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Rob Lavigne *Editors*

Bacteriophages

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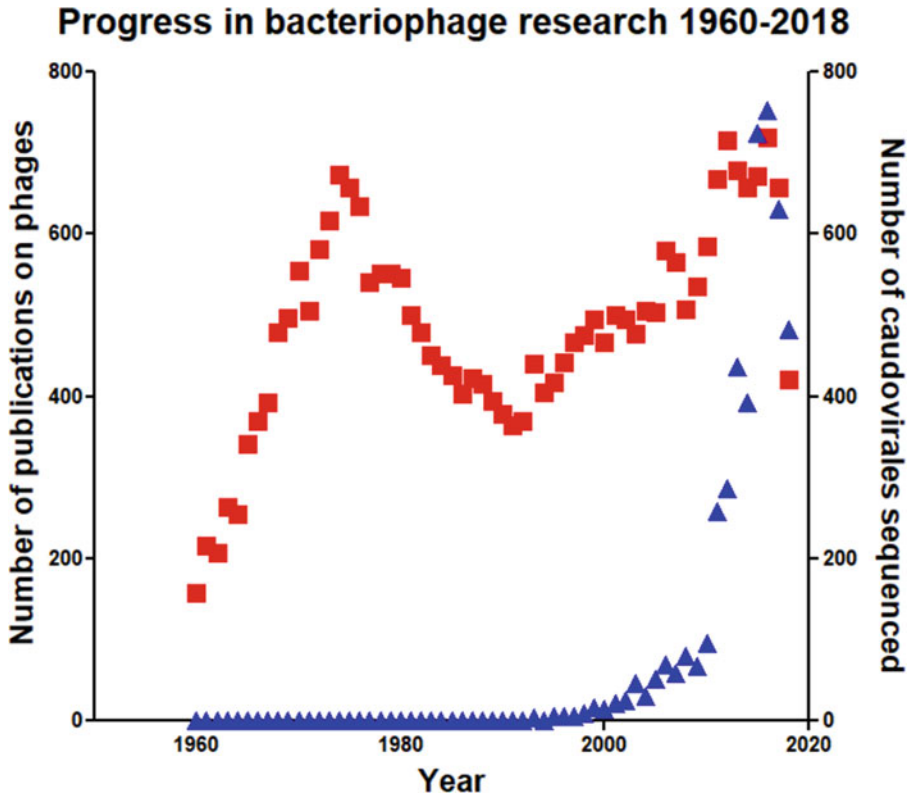
Preface

In 2015 we celebrated the centenary of the first description of bacteriophages by Frederick Twort and then in 2017 by Felix d'Hérelle who independently discovered them. One may wonder how they, or indeed other bacteriophage scientific icons of later generations, such as Emery Ellis and Max Delbrück, Giuseppe Bertani or Martha Chase, would view the current status of "their" research field. Fortunately, this (American) Phage Group laid the foundation of molecular biology and bacterial genetics by focusing on a defined set of phage and host strains. By focusing on just a few phages, significant progress was made in our understanding at a mechanistic level, and work carried out by this group and others in the 1950s and onwards still serves as a bedrock in our understanding of phage biology.

High-throughput sequencing of both phages and phage metagenomes is increasingly revealing the massive phage diversity in our oceans and soils and providing unparalleled clues about the ecological impact phages have at a global scale. One may argue that the availability of advanced technology is defining today's research, generating vast amounts of data, and enabling us to paint a much broader picture than was previously possible. However, perhaps our distinguished predecessors would warn us about not losing creativity and depth. If this were the case, we are taking heed of these warnings in this book, *Bacteriophages: Methods and Protocols, Volume 4*, as several chapters focus on the functional elucidation of phage and their proteins.

A second discernable trend within bacteriophage biology is the drive toward application-driven research, often involving partnerships with industry. The needs for novel therapeutics and diagnostics are driving biotechnology companies, to embrace both phage therapy, designer phage, and phage-derived products. Partnership is a key word here, as cautionary words from our predecessors both early and from the later scientists/entrepreneurs who sought to commercialize phages would likely warn against industrial work leading to secrecy and advocate performing research in an open and communicative manner. It is this drive for application-driven research and the partnerships with industry that are leading toward new innovations reflected here by the chapters dedicated to various novel phage therapy models. It has never been a more exciting and possible time to develop bacteriophages for many applications. The genetic tools are here to manipulate phages; we have systems for many bacterial groups, and our sequencing capacity to help us unravel the mechanics of phage biology has never been as powerful.

As a final note, we would like to thank the contributing authors for their work and patience for the completion of this volume. And, in the end, we are all indebted to the scientists who came before us for their example, creativity, and knowledge. We hope this book can in turn inspire a next generation of phage biologists.



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Chapter 1

PhageFISH for Monitoring Phage Infections at Single Cell Level

Jimena Barrero-Canosa and Cristina Moraru

Abstract

PhageFISH uses the power of fluorescence in situ hybridization to monitor intracellular phage infections at single cell level. It combines host cell identification via rRNA probes and phage identification via phage-specific gene probes, allowing for the quantification of the infected cell fraction and the discrimination between infection stages. This book chapter covers all aspects of the procedure, from phage probe design and synthesis, to the phageFISH protocol itself, to microscopy and image analysis.

Key words PhageFISH, Virus, Phage, Microorganisms, Fluorescence in situ hybridization, FISH, Infection cycle, Infection stages

1 Introduction

PhageFISH [1] is based on the detection of phage genes by fluorescence in situ hybridization (FISH) as a means to quantify viral infections in microorganisms, at single cell level. It has been applied in one-step growth experiments [1, 2] to follow phage–host infection dynamics, wherein it has provided two metrics: the fraction of infected cells (a quantitative metric), and the relative extent of per-cell phage infection (the phage signal area per cell, a semiquantitative metric which allows for discriminations between new and advanced infections). Using the two metrics, the method allowed for modeling of the infection stages (adsorption, replication, assembly, and lysis), and also, discrimination between subsequent waves of infection. Moreover, the protocol was able to detect free phage particles in the lysis stage of infection.

The protocol is based on the geneFISH protocol [3]. It combines host cell identification by rRNA-targeted oligonucleotides (Fig. 1a–c) with phage gene detection (Fig. 1d–f). For gene detection, multiple ~300 bp long dsDNA polynucleotide probes (Fig. 1d) labeled with digoxigenin (Dig) are used. Subsequently, anti-Dig antibodies conjugated with horseradish peroxidase (HRP)

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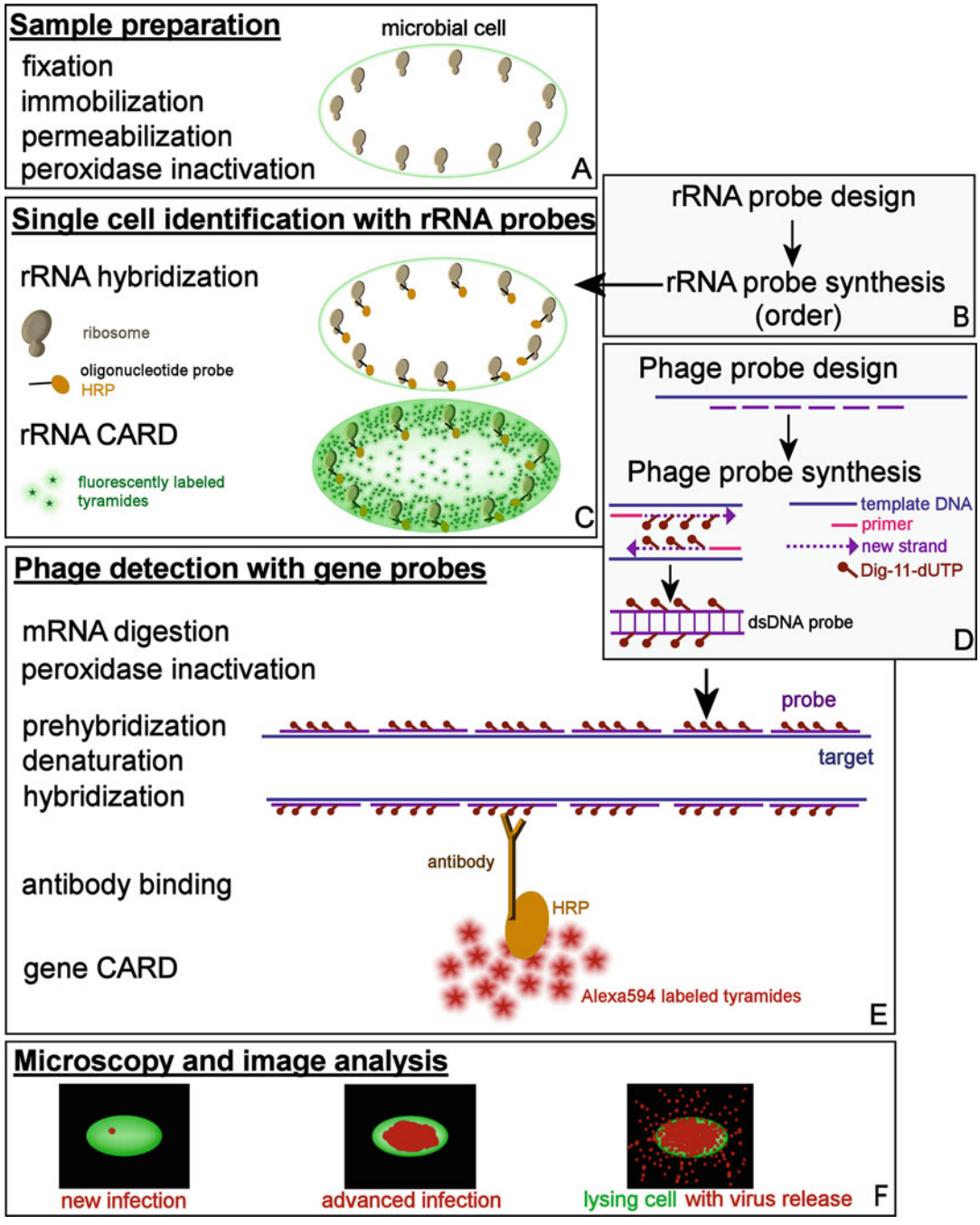

Fig. 1 Workflow of the phageFISH protocol

are applied. The bound HRP enzymes catalyze the covalent binding of multiple fluorochrome-labeled tyramides to cellular proteins in a so-called catalyzed reporter deposition (CARD) step. This results in both signal amplification and fixation of the signal inside the cells (Fig. 1e). By dual color epifluorescence microscopy host cells can be identified in one color and intracellular and extracellular phage particles in another color (Fig. 1f). Since the writing of this book chapter, a new method for gene detection in microorganisms has been developed, called direct-geneFISH [4]. It uses polynucleotide probes directly labeled with fluorochromes and therefore eliminates the antibody binding and CARD steps. As a consequence, the protocol is significantly shorter and simpler. We are currently testing the protocol in our labs on phage infected samples and the results a very promising.

So far phageFISH has been applied to pure cultures, to model the infection dynamics of a lytic phage–host system. However, its use can be extended to the study of lysogenic systems and, since it allows for both host and virus identification, also to the study of more complex environmental systems. It should furthermore be possible to apply phageFISH not only to double-stranded DNA viruses but also to single-stranded DNA viruses and RNA viruses.

2 Materials

Always use ultrapure water, which was 0.22 μm filtered and autoclaved, for the preparation of solutions. Unless indicated otherwise, prepare and store the solutions at room temperature. Avoid exposing the fluorescent reagents to light, by storing them in nontransparent tubes/racks or wrapped in aluminum foil. Several of the chemicals used are toxic and/or volatile. Use appropriate protection measures; for example, always work with formamide and paraformaldehyde in fume hood cabinets equipped with special waste disposal bins.

2.1 Stock Solutions and Chemicals

1. PCR Dig Probe Synthesis Kit (Roche, cat. no. 11636090910). Store at −20 °C.
2. Alternative to the PCR Dig Probe Synthesis Kit: 1 mM Dig-dUTPs (Jena Biosciences, cat. no. NU-803-DIGXS), 5 Prime Master Taq Kit (5 PRIME, cat. no. 2200230), 100 mM dNTP Set, PCR Grade (Invitrogen, cat. no. 10297-117). Store at −20 °C.
3. Gene Clean Turbo kit (Q-Biogene, cat. no. 1102-600) or QIAquick PCR purification kit (Qiagen, cat. no. 28106).
4. 3-aminopropyl-triethoxysilane (TESPA), (Sigma, cat. no. A-3648) or poly-L-lysine (Sigma Cat. No. P-2636).

5. 10× PBS (Ambion, cat. no. AM9625).
6. 1× PBS, pH 7.4: 137 mM NaCl, 2,7 mM KCl, 8 mM Na_2HPO_4, and 2 mM KH_2PO_4, prepared from 10× PBS by mixing 1 part 10× PBS and 9 parts water.
7. 20% paraformaldehyde (PFA), electronic microscope grade (Electron Microscopy Sciences, cat. no. RT 15713).
8. 1 M Tris–HCl, pH 8.0 (Ambion, cat. no. AM9856).
9. 0.5 M EDTA, pH 8.0 (Ambion, cat. no. AM9262).
10. TE, buffer: 5 mM Tris, 1 mM EDTA, pH 8.0.
11. 20% SDS (Ambion, cat. no. AM9820).
12. 20× SSC (Ambion, cat. no. AM9765).
13. 5 M NaCl (Ambion, cat. no. AM9759).
14. 37% HCl (~ 10 M HCl).
15. 96% ethanol, NOT denatured.
16. Lysozyme, powder (AppliChem, cat. no. A4972.0010).
17. 10 mg ml^{-1} sheared salmon sperm DNA (Ambion, cat. no. AM 9680).
18. 10 mg ml^{-1} yeast RNA (Ambion, cat. no. AM 7118).
19. Dextran sulfate (DS), sodium salt (Sigma, cat. no. D8906).
20. Formamide, molecular grade, deionized (Sigma, cat. no. F9037).
21. Nucleic acid blocking reagent (Roche, cat. no. 11096176001).
22. Maleic acid buffer (to dissolve the nucleic acid blocking reagent): 100 mM maleic acid, 150 mM NaCl, pH 7.5, adjusted with concentrated or solid NaOH, sterile.
23. Alexa Fluor 488 (Alexa488) and Alexa Fluor 594 (Alexa594) labeled tyramides (*see* **Note 1**).
24. RNase I (Ambion, cat. no. AM 2295).
25. RNase A (Sigma, cat. no. R4642-10).
26. Anti-Dig-POD Fab fragments (Roche, cat. no. 11207733910): prepare stock solution as indicated by the manufacturer, store at +4 °C. Do not vortex antibody containing solutions!
27. Western Blocking Reagent (WBR), Solution (Roche, cat. no. 11921673001).
28. HRP-labeled 16S rRNA-targeted oligonucleotide probes (Biomers, Germany; http://www.biomers.net/): prepare stock solutions as indicated by the manufacturers. From the stock solutions, prepare working solution to a concentration of 50 ng μl^{-1}. Do not freeze HRP stocks once thawed! Do not vortex HRP probes!

29. SlowFadeGold (Invitrogen, cat. no. S36936) or ProLong Gold antifade reagent (Invitrogen, cat. no. P36930).
30. 4′,6-diamidino-2-phenylindole (DAPI), 5 mg ml^{-1}(Sigma-Aldrich, cat.no. D9542-50MG).

2.2 Glassware and Plasticware

1. Thin forceps, from materials resistant to acids, bases, organic solvents, and temperature (e.g., from Electron Microscopy Sciences, cat. no. 72692-F; https://www.emsdiasum.com/).
2. Petri dishes, various sizes, sterile, DNase free.
3. 15 and 50 ml Falcon tubes, sterile, DNase free.
4. Scalpels: sterile, disposable.
5. Hybridization chambers: any tightly closing, temperature resistant container that seals with a silicone O-ring (e.g., food containers with glass bottom used in the kitchen).
6. Hybridwell sealing chamber (Electron Microscopy Sciences, cat. no. 70328-01). Press-To-Seal silicone isolators (Sigma-Aldrich, cat. no. GBL 664301-25 EA).
7. 0.22 μm sterile syringe filters.
8. 0.2 μm polycarbonate membrane filters (GTTP, Millipore, cat. no. GTTP02500).
9. Diamond Retractable Tip Scriber: for writing on glass, metal and plastic (Electron Microscopy Sciences, cat. no. 70036).
10. Glass slides, frosted end.
11. Poly-L-Lysine Coated Slides (Electron Microscopy Sciences, cat. no. 63410-02).
12. Coverslips, # 1.5, high precision (Marienfeld, cat. no. MARI0107052; http://www.marienfeld-superior.com/home.html).

2.3 Laboratory Equipment

1. Incubators: at different temperatures (e.g., 37 °C, 42 °C, 46 °C, 85–90 °C).
2. Slide Denaturation/Hybridization system (alternative to incubators).
3. Water baths: at different temperatures (e.g., 37 °C, 42 °C, 48 °C).
4. Tabletop centrifuges.
5. Agarose gel electrophoresis devices.
6. Spectrophotometer or other device for measuring nucleic acid concentration (e.g., Nanodrop).
7. Filtration device (e.g., Millipore) and vacuum pump.
8. Epifluorescence microscope: equipped with a 63× or 100× objective, with fluorescent filter sets suitable to discriminate

between Alexa488 and Alexa594 fluorescence, with a black and white CCD camera and with software for image capture. Example of filter sets: for Alexa488 – 472/30 excitation, 520/35 emission and 495 Beam Splitter; for Alexa594 – 562/40 excitation, 624/40 emission and 593 Beam Splitter.

2.4 Software

1. PolyPro [5]: for polynucleotide probe design (https://www.mpi-bremen.de/Binaries/Binary2975/PolyPro.zip).
2. DAIME [6]: for microscopy image analysis (http://www.microbial-ecology.net/daime/).

2.5 Work Solutions

1. Permeabilization solution: 0.5 mg ml^{-1} lysozyme, 1× PBS pH 7.4, 0.1 M Tris–HCl pH 8.0, and 0.05 M EDTA. First, prepare 50 ml of permeabilization buffer, by mixing 5 ml 10× PBS, 5 ml 1 M Tris–HCl pH 8.0, 5 ml 0.5 M EDTA pH 8.0, and 35 ml water. Second, prepare a 5 mg ml^{-1} lysozyme solution (e.g., by dissolving 50 mg lysozyme in 10 ml permeabilization buffer). Warm up at 37 °C to dissolve, if necessary. Finally, mix 1 part of 5 mg ml^{-1} lysozyme solution with nine parts permeabilization buffer, to get a final concentration of 0.5 mg ml^{-1} lysozyme. Always use a freshly made permeabilization solution.
2. 0.01 M HCl: to 50 ml of water add 50 µl 37% HCl, mix.
3. 0.2 M HCl: to 49 ml of water add 1 ml HCl, mix.
4. rRNA hybridization buffer: 35% formamide (*see* **Note 2**), 10% dextran sulfate (DS), 0.9 M NaCl, 20 mM Tris–HCl pH 8.0, 1% nucleic acid blocking reagent, 0.25 mg ml^{-1} sheared salmon sperm DNA, 0.25 mg ml^{-1} yeast RNA, and 0.02% SDS. For preparation of 40 ml hybridization buffer proceed as follows. In a 50 ml Falcon tube add 4 g DS, 7.2 ml 5 M NaCl, 0.8 ml 1 M Tris–HCl pH 8.0, and 4 ml water, close the tube and shake/vortex strongly to disperse the DS in solution. Incubate in a water bath at 37–48 °C to dissolve the DS, vortex and shake from time to time. After the DS has dissolved completely, allow the solution to cool down to RT, and then add 4 ml 10% nucleic acid blocking reagent, 1 ml 10 mg ml^{-1} sheared salmon sperm DNA, 1 ml 10 mg ml^{-1} yeast RNA, 17.5 ml 100% formamide, and 40 µl 20% SDS (*see* **Note 3**). If necessary, adjust with water up to the mark of 40 ml. Vortex to mix components, followed by quick spin. Optionally, the buffer can be filtered through 0.22 µm sterile syringe filters. Aliquot and store at −20 °C. Before use warm up at 37 °C, to redissolve any precipitates.
5. rRNA hybridization buffer – probe mix: to 900 µl rRNA hybridization buffer add 3 µl of 50 ng μl^{-1} HRP labeled 16S, to a final concentration of 0.17 ng μl^{-1} (*see* **Note 4**). Mix by gentle

shaking or up and down pipetting. Do not vortex, to avoid damage to the HRP probes! Prepare freshly, just before use, keep at room temperature until hybridization.

