agency (EMA) after the workshop dedicated to the reacceptance of bacteriophage therapy, held on June 8, 2015 emphasizes the urgency for such clinical trials [4, 5]. However, the pressure caused by the global antibiotic crisis with increasing number of newly emerging antibiotic-resistant superbugs creates a challenging situation with high expectations from bacteriophage therapy [6]. This should not affect the scrutiny of the whole process of bacteriophage therapeutics production and subsequent validation. The development of effective therapeutic bacteriophage cocktails and their application in thoroughly designed standardized clinical trials should be considered as one of the most important issues in order not to provoke irreversible rejection of the bacteriophage therapy concept.

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Felix d'Herelle, one of the discoverers of bacteriophages and the initiator of the idea of applying bacteriophages for therapeutic use, was also the first person to warn that bacteriophage cocktails should be designed and developed by the laboratories having vast knowledge in therapeutic bacteriophage research and not by the companies oriented only on quick profit gain [7].

Current commercialization of bacteriophage therapy should definitely exploit the advances in molecular biology, biotechnology and modern biomedical sciences as well as the specific knowledge of bacteriophage biology and its coevolutionary aspects in order to avoid the failures of the past [8].

In this chapter, we provide recommendations on the selection of candidate bacteriophages and bacterial strains to use in the process of therapeutic bacteriophage cocktail design and production. Our recommendations are based on almost a century of experience in bacteriophage therapeutic production processes as well as on current scientific research evidence confirming the eligibility of each procedure. We also describe a modification of Appelmans' method [9] to evaluate the stability of bacteriophage activity in liquid media cultures to be applied to enhance the activity and broaden the host range of therapeutic bacteriophages and to define their complementary activity in the cocktail.

2 Selection of Bacterial Host Strains

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

The principle of bacteriophage therapy is based on the application of Darwinian ecological-evolutionary theory of controlling pathogenic microbes. Interaction between bacteria and bacteriophages can be described as antagonistic coevolution [10] that argues for considering the "couplet bacteriophage/bacterium". This principle should be considered as one of the main advantages of bacteriophage therapy and should not be compromised on its way of a quick success.

Bacteriophage cocktails cannot be considered as medications with a fixed composition neither as a classic static chemical. At the contrary, they should be produced under a continuous renewal and adaptation process. In this regard, the choice of the bacterial strains used for the development of bacteriophage cocktails is considered as crucial. Laboratories aiming at developing bacteriophage cocktails should have large continuously growing collections of pathogenic bacterial strains. At least one third of such bacterial collections needs to be renewed annually with the new isolates originating from the clinical environments and geographical areas where the bacteriophage therapeutic product will be used.

The preproduction bacterial strains should be virulent strains. The strains used in the bacteriophage selection process should have species typical physiological, biochemical, serological and genetic

features, but also clinically important nontypical strains of the species can be used. Strains should be characterized by state of the art methods in microbiology and molecular biology.

Strains used for selection of bacteriophages should be currently circulating isolates from clinical settings. Therefore, bacteriophage products developing laboratories should be in perpetual interaction with hospitals and routine bacteriology laboratories.

Strains selected with the purpose of the isolation of therapeutic bacteriophages should be evaluated anew 1 year after their introduction into the preproduction process and in case of maintaining the main biological features, including virulence properties, the validity of the strains can be prolonged. Strains should be maintained in a way that ensures they do not lose their virulence and other specific biological features.

As a rule for the isolation and selection of new bacteriophages, at least 10–15 bacterial strains, differing by various features, should be used [11–14].

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

Absolutely different requirements are applied to the strains used directly in the process of bacteriophage production, in particular for the bacteriophage propagation process on a large scale. This type of strains should guarantee maximal safety of the final product. Therefore, the following requirements should be followed strictly:

1. The industrial production strains should be typical representatives of the species. They should have well-documented pedigree history, identified at the species and strain level based on state of the art methodology of microbiology and molecular biology (genotyping methods, such as AFLP, PFGE, MLST alongside with phenotypic characterization). Preferably genome sequences of the industrial strains should be available to avoid different kinds of unexpected outcomes, such as undesirable horizontal genetic exchange(s).
2. The industrial strains should be nonvirulent and nontoxin producing to avoid toxicity in the final bacteriophage product. Therefore, for the production of bacteriophage products active against highly virulent bacteria it is recommended to use so called “surrogate” bacteriophage-sensitive strains with low pathogenicity [8, 15].
3. Industrial strains should preferably be nonlysogenic and in case such strains are not available, they should possess low frequency of spontaneous and induced bacteriophage induction ability in order to have a negligible amount of temperate bacteriophages in the final product and to avoid incurred horizontal genetic exchange. The features can be confirmed by complete genome sequencing and prophage induction methods [16].

4. Industrial strains should be low mutator strains to exclude unpredictable mutations during the production process. This can be confirmed by state of the arts methods (e.g. disk diffusion tests) [17].
5. Industrial strains should preferentially be rapidly growing and easy to culture to ensure low cost of the commercial end product.

3 Selection of Candidate Bacteriophages and Design of a Therapeutic Cocktail

3.1 Recommended Isolation Sources for Therapeutic Bacteriophages

Candidate therapeutic bacteriophages should be naturally occurring bacteriophages (not genetically manipulated) and can be isolated from various environments, such as river water, sewage, soil or rhizospheres as well from clinical samples including patient specimens such as pus, urine, feces, body fluids and others.

Some of newly emerging pathogenic bacterial species are not inhabitants of common environments [18] which challenges the bacteriophage isolation process. In this case bacteriophages should be searched in the specialized environments typical for that particular pathogen species/strains [19].

Candidate bacteriophages are recommended to be isolated by enrichment method [20, 21] using a mixture of several preproduction bacterial strains (Chapter 1, Subheading 1). The enrichment method creates certain kinds of biases by selecting fast-replicating bacteriophages which is considered as an advantageous feature for therapeutic bacteriophages. Using mixtures of bacterial strains in the process also guarantees a broad host range of newly isolated bacteriophages [8, 22].

Laboratories involved in the development of bacteriophage therapeutics should possess large collections of well-characterized candidate bacteriophages that constantly are updated.

3.2 Requirements for Single Therapeutic Bacteriophage Candidates

There are a number of requirements for therapeutic bacteriophages, parts of which can be considered as essential, important, or desirable [23, 24]. However, each requirement should be considered in the light of the application aims of the future bacteriophage product, such as sense of urgency, target infection site, and duration of treatment. Main principles of the candidate bacteriophage choice and the therapeutic cocktail design are presented in Fig. 1.

3.2.1 Bacteriophage Host Range and Ability of Killing Target Bacterial Cells

Candidate therapeutic bacteriophages should ideally have the broadest host range as possible against the target species/strains of bacteria. 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

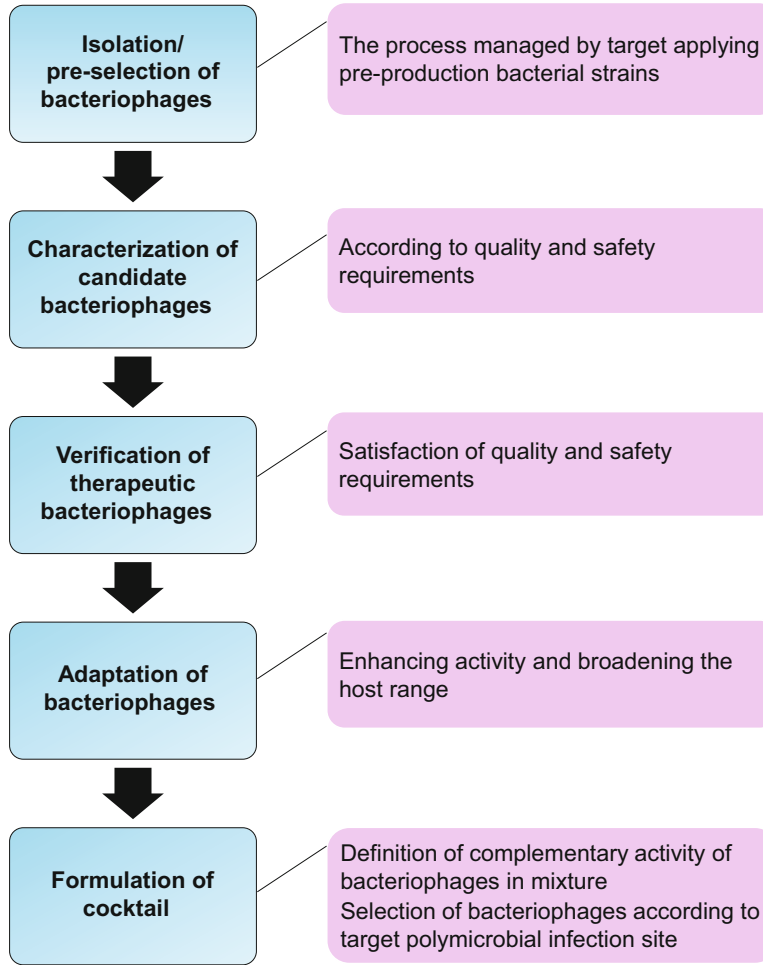

Fig. 1 Main principles of therapeutic bacteriophage cocktail design

each individual bacteriophage does not exceed 40–50% of the clinical strains and therefore a mixture of different bacteriophages (cocktail) should be assembled for application (Chapter 2, Subheading 3).

Therapeutic bacteriophages should act as "active" killers towards the majority of the 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. Bacteriophages which lack these abilities but are able to adsorb on the bacterial cell wall can still kill bacteria by "killing from without" when present in high numbers [25]. However, for successful bacteriophage therapy it is important that at the infection site the increase of the bacteriophage population surpasses that of the bacterial population. The ability of bacteriophages to propagate on target strains should be evaluated by determination of the

efficiency of plating (EOP) [26]. The threshold of EOP should be defined for each bacterial species.

The host range of candidate bacteriophages should be broadened by serial passages (at least 3–4 times per strain) in parallel on 10–15 preproduction bacterial strains in broth cultures by modification of Appelmans' method [9]. Adapting bacteriophages on bacterial strains was already widely applied by d'Herelle in his first therapeutic experiments [27]. The bacteriophage adaptation process is based on coevolution of the bacteriophage–bacterium tandem and allows not only to expand the host range of the candidate bacteriophages but also reduces resistance evolution in intermittent and chronic bacterial strains [28].

Therapeutic bacteriophages should also have high rates of clearance of target strains. It is considered that bacteriophages with high adsorption rate, short latent period and large burst size are good candidates to be applied in therapeutic treatment [23, 24]. The above physiological parameters can be defined by the one-step growth test as described by Adams [16].

Therapeutic bacteriophages should maintain their killing ability during treatment for a period as long as possible. However, emergence of bacteriophage-resistant mutants is unavoidable and therefore another criterion to be taken into account is the low frequency of emergence of resistant bacterial mutants. As a rule, the frequency of mutants' appearance in bacteriophage-sensitive strains should be in the threshold range of 10^{-7}–10^{-8} mutants per generation [11–14]. The frequency of mutants occurrence can be determined directly by the classical method as described also by Adams [16]. Ability to overcome growth of resistant mutants can be checked by coculturing of bacteria and bacteriophages in broth.

3.2.2 Safety Requirements

To preclude any kind of horizontal genetic exchange, therapeutic bacteriophages should be strongly lytic, i.e. so called virulent and nontransducing bacteriophages. Their genomes should not encode for any lysogeny conferring genes, such as integrases, transposases, or repressors. The ability for generalized transduction in lytic bacteriophages depends on DNA packaging organization and can be tested by PCR-based methods [29] or the classic transduction assay [30].

Therapeutic bacteriophages 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 bacteriophages is considered as an essential strategy for assessing their safety.

It is highly recommended that each bacteriophage used for therapeutic purposes has a clearly identified pedigree/history to confirm its naturally evolved origin. Well-defined morphology and classification of the therapeutic bacteriophages also includes their matching identity verification.

3.2.3 Stability Requirements

Candidate therapeutic bacteriophages should have a good shelf life. This means that they could be stored in an application form (galenic formulation) that guaranties their activity as well as their stability during a long-term storage period of at least one year under different conditions, including refrigeration and freeze-drying. The stability of bacteriophages 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 as essential characteristics which have to be, partly empirically, investigated. Those characteristics depend from the type of bacteriophage as well as from the formulation itself and its specific application use. Bacteriophages with such "ideal" characteristics would definitely facilitate the broadening of therapeutic application options.

3.2.4 Desirable Features

There are also some desirable features for candidate bacteriophages, such as bacteriophages having an immuno-modulatory effect in patients [31, 32] or when bacteriophage-resistant bacterial mutants emerged on the expenses of high fitness cost, including virulence reduction [33, 34]. A good example is resistant bacterial mutants of LPS-specific bacteriophages with modified receptors and reduced virulence [35, 36].

3.3 Bacteriophages in Cocktails

3.3.1 Cocktails Targeting One Bacterial Genus/Species

Correctly chosen individual therapeutic bacteriophages with broad spectrum can be applied as mono-phage therapeutics. K-like bacteriophages, active against *Staphylococcus aureus* and *Staphylococcus epidermidis* strains, are good candidates for such kind of therapeutic products [8, 37]. The host range of these bacteriophages as a rule covers more than 90% of *S. aureus* and around 80% of *S. epidermidis* clinical isolates [12, 38–40]. Preparations made with K-like bacteriophages proved to be highly effective against various kinds of infections [37, 41].

However for most bacterial species there is the necessity to apply so called bacteriophage cocktails comprising several bacteriophages 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 bacterial species.

This is a consequence of the specific population structure of a bacterial species which can be very clonal, panmictic, or epidemic. A panel of the clinical control strains for the ready-to-use cocktails should comprise at least 50 currently circulating clinical strains of the target species.

It is important to understand that bacteriophage cocktails should not be random mixtures of different bacteriophages, because a number of well-documented studies show that bacteriophages in combination can interfere with each other upon coinfection [42]. The interference can have synergetic [43, 44] as well

antagonistic effects [45, 46]. Therefore, the compatibility of bacteriophages in mixtures needs to be defined by challenging them against preproduction strains in broth cultures by Appelmans' method. The results can be monitored by measuring OD_{600}. If candidate bacteriophages collectively demonstrate higher efficacy in comparison to individual bacteriophages, the positive interference between bacteriophages of the same cocktail is guaranteed.

Another key factor to be taken into account while designing the cocktail should be the choice of bacteriophages with different bacterial cell wall receptor recognition sites [43]. Application of individual bacteriophages with multiple recognition sites is considered preferable as well, e.g. T-even bacteriophages are a good example of dual-receptor bacteriophages [47, 48]. Therefore it is highly important that bacteriophage cocktails are presented as combinations of bacteriophages prompting various kinds of resistance mechanisms in bacterial mutants.

3.3.2 Cocktails Targeting Several Bacterial Species/Genera

While targeting infection sites with polymicrobial communities, the complex cocktails containing mixtures of mono and/or complementary bacteriophages active against each bacterial genus/species are applied. Pyo- and Intestibacteriophage are the cocktails initially designed by d'Herelle himself [49] and still produced by several bacteriophage companies in different countries (e.g. Eliava BioPreparations LTD, Tbilisi, Georgia) nowadays. These cocktails contain bacteriophages active against five and seven different bacterial genera, respectively [50].

Pyobacteriophage targets various kinds of infections located in different parts of human body (jskin, respiratory tract, urological infections, purulent wounds, infected burn wounds, etc.), while Intestibacteriophage is applied widely in case of intestinal infections (dysentery, salmonellosis, dysbiosis, etc.). While designing such cocktails, first the microbial composition of target infection sites needs to be well defined through epidemiological surveys (incidence and prevalence data) while the frequency of simultaneous occurrence of different bacterial species during various infections at the infection site should be taken into consideration. Antagonistic and synergistic activity between different bacterial species should also be taken into account. For example, during early bacteriophage trials it was defined that application of bacteriophages against *Clostridium perfringens* strains with both prophylactic and therapeutic aims against gas gangrene should be combined with bacteriophages 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* carries a natural antagonism against them [51].

4 Modification of Appelmans' Method for Adaptation of Bacteriophages Against Bacterial Strains and Evaluation of Bacteriophage Complementary Activity in the Therapeutic Cocktail

4.1 Materials

1. OD_{600} spectrophotometer.
2. Centrifuge (available speed of 6000 × *g*).
3. Appropriate sterile centrifuge tubes.
4. 15 mL culture tubes resistant to chloroform.
5. Pipettes for volumes of 5 mL.
6. Variable micropipettes for volumes of 1000 and 200 μL.
7. Sterile tips for micropipettes.
8. Syringes of 5 mL and needles.
9. 0.45 or 0.22 μm syringe filters.
10. Broth media specific for the growth of bacterial species/strains (including supplements).
11. Chloroform.
12. Individual bacteriophages or mixture of candidate bacteriophage suspensions to be tested.
13. Bacterial preproduction strain broth culture in log growth phase at a minimal concentration of 10^8 CFU/mL.

4.2 Method

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

7. Bacterial growth and bacteriophage activity can be monitored by measuring OD_{600} after 24 and 48 h of incubation.
8. Results of measurement should be compared to the control tubes with positive and negative controls.
9. *In case of the bacteriophage adaptation procedure*, the tube with highest bacteriophage dilution and the OD_{600} value similar to negative control is selected and chloroform with final volume 2.0% (vol/vol) is added, tube is shaken and incubated at 4 °C for at least 2 h.
10. After incubation, the upper phase without chloroform can either be centrifuged at 6000 × *g* for 15 min or aspirated through needle into syringe and filtrated through 0.45 or 0.22 μm filters, or both procedures can be applied.
11. The obtained lysate further undergoes several (at least 3–4) above described passages on all bacterial strains from the preproduction panel to obtain adapted individual bacteriophages ready to be included in the future cocktail.
12. *In case of evaluating complementary activity of bacteriophages* the resulting values of OD_{600} measurement of bacteriophage mixture to individual bacteriophages should be compared. Values should be lower in case of mixture that means bacteriophages in mixture demonstrate higher activity than individual bacteriophages. The test can be performed on each bacterial strain or mixture of strains representing preproduction strain panel.

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Part IV

Biodistribution, Host Interaction and Clinical Application

Chapter 10

Interaction of Bacteriophages with Mammalian Cells

Zuzanna Kaźmierczak and Krystyna Dąbrowska

Abstract

Natural bacteriophages (present in the microbiome) and those applied as therapeutic agents may interact with mammalian cells and tissues. Adhesion interactions may define bacteriophage pharmacokinetics and resulting efficiency of bacteriophage agents in therapeutic applications by shaping bacteriophage homing to tissues and organs. Here we propose protocols for testing direct adhesion of bacteriophages or bacteriophage proteins to mammalian cells (in vitro). We further propose an animal model for investigation of accumulation/homing of bacteriophages in tissues (in vivo).

Key words Adhesion, Molecular imaging, Bacteriophage labeling, Mammalian cells, Phage display

1 Introduction

Both natural bacteriophages (composing microbiome) [1] and those applied for medical purposes may enter direct interactions with mammalian cells and tissues. Interactions related to adhesion may impact bacteriophage homing to tissues and organs, and they may further determine bacteriophage pharmacokinetics and resulting bacteriophage efficiency in therapeutic applications [2–4]. Characteristics of bacteriophage adhesiveness or capsid elements (proteins) that allow bacteriophages to interact directly with mammalian cells are little known [5, 6]. Here we present protocols applicable in such studies.

We propose protocols that may be applied independently or as a combination in a more complex study. The protocols comprise testing for direct adhesion of bacteriophages or bacteriophage proteins to mammalian cells (in vitro) and accumulation/homing of bacteriophages in tissues (in vivo). Animal models and visualization of bacteriophage in vivo require a labeled bacteriophage preparation. Here we propose bacteriophage labeling by fluorescent proteins (GFP, RFP, or others). These proteins can be incorporated into the bacteriophage capsid as fusions to bacteriophage proteins by phage display (Fig. 1) [7].

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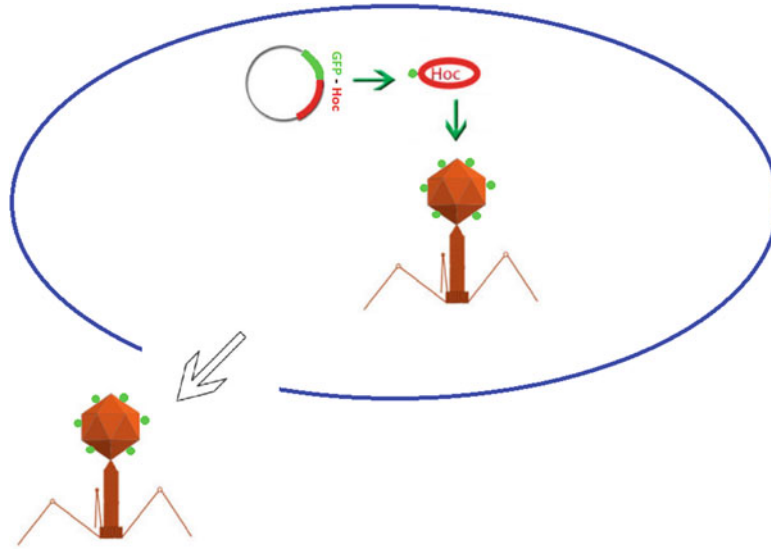

Fig. 1 Bacteriophage labeling with a fluorescent protein by phage display

2 Materials

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

2.1 Adhesion of Bacteriophages or Bacteriophage Proteins to Mammalian Cells

1. Bacteriophage or protein samples for testing: (a) bacteriophages purified in any standard procedures such as chromatography and ultracentrifugation or (b) bacteriophage proteins purified by two independent chromatography methods, e.g. by affinity chromatography and size exclusion chromatography or size exclusion and ion exchange chromatography.
2. 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: at least 300 (protein)–600 (bacteriophage) times more (*see* **Note 1**).
3. 1% (wt/vol) bovine serum albumin.
4. 50% (vol/vol) trichloroacetic acid.
5. Adherent mammalian cells (e.g. Primary Dermal Fibroblasts, FHs 74 Int, or others).
6. Cell culture medium suitable for the tested cell line (*see* **Note 2**).
7. Cell Dissociation Solution (CDS) (Sigma) or other nonenzymatic reagent for cell dissociation.
8. Cell culture plates, sterile.

9. Distilled H_2O.
10. 0.4% (wt/vol) sulforhodamine B (SRB).
11. 1% (vol/vol) acetic acid.
12. 10 nM Tris base solution.
13. Nunc MaxiSorp flat-bottom 96-well plates (Thermo Fisher Scientific) or other plates designed for preferential adsorption of proteins.
14. Cell counting chamber (e.g. Bürker chamber).
15. CO_2 cell incubator (5% CO_2, 37 °C).
16. Centrifuge.
17. Plate reader (540 nm).
18. Inverted light microscope.

2.2 Adhesion of Bacteriophages to Mammalian Cells, Tube Assay

1. Bacteriophage preparation purified by any standard procedure such as chromatography, ultracentrifugation.
2. 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: at least 300 (protein)–600 (bacteriophage) times more (*see* **Note 1**).
3. Adherent mammalian cells (e.g. Primary Dermal Fibroblasts, FHs 74 Int, or others).
4. Cell culture medium suitable for the tested cells.
5. Cell Dissociation Solution (CDS) (Sigma) or other nonenzymatic reagent for cell dissociation.
6. Eppendorf tube.
7. Cell counting chamber (e.g. Bürker chamber).
8. CO_2 cell incubator (5% CO_2, 37 °C).
9. Centrifuge.
10. Inverted light microscope.
11. Culture plates, susceptible bacteria and other materials for bacteriophage titration.

2.3 Accumulation of Bacteriophages in Tissues, Animal Model

In this procedure, competitive phage display was applied to label T4 bacteriophage particles.

1. Expression vector allowing for expression of fusion protein: GFP-Hoc, where GFP is the fluorescent marker (Green Fluorescent Protein) and Hoc is the decorative, structural protein exposed on the T4 bacteriophage capsid [8].
2. Selection of antibiotic suitable for the expression vector used in the procedure (according to vector manufacturer's information).

3. Inductor of protein expression suitable for the expression vector used in the procedure (according to vector manufacturer's information, e.g. IPTG 0.5 M).
4. Host bacteria: *Escherichia coli* expression strain sensitive to T4 bacteriophage (e.g. *Escherichia coli* expression strain B834, Novagen).
5. Tested bacteriophage in a liquid culture.
6. Culture medium LB: casein enzymic hydrolysate 10.0 g/L, yeast extract 5.0 g/L, sodium chloride 10.0 g/L, pH 7.5+/−0.2 at 25 °C, optional: (a) selection of antibiotic suitable for the expression vector used in the procedure, (b) agar 15 g/L for solid media in Petri dishes.
7. Polyacrylamide gel electrophoresis of proteins: standard materials and reagents [9].
8. Baffled Erlenmeyer flasks 1 L.
9. Flask incubator with temperature regulation and shaking.
10. Sterile filters 0.22 μm (Millipore: Steritop bottle top filter) with a vacuum pump and glass bottles.
11. Six-to-ten-week-old mice.
12. Syringes for application of tested preparations of bacteriophages, sterile.
13. Anesthetic drugs.
14. Optional: animal surgery equipment.
15. Molecular imaging hood with necessary equipment (e.g. anesthetic drug supply, light filters).

3 Methods

3.1 Adhesion of Bacteriophages or Bacteriophage Proteins to Mammalian Cells

This method was developed to test cells of an adherent cell line prepared inan in vitro culture (*see* **Note 3**). The assay is suitable for adhesion studies of either bacteriophage proteins or whole bacteriophage.

1. Cover MaxiSorp flat-bottom 96-well plate sterilely, overnight at 4 °C with the bacteriophage (5×10^9 pfu/mL) or the bacteriophage protein (10–20 μg/mL per well) 100 μL per well (*see* **Note 4**).
2. Remove protein preparations and wash the plate five times with PBS (*see* **Note 5**).
3. Block the plate for 45 min with 1% (wt/vol) albumin, 150 μL per well, room temperature (RT), do not extend incubation time.

4. Remove albumin and wash the plate five times with PBS, cover the plate to prevent drying.
5. Prepare cell from the cell culture (2–3 days at 37 °C, 5% CO_2 in appropriate medium). Cell condition and coverage of culture plates should be controlled in the optical microscope before the experiment (*see* **Note 6**).
6. Gently remove the cell culture medium.
7. Using a pipette, add CDS to cover the cell layer (approximately 0.5–1.0 mL per 10 cm^2 plate) and incubate for approximately 2 min, controlling cell dissociation in the optical microscope (*see* **Note 7**).
8. Quickly remove CDS.
9. Add prewarmed complete growth medium. Disperse the medium by pipetting over the cell layer surface several times to detach the cells.
10. Centrifuge the cells to remove residues of CDS (300 × *g* for 5 min), resuspend cells in complete cell culture medium and count in the optical microscope.
11. Dilute cells in appropriate culture medium to 5×10^4–10^5 cell per mL (*see* **Note 8**) and immediately use this cell solution for further steps.
12. Add 100 μL of cell solution to each well, gently shaking to prevent cell sedimentation and incubate the plate at 37 °C, 5% CO_2 for 2 h (*see* **Note 9**).
13. Gently remove the supernatant using a pipette, carefully avoiding scratching well bottoms.
14. Wash wells by gently adding 100 μL of cell culture medium (*see* **Note 10**).
15. Repeat **step 14** six times.
16. Add 200 μL of cell culture medium per well.
17. Add 50 μL of cold 50% (vol/vol) trichloroacetic acid per well and incubate at 4 °C for 1 h (*see* **Note 11**).
18. Wash the plate five times with distilled water.
19. Add 50 μL of 0.4% (wt/vol) sulforhodamine B (SRB) to each well and incubate for 30 min, at RT.
20. Wash the plate five times with 1% (vol/vol) acetic acid.
21. Add 10 nM Tris base solution and incubate for 30 min, at RT.
22. Read the plate: absorbance at 540 nm (strength of adhesive interaction correlates positively with intensity of absorbance).

3.2 Adhesion of Bacteriophages to Mammalian Cells, Tube Assay

1. Prepare cells from the cell culture (2–3 days at 37 °C, 5% CO_2 in appropriate medium). Cell condition and coverage of culture plates should controlled in the optical microscope before the experiment (*see* **Note 6**).
2. Gently remove the cell culture medium.
3. Using a pipette, add CDS (Cell Dissociation Solution or other nonenzymatic cell dissociation agent) to cover the cell layer (approximately 0.5–1.0 mL per 10 cm^2 plate) and incubate for approximately 2 min, controlling cell dissociation in an optical microscope (*see* **Note 7**).
4. Quickly remove CDS.
5. Add prewarmed complete growth medium. Disperse the medium by pipetting over the cell layer surface several times to detach the cells.
6. Centrifuge the cells to remove residues of CDS (300 × *g* for 5 min), resuspend cells in complete cell culture medium and count in the optical microscope.
7. Dilute cells in appropriate culture medium to 6.25×10^5 cells per mL and immediately use this cell solution for further steps.
8. Add 0.8 mL of cell solution to Eppendorf tube.
9. Add 0.2 μL of bacteriophage (bacteriophage 2.5×10^9 pfu/mL) and incubate at 37 °C, 5% CO_2 for 1 h.
10. Centrifuge the tube (300 × *g* for 5 min).
11. Remove supernatant precisely but avoiding disturbing the pellet.
12. Add 100 μL of PBS, mix gently by inversion.
13. Repeat **steps 11–12** twice.
14. Add 100 μL of PBS and mix.
15. Determine bacteriophage titer in the cell solution by the two-layer method of Adams [10] or by routine test dilution (RTD).

3.3 Accumulation of Bacteriophages in Tissues, Animal Model

This procedure allows for visualization of bacteriophages in mammalian organs and tissues, and it is helpful to understand bacteriophage circulation in vivo. Here we present a version of it that was developed for T4 bacteriophage and closely related strains that are labeled by a fluorescent protein and detected in an imaging chamber. Bacteriophage labeling is achieved by competitive phage display (*see* **Note 12**).

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.

1. Transform *E. coli* competent cells with an expression vector containing *gfp-hoc* fusion; you may use the Hanahan method [9], electroporation [9] or another method.
2. Plate the *E. coli* on LB medium with an appropriate selection antibiotic and culture at 37 °C overnight. This plate should not be stored; use the next day.
3. Prepare *initial cultures* for phage display. Start a liquid culture of transformed bacteria in 10 mL of LB medium with a selection antibiotic from a single colony. When the culture reaches $OD_{600} = 0.5$ freeze immediately 0.5 mL portions of the culture in sterile glycerol (final concentration: 25%) at −80 °C.
4. Test one portion of the *initial cultures* for expression of recombined Hoc proteins (GFP-Hoc fusions). Culture the *E. coli* portion in 100–200 mL of LB medium with a selection antibiotic up to $OD_{600} = 0.5$, collect a negative control sample of approx. 20 mL and save harvested cells at −20 °C. Induce expression in the rest of the culture (according to the vector manufacturer's instructions, e.g. by adding IPTG up to 0.5 mM), and culture for a further 3–12 h at 37 °C. Harvest bacteria by centrifugation. Evaluate the expression of GFP-Hoc proteins by SDS-PAGE [9], comparing control bacteria to the induced ones. Use this set of portions of transformed *E. coli* for the further procedure only when recombined Hoc protein is markedly overexpressed.
5. Prepare the culture of expression *E. coli* for phage display. Use one portion of the *initial culture* and add 400 mL into six 1 L baffled Erlenmeyer flasks (400 mL of LB medium, prewarmed to 37 °C, with a selection antibiotic each), and incubate with shaking at 37 °C until OD_{600} is 0.08–0.1.
6. Induce recombinant protein expression in five flasks; the sixth one serves as a negative control of expression. Add the expression inductor to a final concentration equal to 1/10 of the concentration identified as the effective one (*see* **step 3**); incubate with shaking at 37 °C for 1 h.
7. Infect four of five induced flasks with the T4 bacteriophage; the fifth flask serves as a positive expression control. Add 10^6–10^7 pfu per 400 mL flask and incubate with shaking at 37 °C for 8 h. Clarify the lysate, centrifuge 7000 × *g* for 10 min, filter with sterile 0.22 μm filters. Sterile lysates can be stored at 4 °C for at least 3 months and used in portions.
8. Test the preparation for fluorescence in a plate reader.

9. Purify the bacteriophage with any standard procedure, e.g. chromatography, monolith columns, ultracentrifugation.
10. Inject mice intravenously (*i.v.*) with 200 μL of the labeled bacteriophage, 10^{11} pfu per mouse (*see* **Note 13**).
11. Visualize fluorescence in the imaging hood in anesthetized mice (by delivery of anesthetic drugs in accordance to the hood manufacture instruction) in the time course or terminate the experiment according to the experimental design (*see* **Note 14**).
12. Optional: mice can be sacrificed and selected organs can be excised. Fluorescence in selected organs can be visualized in the imaging hood (*see* **Note 15**).

4 Notes

1. Alternatively, when PBS cannot be used, e.g. due to unstable protein of bacteriophage, other buffers, nontoxic for mammalian cells, can be used.
2. DMEM culture media or RPMI culture media with 10% (vol/-vol) Fetal Bovine Serum (FBS), 2 mM glutamine, 100 U penicillin/0.1 mg/mL streptomycin.
3. Any cells can be applied in this method, including those requiring solution cultures or freshly isolated from animal or human tissues, as long as their surface receptors are not destroyed (e.g. by proteolytic agents).
4. Please note that all necessary controls must be included. A proper set of controls depends on the experimental design, but usually: a positive control, e.g. fibronectin or collagen, should be used for optimization of adhesion, and a negative control, e.g. PBS, and control (nonreactive) proteins are recommended (other controls should be considered if applicable).
5. For the whole procedure you may use a multichannel pipette and/or an ELISA plate washer.
6. Cells in poor physiological condition or overgrown are not appropriate for adhesion studies.
7. Cells need to be controlled in an optical microscope to prevent their damage due to too long exposure to CDS or other similar agents. Time of incubation must be shorter in the case of cells that dissociate easily. Visually, dissociation is related to changes in the shape of cells that lose adhesion to the culture plate.
8. Cell density sometimes needs to be optimized according to the cell size, i.e. very small cells should be used for the assay in a concentration up to 2×10^6 cells per mL, and very large ones can be applied at 10^4 cells per mL.

9. Incubation time should be individually optimized; it usually ranges from 30 min to 2 h, depending on individual properties of cells.
10. It is necessary to apply the medium to the side of a well instead of applying it directly onto cells on the bottom. Usually, adhesion is not strong enough to resist intensive rinsing.
11. Here we propose a universal cell density detection by SRB. Other, equivalent methods can be applied here, as well as fluorescently labeled cells (in such case they can be detected by an appropriate reader directly in **step 16**).
12. Other methods for bacteriophage labeling can replace the one proposed here, as long as they do not produce toxic preparations or are unstable in vivo. Here we propose GFP-Hoc fusion to label the bacteriophage. Other fluorescent proteins (e.g. RFP, YFP) or chemical labeling can be used.
13. The dose and route of administration can be modified according to the experimental design. Bacteriophage titer should be high to achieve a good fluorescent signal.
14. For GFP-labeled bacteriophage imaging, the hood must ensure excitation 470 nm and emission 535 nm; for other types of labeling appropriate parameters must be ensured. Time of exposure, acquisition, etc. should be optimized individually. Bacteriophage homing in selected organs can be assessed quantitatively by detection and calculation of light intensity.
15. In cases when the signal is too weak to be observed in the animals, separate organs may elicit a sufficient signal for detection.

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Chapter 11

In Vivo Bacteriophage Biodistribution

Nicolas Dufour, Raphaëlle Delattre, and Laurent Debarbieux

Abstract

At the dawn of the renaissance of bacteriophage therapy, the full acceptation of bacteriophages as antibacterial agents requires the determination of their basic pharmacokinetic parameters. Such data, known for all conventional drugs used in human and veterinary medicine, allow optimizing dose regimens, efficacy, and help to limit toxicity. Here, we describe basic methods to experimentally obtain pharmacokinetic data and give also examples of data calculation to determine key parameters related to the biodistribution and elimination of bacteriophages in vivo.

Key words Bacteriophage, Bacteriophage therapy, Pharmacokinetic, Clearance, Half-life, Volume of distribution

1 Introduction

To penetrate the modern medical field and claim to be a therapeutic agent, a substance has to provide not only evidence of its efficacy but also its basic pharmacokinetic traits regarding its fate when administered in the body (absorption, distribution, metabolism, and elimination). Pharmacokinetic studies are thus a prerequisite of any therapy to optimize dose regimens, route of administration, and to limit toxicity. Despite the current use of therapeutic bacteriophages in some countries, it must be noted that exhaustive data about their pharmacokinetics are still missing. In this chapter we give basic guidelines to study bacteriophage biodistribution to determine how bacteriophages distribute in an organism after their administration.

With conventional drugs, such as antibiotics, the study of biodistribution is traditionally achieved by direct quantitative analysis (determination of the concentration of the molecule) in different organs (targeted organ, blood, urine, etc.). Others methods imply to label the molecule to indirectly quantify and follow its circulation in the body using dedicated imaging systems [1]: while classical radiolabeling is used for more than 50 years (mostly ^{14}C

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and ^{3}H, [2]), more recent technics involving others radioisotopes are now routinely available (Positron Emission Tomography or Single-Photon Emission Computed Tomography [3, 4]) as well as fluorescent [5] or magnetic resonance-based markers [6].

Regarding bacteriophages, data are scarce, even though all the above-mentioned technics could be used in theory. The earliest quantitative study was performed by Geier et al. in 1973 [7] with bacteriophage lambda: following administration of the virus using different routes (intravenous, intramuscular, intraperitoneal, and oral), mice were sacrificed at different time points and PFUs were counted in several organs (peritoneum, blood, liver, spleen, thymus, and kidneys). This study highlighted the ability of bacteriophages to reach the bloodstream regardless of the administration route (except the oral route) and confirm previous studies illustrating the key role of the spleen and the liver to retain bacteriophages [8, 9].

