Chapter 9

Bacteriophage Control of Infectious Biofilms

Luciana Meneses, Sanna Sillankorva, and Joana Azeredo

Abstract

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

Key words Bacteriophage, Biofilm, Control, Quantification

1 Introduction

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

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

Joana Azeredo and Sanna Sillankorva (eds.), *Bacteriophage Therapy: From Lab to Clinical Practice*, Methods in Molecular Biology, vol. 2734, https://doi.org/10.1007/978-1-0716-3523-0_9,

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

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

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

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

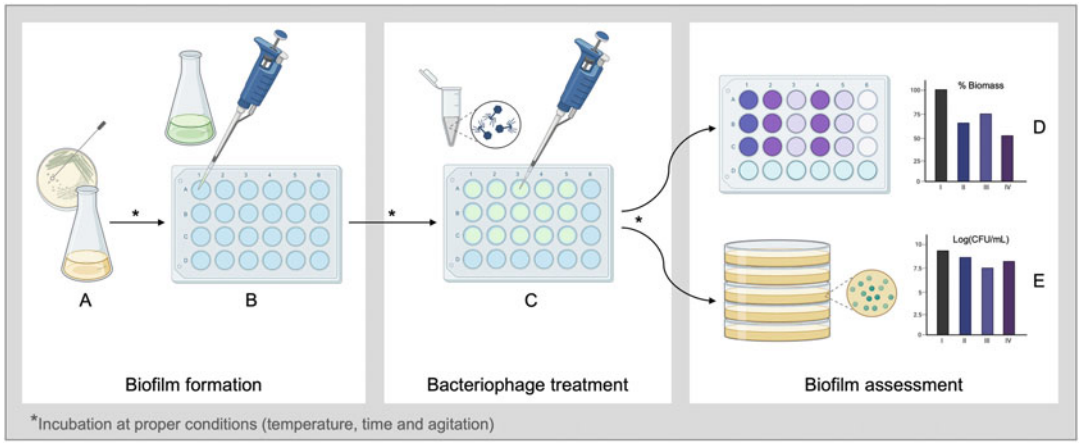

Fig. 1 Schematic of in vitro evaluation of bacteriophage treatment of biofilms. A bacterial inoculum is prepared from a fresh agar plate of the desired strain and incubated at proper conditions (**a**). The microplate is inoculated with overnight grown bacteria diluted with the desired culture medium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

2 Materials

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

2.1 Biofilm Formation

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

2.2 Biofilm Control with Bacteriophages

1. Liquid stocks of bacteriophages stored at 4 °C.
2. Sterile LB (*see* Subheading 2.1).
3. Sterile Saline Magnesium buffer (SM buffer): prepare 1 M Tris-HCl buffer (pH 7.5)—add 6.06 g of Tris-Base into a 250 mL bottle to a final volume of 50 mL in water, adjust the pH to 7.5 with HCl, and sterilize by autoclaving for 15 min at 121 °C. Prepare SM buffer—add 5.8 g of NaCl, 2.0 g of $MgSO_4 7H_2O$, 50 mL of 1 M Tris-HCl (pH 7.5), and water to a final volume of 1 L in a 1 L bottle and sterilize by autoclaving for 15 min at 121 °C.
4. Sterile 15 mL Falcon tubes.
5. 24-well microplate (*see* **Note 3**) with pre-formed PAO1 biofilms (*see* **Note 4**).

2.3 Evaluation of Biofilm Treatment with Bacteriophages

2.3.1 Quantification of Biofilm Culturable Cells

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

2.3.2 Quantification of the Biofilm Biomass

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

2.3.3 Bacteriophage Titration by Drop Plaque Assay

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

3 Methods

Carry out all procedures at room temperature unless stated otherwise.

3.1 Biofilm Formation

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

3.2 Biofilm Control with Bacteriophages

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

$$\begin{aligned}&\text{Phage stock titer (PFU/mL)} \times \text{Volume of phage (mL)}\\&= \text{Phage final concentration (PFU/mL)}\\&\times \text{Volume of the well (mL)}\end{aligned} \tag{1}$$

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

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

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

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

3.3 Evaluation of Biofilm Treatment with Bacteriophages

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

3.3.1 Quantification of Biofilm Culturable Cells

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

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

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

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

3.3.2 Quantification of Biofilm Biomass

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

3.3.3 Bacteriophage Titration by Drop Plaque Assay

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

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

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

4 Notes

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

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

Acknowledgments

L.M. has been funded by the Portuguese Foundation for Science and Technology (FCT) through the PhD grant SFRH/BD/07494/2020. S.S. acknowledges funding by FCT, through the individual scientific employment program contract (2020.03171.CEECIND).

References

1. Flemming HC, Wuertz S (2019) Bacteria and archaea on Earth and their abundance in biofilms. Nat Rev Microbiol 17:247–260
2. Flemming HC, Wingender J, Szewzyk U et al (2016) Biofilms: An emergent form of bacterial life. Nat Rev Microbiol 14:563–575
3. Sauer K, Stoodley P, Goeres DM et al (2022) The biofilm life cycle: expanding the conceptual model of biofilm formation. Nat Rev Microbiol 20:608–620
4. Yin W, Wang Y, Liu L et al (2019) Biofilms: the microbial "protective clothing" in extreme environments. Int J Mol Sci 20:3423
5. Sharma D, Misba L, Khan AU (2019) Antibiotics versus biofilm: an emerging battleground in microbial communities. Antimicrob Resist Infect Control 8:76
6. Lebeaux D, Ghigo J-M, Beloin C (2014) Biofilm-related infections: bridging the gap between clinical management and fundamental aspects of recalcitrance toward antibiotics. Microbiol Mol Biol Rev 78:510–543
7. Bowler P, Murphy C, Wolcott R (2020) Biofilm exacerbates antibiotic resistance: is this a current oversight in antimicrobial stewardship? Antimicrob Resist Infect Control 9:162
8. Pires DP, Melo LDR, Vilas Boas D et al (2017) Phage therapy as an alternative or complementary strategy to prevent and control biofilm-related infections. Curr Opin Microbiol 39:48–56
9. Harper DR, Parracho HMRT, Walker J et al (2014) Bacteriophages and biofilms. Antibiotics 3:270–284
10. Pires DP, Meneses L, Brandão AC et al (2022) An overview of the current state of phage therapy for the treatment of biofilm-related infections. Curr Opin Virol 53:101209
11. Abedon ST, Danis-Wlodarczyk KM, Wozniak DJ et al (2021) Improving phage-biofilm in vitro experimentation. Viruses 13:1175
12. Ferriol-González C, Domingo-Calap P (2020) Phages for biofilm removal. Antibiotics 9:268
13. Azeredo J, Azevedo NF, Briandet R et al (2017) Critical review on biofilm methods. Crit Rev Microbiol 43:313–351