6. rRNA hybridization — washing buffer: 70 mM NaCl (*see* **Note 5**), 5 mM EDTA pH 8.0 (only if >= 20% formamide in the hybridization buffer), 20 mM Tris–HCl, and 0.01% SDS. In a 50 ml Falcon tube, add 700 µl 5 M NaCl, 500 µl 0.5 M EDTA, 1 ml 1 M Tris–HCl, fill with water up to the 50 ml mark and then add 25 µl 20% SDS.
7. rRNA CARD buffer: 1× PBS, 10% DS, 0.1% nucleic acid blocking reagent, and 2 M NaCl. For preparation of 40 ml rRNA CARD buffer proceed as follows. In a 50 ml Falcon tube add 4 g DS, 4 ml 10× PBS pH 7.4, 16 ml 5 M NaCl, and water up to 40 ml, close the tube and shake/vortex strongly to disperse the DS in solution. Incubate on water bath at 37–48 °C to dissolve the DS, vortex and shake from time to time. After the DS has dissolved completely, allow the solution to cool down to RT, and then add 400 µl 10% nucleic acid blocking reagent, vortex, quick spin. Filter through 0.22 µm sterile syringe filters. Aliquot and store at +4 °C. Before use warm up at 37 °C, to redissolve any precipitates.
8. rRNA CARD buffer–Alexa488 tyramide mix: to the rRNA CARD buffer, add H_2O_2 to a final concentration of 0.0015% and Alexa488 tyramides to a concentration of 0.33 µg ml^{-1} (*see* **Note 6**). For example, to 3 ml of buffer add 30 µl of 100× H_2O_2 (always prepare freshly by mixing 1 µl 30% H_2O_2 and 200 µl 1× PBS) and 1 µl of 1 mg ml^{-1} Alexa488 tyramides, vortex, quick spin. Prepare freshly, just before use, and keep at room temperature, in the dark, until rRNA CARD.
9. RNase solution: 0.1 U $µl^{-1}$ RNase, 75 µg ml^{-1} RNase A, 0.1 M Tris–HCl, pH 8.0. For preparation of 12 ml RNase solution, in a 15 ml Falcon tube add 10.8 ml water, 1.2 ml 1 M Tris–HCl pH 8, 15 µl 100 U $µl^{-1}$ RNaseI, and 30 µl 10 mg ml^{-1} RNaseA.
10. Gene hybridization buffer: 35% formamide, 5× SSC, 10% DS, 0.1% SDS, 20 mM EDTA, 1% nucleic acid blocking reagent, 0.25 mg ml^{-1} sheared salmon sperm DNA, and 0.25 mg ml^{-1} yeast RNA. For preparation of 40 ml gene hybridization buffer proceed as follows. In a 50 ml Falcon tube add 4 g DS, 10 ml 20× SSC, 1.6 ml 0.5 M EDTA pH 8.0, and 4.4 ml water. Close the tube and shake/vortex strongly to disperse the DS in solution. Incubate on water bath at 37–48 °C to dissolve the DS, vortex and shake from time to time. After the DS has dissolved completely, allow the solution to cool down to room temperature, and then add 4 ml 10% nucleic acid blocking reagent, 1 ml sheared salmon sperm DNA, 1 ml yeast RNA,

14 ml formamide, and 200 µl 20% SDS. Vortex to mix components, followed by quick spin. Optionally, the buffer can be filtered through 0.22 µm sterile syringe filters. Aliquot and store at −20 °C. Before use warm up at 42 °C, to redissolve any precipitates.

11. Gene hybridization buffer–probe mix: to the gene hybridization buffer add each probe to a final concentration of 5 pg μl^{-1}. For example, to 1 ml gene hybridization buffer add for each probe 1 µl of 5 ng μl^{-1} probe stock. Vortex to mix, followed by quick spin. Prepare freshly, on the same day as the hybridization, and store at room temperature before use. *See* **Note 7** for the concentration of the negative control probe.
12. Gene hybridization washing buffer I: 2× SSC and 0.1% SDS. In a 50 ml Falcon tube, add 5 ml 20× SSC, water up to 50 ml, and 250 µl 20% SDS, vortex. Prepare for use within 1–2 days, keep at 42 °C (*see* **Note 8**).
13. Gene hybridization washing buffer II: 0.1× SSC and 0.1% SDS. In a 50 ml Falcon tube, add 250 µl 20× SSC, water up to 50 ml, and 250 µl 20% SDS, vortex. Prepare for use within 1–2 days; keep at 42 °C (*see* **Note 8**).
14. Antibody blocking/washing solution: 1× PBS and 1% Western Blocking Reagent (WBR) (*see* **Note 9**). To a 50 ml Falcon tube, add 5 ml 10× PBS, 5 ml 10% WBR, and water up to 50 ml, vortex. Prepare on the same day as the antibody step, keep at room temperature until use.
15. Antibody binding solution: 1× PBS, 1% Western Blocking Reagent (*see* **Note 9**) and 0.3 U ml^{-1} (500× dilution of the 150 U/ml stock) anti-Dig HRP-conjugated antibody. To a 15 ml Falcon tube, add 1 ml 10× PBS, 1 ml 10% WBR, 8 ml water, and 20 µl antibody (just before use, centrifuge the antibody stock solution for 10 min at 10,000× *g* and + 4 °C, to deposit any precipitates). Mix gently. Do not vortex solutions containing antibodies! Prepare freshly and keep at room temperature until use.
16. Gene CARD amplification buffer: 1× PBS, 20% DS, 0.1% blocking reagent, and 2 M NaCl. For preparation of 40 ml gene CARD buffer proceed as follows. In a 50 ml Falcon tube add 8 g DS, 4 ml 10× PBS pH 7.4, 16 ml 5 M NaCl, and 15.6 ml water, close the tube and shake/vortex strongly to disperse the DS in solution. Incubate on water bath at 37–48 °C to dissolve the DS, vortex and shake from time to time. After the DS has dissolved completely, allow the solution to cool down to room temperature, and then add 400 µl 10% nucleic acid blocking reagent, vortex, quick spin. Filter through 0.22 µm sterile syringe filters. Aliquot and store at +4 °C. Before use warm up at 37 °C, to redissolve any precipitates.

17. Gene CARD amplification buffer–Alexa594 tyramide mix: to the rRNA CARD buffer, add H_2O_2 to a final concentration of 0.0015% and Alexa594 tyramides to a concentration of 2 μg ml^{-1}. For example, to 1 ml of buffer add 10 μl of 100× H_2O_2 (always prepare freshly by mixing 1 μl 30% H_2O_2 and 200 μl 1× PBS) and 2 μl of 1 mg ml^{-1} Alexa594 tyramides, vortex, quick spin. Prepare freshly, just before use, and keep a room temperature, in dark, until CARD.
18. Antifade reagent — 5 μg ml^{-1} DAPI mix: to 1 ml antifade reagent (SlowFade Gold or ProLong Gold) add 1 μl of 5 mg ml^{-1} DAPI solution, vortex, quick spin. Store at −20 °C.

3 Methods

3.1 Phage Probe Design

1. Use the bioinformatics tools you have in your laboratory to select a phage-specific genomic region (not more than 70% identity with other, non-target, sequences, *see* **Note 10**), which is not found in the other microbial members of the sample being studied (the host, or coinfecting viruses, other bacteria, archaea, viruses, etc., which might be present in the sample). This requires having a priori genomic/metagenomic information for the sample of interest. If such information is not available, then compare your phage genome against the NCBI database.
2. Next, in the selected region identify at least six probes (*see* **Note 11**), each ~300 bp, with similar %GC. In a first step, calculate the variation of the %GC along the selected DNA region, using the bioinformatics tools in your laboratory. One such tool is DAN (http://www.hpa-bioinfotools.org.uk/pise/dan.html), which can calculate the denaturation profile, including the % GC. When using DAN, choose the following parameters: "window size" = 100, "shift increment" = 1, "DNA concentration" = 1 nm, "salt concentration" = 1000 mM, and "output format" = excel. In Excel, plot the base position (for DAN, this would be column "Start" in the output file) versus the % GC (*see* **Note 12**). To select the probes, avoid sequence stretches with high variations (e.g., more than 10 units) in the %GC. Furthermore, if possible, choose sequence stretches with 30–40 %GC (*see* **Note 13**). Perform a Blast search with the individual probes against the database relevant for your samples, to identify potential nonspecific binding sites (regions with more than 80% identity on higher than 20–30 base stretches). If necessary, discard probes with potential for non-specific binding. The last step is to design primers for the ends of each 300 bps probe.

3.2 Phage Probe Synthesis

The phageFISH probes are dsDNA molecules labeled with Dig. They are produced by incorporating Dig into dsDNA during a PCR reaction (*see* Fig. 1d).

1. Optimize the PCR conditions (to avoid expenses you can leave out Dig) for the template and primers of interest. Use either viral DNA or plasmid DNA as PCR template.
2. Proceed to the probe synthesis PCR. A labeling kit is available from Roche—the PCR Dig Probe Synthesis Kit. Follow manufacturer's instructions for probe synthesis. Because Dig incorporation results in lower product yields, you can use two kit reactions per probe and pool them for purification. For an alternative to the kit, *see* **Note 14**.
3. Purify the PCR products using PCR purification kits, as for example the Gene Clean Turbo kit or QIAquick PCR purification kit. At the end of the purification, elute the probes in TE buffer, pH 8.0.
4. Check the probes on 2.5–3% agarose gels. Due to Dig incorporation, the probes will migrate slower than the unlabeled counterparts.
5. Measure the probe concentration using a spectrophotometer.
6. Prepare stocks of 5 ng μl^{-1} each probe, by diluting in TE buffer, pH 8.0.
7. Store at −20 °C.

3.3 Determination of the Stringency Parameters for Gene Hybridization

The hybridization and washing stringency will influence not only the specificity, but also the detection efficiency of the hybridization. The stringency refers to how close to the melting temperature of the probe–target hybrids the hybridization or washing takes place. It can be modulated by modifying the formamide concentration in the hybridization buffer and by changing the hybridization and the washing temperatures.

1. Calculate the formamide concentration which will allow for a hybridization temperature in the range 42–50 °C (*see* **Note 15**). For this, use the HPC module of the PolyPro software to calculate for each probe–target pair the graph of the melting temperature as a function of formamide concentration. As input parameters use DNA–DNA hybridization, Na+ concentration of 975 mM, formamide in between 1 and 100%, and criterion of 25 (distance from T_m, *see* **Note 16**). As output option, choose "Hybridization temperature function of % formamide." Using the graphs for all probe–target pairs, select a formamide concentration that will give a hybridization temperature in the range 42–50 °C for every probe–target pair.
2. Use the chosen formamide concentration to test the detection efficiency at different gene hybridization temperatures (in the

range 42–50 °C), by performing the whole phageFISH protocol. The optimum hybridization temperature will be the one giving the highest detection rate (after correction of the false positives in the negative control—*see* **Note 17**).

3.4 PhageFISH Protocol

During the FISH procedure the cells are immobilized on solid support, most often on 0.22 μm polycarbonate filters or glass slides. *See* **Note 18** for general instructions for handling polycarbonate filters and **Note 19** for glass slides. Avoid excessive light exposure during the procedure. Avoid sample drying, unless specifically instructed in the protocol (*see* **Note 20**). Unless stated otherwise, perform all incubations at room temperature. Prewarm or precool reagents before use, to bring them to the required incubation temperature. Whenever working with toxic substances, e.g. paraformaldehyde during fixation, formamide during hybridization, use a chemical fume hood. Unless otherwise specified, all washing steps should be performed in large volumes, e.g. 50 ml, *see* **Notes 18** and **19**.

1. Sample fixation and immobilization. Add 20% PFA directly to the sample of interest (e.g., culture, seawater), to result in a final concentration of 1–4% paraformaldehyde (PFA). Incubate for 1 h at room temperature or overnight at +4 °C (*see* **Notes 21** and **22**). To remove the PFA and bring the cells on solid support, filter the fixed culture on 0.22 μm polycarbonate filters (*see* **Notes 23** and **24** for other methods to remove PFA, concentrate the cells and immobilize them on glass slides). Apply a vacuum pressure as low as possible and not higher than 0.2 mBar. Initially, test different culture volumes to see which one gives a uniform distribution of cells on the filter, avoiding too little or too many cells. Consider using a volume that will give a denser cell distribution because during the phageFISH protocol some cells will detach from the solid support (*see* **Note 25**). After filtering the fixed culture, filter through 10–15 ml 1× PBS and then 10–15 ml water, to wash. Allow filters to air-dry. Store at −20 °C, or directly proceed further.
2. Permeabilization. Overlay samples with permeabilization solution (e.g., 0.5–1 ml per 25 mm filter or per glass slide), incubate on ice for 1 h, and then wash 5 min with 1× PBS and 1 min with water. Permeabilization can be sample specific. *See* **Notes 18** and **19** for washing and **Note 26** for permeabilization details.
3. Inactivation of endogenous peroxidases. Immerse the samples in 0.01 M HCl (e.g., place filter pieces in a petri dish with 20 ml inactivation solution; alternatively, when working with glass slides, cover the sample area with 1–2 ml inactivation solution) for 10 min. Then, wash with 1× PBS for 5 min, water

for 1 min, and 96% ethanol for 1 min. Allow samples to air-dry. Store at −20 °C, or directly proceed further. Inactivation of peroxidases can be sample specific. *See* **Note 27** for more details.

4. rRNA hybridization. Cover samples with hybridization buffer - probe mix. For example, place filter pieces in a petri dish, sample face up, and cover them with enough mix to completely cover the filters (30–100 μl are enough for a 1/8 piece from a 25 mm filter). Alternatively, when working on glass slides, add the hybridization buffer – probe mix on top of the sample area, in suficient volume to completely cover it. Transfer the petri dish or the glass slides to a humidity chamber (*see* **Note 20**). Incubate at 46 °C for 1.5–3 h. For washing, quickly rinse the samples in rRNA washing buffer, then transfer them in 50 ml of prewarmed rRNA washing buffer and incubate for 15 min at 48 °C (*see* **Notes 18** or **19**). CAUTION: remove samples from the hybridization buffer under a fume food, to avoid exposure to formamide vapors.
5. CARD for rRNA detection. Incubate the samples for 10–15 min in 1× PBS. Further, transfer the samples in rRNA-CARD buffer–Alexa488 tyramide mix (e.g., place ~20 small filter pieces in 10 ml mix in a petri dish) and incubate for 10 min at 37 °C (*see* **Note 28**). For washing, quickly rinse the samples in 1× PBS, then transfer in 1× PBS, 10 min at 46 °C, followed by 1 min with water and 1 min with 96% ethanol. Air-dry and store at −20 °C, or directly proceed further.
6. RNase treatment (*see* **Note 29**). Cover the samples with RNase solution (e.g., place ~20 small filter pieces in 10 ml RNase solution; for glass slides, cover sample area with 0.5–2 ml solution) and incubate for 1h at 37 °C. Wash twice for 5 min in 1× PBS and one for 1 min with water.
7. Inactivation of HRP introduced with the rRNA probe. Immerse the samples in 0.2 M HCl for 10 min. Wash with 1× PBS for 1 and 5 min, then 1 min with water, 1 min with 96% ethanol. Allow samples to air-dry and store at −20 °C, or directly proceed further.
8. Gene hybridization — prehybridization. Cover samples with hybridization buffer. For example, place filters face up on petri dishes and overlay them with 30–100 μl of hybridization buffer. Introduce the petri dish in a humidity chamber (*see* **Note 20**) and incubate for 0.5–1 h at the hybridization temperature (*see* "Determination of the stringency parameters for gene hybridization" section 3.3).
9. Gene hybridization — denaturation and hybridization. On a petri dish, place as many 30–100 μl droplets of gene hybridization buffer–probe mix as the number of filters. Gently, remove

the filters from the prehybridization buffer and place them face down into the droplets of gene hybridization buffer–probe mix. *See* **Note 30** for working on glass slides.

Place the samples back in the humidity chamber and denature for 1 h at 85 °C–90 °C (*see* **Note 31**). Further, quickly move the humidity chambers in an oven set at the hybridization temperature and hybridize for 2 h or overnight (*see* **Note 32**). For washing, first immerse samples in gene washing buffer I, 3× for 1 min at room temperature and for 30 min at 42 °C, followed by gene washing buffer II, 3× for 1 min at room temperature and 1.5 h at 42 °C. The 42°C incubations should be performed in a slow shaking water bath. Finally, wash for 1 min in 1× PBS at RT. CAUTION: remove samples from the hybridization buffer under a fume food, to avoid exposure to formamide vapors.

10. Antibody binding. Incubate samples in antibody blocking solution for 30 min. Use sufficient volume to completely cover the samples. For example, ~20 small filter pieces (1/8 of a 25 mm filter) could fit in 15 ml antibody solution in a Petri Dish. Alternatively, samples on glass slides should be covered with 1–2 ml solution. Transfer samples in antibody binding solution and incubate for 1.5 h. For washing, immerse samples in antibody washing solution for 1 min and then 3× 10 min. During these steps, slow shaking (e.g., 20 rpm) could improve results, but could also result in cell loss.
11. CARD for gene detection. Cover samples with gene CARD amplification buffer–Alexa594 tyramide mix (30–100 μl) and incubate for 45 min at 37 °C. Quick wash for 1 min in 1× PBS at room temperature, and then for 5 min and 2× 10 min with 1× PBS in a 46 °C oven, slow shaking, then 1 min with water, 1 min with 96% ethanol. Allow filters to air-dry and store at −20 °C, or directly proceed further.
12. Embedding and counterstaining. Place the filters face up on a microscopy glass slide, in a droplet (1–2 μl) of embedding reagent. On top of the sample add sufficient embedding reagent (usually 2–5 μl per filter piece), so that when placing next a coverslip on top, the whole sample surface will be covered by the embedding reagent. If a nonhardening medium is used (e.g., SlowFade Gold), then samples can be imaged immediately. If a hardening media is used (e.g., ProLong Gold), then allow the samples to cure for 24 h at room temperature.

3.5 Microscopy and Data Analysis

1. Image acquisition. Use the Alexa488 filter set to image the 16S rRNA signals and the Alexa594 filters set to image the phage signals. Because the phage signals can vary greatly with respect to intensity, take a series of images with increasing exposure

times. Short exposure times will capture the advanced infections, which give strong signals, while overlooking the weak signals. The long exposure times will result in overly saturated signals from the advanced infections, while capturing the weak signals from early infections/free phage particles. Adjust the lowest exposure time such that the advanced infections are visible, but their signal is not oversaturated. Adjust the highest exposure time such that the early infections/free phage particles are visible, but not oversaturated. In between the lowest and highest exposure time, set several time exposures. These will help with image analysis later. For each sample acquire several images representing different fields of view. Each field of view will have images corresponding to different acquisition channels — the Alexa488 channel, and the many Alexa594 channels, each at a different exposure time.

2. Image processing with DAIME (*see* **Note 33**).
   - From the time exposure series, choose the exposure time where the strong signals from advanced infections are not overexposed, but also where the weak signals from early infections/free phage particles are present, albeit almost invisible (*see* **Note 34**). This exposure time will be used for phage signal quantification and thus termed further "phage quantification exposure time."
   - Import images in DAIME grouped in "stacks." One stack will contain all images (all fields of view) from one replicate corresponding to an acquisition channel. Each replicate sample per time point will have three stacks: the rRNA signals, the phage signals from the phage quantification exposure time (the phage quantification stack) and the phage signals from the highest exposure time. To ensure correct correlation between the three channels of one field of view, each field of view should receive a number and this number should be present at the end of all images from that field (*see* **Note 35**).
   - Segment objects in each stack. This will identify each individual cell or phage signal and will transform them in "objects." Test different segmentation algorithms, choosing the one which best identifies individual cells and phage signals (*see* **Notes 33** and **36**). The aim is to define the phage objects in the stacks where they are visible, but not overexposed. Therefore, one should define the objects for advanced infections (bigger and stronger phage signals) in the phage quantification stack and for early infections in the highest exposure time stack. In the next steps, the objects from the two stacks will be transferred to the phage quantification stack.

- Create a fourth stack by replicating the phage quantification stack and removing the mask. Transfer the object layer from the phage highest exposure time stack to the newly created phage quantification stack — further named "single phage quantification stack."
- In "Visualizer" module, create a new Session by adding the following segmented stacks: the 16S rRNA stack, the phage quantification stack and the single phage quantification stack (from the above step). For each field of view, compare the objects in the two phage stacks, removing any duplicated object (if the same object appears in both stacks, remove one of them).
- For each stack, measure the segmented objects ("Analysis menu") and export the data as csv files. The most important parameters are total area, mean intensity, pixels, centroid X (pix), and centroid Y (pix).

3. Data analysis.
   - Import the data in Excel. Calculate the signal intensity of each phage signal by multiplying the mean intensity column with the pixels column.
   - For all time points in the experiment, plot the phage signal intensity versus the phage signal area (total area parameter) in the same graph (data grouped by time points and replicates).
   - Use the graph generated above to define three phage signal size classes. Since the time 0 infections should represent new infections, use their phage signal area range as the definition for the first size class. Further, identify the first time point where both the signal area and the signal intensity are highest. This time point is most likely represented by advanced infections (late replication and encapsidation). Use the phage signal area range in this time point as the definition for the third size class. All the signals in between the first and the third size class can be considered as the second size class (ongoing replication).
   - To calculate the cell fraction infected by phages per each time point, count all the cells displaying phage signals and calculate their percentage relative to the total host cells (the number of objects in the corresponding 16S rRNA stack). Most of the times, to one cell corresponds one phage signal. In the cases where multiple phage signals are presented per one cell, they have to be counted as one (this information can be obtained by comparing the pixel coordinates of the cells with that of phage signals—the centroid X (pix) and centroid Y (pix) columns for each stack).

4. Image processing for "display." Because the difference in signal strength prevents acquisition of images where both early and advanced infections are visible in a meaningful way, image processing algorithms have to be applied on the time exposure series, to merge the information from the different exposures. Such algorithms are called High Dynamic Range algorithms and they often are included in software packages for image processing.