More recent studies have focused on bacteriophages biodistribution (mainly with filamentous bacteriophages as well as liposome-encapsulated *Caudovirales*) with direct assessment of bacteriophage particles [10–12] but also with indirect approach based on PET-imaging [13]. It should be noted that this latter strategy was applied to an extensively modified bacteriophage which was not infectious anymore. With the ongoing development of labels coupled to appropriate imaging systems, new solutions with improved characteristics in terms of sensitivity and three-dimensional spatial resolution may emerge in the near future.

However, indirect tracking has some potential drawbacks that have to be kept in mind. For example, one limit is that the labeling could interfere with the infectivity and/or the way the labeled bacteriophage is distributed and eliminated in the body. Moreover, in such models, it could be uneasy to make the difference between a signal issued from labeled entire bacteriophages and from bacteriophage debris (thus interfering with late assessment). So, in the perspective of bacteriophage therapy, the direct count of PFUs currently remains the most trusted and the easiest way to establish the number of infectious particles in an organ, also avoiding the need for a costly in vivo imaging system (the latter also having its own advantages).

More generally speaking, a bacteriophage-specific property has to be kept in mind when interpreting biodistribution data: while biodistribution of bacteriophages alone is expected to look like other drugs, it is highly probable that the biodistribution of a virulent bacteriophage in an infected animal would be significantly modified due to the adsorption to the susceptible bacterial cells causing the infection. In addition, virulent bacteriophages multiply in their host and as a result, while classical substances decay over time in an organism, bacteriophage titer increases in presence of a susceptible host. Note that when considering temperate

bacteriophages, the rate of lysogeny should also be taken into consideration [14]. The ability to self-amplify confers radical differences between biodistribution and kinetic of bacteriophages when studied in noninfected or in infected animals. Therefore, data obtained with a given bacteriophage without any amplification in vivo (uninfected animal) should not be directly and fully transposed in an infected setting.

Here we propose to describe a basic approach to assess main biodistribution parameters. We voluntarily limit this chapter to methods typically dealing with virulent bacteriophages in uninfected conditions and with a direct assessment of bacteriophage particles. Examples of data calculations are also provided to illustrate the theoretical part of the analysis. When considering similar studies in infected animals we recommend to read the extensive review by S. Abedon to grasp the multiple parameters to be assessed [15]. A particular attention should be taken on the important issue of determining the accurate number of bacteriophages and bacteria at a given time point in samples that contain both, as bacteriophages could infect their hosts during samples preparations increasing chances of contact that could lead to overestimate the amount of bacteriophages and conversely underestimate the number of bacteria.

2 Materials

1. Laboratory animals (*see* **Note 1**).
2. Bacteriophage preparation of known concentration, of suitable purity (*see* **Note 2**).
3. Indicative strain to perform bacteriophage titration.
4. Agar plates with suitable medium for bacteriophage titration (square Petri dishes are more convenient than standard ones when many titrations are required).
5. Sterile PBS buffer for tissue homogenization (for 1 L final, dissolve in distilled water: 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4, adjust pH to 7.4 with HCl).
6. Buffer for bacteriophage dilution (*see* **Note 3**).
7. Timer (needed for short time kinetic studies).
8. Appropriate preweighted and labeled tubes for organ collection and grinding, prefilled with 0.5–2 mL of PBS (depending on the homogenization system).
9. Tissue homogenizer (*see* **Note 4**).
10. Sterile scissors and tweezers for dissection.
11. Ketamine and Xylazine if anesthesia needed, obtained from veterinary suppliers. Prepare a mix of the two molecules in

physiological saline at the following concentrations: 10 mg/mL Ketamine, 2 mg/mL Xylazine. An intramuscular injection (26 G needle) of 100 μL of this mix to a 25 g mouse (i.e. 40 mg/kg Ketamine, 8 mg/kg Xylazine) provides a short anesthesia (20–30 min) deep enough to perform an intranasal instillation.

12. Glass capillaries precoated with sodium heparin for blood collection (Haematocrit capillaries, 75 mm/75 μL, ref. 910 0275, Hirschmann®, Germany).
13. Sodium heparin, needed for blood collection.
14. Precision weighing scale.
15. 96-well plates for serial dilutions (round bottom is better).
16. Ice to preserve samples during procedure.
17. Precooled (4 °C) centrifuge for Eppendorf tubes.
18. Eppendorf tubes (1.5 mL).

3 Methods

3.1 Bacteriophage Administration

1. Biodistribution depends on the route of administration and this route should be chosen according to therapeutic considerations (e.g. the oral route should be used if targeting gut bacteria, the intranasal route for airways infections). This route also influences further sampling time points (i.e. an intravenous injection will provide a very fast diffusion of the bacteriophage while an oral gavage will be slower).
2. In order to accomplish the same time frame for each replicate and decreases the inter-individual variability, it is important to establish a specific time delay for bacteriophage administration between each animal. That time depends on operator skill and on the number of organs to be harvested (usually 5–15 min).
3. Bacteriophage administration (volume, procedure, contention) must be performed in accordance with general guidelines [16] and local regulation on the care and use of laboratory animals for scientific purposes. For intravenous injection in mice, the easiest way is to use the tail vein. Oral route is achieved using a gavage procedure while intraperitoneal and intramuscular injections are routine techniques. For lung delivery, intranasal or intra-tracheal routes could be used, both requiring anesthesia to prevent coughing, swallowing and movements of the animal. The intranasal route is the easiest one for small animals but is associated with a loss of a small fraction of the inoculum (often negligible) within the nose and the nasopharynx of the animal (dead space). A small volume (20–30 μL for a 25 g mouse) is applied slowly on the muzzle of

the anesthetized animal hold in supine position with head up at 45°. While the intra-tracheal route is more difficult to perform and implies expertise for small animals (but guarantees a more precise administration to the lung), this technique turns out to be easier (and particularly recommended) with larger animals.

3.2 Time Points

1. Time points are depending on the administration route. Intravenous injection provides a quick distribution in many tissues only few minutes following injection while oral route could prove to be more impermeable and limit the bacteriophage diffusion to a detectable threshold in peripheral organs, even with a high dose intake [7].
2. As previously mentioned, numerous time points allow the experimenter to have a dynamic overview of the bacteriophage distribution/elimination and to proceed to calculations (*see* Subheading 3.7). But this is not mandatory as results could also be acquired from a unique fixed observation: e.g. "30 min after an intravenous injection, *x*% of the initial dose is located in the spleen, *y*% in the liver and *z*% in the kidneys, etc.".

3.3 Blood Collection

1. Different sites of sampling are available in mouse, more or less difficult to implement, with different yields of blood volume [17]. We recommend to obtain a volume >150 μL to assess bacteriophage titer in triplicate and to perform additional inhibition tests (*see* **Note 5** and Subheading 3.5, **step 6**). The terminal retro-orbital sampling method is quite easy and usually provides a volume of blood greater than 400 μL (i.e. a volume of plasma roughly >200 μL). We recommend using a glass capillary precoated with sodium heparin that is extemporaneously prefilled with 1 μL of additional sodium heparin (5 UI/μL) on the extremity that will be introduced into the animal.
2. Blood should be collected first in unconscious animals (but still with a beating heart to improve recovery, *see* **Note 6**), and quickly poured in an Eppendorf tube containing sodium heparin (final concentration 10–25 UI/mL) to avoid clotting. Collecting blood first help to decrease blood volume present in highly vascularized organs like liver, kidneys, lungs, or spleen and provide a more accurate assessment of viral content in the tissue part of the organ (opposed to its vascular content) [18, 19].
3. As soon as collected, place the blood sample on ice.

3.4 Organs Harvesting and Homogenization

1. Animals must be euthanized according to validated procedure and organs quickly harvested after death using clean tools.
2. As some organs may contain a high amount of bacteriophages, the tweezers and scissors have to be rinsed with large amount of

sterile water or disinfected with an adequate chemical between each organ and each animal (*see* **Note 7**).

3. Paired organs (kidneys, lungs) have to be harvested simultaneously and mixed together in the same tube.
4. Once harvested, put the organs into dedicated preweighted tubes prefilled with PBS and directly place on ice. Then, weigh the tubes and deduce the weight of each organ.
5. Homogenize the samples (*see* **Note 4**) and put them back on ice.

3.5 Determination of Bacteriophage Titer in Samples

1. From the homogenized samples, put a 500 µL aliquot in a 1.5 mL Eppendorf tube and centrifuge at maximum speed (10,000–15,000 × *g*) for 5 min at 4 °C to pellet cells and debris (*see* **Note 8**).
2. For dilution, use a 96-well plate with your usual bacteriophage buffer (*see* **Note 3**). Dispense 200 µL of the supernatant of each sample in the wells located in the upper line (undiluted, A1–A12) and make tenfold serial dilutions with a multichannel pipette using the downstream lines, until a final dilution factor of 10^7 (e.g. aspirate 20 µL from the wells that have to be diluted and dispense them in the downstream wells prefilled with 180 µL of diluent).
3. Once diluted, bacteriophage titration could be performed using standard procedures. Following a preliminary test of your bacteriophage of interest, the bacterial overlay technique could be used instead of the classical top-agar method as it is faster with an identical reliability. It consists in using a standard agar plate where a homogenous bacterial lawn is made with 1 mL of a growing culture ($OD_{600\ nm}$ 0.3–0.5). The excess volume is removed and the surface is allowed to dry (*see* **Note 9**). Then, using an 8-channel pipette, drop 5 µL of each dilution on the bacterial overlay, in triplicate (*see* **Note 10**).
4. Incubate for 6–12 h at optimal temperature and count PFUs (*see* **Note 11**).
5. Determine the titer of each replicate for each organ and each time point (if several ones). Knowing the volume of diluent put in the homogenization tubes and the weight of each organ, express the result in PFU per gram of organ and also calculate the total amount of bacteriophages per organ.
6. To determine the titer of bacteriophages in plasma (*see* **Note 12**), centrifuge the whole blood at low speed (1500–2000 × *g*, 10 min, 4 °C), retrieve the supernatant and proceed to serial dilution and titration as previously detailed. As some

bacteriophages could be inhibited by plasma antibodies, an inhibition test should be performed to unmask it (*see* **Note 5**).

3.6 Determination of Bacterial Titer in Samples (if Infected Animals Are Studied)

1. Bacteria should be tittered according to the same method used for bacteriophage titration. One exception is the need to work with noncentrifuged samples. Nonetheless, this mandatory caution implies that bacteria and bacteriophages will be present simultaneously during the process (dilution, plating, and incubation). Therefore, keep in mind that the bacterial titer that will be obtained will not necessarily reflect the exact number of bacteria present at the sampling time but rather the number of bacteria able to grow when surrounded by bacteriophages. The intensity of this limit depends on the bacteriophage/bacteria ratio in the sample, as well as the intrinsic properties of each bacteriophage such as adsorption to host surface, burst size (*see* ref. 15 for more details). Repeated centrifugation/washing with a high volume of buffer could help to limit the amount of present bacteriophages. PCR-based methods could also be used to indirectly quantify bacteria but have the potential limit to detect both dead and alive cells [20]. Another way to record the amount of bacteria present in animal is to rely on imaging devices when using bioluminescent bacteria, a method that can drastically reduce the number of animals used in the preliminary studies in order to target the most appropriate endpoint to sample organs [21].

3.7 Calculation and Modeling

1. This paragraph provides a basic insight of simple pharmacokinetic calculations and has no ambition to deal with all the available and more complex methods. However, calculations suggested here are robust enough for a first experimental approach and allow comparison between several bacteriophages. Readers could refer to classical pharmacology textbooks for more details [22].
2. Knowing the initial amount of bacteriophages administered and the amount retrieved in each organ, the fraction of the initial dose present at a given time point in each organ could be calculated (study of the distribution of the drug).
3. If several time points are available, the evolution of the viral concentration according to time in a given organ or in blood could be plotted and generally follows, as a first approximation (one-compartment hypothesis), a mono-exponential decay. This exponential decay is defined as $C_t = C_0.e^{-kt}$ where C_t is the concentration at time t, k the elimination constant and C_0 the concentration when $t = 0$ (t_0, injection time). With such equation used to fit experimental data, the half-life of a bacteriophage could be easily calculated as $t_{1/2} = \ln 2/k$ (*see* Subheading 3.8 below).

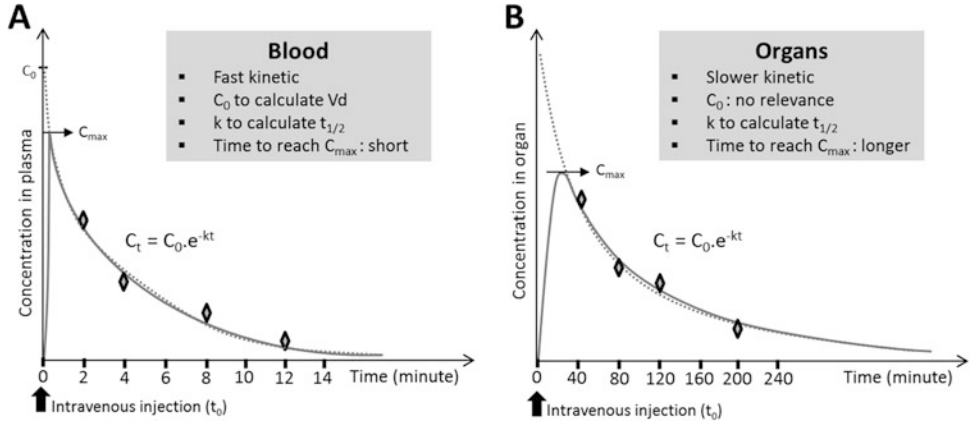

Fig. 1 Examples (fictive data) of kinetics usually observed in blood (Graph **A**, one-compartment hypothesis) and in organs (Graph **B**) for a standard substance. *Diamonds* represent the experimental measurements; the *full line curve* represents the real concentrations (as obtained by a method with a very high sampling frequency) and the *dotted curve* represents the extrapolated evolution of the concentration. Note the difference in timescale and the limits regarding the extrapolation of drug concentration before C_{max}

Nota bene: in the case of the blood compartment (Fig. 1, graph A) where an intravenous injection is almost instantaneously followed by the peak concentration (C_{max}), C_0 could be obtained from regression in order to calculate the volume of distribution, a theoretical blood compartment-related parameter (*see* Subheading 3.7, **step 6**). However, when focusing on organs (where a delay exists between the injection and the peak concentration in the organs), the extrapolation of the value of C_0 has no physiological sense (Fig. 1, graph B). In addition, the portion of the exponential curve (obtained by regression) which is located upstream the C_{max} does not reflect the real evolution of the drug concentration.

4. More complex models may be useful, especially when working on the blood compartment following intravenous administration. In this case, decay generally follows a two steps decrease: a fast slope is observed first, due to passive distribution of the virus in peripheral organs followed by a slower slope due to the elimination process. The global curve is then modeled using an equation having a more complex shape: $C_t = A.e^{-k1t} + B.e^{-k2t}$, where $k1$ and $k2$ are respectively the constant of distribution and elimination, A and B the initial concentration respectively weighted by the fraction of the span accounted for the faster and the slower components.
5. Several programs are dedicated to this kind of analysis such as GraphPad, MedCalc, or Matlab while Microsoft Excel perfectly serves this purpose for a mono-exponential curve without advanced knowledge.

6. Volume of distribution (Vd, *see* **Note 13**) is an important parameter in pharmacology as it gives information about the propensity of a substance to freely diffuse (distribute) outside the blood compartment. The Vd is calculated after an intravenous injection and implies to determine the evolution of the concentration in blood over time.
7. One method to calculate the Vd is to determine C_0, the concentration when $t = 0$. C_0 is a theoretical concentration that should exist instantaneously following an intravenous injection if the total amount of bacteriophages was instantaneously and evenly diffused in all compartments prior to any elimination. C_0 is obtained using mathematical regression from experimental measures. The Vd is then calculated as D/C_0 where D is the dose of injected bacteriophages (in PFU) and C_0 the extrapolated concentration at $t = 0$ in the blood (in PFU/mL).
8. A more reliable (but complex) method requires to calculate the Vd as the ratio of the plasmatic clearance (Cl, *see* **Note 14**) to the elimination constant:

   $\mathrm{Vd} = \mathrm{Cl}/k$.
9. Clearance (in mL/h) is calculated as the ratio of the injected dose (D, in PFU) to the area under the curve (AUC, in PFU/h/mL) linked to the evolution of the concentration over time:

   $\mathrm{Cl} = D/\mathrm{AUC}$.
10. AUC is easily calculated with basic software and should interest the full portion of the curve (from $x = 0$ to the last point, that is x value when C is null; these values being deduced from the regression, *see* Subheading 3.8 below).

3.8 Example of Data Calculation

Below is provided an example of data calculation obtained from an experiment where 10^{10} PFUs of a bacteriophage were administered intranasally in noninfected mice. Bacteriophage titer in the lungs was then followed over time at 12, 24, 48, 96 and 144 h post-administration. The same dataset would also be used as if it was obtained from blood samples (note that in reality, the timescale would be shorter because bacteriophages are more rapidly eliminated from the blood compartment than from the lung).

Table 1 provides experimental data with replicates ($n = 6$) and mean values for each time point. Mean values are plotted and a one-phase exponential regression is performed ($C_t = C_0.\mathrm{e}^{-kt}$, Fig. 2, graph A). Data could be also transformed using a Naperian logarithm (*see* **Note 15**) for linearization purpose and thus allow a linear curve fit ($C_t = kt + C_0$) (Fig. 2, graph b). In this case, the elimination constant (k) is the slope of the linear regression.

Table 1
Evolution of bacteriophage concentrations in lung over time (PFU per gram of organ) after an intranasal administration of 10^{10} PFUs

	Time (hours)	Mouse 1	Mouse 2	Mouse 3	Mouse 4	Mouse 5	Mouse 6	Mean of replicates	Mean (ln transform)
Extrapolated	0	–	–	–	–	–	–	3.8E+09	*22.06*
Measured	12	8.0E+08	9.0E+08	8.0E+08	3.0E+09	9.2E+08	5.0E+08	1.2E+09	*20.9*
Measured	24	2.0E+08	1.0E+08	9.0E+07	1.0E+08	3.0E+08	7.0E+07	1.4E+08	*18.8*
Measured	48	2.0E+07	2.0E+07	9.0E+06	5.0E+07	8.0E+06	2.0E+07	2.1E+07	*16.9*
Measured	96	4.4E+05	3.2E+05	1.2E+05	5.7E+05	1.0E+07	9.2E+04	1.9E+06	*14.5*
Measured	144	4.0E+02	1.0E+02	6.0E+01	5.0E+02	9.0E+02	5.0E+01	3.4E+02	*5.8*
Extrapolated	213	–	–	–	–	–	–	1	*0*

Six mice were used per time point with 5 different time points. The means are shown as well as the extrapolated concentration if t = 0 (C_0) and the extrapolated time when concentration becomes equal to zero. The Naperian logarithm (ln) transformation of raw values is also indicated in the last column

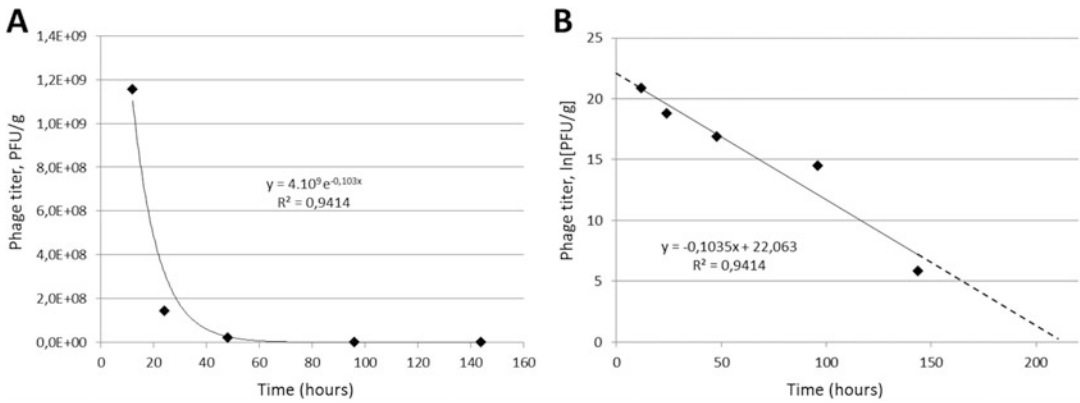

Fig. 2 Graphical representation of data provided in Table 1. Graph **a**: raw data with nonlinear regression using a one-phase exponential decay equation (*see* Subheading 3.7, **step 3**). Graph **b**: same data plotted after Naperian logarithm transformation and linear regression. *Dotted* segments represent the extension of the linear regression in order to determine C_0 and clearance

1. *Half-life*. As soon as the slope of the straight line obtained after the linear regression is known, we can calculate the half-life of the bacteriophages in the lung:
   - $t_{1/2} = \ln 2/k = 0.693/0.103 = 6.7$ h = 6 h and 42 min.

Nota bene: when working with such organ, as already mentioned on Subheading 3.7, **step 3**, the calculation of C_0 (concentration when $t = 0$) from regression is not allowed to estimate the peak concentration in the organ (C_{max} is not C_0).

2. Volume of distribution and clearance. As a second example, we will now consider the same dataset as if it was obtained from blood sampling (bacteriophage titer in plasma, *see* **Note 12**) following an intravenous injection of the same amount of bacteriophage ($D = 10^{10}$ PFUs). The apparent Vd can then be calculated.

First, C_0 is determined: this is the concentration when time (x) is equal to zero.

Starting with $y = -0.1035\times + 22.063$ (Fig. 2, Graph b) and if $x = 0$, we obtain $y = C_0 = 22.063$ PFU/mL (in ln unit). To retrieve the original value, it is necessary to reverse it using the exponential function:

- $C_0 = e^{22.063} = 3.8 \times 10^9$ PFU/mL.

Vd is then calculated:

- $\text{Vd} = D/C_0 = 1 \times 10^{10}/3.8 \times 10^9 = 10/3.8 = 2.62$ mL.

As mentioned above, the Vd could be also calculated using the plasma clearance (Cl) of the bacteriophage, with AUC determination. In this case, the time value (x) when the bacteriophage concentration in blood becomes equal to zero should be determined first.

Starting with $y = -0.1035\times + 22.063$ and if $y = 0$, we obtain $0.1035\times = 22.063$, hence $x = 22.063/0.1035 = 213$ h.

Determination of the AUC requires calculating the integral of the concentration over time from time 0 to 213 h: $\int_0^{213} f(x)\mathrm{d}x$. This could be easily done by online calculators, pocket graphing calculator or classical software such as those previously mentioned.

AUC has to be determined from the raw exponential curve ($y = 4.10^9.e^{-0.103x}$). We obtain AUC $= 4.05 \times 10^{10}$ pfu·h/mL.

Hence, as Cl $= D/$AUC we have:

- $\text{Cl} = 1 \times 10^{10}/4.05 \times 10^{10} = 0.247$ mL/h.

Vd is then deduced:

- $\text{Vd} = \text{Cl}/K = 0.247/0.1035 = 2.39$ mL.

4 Notes

1. Due to its high comfort of use (small size, low cost, easy housing, broadly available), mouse is the most used laboratory animal. However, many differences exist in drugs behavior between mouse and human according to their administration route. As bacteriophage pharmacokinetic data are scarce in the literature, the protocols reported here are focused only on mice but the experimenter should choose the most relevant animal

model according to the goal of the study. For reliability purposes, at least 4–6 animals are needed per time point. Even if optional, several time points should be studied to get a dynamic view of the biodistribution and the kinetic of bacteriophages in different organs. This allows subsequent data calculation such as half-life ($t_{1/2}$) or volume of distribution (Vd). If animals known to be coprophage are used (such as rodents or rabbits), each of them should be housed in separate cages to prevent coprophagy from one individual to another. This point is mostly relevant if a late assessment is performed (>6 h post-administration in mice) because coprophagy may lead to the re-ingestion of bacteriophages eliminated in the feces (particularly, but not only, in case of oral administration), increasing the variability. As the elimination and the distribution strongly depend on gender, age, weight, and species, the experimenter should keep the same animal setting for each experiment, especially if comparisons are required between different bacteriophages.

2. Because biodistribution is influenced by inflammation (which modifies the cardiac output, the local blood flow and recruits phagocytic cells), bacteriophages administered in animals should be pure enough to prevent a significant pro-inflammatory reaction. The degree of purity to reach depends on the route of administration (an intravenous or intraperitoneal injection requires a high purity/low endotoxin preparation while an oral administration could be performed with a diluted bacterial lysate). Purification could be performed using ultracentrifugation [23] while endotoxin removal is easily performed using commercially available affinity chromatography-based kit (e.g. EndoTrap Blue, Hyglos, Germany) or homemade procedures [24].
3. Use your routine or TN buffer (10 mM Tris, 150 mM NaCl, HCl to reach pH 7.5). Keep in mind that some bacteriophages could require divalent cations (Ca^{2+}, Mg^{2+}) to adsorb efficiently.
4. Several systems are available for this purpose, more or less time-consuming. We recommend using mechanized tools (such as Ultra-Turrax™, FastPrep™ or GentleMACS™ Octo Dissociator) rather than fully manual and time-consuming technics (e.g. pestle and mortar, Potter-Elvehjem homogenizer) which give a lesser yield of organ homogenization [25]. If a system having a shared mechanical part between samples is used (like the Ultra-Turrax™ system), take care of avoiding cross-contamination by rinsing the grinding arm between each sample following a pretested procedure to ensure that no viable bacteriophage remains.

5. A rough test could be performed as follows: add 1–5 μL of the bacteriophage solution of known titer to 50 μL of a blank plasma sample (obtained from an animal which has not received any bacteriophages), add the same volume of bacteriophage to 50 μL of a control buffer (e.g. PBS with 2% (wt/vol) BSA) and compare the titer obtained in both cases. A difference in titer greater than 1 Log is in favor of an inhibitory effect mediated by the plasma. If present, such an inhibition prevents the interpretation of bacteriophage titer in plasma samples.
6. For mice, we recommend to euthanize animals using an intraperitoneal injection of pentobarbital while CO_2 asphyxiation and cervical dislocation are not suitable for this purpose. Pentobarbital provides a quick falling asleep, followed by a coma and ultimately a cardiac arrest. Once the mouse is nonreactive towards a strong nociceptive stimulation, blood sampling may be performed while the heart is still beating. CO_2 asphyxiation does not provide this window of time between the coma and the cardiac arrest and the cervical dislocation can provoke vascular lesions in the cervical area, compromising blood supply of the retro-orbital sinus.
7. Classical detergent sprays used for surface disinfection could be used whereas 70% (vol/vol) ethanol has to be excluded due to its very low efficacy on bacteriophages.
8. If an administration of bacteria is also performed (i.e. to study the bacteriophage behavior in presence of its target), this centrifugation step is essential to pellet (and exclude) the infected bacterial cells that might be about to give birth to a viral progeny.
9. One key point with this method is to use correctly dried agar plates to prevent spreading and coalescence of bacteriophage droplets dispensed at the surface. Freshly poured agar plates have to be dried under a biosafety cabinet for at least 2 h, depending of the room hygrometry. Drying is achieved when little wrinkle-like lines start to be present at the surface of the agar medium.
10. This method allows the titration of a large range of bacteriophage concentration at the same time. The detection threshold is $\approx 10^2$ PFU/mL but it could be lowered to 10 PFU/mL by making a 100 μL drop-off of the undiluted sample onto a unique plate and spread this drop by gently shaking. High concentrations can also be numerated up to $\approx 10^{10}$ PFU/mL. If higher concentrations are expected, be sure to proceed with additional dilutions in order to obtain isolated PFUs.
11. If dealing with bacteriophages that produce large PFUs whose diameter increases over time, an early reading could be necessary (implying a shorter incubation time, e.g. 4 h) in order to

avoid coalescence of isolated PFUs and subsequent inaccurate count.

12. By convention in pharmacology, concentration of a substance in the blood compartment is expressed per volume of plasma and not whole blood. This is justified because most molecules do not dilute within the volume occupied by blood cells (which is roughly half of the whole blood volume, depending on the animal). Bacteriophages behave the same and since nonspecific adsorption on blood cells could be considered as negligible, it is preferable to express bacteriophage concentration as PFU per mL of plasma.
13. Volume of distribution (Vd) is a theoretical volume (also called volume of *apparent* distribution). The Vd is an important indicator of the extent of drug distribution into body fluids and tissues. The Vd is defined as that volume of plasma in which the total amount of a drug in the body would be required to be dissolved in order to reflect the drug concentration reached in plasma. If a drug has a large Vd that does not equate to a real volume, e.g. total plasma volume, this suggests that the drug is highly distributed in tissues. On the other hand, if the Vd is similar to the total plasma volume this will suggest that the total amount of drug is poorly distributed and is mainly retained in the plasma [22].
14. Clearance (plasmatic): volume (of plasma) cleared from a given substance per unit of time.
15. The Naperian logarithm (ln, natural logarithm) is the inverse function of the exponential function and is different from the decimal logarithm (Log_{10}, commonly used in biology). Even if both of these logarithms are symmetrical in their progression, a 2.3-fold ratio constantly exists between them (e.g. Log_{10} $10 = 1$ and $\ln 10 = 2.3$), introducing errors in calculation when mixed, whereas the global aspect of the curves will be similar in both cases.

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Chapter 12

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

Krystyna Dąbrowska

Abstract

In all cases when a bacteriophage makes direct contact with a mammalian organism, it may challenge the mammalian immunological system. Its major consequence is production of antibodies specific to the bacteriophage. Here we present protocols applicable in studies of bacteriophage ability to induce specific antibodies. The protocols have been divided into three parts: purification, immunization, and detection (ELISA).

Key words Antibody, Immunogenicity, ELISA, Immune response, Immunization, Bacteriophage

1 Introduction

Antibodies (Ab), also called immunoglobulins (Ig), are immunological proteins produced mainly by plasma cells. Their function is to identify and neutralize pathogens and foreign elements that invade the system. An antibody specifically recognizes a unique molecule that is called an antigen. Precision of their match is often compared to the key-lock match. Antibodies are commonly considered the most spectacular immunological phenomenon.

In all cases when a bacteriophage makes direct contact with a mammalian organism, it may challenge the mammalian immunological system [1, 2]. Its major consequence is production of antibodies specific to the bacteriophage. Antibody production, however, appears to depend on the route of bacteriophage administration and on individual features of a bacteriophage. It further depends on the application schedule and dose. 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 [3, 4] to devastating [5, 6].

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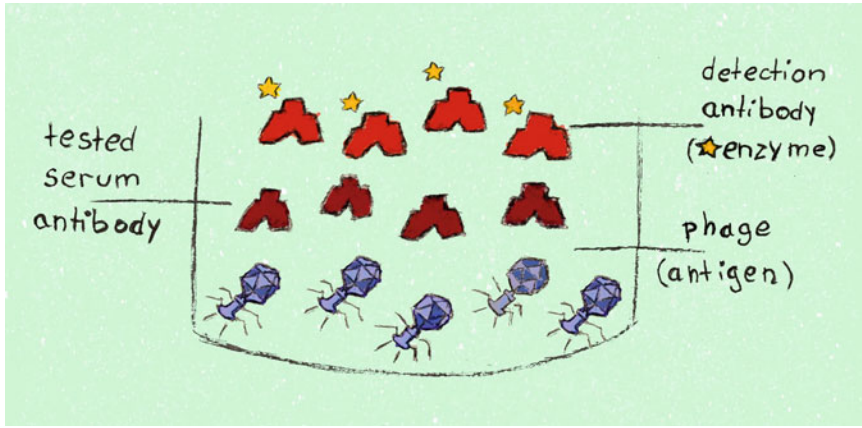

Fig. 1 Detection of bacteriophage-specific antibodies in human or animal serum by indirect ELISA

Here we present protocols applicable in studies of bacteriophage ability to induce specific antibodies. Anti-bacteriophage antibodies can be studies in human sera, as they result from natural contact with these viruses or from bacteriophage treatment. Anti-bacteriophage antibodies can also be studied in animal models, conveniently in murine models, and mouse immunization has been included in the protocols presented herein. Since bacteriophage virions typically are 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 to a bacteriophage protein are not available.

Protocols have been divided into three parts: purification, immunization, and detection (ELISA). Purification is necessary to remove bacterial remains, which are often highly reactive to the immune system and which may cause a false-positive detection or other interference at further steps. Therefore this protocol is recommended in addition to standard procedures that are routinely conducted in many laboratories [7–9].

Immunization protocols are designed to study and to compare bacteriophage ability to induce antibodies. They can be useful for investigation of consequences of antibody induction in therapeutic trials or just for preparation of highly reactive polyclonal antibodies able to detect and/or to neutralize bacteriophage. However, natural antibodies are also investigated in animals and humans. In such cases, only purification and detection protocols will be applicable [10, 11]. In any type of study, serum from a studied individual must be prepared.

Detection of specific antibodies is based on ELISA. Here, a variant of indirect ELISA (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 by 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 quantification of the reaction.

2 Materials

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

2.1 Preparation of Bacteriophages or Bacteriophage Proteins

1. Samples for testing: (a) bacteriophages purified in any standard procedures such as chromatography and ultracentrifugation or (b) bacteriophage proteins purified by 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 (Hyglos GmbH, 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: (a) 300× protein preparation volume, or (b) 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. EndoLISA (Hyglos GmbH, Germany) or Limulus Amebocyte Lysate assay.

2.2 Immunization of Mice

1. Six- to ten-week-old mice 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 Subheading 3.1), for calculation of the amount *see* doses and schedule in Tables 1 and 2.
3. Vehicle (buffer or solvent identical to that used for tested bacteriophage or protein) for treatment of control mice. Preferentially phosphate-buffered saline (PBS): 10 mM Na_2HPO_4,

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

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

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 Blood

1. Serum separated from animal or human blood (by a standard procedure).
2. Nunc MaxiSorp flat-bottom 96-well plates (Thermo Fisher Scientific) or other plates designed for preferential adsorption of proteins.
3. Highly purified preparation of bacteriophage or protein (prepared according to Subheading 3.1).
4. A microtiter plate sealing film or other accessories for covering the plates during incubation.
5. 1% (wt/vol) albumin (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 detection of human IgG; peroxidase-conjugated goat anti-mouse IgM for detection of murine IgM.
9. Substrate/substrates for peroxidase: tetramethylbenzidine (TMB), e.g., TMB X-Treme (ImmunO4, Westminster, MD, USA) or equivalent.
10. 2 N H_2SO_4.
11. Plate reader (450 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 conducted to limit any bacterial remains, which are often highly reactive to the immune system and which may cause a false-positive detection or which may 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 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 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 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 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 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 other solvent, if required) at 4 °C:
    (a) Proteins should be dialyzed at least overnight including three changes of PBS, using a dialysis tube with cut-off 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 cut-off pore size 300–1000 kDa (unless a lower cut-off 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 by 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 production of specific sera containing polyclonal antibodies, suitable, e.g., for detection of 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.

1. Inject mice subcutaneously (s.c.) with 200 μL of bacteriophage or protein preparation (*see* **Note 11**). Control mice need to be injected with vehicle. For recommended schedules *see* Tables 1 and 2. For alternative routes of preparation delivery *see* **Note 13**.
2. Sample murine blood from lateral tail vein and terminate the experiment when ready.

Typical pattern of specific IgM and IgG induction in mice challenged parenterally with bacteriophage (*Caudovirales*) is presented in Fig. 2.

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.
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 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 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.

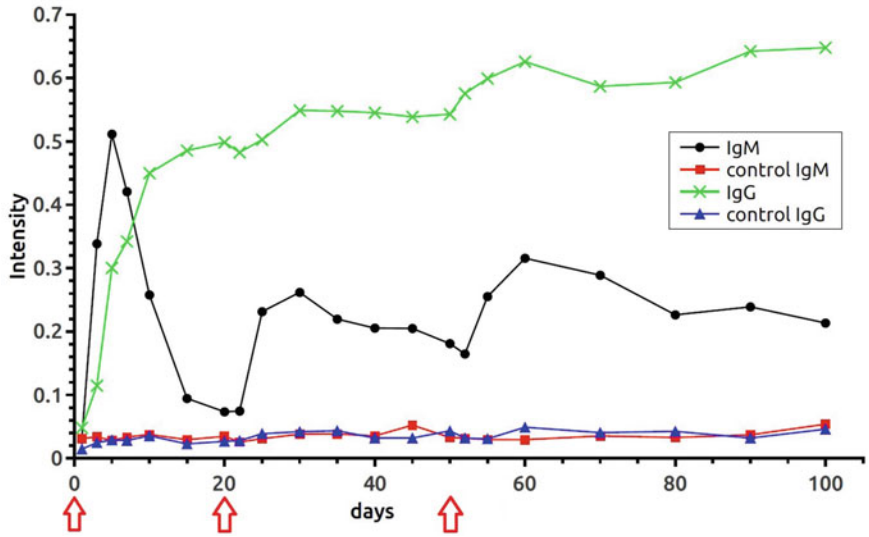

Fig. 2 Induction of specific IgM and IgG in mice challenged with bacteriophage parenterally, example pattern. IgM—serum level of IgM in bacteriophage-challenged mice; control IgM—serum level of IgM in control mice; IgG—serum level of IgG in bacteriophage-challenged mice; control IgG—serum level of IgG in control mice; intensity—relative intensity according to OD ELISA; *red arrows* indicate days of challenge (day 0, 20, 50)

11. Add H_2SO_4 (50 μL per well); the color will turn to 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 in 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 knock-out 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 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 contamination of the culture media and/or contamination of the bacteriophage preparation at various stages of production. Endotoxins are very stable and extremely immune-reactive; 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:

   (a) the amount of endotoxin content in a particular sample and (b) 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). 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 cut-off membranes (300–1000 kDa pores) is crucial for preparation of bacteriophages for immunological testing. It allows for removal of residual peptidoglycan, bacterial DNA, lipopolysaccharides (boosters), and bacterial proteins (non-bacteriophage 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 U/mL or per mouse, respectively.
11. Adjuvants are not recommended unless necessary.
12. Please note that the typical peak of specific IgM in blood is temporary, usually within the 5th–10th day after challenge; then a decrease of IgM level and a significant increase of IgG may be expected (*see* also Fig. 2).
13. Other routes of delivery are also possible although s.c. is widely considered as the most efficient and safe. Other common and convenient routes that can be used are: intraperitoneal (i.p.), intravenous (i.v.) or *per os*. Please use anesthetic drugs for animals when applicable. Please note that bacteriophage administered per os may be much less immunogenic than when administered parenterally [12], and induction of serum IgG by isolated bacteriophage proteins administered *per os* is very improbable. Additionally, a researcher must be cautious when delivering bacteriophage per os, since 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.
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 detection antibody and the antigen, a positive control should be used for 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* also **Note 16**) or a standard curve (*see* also **Note 17**) 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, etc.) to ensure the reliable signal and quality of serum. Many experiments apply serum dilutions between 1/100 and 1/10,000.

17. ELISA units can also be calculated according to Miura et al. [13, 14]. 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 Subheading 3.2. Standard serum dilution series must be included on each of your ELISA plates to obtain a standard curve for 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. Healing of the ulcers is largely delayed by the concomitant infection. Antibiotic treatment of infected ulcers is complicated by formation of microbial biofilms, which are often heterogeneous and resistant to antibiotics. Bacteriophage therapy is considered as an additional approach to the treatment of infected wounds. Here, we describe the basic method of application of bacteriophages for treatment of infected diabetic foot ulcers, including ones that are very large.

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]. About 5% of patients with diabetes develop foot ulcers each year and 1% need amputation, making diabetes the leading cause of nontraumatic lower extremity amputations in many countries [2, 3]. The comprehensive management of diabetic foot ulcers requires offloading the wound by 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 [1]. However, bacterial infections are common in diabetic foot ulcers, which are very susceptible to pathogens. There are local infections or 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 largely delayed or even prevented by the concomitant infection.

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The most common pathogens in diabetic foot ulcers are aerobic gram-positive cocci and gram-negative *Pseudomonas aeruginosa* and representatives of *Enterobacteriaceae* family including *Proteus* spp., *Klebsiella* spp., *Enterobacter* spp., and *Citrobacter* spp. Staphylococci and enterococci are the most frequent causative agents for nonthreatening limb infections, while limb-threatening infections are mostly polymicrobial and gram-negative bacteria are dominating [4, 5].

Antibiotic treatment of infected ulcers is often complicated by low bioavailability of antibacterial drugs because of reduced microcirculation and formation of microbial biofilms, which are often heterogeneous and antibiotic resistant [6]. Therefore, the development of new approaches, alternative or additional to antibiotic therapy, is required. One of the approaches is implementation of lytic bacteriophages for treatment. Here we describe a basic method of bacteriophage treatment of diabetic foot ulcers.

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, and if any of these bacteriophage preparations are active against the tested strain and the bacteriophage titer is high enough, we recommend using them. Otherwise, we use 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 with titer between 10^7 and 10^{10} pfu/mL and must have records of their thorough characterization, including determination and analysis of genome sequence and lytic properties (*see* **Note 1**).
2. Indicator host strains.
3. 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. TSB 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 $MgSO4{\cdot}6H_2O$ to a 1 L bottle and 1 L of

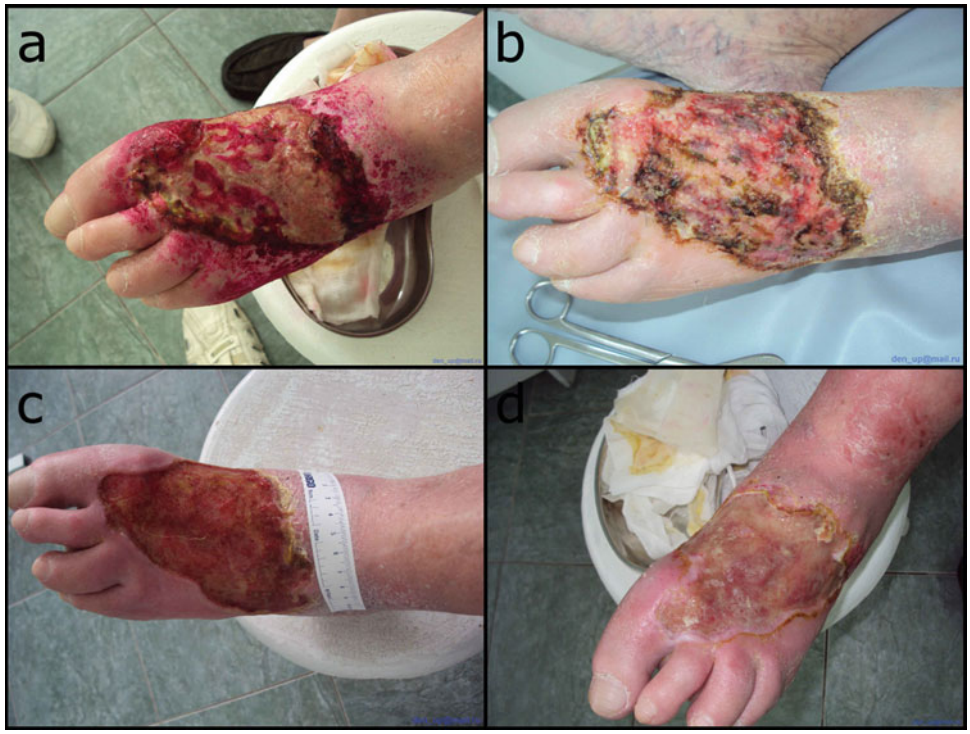

Fig. 1 Clinical case of eradication of MRSA infection with the help of bacteriophage therapy. Patient Sh., 60 years old, history of diabetes with multiple ulcers. (**a**) Beginning of bacteriophage therapy by Piobacteriophage (NPO "Microgen"), 4-12-2013. (**b**) Treatment continues, 14-12-2013. (**c**)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

deionized water. Filter-sterilize or autoclave at 121 °C for 15 min.

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 of deionized water. Autoclave at 121 °C for 15 min.
8. Transport swabs with Amies medium.
9. 0.22 μM filters (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 Strains 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,

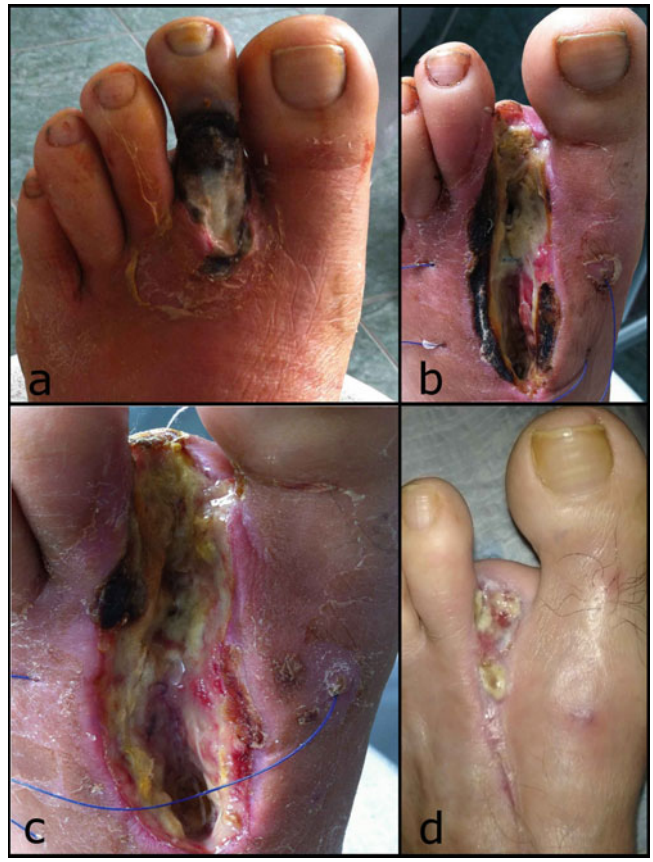

Fig. 2 In this particular case, wound was infected by MRSA resistant to other tested antibiotics. Lytic Staphylococcus bacteriophage preparation was used to prevent further growth of infection and possible subsequent amputations. (**a**) Before elective amputation, 30-05-2013. (**b**) Beginning of bacteriophage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

temperature, pain, tenderness, edema, suppuration, and the presence of suspected discharge.

2. Swab is taken from the ulcer for isolation of bacteria.
3. Bacterial isolation and identification is carried out using a routine microbiological protocol, according to the lab opportunities. Isolated bacteria are grown on selective agar media with subsequent use of Biochemical Analyzer GENIII Omni-Log (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 of 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, which 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 at the next morning. Bacteriophages are considered to be 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. For doing this, make tenfold dilutions of bacteriophage preparations in sterile SM buffer and apply drops of each dilution on the bacterial lawn. To perform accurate determination of the titer, it 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 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 (*see* **Note 6**).

3.4 Bacteriophage Treatment

It is necessary to clarify that bacteriophage therapy is used to eliminate infection from the ulcer, as a part of comprehensive management, as mentioned in the introduction. 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 following is the procedure for bacteriophage treatment:

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 important to use an antiseptic solution, for example, Chlorhexidine or Myramistin, which do not affect the viability of bacteriophages, or apply 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 the minimal volume depending on the size of the wound cavity. Rinse the wound cavity with the bacteriophage preparation, cover with gauze soaked with bacteriophage preparation, wait 10–15 min, and wrap it with dressing. When it becomes dry, replace the gauze with a new one, soaked with bacteriophage preparation. Repeat replacement up to four times per day.
3. Continue bacteriophage treatment for 2–3 weeks. 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 be absent (*see* **Note 7**). Repeat microbiological analysis every 5–6 days of treatment.
4. Protect the wound surface by 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 [7].
2. Some bacterial strains need specific agar medium for bacterial lawn formation. *Enterococcus* strains often grow slowly on

LB-plates and need 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 of 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, **item 2**. Take a large colony, thoroughly resuspend it in 200 μL of 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 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 0.22 μM filter. Determine titer of bacteriophage preparation using 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 to all of them. In this case, while the use of bacteriophages specific to one or two suspected agents leads to the elimination of them, 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), selection of a new bacteriophage preparation is required.

Acknowledgement

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Chapter 14

Compassionate Use of Bacteriophage Therapy for Foot Ulcer Treatment as an Effective Step for Moving Toward Clinical Trials

Randolph Fish, Elizabeth Kutter, Gordon Wheat, Bob Blasdel, Mzia Kutateladze, and Sarah Kuhl

Abstract

We here present detailed descriptions of successful treatment of a series of diabetic toe ulcers using the Eliava BioPreparations' commercial preparation of the very well-studied anti-staphylococcal bacteriophage Sb-1. This chapter outlines what we feel is an appropriate mechanism to speed movement toward full-scale clinical trials with bacteriophage use to treat wound infections and to help address the crisis in antibiotic resistance.

Key words Diabetic foot ulcers, Bacteriophage therapy, Debridement, Amputation, Osteomyelitis

1 Introduction

The early years of antibiotic therapy in treating soft tissue infections were filled with successes, leading to optimism that bacterial infections could finally be conquered. Unfortunately, medical literature soon emerged detailing clinical failures due to resistance. Antibiotic resistance occurs naturally, and has been present for thousands of years [1, 2], but the increase in antibiotic resistance now demands rapid development of alternative therapies. Infections involving diabetic foot ulcers (DFU) are a major public health problem around the world, and have a substantial negative impact on patient outcomes. Here, we describe exploration of the compassionate-use treatment of diabetic toe ulcers as a way to help introduce bacteriophage therapy into western medicine and help lay the groundwork for more formal clinical trials. It is estimated that in the United States, 15% of people with diabetes eventually develop ulcerations, leading to a tenfold risk of being hospitalized with a foot infection compared to those who do not have diabetes [3]. Over 60% of nontraumatic lower extremity amputations in the U.S. occur in

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diabetics. The problem becomes more serious when the underlying bone becomes infected, and often this is the final event in the path toward a lower extremity amputation. The most common organism within these wounds is methicillin-sensitive *Staphylococcus aureus* (MSSA) [4], with methicillin-resistant *Staphylococcus aureus* (MRSA) often further complicating effective treatment.

Wound healing specialists accept the responsibility of diagnosing and treating infections, but desire the most effective methods for safely resolving the issue. Unfortunately, antibiotic treatment is increasingly associated with both resistance and serious secondary complications, such as *Clostridium difficile*-associated diarrhea, as well as rapidly increasing costs. In addition, age-related microcirculation loss and disease-driven vascular dysfunction [5] have greatly increased the difficulty of successful wound treatment.

New approaches to the treatment of infections are needed. Interest is rising in the use of bacteriophages as a treatment for infections complicated by bacterial resistance, including the treatment of osteomyelitis. Literature on treatment of osteomyelitis is divided between surgical vs. nonsurgical methods. Some maintain that osteomyelitis can be resolved without the use of surgical excision of bone beyond basic debridement [6, 7], but unfortunately, no optimal antibiotic, method of delivery or duration of treatment has yet been defined. We looked at the possibility of treating digital osteomyelitis using staph-specific bacteriophage and have found that it is feasible, safe and effective in these circumstances and deserves extensive further investigation [8]. The following describes our method for treating digital osteomyelitis and underlying soft tissue infections on a compassionate-use basis using bacteriophage.

2 Procedures

The patients included in this series are outpatients in a hospital-based wound clinic, which has no associated research laboratory. Clinic cultures generally consist of a swab or small tissue sample sent to the hospital laboratory. In all cases in this series, culture reports showed *Staphylococcus aureus* as the single primary organism, with perhaps a second or third organism included, and the resulting antimicrobial sensitivities. The culture results often also show common skin contaminants, but they are not typically tested for antibiotic sensitivities. Each patient in our study had been treated with appropriate antibiotics based on the sensitivity results from the hospital laboratory, but those treatments had proven ineffective.

The patient selection criteria in this compassionate use series of cases included ulcer location (toes) and severity (IDSA mild to moderate) [9], a clear indication for elective amputation due to

poor response to previous conventional therapy, and the provision of informed consent by the patient or next of kin. Toes were chosen primarily because of consistently poor circulation and the relatively similar character of the ulcers that occur as a result of common toe deformities. Here, muscle imbalance has led to toe contractions commonly called "hammertoes," creating a pressure point at the dorsal proximal interphalangeal joint, and ulcers occur from pressure of the shoe toe box [10] against the joint or at the distal tip from weight bearing. Each of these toes had a clear indication for amputation and poor response to previous treatment; while toe amputation usually creates relatively little deformity, often the toe removal wound doesn't heal, leading to much more severe problems including further amputation.

Our patients were also selected based on their perceived ability to fully understand and follow directions, since one major problem is that patients often change their own treatment plan based on their personal feelings, advice from neighbors and relatives, or internet information [11]. No alternative treatment other than amputation was available in the U.S., nor were these patients' candidates for any established research protocol. Each patient was offered the treatment as a last option before amputation, and each gave informed consent. Thus, all treatments were ethically justified on a compassionate use basis as formalized in Section 37 of the Helsinki protocol and according to the U.S. Food and Drug Administration "compassionate use" or "expanded access" option.

3 The Bacteriophage

The bacteriophage being used is a commercial preparation of staphylococcal bacteriophage Sb-1 [12], isolated in 1977 for detailed characterization from a long-used wound therapy cocktail at the Eliava Institute, located in Tbilisi, Republic of Georgia [13]. It is a staph-specific distant relative of the bacteriophages approved by the FDA and by the European Union for dealing with *Listeria monocytogenes* in ready-to-eat foods [14]. In the 1980s, it was intravenously used in highly purified form for treatment of widespread infant and adult infections caused by *Staphylococcus aureus*. In clinical experiments performed in Moscow and Tbilisi, it was used in treating acute and chronic sepsis, peritonitis, osteomyelitis, mastitis, purulent arthritis, and other severe and chronic infections, which helped to establish its safety [14]. Sb-1 was sequenced and extensively studied under a special US Department of Health and Human Services Biotechnology Engagement Program (DHHS-BTEP) grant [12, 15]. The bacteriophage is grown in minimal medium, column purified and sealed in sterile vials in 10-ml aliquots. It is routinely tested against strains of *Staphylococcus aureus* and approved for sale by the Georgian Ministry of Health [13] and

is being brought into the US as part of a research agreement between Eliava BioPreparations Ltd., Tbilisi and the PhageBiotics Research Foundation in Olympia, Washington for the purpose of this type of compassionate use case studies, which will help establish parameters for more formal clinical trials.

Treatment involved good wound care, including soft tissue debridement when necessary. Softened and obviously infected bone was trimmed out in four cases and left in place in two cases where bone debridement would lead to an unfavorable functional and cosmetic result. The bacteriophage preparation was dripped into the wound cavity, which was then packed with plain packing gauze soaked with bacteriophage preparation (0.1–0.5 cc), (*see* patient #2 in Subheading 5), covered with Xeroform© gauze to prevent the bacteriophage solution from being wicked out of the packing, and then covered with dry gauze. The dressing with packing was kept in place for 48 h, and then the patient performed dressing changes with standard moist dressings for the balance of the week. The patients were seen in clinic weekly, and any debridement, if necessary, was performed at that time. The bacteriophage was then reapplied and the patient reappointed for the next week.

4 Patient #1 Osteomyelitis

Patient #1 was a 48-year-old man with diabetes and hyperlipidemia. His ulcer was originally caused by shoe pressure on the left third proximal interphalangeal joint of the third toe (Fig. 1). The toe cellulitis had been treated with culture-directed oral antibiotics (levofloxacin for ten days) without improvement. Examination revealed the head of the proximal phalanx floating free within the joint space, due to bacteria dissolving the neck of the phalanx, and this nonfunctional bony fragment was removed. No other bone was

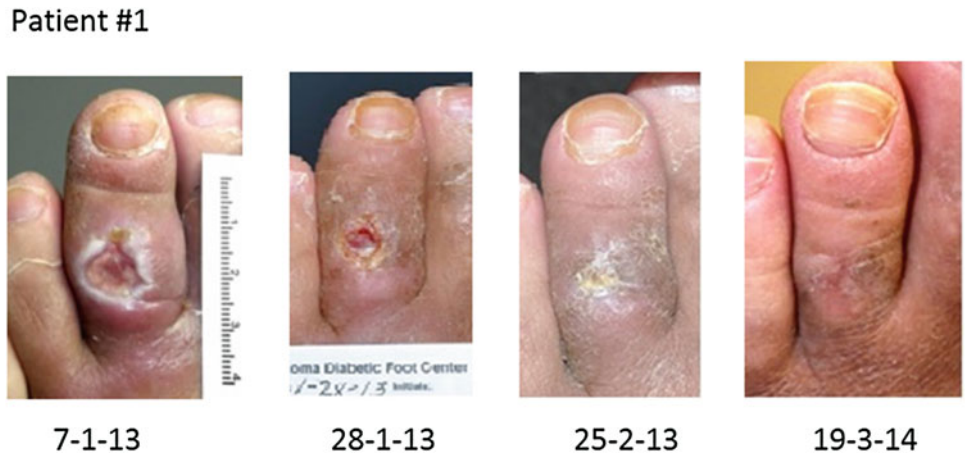

Fig. 1 Bacteriophage therapy in a 48-year-old male osteomyelitis patient. Figure reprinted from [8]

excised. Within 7 weeks of bacteriophage treatment, we observed closure of the wound. The functional result involved some toe stiffness and lack of flexion of the interphalangeal joint associated with the removal of the head of the proximal phalanx.

5 Patient #2 Osteomyelitis

Patient #2 was a 27-year-old woman with a history of spina bifida, paraplegia, hydrocephalus, seizure disorder, and depression who was referred to the wound center for an ulcer on the dorsal left second toe (Fig. 2). She has a history of ulcerations due to her lower extremity neuropathy and had been seen in the wound center on previous occasions, with one admission resulting in the left great toe being amputated for osteomyelitis. She was admitted to the wound center on 9-12-12 with a diagnosis of osteomyelitis of the

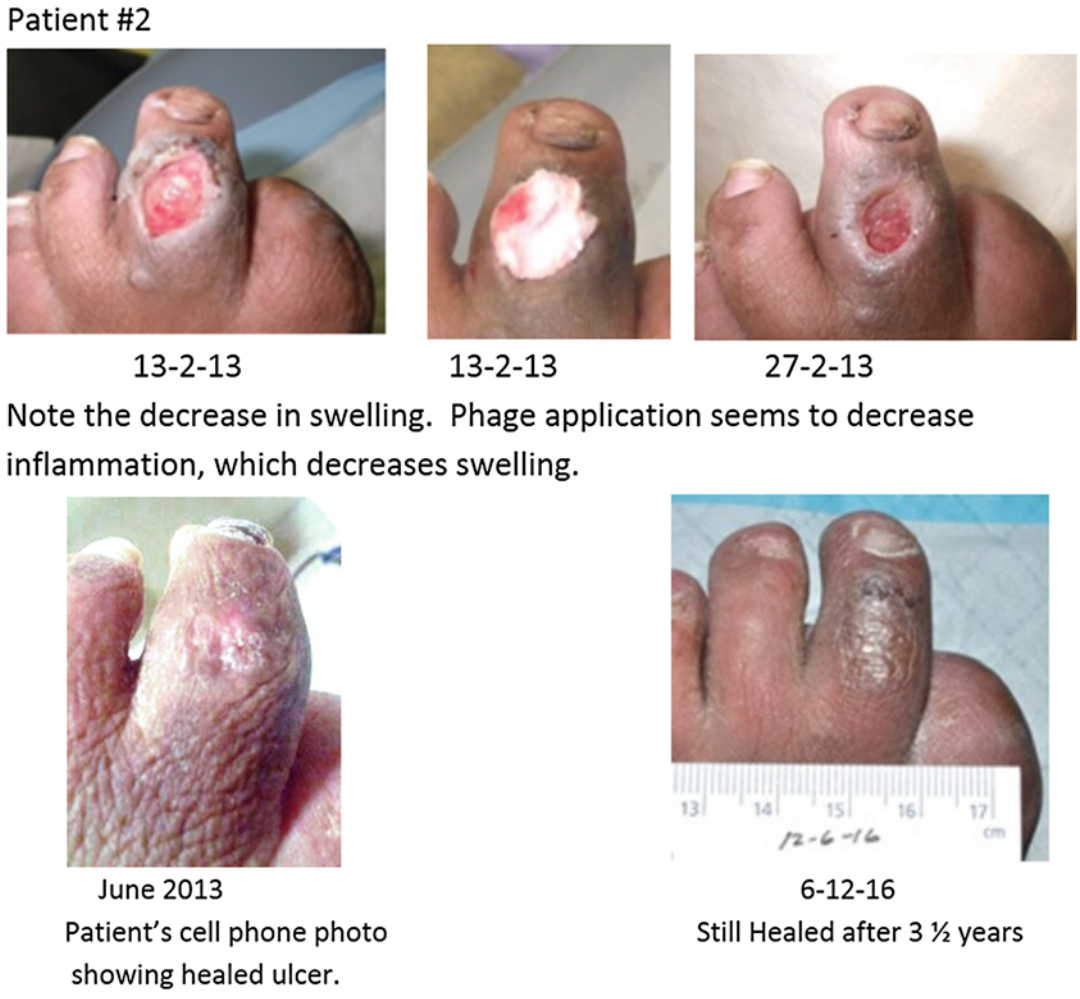

Fig. 2 Bacteriophage therapy in a 27-year-old female osteomyelitis patient. Figure reprinted from [8]

second toe with ulceration, while beginning IV antibiotic treatment as directed by the infectious disease department. After finishing the eight week IV antibiotic course, she continued on oral antibiotics for another two weeks. After weeks of care in the wound center with no change in the ulcer, amputation was considered. The patient did not wish another toe amputation, so bacteriophage treatment was offered as a last resort. The above basic protocol was followed, with the ulcer demonstrating the first signs of healing by the next clinic visit a week later. Unfortunately, after two weeks and only three doses of bacteriophage, the patient stopped wound center visits because of the need to care for her ill mother. She did send a cellphone photo in June 2013, showing that the ulcer was completely healed, and reporting it had healed uneventfully. The patient returned to the wound center in December of 2016 with a new ankle ulcer, and it was clear that the bacteriophage-treated second toe remained healed. Note the decrease in edema (Fig. 2). Bacteriophage application reduces biofilm, which decreases inflammation and edema thereby increasing microcirculation [16, 17].

6 Patient #3 Osteomyelitis

Patient #3 was a 74-year-old man with diabetes who originally injured his foot by accidentally kicking furniture in his home two months before he presented to the clinic. His history included hypertension, recurrent angina, three-vessel CABG and right femoral endarterectomy. He was under treatment, including use of the antibiotic piperacillin/tazobactam, for 7 weeks before referral to our wound care clinic. His right third and fourth toes had recently been amputated by the vascular surgeon as a result of this injury, and he presented for standard treatment of that wound (Fig. 3). In addition, an ulcer on the second toe was covered with eschar, which fell away two weeks later to expose the base of the middle

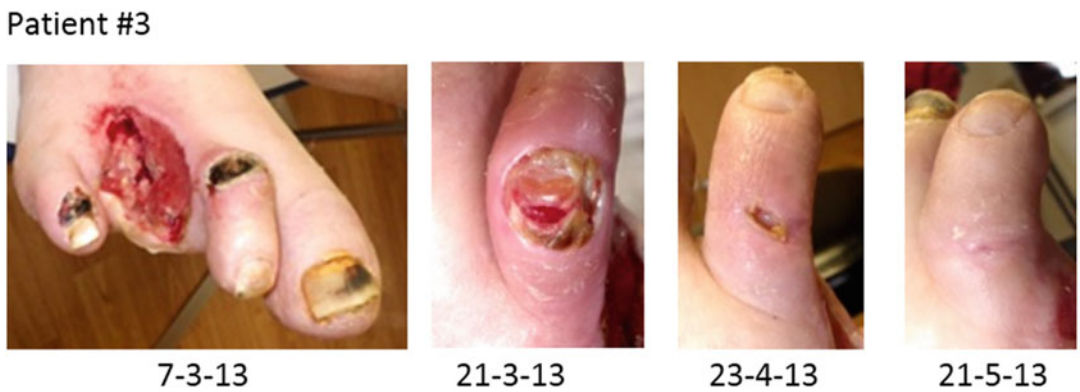

Fig. 3 Bacteriophage therapy in a 74-year-old male osteomyelitis patient. Figure reprinted from [8]

phalanx. The bone appeared soft and discolored, and was obviously infected. A bone nipper was used to excise the infected and necrotic base of the middle phalanx and weekly bacteriophage treatment was initiated. Within eight weeks of bacteriophage treatment, we observed full closure of the toe wound. The functional result included stiffness of the proximal interphalangeal joint of the toe due to joint excision, but scar contraction allowed it to retain its ability to bear weight.

7 Patient #4 Osteomyelitis

Patient #4 was a 60-year-old man with diabetes in fair control after years of poor control. He continued to smoke 1 ppd. He had been treated for 5 months without success, including 6 weeks of antibiotics (oral levofloxacin), before being referred to us for care. His vascular testing demonstrated a left toe brachial index at 0.34, with a flat line photo plethysmography tracing of the hallux microcirculation pressure, indicating extremely poor circulation (Fig. 4). Trans Cutaneous Oxygen levels at the base of the hallux in three locations ranged from 22 to 29 mmHg, below the adequate levels generally necessary for healing. While the exposed bone was obviously unhealthy, in this case it was not removed because of the size of the hallux and the possibility that the bacteriophage could assist healing over intact infected bone and thus let us preserve important great toe function—which in fact happened. Within eighteen weeks of bacteriophage treatment, we observed closure of the wound with the joint preserved. The functional result for the toe involved only mild stiffness due to prolonged immobilization.

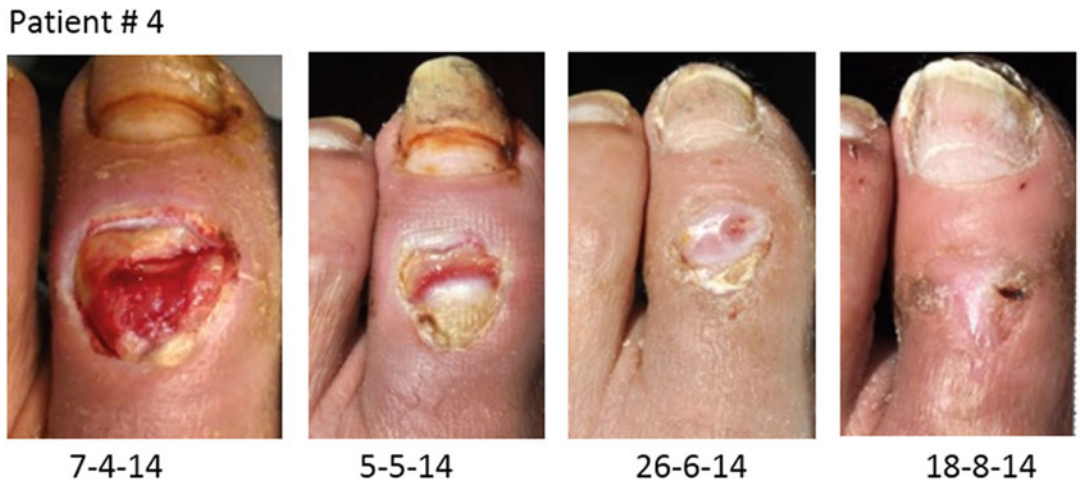

Fig. 4 Bacteriophage therapy in a 60-year-old male osteomyelitis patient. Figure reprinted from [8]

Fig. 5 Bacteriophage therapy in a 61-year-old male patient with gangrene caused by distal embolization. Figure reprinted from [8]

8 Patient #5 Gangrene Caused by Distal Embolization

Patient #5 was a 61-year-old man with a history of type 2 diabetes, hypertension, hyperlipidemia, and hepatitis C, as well as heroin and alcohol abuse. He continued to smoke 1 ppd. His critical limb ischemia was reduced by stenting but still remained significant. This patient was referred to us by his vascular surgeon for toe amputation due to the gangrenous right third toe, caused by embolization from his stenting procedure (Fig. 5). The lower side of the toe appeared healthy; however, so the decision was made to try and salvage the toe using bacteriophage. The gangrenous middle and distal phalanges and the necrotic soft tissue were removed. The remainder of the toe was treated with dehydrated human amniotic chorionic membrane (EpiFix©) rehydrated with the staphylococcal bacteriophage solution. The rehydrated amniotic membrane was reapplied one more time, but the bacteriophage was applied a total of four times. No antibiotics were prescribed as there was no infection present; therefore the bacteriophage was used only for prophylaxis. However, the damage was great enough and the risk of infection was substantial enough that the toe could have been appropriately amputated at the time. The wound healed without infection in seven weeks.

9 Patient #6 Treatment of Osteomyelitis in Patient with *Clostridium difficile* Infection

Patient #6 was a 71-year-old woman with a history of diabetes, hypertension, hyperlipidemia, rheumatoid arthritis, and stroke with expressive aphasia. She had very poor circulation in the right leg, with a toe brachial index varying from 0.17 to 0.23, leading to ulcerations, osteomyelitis, and toe amputations (Fig. 6). She had

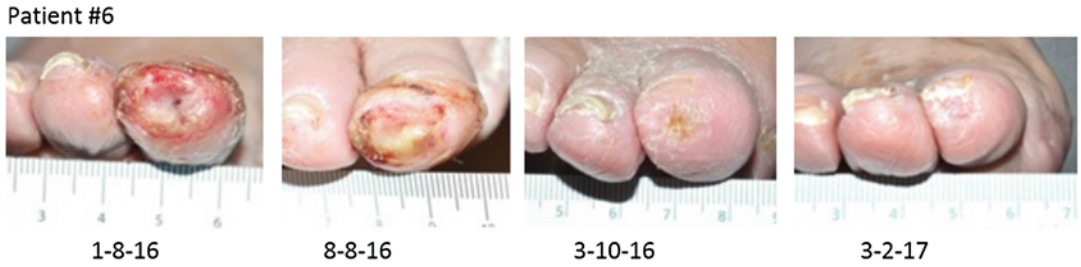

Fig. 6 Bacteriophage therapy in a 71-year-old female with toe ulcer. Figure reprinted from [8]

undergone arterial stenting plus a right hallux amputation for osteomyelitis and necrosis. Long-term antibiotics had caused a *Clostridium difficile* infection, which was being treated with oral vancomycin when she presented with right second toe ulceration and cellulitis. Since any antibiotics on top of the oral vancomycin would likely cause exacerbation of the *C difficile* infection, it was decided to use bacteriophage for the presenting cellulitis. X-ray revealed destruction of the distal phalanx of the second toe, consistent with osteomyelitis, and bone culture demonstrated *Staphylococcus epidermidis* and *Staphylococcus lugdunensis*. On examination, the distal phalanx was visible through a hole in the toe tip, so a rongeur forceps was used to remove the bone and 0.5 cc of bacteriophage was injected into the distal toe on three occasions, 8-1-16, 8-8-16, and 8-29-16. The ulcer was resolved in 8 weeks. She returned for a left fifth toe ulcer on 2-13-17, and we were able to see that the right second toe remained completely healed.

10 Results

These are typical examples from an ongoing series of cases of toe ulcers on compromised patients with exposed and/or infected bone; several of these cases and some others were already published [8]. All patients had a history of poor response to conventional therapy, and to date all patients treated with bacteriophage have successfully healed. In my (RF) experience, resolution of the ulcers would have taken much longer (if healing occurred at all) without using the bacteriophage. No adverse effects, tissue breakdown or recurrence of infection were seen, and the progression to closure was smooth and continuous after initiation of bacteriophage therapy.

11 Conclusions

1. Clinically relevant levels of bacteriophage can be reached using topical application or by injection in localized infections.

2. Topical application of a single bacteriophage targeting staph appeared to be efficacious in difficult-to-close toe ulcers containing contaminated/infected bone.
3. In all these difficult cases, where all the patients had osteomyelitis or exposed and contaminated bone, the progression to closure was complete and much more rapid than expected and none progressed to serious infection requiring amputation.

12 Discussion

The primary criticism of bacteriophage therapy is the lack of modern, double-blinded clinical efficacy trials, but a number of recent studies and reviews have explicitly investigated the question of bacteriophage therapy efficacy during clinical treatment of humans [8, 18–20]. A great deal of unpublished experience from surgeons in Poland, France, Russia, and Georgia has indicated that complicated infected foot ulcers can be safely and effectively treated with bacteriophage therapy. If the addition of well-tested commercially available bacteriophage preparations to standard treatment can be shown to improve outcomes, this would represent a major clinical advance and its broad implementation would result in substantially reduced disability, amputations, and expense.

Further investigations including controlled clinical trials are urgently needed to confirm the potential usefulness of bacteriophage as a complementary, narrow-spectrum topical antibacterial treatment for wounds, both in combination with antibiotics and alone. It is within this context that we see a role for diabetic ulcer treatment using bacteriophage. This form of wound infection treatment can be done in various settings and can be very important in informing full-scale clinical trials, as well as getting both physicians and patients used to the ideas and practice of bacteriophage therapy, and also in saving money and a great many lives.

With this in mind, it is important to use bacteriophage under careful observation, including noting dose volume, methods of application, the number and frequency of dosing, length of treatment, etc. so that we can better understand how bacteriophage are best used. While bacteriophage use in a clinic attached to a research laboratory would be optimal (*see* Chapter 13), there are few facilities within the U.S. where this would be possible. However, compassionate care addition of bacteriophage that have been extensively used elsewhere to standard diabetic ulcer treatment in private offices is a natural stepping stone, giving more flexibility to determine treatment details and collect key data. Safety is, of course, paramount. A U.S. phase I safety trial has helped confirm that this species of staph bacteriophage are safe for human wound care use [21], but more detailed data are badly needed. After

developing better understanding of bacteriophage use, proper clinical trials can be constructed. Unfortunately, officially approved therapeutic bacteriophage preparations are currently only commercially available through Eliava BioPreparations (Eliava BioPreparations, Tbilisi, Georgia) and Microgen (Federal State Scientific-Industrial Company Microgen, Moscow, Russia), but patients in concert with their physician and with consultation from one of the Phage Therapy Centers in Tblisi, Republic of Georgia, may import Eliava bacteriophages patients in concert with their physician and with consultation from the Phage Therapy Center in Tblisi, Republic of Georgia, may import Eliava bacteriophages, and other approved preparations may soon be available from other countries. Our compassionate use case series marks the very beginning of a process designed to reach the goal of developing bacteriophage applications for important clinical infections, especially diabetic wound infections and osteomyelitis.

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Part V

Production, Purification and Bacteriophage Storage

Chapter 15

Bacteriophage Production in Bioreactors

Maryam Agboluaje and Dominic Sauvageau

Abstract

The optimal conditions for the production of virulent bacteriophages in bioreactors can vary greatly depending on the host–bacteriophage system used. We present a general method for the production of virulent bacteriophages in bioreactors that can be adapted to many host–bacteriophage systems and various operating conditions (reactor volume, medium composition, temperature, etc.). The procedures detail how to establish optimal initial infection conditions (infection load and initial multiplicity of infection (MOI)), prepare the host pre-culture and bioreactor, operate the bioreactor, and harvest the bacteriophage product. Batch operation is detailed but a short discussion addresses other modes of operation, namely two-stage continuous bioreactors and two-stage cycling bioreactors.