4 Notes

1. Preparation of fluorescently labeled tyramides has been described by Pernthaler and Pernthaler [7].
2. To ensure specific hybridization, the formamide concentration in the rRNA hybridization buffer is probe specific. Thus, when preparing the rRNA hybridization buffer, both the water and 100% formamide volumes have to be adjusted accordingly. For more information about the formamide concentration of established probes, you can consult the online database http://probebase.csb.univie.ac.at/.
3. To avoid precipitation, always add SDS as the last component.
4. A higher concentration of 16S rRNA probe can be used if the target cells are low in ribosomal content. Do not exceed a probe concentration of 0.5 ng μl^{-1} as this can result in strong background signals.
5. The amount of 5 M NaCl in the washing buffer varies with the probe, more exactly, with the formamide concentration used in the hybridization buffer. NaCl gives the Na ions, which are important for the hybridization stringency. The 5 mM EDTA is also contributing to the Na ions concentration with 10 mM. This has to be considered when calculating the volume of 5 M NaCl added. The formamide (FA) concentrations and the corresponding Na ions concentrations when washing at 48 °C are as follows: 0% FA — 900 mM Na^+, 5% FA — 636 mM Na^+, 10% FA — 450 mM Na^+, 15% FA — 318 mM Na^+, 20% FA — 225 mM Na^+, 25% FA — 159 mM Na^+, 30% FA — 112 mM Na^+, 35% FA — 80 mM Na^+, 40% FA — 56 mM Na^+, 45% FA — 40 mM Na^+, 50% FA — 28 mM Na^+, 55% FA — 20 mM Na^+, 60% FA — 14 mM Na^+.
6. The concentration of the Alexa488 tyramides can be adjusted depending on the requirements of the microbial cells. For fast growing cells, with high number of ribosomes, a lower concentration of tyramides can be used (e.g., 0.33 μg ml^{-1}), while more tyramides are necessary for slow growing cells, with low number of ribosomes. Generally, do not exceed a

concentration of 2 μg ml^{-1}, as it can result in background formation. If you want to preserve sub-cellular localization of the rRNA signal, use less Alexa488 tyramides. Furthermore, too much Alexa488 tyramides in the rRNA step can saturate the tyramide binding sites in the cell, leaving too little biding sites for the Alexa594 tyramides in the gene CARD step, and thus, decreasing phage detection.

7. When using a negative control probe for the gene (*see* **Note 17**), add it to a concentration equivalent to that of all the probes in the positive control probe mix. For example, if the positive control probe mix contains six probes, the final concentration of the negative control probe for the gene should be 5 pg $\mu l^{-1} \times 6 = 30$ pg μl^{-1}.
8. Store washing buffers for the gene hybridization at the washing temperature, if different from 42 °C.
9. The antibody step can result in false positives signals. To reduce the background formation, western blocking reagent is used before and during the antibody binding step. If increased background is a problem for your samples, you can test stronger blocking mixes, for example, by adding bovine serum albumin or sheep serum to the antibody blocking and binding solutions. On the other hand, a too stronger blocking can reduce the detection efficiency, as it interferes with the specific antibody binding sites.
10. When working with a group of similar phages, keep in mind that polynucleotide probes are not able to discriminate between closely related sequences. On the other hand, this enables the use of polynucleotide probes for targeting a larger group of mismatched sequences [3, 5]. One polynucleotide probe can be used to detect targets with 5% mismatches, with a slight decrease in the hybridization rate (and thus detection efficiency). Increasing the mismatches will lead to a further decrease in the hybridization rate, until, at around 20% mismatches, no hybridization will take place. The PolyPro software [5] can be used to design polynucleotide probes for multiple alleles. From a multiple alignment of the allelic regions, select 300 bps stretches which show minimum sequence variation. For each of the 300 bp stretches, the PolyPro software will search for the probe–probe mixes which will enable probe binding to all targeted alleles.
11. The detection efficiency, that is, the percentage of the cells which show a positive gene signal from all cells having the respective target gene, depends both on the number of polynucleotide probes used and on the number of targets per cells. Allers et al. [1] show that, for 3–8 gene copies per cell, one probe gave 70% detection efficiency and an increasing number

of polynucleotides increased the detection efficiency. For example, a hybridization efficiency of >90% was obtained with four polynucleotides and 98% with 12 polynucleotides. For cells with a high number of target copies, one probe was sufficient for 90% detection efficiency, while 3 probes gave 100%. Calculations indicate that, for cells with one gene copy, at least 12 polynucleotides will be necessary for 100% detection.

On the other hand, in a similar approach Matturro et al. [8] reached 100% detection efficiency with a single polynucleotide probe. The factors that could contributed to the increased on the detection efficiency is the higher denaturation temperature (from 85°C for 1 h in a humidity chamber, to 90°C for 20 min in PCR tubes), a better accessibility to chromosomal regions that are actively transcribed because they tend to stand in a decondensed state [9], and thus more accessible to the probes, and second, a higher number of targets per cell often found in dividing bacteria.

12. In the plot, the %GC at a certain base position will correspond to the sequence stretch from the respective base position plus 99 bases (this for cases when the "window size" parameter for calculating the %GC has been set to 100).
13. Target sequences with lower %GC will denature more easily and allow access of the probes to the individual DNA strands.
14. Alternatively, probes can be synthesized by using Digoxigenin-11-dUTP nucleotides, any *Taq* polymerase kit (e.g., 5Prime MasterTaq kit) and unlabeled nucleotides. High fidelity polymerases should be avoided because they do not incorporate modified nucleotides. For synthesis, start preparing a mix of unlabeled nucleotides with a concentration of 2 mM per dATP, dCTP, and dGTP and 1.3 mM dTTP. For example, add 2 μl of 100 mM dATP, 2 μl 100 mM dCTP, 2 μl of 100 mM GTP, and 1.3 μl 100 mM dTTP to 92.7 μl water. Mix by vortexing and store at −20 °C. Next, prepare a 100 μl PCR reaction with the following concentrations: 1× *Taq* buffer, 200 μM of each dATP, dCTP and dGTP, 130 μM dNTP, 70 μM Dig-11-dUTP, 1 μM of each primer, 0.04 U of *Taq* polymerase, and 50 ng of DNA template. The concentration of the DNA template can affect the success of obtaining a high probe concentration, it is recommended to do serial dilutions of 1:10, 1:100, and 1:1000 of the template and run PCR reactions in parallel to find the optimal DNA concentration for the PCR mix.

    For the PCR thermocycling, start with an initial denaturation step of 5 min at 95 °C, followed by 30 cycles of: denaturation (1 min at 95 °C), annealing (1 min at 55–60 °C), and elongation (1–3 min at 72 °C). Finalize the reaction with an elongation step at 72 °C for 10 min. The annealing temperature and the elongation time have to be optimized for the primers and template of interest.

15. Because high temperatures are damaging for the cells, formamide is used in hybridization buffers to lower the melting temperatures and to allow for stringent hybridization at relatively low temperatures (42–50 °C). On the downside, high concentrations of formamide are decreasing the hybridization rate.
16. Theoretical considerations indicate that the highest hybridization rate for polynucleotides is at −25 °C below their Tm. Getting closer to the Tm will decrease the hybridization rate (and thus, detection efficiency), while getting away from the Tm will not only decrease the hybridization rate, but also favor the formation of short mismatched hybrids [5].
17. There are two controls which can be used for phageFISH: (1) the same sample, but not infected, and/or (2) a negative control gene probe, (e.g., NonPolyPro350 [3] or a probe binding to other phage).
18. Working with filters.
    - To label the filters, use a carbon pencil and write preferably on the edge of the filter, where there are no cells. Do not use permanent marker pens, they might interfere with the fluorescent signals.
    - To treat the filters with different reagents:
      – If the reagents used are rather expensive and economical use is preferred (e.g., when working with enzymes or with hybridization buffers): place the filters face up on a petri dish plate, add the reagents on top of the filters while making sure that the filters are completely covered.
      – If the reagents are relatively inexpensive (e.g., when inactivating the endogenous peroxidases with 0.1 M HCl): fill a 25 or 50 ml petri dish (depending on the number of filters, crowding should be avoided) with the reagent of interest and immerse the filters in it. Make sure the filters are completely immersed and they are not floating on top of the solution.
    - To wash the filters, always use large volumes (e.g. 50 ml) of the corresponding washing buffer:
      – If the incubation is performed at room temperature or in the oven, then place the filters in a 50 ml petri dishes filled with the washing solution of interest.
      – If the incubation is performed in the water bath, then place the filters in 50 ml Falcon tubes filled with the washing solution of interest. To remove them from the Falcon tube, either pour the solution in a petri dish and remove the filters from there, or pour the solution through a ceramic sieve which will catch the filters.

- To cut the filters in smaller pieces, use sterile scalpels or cleaned with ethanol when reused.
- To dry the filters, first blot them on chromatography paper to eliminate most of the liquid, and then leave them to air-dry on fresh chromatography paper. An alternative to chromatography paper is the "Kimwipes" paper, from Kimtech Science (https://www.kcprofessional.com/).

19. Working with glass slides.
    - Buy slides already coated, for example, with TESPA or L-polylysine. Make sure that the slides are clean before using them, to avoid background problems.
    - Alternatively, coat your own slides, for example, with L-polylysine or with TESPA, following manufacturer's instructions. Before coating, clean the slides to remove any particles or grease, for example, by incubating for 20 min in a mixture of 10% HCl and 70% ethanol, rinsing with milliQ water followed by 95% ethanol and air-drying at 60 °C.
    - To label the slides, use a carbon pencil to write on the frosted end of the slide. Do not use permanent marker pens, they might interfere with the fluorescent signals.
    - To mark the sample area on the glass slide, use a Diamond Pen to draw a circle or rectangle on the back of the slide. Do not draw on the sample face of the slide, since the scratched glass could result into an uneven surface and interfere with microscopy latter on.
    - To treat the sample on glass slides with different reagents:
        - If economical use is preferred: place the glass slides face up, add the reagents on top of the sample are while making sure that the sample area is completely covered.
        - If the reagents are relatively inexpensive: fill a slide staining jar with the reagent of interest and immerse the slides in it. Make sure that the sample area or any other slide surfaces previously coming into contact with other reagents are completely immersed.
    - To wash the samples on glass slides:
        - If the incubation is performed at room temperature or in the oven, then place the glass slides in staining jars filled with the washing solution of interest.
        - If the incubation is performed in the water bath, then place the glass slides (up to two slides per Falcon tube, back to back) in 50 ml Falcon tubes filled with the washing solution of interest.

20. Avoiding sample drying.
    - Do not allow samples to dry unless indicated in the protocol, otherwise background formation will occur, especially in the steps using DS.
    - Avoid drying during incubations and washing steps by completely immersing the filters in the respective buffers.
    - Drying can especially occur when smaller volumes of buffers are used and/or the incubations are performed at higher temperatures. To avoid drying in these cases, the samples have to be placed in humidity chambers. A humidity chamber is represented by any tightly closing container that seals with a silicone O-ring (e.g., food containers used in the kitchen). Polypropylene containers work well for low temperature incubations (e.g., 46 °C). However, they will get deformed at high temperatures (e.g., 85 °C), and in this case containers with a glass bottom part should be used. To create and maintain humidity in the chamber, line the bottom of the container with tissue paper and soak it with liquid. The soaking liquid is either water when the reagents are not volatile, or a water–volatile reagent (e.g., formamide, paraformaldehyde) mixture when reagents used are volatile. In this case, the volatile reagents should have the same concentration in the soaking mixture as in the buffers themselves. For samples immobilized on filters, the filters can be placed face-up in petri dishes, covered with buffer, and then the petri dishes placed in humidity chambers. For samples immobilized on slides, place a PCR tube rack in the humidity chamber and then place the slides on top. Care should be taken that the humid paper does not to come into contact with the sample area.
21. The fixation procedure can be sample specific. However, most often is performed by using PFA. Generally, avoid long incubation times with PFA, as it can result in increased cell autofluorescence. Another fixative is ethanol, mostly used for fixation of gram-positive bacteria [10].
22. More details about variations in protocols for fixation, permeabilization, and endogenous peroxidase inactivation can be found in the literature [11–14].
23. For cell concentration and PFA removal, three procedures can be used: centrifugation, filtration in Swinnex filter holders (Millipore) containing 0.22 μm polycarbonate filters or direct filtration on 0.2 μm polycarbonate membrane filters, in filter towers (described in the Subheading 3.4 point 1). In all cases, harsh conditions which would promote cell lysis should be avoided.

During centrifugation, use the minimum centrifugal force required to pellet the cells, preferably in a swinging bucket rotor. After centrifugation, remove the supernatant and suspend the cells in 1× PBS. Repeat the wash step, to ensure that all PFA has been removed. You can proceed immediately to the sample immobilization step. Alternatively, for long-term storage, add 96% ethanol in a 1:1 ratio with the 1× PBS. Store the cells at −20 °C.

During filtration in Swinnex filter holders, gently push the liquids through the device using a syringe, first the fixed cells, then at least 20 ml of 1× PBS. This will remove the culture media and the PFA, while concentrating the cells in the liquid left on top of the membrane filter. To recover the cells, invert the Swinnex filter holder, position its cell end in a 2 ml tube and unscrew it. The 1× PBS containing the cells will go into the 2 ml tube. Remove the filter from the holder and place it in the 2 ml tube, then gently wash its surface by pipetting up and down before removing it. You can proceed immediately to the sample immobilization step. Alternatively, for long term storage, add ethanol in a 1:1 ratio with the cell suspension, and store at −20 °C.

24. For the cells which in the fixation step were either centrifuged or processed through a Swinnex filter holder (*see* **Note 23**), a separate step for immobilization on solid support is necessary. The solid support is represented either by 0.2 μm polycarbonate filters, or by coated glass slides (*see* **Notes 18 and 19**). To immobilize on filters, mix different volumes of cell suspension with 10 ml 1× PBS and filter on 0.22 polycarbonate filters, followed by washing with 10–15 ml 1× PBS and 10–15 ml water. Use DAPI staining and microscopy to check which cell suspension volume gives the best distribution of cells on filters. To immobilize on glass slides, spot 10–100 μl of cell suspension in the marked area (*see* **Note 19**) and air-dry at 37 °C. On some slide types, the ethanol used in the cell suspension will cause it to spread too much. To avoid that, the cell suspension could be diluted just before use in 1x PBS, to decrease the ethanol concentration (e.g. to 10–25%). Alternatively, use press-to-seal silicone isolators to create a "well" for the sample. The isolators can be removed when the sample is dried. To remove precipitated salts, wash once in water and then let dry.
25. To mitigate cell loss, agarose embedding is routinely used in rRNA CARD-FISH protocols [13]. However, we have found that it significantly decreases detection efficiency of the phage genes.
26. Permeabilization is necessary to allow intracellular diffusion of the high molecular reagents used during phageFISH (e.g., HRP-oligonucleotide probes, polynucleotide probes, HRP–antibody conjugates). Depending on the cell type,

different permeabilization reagents can be used (e.g., achromopeptidase for gram-positive bacteria, lysozyme for gram-negative bacteria, detergents, acids, and/or proteinases for archaea; *see* **Note 22** for literature references).

27. For most samples, 0.1 M HCl will be sufficient to inactivate endogenous peroxidases. However, there are samples for which a stronger inactivation is necessary, e.g., by using H_2O_2 and/or methanol, *see* **Note 22** for literature references. The efficiency of the inactivation can be tested by performing only the rRNA CARD-FISH part of the phageFISH protocol, both with a 16S probe (as positive control) and without any probe (as negative control). The rRNA CARD-FISH part includes sample fixation, immobilization, permeabilization, inactivation of endogenous peroxidases, rRNA hybridization, CARD for rRNA detection, and embedding and counterstaining. If microscopic evaluation reveals no signals in the negative control and signals in the positive control, then the inactivation procedure has worked. If not, further optimizations have to be performed.
28. For cells with low ribosomes content, the signal intensity of the rRNA can be enhanced by increasing Alexa488 tyramide concentration (*see* **Note 6**) and also, by performing the incubation with Alexa488 tyramides for longer times (e.g., 20–30 min), and/or, at 46 °C.
29. RNase treatment is necessary when the discrimination of the different infection stages based on the per cell phage signal area is desired. Otherwise, binding of the phage probes to the corresponding mRNA would result in overestimation of the phage signal area.
30. If the samples are on glass slides, add the same volume of hybridization mix as for prehybridization, however, with a double probe concentration than the intended one.
31. The temperature control during the denaturation/hybridization steps is very important for the success of the method, as it influences both detection efficiency (to access the target gene, high temperature for longer times are necessary) and the cell morphology and loss (most often, high temperatures for prolonged times are damaging for the cells, especially for such fragile ones as virus-infected cells). When working with humidity chambers and ovens, decreasing the denaturation time from 1 h at 85 °C to 30 min at 85 °C led to a decrease in the detection efficiency for early infection stages (low numbers of virus genomes per cell), while it had no influence on the late infection stages (high numbers of virus genomes per cell). On the other hand, the cells looked more damaged after 1 h denaturation. A more precise control of the temperature can be

obtained in a PCR thermocycler, as Matturro et al. [8] have shown, by immersing the filters in PCR tubes with hybridization buffer. In this case, only 20 min at 90 °C where sufficient to obtain 100% detection efficiency (*see* **Note 11**). To obtain a similar control of the temperature when working with slides, a slide thermocycling machine can be used, as exemplified below.

Note that the slide thermocycling machines vary in their operation mode. For illustration, we describe the conditions used with the TDH-500 Slide denaturation/hybridization system from Hangzhou Allsheng Instruments Co. (http://www.allsheng.com/).

- Prepare glass slides with the samples as described in **Note 19**.
- To avoid evaporation of the hybridization mix, use Hybriwell sealing chambers. Put the chamber on top of the sample region on the glass slide (*see* **Note 19**). Apply pressure to seal the secure chamber to the slide.
- Add the hybridization mix, making sure that it covers the sample entirely. About 20 μl are needed for one sample.
- Close the slide thermocycling machine and verify that it is tightly sealed.
- Incubate for 5–15 min at the denaturation temperature (85–95 °C) and then for 2 h or overnight at the hybridization temperature. The optimal denaturation conditions will vary depending on the sample and therefore they need to be optimized for each specific viral–host system.

32. A significant decrease in the gene hybridization time is obtained by increasing the DS concentration in the hybridization buffer from 10% to 20%. Prepare the gene hybridization buffer as follows: in a 50 ml Falcon tube add 8 g DS and 10 ml of 20 SSC, vortex vigorously. Add 1.6 ml 5 mM EDTA pH 8 and 4 ml water. Close the tube and shake/vortex strongly to disperse the DS in solution. Incubate on water bath at 46–48 °C to dissolve the DS, vortex and shake from time to time. It might take a long time (e.g., overnight) to dissolve all DS. After DS has dissolved completely, allow the solution to cool down to room temperature, and then add 4 ml 10% nucleic acid blocking reagent, 1 ml 10 mg ml^{-1} sheared salmon sperm DNA, 1 ml 10 mg ml^{-1} yeast RNA, 14 ml 100% formamide, and 200 μl 20% SDS. Vortex to mix components. The buffer needs to be filtered through 0.22 μm sterile syringe filters. Aliquot and store at −20 °C.
33. DAIME will be efficient for image analysis every time when the cells are sufficiently separated so that they will be recognized by the software (through the use of segmentation algorithms) as separate objects. When the segmentation algorithm cannot

recognize individual cells, then the image analysis has to be performed in a semimanual way. For example, counting of the phage infected cells can be performed using the AxioVision software (from Zeiss), by manually marking the cells in the Alexa488 channel and the corresponding gene signals in the Alexa594 channel with the "Events" tool from the "Measure" menu. The number of events can be determined using the "measure events" function. Similarly, when the phage signals cannot be segmented by DAIME, their signal intensity has to be measured again semimanually, with the help of image analysis software. For example, the free hand tool in Zen Lite 2011 (Blue Edition; Carl Zeiss, Germany) software can be used to mark the phage signals and measure their signal intensity.

34. Most of the time, the early infections will also be recorded in the low-exposure images, but at a very low intensity. They will become visible to the eye only after image enhancement (e.g., increased brightness and magnification) and comparison with the same field of view, but from a higher-exposure time (which indicates where to look).
35. The images could be labeled for example using the following pattern: replicate_timepoint_channel__XX (where XX is the number of the field of view). See DAIME manual for further information regarding naming of files for import into stacks.
36. In our hands, the edge thresholding algorithm worked best.

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Chapter 2

Fluoromycobacteriophages for Drug Susceptibility Testing (DST) of Mycobacteria

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Abstract

Fluoromycobacteriophages are a new class of reporter phages that contain Laboratorio fluorescent reporter genes (*gfp, ZsYellow*, and *mCherry*) and provide a simple means of revealing the metabolic state of mycobacterial cells and therefore their response to antibiotics. Here we described a simple and rapid method for drug susceptibility testing (DST) of *Mycobacterium spp* using a fluorescence microscope, a flow cytometer, or a fluorimeter in a convenient multiwell format.

Key words Reporter phage, Mycobacteria, DST, Fluoromycobacteriophage, Antibiotics, Resistance

1 Introduction

Tuberculosis (TB) is a major cause of human mortality with nine million new cases and nearly two million deaths annually; approximately two billion people are infected with the causative agent *Mycobacterium tuberculosis* [1]. While *M. tuberculosis* infections can be effectively resolved with a 6–9 month course of antibiotics with at least three drugs, the emergence of drug resistant strains greatly complicates treatment. Of particular concern are those strains resistant to two or more of the first-line antituberculosis drugs, including multidrug-resistant (MDR) strains that are resistant to rifampicin and isoniazid, and extensively drug resistant (XDR) strains that in addition are resistant to a second line injectable drug—such as capreomycin, kanamycin or amikacin—and a fluoroquinolone [2]. Drug resistance profiles can be readily determined using sensitive and automated methods or by DNA-based technologies, but these can be expensive, limiting their applicability in the developing world where the vast majority of TB cases occur [3–6]. There is, therefore, a need for new diagnostic approaches that combine speed (time-to-detection), sensitivity, specificity,

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biosafety, and cost, to determine resistance to the commonly used antituberculosis drugs.

Mycobacteriophages are excellent candidates for the development of diagnostic tools since they efficiently and specifically infect and replicate in mycobacterial hosts. We have described the development of fluoromycobacteriophages as a new class of reporter phages that contain the fluorescent reporter genes *gfp* or *ZsYellow* [7, 8]. These fluoromycobacteriophages have potentially significant advantages over existing phage-based tuberculosis diagnostic methods. The commercially available phage amplification biological assay (FASTPlaque™, Biotec; http://www.tinhangtech.com/) utilizes *M. tuberculosis*-dependent reproduction of phage D29 and determination of viral particle counts on the fast-growing *Mycobacterium smegmatis* [9, 10], and has been adapted for determining resistance to rifampicin [11]. The luciferase reporter phage assay uses recombinant mycobacteriophages carrying the firefly luciferase gene to detect *M. tuberculosis* by luminescence, coupled with empirical determination of drug resistance by light emission in the presence of antibiotic [12, 13]. These methods are rapid, accurate, and simple, but are not well-suited to detection of partially resistant cultures and require the propagation of live potentially infectious cultures.

Fluoromycobacteriophages are simple and rapid to use and have high levels of sensitivity, restricted primarily only by the efficiency with which cells can be recovered for analysis by fluorescent microscopy or flow cytometry. While detection using fluorescence microscopy might not seem the best option for inexpensive diagnosis, the use of low-cost LED (light-emitting diode) fluorescence adapters to microscopes well-suited for developing countries has been reported [14–16].

Moreover, recently we have developed a second generation of Fluoromycobacteriophages (carrying a *mCherry* gene with a codon optimized usage in mycobacteria) with higher sensitivity and a shorter time to detection of signal in *M. tuberculosis* [17]. We have also established the conditions for infection in a multiwell format in the presence of drugs. This innovative methodology could not only be used for DST of clinical isolates but also for further application in HTS (high throughput screening) of novel antitubercular compounds.

2 Materials

2.1 Preparation of Fluorophage Stocks

1. Phage buffer: 50 mM Tris–HCl pH 7.5; 150 mM NaCl; 10 mM $MgSO_4$.
2. 7H10 plates, containing 10% (v/v) ADC (2 g/l D-glucose, 5 g/l bovine serum albumin fraction V, 0.85 g/l NaCl),

carbenicillin (CB 50 μg/ml), cycloheximide (CHX 10 μg/ml). 7H10 media is prepared following manufacturer instructions, autoclaved and supplemented with ADC, CB, and CHX.

3. Mycobacterial top agar (MBTA): 7H9 with 0.7% Bacto agar, autoclaved to sterilize.
4. 0.1 M stock $CaCl_2$ sterilized by autoclave.

2.2 Preparation of Bacterial Cultures

1. 7H9 liquid media is prepared following manufacturer instructions and autoclaved. 7H9 is supplemented with 10% (v/v) ADC (Albumin Dextrose Catalase Supplement) for *M. smegmatis* or OADC (Oleic Albumin Dextrose Catalase Supplement) for *M. tuberculosis* (*see* **Note 1**).
2. Tween 80. Prepare a 20% solution and sterilize by filtration.
3. Glass tubes.
4. Baffled flasks.
5. Screw cap tubes.

2.3 Preparation of Antibiotic Stock Solutions

Rifampicin (RIF) (50 mg/ml) in DMSO and ofloxacin (OFLO) (10 mg/ml) in NaOH 1 N, further dilutions starting at 5 mg/ml can be prepared in water. Isoniazid (INH) (5 mg/ml), ethionamide (ETH) (10 mg/ml), ethambutol (EMB) (10 mg/ml), kanamycin (KAN) (5 mg/ml), streptomycin (STR) (10 mg/ml) carbenicillin (50 mg/ml), and cycloheximide (10 mg/ml) prepared in distilled deionized water and filter sterilized. Antibiotic stocks can be stored at −20 °C.

2.4 Cell Fixation

PBS (0.1 M sodium phosphate, 0.15 M sodium chloride).

Paraformaldehyde 4% in PBS.

2.5 Fluorimetric Assays

Black, flat, clear bottom 96-well microplates (Greiner Bio-One; https://www.gbo.com/en_US.html).

Black light-absorbing sealing film (AbsorbMax, Excel Scientific Inc.; http://www.excelscientific.com/blackwhite_content.html).

3 Methods

3.1 Preparation of Fluorophage Stocks

It is not convenient to amplify fluorophages from stocks more than two times since phage mutants that render less fluorescence could be obtained. Ideally, *M. smegmatis* mc^2155 cells are electroporated with phasmid DNA and individual plaques are amplified to further obtain a high titer stock (*see* below).

1. Electroporate *M. smegmatis* mc^2155 competent cells with 200–300 ng of the appropriate phasmid (Bio-Rad Gene Pulser; settings: 2500 mV, 1000 Ω, 25 μF for a 0.2 cm cuvette) (*see* **Note 2**).
2. Recover cells at 30 °C for 30 min in 1 ml 7H9 + ADC.
3. Mix the cells with 200 μl of an exponentially growing culture of *M. smegmatis* mc^2155 (*see* below) and 3 ml of MBTA + $CaCl_2$ (final concentration 1 mM). Pour on a 7H10/ADC/CB/CHX plate.
4. Incubate the plate at 30 °C for 48 h (*see* **Note 3**).
5. Cover the plate with 3 ml of phage buffer (if you have got between 100 and 200 plaques) or pick about 10 plaques using a sterile tip and combine them in 500 μl of phage buffer (if you have got between 20 and 50 plaques). Incubate for 2 h to ON at 4 °C. You can directly calculate the titer of this small stock by the double agar overlay method.
6. If you covered the plate with buffer, recover the buffer (scrapping part of the top agar too), centrifuge at 3500 × *g* at 4 °C for 5 min to remove cell debris and agar.
7. Filter the supernatant through a 0.45 μm pore and calculate the titer of the plate stock by the double agar overlay method (*see* **Note 4**).
8. Make phage dilutions of the stock in order to get about 5.10^4–1.10^5 PFU/ml.
9. Mix 2 ml of the appropriate dilution with 20 ml of an exponential phase culture of *M. smegmatis* mc^2155. Incubate at RT for 15 min.
10. Mix the bacteria–phage suspension with 180 ml of MBTA/ADC containing 1 mM $CaCl_2$.
11. Pour 10 ml of the mix per 7H10/ADC/CB/CHX plate (150 × 15 mm petri dish) (Total of 20 big plates).
12. Incubate at 30 °C for 48 h. You should get almost but not complete lysis of the bacterial lawn in order to get a high titer stock.
13. Cover each plate with 10 ml of phage buffer +1 mM $CaCl_2$ and incubate at 4 °C overnight.
14. Recover and combine the buffer from the plates and remove cells and cell debris by centrifugation at 3500 × *g* at 4 °C for 15 min.
15. Filter the supernatant through a 0.45 μm pore.
16. To concentrate the phage stock, centrifuge at 100,000 × *g* for 2 h at 4 °C (*see* **Note 5**).
17. Discard the supernatant and cover the pellet with 1 ml of phage buffer + 1 mM $CaCl_2$. Incubate at 4 °C overnight.
18. Completely resuspend the pellet.

19. Filter the phage suspension using a 0.45 μM filter.
20. Calculate the titer of the stock by the double agar overlay method.

3.2 Detection by Fluorescence Microscopy or Flow Cytometry

3.2.1 Infection of M. smegmatis Cultures

1. Inoculate a colony of *M. smegmatis* mc^2155 into 3 ml 7H9 broth (containing ADC, CB, CHX, and 0,05% Tween) in a sterile glass tube and grow shaking at 37 °C until culture is saturated (about 2 days). This is subcultured into 15 ml of the same media but without Tween and CB, CHX in a 125 ml baffled sterile flask to a final OD_{600nm} = 0.020 and grow shaking at 37 °C overnight (*see* **Note 6**).
2. Once the cells have reached OD_{600nm} = 0.800-1 add 250 μl of cells into a microcentrifuge tube and infect with 250 μl of a phage dilution to obtain a multiplicity of infection (MOI) of 100 (*see* **Note 7**). Always include a mock-infected control.
3. If performing DST, add the antibiotics at the appropriate concentration simultaneously with the phage. For some antibiotics a 4 h preincubation is necessary before addition of the phage (*see* **Note 8**).
4. Incubate the phage–cell mix for 15 min standing and then for 3 h (for $mCherry_{bomb}$ phage) or 5 h (for phAE87::*hsp60-EGFP*) at 37 °C with moderate shaking (*see* **Note 3**).
5. Add 500 μl of paraformaldehyde 4% in PBS and incubate for 1 h at room temperature (*see* **Note 9**).
6. Spin down the cells using a microcentrifuge (3 min at max. speed) and wash the pellet using 500 μl of PBS (*see* **Note 10**).
7. Resuspend the cells in 25 μl (for microscopy) or 300 μl (for flow cytometry) of PBS. Cells can be stored at 4 °C until use.
8. For microscopy detection spot 4.5 μl on a slide and cover with a coverslip, remove the excess of liquid with a tissue and seal (*see* **Note 11**).
9. Observe the cells using a fluorescence microscope (*see* **Note 12**).

3.2.2 Infection of M. tuberculosis Cultures

1. Inoculate a colony of *M. tuberculosis* into 3 ml 7H9 broth (containing OADC, CB, CHX and 0.05% Tween) in a sterile screw cap plastic tube and grow standing at 37 °C until culture is saturated (about 10 days). Use those 3 ml to inoculate 15 ml of the same media in a 50 ml sterile plastic tube and incubate standing at 37 °C.
2. Once the cells have reached OD_{600nm} = 0.800-1 (corresponding to a turbidity of McFarland 4; https://catalog.hardydiagnostics.com/) washed them two times with 7H9 and resuspend in 7H9+ OADC (without Tween and

CB, CHX) (*see* **Note 13**). Incubate the cells at 37 °C for 24 h (*see* **Note 14**).

3. Add 250 μl of cells into a microcentrifuge screw cap tube and infect with 250 μl of a phage dilution to obtain a multiplicity of infection (MOI) of 100 (*see* **Note 15**).
4. When performing DST, add the antibiotics at the appropriate concentration simultaneously with the phage. For some antibiotics a 24 h preincubation before addition of phage is required (*see* **Note 16**).
5. Incubate the phage–cell mix for 15 min standing and then for 5 h (for $mCherry_{bomb}$ phage) or 16 h (for phAE87::*hsp60-EGFP*) at 37 °C, with moderate shaking (*see* **Note 3**).
6. Add 500 μl of paraformaldehyde 4% in PBS and incubate for 3 h (*see* **Note 17**) at room temperature (*see* **Note 9**).
7. Spin down the cells using a microcentrifuge (3 min at max. speed) and wash the pellet using 500 μl of PBS (*see* **Note 10**).
8. Resuspend the cells in 25 μl (for microscopy) or 300 μl (for flow cytometry) of PBS. Cells can be stored at 4 °C until use.
9. For microscopy detection spot 4.5 μl on a slide and cover with a coverslip, remove the excess of liquid with a tissue and seal (*see* **Note 11**).
10. Observe the cells using a fluorescence microscope (*see* **Note 12**).

3.3 Detection Using a Fluorimeter in a Multiwell Format

1. Grow *M. smegmatis* or *M. tuberculosis* cells as described in Subheading 3.2.1, **steps 1** and **2** or 3.2.2, **steps 1** and **2**, respectively. When testing clinical isolates, alternatively resuspend a few colonies in 7H9/OADC to a turbidity of McFarland 4.
2. In a black, flat, clear bottom 96-well microplate prepare a range of twofold dilutions of the drug to be tested in 7H9/ADC (for *M. smegmatis*) and 7H9/OADC (for *M. tuberculosis*) in a final volume of 100 μl. Leave the last well with 100 μl of media only as control.
3. Add 100 μl of *M. smegmatis* mc^2155 or *M. tuberculosis* cells to each well (*see* **Notes 18** and **19**).
4. Add 10 μl of a phage dilution to obtain a multiplicity of infection of 100 to each well (*see* **Note 20**).
5. Cover the top of the microplate with a black light-absorbing sealing film (*see* **Note 21**).
6. Introduce the sealed microplate in the fluorimeter previously set at 37 °C.

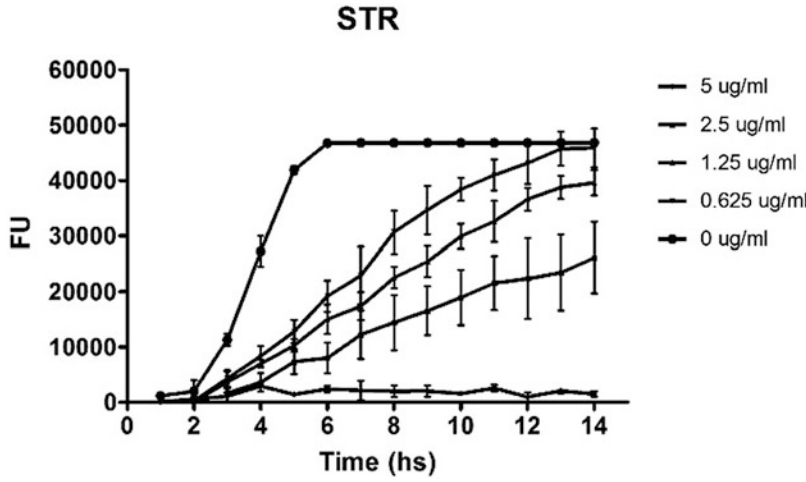

Fig. 1 Kinetics of expression of fluorescent genes after infection of *M. tuberculosis* mc^{2}6230 with *mCherry-bomb* phage in the presence of different concentrations of streptomycin

7. Set up a program that allows for monitoring fluorescence as a function of time at 37 °C (*see* **Note 22**). Double orbital shaking is recommended before each reading.
8. When retrieving your results subtract the background (cells plus phage at time zero) to each time point. A typical curve obtained for *M. tuberculosis* mc^{2}6230 in the presence of different concentrations of the drug is shown in Fig. 1.

4 Notes

1. ADC and OADC are prepared as 10X stock solutions. Combine 925 ml deionized water with 8.5 g NaCl and 20 g of glucose in a flask. Stir with a magnetic bar. Add 50 g BSA and stir until completely dissolved. If preparing OADC, add 50 ml of 1% sodium oleate solution* and stir until completely mixed. The pH should be 6.9–7. Filter sterilize through a 0.22 μm pore membrane and store at 4 °C.

   * To prepare 500 ml of 1% sodium oleate solution, mix 12.5 ml of 2 M NaOH, 6 ml of oleic acid (5 g), and 481.5 ml of water.
2. Details for preparation of *M. smegmatis* mc^{2}155 electrocompetent cells and transformation with phage DNA are given in Chapter 6 (BRED chapter). Overall, cells are grown as indicated in Subheading 3.2.1, **steps 1** and **2** and washed several times with 10% sterile ice-cold glycerol.
3. Fluoromycobacteriophages are derivatives of pH 101 that has a thermosensitive phenotype. After infection cells are lysed at 30 °C but not at 37 °C. So it is crucial to incubate plates at

30 °C to obtain plaques and cell suspensions at 37 °C to visualize fluorescent cells.

4. Depending of the number of plaques obtained after electroporation, titer of the stock will be around 10^7–10^8 PFU/ml.
5. This centrifugation step has to be done using an ultracentrifuge. Alternative you can follow Sarkis and Hatfull (Methods in Molecular Biology, Vol 101: Mycobacteria Protocols, Chapter 12, 3.3.2. High Titer CsCl Phage Stocks).
6. Cells are not infected in the presence of Tween so *M. smegmatis* cells should be grown in the absence of the detergent or in the case of *M. tuberculosis,* washed thoroughly and incubate without detergent for at least 8 h before infection.
7. An $OD_{600nm} = 1$ of *M. smegmatis* mc^2155 corresponds to 10^8 cells/ml. 250 μl contain about 2.5 10^7 cells that have to be infected with 2.5 10^9 phages to obtain a MOI of 100.
8. When the target of the tested drug is gene expression (transcription or translation), phage and antibiotics can be added simultaneously. In contrast, when the antibiotic has a different target (e.g., cell wall synthesis), a preincubation with the drug is necessary prior to addition of phages. RIF (50 μg/ml), KAN (10–20 μg/ml), OFLO (12.5 μg/ml) and STR (12.5–25 μg/ml) can be added simultaneously with the phage. For ETH (20 μg/ml), EMB (10 μg/ml) and INH (25–50 μg/ml) cells should be preincubated for 4 h at 37 °C before addition of phage. These concentrations were tested to inhibit fluorescence when using *M. smegmatis* mc^2155 strain. These values might need to be modified when using a different strain.
9. It is better to do this incubation in a roller rotator or similar to ensure the contact between the cells and the paraformaldehyde solution.
10. You can repeat this wash one more time to get a cleaner sample but keep in mind you are going to lose cells in each wash.
11. Avoid using nail polish, it quenches the fluorescence. We suggest the following sealer: 1:1:1 vaseline–lanolin–paraffin melted in a beaker on a hot plate set to low (you can reuse this mix several times).
12. Look at the cells in bright field first and then switch to fluorescence. We use the following filters: for EGFP, CLON ZsGreen1 (42002- HQ 470/30×, HQ 520/40 m, Q495LP), for mCherry, 64 HE m Plum shift free (E).
13. Check that turbidity remains similar to McFarland 4.
14. If performing DST use this 24 h to preincubate with the antibiotics (e.g., ETH and INH).

15. An $OD_{600nm} = 1$ of *M. tuberculosis* corresponds to 10^9 cells/ml. 250 μl contain about 2.5 10^8 cells that have to be infected with 2.5 10^{10} phages to obtain an MOI of 100.
16. RIF (2 μg/ml), KAN (4 μg/ml), STR (6 μg/ml), and OFLO (10 μg/ml) can be added simultaneously with the phage. For ETH (10 μg/ml), EMB (5 μg/ml), and INH (0.4 μg/ml) cells should be preincubated for 24 h at 37 °C before addition of the phage. These concentrations were tested to inhibit fluorescence and discriminate between sensitive and resistant strains when using *M. tuberculosis* mc^26230 strain (a derivative of H37Rv). These values might need to be optimized when using a different strain.
17. Three hours incubation is sufficient to kill all viable bacteria when using *M. tuberculosis* mc^26230 strain but we suggest testing this when using other strains. After fixation sample manipulation is safe and is not necessary to work under BSL2/3 conditions.
18. We add the cells using a multichannel pipette changing tips between each drug.
19. If you are testing antibiotics that require a preincubation (*see* **Notes 8** and **16**), cover the plate with a lid and incubate the cells for 4 h (*M. smegmatis*) and 24 h (*M. tuberculosis*) at 37 °C before addition of phage. When doing 24 h preincubation, fill some of the empty wells in the microplate with water to avoid evaporation. Alternatively incubate in a "wet chamber".
20. An $OD_{600nm} = 1$ of *M. smegmatis* mc^2155 corresponds to 10^8 cells/ml. 100 μl contain about 10^7 cells that have to be infected with 10^9 phages to obtain an MOI of 100. An $OD_{600nm} = 1$ of *M. tuberculosis* corresponds to 10^9 cells/ml. 100 μl contain about 10^8 cells that have to be infected with 10^{10} phages to obtain an MOI of 100.
21. Carefully clean the outside of the microplate using a 10% sodium hypochlorite solution.
22. Set up conditions to measure fluorescence each hour for at least 8 h. The presence of the black light-absorbing sealing film only allows for bottom readings, so be sure you have that option in your equipment. For convenience, plates can be read overnight.

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Chapter 3

Engineering Bacteriophage-Based Biosensors

Daniel Brownell, John King, Brian Caliando, Lada Sycheva, and Michael Koeris

Abstract

Bacteriophages have been used for diagnostic purposes in the past, but a lack of parallelizable engineering methods had limited their applicability to a narrow subset of diagnostic settings. More recently, however, advances in DNA sequencing and the introduction of more sensitive reporter systems have enabled novel engineering methods, which in turn have broadened the scope of modern phage diagnostics. Here we describe advanced methods to engineer the genomes of bacteriophages in a modular and rapid fashion.

Key words Bacteriophage engineering, Genome engineering, Reporter systems, Diagnostics, Luciferase

1 Introduction

Bacteriophages have been engineered to express reporter proteins that aid in detecting the presence or absence of a particular type of bacterial cell, which is susceptible to infection by this strain of bacteriophage [1–4]. The natural host range of a particular bacteriophage isolate is limited, and the combined host ranges of all previously characterized bacteriophage strains still may not encompass a sufficient fraction of the target bacteria of interest for a given diagnostic application. Methods of engineering bacteriophages with novel, designed-to-specification host ranges will contribute to expansion of the use of bacteriophages as diagnostic entities. Before this can occur, however, high-throughput methods of engineering of bacteriophages with specified properties are required [4].

Engineering bacteriophages is inherently difficult due to certain properties of bacteriophage genomes. For example, bacteriophage genomes have evolved to contain relatively fewer restriction sites, and their DNA is heavily chemically modified, making use of traditional cloning techniques with bacteriophages challenging [5, 6]. Bacteriophages also have compact genomes with many

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essential genes and very little noncoding DNA. This feature can make it difficult to find acceptable sites for engineering modifications that are aimed at either inserting heterologous sequence or replacing parts of the genome [7].

One approach for cloning bacteriophage DNA relies on isolating bacteriophage DNA, cutting the DNA with restriction enzymes, ligating the heterologous sequence and transforming the DNA back into the host either for assembly of the engineered phage [8]. A second approach is to clone a smaller segment of a bacteriophage genome into a plasmid, modify this segment by adding a heterologous sequence, transform this modified plasmid into the appropriate bacterial host strain and then infect the host with wild type phage. At some low frequency, homologous recombination between the bacteriophage genome and the plasmid will occur resulting in the insertion of the heterologous sequence into the wild-type phage genome [9]. Screening the phage progeny for the recombinant phenotype will reveal the engineered phages.