Key words Bacteriophage production, Bioreactors, Batch reactors, Optimization, Two-stage production

1 Introduction

Considering the rapidly increasing number of bacteriophage-based technologies being developed for applications in medicine (bacteriophage therapy, diagnostics, etc.), food safety, and even as nanomaterials, to name just a few, there is a growing demand for efficient strategies for bacteriophage production. Most notably, clinical studies currently conducted for bacteriophage therapy and large-scale utilization of bacteriophages for biocontrol in agriculture and water treatment suggest significant improvements in production capacity and efficiency will be required in the near future.

Since their discovery, the production of virulent bacteriophages—either strictly virulent bacteriophages or temperate bacteriophages undergoing lytic cycle (*see* **Note 1**)—has played an important role in the development and commercialization of bacteriophage-based products, mostly notably for bacteriophage therapy. This being said, bacteriophage production has been, at least in the West, little more than an afterthought in the scientific literature. In fact, many studies referring to bacteriophage

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production investigated bacteriophage assembly, intracellular mechanisms or the induction of the lytic cycle in lysogenized hosts (e.g. [1–3]), and only a small number of publications have directly dealt with production in bioreactors (e.g. [4–8]). Recently, Grieco et al. [9] conducted one of the few studies concerned with optimizing bacteriophage production in bioreactors through a systematic approach. Although this study pertained to the production of a temperate bacteriophage, a similar methodology can be used for the optimization of virulent bacteriophage production. In this context, expertise on bacteriophage production in bioreactors has mostly been developed in industrial settings without much information seeping into the public domain. Hence, methods such as the low MOI and high MOI bacteriophage amplification protocols described in [8] often serve as templates to scale up virulent bacteriophage production, even if they were developed for small-scale amplification. While this can be useful for preliminary production, more concerted approaches can be used to improve bacteriophage productivity and bacteriophage titers at larger scales.

1.1 Challenges and Parameters Affecting Bacteriophage Production in Bioreactors

In order to achieve optimized production, it is important to understand the context and challenges related to bacteriophage production in bioreactors.

Perhaps the most important aspect to grasp is that the conditions for optimal bacteriophage production are not necessarily the same as the conditions for optimal virulence or optimal host growth. In fact, to obtain the maximum amount of bacteriophages out of a production batch, it is important to allow the host concentration to reach a value near its theoretical maximum (based on the maximum yield in the medium) before population-wide lysis takes place. Moreover, since for many bacteriophages the burst size is greater in faster growing cells [10], population-wide lysis should ideally take place just before the population enters the transition phase between exponential growth and stationary phase. This requires a fine balance between the dynamics of the bacteriophage and host populations.

Multiple factors affect population dynamics, and by relation bacteriophage production. These can be associated with the host—growth rate, density of bacteriophage receptors on cell surface, metabolic activity, and stage in cell life cycle—or the bacteriophage—adsorption rate, burst size, lysis time—or can be impacting population dynamics in other ways—infection load, initial MOI. In addition, process conditions such as temperature, pH, medium composition, aeration rate, agitation, or the presence of ions or cofactors can all impact the outcome of infections and, consequently, the yield of bacteriophage in bioreactor operation.

For example, Fig. 1 shows the impact of changing the initial MOI on the final titer for bacteriophage T4 infecting *Escherichia coli* at a given infection load and set of processing conditions. As can

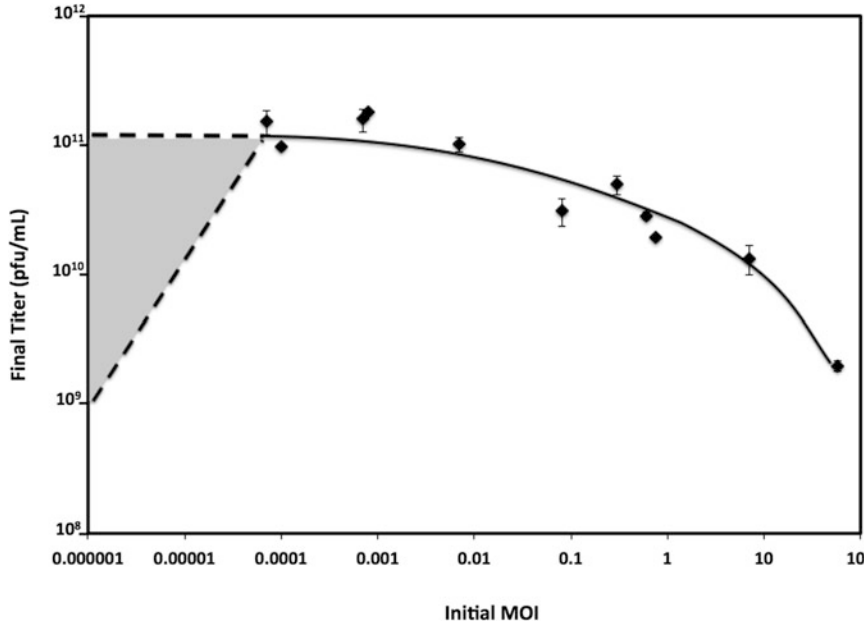

Fig. 1 Final titer as a function of initial MOI for bacteriophage T4 infecting *E. coli*. Experiments were conducted in a 1-L bioreactor at 37 °C with agitation at 200 rpm (Rushton impeller) and aeration at 0.4 L/min. The medium was a minimum mineral salt medium. The infection load was 4×10^7 colony forming units (cfu) per mL. The gray-shaded area represents the potential response at initial MOIs lower than 7×10^{-5}

be seen, decreasing the initial MOI increases the final titer up to a maximum titer of 10^{11} plaque forming units (pfu) per ml attained at initial MOIs below 0.01. At first glance, it would thus be desirable to operate the bioreactor at initial MOIs at or below this value. Further decreasing the initial MOI below 10^{-4} would eventually lead to a decrease in final titer, as a significant portion of the host population would be able to reach stationary phase before being infected—which leads to inefficient infections. Another important point to consider for bacteriophage production is the amount of bacteriophages produced per amount of cell debris. Obtaining a high titer with fewer cells should lead to cheaper, easier downstream processing (bacteriophage purification and concentration). Considering these factors, in the case presented in Fig. 1, selecting an initial MOI between 0.001 and 0.01 would be preferred for bacteriophage production.

It should also be noted that changing any of the physiological or processing parameters discussed above would change the relationship between final titer and initial MOI; thus changing the optimal infection conditions. For example, if conditions increase bacteriophage virulence, a reduction in initial MOI would likely be required.

A description of mathematical models describing bacteriophage and host population dynamics in various bioreactor systems

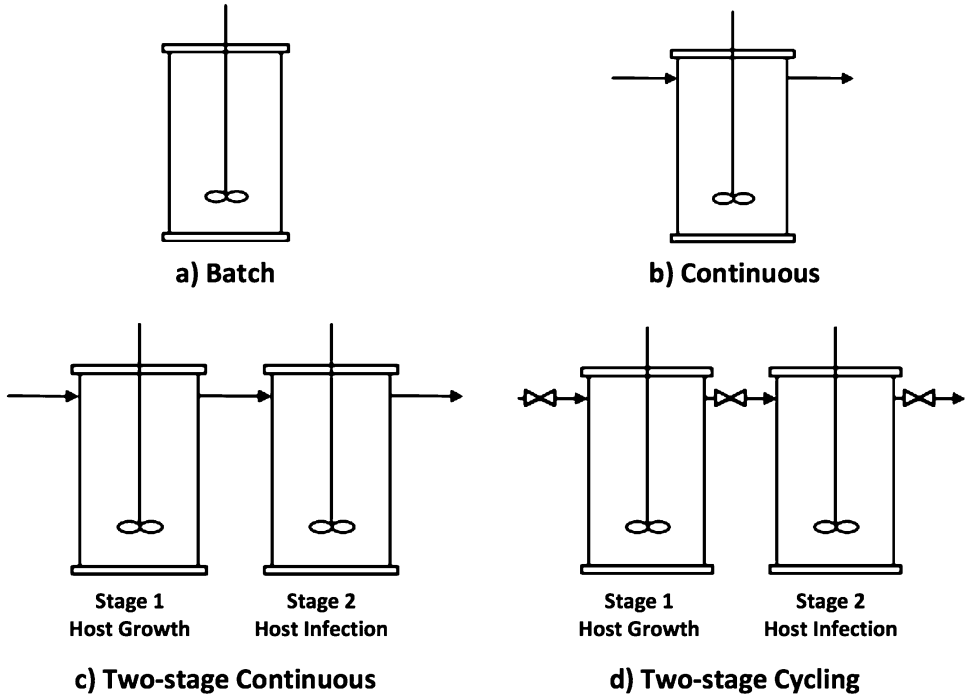

Fig. 2 Simplified schematics of reactor types used for bacteriophage production: (**a**) batch bioreactor, (**b**) single-stage continuous bioreactor (chemostat or turbidostat), (**c**) two-stage continuous bioreactor setup, (**d**) two-stage semi-continuous bioreactor setup

is found in Chapter 16. These models can be used to determine optimal infection and processing conditions.

1.2 Types of Bioreactors for Bacteriophage Production

The type of bioreactor used plays an important role in establishing the infection and operating conditions that will lead to optimal bacteriophage titers. The general types of bioreactors or bioreactor arrangements that have been used for bacteriophage production are batch, continuous, two-stage continuous and two-stage cycling (Fig. 2). The pros and cons of each strategy are discussed briefly below.

1.2.1 Batch Operation

By far, most bacteriophage production schemes rely on batch operation (Fig. 2a). In this approach, the infection is initiated at a given initial MOI, and the host and bacteriophage populations grow until the bacteriophage population takes over and population-wide lysis occurs. Since this process is highly dependent on transient conditions and population dynamics, the outcome of production (final titer) for a given set of processing conditions (temperature, medium composition, aeration, etc.) is essentially dictated by the initial conditions of infection (infection load, initial MOI, infection volume). It is thus important to identify optimal infection conditions that will lead to the highest titer—ideally with the least amount of host cell debris.

One of two approaches is generally used. In the first one, the infection is initiated at low host infection load and low initial MOI. This allows the host population to grow to a high cell number before population-wide lysis takes place. In the second approach, a large infection load is used with a high initial MOI. In this case, the goal is to infect a large portion of the host population rapidly and obtain a rapid population-wide lysis—within one to three bacteriophage replication cycles (or lysis times). While the high load/high MOI approach leads to shorter batches, the low load/low MOI approach is often favored for production because it can lead to greater final titers and lower levels of cell debris.

The main advantages of batch operation are the relatively high titers obtained, the robustness of the process, and the relative ease of operation and control [11]. On the other hand, as with most batch bioreactor processes, the disadvantages lie in the downtime required for preparation, sterilization, and cleaning of the bioreactor relative to the production time, low throughput, relatively large equipment volumes and footprints, and potential batch-to-batch variations [12].

Despite these disadvantages, batch operation remains most common, and the methods discussed in this chapter are based on this type of operation.

1.2.2 Single-Stage Continuous Bioreactor Operation

Perhaps the best-known continuous modes of operation for bacteriophage proliferation are the chemostat and the turbidostat (Fig. 2b). While they can indeed lead to bacteriophage progeny at high volumetric throughput, these single-stage continuous processes are not recommended for bacteriophage production. In fact, the dynamic nature of host–bacteriophage interactions—mostly notable by the rise of mutations and host–bacteriophage coevolution—makes it very difficult to operate the system at steady state, a prerequisite for reliable continuous production. Fluctuations in host and bacteriophage populations have been shown time and time again (e.g. [13, 14]). As demonstrated through notable work by many researchers, chemostats are great tools for evolutionary studies (e.g. [15–20]). The underlying selective pressures existing in chemostats are the basis for the "arms-race" taking place between host and bacteriophage [21] and often result in the selection of significant mutations in the bacteriophage genome; this in turn can create significant hurdles from a product quality and regulatory stand-point.

Two other factors play an important role in continuous production of bacteriophages. The first one is the existence of residence time distributions in continuous stirred-tank reactors [12]: different host cells will spend different amounts of time in the bioreactor, some of them leaving the vessel without being infected. There is thus a fine balance between the rate of addition of nutrients

(dilution rate), the rate of host cell proliferation, the rate of host cell infection and of cell lysis. Any fluctuation in one of these parameters leads to, at best, a different steady state (different host concentration, different bacteriophage outlet titer) or, at worst, washout and loss of production. The second factor is the threshold population density, the host population density for which the infection is sustained [22–24]. Below this value, the infection transmission rate is too low and the infection will die out; above it, the infection will become endemic and completely overtake the host population (leading to bacteriophage washout in continuous operation). Unfortunately, in many systems, the threshold population density occurs at fairly low concentrations and thus leads to relatively low bacteriophage titers.

1.2.3 Multi-stage Continuous Bioreactor Operation

Other bioreactor schemes, such as two-stage (Fig. 2c) and multi-stage continuous processes [25–29], enable more effective continuous bacteriophage production. The general strategy adopted in these cases is to decouple the growth of the host from the infection process. The host is thus grown in the absence of the bacteriophage in a first bioreactor (stage 1) and sent to a second bioreactor for infection (stage 2). Extra nutrients can also be added between stages 1 and 2. This strategy improves on many aspects of the single-stage continuous process but still has many limitations. (1) It generally leads to greater threshold population densities, and thus greater titers than the single-stage operation. (2) It improves the robustness of the process—however the threshold population density must still be maintained to ensure the sustenance of the infection in the second stage. So the host cells must be fed to the second stage at a rate proportional to the infection rate to maintain steady state infection. If too few hosts are fed to this stage, a larger proportion of bacteriophages will leave the bioreactor without infecting a host, leading to bacteriophage washout. (3) It lowers the probability of coevolution—since most host cell growth takes place in the first stage, where the bacteriophage is not present, the probability of encountering a mutation leading to bacteriophage resistance is greatly reduced. However, in both stages, there is still a residence time distribution, meaning some host cells can be replicating in the second stage and the rise of a resistant host is still possible. The residence time distribution also means that uninfected host cells are present in the outlet stream, which is not optimal. (4) The two stages can be operated under different sets of conditions, each optimal for their own function (optimal growth in stage 1, optimal infection in stage 2). A good example of the usefulness of this strategy was shown for the production of a recombinant protein from a inducible lysogenized bacteriophage vector [26–28, 30].

1.2.4 Two-Stage Semi-continuous Bioreactor Operation

Multi-stage semi-continuous bioreactor systems (Fig. 2d) have also been studied for the production of bacteriophages or of recombinant proteins using a bacteriophage vector [11, 31, 32]. In these cases, as in the two-stage continuous processes, the host is grown in the first stage—operated as a sequential batch reactor or as a self-cycling fermenter—and infection takes place in the second stage—where a small fraction of the bacteriophages produced in the previous cycle is used to infect host fed from the first stage. This mode of operation aims to take advantage of the robustness and high titers of batch processes, and of the high throughput, smaller downtime to production time ratios and smaller equipment footprints of the continuous processes. In addition, unlike continuous processes, there are no residence time distribution issues and, since essentially all host cells are killed and removed between cycles, there is a significant decrease in the probability of coevolution. This mode of operation has many advantages over the other types of bioreactors but it also requires a more sophisticated monitoring and control strategy.

Regardless of the bioreactor type used, the exact conditions of infection and operation will change for different host–bacteriophage systems and media. Below is an example of the development of a batch production scheme for the virulent bacteriophage T4 infecting *E. coli* in a defined minimum mineral salt medium conducted in a batch bioreactor of working volume 10 L. The procedures involve a rapid study to determine optimal conditions of infection, growth of host pre-cultures, and bioreactor preparation, operation and harvesting. The volumes and conditions can be adapted to smaller and larger production volumes.

2 Materials

2.1 Growth Medium

The selection of medium for bacteriophage production will depend on the host–bacteriophage system of interest; some complex media favor the growth of the host—which often affects positively the burst size—while, on the other hand, defined media may simplify downstream processing procedures—reducing the demand for the removal of undesirable media components (e.g. ingredients of animal origin). The present procedure details the production of 1 L of a defined minimum mineral salt medium used for production. The quantities can be adjusted to produce any desired volume of medium.

1. Minimum mineral salt medium (1.1×): Add 800 mL of deionized, distilled water to a 1 L graduated cylinder before being transferred to a 2 L glass media bottle containing a magnetic stir bar. Place the media bottle on a magnetic stirrer hot plate. Add—in order and leaving time for one salt to dissolve before

adding the next—6 g sodium phosphate dibasic, 4 g ammonium nitrate, 4 g potassium phosphate, 0.014 g disodium EDTA, 0.01 g calcium chloride dihydrate, 0.01 g iron sulfate heptahydrate and 0.05 g yeast extract. Mix until all salts are dissolved. Transfer the solution back to the 1 L graduated cylinder and bring the volume to 900 mL by adding deionized, distilled water. Transfer the solution to the glass media bottle (*see* **Note 2**). Sterilize the solution by autoclaving for 45 min at 121 °C and 204.7 kPa (15 psig) (*see* **Note 3**).

2. Glucose solution (10×): Add 80 mL of deionized, distilled water to a 100 mL graduated cylinder before transferring to a 500 mL glass media bottle containing a magnetic stir bar. Place the media bottle on a magnetic stirrer hot plate. Add, in order, 0.2 g magnesium sulfate heptahydrate and 2 g glucose. Mix until all components are dissolved. Transfer the solution back to the 100 mL graduated cylinder and bring the volume to 100 mL by adding deionized, distilled water. Transfer the solution to the glass media bottle. Sterilize the solution by autoclaving for 45 min at 121 °C and 204.7 kPa (15 psig) (*see* **Note 3**).
3. Minimum mineral salt medium (1×): Once the solutions have cooled down (*see* **Note 4**), add the glucose solution (10×) to the glass media bottle containing the minimum mineral salt medium (1.1×) in a sterile environment (in a laminar flow biological safety cabinet or near a flame, or directly to the bioreactor for larger volumes) to obtain the complete minimum mineral salt medium.
4. 1 L and 100 mL graduated cylinders.
5. 2 L and 500 mL glass media bottles.
6. Magnetic stir bars.
7. Magnetic stirrer hot plate.

2.2 Determination of Initial Infection Conditions for Production

1. 400 mL of sterilized growth medium.
2. Forty (40) sterilized 50 mL shake flasks.
3. Incubator-shaker with temperature control.
4. Forty (40) sterilized 1.5 mL microcentrifuge tubes.
5. 20 mL of host culture: a growing culture of the host of interest at an optical density at 600 nm (OD_{600}) of 0.6 (cell concentration of approximately 5×10^8 cells/mL) in the medium that will be used for bacteriophage production.
6. Bacteriophage stock of known titer (ideally 1×10^{10} pfu/mL or greater).
7. Double-layer agar plates: Prepared according to standard techniques as detailed in [33] (*see* **Note 5**).

8. Incubator for agar plates.
9. Forty (40) 5 mL sterile syringes.
10. Forty (40) 0.2 μm syringe filters.

2.3 Host Pre-culture

The following material is recommended for a pre-culture to be used to initiate a 10-L bioreactor production batch. The quantities can be modified to accommodate any desired production volume (*see* **Note 6**). Two flasks are prepared to account for any potential problem with one of the pre-cultures.

1. 200 mL of sterilized growth medium.
2. Two (2) sterilized 500 mL shake flasks.
3. Incubator-shaker.
4. Host culture: The host can be obtained from colonies on an agar plate, an agar slant, from a culture growing in suspension, or from a frozen culture aliquot.

2.4 Bioreactor

The following material is described for a typical bioreactor. This should be taken as an example and can be adapted depending on the required condition for growth and infection, and on the desired monitored parameters.

1. Bioreactor: Includes vessel, Rushton impeller and motor for agitation, sampling/harvesting port, aeration port with 0.2 μm in-line HEPA filter and sparger, outlet gas stream port with condenser and 0.2 μm in-line HEPA filter, in-line CO_2 and O_2 sensors in outlet gas stream, ports for in situ probes (typically pH probe, dissolved oxygen (DO) probe), port for thermowell/thermocouple, electrical bioreactor heating blanket and cooling coils, ports for acid and base addition for pH control, ports for level/foam detector and antifoam addition, and port with septum for inoculum addition, peristaltic pumps for antifoam, acid and base addition (*see* **Note 7**). Refer to Fig. 3 for a simplified schematic of the bioreactor set up.
2. 9 L of minimum mineral salt medium (1.1×).
3. 1 L of glucose solution (10×).
4. Air source with regulated flow rate for aeration (*see* **Note 7**).
5. 5 N NaOH solution (base).
6. 2 N H_2SO_4 solution (acid).
7. Antifoam agent (e.g. antifoam A).
8. Autoclavable silicon tubing.
9. Tubing clamps.
10. Peristaltic pump with sterilized tubing.
11. Sterilized sample vials.

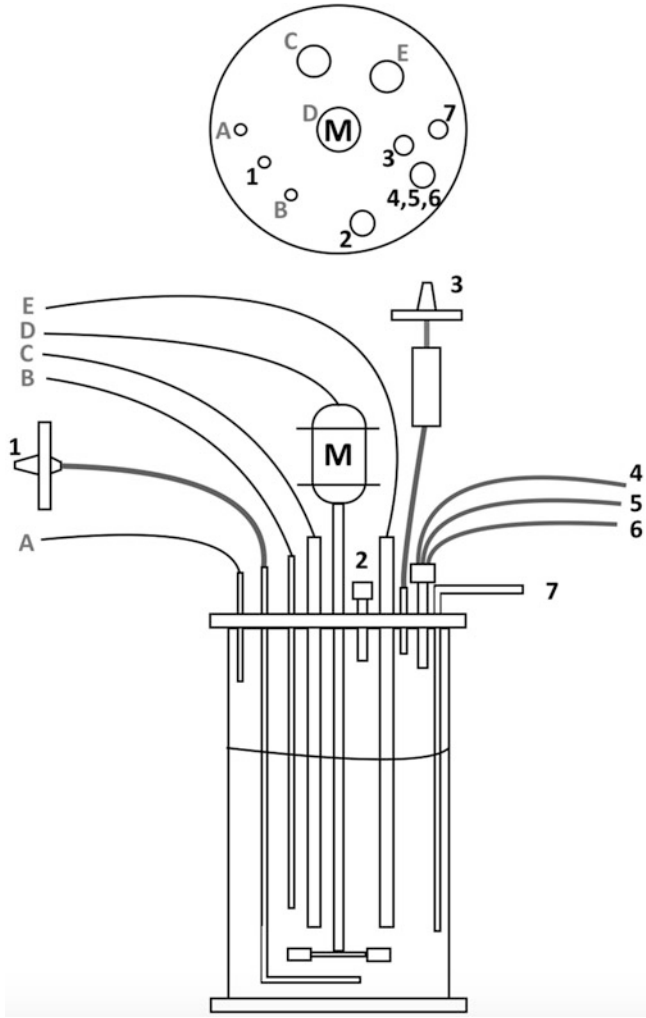

Fig. 3 Simplified schematic of a batch bioreactor setup with some accessories. *Black numbered ports* indicate process *lines* while *gray lettered ports* indicate connections to the data acquisition and control system. Top- and side-views of the bioreactor are shown. Port *1*: air-line in connecting to in-line HEPA filter; *2*: inoculation/injection port with septum; *3*: off-gas line to condenser and in-line HEPA filter (this line can further be connected to a gas analyzer for Oxygen Uptake Rate or Carbon Dioxide Evolution Rate determination; *4*: antifoam addition port; *5*: acid addition port; *6*: base addition port (antifoam, acid and base addition can be performed in a single port); *7*: sampling/harvesting port; *A*: level sensor for detection of foam; *B*: thermocouple; *C*: dissolved oxygen probe; *D*: motor for agitation; *E*: pH probe. Note that ports and accessories may be removed or added as required

12. Host pre-culture at an OD_{600} of 0.6 (cell concentration of approximately 5×10^8 cells/mL); obtained in Subheading 3.2 below. The volume of pre-culture required is determined based on the results obtained in Subheading 3.1 below. In the present case, 100 mL of pre-culture are required (1% inoculum).

13. Bacteriophage stock of known titer (ideally 1×10^{10} pfu/mL or greater).
14. One (1) sterile 20-mL luer-lock syringe with one (1) sterile 20 G luer-lock needle for the addition of bacteriophages.
15. Spectrophotometer.
16. Aluminum foil.
17. 10 mL of glycerol for thermowell.

2.5 Harvesting

1. Sterilized harvesting vessels (media bottles or carboys).
2. Harvesting peristaltic pump with sterilized tubing.
3. 0.45 μm in-line cartridge filter.
4. Double layer agar plates: Prepared according to standard techniques as detailed in [33] (*see* **Note 5**).
5. Incubator for agar plates.

3 Methods

3.1 Determination of Initial Infection Conditions for Production

Since the outcome of the infection process is greatly determined by the populations dynamics, it is crucial to clearly define the initial and operating conditions in order to optimize bacteriophage production. The initial infection conditions include the infection load (number of host cells present), the initial MOI and the infection procedure itself (are host and bacteriophages added directly to the operating volume or premixed for a short period in a pre-culture volume); while the operating conditions include temperature, aeration rate, agitation rate, medium composition, etc. Typically, the operating conditions are selected to favor the growth of the host; but other considerations may prevail, for example a simple synthetic medium may be selected to facilitate downstream processing.

The method detailed here aims to select the optimal initial infection conditions that will lead to the highest bacteriophage titer for a given set of operating conditions. It is important to perform this short preliminary study in conditions as similar to those of the bioreactor (for example, same infection procedure, temperature and medium composition) to ensure the results will be directly transferable to larger scale operation.

1. Prepare forty (40) 50 mL shake flasks each containing 10 mL of culture medium (*see* **Note 8**).
2. Establish a range of infection loads and initial MOIs to be tested. Typical ranges are 1% (vol/vol) to 5% (vol/vol) (0.1–0.5 mL in 10 mL cultures) of host cultures at an OD_{600} of 0.6 (cell concentration of approximately 5×10^8 cells/mL) for infection loads, and 1×10^{-7}–1×10^{1} for initial MOIs.

3. Preheat the shake flasks with their contents to the desired processing temperature to be tested (in the present case, 37 °C).
4. Using a pipetter, place the appropriate amount of host cell cultures based on the desired infection loads to be tested in each of the shake flasks.
5. Add bacteriophage stock to appropriate initial MOI for each of the shake flasks (*see* **Note 9**).
6. Incubate the shake flasks at a temperature (37 °C) and agitation rate (250 rpm) representative of the expected bioreactor operating conditions (*see* **Note 10**) for 8 h or until population-wide lysis occurs (detected by a noticeable reduction in the optical density of the culture).
7. Upon lysis, using the 5 mL syringes and 0.2 μm syringe filters, filter 1 mL of each lysate in individual sterile microcentrifuge tubes.
8. Determine the titer of each condition tested by using a dilution/spotting assay with the double-layer agar plating technique and incubating the plates.
9. Prepare a plot of the final titer obtained as a function of both infection load and initial MOI (*see* Fig. 4) and identify the region of infection conditions for which the optimal titers were obtained. Based on the results observed in Fig. 4, for the *E. coli*-bacteriophage T4 system grown in minimum mineral salt medium under the given processing conditions, the optimal titers were obtained for infection loads between 1% and 2% and initial MOIs between 1×10^{-7} and 1×10^{-6}. For practical reasons, and to minimize cell debris after cell lysis, an infection load of 1% with an initial MOI of 1×10^{-6} should be selected. These initial infection conditions can then be used in the bioreactor operation for optimal bacteriophage production.

3.2 Host Pre-culture

The following method describes the preparation of 100 mL of host pre-culture for a 10-L bioreactor production batch based on the optimal infection load found in Subheading 3.1. The same approach can be used with larger volumes for larger production scales. In the eventuality of using production batches of much larger volumes, it would be recommended to prepare the pre-culture in a smaller bioreactor (rather than in shake flasks).

1. Place 100 mL of sterilized growth medium in each of the two sterilized 500-mL shake flasks.
2. Preheat the shake flasks with their contents to the desired processing temperature (in the present case, 37 °C).

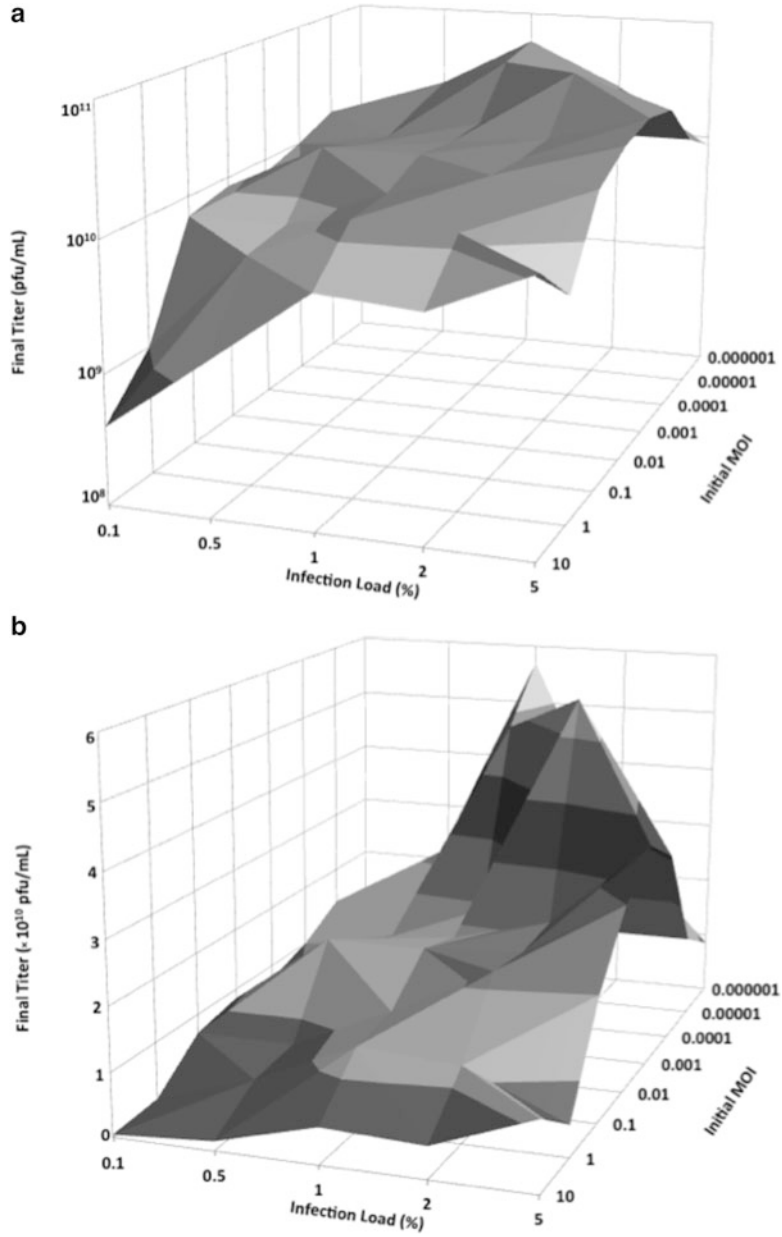

Fig. 4 Results from experiments conducted to determine the optimal initial infection conditions for production. Final titer as a function of infection load and initial MOI. (**a**) Final titer on logarithmic scale, and (**b**) final titer on linear scale. Both (**a**) and (**b**) show the same data to highlight the potential differences in interpretation

3. Add the host culture. If starting the culture from an agar plate or slant, deposit a streak or one to three colonies picked from a plate using a sterile loop. If starting from a suspended culture, add 10% (vol/vol) inoculum of a growing culture to each of the shake flasks.

4. Incubate the shake flasks in an incubator-shaker at the desired temperature and agitation rate (in the present case, 37 °C and 250 rpm).
5. Monitor the growth of the population by taking periodical samples and measuring their OD_{600} using a spectrophotometer. Once the population has reached an OD_{600} of 0.6 (cell count of approximately 5×10^8 cells/mL), it is ready to be used to initiate the production batch (*see* Subheading 3.4 below).

3.3 Bioreactor Preparation

The following method describes the preparation of a batch bioreactor with a 10-L working volume. Note that the volumes and methods can be adapted for any desired volume (*see* **Note 6**). Also, the preparation and sterilization of sterilize-in-place (SIP) bioreactors will differ and depend on the available infrastructure.

1. Add 9 L of minimal mineral salt medium (1.1×) solution to the bioreactor.
2. Prepare all connections for tubing. Connect tubing for inlet gas to the sparger of the bioreactor on one end and an in-line HEPA filter on the other. Connect the off-gas port to the condenser followed by an in-line HEPA filter. Insert sparger, autoclavable probes, thermowell, level sensor for foam detection, sampling port in their respective ports. Tighten the reactor lid shut.
3. Close all tubing lines using tubing clamps, except for the off-gas line, which should be kept open to allow for venting during the sterilization process in the autoclave.
4. Cover the inoculation port with its septum. Cover all free ends of tubing, HEPA filters and sampling port with aluminum foil. Ensure that all ports and tubing are covered.
5. Sterilize bioreactor containing minimal mineral salt (1.1×) solution in vertical position, 1 L glucose solution (10×), antifoam solution in bottle with tubing, tubing for acid and base, and sampling bottles for 1.5 h at 121 °C and 204.7 kPa (15 psig).
6. Upon sterilization, connect all tubing to their respective lines (air source to in-line HEPA filter to sparger; acid addition tubing to reactor, peristaltic pump and acid bottle; base addition tubing to reactor, peristaltic pump and base bottle; antifoam addition tubing to reactor, peristaltic pump and antifoam bottle; cooling water line in and out of condenser, etc.)
7. Add a few mL of glycerol to the thermowell and insert the thermocouple.

8. Connect probes, motor and pumps to their respective connectors. Attach the blanket heater to the reactor and connect it to the temperature control unit.
9. Let the minimal mineral salt (1.1×) solution (in the bioreactor) and the glucose solution (10×) cool to a temperature below 60 °C. To speed up this process, aeration and agitation can be turned on in the bioreactor. Once cooled, add the glucose solution to the bioreactor through the inoculation port.
10. Set the operating conditions (temperature, pH, dissolved O_2, etc.) and initiate the controllers. Let the conditions stabilize.

3.4 Bioreactor Operation

1. Once the operating conditions of the bioreactor are reached and stable, place one sterile end of the inoculation tubing in the pre-culture flasks and connect the other end to the inoculation port. Initiate the inoculation peristaltic pump to transfer the pre-culture to the bioreactor. In the present case, the desired infection load is 1% (vol/vol) of the total volume (100 mL of a culture at an OD_{600} of 0.6—cell count of approximately 5×10^8 cells/mL—in a total volume of approximately 10 L) as determined in Subheading 3.1.
2. Within minutes, add the bacteriophage to the desired initial MOI (in this case MOI of 1×10^{-6}) as determined in Subheading 3.1 by injecting the appropriated amount of bacteriophage stock using a syringe and needle through the inoculation port (*see* **Note 11**). This step initiates the infection.
3. Monitor the parameters of interest either through periodical sampling (e.g. OD_{600} and titer at intervals) or in real-time through the bioreactor data acquisition system (dissolved oxygen (DO) and pH using in situ probe, oxygen uptake rate (OUR) and carbon dioxide evolution rate (CER) through in-line probes, etc.) Fig. 5 shows an example of data of OD_{600}, titer and CER for a typical batch operation.
4. The bioreactor should be operated until population-wide lysis is observed—most often identified by a significant reduction, or at least halt, in optical density. Other noticeable changes in various parameters can also be observed during this stage of the infection: sharp increase in dissolved oxygen levels, significant decrease in OUR and CER, changes in pH, and often rapid rise of foam.
5. Samples are taken and assessed to determine the final titer of the batch run.

3.5 Harvesting and Cleaning

1. Once population-wide lysis has taken place, the bioreactor can be shut down: the temperature control, pH control, antifoam addition, and agitation can all be stopped. It is recommended to keep aeration on but reduce significantly the flow rate to the

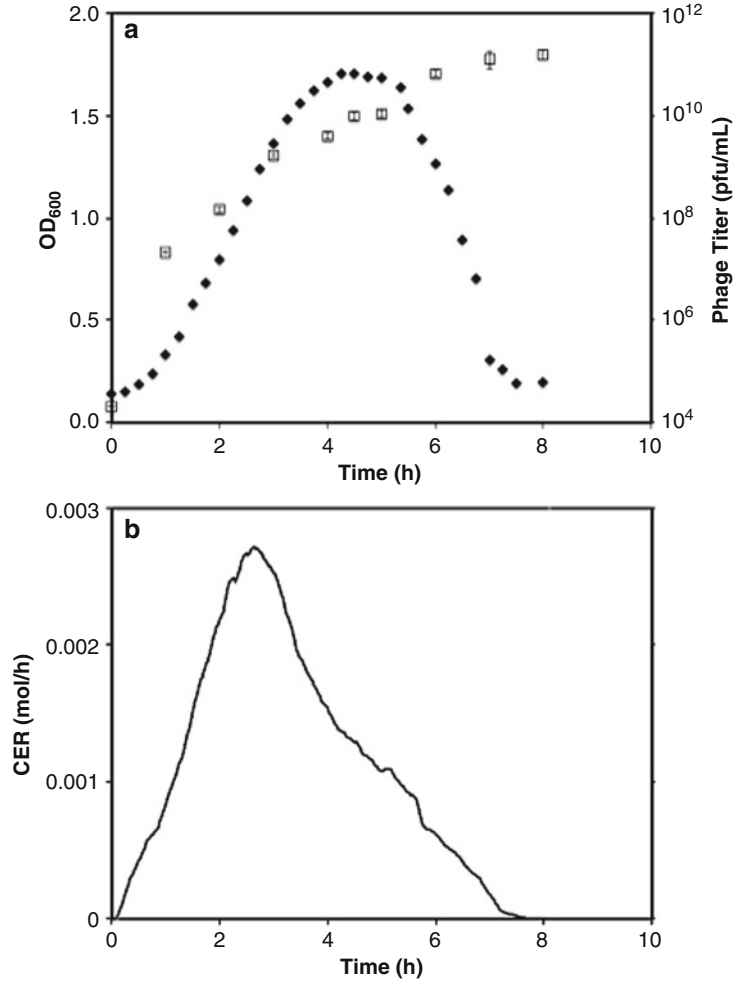

Fig. 5 Monitoring a batch production run performed in a batch bioreactor at an infection load of 1% and initial MOI of 0.001. Results are shown for (**a**) optical density at a wavelength of 600 nm (OD_{600}—*black diamonds*) and bacteriophage titer (*open squares*), and (**b**) carbon dioxide evolution rate (CER—full line)

bioreactor—this will avoid any backflow of the lysate in the sparger and air stream, avoiding the potential accumulation of bacteriophages in the equipment (*see* **Note 12**).