While these basic techniques have succeeded in a number of isolated instances, they also have a number of limitations. For instance, prior to functionally testing a recombinant phage isolate's diagnostic properties, the phage sample must undergo thorough genotypic characterization to ensure that its DNA was modified as intended. In addition, for each engineered variant created or new insertion site tested the whole engineering process must be repeated from beginning to end [1, 9]. To overcome these limitations, we have introduced several improvements into this engineering protocol to make it faster and more efficient [10, 11]. This improved protocol we call Phage-Infective Engineering (PIE, Fig. 1) [10, 11].

To engineer the phage, we create a Phage Targeting Vector (PTV), which consists of a reporter gene (luciferase) flanked by about 1 kb of bacteriophage genomic sequence, corresponding to the loci directly upstream and downstream of the desired insertion site. The PTVs are assembled from PCR fragments, which are amplified using primers that deliberately incorporate 20 bp of overlapping sequence into each pair of adjacent insert fragments in order to facilitate assembly via recombination-based cloning methods [12].

2 Materials

Prepare all solutions using ultrapure water (prepared by purifying deionized water to attain a sensitivity of 18 MΩ·cm at 25 °C) and analytical grade reagents. Prepare and store all reagents at the appropriate storage temperature (unless indicated otherwise). Follow all local and federal waste disposal regulations when disposing waste materials.

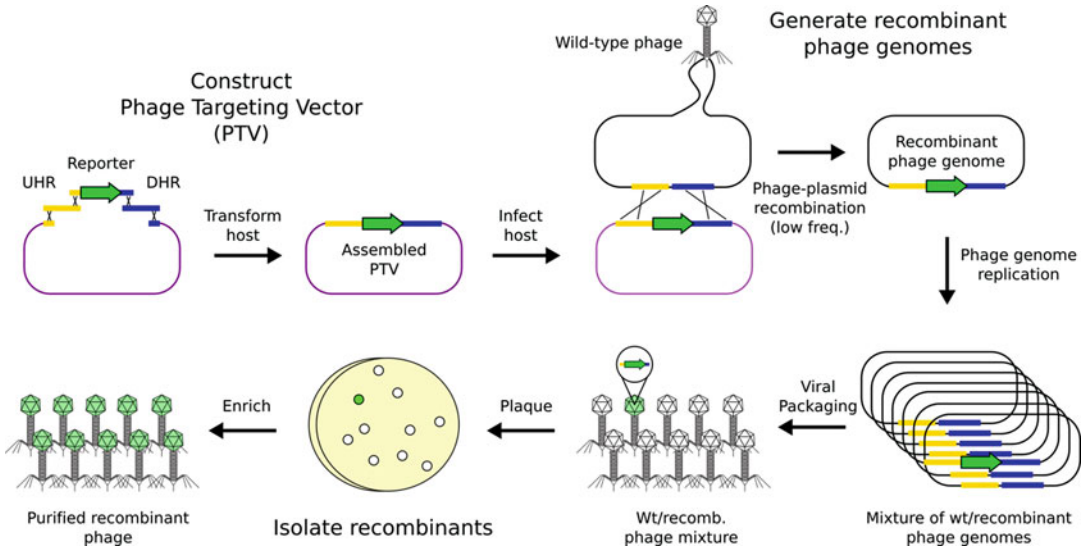

Fig. 1 Schematic of phage infective engineering (PIE) workflow. Recombination-based cloning methods are used to construct the phage targeting vector (PTV) in which a reporter gene (green arrow), such as luciferase, is flanked by ~1 kb of DNA sequence homologous to the upstream (UHR, yellow rectangle) and downstream (DHR, blue rectangle) regions of the phage chromosome that is targeted for modification. This vector is transformed into a permissive bacterial host strain. Subsequently, the strain is infected with wild-type phage. During the course of infection, there is a low probability that a double-crossover homologous recombination event occurs between the PTV and the wild-type phage DNA that results in the insertion of reporter gene sequence into the phage genome. These recombinant phage genomes, which express the reporter gene phenotype, are typically a minority among those present during the cycle of infection, and so viral packaging yields a mixed pool containing few recombinant phage virions among mostly wild-type phage virions. Iterative rounds of screening and enrichment for the recombinant phages are then performed until a pure monoclonal sample is obtained and subsequently amplified (*see* Fig. 2 for greater detail on enrichment procedures)

2.1 Materials for Construct Design and Assembly

- Phusion® High-Fidelity PCR Master Mix with HF Buffer.
  - M0531S, New England Biolabs, USA.
- Primers, 25 nM, standard desalting.
  - Integrated DNA Technologies, USA.
- Mastercycler pro and Control Panel.
  - 6321 000.515, Eppendorf, USA
- GeneArt® Seamless Cloning and Assembly Kit.
  - A13288, Thermo Fisher Life Technologies, USA.
- Base plasmid pMK4 or other gram-negative/gram-positive shuttle vector.
  - ATCC 37315.
- One Shot® TOP10 Chemically Competent *E. coli*.
  - C4040-10, Thermo Fisher Life Technologies, USA.

- Gene Pulser Xcell™ Electroporation Microbial System.
  - 165-2662, Bio-Rad Laboratories, USA
- Gene Pulser/MicroPulser Cuvettes.
  - 165-2083, Bio-Rad, USA
- BHI agar plates.
  - W15, Hardy Diagnostics, USA.
- BHI + 500 mM Sucrose Broth (autoclaved).
  - 90,003-032, VWR, USA
- Penicillin *g* (50 mg/mL).
  - AAJ63901-22, VWR, USA.
- Sucrose Glycerol Wash Buffer (500 mM sucrose, 10% glycerol pH 7.0, filter sterilized).
  - Sucrose.

    P-908, Boston Bio Products, USA (https://bostonbioproducts.com/)
  - Glycerol.

    G5516-100 ML, Sigma Aldrich, USA.
- Lysozyme (50 mg/mL).
  - 470301-618, VWR, USA.

2.2 Materials for Screening

- GloMax® 96 Microplate Luminometer w/Dual Injectors.
  - E6521, Promega, USA.
- ChemiDoc™ XRS+ System with Image Lab™ Software.
  - 170-8265, Bio-Rad, USA.
- Nano-Glo® Luciferase Assay.
  - N1110, Promega, USA.
- RC-5C Superspeed Centrifuge.
  - Beckman Coulter, USA.
- SLA-1500 rotor.
  - Beckman Coulter, USA.
- UltraPure™ Low Melting Point Agarose.
  - 16520-050.
- 500 mM sucrose solution
  - Sucrose.

    P-908, Boston BiopProducts, USA
- Greiner Bio-One Lumitrac 96-well Assay Plates.
  - 655,075.

3 Methods

Carry out all the procedures at room temperature unless indicated otherwise.

3.1 Targeting DNA Vector Design and Assembly

3.1.1 Design

To target the reporter to a specific location in the phage genome, a plasmid must be created that contains the reporter gene flanked by specific upstream and downstream homology regions. To maximize recombination efficiency, homology fragments are typically designed to be ~1 kb in length. Desired promoter(s), ribosome binding site(s), and spacer region(s) can be easily introduced into the junctions between upstream, reporter, and downstream DNA fragments with PCR primers. This plasmid, once assembled, will allow site-specific recombination of the reporter gene into the locus of interest to occur. In this protocol we describe the introduction of a promoterless luciferase gene into the phage genome downstream of the major capsid protein of the *Listeria* phage A511 [10, 11, 13].

3.2 Assembly, Transformation and Propagation in Host Strains

In this section, we describe the propagation of the PTV in the *E. coli* host followed by transfer to the *Listeria* host.

3.2.1 Assembly

- Amplify the required fragments using PCR according to 2× Phusion Master Mix protocol.
- Linearize the vector (pMK4) by digestion with SmaI and PstI.
- Assemble the final vector by mixing three amplified fragments with the linearized vector using a homology-based assembly kit (GeneArt Seamless Cloning kit, Thermo Fisher Scientific).

3.2.2 Transformation

The GeneArt Seamless cloning kit includes TOP10 chemically competent cells. If using a different assembly kit follow the instructions for that kit.

- Add 6–8 μL of the seamless cloning and assembly reaction into a vial of One Shot® TOP10 chemically competent *E. coli* and mix gently by swirling.
- IMPORTANT! Do not mix by pipetting up and down. Note: If you are performing transformation control, add 2.5 μL of pUC19 Control DNA into a separate vial of One Shot® TOP10 chemically competent *E. coli* and follow the transformation procedure.
- Incubate the transformation mix on ice for 20 to 30 min.
- Heat-shock the cells for 30 s at 42 °C without shaking.
- Immediately transfer the tubes to ice and incubate on ice for 2 min.

- Add 250 μL of room temperature S.O.C. medium to the transformation mix.
- Cap the tube tightly and shake it horizontally (200 rpm) at 37 °C for 1 h.
- After incubation, dilute the transformations 1:10 in S.O.C. medium and spread 10–50 μL from each transformation on a prewarmed selective plate. If you have performed a 4-fragment assembly, plate the transformations undiluted. We recommend plating two different volumes to ensure that at least one plate has well-spaced colonies.
- Incubate the plates overnight at 37 °C.

3.2.3 E. coli: PTV Screening

Screening

To screen for colonies containing the correctly assembled vector, it is expedient to carry out a colony PCR with the primers flanking the desired insert. However, we routinely use a faster, more higher-throughput method for the initial screening of transformants that takes advantage of the strong and constitutive expression of the reporter NanoLuc luciferase gene with in the *E. coli* host.

- Prepare a microcentrifuge tube with 50 μL of LB medium for each of the colony to be screened.
- Pick each colony to be tested with a sterile P200 pipette tip and place it into the prepared microcentrifuge tube. Mix the content of the tube well by vortexing for 10 s. Use the obtained colony suspension for all subsequent steps.
- Transfer 5 μL of each of the resuspended colonies into its own well of a Lumitrac 200 medium 96-well binding plate, retain the remainder colony suspension for future steps.
- Prepare a Nano-Glo® reagent according to the manufacturer instructions.
- Add 5 μL of Nano-Glo® to each well.
- Measure the luminescence on a Promega GloMax96 luminometer using the built-in "SteadyGlo" protocol (1 s integration), or equivalent.
- Those colonies that exhibit prominent brightness during this screen are likely to harbor the correctly assembled vector. This shall be confirmed by colony PCR and sequencing using the reserved colony suspension as the template.
- Culture those clones for which sequencing confirms that their plasmids had assembled correctly by inoculating LB supplemented with the appropriate antibiotic.
- Prepare the plasmid using a QIAGEN maxi-prep kit.

3.2.4 *Listeria: In Vivo Recombination*

After assembly and sequence verification of the recombination plasmid, it must be transferred in to an appropriate host for the phage of interest. In this example, A511 can infect the *Listeria monocytogenes* strain EGD-e (ATCC BAA-679). Competent cells must be prepared and transformed prior to recombination.

Generating Electrocompetent Cells [14]

- On day 1, streak EGD-e from glycerol stock at −80 °C onto BHI plates.
- Grow plate at 30 °C overnight.
- On day 2, pick an isolated single colony from the plate and inoculate 6 mL BHI supplemented with 500 mM sucrose (autoclaved).
- Grow culture overnight at 30 °C in an orbital shaker at 200 rpm.
- On day 3, dilute the overnight culture of EGD-e 1:100 into 500 mL of BHI supplemented with 500 mM sucrose.
- Grow cells at 37 °C shaking at 200 rpm until the cells reach an OD_{600} of 0.25.
- Add 100 μL of 50 mg/mL Penicillin *g* (final concentration 10 μg/mL), return to incubator, and grow for 1 h shaking at 150 rpm, 37 °C.
- Remove cells from incubator and chill for 10 min on ice.
- Spin cells at 5000 × *g* (5700 rpm) for 10 min in a SLA-1500 rotor at 4 °C.
- Remove supernatant and resuspend cells carefully by gently swirling in 500 mL of ice-cold Sucrose Glycerol Wash Buffer ("SGWB," 500 mM sucrose, 10% glycerol pH 7.0, filter sterilized). It is extremely important to resuspend the cells gently in these steps. It is not uncommon for the initial resuspension to take 30 min or more.
- Spin cells at 5000 × *g* for 10 min at 4 °C.
- Remove supernatant and resuspend cells carefully in 250 mL of ice-cold SGWB.
- Spin cells at 5000 *g* for 10 min at 4 °C.
- Resuspend cells in 50 mL of ice-cold SGWB.
- Transfer cells to 50 mL conical tube and add 10 μL of 50 mg/mL lysozyme (final concentration 10 μg/mL) and incubate cells statically at 37 °C for 20 min.
- Transfer cells back to centrifuge tube and pellet at 3000 × *g* (3000 × *g* in SLA1500) for 10 min at 4 °C.
- Discard supernatant and resuspend cells in 20 mL of ice-cold SGWB.
- Pellet cells at 3000 × *g* for 10 min at 4 °C.

- Discard supernatant and resuspend cells in 2 mL of ice-cold SGWB.
- Aliquot 50 μL of cells into prechilled microtubes.
- Freeze cells at −80 °C.

Electroporation Conditions

- Thaw the electrocompetent Listeria EGD-e cells on ice.
- Add 4 μL of PTV plasmid DNA (typically 250–1000 ng). Incubate on ice for 30 min.
- Transfer cells to a 0.1 cm electroporation cuvette (Bio-Rad or equivalent).
- Electroporate cells at 1.0 kV, 400 Ohms, 25 μF.
- Add 1 mL of BHI supplemented with 500 mM sucrose (filter sterilized).
- Incubate statically at 30 °C for 3 h.
- Plate entire transformation mixture onto solid BHI media supplemented with 10 μg/mL of chloramphenicol.
- Grow plates for 2–4 days at 30 °C.
- Similar to *E. coli* PTV transformants positive *Listeria* transformants can be screened for their ability to produce light in the presence of NanoLuc in accordance with the aforementioned protocol (*see* "Screening" in Subheading 3.2.3) and should be similarly confirmed by colony PCR and sequencing.

3.3 Recombination

- Inoculate a 5 mL overnight culture of the verified PTV-positive *Listeria* transformant and incubate at 30 °C for 16–24 h shaking at 200 rpm.
- Dilute the overnight culture to an OD_{600} of 0.02 in 5 mL 0.5× BHI medium.
- Add 10^4–10^7 PFU/mL of wild-type phage to the culture and mix gently. Incubate overnight at 26 °C, shaking at 50 rpm.
- Inoculate a culture of *L. monocytogenes* EGD-e in 5 mL 0.5× BHI.
- The following day, filter-sterilize the recombinant lysate using a 0.22 μm vacuum filtration device or equivalent syringe filter.

3.4 Test Infection

To determine if the marker has recombined into the phage, a test infection must be performed. If the recombinant lysate can produce signal upon infection of wild-type cells, recombination has occurred and the enrichment process can begin.

- Prepare an infection of wild-type EGD-e cells by mixing 190 μL of 0.5× BHI with 5 μL of the recombinant lysate from Subheading 3.3 and 5 μL of an overnight culture of EGD-e.

- Prepare phage-free and cell-free controls by mixing 5 μL of cells or recombinant lysate, respectively, with 195 μL of 0.5× BHI.
- Measure the luminescence of 10 μL from each tube mixed with 10 μL of Nano-Glo® reagent on the Glomax 96 luminometer. Save the measured values as first data points ($T = 0$) in the monitoring of luminescence of test infection over time.
- Incubate all tubes statically for 6 h at 30 °C.
- Measure the luminescence of all tubes again after 6 h ($T = 6$).
- If the luminescence of the test infection is more than tenfold of the background luminescence (the value measured for the controls), recombinant phage particles are present.

3.5 Enrichment

- Prepare the phage bacterial host cell culture in advance, so that it reaches log phase (an OD_{600} of 0.2) prior to the start of enrichment procedure.
- Prepare different phage dilutions according to Table 1. Disposable reservoirs are a good choice for preparation of the mixtures if multichannel pipettes are to be used.
- Pipette 200 μL of the solution containing the highest C[phage] into each well of the top three rows of the first 96-well plate.
- Pipette 200 μL of the solution containing the intermediate C [phage] into each well of the bottom five rows of the first 96-well plate.
- Pipette 200 μL of the solution containing the lowest C[phage] into each well of the second plate (Fig. 2).
- Cover the plates to minimize evaporation and incubate overnight at 26–28 °C.
- On the next day, follow the screening protocol for luminescence; mix 5 μL from the test well with 25 μL of Nano-Glo® substrate. Measure the bioluminescence using the GloMax 96 luminometer as before.

Table 1
Recipe for preparation of different phage dilutions

	C[phage] = 10^8, PFU/mL	C[phage] = 10^7, PFU/mL	C[phage] = 10^6, PFU/mL
V[phage]	$\frac{20\ \text{mL} \times 108\ \text{PFU/mL}}{\text{X PFU/mL}}$	$\frac{20\ \text{mL} \times 107\ \text{PFU/mL}}{\text{X PFU/mL}}$	$\frac{20\ \text{mL} \times 106\ \text{PFU/mL}}{\text{X PFU/mL}}$
V[bacterial culture]	$\frac{20\ \text{mL} \times 0.02\ \text{AU}}{\text{Y AU}}$	$\frac{20\ \text{mL} \times 0.02\ \text{AU}}{\text{Y AU}}$	$\frac{20\ \text{mL} \times 0.02\ \text{AU}}{\text{Y AU}}$
V[medium]	To 20 mL	To 20 mL	To 20 mL

X—phage concentration C[phage] in PFU/mL, Y—OD_{600} of bacterial culture in AU. V[phage], V[bacterial culture] and V[medium]—volumes of respective components of enrichment mixtures

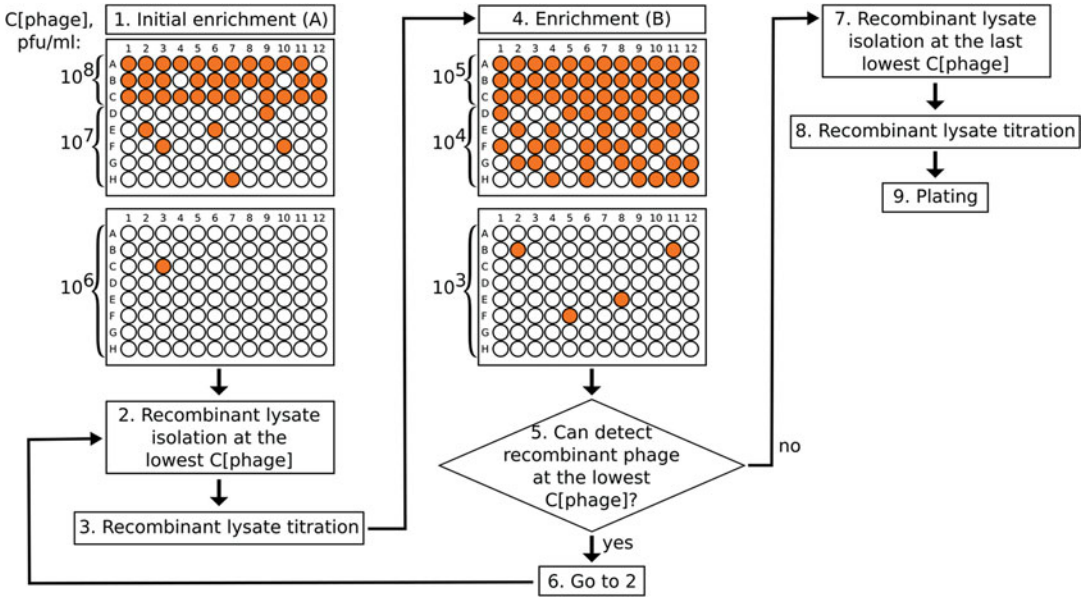

Fig. 2 Schematic representation of iterations of enrichment procedure. Orange wells represent bioluminescent wells that contain recombinant phage. Before the sufficient dilution of phage is achieved the enrichment has to be repeated decreasing the phage concentration in each round as depicted here: the highest phage concentration used in enrichment (B) is 10× lower compare to the lowest phage concentration of the initial enrichment (A). As more rounds of enrichment are performed, the frequency of occurrence of wells with recombinant phage shall increase. As depicted on the schematic, the number of wells showing bright bioluminescence has increased in enrichment B compared to initial enrichment A

- Identify the well with the brightest bioluminescence and the lowest phage concentration. A positive signal indicating the presence of recombinant phage is typically more than 10–100 fold brighter than values indicating the presence of pure wild-type phage. As the recombinant phage is enriched further, the positive signals will increase.
- Transfer the material from this well to a microcentrifuge tube and save the recombinant lysate for subsequent rounds of enrichment.
- Remove a sample of this recombinant lysate to determine the titer of phage present.
- Set up the next round of enrichment following the procedure described above except decrease the phage concentrations tested by further diluting the recombinant lysate (Fig. 2).
- Perform as many rounds of liquid enrichments as needed until luminescent wells can be seen from lysates made from 10-100 PFU.
- Plate obtained recombinant lysate to isolate single plaques (*see* Subheading 3.5 below).

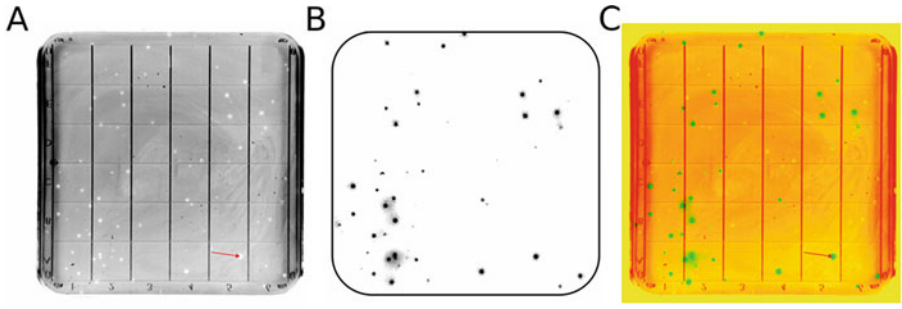

Fig. 3 Images of the plated plaques. (**a**) Colorimetric image, plaques present as white/clear spots in the image, while the nonlysed bacterial lawn is opaque and shows shading due to polymerization artifacts of the top agar. (**b**) Chemiluminescent image, exposed for 10 s using the Bio-Rad XRS+ ChemiDoc system. (**c**) Composite image, artificially colored using the ImageLab software package. This image highlights the difference between wild-type (nonluminescent) and recombinant (luminescent) plaques. The arrow on panels (**a**) and (**c**) shows a well-isolated, recombinant plaque

3.5.1 Plating of Recombinant Lysate

- Prepare serial dilutions of the recombinant lysate (range of dilutions) and plate those onto 0.5× BHI agar plates using the top agar overlay method.
- Incubate plates overnight at 30 °C.

3.5.2 Detection of Recombinant Plaques

- Prepare a sufficient volume of the "detection solution" by mixing of 1 mL of Nano-Glo® substrate with 4 mL of 0.5× BHI low-melt top agarose for each plate to be screened.
- Overlay each plate with the detection solution and leave if to solidify (~5 min).
- Image the plate using the Bio-Rad Chemidoc system in two different regimes: (1) chemiluminescent regime (typically a 10 s exposure); (2) colorimetric regime (typically a 5 s exposure). The Bio-Rad Chemidoc software can overlay the two images allowing one to more easily identify the glowing plaques from the resulting composite image (Fig. 3).

3.5.3 Isolation of Recombinant Plaques

- Using a P1000 pipet tip attached to a P1000 pipet, puncture the agar to isolate the desired plaque (or zone of lysis), and then eject the isolated plaque material in a microcentrifuge tube containing 100 μL of 0.5× BHI broth.
- Pipet the mixture up and down to break up the agar and homogenize the mixture.
- Incubate the mixture for 10 min at room temperature to allow the phages to diffuse into the broth.
- Remove bacterial cells and debris by filtration through a 0.22 μm syringe filter or by adding 10 μL of chloroform to the tube.
- Titer the filtrate to determine the phage concentration.

4 Notes

1. Targeting DNA Vector
   (a) We have seen multiple instances of missing fragments during assembly, i.e., plasmids containing only the UHR and NanoLuc fragments but lacking the DHR. In these cases, we have had success using the following techniques:
      - Repeating the assembly with an increased concentration of the missing fragment. For example, if the DHR is missing from the assembly, increase the amount of this fragment in the assembly mixture by 2–3 ×.
      - Prior to assembly, the UHR, reporter, and DHR fragments can be joined into a single larger insert using crossover PCR. The subsequent assembly with a smaller number of components is more likely to be successful compared to the initial assembly.
      - The assembly reaction volume and reagents can be cut in half and still yield a successful vector. This saves reagents.
   (b) Design of upstream and downstream homology fragments:
      - In general, longer stretches of homologous sequence between the phage and PTV increase the efficiency of recombination. However, longer fragments also increase the likelihood of intact viral genes being present in the PTV plasmid and expression of some viral can have toxic effects during assembly. We have found a successful balance between these competing constraints using homology region fragments between 500 and 1200 bp.
   (c) Linearization of vector.
      - To simplify PTV construction, the vector backbone is typically linearized by digestion with one or more restriction enzymes. However, if digestion is incomplete, uncut plasmid or plasmid with a single cut can recircularize to yield background colonies during transformation. To prevent this amplify a linearized vector using PCR with primers that face opposite the desired insertion site.
2. Transformation
   (a) Chemically competent *E. coli* are typically sufficient to obtain clones from the PTV assembly reaction. Electroporation is usually not necessary and requires additional steps to remove salt from the assembly reaction prior to

transformation. Refer to the instructions for the particular assembly kit being used.

(b) DNA quantification of assembly products does not reflect properly assembled vectors, because the reaction mixture contains mostly unassembled or incompletely assembled linear fragments.

(c) Competent cell preparation:

- If using Listeria strains other than EGD-e, different competent cell preparation and electroporation protocols may apply. There are a number of conditions that may need to be optimized for a given host, including the concentration of Pencillin G, lysozyme, etc.

(d) Listeria transformations are not as efficient as *E. coli*. Transforming with more DNA will increase the likelihood of a successful transformation, but must be balanced against the addition of extraneous volume, which dilutes the osmoprotectants, and additional salt, which can cause arcing, to the electroporation sample.

3. Recombination

(a) An abbreviated 6-h recombination has been shown to be sufficient in some cases. This may require a more conservative dilution series when performing the subsequent enrichments.

(b) If using different phage/host combinations, different concentrations of each may be required to successfully recombine. In addition, different recombination efficiencies may be observed.

4. Enrichment

(a) Enrichment of the initial recombinant lysate can be abbreviated by plating 100 μL of undiluted recombinant lysate, following the plaque plating protocol (*see* Subheading 3.5.1). Positive glowing zones can be isolated and replated to isolate individual plaques.

(b) When using the Glomax96 luminometer, signal can bleed through when the neighboring well has extremely high signal. We typically observe 10^5 reduction of the signal in adjacent wells.

(c) 96-well plate-based enrichments can be abbreviated to a 6 h same day protocol, depending on strain/phage. In most cases, the signal levels observed will be lower than with an overnight incubation.

5. Screening

(a) Top agar is prepared using low-melt agarose to allow the molten material to stay in the liquid phase at 42 °C. This

temperature is low enough to neither damage the Nano-Glo® substrate, nor disrupt the already solidified top agar from the initial plating.

(b) Same day plaque isolation is possible depending on cells/phage.

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Chapter 4

Introduction of Phage Genome into *Escherichia coli* by Electroporation

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Abstract

Electroporation has been an established tool for DNA delivery into prokaryotic and eukaryotic cells, thus facilitating basic research studies and improving medical treatments. Here we describe its use for introduction of phage genomic DNA into *Escherichia coli* cells, including preparation of electrocompetent cells, electric pulse optimization and recovery of electrotransformed cells. The technique can also be adapted for other bacterial species.

Key words Phage genome, Electrotransformation, Bacteria

1 Introduction

Electroporation-assisted uptake of DNA relies on delivery of short high-voltage pulses that cause reversible permeabilization of cellular membrane. During this transient state, cells can be loaded with DNA of various sizes and origins. Although the mechanism behind electrotransformation is still not entirely clear, it is known that when a biological membrane is exposed to electrical pulses of sufficient strength, transmembrane voltage exceeds a certain value and membrane becomes permeable for small or large molecules [1]. Electroporation has been successfully used to transform a variety of prokaryotic, fungal, yeast, and mammalian cells [2–4]. Parameters affecting electrotransformation outcome are cell competency, electric pulse parameters (pulse amplitude, duration, number, and electric field strength), and postpulse manipulation. Gram-negative bacterial species can be treated using very similar transformation protocols, whereas gram-positive species many require additional steps to achieve satisfactory results [5]. It is advised to follow protocols for preparation of electrocompetent cells consistently, paying attention to harvest point, lower temperatures, sufficient

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washing in low ionic strength buffer, and finally adjusting cell concentration to 10^8–10^{10} CFU/mL. Large phage DNA is delivered to bacterial cells using pulse protocols that simultaneously enable DNA transfer and preserve cell viability. The degree of bacterial membrane permeabilization depends on electric field strength, pulse duration, number of pulses, and pulse repetition frequency [6]. The applied electric field strength has to be large for bacteria due to their small cell radius and many currently commercially available pulse generators may generate only limited range of pulse parameters [7]. After permeabilization bacterial cells have to be allowed to recover and express phage DNA. This step may be a matter of additional optimization, as it affects number of plaques significantly.

In this chapter, a reliable method to deliver lambda phage DNA to *E. coli* by electroporation is presented, however transformation efficiency is lower than with small plasmid DNA. Nevertheless, phages from obtained plaques can be easily analyzed using electron microscopy (Fig. 1c) and propagated using conventional techniques (Fig. 1b). The protocol described here may be extended to other bacterial species and their phages, but it should be modified corresponding to the relevant system under study.

2 Materials

2.1 Preparation of Electrocompetent Cells

1. Suitable host *E. coli* strain, e.g., DSM 4230 (*see* **Note 1**).
2. Lysogeny Broth (LB) agar plates: prepare the medium according to the manufacturer's instructions, add 15 g/L technical agar and heat-sterilize. Aliquot 18–25 mL to sterile petri dishes and allow to solidify at room temperature.
3. Prewarmed LB broth (*see* above Subheading 2.)
4. Cultivation flasks.
5. Incubator or warm water bath set to 37 °C with shaker.
6. Spectrophotometer to measure OD_{600}.
7. Ice.
8. 50 mL sterile plastic tubes suitable for centrifugation
9. Centrifuge.
10. Ice-cold sterile distilled water.
11. 10% (v/v) ice-cold sterile glycerol
12. Cryovials.
13. Freezer −20 °C (for short time storage) or −80 °C (for long time storage).

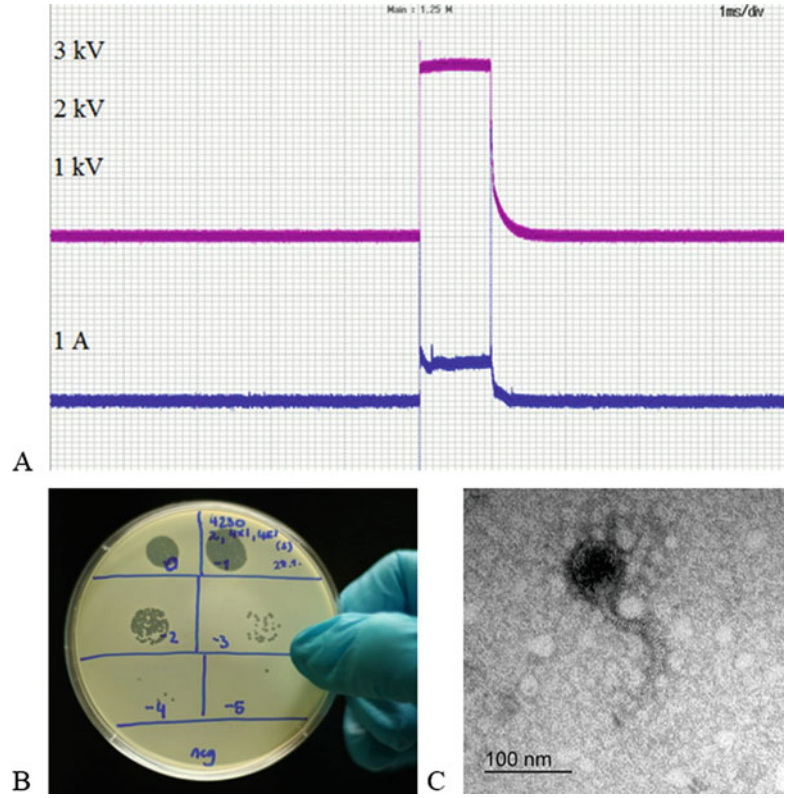

Fig. 1 (**a**) 4 × 1 ms, 3 kV, 1 Hz pulse measured by oscilloscope (DLM 2024, Yokogawa, Japan) using a high-voltage probe (P6015A,Tektronix, USA) and current probe (Tektronix TCP0150). Purple line: voltage (U) = 3 kV and blue line: electric current = 1 (**a**, **b**) serial tenfold dilutions of phage extract, obtained directly from a plaque, plated on *E. coli* DSM 4230, (**c**) lambda phages expressed by electrotransformed *E. coli* DSM 4230

2.2 Electroporation, Postpulse Manipulation, and Transformant Recovery

1. Electroporation cuvettes with 0.2 cm electrode gap.
2. Ice.
3. Lambda phage DNA (for one transformation 1.5 μg) in TE buffer: 10 mM Tris–HCl, 1 mM EDTA, pH 7.6.
4. Electrocompetent cells (*see* above Subheading 3.1).
5. Pulse generator (*see* **Note 2**).
6. Prewarmed commercially available S.O.C. medium or S.O.B. medium supplemented by 20 mM glucose: 2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose.
7. 1–2 mL sterile plastic tubes.
8. Incubator or warm water bath set to 37 °C with shaker.
9. Centrifuge.

10. Sterile 10 mM $MgSO_4$.
11. LB bottom agar: prepare the medium according to the manufacturer's instructions and add 15 g/L technical agar and heat sterilize. Aliquot 18–25 mL to sterile Petri dishes and allow to solidify at room temperature.
12. Overlay LB agar: prepare the medium according to the manufacturer's instructions and add 6 g/L technical agar and heat sterilize. Aliquot 3–5 mL overlay LB agar in sterile tubes and keep at 54 °C until use.

3 Methods

3.1 Preparation of Electrocompetent Cells

1. Inoculate 150 mL LB broth with colonies of suitable *E. coli* grown overnight on a LB agar plate (*see* **Note 3**). Incubate at 37 °C with agitation until the cultures reach $OD_{600\ nm}$ ~0.25 (*see* **Note 4**).
2. Split and transfer bacterial cultures to four sterile tubes. Cool them on ice for 20 min.

   From this point on, keep cells as much as possible on ice or at 4 °C.
3. Centrifuge 3000 × *g* for 30 min at 4 °C. Discard supernatant.
4. Resuspend pellets in 37.5 mL (together 150 mL) cold sterile distilled water and centrifuge 3000 × *g* for 30 min at 4 °C. Discard supernatant.
5. Resuspend pellets in 18.75 mL cold distilled water and combine them together into two tubes (together 75 mL).
6. Centrifuge 3000 × *g* for 30 min at 4 °C. Discard supernatant.
7. Resuspend cell pellets in 10 mL ice-cold 10% glycerol and pool them together into one tube.
8. Centrifuge 3000 × *g* for 30 min at 4 °C. Remove supernatant as much as possible.
9. Resuspend cell pellet in 3 mL ice-cold glycerol and aliquot cell suspension 200 μL per cryovial. Transfer them immediately to a freezer. Store competent cells at −80 °C or at −20 °C for a shorter period.

3.2 Electroporation, Postpulse Manipulation, and Transformant Recovery

1. Precool cuvette at 4 °C and thaw competent cells on ice (prepared in 3.1).
2. Transfer 1.5 μg DNA (dissolved in 5–10 μL TE buffer) into the tube with competent cells and mix gently.
3. Incubate on ice 2 min and transfer the mixture to the cuvette (*see* **Note 5**). Place the cuvette in the chamber of pulse generator. Deliver a pulse 4 × 1 ms, 3 kV, 1 Hz (*see* **Note 6**).

4. Take the cuvette from the chamber and add immediately 0.8 mL S.O.C. medium. Mix gently and transfer all the liquid from the cuvette to a sterile 1 mL tube. Incubate at 37 °C for 45 min vigorously shaking.
5. Centrifuge 8000 × *g*, 5 min at room temperature.
6. Remove supernatant as much as possible and resuspend gently cell pellet in 1 mL 10 mM $MgSO_4$.
7. Add 500 μL of this suspension (from point 6.) to 3–5 mL molten LB top agar, mix and then pour onto an LB agar plate (*see* **Note 7**).
8. When top agar solidifies, turn the plate upside-down and incubate for 24 h at 37 °C.
9. After 24 h successful transformation will result in easily visualized plaques. For further analysis plaques can picked from the overlay agar and homogenized in 50–100 μL LB broth. Liquid extracts can be used directly for electron microscopy examination or propagation using double agar overlay technique (plaque assay) (Fig. 1b, c).

4 Notes

1. Electrotransformation efficiency may be affected also by parameters independent from competency, pulse or postpulse manipulated, e.g., DNases or genetically incompatible phage–-host system.
2. In this chapter we describe pulse protocol optimized using prototype square wave (Fig. 1a) pulse generator. The latter operates over a wide range of pulse parameters and delivers a square wave pulses with higher amplitude [8]. Equipment suitable for electroporation of bacteria can be purchased from Bio-Rad (Gene Pulser; http://www.bio-rad.com), Tritech Research (BactoZapper; http://www.tritechresearch.com/), or BTX (ECM 630, Gemini SC, or Gemini X2; http://www.btxonline.com/).
3. Using bacterial colonies from agar plate as inoculum will result in higher electrocompetency than growing bacterial culture from liquid overnight culture as often used to prepare exponentially growing broth culture.
4. Bacterial cultures have to reach early exponential growth phase. If an unknown bacterial strain is used, its growth parameters should be determined prior to preparation of electrocompetent cells. Growth phase highly effects electrotransformation efficiency.

5. Make sure that cuvette is dry from outside and sample equally distributed between electrodes in the cuvette, without bubbles that may be formed during pipetting. It is advised to use each time a new cuvette, because cleaning may cause changes on the electrodes and subsequently affect pulse application.
6. To electrotransform the same *E. coli* strain with 4 kb plasmid 1 × 1 ms, 2 kV, 1 Hz pulse is applied.
7. Here bacterial culture is mixed with overlay agar similarly as in protocol for double agar overlay technique. Please see for details reference [9].

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Chapter 5

Site-Specific Mutagenesis of *Bacillus subtilis* Phage SPO1

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Abstract

This chapter describes the procedure that we have used to introduce suppressible nonsense mutations into various genes of *Bacillus subtilis* bacteriophage SPO1. The targeted gene is cloned in a *B. subtilis/Escherichia coli* shuttle vector. Using an in vitro enzymatic procedure dependent on mutant oligonucleotide primers, a mutation is inserted into the cloned gene, replacing an early lysine codon (AAA or AAG) with a nonsense codon (TAG or TAA). The mutant plasmid is recovered by transformation into *E. coli*, and is then transformed into *B. subtilis* carrying a suppressor that inserts lysine at TAG or TAA codons. Recombination is allowed between the mutant plasmid and superinfecting wild-type SPO1, and mutant progeny phage are identified by plaque-lift hybridization to labeled oligonucleotides having the mutant sequence. This procedure is adaptable for other types of mutations, and for other phage–bacteria combinations for which appropriate strains and plasmids are available.

Key words Site-specific mutagenesis, Bacteriophage SPO1, *Bacillus subtilis*, Primer-directed mutagenesis, Recombination, Hybridization

1 Introduction

Inactivating a gene by mutation is often the best way to elucidate the role of the gene product. Classical phage genetics isolated mutants affected in essential genes by screening for conditional lethal mutants, which could grow at one temperature but not another, or on one strain but not another [1, 2]. However, that approach is not available for genes that are not essential for phage growth. Here we describe the procedure that we have used for site-specific mutagenesis of nonessential genes in bacteriophage SPO1. The steps of the procedure are as follows: (a) Clone the gene to be mutated. (b) Mutate the cloned gene in vitro. The plasmid carrying the cloned gene is amplified, making use of mutant oligonucleotides as primers for the amplification reaction, thus producing plasmids carrying the desired mutation in the targeted gene. (c) Transform the mutant plasmid into the *B. subtilis* host. (d) Recombine the mutation into the SPO1 genome. The *B. subtilis* strain carrying the mutant plasmid is infected with wild-type SPO1,

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permitting recombination between the plasmid and the phage genome, which inserts the mutation in place of its wild-type allele in the SPO1 genome. (e) Identify the mutant phage. The mutant phage are identified among the progeny of that infection, by plaque-lift hybridization to oligonucleotides with the mutant sequence. An outline of this procedure has been published [3].

2 Materials

2.1 *B. subtilis* Strains

CB313 and CB10 are the SU^+ (suppressor plus) and Su^- strains, respectively [4]. The suppressor in CB313 inserts lysine at nonsense codons [5].

2.2 Cloning Vector

The primary *B. subtilis/E. coli* shuttle vector that we have used is pPW19, previously described by Wei and Stewart [6]. It has a selectable chloramphenicol-resistance gene, and an IPTG-inducible promoter just upstream of its polylinker.

2.3 Growth Media

1. TSA plates: 40 g trypticase soy agar (BBL), 1 L water. This is used for plating for colonies and as bottom agar for plating phage.
2. TC plates: TSA plates containing 10 μg/ml of chloramphenicol (Cm).
3. TC2 plates: TSA plates containing 20 μg/ml of Cm.
4. TBAB top agar: 15.4 g Tryptose Blood Agar Base (Difco), 1 L water. This is used as top agar for plating phage for plaque formation.
5. VY broth: 25 g Veal Infusion Broth (Difco), 5 mg yeast extract (Difco), 1000 ml water.
6. Penassay Broth: 17.5 mg Antibiotic Medium 3 (Difco), 1000 ml water.

2.4 Solutions

1. 1.0 M Tris pH 7.4: 132.2 mg Trizma Hydrochloride (Sigma-Aldrich), 19.4 g Trizma Base (Sigma-Aldrich), 1.0 L water (*see* **Note 1**).
2. 0.5 M Tris pH 7.4: 66.1 mg Trizma Hydrochloride (Sigma-Aldrich), 9.7 g Trizma Base (Sigma-Aldrich), 1.0 L water.
3. SSC (Standard saline citrate): 0.15 M NaCl, 0.015 M sodium citrate.
4. CSC (Concentrated saline citrate): 1.5 M NaCl, 0.15 M sodium citrate.
5. 10% SDS (Sodium dodecyl sulfate): 10 gm SDS, 90 ml water.
6. 5× SSC, 0.1% SDS: 10 ml of 10% SDS, 500 ml CSC, 490 ml water.