2. Connect the tubing of the harvesting peristaltic pump to the sampling or harvesting port on one end, and to the 0.45 μm in-line cartridge filter—itself connected to the harvesting vessel through sterile tubing—on the other end (*see* Fig. 6).
3. Start the harvesting pump at a flow rate of 0.5 L/min. The flow rate can be modulated depending on the performance of the filter. Lysates have a tendency to clog filters fairly rapidly and initially keeping a lower flow rate should help ensure the trans-

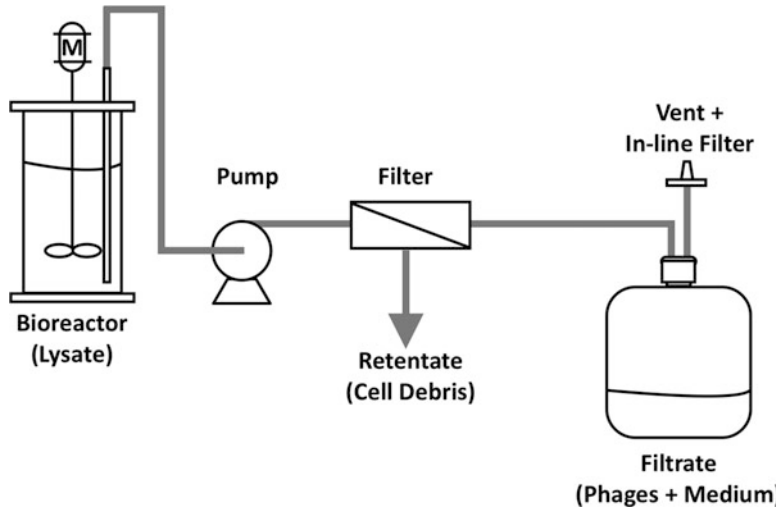

Fig. 6 Simplified schematic of harvesting setup. The bioreactor is connected to a pump by its harvesting port. The pump connects to an in-line filter. The filtrate is recovered in a sterile bottle or carboy vented (through an in-line HEPA filter)

membrane pressure does not increase too rapidly. It may, however, be required to increase the flow rate gradually.

4. Once the harvesting is complete, sterilize and clean the bioreactor vessel thoroughly before the next batch. This will help avoid any left-over bacteriophage or resistant host mutants to be carried over to subsequent batches (*see* **Note 12**).

4 Notes

1. While lysogenic and chronic infections are not covered in the present chapter, bacteriophages undergoing the former can be produced in bioreactors under optimal conditions for host growth (avoiding conditions that could induce the lytic cycle), while bacteriophages undergoing the latter can be grown under conditions favoring bacteriophage production and excretion. *See* [9] for an example of a study based on chronic infection optimization through a surface response analysis approach.
2. In the case of production in larger volumes, the solutions can be prepared in carboys of appropriate sizes or in mixing tanks.
3. In the case of production of volumes up to approximately 20 L, the sterilization time should be increased appropriately. For volumes larger than 20 L, or when the equipment allows, the unsterilized minimal mineral salt medium (1.1×) can be added to the bioreactor and sterilize-in-place (SIP) protocols should be followed. The glucose solution (10×) should be sterilized

independently and added to the bioreactor once cooled. It should also be noted that the glucose solution can also be filter-sterilized rather than sterilized by autoclave.

4. We recommend mixing the solutions once their respective temperatures are below 60 °C.
5. We generally use 1.5% (wt/vol) agar in the bottom layer and 0.75% agar in the top layer. Similarly, different quantities of host can be added to the top layer. For most host–bacteriophage systems, we add 10 μL of overnight host culture for 3 mL of top layer agar.
6. For production batches in bioreactors of working volumes of 10 L or more, it may be preferred to prepare the host pre-culture in its own smaller bioreactor. In such cases, the materials required span Subheadings 2.3 and 2.4 and the procedures mirror those found in Subheadings 3.3 and 3.4, omitting the addition of bacteriophages.
7. Many of the features of the bioreactor setup are facultative and will depend on the desired controlled conditions, the equipment available, the data of interest and the level of monitoring desired.

   For example, in cases where high levels of aeration are required, a feedback control loop based on the DO levels (with control over the impeller motor speed and air flow rate to the bioreactor) would be desirable. Werquin et al. [7] observed that, for their bacteriophage-host system, when the oxygenation of the cells was not sufficient, the yield of bacteriophages was greatly decreased. On the other hand, while conducting a factorial design experiment to determine the optimal processing condition for the production of a bacteriophage through a chronic infection, Grieco et al. [9] concluded that DO was not a significant variable for bacteriophage production in their experiment. These contrasting results highlight the specificity of optimal conditions for each bacteriophage-host system.
   In the case of anaerobic bacterial hosts, aeration, DO, and O_2 off-gas sensing would be removed from the installation.
   Similarly, pH control (acid and base addition) is not a requirement for some bacteriophage production schemes.
   Another factor to consider is the lysis of the host population, which can lead to significant foaming and may thus require the addition of an antifoam agent (e.g. Antifoam A). However, depending on the host–bacteriophage system, the concentration of host cells in the bioreactor, the levels of agitation and aeration, and the geometry of the reactor, the addition of an antifoam agent is not always a requirement. It should also be considered that the presence of antifoam agents in the lysate can lead to more difficult filtration steps following the production stage.

8. The actual number of shake flasks to be prepared will depend on the number of conditions tested (in this case, 5 infection loads × 8 initial MOIs = 40 conditions to be tested). This number can be reduced if the number of infection loads and/or the number of initial MOIs tested is reduced.
9. Alternatively, the infections can be initiated in a smaller volume outside the shake flask prior to addition to the medium. In such a case, the volume of the infection load (e.g. 0.1 mL of host culture at OD_{600} of 0.6—cell concentration of approximately 5×10^8 cells/mL) is added to a microcentrifuge tube. The appropriate volume of bacteriophage stock is added to reach the MOI desired (e.g. 0.1). The infection mixture is then vortexed for 10 s, left to sit for 5 min, before the full volume is added to the shake flask containing the culture medium.

   Two important points should be noted. (1) The same infection protocol should be used in this preliminary study and in the bioreactor operation (with scaled quantities), otherwise the results will not be good predictors of bioreactor operation. (2) The method used to initiate infection will have an impact on the relationship between initial MOI and final titer. Infections initiated in smaller volumes (at higher concentrations) before addition to the bioreactor typically require lower MOIs to obtain a given final titer.
10. The selected temperature of operation is generally the optimal temperature for the growth of the host as it generally corresponds to greater burst sizes (faster growth and infections reduce the batch production time). Since aeration can play an important role in the host growth rate and bacteriophage production [7], it is important to ensure the aeration level in the shake flasks (which depends greatly on agitation) is similar to the aeration levels in the bioreactor operation.
11. As described above, the inoculation and initial infection procedure could also be carried in a small volume outside the bioreactor before adding the infection mixture (host and bacteriophage) at once in the bioreactor.
12. It is important to limit the potential accumulation of bacteriophages in the equipment between batches. Bacteriophages are renowned for being difficult to remove from processing equipment. This is the cause of many cases of contamination of bacterial processes in industry. It is thus essential to place special care in the sterilization and cleaning processes post-production. Carrying over bacteriophages from a previous batch could lead to suboptimal production batches, as one of many possible negative consequences.

    Likewise, carrying over surviving hosts from a previous batch to a new batch would open the door to the rise of resistance to the bacteriophage. It goes without saying that this is to be avoided.

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Chapter 16

Computational Modeling of Bacteriophage Production for Process Optimization

Konrad Krysiak-Baltyn, Gregory J.O. Martin, and Sally L. Gras

Abstract

Computational models can be used to optimize the production of bacteriophages. Here a model is described for production in a two-stage self-cycling process. Theoretical and practical considerations for modeling bacteriophage production are first introduced. The key experimental protocols required to estimate key kinetic parameters for the model, including determining variable infection rates as a function of substrate concentration, are described. ppSim is an open-source R-script that can simulate bacteriophage production to optimize productivity or minimize costs. The steps included to run the simulation using the experimentally determined infection parameters are described. An example is also presented, where a level sensor and cycle time are optimized to maximize bacteriophage productivity in two sequential 1-L bioreactors, resulting in a production rate of 4.46×10^{10} bacteriophage particles/hour. The protocols and programs described here will allow users to potentially optimize production of their own bacteriophage–bacteria pairing by effectively applying bacteriophage modeling.

Key words Bacteriophage dynamics, Computational modeling, Process optimization

1 Background

Bacteriophages have proven useful as tools to control and reduce problematic bacteria in several fields. This includes medicine, where bacteriophages may treat bacterial infections [1] or food science where they can prevent meat contamination [2]. They also have further potential to treat pipe corrosion [3] or to target problematic bacteria in wastewater treatment processes to prevent or reduce foaming [4, 5]. The challenge of large-scale bacteriophage production arises as commercial demand increases and as effective production methods need to be developed and optimized to satisfy global demand.

The interactions between bacteriophages and bacteria determine both the cost and timescale of bacteriophage production. This includes bacteriophage infection and lysis, as well as the rate of bacterial growth. These phenomena follow nonlinear dynamics,

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which may be difficult to intuitively predict, making computational models an essential tool for determining optimal conditions for bacteriophage production. As with any model, the accuracy and usefulness is highly dependent on the underlying assumptions; while more complex models are more likely to be accurate, they contain a larger number of parameters and consequently require more experimental work to calibrate. Depending on the scenario under consideration, simplifications can be made that do not detract from the usefulness of the model yet simplify the calibration process. In this chapter, we provide a short background on population models describing bacteria and bacteriophages and outline the simplifications and assumptions that could reasonably be made in the context of bacteriophage production.

2 Population Models of Bacteria and Bacteriophages

Models of population dynamics of bacteria and bacteriophages were first studied in the 1960s and 1970s [6, 7] and retained their basic form for roughly three decades without any major changes [5]. These basic models take into account four basic processes: bacterial growth, bacterial decay, bacteriophage decay and the infection of bacteria by bacteriophages. These processes are formulated into a system of *Delay Differential Equations* (DDEs). An example of a fairly simple model consisting of one species of bacteria, one bacteriophage and one substrate grown in a single well-mixed chemostat is given by Eqs. 1–4:

$$\frac{\mathrm{d}S}{\mathrm{d}t} = D\cdot(S_0 - S) - X_\mathrm{S}\cdot\mu\cdot e, \tag{1}$$

$$\frac{\mathrm{d}X_\mathrm{S}}{\mathrm{d}t} = X_\mathrm{S}\cdot\mu - K_\mathrm{i}X_\mathrm{S}P - (d_\mathrm{X} + D)X_\mathrm{S}, \tag{2}$$

$$\frac{\mathrm{d}X_I}{\mathrm{d}t} = K_\mathrm{i}X_\mathrm{S}P - e^{-D\cdot T}K_\mathrm{i}X_S(t-T)P(t-T) - (d_X + D)\cdot X_I, \tag{3}$$

$$\frac{dP}{dt} = b\cdot e^{-D\cdot T}\cdot K_\mathrm{i}X_\mathrm{S}(t-T)P(t-T) - K_iX_\mathrm{S}P - (d_\mathrm{P} + D)\cdot P, \tag{4}$$

where

S = substrate concentration (μg/mL)	S_0 = concentration of substrate in influent (μg/mL)
X_S = concentration of susceptible bacteria (cells.mL^{-1})	P = concentration of bacteriophages (bacteriophages.mL^{-1})

(continued)

X_I = concentration of infected bacteria (cells.mL^{-1})	D = dilution rate of chemostat (h^{-1})
b = burst size (bacteriophages/cell)	K_i = adsorption rate constant (mL/h)
T = latency time (h)	e = substrate required per bacterial cell (μg/cell)
d_X = decay rate of bacteria (h^{-1})	d_P = decay rate of bacteriophages (h^{-1})
μ = bacterial specific growth rate as a function of S (h^{-1})	

The bacterial specific growth rate μ is assumed to follow the Monod growth expression:

$$\mu = \frac{\mu_{\max} \cdot S}{K_m + S}. \tag{5}$$

Three key parameters for characterizing a bacteriophage are:

1. Adsorption rate K_i.
2. Burst size b.
3. Latency time T.

The rate of infection of bacteria by bacteriophages is assumed to follow mass action kinetics and is given by the term $K \cdot X_S \cdot P$. Immediately after a bacteriophage infects a bacterial cell, the bacteriophage DNA or RNA is injected and takes over the metabolic machinery so as to produce new bacteriophage progeny. After a certain amount of time, termed the latency time T, the bacterial cell lyses and releases the newly produced bacteriophage progeny. The number of bacteriophage particles released per bacterial cell is termed burst size b. The term $K_i X_S(t - T)P(t - T)$ is of particular interest, as it includes the delay term T. The term describes the rate of infection T time units prior to the current time t.

Although the model described by Eqs. 1–4 can describe key behaviors of bacterial and bacteriophage populations also observed in experimental systems, a number of known biological mechanisms are not included. Many of these have been explored relatively recently during the last 1–2 decades and include bacterial resistance against bacteriophages, coevolution, lysogeny, multiple host binding sites, change in infection rate as a function of bacterial growth rate and release of substrate upon bacterial lysis. The reader is referred to a comprehensive review paper on this subject for more details [5]. For the purpose of the modeling in this chapter, many of these mechanisms are assumed to have limited influence, as discussed further below.

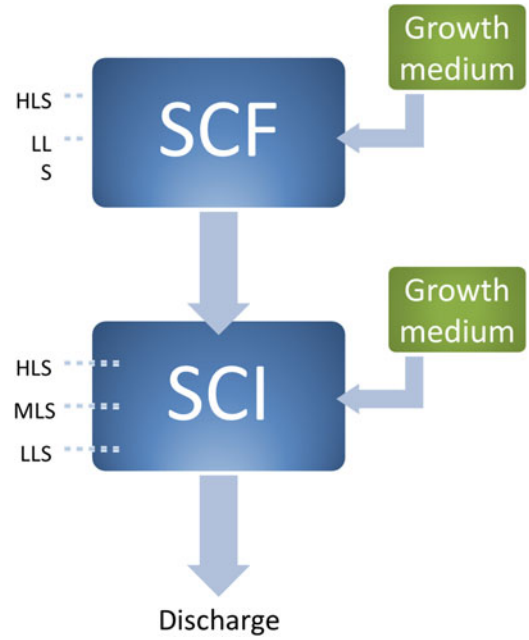

Fig. 1 Schematic image of the two-stage self-cycling process. See text for details

2.1 The Two-Stage Self-Cycling Process

The modeling example provided in this chapter will simulate a two-stage self-cycling process [8], which is a relatively simple approach for automated bacteriophage production on a large scale (Fig. 1). The setup consists of two reactors; the self-cycling fermentation reactor (SCF) and self-cycling infection reactor (SCI). Each reactor is supplied with sterile growth medium, indicated by "growth medium" in Fig. 1. The process starts by growing a pure culture of bacteria in the SCF reactor, while the SCI reactor is filled to the low-level sensor (LLS) with a solution of viable bacteriophages. At a point in time known as the cycling-time, a volume of culture is removed from the SCF reactor and transferred to the SCI reactor. The liquid level in the SCF reactor is drained to the LLS whilst the liquid level in the SCI reaches the mid-level sensor (MLS), any excess liquid is discarded via the discharge stream. At this point, the newly added bacteria are infected by the bacteriophages, initiating bacteriophage production. The SCI tank is further filled with fresh growth medium until the level reaches the high-level sensor (HLS). Fresh growth medium is also added to the SCF until the level reaches the HLS, starting a new cycle of bacterial growth. After a certain period of time, the bacteriophages are harvested from the SCI tank by removing the liquid until the LLS is reached. The entire process is then repeated with the transfer of more bacteria from the SCF reactor to the SCI reactor. The solution of bacteriophages removed from the SCI reactor at each cycling event is transferred for downstream processing and

purification, which typically involve polyethylene glycol precipitation followed by CsCl gradient centrifugation.

The two-stage self-cycling process is a type of modified batch culture; while it operates as batch most of the time, at cycling times a volume of liquid corresponding to the LLS is always retained in both the SCF and SCI reactors. Therefore, there is an element of continuity in the process. An understanding of the mode of operation is important, as it guides the design process of the computational model, including any assumptions that can be made to simplify the model and decrease computational load.

There are a number of key factors that have been considered in the development of a suitable computational model for a two-stage self-cycling process:

1. We assume only one species of bacteriophages and bacteria are used. In bacteriophage production, the output needs to be controlled. Having more species of bacteria or bacteriophages adds a great degree of uncertainty that does not necessarily assist production.
2. We use a batch or modified batch culture, as opposed to continuous culture. This is the most commonly applied approach that minimizes substrate consumption and costs but more importantly helps avoid the emergence of bacteria that are resistant to infection by the bacteriophage strain. Bacteria naturally develop some resistance to bacteriophages [9]. In continuous culture, this process results in a type of natural selection where resistant bacteria dominate the population and will likely interfere with optimal bacteriophage production. The two-stage self-cycling process described here is a modified batch process, which has been shown to avoid the selection of bacterial resistance [10].
3. Coevolution is ignored, as the two-stage self-cycling process is purposefully designed to minimize the risk of resistant bacteria developing.
4. A system of *Ordinary Differential Equations* (ODEs) is applied to assist modeling multiple interconnected reactors [11], avoiding the algorithmic challenge introduced by multiple vessels.
5. The bacteriophage infection rate is assumed to vary as a function of the bacterial specific growth rate μ (or indirectly by substrate availability). In many models published to date, the infection parameters of K_i, T and b are assumed constant throughout the simulation [6, 9, 12–15]. This assumption may be appropriate if simulating a continuous culture where the substrate concentration remains fairly constant. Bacteriophage infection parameters have been shown to vary considerably in experiments, however, as a function of specific growth

rate. Specifically, the adsorption rate has been observed to vary by 1–2 orders of magnitude, the burst size to vary from 0 to 150 depending on the species and the latency period to exhibit at least a two-fold difference [16–18]. Therefore, if the substrate concentration changes significantly during the course of simulation, as occurs in a batch reactor, variable infection parameters must be implemented.

Points 4 and 5 above are the major factors that add greater complexity to the basic model outlined in Eqs. 1–4. Point 4 has been described in a prior publication [11] and involves subdividing the infected bacteria X_I into N subpopulations ($X_{I,1}, X_{I,2} \ldots X_{I,N}$), where each subpopulation represents a certain "age" after infection. In this notation, $X_{I,1}$ would be the youngest bacteria immediately after infection, while $X_{I,N}$ would be the oldest population immediately before lysis. The idea is that the bacterial populations $X_{I,1}$ undergoes ageing by being "transferred" into population $X_{I,2}$, and so on. The rate of this "transfer", which is equivalent to an ageing rate, is given by:

$$D_T = \frac{N}{T}, \tag{6}$$

where:

T = latency time	N = number of subgroups of the infected bacteria
D_T = rate of ageing, i.e., a "dilution rate" through time	

The time derivative for the infected populations $X_{I,1} \ldots X_{I,N}$ and bacteriophage P would then be expressed as:

$$\frac{dX_{I,1}}{dt} = K_i \cdot X_S(t) \cdot P(t) - (d_X + D + D_T) \cdot X_{I,1}(t), \tag{7}$$

$$\frac{dX_{I,k}}{dt} = D_T \cdot X_{I,k-1} - (d_X + D + D_T) \cdot X_{I,k}(t) \text{ for}$$

$$2 \leq n \leq N \tag{8}$$

$$\frac{dP}{dt} = -K_i \cdot X_S(t) \cdot P(t) + b \cdot D_T \cdot X_{I,N} - (d_P + D) \cdot P(t), \tag{9}$$

where:

d_P = decay rate of bacteriophages (h^{-1})	d_X = decay rate of bacteria (h^{-1}).
b = burst size (bacteriophages.cell^{-1})	D = dilution rate of chemostat (h^{-1}).
$X_S(t)$ = the concentration of bacteria at time t (cells.mL^{-1}).	
$P(t)$ = the concentration of bacteriophages at time t (bacteriophages. mL^{-1}).	

(continued)

$X_{I,k}(t)$ = the concentration of infected bacterial subgroup k at time t with $1 \leq k \leq N$ (cells.mL^{-1})
N = number of subgroups representing the total population of infected bacteria
K_i = rate of adsorption between bacteriophages and uninfected bacteria (mL.h^{-1})
D_T = rate of ageing of the infected bacterial population (h^{-1})

With this approach, no delay terms $(T - t)$ are necessary and the higher the value of N (the number of subgroups of infected bacteria in the population) the better the approximation of the DDE model.

Point 5 can be addressed by another mechanism. The bacteriophage infection rate, which varies as a function of the specific growth rate μ, can be modeled by subdividing each infected bacterial population X_I into $M \times N$ subpopulations, with each subpopulation denoted by $X_{I,m,n}$ where $1 \leq m \leq M$ and $1 \leq n \leq N$. As in Eqs. 7–9 above, the index of n indicates the age group of the infected population. In contrast, the index of m indicates subpopulations with different infection parameters, $K_{i,m}$, T_m and b_m.

During each iteration of the simulation, the bacterial specific growth rate μ at the current time point t dictates the value that should be assigned to m. Let us define the function $\sigma(\mu)$ that depends on the specific growth rate and outputs an integer between 1 and M. During each iteration, only the infected population $X_{I,\sigma(\mu),1}$ will increase in concentration due to bacteriophage infection at a rate of $K_{i,\sigma(\mu)} \cdot X_S \cdot P$. The population $X_{I,\sigma(\mu),1}$ will then continuously age over the course of the latency time $T_{\sigma(\mu)}$ and finally lyse to produce a number of bacteriophages equal to the burst size $b_{\sigma(\mu)}$.

The relationship between specific growth rate μ and infection parameters needs to be established experimentally for each situation and depends on the selected species of bacteria and bacteriophages, as well as type of growth medium used (discussed in greater detail in Subheading 3 of this chapter). In past studies, experiments have indicated that an increase in the growth rate μ is expected to increase the infection rate K_i (due to an increase in the number of cell membrane receptors that are available to facilitate adsorption). The growth rate μ has also been shown to affect the burst size b and result in a decrease the latency time T [15–18]. Using the terminology defined in the previous two paragraphs, this relationship can be expressed as:

$$K_{i,1} < K_{i,2} < \ldots < K_{i,M}. \tag{10}$$

$$b_1 < b_2 < \ldots < b_M. \tag{11}$$

$$T_1 > T_2 > \ldots > T_M. \tag{12}$$

The complete model describing all microbial populations in a single reactor is described by Eqs. 13–18. For substrate S, the time derivative remains unchanged from the basic model:

$$\frac{\mathrm{d}S}{\mathrm{d}t} = D{\cdot}(S_0 - S) - X_{\mathrm{S}}{\cdot}\mu{\cdot}e. \tag{13}$$

For all other species significant changes are made. The rate of change for susceptible bacteria X_{S} becomes:

$$\frac{\mathrm{d}X_{\mathrm{S}}}{\mathrm{d}t} = X_{\mathrm{S}}{\cdot}\mu - K_{\mathrm{i},\sigma(\mu)}X_{\mathrm{S}}P. \tag{14}$$

For the "young" infected population $X_{I\,,\,\sigma(\mu)\,,\,1}$ we have:

$$\frac{\mathrm{d}X_{\mathrm{I},\sigma(\mu),1}}{\mathrm{d}t} = K_{\mathrm{i},\sigma(\mu)}X_{\mathrm{S}}(t)P(t) - \left(D + D_{\mathrm{T},\sigma(\mu)}\right){\cdot}X_{\mathrm{I},\sigma(\mu),1}(t). \tag{15}$$

And for all the other "young" infected populations $X_{\mathrm{I}\,,\,m\,,\,1}$ where $m \neq \sigma(\mu)$ and $1 \leq m \leq M$ we have:

$\frac{\mathrm{d}X_{\mathrm{I},m,1}}{\mathrm{d}t} = -\left(d_{\mathrm{X}} + D + D_{\mathrm{T},m}\right){\cdot}X_{\mathrm{I},m,1}(t)$ for $1 \leq m \leq M$

and

$$m \neq \sigma(\mu). \tag{16}$$

For the "older" infected populations ($n > 1$), we have:

$\frac{\mathrm{d}X_{\mathrm{I},m,n}}{\mathrm{d}t} = D_{\mathrm{T}}{\cdot}X_{\mathrm{I},m,n-1} - \left(d_{\mathrm{X}} + D + D_{\mathrm{T},m}\right){\cdot}X_{\mathrm{I},m,n}(t)$ for $2 \leq n \leq N$

and

$$1 \leq m \leq M. \tag{17}$$

And for the bacteriophage population:

$$\frac{\mathrm{d}P}{\mathrm{d}t} = -K_{\mathrm{i},\sigma(\mu)}X_{\mathrm{S}}P + \sum_{m=1}^{M} b_m{\cdot}D_{\mathrm{T},m}{\cdot}X_{\mathrm{I},m,N} - (d_{\mathrm{P}} + D){\cdot}P(t) \tag{18}$$

and:

$$D_{\mathrm{T},m} = \frac{N}{T_m} \tag{19}$$

where:

S = substrate concentration (μg. mL^{-1})	S_0 = concentration of substrate in influent (μg.mL^{-1})
X_{S} = concentration of susceptible bacteria (mL^{-1})	P = concentration bacteriophages (mL^{-1})
b = burst size (bacteriophages. cell^{-1})	$K_{\mathrm{i}\,,\,m}$ = adsorption rate constant (mL. h^{-1})

(continued)

T = latency time (h)	e = substrate required per bacterial cell ($\mu g.cell^{-1}$)
d_X = decay rate of bacteria (h^{-1})	d_P = decay rate of bacteriophages (h^{-1})
D = dilution rate of chemostat (h^{-1})	$D_{T,m}$ = ageing rate of infected bacteria m (h^{-1})
μ = bacterial specific growth rate as a function of substrate $S(h^{-1})$	
N = number of discrete steps to represent the course of latency time	
M = number of discrete populations to represent $K_{i,m}$, T_m and b_m as function of μ	
$X_{I,m,n}$ = concentration of infected bacteria population m at age n (mL^{-1})	
$\sigma(\mu)$ = a function specifying which young infected population $X_{I,m,1}$ should increase in concentration due to infection, with $1 \leq \sigma(\mu) \leq M$	

Once a suitable model has been formulated, it can be used to estimate the best conditions for optimal bacteriophage production. In the case of the two-stage self-cycling approach, the major operational parameters that may influence optimal bacteriophage production are the cycling time, levels of the *high-*, *mid-*, and *low-level sensors* (HLS, MLS, and LLS) and the concentration of substrate in the growth medium.

To facilitate simulation and optimization of bacteriophage production, we include a script (ppSim) written in the R statistical language for simulating bacteriophage production using the two-stage self-cycling process. The script ppSim can simulate bacteriophage production under a range of different conditions. These include varied cycling time, varied heights for the three level sensors (HLS, MLS, and LLS) on the two reactors and varied substrate concentration. To simplify the modeling, this script assumes that all transfer of liquids during each cycling event is instantaneous. This assumption is reasonable if the liquid flow rate in corresponding experimental or production systems is high enough to complete the cycling event in a few minutes.

This script can be used to pinpoint the optimal condition for either maximum productivity (measured in terms of bacteriophages produced/hour) or minimum cost (measured in terms of cost/bacteriophage particle). ppSim is freely available for noncommercial use and can be downloaded from http://people.eng.unimelb.edu.au/sgras/. The source code is available upon request via email and will be provided under the LGPL license agreement.

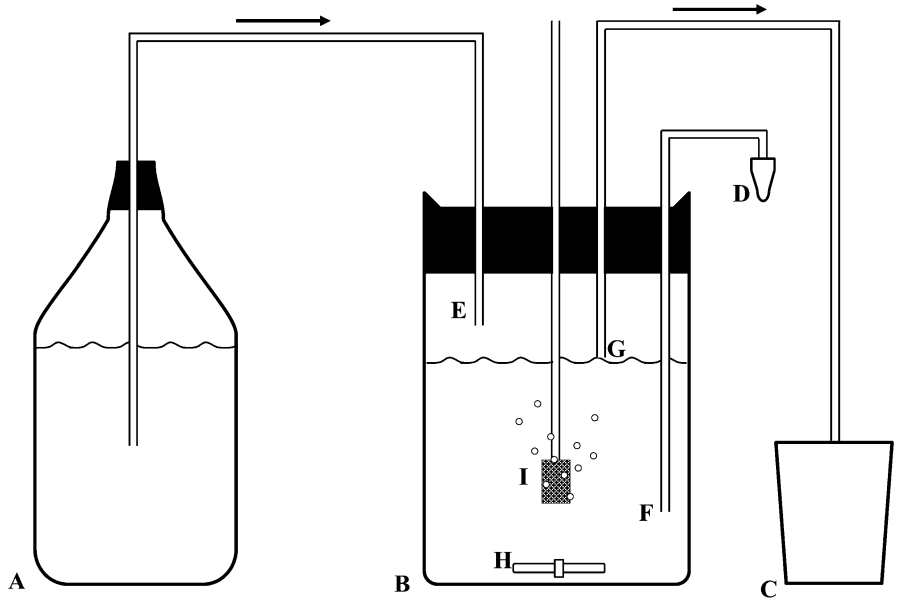

Fig. 2 Schematic of a chemostat (continuous culture) experiment. (*A*) Growth medium vessel, (*B*) chemostat, (*C*) effluent collection vessel, (*D*) sampling port, (*E*) influent pipe, (*F*) sampling pipe, (*G*) effluent pipe, (*I*) aeration, *(H)* magnetic stirrer bar

3 Characterizing the System

3.1 Bacterial Growth Parameters

Before simulating a system of interest, such as the two-stage self-cycling process, the relevant kinetic parameters must be experimentally characterized. In the case of modeling bacteriophage production, this includes the parameters for bacterial growth ($V_{\max}$ and K_m) and rates of decay for bacteria and bacteriophages (d_X and d_P). It is also necessary to understand how the bacteriophage infection parameters (K_i, T, and b) vary with the bacterial growth rate.

The parameters for bacterial growth and decay can best be estimated via a series of chemostat experiments using the experimental setup described in Fig. 2. Different conditions can be applied where the dilution rate D is varied and the systems are allowed to reach steady-state, i.e. a state where a constant growth rate of bacteria is maintained. The recorded concentrations of substrate S and X_S can then be used to estimate the necessary parameters. Typical dilution rates can vary between 0.1 and 1.5 h^{-1}, depending on the bacterial species. As it can normally take 2–4 days to establish a steady-state, it is recommended to use fairly small chemostats with volumes of around 100–200 mL, although the findings are still applicable to larger volumes. With larger chemostats, larger quantities of fresh growth media are required to continuously maintain a given dilution rate through the system, which is costly and not necessary to accurately determine the parameters needed for modeling.

The system shown in Fig. 2 is described by the following set of ODEs:

$$\frac{dS}{dt} = D\cdot(S_0 - S) - X_S\cdot\mu\cdot\frac{1}{\Upsilon}. \tag{20}$$

$$\frac{dX_S}{dt} = X_S\cdot\mu - (d_X + D)\cdot X_S. \tag{21}$$

By assuming steady-state, i.e., $dS/dt = 0$ and $dX_S/dt = 0$ and substituting Eq. 5 into Eqs. 20 and 21, the expressions can be linearized to give Eqs. 22 and 23:

$$\frac{D\cdot(S_0 - S)}{X_S} = \frac{d_X}{\Upsilon} + \frac{D}{\Upsilon}. \tag{22}$$

$$\frac{1}{D + d_X} = \frac{K_m}{\mu_{max}}\cdot\frac{1}{S} + \frac{1}{\mu_{max}}. \tag{23}$$

By plotting the *left hand side (LHS)* of Eq. 22 using the experimental data, a straight line can be fitted. The parameters d_X and Υ can then be estimated from the slope and intercept (Fig. 3a). Similarly, a straight line can be fitted to a plot of the *LHS* of Eq. 23 using the experimental data points. In this case, parameters K_m and μ_{max} can be estimated from the slope and intercept (Fig. 3b).

3.2 Bacteriophage Infection Parameters

Once the bacterial growth parameters have been estimated, as outlined in Subheading 3.1 above, the bacteriophage infection parameters at different bacterial growth rates can be estimated. Bacterial liquid cultures should be grown in chemostats where the steady-state is reached at different dilution rates D. The specific growth rate can be calculated from Eq. 21 at steady-state and a specific growth rate D, by setting the time derivative to zero and solving for $\mu(S)$:

$$\mu = D + d_X, \tag{24}$$

where the decay rate d_X is known, as it was estimated in Subheading 3.1. Once the system reaches steady-state, a sample should be withdrawn and used to estimate the bacteriophage infection parameters K_i, T and b according to suitable protocols, such as those included in Subheading 4 below. These parameters can then be related to the specific bacterial growth rate.

According to a study by Hadas et al. [16], the bacteriophage infection is expected to approach zero when the bacteria reach the stationary phase and all the substrate is depleted. The bacteriophage infection parameters can be formulated as a function of bacterial growth rate; $K_i(\mu)$, $T(\mu)$, and $b(\mu)$. In the simplest case, these parameters can be formulated as linear functions of μ but this may depend on the species of interest, as well as composition of growth medium.

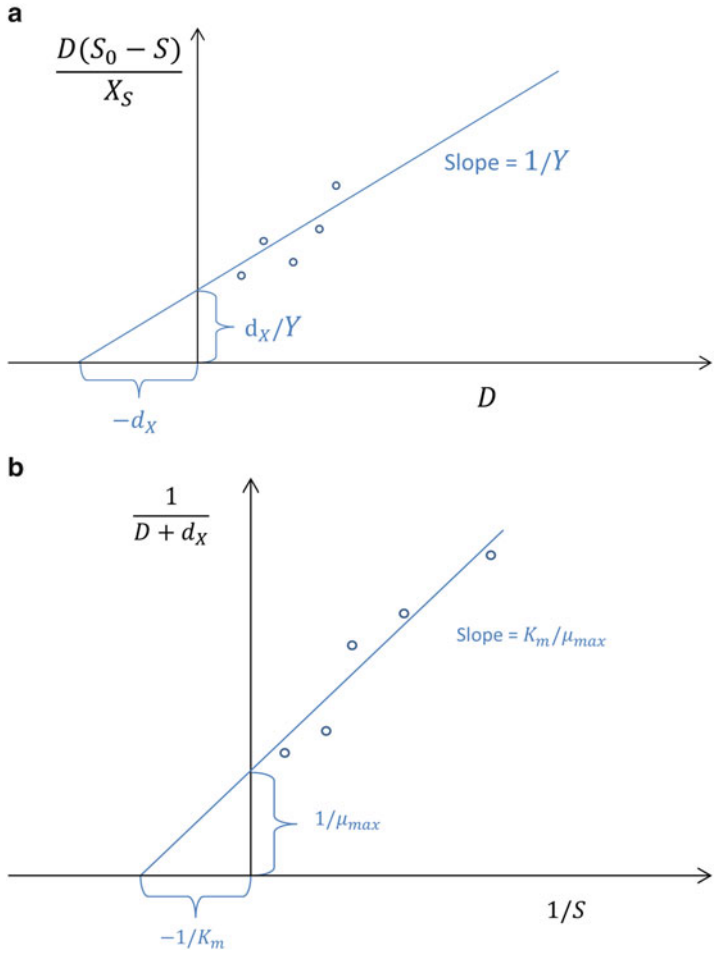

Fig. 3 Linearization of the equations describing substrate consumption and bacterial growth in a chemostat to give the relevant bacterial growth parameters

4 Experimental Protocols for Measuring Bacteriophage Kinetic Parameters

Experiments for measuring bacteriophage kinetic parameters must be conducted under sterile conditions, close to a flame or inside a biosafety cabinet. The experiments outlined below also assume that bacterial cultures have already been grown in chemostats under controlled conditions, such that the bacterial specific growth rates are known, as described in Subheading 3. Here, plating is used to estimate the bacterial concentration by counting colony forming units (cfus) and the bacteriophage concentration by counting plaque forming units (pfus), as described in detail in Subheading 4.3 below. These protocols have been adapted from the literature ([19, 20]).

4.1 Protocol for Measuring Burst Size and Latency Time

This protocol is a variant of the one-step growth curve experiments described in many different microbiology sources. This protocol has been simplified to reduce the workload by monitoring the progress of infection via absorbance measurements, instead of platings, where possible.