7. 50× Denhardt's solution: 500 mg Ficoll 400, 500 mg polyvinylpyrrolidone (360,000 molecular weight), 500 mg bovine serum albumin (BSA), 50 ml water.
8. Liquid Block. Obtained from GE Healthcare as part of the ECL kit. It includes 0.1 M Tris, 0.6 M NaCl, 10% irradiated casein, 0.1% Kathon (Methylchloroisothiazolinone; 5-chloro-2-methyl-1,2-thiazol-3(2*H*)-one; Sigma-Aldrich), 0.05% Antifoam A (Sigma-Aldrich).
9. Hybridization Buffer A: 30.0 ml 10× SSC, 2.0 ml 2.5% sodium dodecyl sulfate (SDS), 300 mg dextran sulfate (500,000 molecular weight), 3.0 ml Liquid Block, 25.2 ml water.
10. Hybridization Buffer B: 6.0 ml 10× SSC, 0.6 ml 10% SDS, 1 ml 50× Denhardt's solution, 1.0 ml water.

3 Methods

3.1 Clone the Gene to Be Mutated

The cloning vector used is pPW19, as described above. We have usually made the fragment to be cloned by PCR amplification from an SPO1 genomic DNA template. The restriction site to be used for cloning is included near the 5′ end of each PCR primer. The longer the distance from the mutation site to the ends of the PCR fragment, the greater will be the frequency of recombination. As a result a smaller number of plaques need to be tested for the presence of the mutation. However, several of our genes had nearby promoters whose presence on a fragment precluded its cloning, so quite short distances have been used successfully. In one case, a fragment with 108 and 760 bp to the left and right of the mutation site, respectively, produced a frequency of 0.4% mutants among the plaques tested. We have obtained significant recombination frequencies with one end as short as 63 bp.

3.2 Mutate the Cloned Gene In Vitro

We have used the QuikChange II Site-Directed Mutagenesis Kit, available from Agilent Technologies (formerly Stratagene). Oligonucleotides including the mutant codon are used as primers for in vitro amplification of the plasmid. Any remaining wild-type plasmids are inactivated by cleavage by DpnI, which is specific for methylated and hemimethylated DNA. The mutant plasmids are then transformed into *E. coli* XL1-Blue (competent cells are supplied with the kit), where the nicks are repaired. Since detailed protocols are provided with the kit, I will only describe modifications in the protocol that have given us improved results.

1. Choice of mutant codon. Each of our mutations has converted an early lysine codon (AAA or AAG) into a nonsense codon (TAG or TAA). We have usually made the mutation that involves changing two nucleotides, to maximize the

discrimination between wild type and mutant in hybridization assays. Even though the middle nucleotide in the mutant codon remains unchanged, it cannot base-pair when there are mismatched bases at either side. As such, it is effectively mismatched from the perspective of contributing to stability of the duplex. Lysine codons are chosen because our suppressor strain inserts lysine at nonsense codons.

2. Selection of primers. Although general protocols call for 10–15 bases of correct sequence on either side of the mutant codon, we have had better success with 16–17 bases, even with oligos having 50% GC, and with as many as 20 bases when an oligo had a GC value as low as 36.4%.
3. Thermal cycler reactions. We have always used 50 ng of the pPW19-derived plasmid in each reaction. This is at the high end of the recommended range. We have used an extension time of 2 min/kb of plasmid length, rather than the 1 min recommended.
4. Transformation. For selection of transformants having the Cm-resistant plasmid, we use TC2 plates containing 20 μg/ml Cm. This is twice the concentration needed for other *E. coli* strains, but 10 μg/ml can result in background growth for *E. coli* XL1-Blue.

3.3 Transformation of the Mutant Plasmid into the B. subtilis Host

The mutant plasmids are transferred from the *E. coli* XL1-Blue cells into *B. subtilis* CB313, the Su^+ strain, for use in recombination with superinfecting SPO1.

3.4 Recombine the Mutation into the SPO1 Genome

The procedure for recombination between the mutant plasmid and wild-type SPO1 is a modification of the procedure first described by Sayre and Geiduschek [7]. For production of multiple mutants, it can be modified by using a mutant strain of SPO1 as the infecting phage.

1. Grow CB313 carrying the cloned mutant gene, on a 37° shaker, in 10 ml VY plus 5 μg/ml Cm, in a 250 ml Klett flask, labeled flask 1, to a cell density of about 5×10^7/ml. Plate an appropriate dilution for colonies on TC plates to get a precise count (*see* **Notes 2** and **3**).
2. As soon as the first dilution has been prepared for that plating, infect flask 1 with about 5×10^8 SPO1 wt. The objective is to have the multiplicity of infection (MOI) be approximately 1.
3. After shaking 5 min at 37 °C, plate again for colonies as in paragraph 1, to determine the number of surviving cells, and thus the actual effective MOI (*see* **Note 4**).
4. Continue shaking 10 more minutes at 37 °C. Dilute 0.2 ml from flask 1 into flask 2, a 250 ml Erlenmeyer containing

9.8 ml VY plus 5 μg/ml Cm, prewarmed to 37°. Shake both flasks at 37 °C.

5. Immediately, dilute flask 2 1000-fold, and plate two 0.1 ml aliquots of the diluted culture for plaque-forming units (PFU). All platings of phage or phage-infected cultures for PFU in this procedure are performed with TSA plates as bottom agar, 15.4 g/L TBAB as top agar, and CB313 as lawn.
6. Continue shaking flask 1 until lysis is complete. Centrifuge the lysate for 10 min at RCF 3020 × *g* (Sorvall SS34 rotor at 5000 rpm), to remove uninfected cells. Dilute supernate 10^7-fold, and plate two 0.1 ml aliquots of the diluted culture for PFU to determine titer of lysate.
7. Continue shaking flask 2 until 60 min after infection (i.e., until 45 min after flask 1 was diluted into flask 2). Then add 0.2 ml chloroform and shake one more minute. Allow the chloroform to settle to bottom of flask (for just a few seconds). Immediately dilute 0.1 ml into 9.9 ml Penassay, being careful not to pick up the chloroform in the pipette. From this 10^{-2} dilution, dilute 1.0 ml into 9 ml Penassay, and 0.1 ml into 9.9 ml Penassay, to prepare 10 ml of each of 10^{-3} and 10^{-4} dilutions.
8. Plate two 0.1 ml aliquots of each of the 10^{-3} and 10^{-4} dilutions for PFU. The ratio of PFU at 60 min to PFU at 15 min is the burst size, which is typically around 100 for a successful infection.
9. The plaques formed in paragraphs 6 and 8 can now be tested for the presence of the mutation by plaque-lift hybridization. Once the titer of the lysates has been determined, more plates can be made, at appropriately adjusted dilutions, to maximize the chance of finding mutant plaques (*see* **Note 5**).

3.5 Identify the Mutant Phage by Plaque-Lift Hybridization

If the targeted mutation has a predictable and readily testable phenotype, it would be easiest to identify mutant plaques by testing for that phenotype. Most of our mutations were not in that category. Since comprehensive searches for conditional lethal mutants had previously identified most essential SPO1 genes, we assumed (correctly as it turned out) that the additional genes that we were targeting would not be essential, and thus not readily identifiable by their plaque-forming capabilities. Therefore, we have used sequence-specific hybridization to identify unequivocally the plaques having the mutant sequence, and growth on the suppressor strain to assure that the mutations did not restrict plaque formation.

1. *Preparation of plaque-lift filters* (*see* **Note 6**).
   (a) We use the BA85, 82 mm diameter, nitrocellulose membrane filters, available from GE Healthcare. Preferably, use

the filters that come with three small, asymmetrically located indentations around the edge of the circle. If not already present, these can be cut with a scissors. Write the number at the top of one side of the filter, using ball point pen, before putting the filter on the plate.

(b) After pouring, the TSA plates to be used as bottom agar are allowed to solidify in a monolayer for 3 to 4 h. (The pouring should be done early in the day, so the plating can be done during the middle of the next day). Next the plates are dried in a monolayer at 37 °C for 24 h, and then used for plating within a few hours. Number the plates, putting the numbers near the edge of the plate. The position of the number defines the top edge of the plate.

(c) Use 2.5 to 3.0 ml of 15.4 g/L TBAB top agar for each plate. Estimate to obtain about 1000 plaques per plate, which enables testing a maximum number of plaques without crowding them together too much. For growth of plaques, incubate the plates overnight at 37 °C. Do not incubate for more than 24 h. After incubation at 37 °C, keep plates at room temperature for 8–24 h. Then place in a 4 °C refrigerator for at least overnight before making lifts (*see* **Note 7**).

(d) Take plates from the refrigerator one at a time, when needed. Holding filter with a stainless steel forceps, set it gently down onto the plate, with its number facing upward at the same position as the same number on the plate. Set the filter down centered within the plate, without any lateral movement once the filter is touching the agar. It's easiest to grasp the filter by the edge away from the number, set the numbered edge down first at its correct position, then let the rest of the filter settle onto the plate, holding the edge opposite the number until it has settled last. Mark the plate with India Ink at the positions of the three indentations in the filter. Leave the filter in place until it is evenly moist, but no longer. Use forceps to lift the filter by one edge. Lift slowly but steadily, avoiding any lateral movement of the part of the filter still in contact with the agar. Place the filter with its plaque side up (ink side down) on a tube rack to dry for >60 min.

2. *Denaturation, neutralization, and cross-linking*

(a) Prepare three piles of 3 MM paper (Whatman) as follows: three sheets per pile, $10'' \times 7''$ on large sheet of aluminum foil. Saturate each pile with 70 ml of the indicated solution:

1. 0.5 M NaOH, 1.5 M NaCl.
2. 1.0 M Tris, pH 7.4.
3. 0.5 M Tris, pH 7.4.

Smooth the liquid out of the pile by gently rolling a 10 ml pipette across it. Use paper towel to collect excess at one end of pile. No puddles should remain, but it should still be as wet as it can be, without having puddles.

(b) Lay six filters, plaque side up, onto pile 1, using forceps. No lateral movement should be permitted once filter is in contact with the pile. Do not overlap the filters. Let stand for 5 min.

(c) Transfer filters to pile 2, as in paragraph b, allowing no lateral movement while filter is in contact with either pile. Let stand for 5 min.

(d) Transfer filters to pile 3, as in paragraph c, allowing no lateral movement while filter is in contact with either pile. Let stand for 5 min. Transfer filters to moistened (solution 3) paper towels in a large tray, for transport to the UV cross-linker. Again, permit no lateral movement of the filters with respect to the pile or the paper towels.

(e) The above piles can be reused for multiple sets of plaques. Add 10 ml of appropriate solution, and smooth out again with pipette before each reuse.

(f) The denatured DNA is then bound to the filter by UV cross-linking. We use the UV Stratalinker 2400, from Stratagene. The damp filters are placed on damp paper towels on the floor of the chamber, with DNA side up, and exposed to UV irradiation for about 1 min.

(g) The damp filters are wrapped in Saran Wrap and stored at 4 °C until used for hybridization.

3. *Selection of oligonucleotide probes.* In principle, a 15 base oligonucleotide with 40–60% GC content should hybridize to its exact complement, but not to a sequence with a centrally located mismatch [8]. As discussed above, we have used a 3-base mismatch to maximize the distinction between mutant and wild-type sequences. A typical mutant probe would include six nucleotides on each side of the mutant codon, and the corresponding wild-type probe would be the same except for the wild-type codon (*see* **Note 8**).
4. *Labeling of oligonucleotides.* Many techniques are available for labeling the oligonucleotides, to enable identification of the plaques to which they hybridize. I will summarize the three techniques that we have used successfully, but will not provide detailed protocols, because each of these has a problem that makes its use less than optimal.

(a) P^{32} labeling. This is the classical technique. P^{32}-labeled phosphate is added to the 5′ end of the oligonucleotide by action of the enzyme polynucleotide kinase. After hybridizing to the plaque-lift filters, the filters are placed against a photographic film, and the P^{32} emissions produce dark spots on the film at positions corresponding to the positions of labeled plaques. This procedure works very well, but we prefer not to use it because of the hazards of working with P^{32}, and the consequent need to take elaborate precautions to avoid exposure.

(b) ECL 3′ oligolabeling and detection. This is based on a kit originally provided by Amersham (now part of GE Healthcare). Fluorescein-11-dUTP is added to the 3′ end of the oligonucleotide by the enzyme terminal transferase. After hybridization, the filters are treated with an anti-fluorescein horseradish peroxidase conjugate, binding the peroxidase to plaques that have hybridized the oligonucleotide. The presence of the peroxidase at that spot is revealed by a chemiluminescent reaction catalyzed by the peroxidase, emitting light that makes a dark spot on the corresponding position on photographic film. This procedure also works very well, but Amersham no longer sells the complete kits, and, although the individual components can be purchased from them and/or several other companies, we are not aware of a consistently reliable source for all of the components.

(c) AlkPhos direct labeling and detection. Also provided by the Amersham division of GE Healthcare. The enzyme alkaline phosphatase is covalently linked to the oligonucleotide, which does not interfere with its hybridization capabilities. After hybridization, the phosphatase catalyzes a chemiluminescent reaction, emitting light that makes a dark spot on the corresponding position on photographic film. This system has worked well with some of our probes, but with others it produced enough nonspecific background to prevent accurate reading.

5. *Hybridization*

(a) Hybridization tubes. We use a Robbins Scientific Model 400 Hybridization Incubator, with the 12 inch tubes provided by Robbins for that incubator. Using stainless steel forceps, place the filters flatly against the wall of the tubes, with the DNA side toward the inside of the tube (i.e., ink side outward). Place the filters so as to minimize overlap, no more than four filters per tube. The filters at the top and bottom of the tube should be on opposite sides of the tube, and the tube should be arranged in the

rotator so each filter is on the bottom when its end is lowest and therefore has most of the buffer.

(b) Prehybridization**.** Once the filters are in place, add 26.4 ml of Hybridization Buffer A (*see* **Note 9**). Roll the buffer slowly up and down in the tube, so it passes over all surfaces. Rotate slowly (4 RPM) in the hybridization incubator for 60 min at 27 °C. This coats the filters with the protective polymers, to prevent nonspecific binding of the oligonucleotide probe.

(c) Hybridization. Add 2 μg of labeled oligonucleotide to the hybridization buffer in the tube. (For oligos with more than 15 nucleotides, increase the 2 μg proportionately to the increase in length of oligo, not counting the labeled compound that has been added.) To make this addition: Remove 1 ml of the hybridization buffer from the hybridization tube, and place it into a microfuge tube. Add the labeled oligonucleotide to that and mix. Then pipet the entire 1 ml back into the hybridization tube, remaining mindful to pipette it into the buffer and not directly onto a filter (This is to avoid artifacts due to the concentrated oligo being placed directly onto a filter). Mix well and then roll the buffer slowly up and down in the tube, so it passes over all surfaces. With tubes in the same arrangement as above, rotate slowly overnight at 27 °C in the hybridization incubator (*see* **Note 10**).

(d) Low stringency wash. Discard the hybridization solution. Place 20 ml of 5× SSC, 0.1% SDS in each tube, seal cover tightly, and roll the liquid around in the tube enough to thoroughly distribute it. Pour out the liquid. Remove the filters into a glass tray. Cover with (106 × N) ml of 5× SSC, 0.1% SDS, where *N* is the number of filters. Agitate gently for about 5 min, turning filters upside down with forceps several times. Pour off liquid, and repeat wash with an equal volume of 5× SSC, 0.1% SDS, for another 5 min. Pour off liquid (*see* **Notes 11** and **12**).

4 Notes

1. Tris solutions are prepared according to the tables provided by Sigma-Aldrich, for pH 7.4 at 25 °C.
2. We have found that VY supports maximum growth rates of *B. subtilis*, and maximum burst sizes from SPO1 infection, both of which also require vigorous shaking (e.g., 200 rpm).
3. We estimate cell density by measurements of turbidity, using a side-arm flask (Klett flask), and a Klett-Summerson colorimeter. A cell density of 5×10^7/ml results in a reading of about

25 on our colorimeter, which corresponds to an optical density at 500 nm of about 0.45.

4. The MOI is calculated using the Poisson probability function. A colony count after infection that is between 30 and 45% of that before infection implies that the MOI was between 0.8 and 1.2.
5. Flask 2 adheres more closely to the original Sayre and Geiduschek protocol [7]. Sometimes it has been more convenient to use the flask 1 lysate, and we have not noticed any decrease in the frequency of mutant plaques obtained from that lysate.
6. Clean lifts require plates that are neither too wet nor too dry. The procedure described has worked well consistently, although rigid adherence to all details is not required.
7. Up to 3 days in the refrigerator also works well. Storage for any longer before using should be avoided.
8. With lower GC regions, we have extended the length of the oligos to as much as 20. We found that oligos with Tm values as low as 35 and as high as 45 provided effective discrimination, where Tm was estimated using the 4(G + C) + 2(T) + 1(A) formula [8]. The value of 1 for each A residue in the oligo is because of the fact that SPO1 DNA contains hydroxymethyluracil (hmUra) in place of thymine. The A:hmUra pair is not as stable as the A:T pair.
9. The procedures described here were used for hybridizations with the ECL- or AlkPhos-labeled oligos. By historical accident, hybridizations with P^{32}-labeled oligos used a higher concentration hybridization buffer (Hybridization Buffer B) and a higher temperature. However, we have no reason to doubt that the procedures described would work equally well for P^{32}-labeled oligos.
10. If the ambient temperature is too high, the incubator may not be able to maintain the temperature at exactly 27 °C, but this doesn't matter. We have obtained satisfactory results with temperatures substantially higher than 27 °C.
11. General protocols call for a high-stringency wash (e.g., 2 min in 300 ml 5× SSC at 36 °C), but we have not found that to be necessary. For every gene tested, we were able to design an oligonucleotide probe that would hybridize well to the mutant sequence at 27 °C, but much less well to the corresponding wild-type sequence.
12. Since many of our targeted genes were in the terminal redundancy, the initial recombinant was usually a heterozygote, which had to go through another growth cycle to segregate homozygous mutants, which were identified by the same hybridization procedure as above [6].

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Chapter 6

Genetic Manipulation of Lytic Bacteriophages with BRED: Bacteriophage Recombineering of Electroporated DNA

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Abstract

We describe a recombineering-based method for the genetic manipulation of lytically replicating bacteriophages, focusing on mycobacteriophages. The approach utilizes recombineering-proficient strains of *Mycobacterium smegmatis* and employs a cotransformation strategy with purified phage genomic DNA and a mutagenic substrate, which selects for only those cells that are competent to take up DNA. The cotransformation method, combined with the high rates of recombination obtained in *M. smegmatis* recombineering strains, allows for the efficient and rapid generation of bacteriophage mutants.

Key words BRED, Recombineering, Electroporation, Mycobacteria, Mycobacteriophage

1 Introduction

Bacteriophages likely represent the largest reservoir of sequence diversity on earth, and it is clear that we have only scratched the surface in our attempts to understand these organisms and how they interact with, and influence, their bacterial hosts [1]. Critical to these endeavors is the ability to manipulate bacteriophage genomes and construct mutants. Although several different strategies have been described for genetic manipulation of phage genomes—including engineering of prophages and phage crosses—efficient introduction of precise mutations, including unmarked nonpolar deletions, is not feasible in most microbial systems [2–10]. Here we describe the use of recombineering strains of *Mycobacterium smegmatis* to construct deletions, insertions, and point mutations in mycobacteriophage genomes.

Functional genomic studies of the mycobacteria have been facilitated by the development of recombineering-based techniques [11–14]. Recombineering, meaning genetic engineering with recombination proteins [15], was developed in *Escherichia coli* by utilizing the bacteriophage λ red recombination proteins (Exo and Beta) or the Rac Prophage RecE and RecT proteins. Exo/RecE is a

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5′ → 3′exonuclease [16–19], and Beta/RecT is a single-stranded binding protein (SSB) that promotes homologous recombination by catalyzing strand annealing, strand exchange, or strand invasion [20–24]. Recombineering systems utilize substrates with short regions of homology and efficiently promote homologous recombination between double-stranded DNA (dsDNA) or single-stranded DNA (ssDNA) substrates and homologous targets in the bacterial chromosome, allowing for high frequency generation of targeted gene replacements, deletions, and point mutations in the chromosome, as well as on replicating plasmids [19, 25–30] (Reviewed in [15, 31]). Mycobacteriophage homologs of the Rac prophage recombineering proteins RecE and RecT—gp60 and gp61, respectively, from mycobacteriophage Che9c—have been utilized to develop mycobacterial recombineering strains, which greatly facilitate mutant construction both in *M. smegmatis* and in the slow-growing pathogen, *Mycobacterium tuberculosis* [11–14].

We have adapted the mycobacterial recombineering system to develop a strategy for generating mutations in lytically replicating mycobacteriophages, which we have termed bacteriophage recombineering with electroporated DNA (BRED; [14, 32, 33]). This method utilizes recombineering-proficient strains of *M. smegmatis* expressing Che9c gp60 and gp61 under control of the inducible acetamidase promoter. BRED enables the construction of a number of different types of mutations, including unmarked deletions, point mutations, small insertions, and gene replacements in lytically replicating mycobacteriophages [14, 32, 34–42]. It has also subsequently been adapted to construct mutations in lytic phages infecting other bacterial species, including *E. coli* and *Salmonella enterica* [43, 44].