4.1.1 Materials

1. Shaker incubator.
2. Biosafety cabinet and/or Bunsen burner.
3. UV-VIS spectrophotometer.
4. Table-top centrifuge.
5. Pipettes for 0.1–1.0 mL.
6. Glass hockey-stick.
7. Erlenmeyer flask (250 mL).
8. Sterilized pipette tips (1 mL).
9. Cuvettes for UV-VIS spectrophotometer.
10. Sterilized micro tubes (1.5–2.0 mL).
11. Sterilized falcon tubes (30–50 mL).
12. Growth medium suitable for growing the bacterial and bacteriophage strain selected in liquid culture.
13. Petri dishes 90–120 mm (gamma-sterilized plastic is recommended) with solidified agar medium suitable for the bacterial strain selected.
14. Ethanol.

4.1.2 Method

1. Grow bacterial cultures in chemostats until steady-state is reached.
2. To two 1.5 mL micro tubes, add 0.9 mL of bacterial culture from the chemostat.
3. Add 0.1 mL of bacteriophage solution (a high concentration of 10^9 pfu/mL or more is desirable).
4. Mix tubes with gentle inversion.
5. Leave tubes for 4 min in a water bath at 30 °C (this promotes bacteriophage infection).
6. Dilute the bacterial–bacteriophage mixtures 10× by adding 0.1 mL of the mixtures to 0.9 mL of broth in two individual microcentrifuge tubes (this will slow down the infection rate by a factor of 100 for any remaining free bacteriophages).
7. Centrifuge gently at 1000–1500 × g for 4 min in a table-top centrifuge. Remove supernatant, and resuspend in 1.1 mL of broth (this will remove the majority of free unadsorbed bacteriophages).

8. Take 1 mL of the resuspended cells from each tube and add to a single falcon tube.
9. Add 18 mL of sterile broth and mix gently with inversion (the final volume of bacteria–bacteriophage mix in the falcon tube will be 20 mL).
10. Keep tubes in shaker incubator at a suitable 30 °C.
11. Immediately perform plating as described further below.
12. Once every 5 min, take a sample volume of 1.0 mL and add to a semi-micro cuvette. Measure absorbance at 600 nm. If absorbance is higher than 1.0, dilute ten times and make a new measurement (this will ensure measurements are in the linear region).
13. At some point, absorbance measurements should exhibit a dramatic fall. Once this rapid decrease has stabilized, take a sample and perform plating.

4.1.3 Data Analysis

Latency time is the time at which the absorbance measurements show a dramatic fall. The burst size b is given by:

$$b = \mathrm{PFU_P}/\mathrm{PFU_I} \tag{25}$$

where:

$\mathrm{PFU_I}$ = the pfus estimated 5–10 min post-infection. In actuality, this number represents the number of infected cells
$\mathrm{PFU_P}$ = is the pfu count observed after the latency time and lysis has stabilized. This represents the number of free bacteriophages in solution

4.1.4 Notes About the Protocol

- Ideally, **steps 1–10** should not take more than 10 min.
- At least two platings are performed in this protocol; one immediately after mixing bacteriophages and bacteria and one towards the end of the experiment after lysis has been observed (where the rapid decrease in absorbance is noted).
- During the course of bacteriophage infection, absorbance is measured once every 5–10 min to monitor the onset of bacterial lysis. Measurement at 600 nm in a UV-VIS spectrophotometer is fine in most cases; however, 550 and 660 nm have also been proposed as suitable and may depend on the bacterial species used. If the turbidity is not high enough to be properly detected by the UV-VIS (an absorbance of at least 0.05 is recommended) then only plating should be used.

4.2 Protocol for Measuring Adsorption Rate

4.2.1 Materials

1. UV-VIS spectrophotometer.
2. Table-top centrifuge.
3. Biosafety cabinet and/or Bunsen burner.
4. Pipettes for 0.1–1.0 mL.
5. Glass hockey stick.
6. Water bath.
7. Erlenmeyer flask (250 mL).
8. Sterilized pipette tips (1 mL).
9. Cuvettes for UV-VIS spectrophotometer.
10. Sterilized micro tubes (1.5–2.0 mL).
11. Sterilized tubes (15 mL).
12. Growth medium suitable for growing the bacteria and bacteriophages selected in liquid culture.
13. Petri dishes 90–120 mm (gamma-sterilized plastic is recommended) with solidified agar medium suitable for the bacteria selected.
14. Ethanol.

4.2.2 Method

Estimation of bacterial concentration in a chemostat.

1. Grow bacterial cultures in a chemostat until steady-state is reached.
2. Take a sample from the chemostat and estimate the bacterial concentration using plating, as described in Subheading 4.3.
3. Incubate plates at an appropriate temperature for 1–7 days (the optimum temperature and time will depend on the bacterial species). These plates will be used later to estimate bacterial concentration in the chemostat by counting colony forming units (cfus).

Preparation of the bacterial culture for estimation of adsorption rate.

4. Take a sample from the chemostat and measure the absorbance at 600 nm.
5. Ideally, the absorbance should be between 0.2 and 0.4 (which commonly corresponds to roughly 10^8 cells.mL^{-1}). If possible, dilute the sample to obtain the desired absorbance. Make sure to write down this dilution for later.

Measuring adsorption rate.

6. Prepare 12 sterile micro tubes (1.5 mL) and label them A1–10, and B1 and B2.

7. Add 0.95 mL of sterilized growth media to tubes A1–10, B1 and B2.
8. Cool tubes A1–10, C1 and C2 for at least 10 min.
9. Prepare two sterile 15 mL tubes and label them C1 and C2.
10. Place 3 mL of bacteriophage stock solution (roughly 10^7 pfu. mL^{-1}) for 10 min in a water bath set to the same temperature as the chemostats.
11. Add 9 mL of bacterial culture (from **step 5**) to tube C1.
12. Add 9 mL of sterile growth medium to tube C2.
13. Place tube C1 in the water bath for 10 min.
14. Add 1 mL of preheated bacteriophage solution (from **step 10**) to tube C1 and start the timer.
15. Immediately afterwards, add 1 mL the preheated bacteriophage solution to tube C2.
16. After 1 min, take a 0.05 mL aliquot from tube C1 and add to the chilled tube A1 (mix the contents vigorously by inverting the tube and place back on ice). This dilution will reduce the adsorption rate by a factor of 400.
17. Repeat **step 16** every minute (i.e., after 2 min, add 0.05 mL to tube A2, and so on) until 10 min have passed.
18. Add 0.05 mL from tube C2 to tubes B1 and B2 each. Mix and place on ice.
19. Centrifuge tubes A1–10 and B1–2 at 1000 × *g* for 5–10 min.
20. In the meantime, label ten new micro tubes D1–10 and two new tubes E1–2 and add 200 μL of bacterial culture (**step 5**).
21. After centrifugation is complete, remove 100 μL of supernatant from tube A1 and add to tube D1. Do this for the remaining tubes, i.e., transfer supernatant from A2 to D2 and so on. Also add supernatant from B1 and B2 to E1 and E2, respectively.
22. Mix the tubes and let them stand for 5 min.
23. Take 100 μL from each of the tubes D1–10 and E1–2 and estimate bacteriophage concentration via dilution series and plating (*see* Subheading 4.3).

4.2.3 Data Analysis

1. The result from **step 3** in Subheading 4.2.2 is the estimated bacterial density, i.e., the colony forming units (cfu).
2. The results from **step 23** in Subheading 4.2.2 are the free bacteriophage concentrations at the various time points. Calculate the average bacteriophage concentration estimated from tubes E1 and E2. This will correspond to time $t = 0$ min. Estimates from tubes D1–10 will correspond to the free bacteriophage concentration at times $t = 1, 2, \ldots 10$ min.

3. Using Microsoft Excel, or any other capable software, create a plot of the log of the free bacteriophage concentration on the *y*-axis and time (in minutes) on the *x*-axis. Use the software to fit a straight line through the data points and note the slope of the line.
4. Calculate the adsorption rate K_i with the following formula:

$$K_i = -\frac{k}{B} \tag{26}$$

where:

K_i = the adsorption rate.
k = the slope of the straight line obtained in **step 26**.
B = concentration of bacteria in tube C1 in **step 14**.

Note: the bacterial concentration B can be obtained from the estimate of the bacterial concentration in the chemostat (cfus obtained in **step 3** in Subheading 4.2.2 above). Any dilutions made (*see* **steps 5** and **14**, Subheading 4.2.2 above) prior to mixing the bacteria with bacteriophages (**step 14** in Subheading 4.2.2) should also be considered in these calculations.

4.3 Plating Techniques

Plating to estimate bacterial concentration.

1. Ahead of time, prepare ten microtubes with 0.9 mL of broth each. Label the tubes 1–10.
2. Add 0.1 mL of a pure bacterial solution to microtube #1 (mix thoroughly but gently, by pipetting up and down).
3. Take 0.1 mL from tube #1 and add to tube #2 and mix. Repeat the process for all remaining tubes (this will make a tenfold dilution series).
4. Take 0.1 mL from each microtube and add to individual plates and spread out the solutions evenly using a sterilized hockey stick (dipped in ethanol and flamed).
5. Incubate plates at suitable growth conditions (usually 25–37 °C) for 1–3 days. Monitor the development of cfus. For some slow growing bacterial strains, up to a week may be required before bacterial colonies are of sufficient size to be observed and counted for cfus.

Plating to estimate bacteriophage concentration.

1. Ahead of time, label ten microtubes 1–10 and add 0.9 mL of pure growth medium to each tube.
2. Add 0.1 mL of a solution containing bacteriophages (this may be pure or a mixed with bacteria) to microtube #1 (mix thoroughly, but gently, by pipetting up and down).

3. Take 0.1 mL from tube #1 and add to tube #2 and mix gently. Repeat this process for all remaining tubes (this will create a tenfold dilution series).
4. To each tube, add 0.2 mL of pure bacterial culture. Mix thoroughly but gently.
5. Take 0.1 mL from each microtube and add to individual plates and spread out the solution evenly using a sterilized hockey stick (dipped in ethanol and flamed).
6. Incubate plates at suitable growth conditions (usually 25–37 °C) for 1–3 days. Monitor development of plaque forming units (pfus). For some slow growing bacterial strains, up to a week may be required before visible plaques occur.

5 Simulating the Model

The program ppSim, which can simulate bacteriophage production using the two-state self-cycling process, has been implemented in the R-statistical language. R is open source and freely available at https://www.r-project.org/. Once R has been downloaded and installed on a computer, starting the program will open a command window. Before running the bacteriophage production simulation itself, some additional R-packages must be installed. This is undertaken by entering the following commands in the R-command window:

$$\text{install.packages}\left(\text{c}\left(\text{"deSolve"}, \text{compiler}, \text{foreach}, \text{doParallel}\right)\right). \tag{27}$$

The R program will attempt to download these packages from an online repository. The user will be prompted to choose the nearest server.

The bacteriophage production model is supplied in three files:

1. run_model.R
2. user_param.R
3. phage_prod_sim.R

Every time a new model is simulated, these files should be copied into a new empty directory.

The file "run_model.R" contains instructions on how to run the script. In addition, it contains four sections: the first section contains code installing additional packages (only executed once), the second section contains code for loading the additional packages, the third for specifying the directory where the script files are located. The fourth section contains the command to start the simulations. For those who are not familiar with the R-language, note that the symbol '#' denotes a comment. The

R-compiler will ignore anything after this symbol. These comments are convenient for describing the code in easily readable terms.

Step-by-step instructions to successfully execute the phage production simulation, using the experimentally determined values described in Subheadings 3 and 4, are as follows:

1. Create a directory (with any suitable name) that will contain the script files and result output from your particular model. Copy the three files "run_model.R", "user_param.R" and "phage_prod_sim.R" into this folder.
2. In the file "run_model.R", in Subheading 3, specify the directory where the files have been copied. Note that forward slash '/' should be used instead of back slash '\', even if running on a Windows system.
3. Open the file "user_param.R" and edit the parameters for the model. These parameters include kinetic parameters for bacterial growth and phage infection as well as the operational parameters. More details are included within this file.
4. Copy the contents from the file "run_model.R" and paste them into the R-command window. This will execute the phage production model. Upon completion, results will be output into the same folder where the script-files are located.

The current version of ppSim can optimize for either cost (minimizing both the cost of growth media and operation) or for productivity (maximizing the number of bacteriophages per unit time).

6 Example Simulation

In our example simulation, we set the goal to optimize productivity (i.e., number of bacteriophages per hour for the given reactor volume) by attempting to find the optimal cycling time and MLS-level in the SCI reactor. For a more extensive example of modeling, which explores variation in a greater number of model parameters and optimizes production costs. The cycling time determines the concentration of bacteria added to the SCI reactor and the length of time the bacteria spend in contact with the bacteriophages. In contrast, the MLS determines only the concentration of bacteria added to the bacteriophages. We assume that the production is performed on a laboratory scale, where the glucose concentration in the influent is 3 mg/L and where both the SCF and SCI reactors have a maximum volume of 1.0 L. The ppSim script can be configured to simulate reactors of any size by setting the corresponding HLS values.

The first step is to input the biological parameters. The bacterial growth parameters and infection parameters applied here are based

Table 1
Infection parameters K_i (adsorption rate), b (burst size), and T (latency time) as a function of the relative specific growth rate

Relative specific growth rate	K_i (mL.h^{-1})	b (bacteriophages.cell^{-1})	T (h)
0.010	0		
0.109	1.0×10^{-9}	10	0.80
0.207	1.2×10^{-8}	20	0.76
0.307	2.3×10^{-8}	30	0.71
0.405	3.4×10^{-8}	40	0.67
0.505	4.5×10^{-8}	50	0.62
0.604	5.6×10^{-8}	60	0.58
0.702	6.7×10^{-8}	70	0.53
0.802	7.8×10^{-8}	80	0.49
0.906	8.9×10^{-8}	90	0.44
1.000	1.0×10^{-7}	100	0.40

on past studies [15–18] as an example, with $\mu_{max} = 0.7726$ h^{-1}, $K_m = 0.0727$ μg.mL^{-1} and $e = 10^{-6}$ μg.cell^{-1}. These parameters were directly entered in the "Species parameters"-section in the user parameter-file. Infection parameters were entered into a table format (Table 1) in a comma separated file using excel. Once the ppSim program is executed, it automatically reads this file and incorporates the specified values. The table follows a specific format, where the first column specifies the relative specific growth rate μ_r, defined as the specific growth rate μ relative to μ_{max}:

$$\mu_r = \frac{\mu}{\mu_{max}}. \tag{28}$$

The remaining columns in Table 1 correspond to the infection parameters. The first row in Table 1 indicates that if $0 < \mu_r \leq 0.01$, then the adsorption rate $K_i = 0$. In a similar fashion, the second row indicates that if $0.01 < \mu_r \leq 0.109$, then $K_i = 10^{-9}$, $b = 10$ and $T = 0.8$. To our knowledge, no prior study has published the relationship between μ_r and the infection parameters over the entire range $0 \leq \mu_r \leq 1.0$ for any given bacterial–bacteriophage pairing. We therefore generated our own ranges based on the small amount of data that is available in the literature [15–18].

The adsorption rate K_i can vary over two orders of magnitude. In this example K_i was assumed to vary logarithmically with bacterial specific growth rate μ, with infection rate $K_i = 0$ at $\mu(S) \approx 0$ and $K_i = 10^{-7}$ mL.h^{-1} at $\mu(S) \approx \mu_{max}$. Burst size b was varied linearly

between 10 and 100 bacteriophages.cell^{-1} and latency time T was varied linearly from 0.8 to 0.4 h (decreasing with increasing μ). These values of infection parameters are a simplification used here to illustrate the dynamics of the model and would normally be replaced by specific values determined experimentally by the user to describe the specific bacteria–bacteriophage pairing of interest, as discussed in Subheadings 3 and 4.

In order to get a good estimate of the number of bacteriophages produced in each cycle, we need to run the model for a long enough time to reach a steady-state, where each cycle does not change much from the other. Test-runs on the model presented here indicated that ten cycles was sufficient for the cycles to be fairly consistent although for some conditions up to 30 cycles had to be simulated before stabilization (data not shown).

In this example we sought to find the optimal length for the cycling times and the optimal level of the mid-level sensor (MLS) in the SCI tank, which determine the bacteria–bacteriophage ratio and contact time for the bacteria and bacteriophages. Past experimental studies using *E. coli* bacteria and T4 bacteriophages estimated that the optimal cycling time was 2.47 h [10]; therefore we configured the program to vary the cycling time between 0.5 and 5 h. No optimal value for the MLS has been found experimentally but this sensor has previously been set to 80 mL [8]. We therefore configured the program to vary the MLS between 50 and 800 mL. The search boundaries for both the cycling time and MLS can be specified in the "Operation and Optimization"-section in the user parameter-file.

We also configured the program to test ten values for each operational parameter, which will total $10 \times 10 = 100$ simulations in this case. In general, testing more values of the chosen parameters will allow for greater precision but at the cost of computational time. One way to improve precision while keeping computational time low is to "zoom in" around the best model obtained from simulating the first 100 models and vary the operational parameters locally in a smaller region. This is done by setting the number of passes to two or higher. Each pass will successively zoom in closer around the optimal model, fine-tuning the parameter values. In this example, we set the number of passes to 2, which will lead to a total of $2 \times 10 \times 10 = 200$ simulations. Finally, we assume that the SCF and SCI tanks have a maximum volume of 1000 mL, by setting the HLS sensors for both tanks accordingly. Running these simulations on a typical office laptop with an Intel i7 2.90 GHz processor took roughly 60 min.

The optimal model has a cycling time set to 2.5 h and the MLS in the SCI set to 467 mL. A time-course of the simulation is illustrated in Figs. 4 and 5, which show how the concentrations of susceptible bacteria X_S, infected bacteria X_I, bacteriophages P and substrate S change over the course of the simulation. Under these

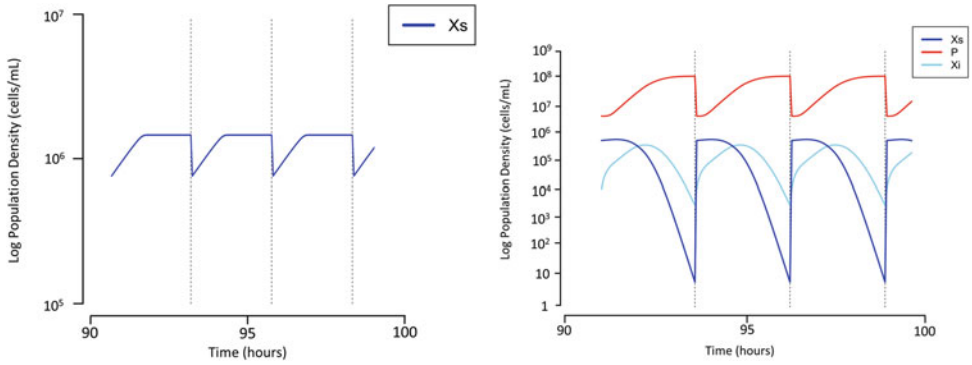

Fig. 4 The concentration of bacteria and bacteriophages during simulation of the two-stage self-cycling process for bacteriophage production under optimal conditions, with a cycling time of 2.5 h and MLS in the SCI reactor set to 467 mL. (**a**) SCF-tank where only susceptible bacteria X_S are grown. (**b**) SCI tank where susceptible bacteria X_S, infected bacteria X_I, and bacteriophages P are grown. The *vertical black dotted lines* represent cycling events, where bacteriophages are harvested from the SCI tank and bacteria are transferred from the SCF to SCI tank. These transfers are assumed within the model to occur instantaneously in a simultaneous way for both reactors

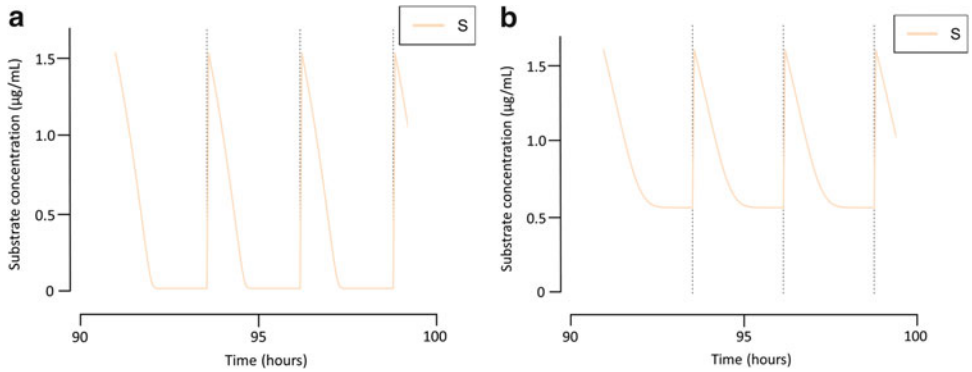

Fig. 5 Concentrations of substrate during the simulation of the two-stage self-cycling process for phage production under optimal conditions, with a cycling time of 2.5 h and MLS in the SCI reactor set to 467 mL. (**a**) SCF-tank (**b**) SCI tank. The *vertical black dashed lines* represent cycling events, where phages are harvested from the SCI reactor and bacteria are transferred from the SCF to the SCI reactor. These transfers are assumed within the model to occur instantaneously and simultaneously for both reactors

conditions, the setup is capable of producing 4.46×10^{10} bacteriophage particles per hour, which is equivalent to 1.11×10^{11} bacteriophage particles for every cycling event. The bacteriophage concentration in the harvested liquid is roughly 10^8 bacteriophage particles.mL^{-1}. This bacteriophage production process would be adequate for bacteriophage therapy, where around $10^6 - 10^8$ bacteriophage particles per dose are typically administered to patients [21, 22], although subsequent processing would be needed to ensure sufficient purity appropriate for administration. Under optimal conditions, the model predicts that bacteriophage productivity

is roughly twice as sensitive to small changes in cycle time compared to small changes in the MLS level. It is recommended based on this simulation that the optimal cycling time (2.5 h) and MLS in the SCI (467 mL) be adopted and that different cycling times could be further varied at a laboratory scale if greater optimization of the production process was required.

7 Conclusion

Computational models of bacteriophage production can be useful tools to understand the dynamics of bacteriophage production and optimize the production process, pinpointing operational parameters that have the greatest influence and determining the optimal conditions for the highest productivity. To create a suitable model, the chosen pairing of bacteria and bacteriophage must first be characterized in terms of the bacterial growth parameters and the bacteriophage infection parameters; a process best performed using a continuous chemostat. Here, the steps involved in determining these experimental parameters and modeling a two-stage self-cycling process using these parameters are outlined. The scripts are also provided to enable modeling. An example bacteriophage production process was then simulated under different operational conditions; determining an optimal cycling time and MLS, the cycle time was also identified as having a greater effect on bacteriophage productivity. Similar simulations can be performed by the reader using the experimental and modeling protocols provided, in order to optimize the production of a specific bacteriophage using a selected bacteria–bacteriophage pairing. This approach can also be used to efficiently direct further laboratory based experiments to enable optimal bacteriophage production.

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Chapter 17

Methods for Bacteriophage Preservation

Małgorzata B. Łobocka, Aleksandra Głowacka, and Piotr Golec

Abstract

In a view of growing interest in bacteriophages as the most abundant members of microbial communities and as antibacterial agents, reliable methods for bacteriophage long-term preservation, that warrant the access to original or mutant stocks of unchanged properties, have become of crucial importance. A storage method that retains the infectivity of any kind of bacteriophage virions, either in a cell lysate or in a purified suspension, does not exist, due to the enormous diversity of bacteriophages and hence the differentiation of their sensitivity to various storage conditions. Here, we describe a method of long-term bacteriophage preservation, which is based on freezing of freshly infected susceptible bacteria at early stages of bacteriophage development. The infected bacteria release mature bacteriophages upon melting enabling the recovery of bacteriophage virions with high efficiency. The only limitation of this method is the sensitivity of bacteriophage host to deep-freezing, and thus it can be used for the long-term preservation of the vast majority of bacteriophages.

Key words Bacteriophage preservation, Deep-freezing, Bacteriophage stability, Bacteria, Bacteriophage storage, Microbiological methods

1 Introduction

The problem of bacteriophage preservation is as old as the history of bacteriophage research. Typically bacteriophages have been stored in a form of free virions. The sensitivity to storage conditions and to the content of storage media may differ significantly between bacteriophages. Therefore, a one universal method of virion preservation does not exist. Storage of lysates at 4 °C, freezing and storage at −80 °C or in liquid nitrogen, and storage of dried or lyophilized bacteriophages, are used most commonly. The applicability of these methods for the long-term preservation of various bacteriophages varies depending on a bacteriophage ([1–4] and references therein). Some virions are especially sensitive to freezing, other to cooling at 4 °C, or to freeze-drying [1, 5–7]. Certain single-stranded DNA or RNA (ssDNA or ssRNA) bacteriophages appeared to be more resistant to freeze-drying than to slow-cooling followed by deep-freezing [8].

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In general, either deep-freezing or storage of lysate at 4 °C appeared to be the most effective in the long-term preservation of bacteriophage virion infectivity [3]. The storage at 4 °C is the simplest method. Several bacteriophages, like P1, T4, and lambda, as well as cocktails of bacteriophages against *Shigella flexneri* and *Shigella sonnei* stored in this way retained their lytic activity and high titer even after 30 years ([1, 9]; our unpublished data). The titer of certain *Staphylococcus aureus* and *Pseudomonas aeruginosa* bacteriophages in a cocktail did not change significantly after 12 months of storage at 4 °C [10]. Often, the protection of lysates from evaporation or contamination is sufficient to minimize the decrease in their titer with time, if only cell debris were removed shortly after the lysate preparation. Adsorption of bacteriophages to cell remnants may cause a rapid drop in bacteriophage titer even by several orders of magnitude. The method of viable cell removal from a crude lysate is important as well. We noticed as a rule an about one order of magnitude drop in titer of bacteriophages that are known as chloroform resistant, when they were purified from viable bacteria by chloroform treatment and centrifugation, as compared to filtered lysates. About 30% of bacteriophages are sensitive to chloroform. In the case of these bacteriophages filtering is the method of choice in the purification of lysate from viable bacteria. A more laborious alternative is a purification of a bacteriophage from other lysate components by anion-exchange chromatography through a CIM^{R} monolithic column [11]. It requires optimization for each bacteriophage or for groups of related bacteriophages, but allows the concentration of bacteriophages, whose titers in lysates are low.

Various additives to lysates or to purified bacteriophage suspensions may prevent the loss of bacteriophage infectivity or delay it. Mg^{2+} and Ca^{2+} ions are the commonly used supplements. They are added to a medium prior to infection of bacteria with a bacteriophage to facilitate bacteriophage adsorption and are present in a lysate. Typical Mg^{2+} and/or Ca^{2+} concentrations are 10 mM each. Mg^{2+} ions at 10 mM or slightly higher concentration could even delay a loss of bacteriophage T4 infectivity in suspensions stored at 30 °C [12].

A preferred method of bacteriophage virion storage in large collections that maintain bacteriophage deposits is freeze-drying, if only it is applicable for a given phage [3, 13]. It appeared to be effective for most of the bacteriophages when done properly, and when vials with freeze-dried specimens are protected from the vacuum loss. It does not require any storage space in freezers, which makes it independent of electricity. A detailed procedure of bacteriophage specimen freeze-drying has been described [3]. Its serious disadvantages include inaccessibility for laboratories that do not possess lyophilizers, as well as the necessity to find out an optimal freeze-drying procedure for a given bacteriophage and its

bacterial host. Standard procedures do exist but they are often inappropriate or not optimal for certain bacteriophages [1, 4, 6]. The observed decreases in bacteriophage titers after storage of freeze-dried stocks may vary from one to several orders of magnitude, depending on subtle differences in freeze-drying procedure or the cryoprotectants used [14]. Thus, it is recommended to test how different lyophilization media work with particular bacteriophages and to verify the infectivity of any bacteriophage from a lyophilized stock directly after freeze-drying and in longer time intervals [3]. This makes freeze-drying too laborious and too uncertain as a handy method of bacteriophage preservation, especially if many bacteriophages of unknown properties have to be preserved at once.

A problem that is associated with the storage of bacteriophage virions is a decrease in bacteriophage titer with time [1]. The refreshment of bacteriophage deposits requires de novo bacteriophage propagation. Multiple rounds of propagation combined with high mutation frequencies in bacteriophage genomes may lead to genetic differences between the original bacteriophage isolate and its preserved progeny [3, 15–18]. Thus, minimization of the number of bacteriophage stock refreshments is an important issue in the choice of bacteriophage storage method.

A method that is superior over the bacteriophage virion storage methods in that it can be nearly blindly applied to all bacteriophages, without a knowledge of a given bacteriophage properties, is a preservation of bacteriophages in a form of nucleic acid in freshly infected, deeply frozen bacteria [19]. We have been routinely using this method for the preservation of tailed bacteriophages of different Gram-negative as well as Gram-positive bacteria and have never encountered problems with a recovery of viable bacteriophages. In detailed studies, all 11 randomly selected bacteriophages that were stored that way could be recovered after several months without a significant decrease in the number of pfu, while the preservation of at least half of these bacteriophages in deeply frozen lysates caused a significant decrease in the number of pfu. Even ssRNA bacteriophages Qß and MS2 could be recovered after several months of storage in frozen infected cells without a loss of the number of pfu. The ssDNA bacteriophages, e.g., M13 and fd are converted to double-stranded replicative forms upon cell infection. Thus, their storage in deeply frozen cells in this stage applies to the same rules as the storage of plasmid containing cells, which does not differ from the storage of cells without plasmids [20]. The method works under any circumstances that ensure bacteriophage adsorption and injection of bacteriophage DNA to a bacterium, and the efficient recovery of viable bacterial cells from frozen stocks. Thus, it can be applied to any newly isolated bacteriophage. Additionally, it can serve as a high-throughput method of bacteriophage storage in situations in which a large number of bacteriophages of

unknown properties (e.g., new isolates or mutants) is acquired in a short time and has to be quickly deposited waiting for further analysis. Optimization of the recovery of viable bacteriophages in this method requires:

1. The choice of bacterial cell culture medium and cryoprotectants that are optimal for the high yield of viable bacterial cell recovery from frozen stocks.
2. The choice of appropriate bacteriophage adsorption time.

2 Materials

1. Rich liquid medium for bacterial growth and for bacteriophage propagation in bacteria. The same medium supplemented with 0.7% (wt/vol) agar (*see* **Note 1**).
2. Incubator, water bath with shaking or orbital shaker with regulated temperature.
3. Solution of 50 mM $MgSO_4$ (*see* **Note 2**).
4. Solution of 50 mM $CaCl_2$ (*see* **Note 2**).
5. Spectrophotometer for visual light.
6. Petri dishes (90 mm in diameter).
7. Cryogenic, 2 mL vials with 150 μL of glycerol (*see* **Notes 3** and **4**).
8. Appropriate solid culture media plates (90 mm Petri dishes).
9. Liquid nitrogen or isopropanol-, ethanol-, or ethylene glycol-dry ice bath.
10. Deep-freezer (−70 °C or below).

3 Methods

1. Inoculate 5 mL of rich medium that is appropriate for growth of bacteria that are hosts of a given bacteriophage with cells of a freshly grown colony, and incubate overnight at appropriate temperature (*see* **Note 1**).
2. Dilute an overnight culture in a fresh medium supplemented with 10 mM $MgSO_4$ and 5–10 mM $CaCl_2$ (optionally) to OD_{600} of approximately 0.05–0.1 and grow with or without shaking until the late exponential or early stationary phase (*see* **Notes 5**–**7**).
3. Briefly harvest cells, e.g., by centrifugation of cell portions in a microcentrifuge for ~2 min and resuspend them in the same volume of the fresh medium, cooled to room temperature. Alternatively cool the cell culture to room temperature or use

the bacterial culture in late exponential or early stationary phase immediately (*see* **Notes 8** and **9**).

4. Infect cells with a bacteriophage at the multiplicity of infection (MOI) of about 0.5–1, mix gently and keep without shaking for 2–15 min (*see* **Notes 10** and **11**).
5. Immediately aliquot 850 μL of infected bacteria suspension to cooled cryogenic vials containing 150 μL of glycerol, quickly close the vials with screw caps, mix gently by pipetting (*see* **Note 12**), and immediately deep-freeze in liquid nitrogen or in isopropanol-, ethanol-, or ethylene glycol-dry ice bath (*see* **Note 13**).
6. Upon freezing transfer the vials into a deep-freezer (−70 °C or below) for storage. Use one portion of infected cells with a bacteriophage to assay the number of pfu/mL before freezing (*see* **Note 14**).
7. Melt one portion of frozen infected cell suspension to test the percentage of loss of the number of pfu as above.
8. In addition to bacteriophage infected cells prepare and freeze noninfected cells to use them as hosts for propagation of bacteriophages released from infected cells upon melting. Bacteria for deep-freezing can be taken from the surface of a solid medium or from a liquid culture. In the first case streak bacteria from a frozen stock or from a solid medium on an LB (or other rich medium) agar plate to obtain single colonies. Incubate plates at a temperature of growth that is optimal for a given bacterial species, until colonies appear. Restreak bacteria from a top of single colony on a fresh plate with a similar solid medium to get a dense growth of colonies, and incubate as previously. When the medium is covered by grown colonies take a 2 mL cryogenic vial with 150 μL of sterile glycerol (*see* **Notes 3** and **4**) and add 850 μL of sterile LB, TSB or other medium appropriate for a given bacterium preservation. Mix the content by pipetting and place in ice. Collect as much bacteria as possible from the surface of the plate with a sterile toothpick or a pipette tip and suspend in the medium with glycerol in the cryogenic vial. Mix thoroughly by pipetting and leave for 15 min to allow the cryoprotectant to penetrate cells. Then freeze the vials by immersing in liquid nitrogen or in an ethanol-dry ice bath, and place them in a deep-freezer (−70 °C or below). To take bacteria for deep-freezing from a liquid culture inoculate 5 mL of rich medium with bacteria from a freshly grown colony, and grow them with shaking until the early stationary phase (usually overnight cultures are appropriate). Add 850 μL of bacterial culture to the cryogenic vial with 150 μL of sterile glycerol, mix the vial content by pipetting and deep-freeze as described above (*see* **Note 15**).

9. To recover the bacteriophage from the infected cells one can either melt the whole content of the vial at room temperature and mix aliquots or dilutions with sensitive cells or to scrape a small amount of frozen infected bacteria without melting the whole vial content and add them to sensitive cells. In the latter case one vial can be repeatedly used as a source of bacteriophage.
10. When the large number of frozen stocks of cells freshly infected with bacteriophages of unknown properties (e.g., new bacteriophage isolates) have to be prepared in a short time, one can omit crude assays of adsorption time and latent period and simply prepare 2–3 variants of frozen stocks, using different bacteriophage adsorption time before freezing of the infected cells. We never had problems with the recovery of bacteriophages from one or more stocks prepared in this way.

4 Notes

1. A commonly accessible medium, which is appropriate for growth of a given bacterium can be used for the preparation of bacterial culture. For instance *Staphylococcus*, *Pseudomonas*, *Klebsiella* or *Escherichia coli* cells for bacteriophage propagation can grow in Luria-Broth (10 g/L bacto tryptone, 5 g/L yeast extract, 5 g/L NaCl, pH 7.0) or TSB medium (17 g/L casein peptone, 2.5 g/L dipotassium hydrogen phosphate, 2.5 g/L glucose, 5 g/L NaCl, 3 g/L soya peptone, pH 7.3). If a bacterium has any special requirements for growth, the medium should be changed or supplemented appropriately.
2. Solutions of $MgSO_4$ and $CaCl_2$ have to be sterilized by autoclaving. Filtering through filters removes bacteria but does not remove contaminating bacteriophages if any are present in solutions.
3. The quality of cryogenic vials is crucial for the long-term survival of deeply frozen bacteria. Although commonly available plastic cryogenic vials are good enough for the preservation of the majority of strains, certain bacterial mutants of decreased viability survive better when they are kept deeply frozen in glass cryogenic vials.
4. Cryogenic vials with glycerol can be prepared in advance, sterilized by autoclaving, tightly closed with screw caps and kept at room temperature or at 4 °C to have them on hand. The quality of glycerol is of crucial importance. While Difco glycerol allowed us to preserve the majority of specimens with retaining their good viability, the use of glycerol of certain

other companies, even of the highest purity may cause problems.

5. Infection of bacterial cultures of final optical density OD_{600} of about 0.5 works fine with bacteria of various genera, despite that the number of cells per mL of the culture of different bacteria can vary significantly at the same optical densities. The method works also with cultures of lower or higher densities, e.g., with fresh overnight cultures. However, the density of cells in a culture may influence the cryotolerance and hence the recovery of viable cells from frozen stocks, and the infection efficiency of cells with a bacteriophage. Cells in the late exponential or early stationary phase of growth are the least sensitive to freezing-associated damages [21, 22]. In general, the greater the cell density, the better the recovery of viable cells after deep-freezing and thawing. Additionally, the infectivity of certain bacteriophages to cells in stationary phase of growth is quite efficient [23–25]. However, using cells of late stationary phase is not recommended. Some bacteriophages may infect such cells less efficiently, due to the thickening of cell wall peptidoglycan or other changes in cell envelopes and due to the presence in late stationary phase cultures of a significant proportion of death bacteria or bacterial cells debris. Cultures of low cell densities should not be used either. Although for most bacteria, cell densities of about 10^7 mL^{-1} are already sufficient to ensure viable cell recovery [26], too low cell densities may result in the decreased proportion of infected cells due to the decreased probability of bacteriophage encounters with bacteria.
6. Cultures of aerobic bacteria or facultative anaerobes should grow with aeration before infection, as cells of aerated cultures are more resistant to freezing-associated damages [27].
7. Different supplements to the medium in which bacteria grow can facilitate bacteriophage adsorption by stabilizing the interaction of bacteriophage with bacteriophage receptors (like Mg^{2+} in general, Ca^{2+} in the case of certain bacteriophages; [28, 29] and references therein). Calcium ions may increase the efficiency of plating of certain bacteriophages even by one order of magnitude [29, 30]. In the majority of cases when Ca^{2+} influences the efficiency of bacteriophage adsorption 10 mM final concentration suffices. However, the sufficient or optimal concentrations may vary from bacteriophage to bacteriophage between 4–15 mM [31–33]. Other supplements may facilitate bacteriophage adsorption by increasing the number of receptors for a given bacteriophage on a bacterial cell. An example of the latter is maltose, which induces the synthesis of an outer membrane porin LamB, a receptor for lambda bacteriophage. While ions facilitating

bacteriophage adsorption can be added to the bacterial growth medium, or alternatively just before the addition of bacteriophage to bacteria at the beginning of bacteriophage adsorption step, compounds inducing the synthesis of proteins that are receptors for bacteriophages should be added to the bacterial growth medium at least 1–2 h before the addition of bacteriophage. The addition of L-tryptophan may also increase the adsorption efficiency of certain bacteriophages [34, 35].

8. Harvesting cells and their resuspension in a fresh medium is not obligatory. However, it allows the removal from the culture of toxic metabolites and can increase the survivability of freezing and thawing by cells.
9. Cooling cells to room temperature before their mixing with a bacteriophage slightly slows down cell metabolism and bacteriophage development in those cells that were infected early upon the bacteriophage addition, and could be damaged too early by bacteriophage lytic enzymes to survive freezing. Some bacteriophages, e.g., enterobacteria phage P1 can adsorb to cells at 4 °C, but cannot inject their DNA at this temperature. Thus, one can cool bacterial culture to 4 °C, before the addition of phage, keep the mixture of bacteriophage with bacteria at 4 °C to let the bacteriophage to adsorb to cells for the appropriate time (estimated in separate adsorption assays), and then start synchronous bacteriophage DNA injection by transferring the suspension to room temperature, 30 or 37 °C, dependent on the bacteriophage. Although the synchronization of bacteriophage DNA injection may be especially useful in the case of bacteriophages of short adsorption time and short latent period, this strategy can be used only for bacteriophages that cannot inject their DNA to cells at low temperature. Additionally, cooling cells to 4 °C, and then warming again may decrease their survivability of deep-freezing. Thus, the cryotolerance of cells that were treated this way as well as the optimal bacteriophage adsorption and DNA injection time should be estimated individually in each case. If bacteria are not cooled before bacteriophage addition, use them immediately, to prevent the culture overgrowth.
10. The optimal MOI and time of bacteriophage adsorption to bacteria prior to deep-freezing should be estimated individually for each bacteriophage, dependent on the kinetics of bacteriophage adsorption and a bacteriophage latent period. Optimally, deep-freezing should meet bacteriophage nucleic acid inside a cell but at an early stage of bacteriophage development/eclipse period. Thus, for bacteriophages of short latent period the adsorption time should be short to avoid damaging of cytoplasmic membrane and cell wall by bacteriophage lytic enzymes or completion of bacteriophage development and

release of mature bacteriophages. At advanced stages of bacteriophage development, cells may have the increased sensitivity to freezing and thawing. The release of bacteriophage progeny from the infected cells can decrease the efficiency of active bacteriophage recovery, if bacteriophage virions are sensitive to deep-freezing. Certain bacteriophages can have a very short latent period (<10 min). If they have a short adsorption time, one can manage to freeze cells infected with these bacteriophages at early/middle stages of bacteriophage development. In the case of longer adsorption times the release of bacteriophages from a fraction of cell population that was infected early is unavoidable. One solution of this problem is to infect cells with these bacteriophage using low MOI (0.01 or lower), so that the progeny of early infected cells will have enough new hosts to start new infections. In our laboratory practice, if a bacteriophage has a short latent period and a short adsorption time we use low MOI and incubate the bacteriophage with bacteria for 2–5 min. In the case of bacteriophages of eclipse time over 10 min and a short adsorption time (at least 80% in 5 min) we infect bacteria with a bacteriophage with MOI about 1 and incubate them for 5 min. In the case of bacteriophages of eclipse time over 10 min and of long adsorption time (less than 60% in 10 min) we infect bacteria with a bacteriophage at MOI = 0.5–1.0 and extend the incubation to 10–15 min but no longer than the duration of bacteriophage latent period. In the case of bacteriophages of very long adsorption time the MOI can be increased even to 5.

11. Detailed protocols concerning the assay of bacteriophage adsorption time and latent period have been published in a previous volume of Methods in Molecular Biology [36]. In practice, a rough estimation of bacteriophage adsorption efficiency in time and of bacteriophage latent period can be used to find out what MOI and what adsorption time will be appropriate. To do that take a fresh overnight culture of host bacteria for a given bacteriophage, add Mg^{2+} and Ca^{2+} to the final concentration of 10 mM and infect the bacteria with a MOI of about 0.1. Each 2 min take a portion of the culture, filter it with 0.22 μM filter, to remove bacterial cells and enumerate the unadsorbed bacteriophages with plaque assay using the traditional agar-overlay method [37]. If a bacteriophage is resistant to chloroform, to recover unadsorbed bacteriophages one can treat the culture with chloroform to kill bacteria instead of separating bacteria from free bacteriophages by filtering. For this purpose, it is sufficient to add 1–2 drops of chloroform to a 0.5–1.0 mL of a sample taken from the culture, vortex the sample with chloroform by vigorous vortexing and leave for 2–5 min to allow chloroform to penetrate cells. Bacteriophage

adsorption will decrease the number of free bacteriophages until the end of latent period, when the number of free bacteriophages will start to increase. The optimal adsorption time selected to start mixing the cells with glycerol and their freezing should ensure the adsorption of at least 60% of bacteriophages, if possible.

12. Due to the high density of glycerol, pipetting ensures better mixing of the bacteria suspension with glycerol than vortexing, which is important for the efficient recovery of viable bacteria from frozen stocks upon melting.
13. Deep-freezing of bacteriophage infected cells after the appropriate time of bacteriophage adsorption should be as quick as possible and should be done at the time that do not exceed the eclipse period of used bacteriophage.
14. It is recommended to measure the total number of bacteriophages (free bacteriophages and bacteriophages in infected cells) in one sample or its portion before freezing. This can be done by the measurement of the number of infective centers that can be obtained in a standard plaque assay upon mixing serially diluted portions of the tested sample with sensitive bacteria. By adding chloroform (in the case of bacteriophages resistant to chloroform) to a half of the tested sample or by filtering the sample through 0.22 μM filter one can measure the number of infective centers that are formed by free bacteriophages. By subtracting the number of infective centers that are formed by free bacteriophages in a sample from the total number of infective centers one can easily estimate the number of infected cells in the sample.
15. Although the protocol of bacterial cells deep-freezing that is provided here is applicable for many laboratory bacteria and clinical bacterial isolates, bacteria of some species, such as *Haemophilus* spp., *Helicobacter* spp., *Neiseria gonorrhoeae*, *Campylobacter* spp., *Azotobacter* spp., and others may require the use of certain cryoprotectants, and certain optimization of freezing procedure. The detailed descriptions of different freezing strategies as well as of the suitability of various cryoprotectants for deep-freezing of such bacteria can be found in the literature concerning bacteria preservation by deep-freezing (*see* e.g. [13, 22, 38–40]).

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Part VI

Safety and Regulation

Chapter 18

Bacteriophage Production in Compliance with Regulatory Requirements

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

Abstract

In this chapter we review bacteriophage production requirements to help institutions, which wish to manufacture bacteriophage products for human use in compliance with the applicable regulatory expectancies, defining production processes and implementing relevant controls ensuring 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 development of expedited bacteriophage product development and licensing pathways, including relevant and pragmatic requirements, and allowing for the full exploitation of bacteriophages as natural controllers of bacterial populations.

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 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 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 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

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eliminated by the patient's immune system and especially by the reticulo-endothelial 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]. Next, selected bacteriophages should be produced according to Good Manufacturing Practice (GMP), a challenging task. Bacteriophage products will often consist of several bacteriophages. There are two main purposes for using cocktails of bacteriophages in bacteriophage therapy: (1) to try to overcome bacterial resistance, although in vitro resistance to bacteriophage cocktail was shown to readily emerge in vitro [2], and (2) to streamline "real-world" clinical therapy [3]. For certain clinical indications, bacteriophage cocktails that contain bacteriophages able to lyse a wide variety of pathogenic bacteria—so-called poly-phage cocktails—have been (and still are) 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 MDR infection situations (empirical treatments). However, all bacteriophages that make up a bacteriophage product should have been shown to be individually acceptable. 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–IV clinical trials for clinical efficacy measurement. Finally, an elaborate application dossier for marketing authorization should be compiled and submitted for assessment.

EU regulators believe that the current medicinal product regulation is adequate for bacteriophage therapy products and that no dedicated regulatory framework is needed [4]. They might be right with respect to industrially produced single bacteriophages or predefined bacteriophage cocktails, providing some modifications and logical exemptions. But, these stationary bacteriophage products are less flexible to deal with geographical diversities and time bound shifts in bacterial populations or with the emergence of 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 bacteriophage 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 frequency to that of antibiotic resistance [5]. Because the efficacy of predefined bacteriophage cocktails is likely to decrease over time, they would need to be regularly adapted and reapproved for use. Today, individual approvals should be obtained for each cocktail variant, due to their unique composition [1].

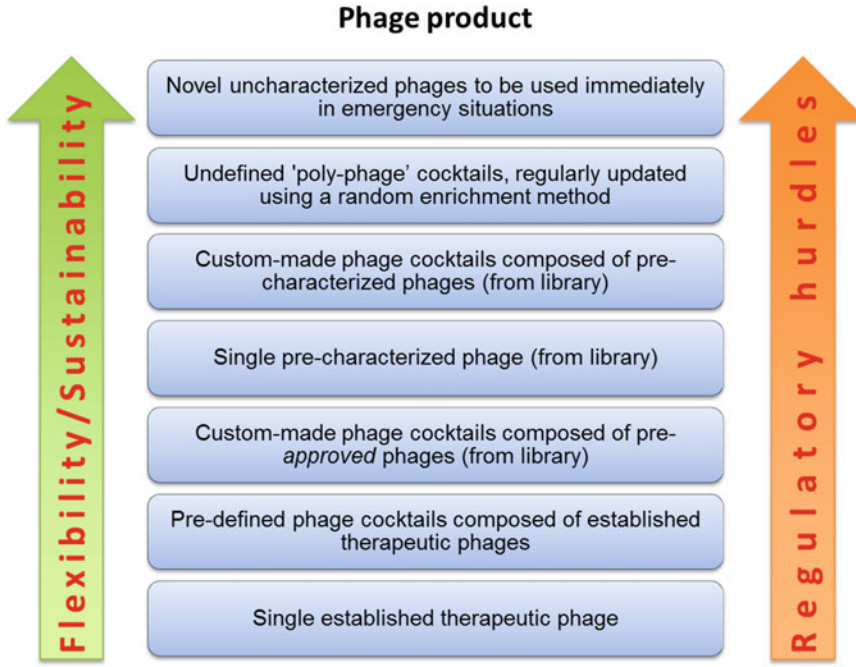

Fig. 1 The more flexible and sustainable the phage product, the more regulatory hurdles the product needs to overcome

According to most bacteriophage researchers, the current medicinal product (a drug in the US) development and approval pathways are not suitable (too rigid and unnecessarily costly) for customized or sustainable bacteriophage therapy approaches [6]. Bacteriophages coevolve with their host bacteria and insure 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, it appears that the more flexible and sustainable the bacteriophage product, the more difficult it is to get it through the current medicinal product (drug) development and approval system (Fig. 1), which was developed to cater for widely used and industrially prepared chemical molecules such as aspirin and antibiotics.

In addition, the established pharmaceutical industry is not ready to invest in bacteriophage therapy products. Weak intellectual property protection (IPP) of natural 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, would compromise their

substantial investments [7]. Bacteriophages are supposed to be 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 [8]. It is estimated to require 2.6 billion USD to bring a new drug to the market [9]. Bringing a typical antibiotic to the market is estimated to cost 400–800 million USD, most of that being regulatory [10].

A few small and medium-sized enterprises (SMEs) did pick up the gauntlet and are now slowly moving along the elaborate and expensive conventional medicinal product (drug) licensing pathway. But, some issues are starting to materialize, for instance during the European Commission sponsored PhagoBurn study, the first major trial under modern medicinal product regulatory standards in the EU. Manufacturing of the investigational products conform to the guidelines recommended by the competent authorities took 20 months and the largest part of the study budget. Even though complex cocktails of 12 *Pseudomonas aeruginosa* and 13 *Escherichia coli* bacteriophages were developed to ensure an acceptable host range, bacteriophage specificity issues turned out to complicate the recruitment of patients [11]. Because the study products target only two of the multiple bacterial species that are known to cause burn wound infections, physicians were reluctant to include patients. Irrespective of the results of the clinical trial, the preliminary phase of the *PhagoBurn* study showed that adapted production and documentation requirements are urgently needed to enable a timely supply of 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 *E. coli* STEC O104:H4 outbreak in Germany in 2011 [12].

Competent authorities for medicines are increasingly aware of these issues, but tend to believe that they will be able to find a solution in consultation with developers and manufacturers. Although they seem to be more or less on the right course for predefined cocktails, it is highly improbable that they will be able to squeeze variable or custom-made bacteriophage cocktails through the conventional medicinal product funnel in a timely way.

The set-up of bacteriophage libraries (bacteriophage banks) of preapproved bacteriophages could allow cocktails to be assembled and timely approved [1]. 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 the emphasis on process controls instead of characterizing each single bacteriophage strain could also make it easier and cheaper to update bacteriophage

cocktails [13]. 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 [14]. 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.

To date, no bacteriophage products have been approved for human use in the EU or in 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 [15]. In acute bacterial infections, broad-spectrum antbiotics are the first choice for rapid (empirical) treatment, but for infections where antibiotics have failed and the patient is still alive, bacteriophages may increasingly come into play [8]. Several EU and US patients suffering from difficult to treat bacterial infections are known to have travelled abroad for bacteriophage therapy.

In some Western countries, policymakers are now entering into discussion with stakeholders on setting a dedicated bacteriophage therapy framework with adapted manufacturing requirements to exploit and further explore the specific nature of bacteriophages as antibacterials. In the near future, some countries may allow customized preparations to be used on a named-patient basis, in consultation with competent authorities and with extensive monitoring. 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 [13].

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.

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 [3].

We will review issues with regard to bacteriophage identification and characterization, bacteriophage cultivation, relevant quality, safety and efficacy controls and bacteriophage product storage. This chapter is meant to help institutions, which wish to manufacture bacteriophage products for human use, defining production processes and implementing relevant controls ensuring quality, safety and efficacy of the final products and meeting 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 the conventional medicinal product or drug pathways. Therefore, we will only focus on products that contain natural whole (intact) bacteriophages.

2 Production Processes

2.1 Bacteriophage Cultivation

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 towards GMP compliance [16]. 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. The dissemination of bacteriophages in the laboratory or production facility where they might induce unwanted lysis of bacteria for months to years after their initial use [17]. Where the production process is not fully closed (e.g. when connecting tubings), GMP grade A cabinets are required for manipulation. The use of entirely closed production systems minimizes the risks of contamination and could allow for bacteriophage production 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 [18].

Bourdin et al. investigated the pilot scale amplification of *E. coli* bacteriophages for bacteriophage therapy [19]. Using various production systems such as wave bags, stirred-tank reactors, and even simple Erlenmeyer flasks, acceptable peak titers of 10^9–10^{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 [19]. The choice of broth had only weak effect on the final bacteriophage titer.

Using well-defined or completely defined media will however increase safety and reproducibility [16]. It is essential that the media used for bacteriophage propagation are free of potentially dangerous contaminants such as prions, viruses and allergens [20]. Vieu (reviewed in [21]) 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 [22]. Montclos (reviewed in [21]) recommended against cultivation of bacteriophages in media

containing animal extracts [23]. Traditional growth media for bacteria often do contain animal extracts (implying a risk of transmission of infectious agents such as BSE) ([23]. 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 [24]. 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 theoretical final concentrations of these remnants, before endotoxin removal, were 25 and 125 mg/ml respectively. Sauve (reviewed in [21]) described the cultivation of bacteriophages in broth without peptone, to avoid the shock induced by peptone [25]. It should be noted that some defined media are *for research use only* and require upgrading to *for further manufacturing use* or to *for 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 GMP [19]. Relatively inexpensive closed cultivation systems could be used in combination with inexpensive microbiological media containing only food-grade supplements can be used for growing bacteria and bacteriophages.

2.2 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 Food and Drug Administration (FDA) and the European Medicines Agency (EMA) mandate that any modern bacteriophage therapy product be made to GMP standards. These standards include 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 [26]. Gill and Hyman discussed the small-scale purification of bacteriophage therapy products [3]. 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 a 0.45 or 0.22 μm membrane.

In the case of Gram-negative bacteria, crude lysates also contain bacterial endotoxin, which is strongly proinflammatory. Therefore,

most Western bacteriophage therapy studies have opted to purify bacteriophage preparations more thoroughly. The degree of further purification s largely function of the type of application the bacteriophages will be used for [3]. A more thorough purification is especially warranted in systemic 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, 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 [3]. 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 [3]. For large volumes, continuous flow centrifugation or 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 [3]. 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 [21]) 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 [27]. Bourdin *et al.* investigated the pilot scale purification of *E. coli* bacteriophages for bacteriophage therapy [19]. 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- 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, while neither ultrafiltration nor chromatography diminished the endotoxin loads. Merabishvili *et al.* used a commercially available chromatographic column that specifically binds endotoxins [24].

Endotoxin limits have been established by the FDA [28] for injectable and parenteral drugs and for medical devices (discussed in [19]). Endotoxin levels in bacteriophage preparations for IV use should not exceed 5 endotoxin units (EU) per kilogram of bodyweight per hour [20]. There is no defined oral endotoxin limit dose, but oral administration to mice of 10^6 EU of *E. coli* endotoxin/mouse elicited no toxicity [19].

3 Quality, Safety, and Efficacy Requirements for Bacteriophage Products

Recently, an international panel consisting of 29 "bacteriophage experts" from ten countries elaborated quality, safety, and efficacy requirements for sustainable bacteriophage therapy products [29]. 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 Directive (EUTCD), which is made up of three Directives, the parent Directive (2004/23/EC), which provides the framework legislation, and two technical directives (2006/17/EC and 2006/86/EC), which provide the detailed requirements of the EUTCD. The EUTCD 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.

The clinical efficacy of bacteriophage therapy products should be evaluated using clinical trials and are not addressed in this chapter. Yet, bacteriophage therapy product manufacturers should demonstrate "efficacy." For 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) [16], 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. The anticipated antibacterial clinical efficacy of bacteriophage products could also be tested in vitro, for instance using validated 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*). Activities are measured under defined test conditions. Unfortunately, no such standard criteria currently exist for bacteriophages [3]. Suitable criteria, for instance based upon a defined lower kill level, should be established [3].

Table 1
Quality, safety, and efficacy requirements for 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 [17]. 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 (BSL) levels will be determined by the bacterial host strain. A well-characterized lytic *E. 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 possible 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, 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 14 June 1993 concerning medical devices and Directive 98/79/EC of the European Parliament and of the Council of 27 October 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

(continued)

Table 1
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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 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 [30] 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 at 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, Rep-PCR,..)
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 phage-like elements of genetic exchange [31, 32] as possible	• 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[a]	• In vitro induction methods (Mitomycine C [33] or UV induction) • State-of-the-art DNA sequencing and analysis (bioinformatics) procedures

(continued)

Table 1
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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) [34]
Validated preservation/storage (cryopreservation, freeze-drying, etc.)	Monitor storage conditions (e.g. temperature)	Variable, depending on the preservation method	Variable (e.g. temperature probes, temperature indicator labels, etc.)
(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 at 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[b] • Highly discriminating genotyping techniques (*e.g.* AFLP, fRFLP (15))[c] • Matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometer [19] • State-of-the-art classification according to the International Committee on Taxonomy of Viruses (ICTV) • State-of-the-art electron microscopy (optional)[d] • Genome size [3] (optional) • One step growth curve [35]
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[e]	• State-of-the-art DNA or RNA sequencing and genome analysis (bioinformatics) procedures[b]

(continued)

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Nontransducing (optional) [36][f]	Screen for "general transduction"	Does not pack random host DNA in a portion of progeny phage particles	Transduction assay [37]
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*)	• Titration of phages against target bacteria according to the soft-agar overlay method [38] • Spot test [35]
	Stability of lysis (optional)[g]	Stable lysis in broth culture for 24–48 h	Appelmans method [39]
	Efficiency of plating (EOP) under conditions similar to eventual clinical application (optional)	Threshold EOP value	EOP determination [38]
	Determination of frequency of emergence of phage-resistant bacteria	Low frequency of emergence of resistance	Method described by Adams [38]
	Determination of growth parameters such as optimal replication temperature and pH, duration of the latent period, average burst size and binding rate (optional) [3]	Threshold values	One step growth curve [35]
	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 [3]	State-of-the-art molecular biology methods
Improvement/ adaptation/ "training" (optional)	Optimization of host range	Broadened and stable host range	• Sequential titrations of phages against target bacteria according to the soft-agar overlay method [35] • Spot test [35]

(continued)

Table 1
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	Shifting optimal growth parameters (temperature, pH, etc.) towards the physiological conditions of the clinical application site	Threshold values	Sequential titrations of phages against target bacteria under gradually changing conditions [35]
	Optimization of phage cocktails towards reducing phages hampering each other upon coinfection [35]	Reduced hampering	Cocultivation of phages [39]
Validated preservation/storage (cryopreservation, freeze-drying, etc.)	Monitor storage conditions (e.g. temperature)	Variable, depending on the preservation method	Variable (e.g. temperature probes, temperature indicator labels, etc.)
(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 [38]
Identification of active substance	Genomic fingerprinting	Matching genomic fingerprint (max. Deviation depends on method)	• State-of-the-art genotyping techniques (e.g. AFLP, fRFLP [40]) • MALDI-TOF mass spectrometer for phage identification [19]
Microbial contamination	Sterility (when there is no sense of urgency)[h]	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 [41]	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)

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Bacterial DNA contamination[i]	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)[j]
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, etc.)	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 14 June 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; (e) how to dispose of unused (expired) phage products			
Validated storage (cold storage, etc.)	Monitor/record/demonstrate storage conditions (temperature, etc.)	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 [38] • CPMP-ICH guideline, Q5C, Q1A • Membrane filtration method (EP method) • pH test (EP method)

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(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

[a] *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 [19]. 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

[b] 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 [42]

[c] 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

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

[e] 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 nontargeted 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 [43]. 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 [43]. 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 [44]. In the future, temperate phages might specifically be used in therapy, e.g. to introduce, by lysogenization, genes conferring sensitivity to antimicrobials [45] or to inhibit virulence traits [46]. Finally, antibiotic stress was also shown to induce genetic transformability in human pathogens [47]

[f] 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)

[g] 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

[h] In some cases, sterility may not be required (e.g. "nonsterile for topical application")

[i] 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 nonpathogenic 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 nonpathogenic host bacterial strains also reduces the potential hazard to the personnel involved in the production of therapeutic phages

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

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.

Therefore, we would like to stress that for bacteriophages intended to deal with urgent public health issues or medical emergencies; less stringent requirements should be considered, 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 preparation [48]:

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. Sterile.
6. Absence of pyrogens (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 [48]. Bacteriophage products 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 *Staphylococcus aureus* infections in burn wound patients [24]. This cocktail, consisting of three carefully selected bacteriophages, was "sterilized" by filtration and purified of endotoxin using a chromatographic column that specifically binds endotoxins. Quality controls included stability (shelf life), determination of pyrogenicity (in rabbits), sterility and cytotoxicity (towards 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, 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 [49]. 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 [50].

4 Conservation, Storage, and Stability

According to most bacteriophage researchers, the best way to store bacteriophages is cooling [20]. 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 [19] and *P. aeruginosa* and *S. aureus* bacteriophages for more than 1 year [24]. 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 [19]. 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 [19]. Bacteriophages can also be preserved by freeze-drying, spray drying, or encapsulation [20].

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Chapter 19

Guidelines for Bacteriophage Product Certification

Alan Fauconnier

Abstract

Following decades in the wilderness, bacteriophage therapy is now appearing as a credible antimicrobial strategy. However, this reemerging therapy does not rekindle without raising sensitive regulatory concerns. Indeed, whereas the European regulatory framework has been basically implemented to tackle ready-to-use pharmaceuticals produced on a large scale, bacteriophage therapy relies on a dynamic approach requiring a regulation on personalized medicine, nonexistent at present. Because of this, no guideline are currently available for addressing the scientific and regulatory issues specifically related to phage therapy medicinal products (PTMP).

Pending to the implementation of an appropriate regulatory framework and to the development of ensuing guidelines, several avenues which might lead to PTMP regulatory compliance are explored here. Insights might come from the multi-strain dossier approach set up for particular animal vaccines, from the homologous group concept developed for the allergen products or from the licensing process for veterinary autogenous vaccines. Depending on national legislations, customized preparations prescribed as magistral formulas or to be used on a named-patient basis are possible regulatory approaches to be considered. However, these schemes are not optimal and should thus be regarded as transitional.

Key words Phage therapy medicinal product, Magistral formula, Named-patient, Autogenous vaccine, Homologous group, Multi-strain dossier, Regulatory framework, Personalized medicine

1 Introduction

After being disregarded for several decades, bacteriophage (or phage) therapy is today experiencing a renewed interest. However, this re-emerging therapy now faces the regulation of health products which was implemented since, entailing serious difficulties. In the European Union (EU), the regulation of human medicinal products is enshrined in Directive 2001/83/EC as amended [1]. This directive stipulates that medicinal products which are "either prepared industrially or manufactured by a method involving an industrial process" are subject to marketing authorization (MA) procedures. Conversely, the handmade "medicinal products prepared in a pharmacy in accordance with a medical prescription

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for an individual patient," usually referred to as magistral formulas do not require registration [1].

One of the most promising approaches of phage therapy is based on the tailored preparation of medicinal products specifically designed for each patient and for each patient's bacterial infection [2, 3], a more personalized "sur-mesure" treatment which guaranties the intrinsic sustainability of the treatment approach [4]. Under this scenario, a customized phage therapy medicinal product (PTMP) would be formulated by mixing suspensions of phages selected from a library and shown to drive lysis of the infecting bacteria. The susceptibility profile of the bacteria should be previously determined through the establishment of a "phagogram." Up to a dozen of different phages used as active ingredients—or "drug substances"—could be then formulated in an ad hoc polyphage [5] finished product—or "drug product"—for subsequent administration to a given patient. Ideally, the phage stocks to be used as the PTMP active ingredients should be manufactured according to approved industrial standards. In this regard, drug substances intended for the formulation of an authorized medicinal product must comply with several regulatory requirements. For instance, their manufacturing process is subject to the GMP requirements for active pharmaceutical ingredients [6, 7] and compliance with these requirements must be attested by a declaration of the qualified person (QP) [8] as set out Art. 8(3) (ha) of the Directive [1]. In contrast, the formulation of the patient-specific finished product is more akin to the preparation of a magistral formula. Hence, the tailor-made PTMP find themselves in an intermediate regulatory context: whereas it would make sense to apply industrial standards and related regulatory requirements to the manufacture of the drug substances, the flexibility offered by the magistral formula scheme would be needed for the formulation of personalized patient-specific drug products. However, the European legislation in force does not lay down any procedure allowing to tackle such an in-between situation. For this reason, the current Community regulatory framework was often singled out as a major hurdle in the development of phage therapy [3, 9, 10]. Accordingly, several publications call for the development of a dedicated regulatory framework specifically suited for phage therapy [2, 11, 12]. Cooper and collaborators made an interesting proposal to address the concern from a new perspective [13]. They appeal for the licensing of pre-approved phage libraries, pointing out that such a pre-approval "would require a radical shift in the thought processes of regulatory agencies." In the same way, a global approach under the concept of the Biological Master File (BMF) was also put forward in order to overcome the limitations currently related to the regulation of customized medicines in general. The BMF procedure, which does not exist in the European legislation, could prove especially valuable for licensing

PTMP [14]. On the 8th of June 2015, a workshop on the therapeutic use of phages was organized by the European Medicines Agency (EMA) [15, 16] with the aim of discussing issues related to this therapeutic approach. This open-minded meeting provided food for thought and gave rise to new ideas primarily based on the legislation currently in force within the European Union (EU). Some of the reflections addressed during this meeting are further developed below. Hence, the regulatory concepts and procedures discussed here refer to the European Community provisions. In addition to this, practical approaches laid down in the legislation of three Member States will be presented. Because of their national character, these approaches cannot be automatically applicable in other Members States of the EU.

2 Existing European and International Regulations and Guidelines

No PTMP has been granted a MA yet in the EU, mainly because these products do not fit well with the current European regulatory framework. Accordingly, there is neither guidance nor monographs which specifically address the PTMP licensing. There are, however, a number of documents which could serve as inspirational model and provide guidance in the assembly of a marketing authorization application (MAA) for a PTMP.

MAA encompasses three main sections usually referred to as quality, nonclinical study reports and clinical study reports, respectively reported in the Modules 3, 4, and 5 of the Common Technical Document (CTD), an international set of specifications for the registration of medicines [17]. Since the singularity of PTMP, as compared to other antimicrobial agents, is mainly relying in their quality features (and much less in the therapeutic area they are intended for), the present chapter is essentially dealing with quality and, in a lesser extent, with nonclinical aspects.

Preparation of a phage suspension necessarily requires a phage seed and a bacterial cell substrate. The international guideline ICH Q5D *Derivation and Characterization of Cell Substrates Used for Production of Biotechnological/Biological Products* [18] may provide insight in this regard. This guideline lists a number of requirements which could be applicable to the bacteria supporting phage growth such as the source, history and generation of the cell substrate, the cell banking system as well as the general principles of characterization and testing of microbial cell banks. Those principles are also outlined in the Food and Drug Administration (FDA) guidance for industry *Characterization and Qualification of Cell Substrates and Other Biological Materials Used in the Production of Viral Vaccines for Infectious Disease Indications* [19]. Moreover, the FDA guidance also provides recommendations related to the viral seeds to be

used in the production of vaccines which could be useful for the derivation of phage seeds intended for PTMP. In these guidelines, it is recommended to organize the cell banks and the virus seeds in two-tiered systems, consisting of a master cell bank/virus seed and a working cell bank/virus seed. This makes sense when it is anticipated that a substantial number of batches will be repeatedly manufactured. However, in the particular case of patient-customized medicinal products, mass production could be avoided. As a consequence, single tier systems, i.e. including a master lot only without working lot, might prove sufficient. With no claim to exhaustiveness and without prejudging what requirements would eventually be requested to comply with regulatory authorities (RA) expectations, one can reasonably anticipate that the bacterial cell substrate will have to be tested for identity, and, unless thoroughly justified, for absence of prophages, antibiotic-resistance genes and virulence factors. As for the phage seeds, they should undergo both genotypic and phenotypic characterization. Phage genome sequencing would allow addressing their identity as well as the absence of virulence factors/toxins and absence of genes necessary for temperate lifestyle. The genetic stability profile of the phages could possibly be addressed by genomic comparison of the master seed and "end of production" phages (i.e. samples collected at the end of the upstream process). Phenotypic characterization of the seeds could include a test for target bacteria specificity (i.e. phages should specifically infect the patient isolate or only a limited number of closely related strains), a test for absence of lysogeny and the determination of morphology by electron microscopy.

Customized polyphage PTMP differ from conventional medicinal products in a major aspect. In contrast to the latter, which have a fixed predetermined qualitative and quantitative composition, the composition of the former varies tremendously since it results from a combinatory process. A number of tests and studies which are usually performed at the drug product (i.e. finished product) level cannot be reasonably conducted for all possible combinations. For instance, a validated potency test including specification limits, which allows to specifically quantify each active ingredient present in the drug product is normally required for any medicinal product. As for the stability of the medicines, studies should be conducted on both the drug substance and the drug product. However, achieving real-time stability studies as well as developing and validating a method according to the recommended standard [20] are time-consuming operations. Repeating these exercises for all possible polyphage customized preparations is not realistic. Similarly, conducting nonclinical animal studies on all drug product combinations is neither technically achievable nor ethically defensible. As a consequence, several tests and studies normally carried out on the drug product should be performed on the individual drug

substances. And not even with this particular disposition could a complete testing of all phages be achieved. Actually, even if the drug product combinatory escalation is bypassed by performing analyses at the drug substance level, not all particular phage preparations might be individually subjected to nonclinical studies. Indeed, the library could gather dozens if not hundreds of singular phages, this being too much for testing them all in vivo. Another example where systematic studies would not be achievable on each drug product combination is the Environmental Risk Assessment (ERA). Should a PTMP contain recombinant phages, then it would be subject to the provision of Art 8(3) (ca) and (g) of Directive 2001/83/EC. The related information should be presented in accordance with the provisions of Directive 2001/18/EC related to the deliberate release of Genetically Modified Organisms (GMO) [21]. Again, conducting and reporting an ERA for each drug product combination and even for each individual phage stock is not reasonably achievable.

Interestingly, two existing regulatory concepts might be helpful for addressing this issue. Strictly speaking, none of these is legally applicable to PTMP but they can likely foster reflections.

The "multi-strain dossier" approach, implemented for the regulation of certain veterinary vaccines, is one of these. The *Guideline on Data Requirements for Multi-Strain Dossiers for Inactivated Vaccines against Avian Influenza, Bluetongue and Foot-and-Mouth Disease* [22] is providing a regulatory framework for vaccines which need rapid and frequent changes in their strains composition and therefore, do not fit well within the general regulatory model for vaccines. As mentioned in this guideline, "the advantages to the applicant (and authorities) of a multi-strain dossier as proposed are the need to maintain only one dossier which can cover a wide range of vaccine strains. Although some specific information will be needed for each strain, other aspects can be dealt with "globally" where the same information is relevant for vaccines produced using any of the strains. This will avoid the need for a separate authorization for each vaccine strain and also each possible combination of vaccine strains that might be envisaged." This approach is not so far from a possible answer to PTMP conundrum and covers objectives at both the regulatory and the scientific levels. From a regulatory point of view, the marketing authorization for a multi-strain dossier allows some flexibility regarding the description of the medicinal product's composition. According to Art 8 (3) (c) of Directive 2001/83/EC, qualitative and quantitative particulars of all the constituents of the medicinal product should be provided in the MAA. This requirement is difficult, not to say impossible, to fulfill for a customized PTMP whose composition is expected to change from patient to patient. Instead, the medicinal product composition within a multi-strain dossier specifies the strains that may be included in the final product but the number

and type of strains actually included in the final product may be adapted to the current epidemiological situation at the time of formulation. Applying this provision to the PTMP could allow granting a MAA on the basis of a generic composition whereas the actual composition could be tailored for one patient/infectious episode. The multi-strain dossier procedure could thus be used as a model for overcoming the regulatory sticking point linked to the absence of a predetermined qualitative and quantitative composition for PTMP. Under this view, PTMP could be approved with a generic composition stating the maximum number of phages and their highest titer. The batch release of the product would be then performed with a customized labeling specifying the actual composition of each specific lot.

The multi-strain dossier approach is interesting in more ways since it can provide scientific and technical guidance putatively applicable to polyphage PTMP. As for the method of preparation, it is recommended to establish the maximum number of strains to be incorporated in the blending of the final product. The quantity of excipients as well as the volume of one dose should be the same regardless of the number of active ingredients and their respective quantities. The control tests during production should preferably be the same for all strains. The multi-strain guideline also suggests an interesting approach which consists of performing studies, validating methods and establishing specifications on mono-strain preparations of finished product. Such an approach appears as an intermediary between performing studies at the level of the phage drug substances on the one hand, and at the level of the polyphage drug product on the other. With this in mind, data could be collected from mock-up monophage drug products and then further extrapolated to any polyphage drug product combining these phages. For instance, stability testing or efficacy studies might possibly be achieved on monophage mock-up preparations. It should be noted, however, that the guideline prescribes that other tests, such as those aimed at addressing the safety, should be carried out using a batch with the maximum number of strains proposed for the finished product.

The multi-strain guidance thus brings some insight into the way the PTMP regulation could possibly be dealt with.

The "homologous group" approach is a second concept which could be usefully exploited for PTMP regulation. This concept was raised in the *Guideline on Allergen Products* [23] where it is stated: "it is impossible to determine all relevant parameters for the allergens within a given extract or a defined allergen extract mixture. Therefore… extrapolation of stability data among members of taxonomic families were… used by the applicants. The concept of homologous groups introduced here replaces the concept of taxonomic families." This concept is more elaborated in the guideline where it is further mentioned that "to a limited extent, data on

quality, safety and efficacy can be extrapolated from the representative source to other members of the homologous group." As can be seen, the principle of selecting a representative source from which not only stability data but also other quality as well as safety and efficacy features can be extrapolated to a larger group already exists for certain medicinal products. Obviously, the grouping should be scientifically justified. In this regard, it is worth noting that in the revised guideline on allergens, the former principle of taxonomic families was replaced by the concept of homologous group [24], suggesting that a grouping based on the sole criterion of taxonomy might be not robust enough. This emphasizes the importance of the above-mentioned phenotypic characterization of the phage seeds, proving that the genome sequencing alone is likely insufficient. In our opinion, the concept of homologous group could be advantageously used for tackling the problem raised by the considerable number of species and strains making up the library of phages. A full set of data (e.g. validation of manufacturing process, stability data, safety assessment in nonclinical studies, ERA for GMO, ...) would be needed for the representative phage whereas only limited amount of data would be required for the other members of the homologous group. Such a strategy would also make the inclusion of new phages or variants/mutants within the library expected to grow over time easier.

There is at least one precedent of a medicinal product, for veterinary use in this case, which was authorized in the EU on the basis of an adaptive composition to be customized for a given patient [25]. It is interesting to note that the product in question is an allergen medicinal product and that the description of its composition, i.e. "contains at most eight allergens," is congruous with the recommendations formulated in the guideline on multi-strain dossiers. As can be seen, the principles outlined in the guidelines on allergens and on multi-strain dossiers have already been diverted in order to provide a pragmatic answer to an unforeseen regulatory situation.

Another feature which distinguishes PTMP from conventional medicinal products is the shortened time-span between the manufacture of the final product and its use. Indeed, since bacterial infections are often fast evolving diseases, fighting an isolated infection event usually requires prompt therapeutic measures. This implies that the delays between the formulation of the customized drug product and its administration to the patient are tight, likely too short for achieving all the tests usually required for releasing a conventional medicinal product. This particular feature of PTMP is shared with certain autologous cell therapy medicinal products which are intended to be used in a patient-specific manner. In this respect, the *Guideline on Human Cell-Based Medicinal Products* [26] foresees exceptions to the rule of the exhaustive release testing of drug products. One of these exceptions is the impracticability of

performing a complete testing due to time restrictions. Accordingly, the testing at one level (e.g. drug product) may be reduced provided that it is balanced by an exhaustive control at the other (e.g. drug substance). Following this principle, the release of a given PTMP could be mainly based on upstream process control data (e.g. phage titer, endotoxin, sterility/bioburden, ...), determined on the drug substances before blending, instead of end product testing as normally required for conventional medicinal products. However, as stated in the guideline, it should be noted that "a critical set of essential tests that can be performed in the limited time prior to clinical use must be defined and justified. Whenever feasible, retention samples should be stored for future analysis." In our opinion, the individualized nature of polyphage PTMP and the consequent need for their nearly bedside manufacturing make them prone to implementation of real-time release testing, an approach set out in the ICH guideline Q8 (R2) on Pharmaceutical Development [27]. This strategy assumes that the evaluation and assurance of the quality of a final product can be based on measured material attributes and process controls. In other words, the quality controls can be shifted upstream in the manufacturing process whereas the end-product testing will be reduced accordingly. Such measures could possibly be relevantly applied to the PTMP licensing process.

3 Accommodating Regulatory Strategies Developed at the National Level

In certain circumstances, the Community code leaves the door open to some flexibility in the setting of the regulatory framework at the national level. Three national regulatory strategies putatively relevant for phage therapy are presented below. However, by nature, transposition of the European provisions is subjected to national accommodations and may vary between Member States. These approaches are thus not necessarily applicable in all Member States.

3.1 The "Specials" Scheme

The named-patient use programs as meant in Article 5.1 of Directive 2001/83/EC, also referred to as "Specials" scheme, allows supplying unlicensed medicinal products. This article lays down that medicinal products, even prepared industrially or manufactured by a method involving an industrial process, may be exempted from the need for a marketing authorization provided it is: "supplied in response to a bona fide unsolicited order, formulated in accordance with the specifications of an authorized health-care professional and for use by an individual patient under his direct personal responsibility" [1].

The United Kingdom (UK) developed the relevant legislation to implement this provision and to allow the supply of unlicensed

medicinal products. The Medicines and Healthcare Products Regulatory Agency (MHRA) as the UK's competent authority which regulates medicines, issued guidance documents on the supply and manufacturing of "Specials" [28, 29]. In line with the Directive and the applicable legislation, these documents point out that unlicensed medicines can be supplied in order to meet the special needs of an individual patient. The medicinal product must be manufactured and assembled in accordance with the specification of a health-care professional in response to a bona fide unsolicited order. This means that the health care professional will contact the supplier directly and not the reverse, and that "the medicinal product must have been prescribed by the doctor as a result of an actual examination of his patients and on the basis of solely therapeutic considerations" [30]. The medicinal product should be manufactured by a supplier which holds a Manufacturer's "Specials" license. The manufacturing site will be "inspected for compliance with Good Manufacturing Practice (GMP) and the conditions of the license. These require that manufacture or assembly is carried out under the supervision of appropriately qualified staff, including a named quality controller and production manager, who are acceptable to the Licensing Authority" [28]. It should be emphasized that the licensing applies to the manufacturing site and its operations and not to the product itself which is unlicensed. In contrast to medicines having a marketing authorization, a qualified person (QP) is not required to sign off the batch release of "Specials." Instead, their release is to be performed by a quality controller or a nominated deputy. Furthermore, the "Specials" are not submitted to the pharmacovigilance requirements normally applied to licensed medicinal products.

Several authors invoked the named-patient program for regulating personalized medicines [31, 32] including PTMP [10]. In absolute terms, there is no formal objection to applying this program to the PTMP regulation. However, this scheme was originally designed to focus on an individual patient and not to reach a broader patient population. Whereas a particular PTMP is actually intended for a given patient, the bacteriophage therapy as an alternative to antibiotics has more in common with a sizable therapeutic approach. It should also be noted that in practice, the "specials" scheme as it applies in UK is mostly used to reformulate licensed medicines [32].

3.2 Autogenous Vaccines

The so-called autogenous vaccines are defined in Directive 2001/82/EC on the Community code relating to veterinary medicinal products as "inactivated immunological veterinary medicinal products which are manufactured from pathogens and antigens obtained from an animal or animals from a holding and used for the treatment of that animal or the animals of that holding in the same locality." However, they are excluded from the scope of this

Directive. Licensing procedures of these products, if any, are thus within the remit of national competent authorities. These medicinal products share with the PTMP the fact that they are tailored for a particular infectious event. Therefore, regulatory prescriptions for autogenous vaccines could putatively serve as a model for PTMP regulation.

The terms of use of autogenous vaccines in France, as comprehensively reviewed by Lacroix [33], are placed within a well-defined regulatory framework, which encompasses a specific authorization pathway distinct from the MA. The authorization is granted not to the vaccines themselves but instead to a qualified person or to an establishment (e.g. a company) employing a QP [34]. The vaccine preparation must be carried out under his responsibility and he must be a pharmacist or a veterinarian that has been authorized by the regulatory authorities [35]. Autogenous vaccines must be prepared in accordance with the Good Preparation Practices (GPP) [36] and compliance to this quality standard is ensured by inspections conducted by the authorities. They must be prescribed by a veterinarian which shall assume responsibility in the same way and within the same limits as off-label prescription. Adverse reactions and/or lack of therapeutic efficacy should be reported in a pharmacovigilance declaration by the prescribing veterinarian.

Needless to say that the autogenous vaccines procedure is not applicable to PTMP per se. Indeed, by nature, autogenous vaccines are veterinary medicinal products. Being vaccines, they are exerting an immunologic action, fundamentally different from the PTMP mechanism of action. They are made of inactivated pathogenic bacteria and not live viruses. However, they can serve as an interesting regulatory model. Indeed, whereas the provisions set up for autogenous vaccines tend toward the requirements enforced for industry-made medicinal products, from a legal perspective they are definitely counted as magistral formulas. This intermediate situation could inspire the setting of ad hoc regulatory processes aimed at providing a legal framework for PTMP use, as exemplified by the Belgian experience outlined below.

3.3 The Magistral "Premium" Formula

As said above, customized PTMP lie somewhere between industry-made medicinal products (subjected to MA) and magistral formulas (i.e. not subjected to MA). This intermediate situation makes unenforceable all the provisions of Directive 2001/83/EC, making MAA unfeasible. In contrast, the less restrictive regulatory requirements of magistral formulas can be met in their entirety whereas, based on the model of autogenous vaccines, inclusion of additional requirements may be considered in order to reach an enhanced regulatory framework unofficially referred to as the magistral "premium" formula.

This option, currently in development in Belgium, could provide a convenient legal framework for PTMP use at the national

level. According to the current legislation [37], the active substances used as starting materials in the manufacture of unlicensed medicinal products, namely officinal and magistral preparations, must meet the requirements of the European Pharmacopoeia (Ph. Eur.), of the Belgian Pharmacopoeia or, failing this, of an official pharmacopoeia. If no compendial document exists, then the starting material in question must be authorized by the Minister of Public Health following a favorable opinion of the national Pharmacopoeia Commission. Though in the case of magistral preparations, to the exclusion of officinal formulas, nonauthorized active substances may also be used on the condition that they are provided with a certificate of analysis issued by a Belgian Approved Laboratory. The certificate should contain identity and quality control data obtained from methods in line with the current state of technical and scientific knowledge. This provision allowing use of nonauthorized active substances was introduced in order to avoid any derogation from the principle of therapeutic freedom and professional autonomy of physicians. Since dozens, if not hundreds, of different bacteriophages could eventually be gathered in the library, an authorization issued by the Minister for each bacteriophage individually is practically not feasible. For this reason, the option of the nonauthorized active substance process was finally retained.

Within the list of Belgian approved laboratories available on the website of the Federal Agency for Medicines and Health Products (FAMHP) [38], the Scientific Institute of Public Health [39] appears as a likely candidate laboratory for testing bacteriophage lots and issuing valid certificates of analysis.

The qualifier "premium," unorthodoxly intertwined to the denomination "magistral formula," actually refers to an enhanced approach. In the standard procedure for nonauthorized active substances to be used in magistral formulas, the FAMHP is not involved, despite being the national competent authority for medicines. Normally the process takes place between the physician and his patient, the approved laboratory and the pharmacist plus, if applicable, the manufacturer of the active ingredient. In the present case, however, because of the innovative nature and the unconventionality of phage therapy, the FAMHP called for a joined-reflection and a collaborative work between all the stakeholders, which was welcomed by the parties. Since there is no dedicated regulatory framework for such a process, it should take place within the context of the existing national Scientific-Technical Advice (STA) procedure [40]. However, it should be highlighted that the STA procedure is initiated on a voluntary basis. Moreover, the provided advice is to be considered as not legally binding, neither towards the FAMHP, nor to the applicant. In such a context, a trust relationship and a transparent communication between each party appear as key assets for coping with the singularity of phage therapy and its regulation.

3.4 Weaknesses of the Regulatory Strategies Developed at the National Level

The above-described national procedures suffer from the same weakness: none leads to a marketing authorization. Granting a MA is coupled with several legal requirements such as the approval of the Summary of Product Characteristics (SmPC). The SmPC must be completed by the applicant and submitted as an inherent part of the MAA. It is a legally binding document subject to the approval by the competent authorities. It gathers the information for healthcare professionals on how to use a medicine safely and effectively. Practically, this means that if a physician prescribes a medicinal product within the terms of the SmPC (therapeutic indication, dose regimen, target population, ...), untoward effects other than those indicated in the SmPC are then the legal responsibility of the manufacturer. In contrast, as there is no SmPC for unlicensed medicinal products, the prescriber takes full responsibility in law for any adverse reaction caused by the medicine unless it can be shown that there was a pharmaceutical malpractice.

Besides the SmPC, the package leaflet (PL) also forms part of the product information legally required for medicines that are granted a MA. Less information to the patient is available for unlicensed products since PL is not required in this case.

The establishment of the benefit–risk balance is another legal requirement of the licensing process. This exercise, which forms a cornerstone of the MA procedure is lacking for unlicensed medicines. For these latter, the body of data is predominantly focusing on quality while leaving aside safety and efficacy aspects. And even with respect to quality, the data package is expected to be far less comprehensive as what is required for a MAA. The quality of the products may thus be variable.

As mentioned previously, several other legal requirements for authorized products, such as compliance to GMP, QP release, pharmacovigilance monitoring, and so on, are either not strictly or simply not applied to unlicensed products.

4 Conclusion

Phage therapy poses challenges to the European regulation for medicinal product. No specific guidance is currently available for PTMP. More fundamentally, the current EU legal framework is not suited for personalized medicines in general. It is noteworthy that on several occasions, the legislature demonstrated adaptability by developing particular provisions and by providing exceptions for the purpose of regulating atypical medicinal products. The seasonal change of influenza vaccine composition [41], the core dossier for pandemic influenza vaccines [41] or the hospital exemption granted to certain advanced therapy medicinal products as meant in Art. 3(7) of the Directive [1] are just a few precedents.

We especially addressed here some interesting examples of ad hoc regulations such as the multi-strain dossier approach set up for particular animal vaccines, the homologous group concept developed for the allergen products and the licensing process for veterinary autogenous vaccines. None of these are applicable to PTMP as such but they introduce concepts which could be recruited for designing what might be a suitable regulatory framework for bacteriophage therapy. These deviations from the basic MA pattern show that there is room for defensible exceptions and reason to believe that the EU legislation will eventually be adapted in order to meet the PTMP regulatory challenges, especially seeing the urgency and negative societal impact of the global antibiotic crisis.

In the meantime, phage therapy potential should be fulfilled by relying on existing regulatory schemes, even if these are not entirely satisfactory. As exemplified in this chapter, customized preparations prescribed as magistral formulas or to be used on a named-patient basis are certainly avenues to be worth exploring in close collaboration with the national regulatory authorities of the respective EU Member States. However, it should be remembered that these procedures were originally set up for individual patients. Their diversion as regulatory processes intended for a large scale therapeutic use is likely not the most appropriate option but at least, this option exists. In our opinion, these schemes should thus be regarded as transitional, pending the establishment of a more robust regulatory framework, well-designed for personalized medicines including PTMP.

Confronted with the threat of antimicrobial resistance and the major public health predicament it entails, we believe that phage therapy has a place within the therapeutic armamentarium against bacterial infections. Based on the premise that the interest of the patient should prevail over strictly legalistic considerations, we think that the regulation needs to be adapted to phage therapy rather than the other way around. This of course cannot be done at the expense of quality, safety, and efficacy. But eventually, this should be achieved.

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Part VII

New Phage Therapy Approaches

Chapter 20

Nano/Micro Formulations 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, a purified lysate highly concentrated (around 10^{10}–10^{11} pfu/mL) is necessary, and to dispose of 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 also be adapted to any bacteriophage and possibly to any delivery route for bacteriophage therapy.

Key words Bacteriophages, Liposomes, Alginate, Nanoparticles, Microparticles

1 Introduction

The benefits of using bacteriophages against the most common food-borne pathogens along the entire food chain are now well known [1]. Similarly, its application for the treatment of humans and animals' infections as an alternative to antibiotics [2], or in combination with these molecules [3], is being studied intensively with very promising results. However, bacteriophages are sensitive to different environmental factors such as pH or temperature, which causes their breakdown and subsequent loss of infectivity and a reduced therapeutic efficacy. Perhaps, it is in the oral bacteriophage therapy against intestinal pathogens where the pH effect is most apparent, because bacteriophages typically lack stability in the extremely acidic environment of the chicken stomach [4]. Additionally, the residence time of bacteriophages in the intestinal tract is very short [5].

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Encapsulation methodologies protect the bacteriophages for overcoming critical environmental conditions [6–8]. For bacteriophage encapsulation, the following aspects should be addressed: (1) the use of biocompatible materials for allowing their administration to living beings or foods; (2) the choice of encapsulation technologies that do not inactivate bacteriophages, and (3) appropriate size of encapsulated bacteriophages according to their application.

Among different biomaterials, alginate is one of the most commonly used for bacteriophage encapsulation. This is due to its high viscosity which confers mucoadhesive properties [9]. Moreover, microcapsules of alginate in combination with other materials [chitosan [10], $CaCO_3$ [11], pectin [12], whey protein [13]] 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 as egg-box suitable to entrap molecules higher than 10,000 Da 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 allow the polymerization of the alginate [14].

Cationic lipids are another biocompatible material for bacteriophage encapsulation as they readily allow the encapsulation of such biological entities which are negatively charged [15, 16]. The liposomes provide the following properties: (1) barrier to protons, thus protecting bacteriophages against gastric acidic pH [17], (2) promoters of mucoadhesiveness, owing their positively charged surfaces [18, 19], which would prolong the intestinal residence time of bacteriophages; (3) in oral therapy against intestinal pathogens, they are degraded upon contact with intestinal bile salts [20], allowing the delivery of bacteriophages in the desired site; (4) their nanometric size facilitates their administration; (5) their capacity of diffusion through the mucosa allows their interaction and possible absorption [21]; and (6) liposomes protect bacteriophages from the neutralizing antibodies [22].

For lipid encapsulation thin-film hydration method [23] is used, since hydrophobic reactions allow obtaining multilamellar, unilamellar vesicles or liposomes. For vesicle formation, a lipid mixture dissolved in an organic solvent is dehydrated on a glass surface, forming a thin lipid layer which is after hydrated with water, sucrose, or other electroless solution and stirred gently.

This causes the separation of lipid sheets from the surface and eventually ends up forming 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 for increasing the encapsulation effectiveness. However, the reduction of the vesicles' size by extrusion confers higher stability to the capsules [24]. Other modifications that can be used include electroforming [25] or gel-assisted hydration [26].

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, rends capsules of nanometer size (from 309 to 326 nm) with an encapsulation efficiency of around 50% [24]. 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% [27]. To perform both methodologies a purified lysate at high concentration is necessary (around 10^{10}–10^{11} pfu/mL) and to dispose of the adequate equipment. *Salmonella* bacteriophages with short and long tail 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 [24, 27]. 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 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 a bacteriophage suspension in 10 mM MgSO4 at a concentration of 10^{11} pfu/mL.
2. Alginate.
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)).
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 Icn., 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 50K (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 in thin-film hydration method [28]. Methodology described here is protected by the patent EP15705303.4.

1. Hydrate the 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 4**).
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 passing through the extruder ten times using a 1 mL syringe.
3. The capsules obtained are stable at 4 °C for at least 6 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 [11].

1. Add 0.5 g of $CaCO_3$ and 0.9 g of alginate to 50 mL of the bacteriophage suspension for obtaining 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 bar.
4. Harden the gelified capsules by maintaining 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 removing the supernatant.
6. Add 30 mL of 10 mM $MgSO_4$, and resuspend the pellet by vortexing.
7. Repeat the centrifugation step three times 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 6 months.

3.3 Characterization of the Nano/Micro Particles

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 noninvasive backscatter technology.

3.3.1 Lipidic Nanoparticles

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 ten 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 5**).

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.

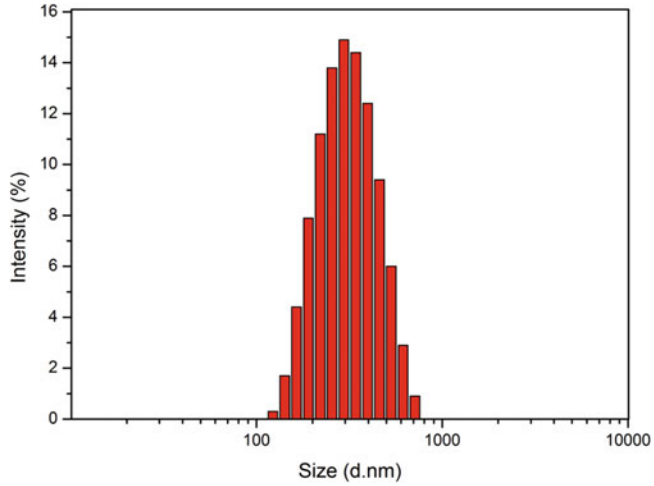

Fig. 1 Example of the particle size distribution of liposome-encapsulated bacteriophage of the *Podoviridae* family, as measured by dynamic light scattering

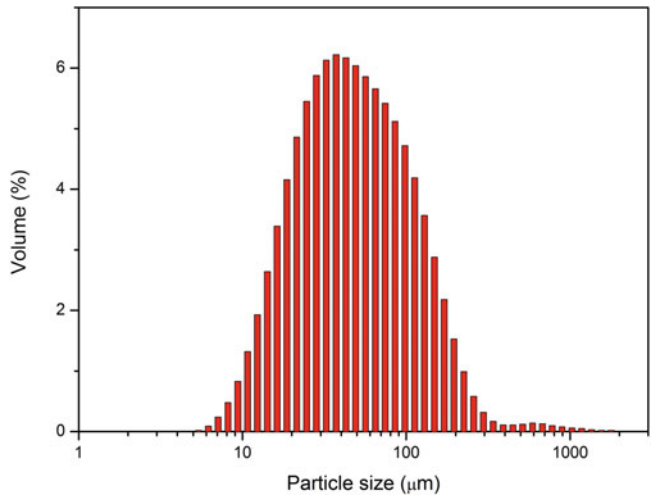

Fig. 2 Example of the particle size distribution of alginate/$CaCO_3$ encapsulated bacteriophage of the *Podoviridae* family, as measured by laser diffraction

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 polystyrene sterile 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 [29], using the appropriate bacterial tester strain.
3. Put the plates in an incubator at 37 °C for 18 h.

   The bacteriophage concentration obtained corresponds to the concentration of free bacteriophages (C_{free}).
4. Treat 0.5 mL of the result of encapsulation with 0.5 mL of 50 mM bile salts to disrupt the liposomes (*see* **Note 6**). Put the mixture in 1.5 mL microcentrifuge tubes.
5. 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 [29], similarly as described before. The bacteriophage concentration obtained corresponds to the concentration of total bacteriophages (C_{total}).
6. Repeat the experiment at least three times, and calculate the mean of C_{free} and C_{total}.
7. Apply the formula:

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

3.4.2 Alginate/$CaCO_3$ Microparticles

1. Prepare polystyrene sterile 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 [29], using the appropriate bacterial tester strain
3. Put the plates in an incubator at 37 °C for 18 h.

   The bacteriophage concentration obtained corresponds to the concentration of total bacteriophages (C_{total}) (*see* **Note 7**).
4. 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.
5. Make the appropriate dilutions of the filtered and plate them onto agar plates, similarly as described before. The bacteriophage concentration obtained corresponds to the concentration of free bacteriophages (C_{free}).

6. Repeat the experiment at least three times, and calculate the mean of C_{free} and C_{total}.
7. Apply the 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 8**)
3. Plunge the grid into liquid ethane at −180 °C and then transfer 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).

Confocal Microscopy

To confirm that the bacteriophages have indeed been encapsulated within liposomes, fluorescently labeled samples are observed by laser confocal microscopy.

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 [30], and purify using Amicon Ultra-15 Centrifugal Filters 50 K three times (*see* **Note 9**).
3. Prepare fluorescent labeled lipid thin film (*see* **Note 10**) 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 same protocol as 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 11**) (*see* Fig. 3b).

3.5.2 Alginate/$CaCO_3$ Microparticles

Morphology of alginate microcapsules is examined in the Axio observer.Z1 microscope.

Optical Microscopy

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

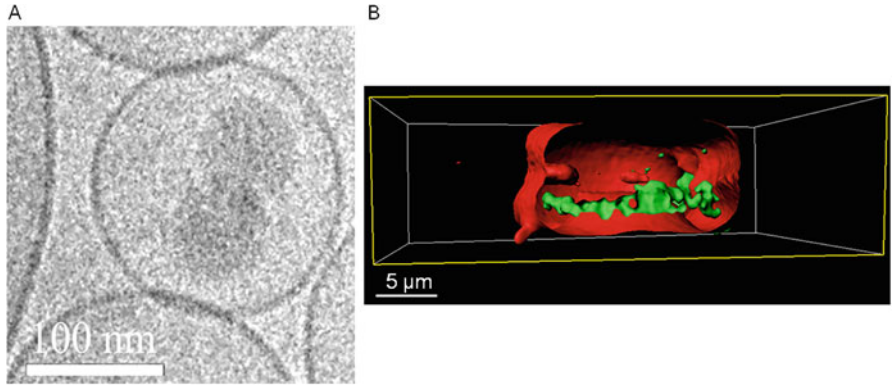

Fig. 3 Example of Cryo-TEM image of a liposome-encapsulated bacteriophage of the *Podoviridae* family (scale bar, 100 nm) (**a**), and 3D confocal microscopy cross-sectional image of this bacteriophage, labeled with SYBR gold (*green*) and encapsulated into fluorescent DiI-labeled liposomes (*red*),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

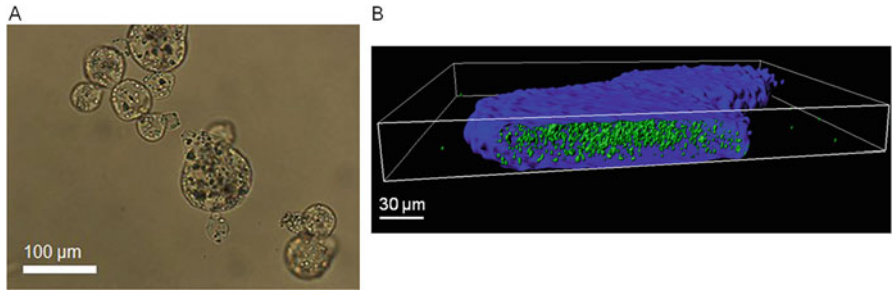

Fig. 4 Example of optical microscopy image of an alginate/$CaCO_3$ encapsulated bacteriophage of the *Podoviridae* family (scale bar, 100 µm) (**a**), and 3D confocal microscopy cross-sectional image of this bacteriophage, labeled with SYBR gold (*green*) and encapsulated into DAPI-labeled alginate/$CaCO_3$ (*blue*), (scale bar, 30 µm) (**b**)

Confocal Microscopy

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

1. Label the bacteriophage with SYBR-gold as it is described at Subheading 3.5.1.
2. Fluorescent label the alginate polymer with 4′,6-diamidino-2′-phenylindole dihydrochloride (DAPI) following a previously described protocol [31].
3. Encapsulate SYBR-gold labeled bacteriophages into DAPI-alginate matrix, following the protocol described above (Subheading 3.2).
4. Add 30 µL of sample onto a coated glass slide and observed in the Leica TCS SP5 confocal microscope (*see* **Note 12**) (*see* Fig. 4b).

4 Notes

1. Bacteriophages must be used after purification by ultracentrifugation applying the appropriated speed according to the size of the bacteriophage. After ultracentrifugation, it is recommended the ultrafiltration of the bacteriophage lysate using the Amicon Ultra-15 Centrifugal Filters de 100 K (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. These conditions were validated for a maximum vesicle volume of 60 mL per batch.
5. Typical values of potential zeta are ranging between +31 mV and +35 mV.
6. 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.
7. In this case, total concentration of bacteriophages is obtained without additional treatments due to divalent ions (Ca^{2+}) 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 gelification of the double agar layer.
8. Appropriate film thickness would be between 20 and 400 nm.
9. It is important to adjust the volume with 10 mM $MgSO_4$ between each purification step.
10. The mixture is made adding 10 μL of Vybrant DiI solution per 20 mg of lipid mixture.
11. Resonance scanning mode of Leica TCS SP5 confocal microscope is applied due to the fast Brownian movement of the particles due to their low size.
12. In this case, resonance scanning mode is not necessary as the size of this type of capsules is big enough to reduce their Brownian movement.

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Chapter 21

Synthetic Biology to Engineer Bacteriophage Genomes

Ana Rita Costa, Catarina Milho, 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 towards 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 are addressed: a yeast-based platform and bacteriophage recombineering of electroporated DNA.

Key words Bacteriophage, Bacteriophage engineering, YAC, BRED

1 Introduction

Bacteriophages have long been praised 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 one of the most promising alternatives to antibiotics in areas from healthcare to food-processing, agricultural, and veterinary fields [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) [6–9].

The ever-expanding collection of bacteriophage genomes deposited in the National Center for Biotechnology Information (NCBI), either fully or partially sequenced, has revealed that a vast number of genes encoded in the bacteriophage genomes have yet to be assigned a function. This suggests that further understanding of their basic biology is required. In this regard, the ability to construct bacteriophage mutants is pivotal for the assessment of gene/protein function. The genome modification of a temperate bacteriophage is relatively simple using homologous recombination

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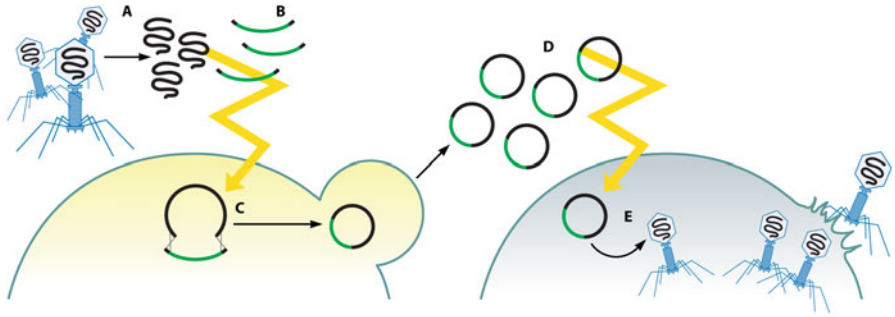

Fig. 1 Yeast-based assembly of phage genomes. Purified phage DNA (**a**) is electroporated into *S. cerevisiae* together with linear YAC molecules with overhangs (in *black*) homologous to the 5′ and 3′ ends of the linear phage genome (**b**). Recombination in the yeast cell enables genomic subcloning (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. (2016) [11]

(it is treated as a bacterial chromosome) [10], but the same cannot be said for virulent bacteriophages. As a consequence, few recombination-based methods have been implemented so far for the purpose of engineering lytic bacteriophages [11].

In order to genetically manipulate bacteriophages without inflicting toxic effects on the host cell, an yeast-based platform for the assembly of bacteriophage genomes (Fig. 1) was developed [12, 13]. In this method, *Saccharomyces cerevisiae* is used as a surrogate for genetic manipulation, which requires a yeast artificial chromosome (YAC). 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 nonetheless share regions of homology, which can be added by PCR, using primers with shared overhangs. Alternatively, purified (linear) bacteriophage DNA can be used instead, being in this case the homologous overhangs added to the linearized YAC. The bacteriophage amplicons (or genomic DNA) are then cotransformed 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 picked, amplified, and sequenced to confirm the introduction of the desired mutations. Several bacteriophages, such as T3 and T7, have been genetically modified using this method [12, 13].

Other methods have been developed for the genetic manipulation of bacteriophage genomes, namely the Bacteriophage

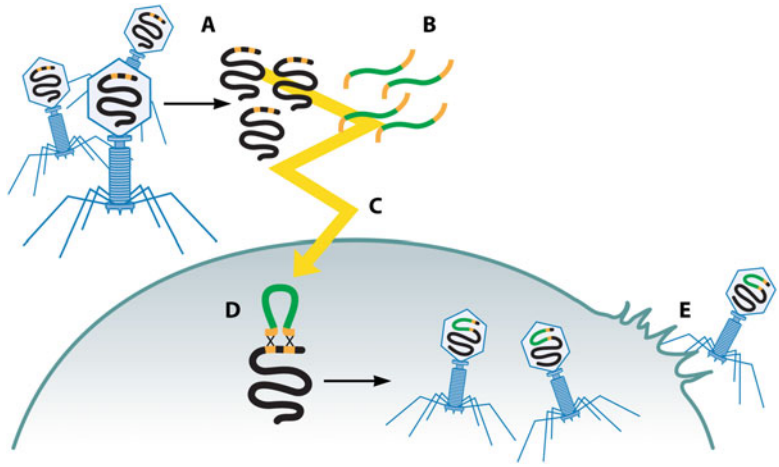

Fig. 2 Bacteriophage recombineering of electroporated DNA. Purified phage DNA (**a**) and dsDNA recombineering substrates (**b**) are coelectroporated into cells (**c**). Recombination between their homologous regions (in *orange*) (**d**) results in recombinant phage particles (containing DNA fragments in green) (**e**). Figure reprinted from Pires et al. (2016) [11]

Recombineering of Electroporated DNA (BRED, Fig. 2). This technique was originally created to generate point mutations, insertions, deletions, and gene replacements in lytic mycobacteriophages [14, 15]. 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 [14]. BRED has also been used to genetically engineer *Escherichia coli* bacteriophages [10] and it has been suggested that, with slight modifications to the protocols and the appropriate recombineering systems, this approach can be applied to many other bacteriophages.

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 as well, according to the requirements of the host bacterium.

1. 150 mL of bacteriophage lysate filtered through 0.22 μm filters (*see* **Note 1**).
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

2.1 Yeast-Based Assembly of Bacteriophage Genomes

2.1.1 Bacteriophage DNA Isolation

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 the 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% (vol/vol)).
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.

2.1.2 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 2**).
3. Sterile 50 mL centrifuge tubes.
4. Sterile 1.5 mL microcentrifuge tubes.
5. Sterile 250 mL flasks.
6. Sterile distilled water

2.1.3 Yeast Transformation

1. 100–200 ng of linearized YAC obtained by PCR (*see* **Note 3**).
2. 0.5–4.0 μg of each sample DNA: each bacteriophage DNA amplicon or purified bacteriophage genomic DNA (*see* **Note 4**).
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 5**).

2.1.4 Yeast Colony PCR

1. Yeast colonies growing on the appropriate agar plates.

2. Confirmation primer sets: to confirm the correct assembly of the construct, a set of primers to amplify all the connections between adjacent fragments should be used.
3. PCR tubes.
4. 0.02 M sodium hydroxide (NaOH).

2.1.5 Plaque Formation Assays

1. LB agar (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 7**).
4. Sterile electroporation cuvettes (*see* **Note 8**).
5. Sterile Super Optimal broth with Catabolite repression (SOC) medium (*see* **Note 9**): 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* **Note 10**). After autoclaving store accordingly (*see* **Note 11**).
7. Sterile 15 mL centrifuge tubes.

2.2 Bacteriophage Recombineering of Electroporated DNA (BRED)

2.2.1 Preparation of Recombineering Cells

1. Electrocompetent cells of the bacterial host (*see* **Note 7**).
2. Plasmid encoding recombineering functions, e.g. pKD46 (*see* **Notes 12** and **13**).
3. Sterile electroporation cuvettes (*see* **Note 8**).
4. SOC medium (*see* **Note 9**): prepare according to the manufacturer's instructions.
5. Ampicillin 1000× stock solution at 100 mg/mL (*see* **Note 13**). Sterilize by filtration using a 0.22 μm filter.
6. LBA plates containing 100 μg/mL ampicillin: prepare LBA, autoclave 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 14**).
8. LB containing 100 μg/mL ampicillin.
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 15**).
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.

2.2.2 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.
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 16**).
6. Sterile 10% (wt/vol) glycerol.
7. Sterile 1.5 mL microcentrifuge tubes.
8. Recombineering DNA substrate (*see* **Note 17**).
9. Sterile electroporation cuvettes (*see* **Note 8**).
10. SOC medium.
11. LB soft agar.

2.2.3 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 14**).
4. Chloroform.
5. Confirmation primer sets (*see* **Note 18**).
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.
9. LBA plates.

3 Methods

Carry out all procedures at room temperature unless otherwise specified.

3.1 Yeast-Based Assembly of Bacteriophage Genomes

This protocol was adapted from Ando et al. [13], with some minor modifications.

*3.1.1 Bacteriophage DNA Isolation (See **Note 19**)*

1. Add 216 μL of buffer L1 to the 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 of 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 over dry as this results in loss of recoverable DNA.
15. After completely air-dried, resuspend the pellet in 100 μL of sterile water and store at −20 °C.

3.1.2 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 last step: 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.

3.1.3 Yeast Transformation

1. Combine all DNA samples (bacteriophage genomic DNA or 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 denatured in a boiling water bath for 5 min (lock the tube to prevent it from opening while boiling).
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 sterile water.
5. Spread the cells on the appropriate agar plates (*see* **Note 5**).
6. Incubate the plates at 30 °C for 3 days and check for yeast transformants.

3.1.4 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 interfere with the PCR reaction) .

3.1.5 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 20**) in a 0.1–0.2 cm gap electroporation cuvette (*see* **Note 8**) and transform the cells via electroporation at the appropriate settings (*see* **Note 21**).

3. Add 1 mL of SOC immediately after the pulse.
4. Transfer to a sterile 15 mL culture tube and incubate for 1–2 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 22**).

3.2 Bacteriophage Recombineering of Electroporated DNA

This protocol was adapted from Marinelli et al. [14, 15] with some minor modifications. Plasmid pKD46 will be used as an example to provide recombineering functions to the cells.

3.2.1 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 0.1–0.2 cm electroporation cuvette and transform the cells via electroporation at appropriate settings (*see* **Note 21**).
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 protocol. 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.

3.2.2 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 23**) to the medium and incubate for 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 infection occur for the duration of the eclipse time of the bacteriophage growth curve (*see* **Note 24**).
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.

3.2.3 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 ten 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 25**).
6. Add 100 μL of a host bacterial culture and 100 μL of serial dilutions of the positive bacteriophage mixture (made in SM buffer) to about 3 mL of LB soft agar and pour onto a 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.

4 Notes

1. 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.
2. 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).
3. The YAC amplicon is amplified by PCR and gel extracted before yeast transformation.
4. The bacteriophage DNA can be directly used for yeast transformation, or 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. In the second case, 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 cotransformed and assembled in the yeast along with the YAC DNA.

5. 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).
6. Alternatively, commercially available LBA, which corresponds to LB plus agar, can be used according to the manufacturer's instructions.
7. 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.
8. According to the bacterial host and cell volume, 0.1 or 0.2 cm gap electroporation cuvettes can be used.
9. SOC is a nutrient-rich bacterial growth medium used for microbiological cultures. It was developed by Douglas Hanahan in 1983 [16] and is an adjusted version of the commonly used LB. 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.
10. 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.
11. 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.
12. 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

[17, 18]. 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 [19]. 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.

13. Plasmid pKD46 and other recombineering plasmids typically confer resistance to ampicillin or kanamycin. If using ampicillin/kanamycin-resistant bacteria, replace the selection marker as appropriate.
14. 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.
15. 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.
16. 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 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 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.
17. 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 [20]. 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.
18. 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 [21].

19. Commercially available kits or alternative protocols for bacteriophage DNA isolation can be used.
20. The concentration of DNA needed to generate bacteriophage plaques is variable and depends on the bacterial host and the transformation efficiencies achieved.
21. The settings used for electroporation should be adjusted according to the bacterial host used.
22. 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.
23. 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.
24. 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.
25. The process of selection 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.

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INDEX

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