The BRED approach requires the coelectroporation of two DNA substrates. One of these is simply phage genomic DNA and can be prepared from relatively small quantities of phage particles. The second is a small linear dsDNA substrate containing the mutation to be introduced and can be readily generated either by synthesis or by PCR. Mycobacterial recombineering frequencies are influenced by length of the targeting substrate homology, and when making insertions, deletions, or gene replacements, 100 bp of homology on either end is usually required. For deletions, the substrate should contain the regions of upstream and downstream homology, immediately flanking the region to be deleted. For small insertions and gene replacements, the substrate should contain the sequence to be inserted, flanked by approximately 100 bp of sequence homologous to either end of the region to be targeted. Mutants containing one or more point mutations can be generated using two synthetic complementary oligonucleotides (oligos), typically 70 nucleotides (nt), which contain the mutation to be introduced (centrally located). Following coelectroporation and a short recovery, transfected cells are plated with additional bacterial cells

(plating cells) and soft agar. Plaques formed from the infectious centers can then be screened by PCR to identify those containing the mutant allele. Because recombination appears to use replicating substrates, all plaques contain the wild-type allele, but mutant and wild-type progeny can be easily separated by plaque isolation and PCR. The efficiency of recombination is sufficiently high that, provided the mutation does not interfere with lytic growth, only 12–18 plaques need to be tested in each of the two rounds of PCR.

2 Materials

Make all solutions using ultrapure double-deionized water (ddH_2O), and store all reagents at room temperature, unless otherwise indicated.

2.1 Generation of Recombineering Substrate and PCR Screening

1. Tris–EDTA (TE).
2. *Pfu* DNA polymerase (or a comparable high fidelity polymerase) and 10× *Pfu* buffer, store at −20 °C.
3. 10 mM dNTP stock, containing 2.5 mM each dNTP (dATP, dTTP, dCTP, and dGTP), store at −20 °C
4. Dimethyl sulfoxide (DMSO).
5. Agarose/gel box/TBE/ethidium bromide (or other DNA stain).
6. 0.1 M stock $CaCl_2$, autoclave to sterilize.
7. Phage buffer: 10 mM Tris-Cl, pH 7.5; 10 mM $MgSO_4$; 68.5 mM NaCl; 1 mM $CaCl_2$ (*see* **Note 1**).

2.2 Preparation of Bacterial Cultures

1. 7H9 liquid media, prepared according to manufacturer instructions and autoclaved; supplemented with 10% (v/v) ADC (albumin dextrose catalase supplement; 2 g/l D-glucose, 5 g/l bovine serum albumin fraction V, 0.85 g/l NaCl) (*see* **Note 2**).
2. Antibiotic stock solutions: carbenicillin (CB; 50 mg/ml), cycloheximide (CHX; 10 mg/ml), and kanamycin (Kan; 50 mg/ml), sterilize by filtration and store at 4 °C.
3. 20% Tween 80, sterilize by filtration and store at 4 °C.
4. 0.1 M stock $CaCl_2$, autoclave to sterilize
5. Glass tubes, sterilized by autoclaving.
6. Baffled and nonbaffled flasks, sterilized by autoclaving.

2.3 Electrocompetent Cell Preparation

1. The recombineering plasmid pJV53 [11] (or another recombineering proficient plasmid).
2. *M. smegmatis* mc^2155.

3. 10% glycerol, filter-sterilized and chilled on ice before use.
4. 20% succinate, sterilize by filtration and store at 4 °C.
5. 20% acetamide, sterilize by filtration and store at 4 °C.
6. Glass tubes, sterilized by autoclaving.
7. Sterile microcentrifuge tubes.

2.4 Transformation and Mutant Recovery

1. Recombineering substrate and purified DNA from the phage to be modified.
2. Microcentrifuge tubes and electroporation cuvettes.
3. Electroporator.
4. 7H9 liquid media.
5. 0.1 M stock $CaCl_2$, autoclave to sterilize.
6. Sterile Pasteur pipettes.
7. Mycobacterial top agar (MBTA): 7H9 with 0.7% Bacto agar, autoclaved to sterilize.
8. 7H10 plates, containing 10% (v/v) ADC, CB (50 μg/ml), CHX (10 μg/ml), and 1 mM $CaCl_2$. Prepare media according to manufacturer instructions, autoclave, and supplement with ADC, CB, CHX, and $CaCl_2$.

3 Methods

3.1 Design Oligonucleotides (Oligos) to Construct the Recombineering Substrate (see Note 3)

1. To construct a deletion, design an approximately 100 nt oligo with 50 nt of homology upstream and downstream of the region to be deleted, making sure the deletion will be in-frame if necessary. For small insertions, design an oligo with the region to be inserted centrally located and ~40–45 nt of flanking homology on either side (*see* **Note 4**).
2. Order two 75 nt "Extender Primers" for making the dsDNA substrate. Design Extender 1 such that it matches the first 25 nt of the 100 nt oligo and has 50 nt additional homology upstream of the deletion/insertion on the 5′ end of the primer. Design Extender 2 such that it matches the last 25 nt of the 100 nt oligo and has 50 nt additional homology downstream of the deletion/insertion on the 3′ end of the primer. Then order the reverse complement of this sequence.
3. For gene replacements, order two sets of 75 nt primers: design the first set such that it will PCR amplify the replacement cassette and add 50 bp of homology flanking the target gene on each end. The second set should add an additional 50 bp of homology flanking the target gene to the substrate generated by the first set of primers, such that the final product has 100 bp of homology flanking the target gene on either end.

4. To incorporate point mutations, order two ~70 nt oligos. The first should be homologous to the genomic region to be altered and contain the desired point mutation in its center. The second is the reverse complement of the first.

3.2 Design Primers for Mutant Screening

1. Primers should be approximately 25–30 nt, with a melting temperature $\geq$ 60 °C.
2. Flanking primers should anneal upstream and downstream of deletion, insertion, or replacement in the phage genome and should generate products, such that the mutant product can be easily distinguished from the wild type (i.e., that are not too similar in size) and neither product is too small (i.e., > 300 bp).
3. You may also order more selective primers that anneal either to a specific tag sequence (inserted by the substrate), or across the new junction created by the mutation, termed deletion amplification detection assay (DADA)-PCR [32]. Point mutations can be engineered to insert a unique restriction site or can be found using mismatch amplification mutation assay (MAMA)-PCR [45].
4. Resuspend flanking primers to 100 μM in TE and dilute in nuclease-free dH_2O to make 10 μM stock.

3.3 PCR to Generate Recombineering Substrate

1. Resuspend deletion/insertion oligo to 1 μg/μl in TE buffer, then dilute in nuclease-free dH_2O to make a 20 ng/μl solution. Resuspend extender primers to 10 μM in TE buffer. Store all at −20 °C.
2. Set up four PCR reactions for each substrate; these should contain 1 μl (20 ng) diluted oligo and the following components: 69 μl nuclease-free dH_2O, 10 μl 10× *Pfu* Buffer, 10 μl dNTP (10 mM stock), 5 μl DMSO, 2 μl each Extender Primers (10 μM stock), 1 μl *Pfu* polymerase.
3. Run reactions in a thermocycler with the following parameters:

   96 °C–5 min.

   96 °C–30 s; 55 °C–30 s; 72 °C–45 s—For 5 cycles.

   96 °C–30 s; 60 °C–30 s; 72 °C–45 s—For 25 to 35 cycles.

   72 °C–7 min.

   4°–∞ (*see* **Note 5**).
4. Check the PCR yield by running ~5–10 μl of each reaction on a 1–1.2% agarose gel.
5. Pool reactions and cleanup with the QIAquick PCR Purification Kit (QIAGEN) or MinElute Reaction Clean-up Kit (QIAGEN). Resuspend in 30 μl sterile dH_2O, and store at −20 °C.
6. Quantify cleaned substrate; ideal concentration is ~100 ng/μl or greater, as you want to be able to add a significant amount of

the substrate (~200–400 ng) to the transformation without adding too much volume to the reaction.

7. To construct a gene replacement, resuspend all primers to 10 μM in TE. Perform the first round of PCR as described above, using 20 ng of DNA containing the replacement cassette as a template per reaction. Check, pool, and clean-up the PCR amplification as described above, although it may be necessary to gel purify if there are nonspecific products. Quantify the purified product, and perform a second round of PCR with the second set of Extender Primers, using the product of the first reaction (~50–100 ng) as a template.
8. When creating a point mutation, resuspend each oligo to 1 μg/μl and dilute to 200 ng/μl in dH_2O; transform 1 μl of each oligo. It is not necessary to anneal prior to transformation.

3.4 Preparation of Recombineering Strain Stocks

1. Transform pJV53 DNA into electrocompetent *M. smegmatis* mc^2155 cells by electroporation (*see* **Note 6**). Electroporate at 2.5 kV, 1000 Ω, and 25 μF, and select for kanamycin-resistant transformants.
2. Grow a small culture of the recombineering strain, mc^2155:pJV53 [11], in 7H9, 10% ADC, 0.05% Tween 80, and KAN (20 μg/ml). Freeze aliquots in 20% glycerol, and store at −80 °C for future use.

3.5 Preparation of Electrocompetent Cells of the Recombineering Strain mc^2155:pJV53 (see Note 7)

1. Grow a 3 ml culture of the recombineering strain, mc^2155:pJV53 [11], in 7H9, 10% ADC, KAN (20 μg/ml), and 0.05% Tween 80 to saturation; incubate at 37 °C shaking at 250 RPM (approximately 2 days).
2. Inoculate 100 ml 7H9 induction medium (7H9, 0.05% Tween 80, 0.2% succinate, KAN (20 μg/ml), and 1 mM $CaCl_2$) to an OD_{600} ~0.02 and incubate shaking at 250 rpm overnight at 37 °C; do not add ADC, and use a nonbaffled flask (*see* **Note 8**).
3. When culture reaches OD_{600} of ~0.4, add acetamide to a final concentration of 0.2% and incubate shaking at 37 °C for 3 h.
4. Divide culture into four sterile tubes (25 ml each) and place cells on ice for 30 min to 2 h.
5. Centrifuge and pellet cells at 5000 RPM for 10 min at 4 °C.
6. Wash cells with 1/2 vol. (~12.5 ml per tube, 50 ml total) 10% sterile ice-cold glycerol (*see* **Note 9**).
7. Pellet cells as before; wash with 1/2 vol. (~12.5 ml per tube, 50 ml total) 10% sterile ice-cold glycerol. Combine cells into two tubes (~25 ml each).
8. Pellet cells as before; wash with 1/4 vol. (~12.5 ml per tube, ~25 ml total) 10% sterile ice-cold glycerol.

9. Pellet cells as before; resuspend in ~1/20 vol (4–5 ml total) 10% sterile ice-cold glycerol (*see* **Note 10**).
10. Aliquot 100 µl cells into chilled 1.5 ml microcentrifuge tubes. Freeze on dry ice and store at −80 °C; thaw on ice when ready to use.
11. Test competency of the cells with control plasmid and phage DNA (*see* **Notes 11** and **12**).

3.6 Transformation

1. Grow a culture of *M. smegmatis* mc^2155 to early stationary phase in 7H9, 10% ADC, CB (50 µg/ml), CHX (10 µg/ml), and 1 mM $CaCl_2$ (no Tween), shaking at 37 °C in a baffled flask.
2. Thaw tubes of electrocompetent cells on wet ice, ~100 µl cells per transformation, for ~10 min.
3. While (or before) cells are thawing, add 900 µl 7H9/10% ADC/1 mM $CaCl_2$ to sterile test tubes (one tube per transformation) for recovery and label.
4. Pipet DNA into thawed cells and mix gently; add 50–150 ng of phage DNA and 100–400 ng of 200 bp recombineering substrate, but no more than ~5 µl total (*see* **Note 13**).
5. Incubate on ice for ~10 min, then pipet cells and DNA into chilled cuvette.
6. Wipe off cuvette, and electroporate at 2.5 kV, 1000 Ω, and 25 µF.
7. Using a sterile Pasteur pipette, carefully remove some of the 900 µl of media from one of the test tubes and add this to the cells in the cuvette; then pipet all of this back into the test tube.
8. Incubate at 37 °C for 30 min to 2 h shaking at 250 rpm; do not recover longer, as cells will lyse.
9. Label one 7H10 plate for each transformation and prepare the plating mix: (1) Carefully melt MBTA. (2) Mix 1.5 ml 7H9, 50 µl 0.1 M $CaCl_2$ (final conc. 1 mM), and 2.5 ml molten MBTA per plate (in appropriately sized sterile tube or flask) and cool for a few seconds. (3) Add ~250–300 µl fresh *M. smegmatis* culture (no Tween) per plate (*see* **Note 14**).
10. Plate transformations as top agar lawns: (1) Using 5 ml pipette, transfer 4 ml plating mix to the tube containing your electroporation recovery. (2) Carefully pour a thin layer onto the surface of a 7H10 plate, and swirl gently to distribute evenly.
11. Incubate plates overnight (~24–36 h) at 37 °C.

3.7 Screen Plaques (See Note 15)

1. Grow a culture of *M. smegmatis* mc^2155 to early stationary phase in 7H9, 10% ADC, CB (50 µg/ml), CHX (10 µg/ml), and 1 mM $CaCl_2$ (no Tween), shaking at 37 °C in a baffled flask.

2. Pick 18–25 individual plaques into 100 µl phage buffer. Incubate at room temperature for 1–2 h or at 4 °C overnight; store plaques at 4 °C.
3. Aliquot 1 µl of each plaque–phage buffer mixture to use as a template for PCR with the flanking primers, making sure to set up a control reaction with WT phage DNA (5–10 ng) (*see* **Note 16**).
4. Make a master mix containing the following components (per reaction), 12.5 µl dH_2O, 2 µl 10× *Pfu* Buffer, 2 µl dNTP stock (10 mM), 1 µl DMSO, 0.5 µl each extender primer (10 µM stock), 0.5 µl *Pfu* polymerase. Aliquot 19 µl master mix per tube:
5. Run PCR reactions in Thermocycler with the following parameters:

   96 °C–5 min.

   96 °C–1 min; X °C–1 min; 72 °C–1.5 min per kb of product—30 to 35 cycles.

   72 °C–7 min.

   4 °C–∞ (*see* **Note 17**).
6. Run PCR reactions on an agarose gel. Positive "mixed" plaques will have a faint mutant band of the expected size in addition to the wild-type band.
7. If mixed plaques containing the deletion are identified, plate to plaque-purify the mutant, as follows. Serially dilute plaque (to 10^{-3}, 10^{-4}, and, 10^{-5}) in phage buffer, and infect ~300 µl fresh *M. smegmatis* (no Tween) with 10 µl of each dilution in sterile tubes. Adsorb at room temperature for 30 min and make top agar overlays: (1) In sterile vessel combine 2.5 ml molten MBTA +2.5 ml 7H9 + 50 µl $CaCl_2$ per plate. (2) Add ~5 ml to each tube and plate on 7H10 plates; incubate plates for 24–36 h at 37 °C.
8. Pick at least 18 individual "secondary" plaques into 100 µl phage buffer each, and make a lysate from the plate containing ~1000 plaques (typically the 10^{-3} dilution plate) (*see* **Note 18**).
9. Perform flanking primer PCR using 1 µl of each purified plaque and 1 µl of lysate as template. If the mutant is viable, a number of secondary plaques will contain only the mutant product, indicating that this plaque arose from a pure mutant. If the mutant band is not present in the lysate PCR, the mutant is likely not viable (*see* **Note 15**). However, if the mutant product is present in the lysate, but all of the secondary plaques are WT, screen additional secondary plaques (*see* **Note 19**).
10. Once a pure mutant plaque is identified, replate and make a plate lysate. This lysate should be filter-sterilized and confirmed to contain only mutant phage by PCR.

4 Notes

1. Add $CaCl_2$ from sterile 0.1 M stock solution after autoclaving.
2. ADC is prepared as a 10× stock solution; combine 924 ml deionized water with 8.5 g NaCl and 20 g glucose and stir with a magnetic bar. Add 50 g bovine serum albumin (BSA) and stir until completely dissolved. The pH should be 6.9–7. Filter-sterilize through a 0.22 μm pore membrane and store at 4 °C.
3. As an alternative to PCR synthesis, sequence-verified, synthetic dsDNA fragments known as gBlocks, can be purchased from Integrated DNA Technologies (IDT) and utilized as the recombineering substrate.
4. Oligos longer than 40 bases should be PAGE-purified.
5. The annealing temperature for the first five cycles is listed as 55 °C; however, prior to running PCR, determine the melting temperature of Extender Primers, checking just the 25 bp that match the deletion/insertion oligo. If lower than 57 °C, decrease the annealing temperature to 2 °C lower than the melting temperature of the oligo with the lowest melting temperature. Also check the temperature for extension cycles according to enzyme manufacturer instructions.
6. To prepare electrocompetent cells of *M. smegmatis* mc^2155, follow the protocol outlined in Subheading 3.5 (Preparation of electrocompetent cells of the recombineering strain mc^2155: pJV53) with the following modifications; (1) omit Kan from all media, (2) in **step 2**, dilute cells in 7H9, 10% ADC, CB (50 μg/ml), CHX (10 μg/ml), and 0.05% Tween 80, (3) omit **step 3**, and (4) prepare cells when culture OD_{600} reaches 0.8–1.0.
7. It is important that competent cells are prepared on ice, centrifuged at 4 °C, and that the 10% glycerol is ice-cold. Any volume of cells can be prepared; 100 ml yields approximately 40 aliquots of competent cells.
8. You may wish to inoculate one or two different initial ODs to ensure cultures do not overgrow.
9. During washing, pipet up and down until the clumps of cells are dispersed.
10. The cells should not be thick or yellowish, but they should not be too dilute either. If unsure, error on the side of "too thick" as they can be diluted later. If the time-constants from the transformation are consistently low (<18 ms), this is an indication that the cells are too thick (*see* **Note 12**). If the cells are too thin, the time constants will be good (19–22 ms), but the number of plaques obtained will be low (<100 plaques from 150 ng phage DNA).

11. This protocol routinely generates cells that yield 10^6 transformants/μg extrachromosomally replicating plasmid DNA. It is also important to check the competency of the cells using the DNA of the phage to be mutated. Ideally, 100–300 plaques should be obtained from a transformation with 50–150 ng phage DNA alone. The amount of phage DNA can be increased to generate more plaques if necessary, but this should be done without adding large volumes of DNA to the transformation.
12. The time constant is important, particularly when transforming large DNAs, such as phage genomic DNA. This should be >18 ms; 19–21 ms is best. Time constants <18 ms will result in very few to no plaques. Inadequate washing of the competent cells or the presence of salt in either the phage DNA or the substrate will adversely affect time constants, which is why all DNA used in transformations should be resuspended in sterile dH_2O. Ideal time constants range from 19–22 ms but will decrease as larger volumes of DNA are added to the reaction, likely due the presence of residual salts. If addition of substrate is lowering time constants below 18 ms, there are a few troubleshooting options: (1) add less substrate (deletions can be made with as little as 100 ng of substrate DNA; although it is somewhat less efficient); (2) dilute the competent cells with ice-cold water (low time constants may be a result of cells that are too concentrated); (3) remake the substrate so that it is more concentrated.
13. BRED employs a cotransformation strategy that uses phage DNA to select against nontransformable cells, and transformations are plated prior to host cell lysis. This ensures only cells competent to take up DNA will give rise to infectious centers (termed "primary plaques"). Depending on the method of screening, ~5–50% of the primary plaques will contain a mixture of mutant and wild-type DNA.
14. Plating mix amounts given are for one plate; this should be scaled up for multiple recoveries.
15. Mutant phage are isolated by replating one of the primary "mixed" plaques containing a high proportion of the mutant allele, and screening the "secondary" plaques by PCR. If the mutant is viable, a proportion of these will contain a pure population of mutant. It is also helpful to make a lysate from a plate containing approximately 1000–5000 secondary plaques. If the mutation is in an essential gene, the mutant product will no longer be present when this lysate is analyzed by flanking primer PCR. Mutants in essential genes can often be isolated by complementation using a strain of *M. smegmatis* expressing a wild-type copy of the mutated gene [32].
16. Mutant products that are larger than the WT product (such as insertions) may be difficult to detect with flanking primers, and

their identification may require the use of a primer specific to the mutant allele.

17. Anneal at 2° lower than the melting temperature of the primer with the lowest melting temperature.
18. Lysates are made by flooding plate with 3–5 ml phage buffer, allowing this to stand for at least 1 h, then collecting the buffer in a sterile tube.
19. For difficult to isolate mutants, it may be helpful to pick pools of 5–10 secondary plaques (in at least 250 μl of buffer). When a pool containing the mutant is found, it can be replated, and again, individual plaques should be screened by PCR.

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Chapter 7

Isolation of Competitive Phage Display-Modified Bacteriophage T4 with Affinity Chromatography

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Abstract

Phage recovery from various solutions, including physiological samples, as well as phage purification from crude lysates often requires a specific isolation method. Here, we demonstrate that T4-like phages can be efficiently isolated by affinity chromatography. This approach employs specific affinity tags (GST (glutathione S-transferase) or His-tag) that allow for the isolation of the phage. These affinity tags are exposed on the phage head using phage display. By combining competitive phage display and affinity chromatography, wild-type phages can be specifically recovered from mixtures with other phage/s, from solutions of very low phage concentration, or purified from crude phage lysates.

Key words Phage purification, Affinity chromatography, Competitive phage display, T4 bacteriophage, Hoc protein, *Escherichia coli*

1 Introduction

Phage recovery from various solutions, including physiological samples, as well as phage purification from crude lysates always require a specific isolation technique. Traditional methods for phage isolation use gradient centrifugation [1–3], whereas new approaches generally make use of chromatography, including size-exclusion chromatography [4], chromatofocusing [5], or monolithic anion-exchange chromatography [6–8]. Chromatography offers safe and straightforward procedures which can be easily scaled for industrial use but require individual optimization. They allow for removal of the bacterial medium along with bacterial proteins, DNA, lipopolysaccharide, peptidoglycan, etc.

Here, we demonstrate that T4-like phages can be purified (or isolated from a variety of solutions) by affinity chromatography (Fig. 1). This approach employs the affinity tags GST (glutathione S-transferase) or His-tag, enabling specific isolation of the phage. These affinity tags are exposed on the phage head by phage display. Importantly, the phage does not require any genetic modifications

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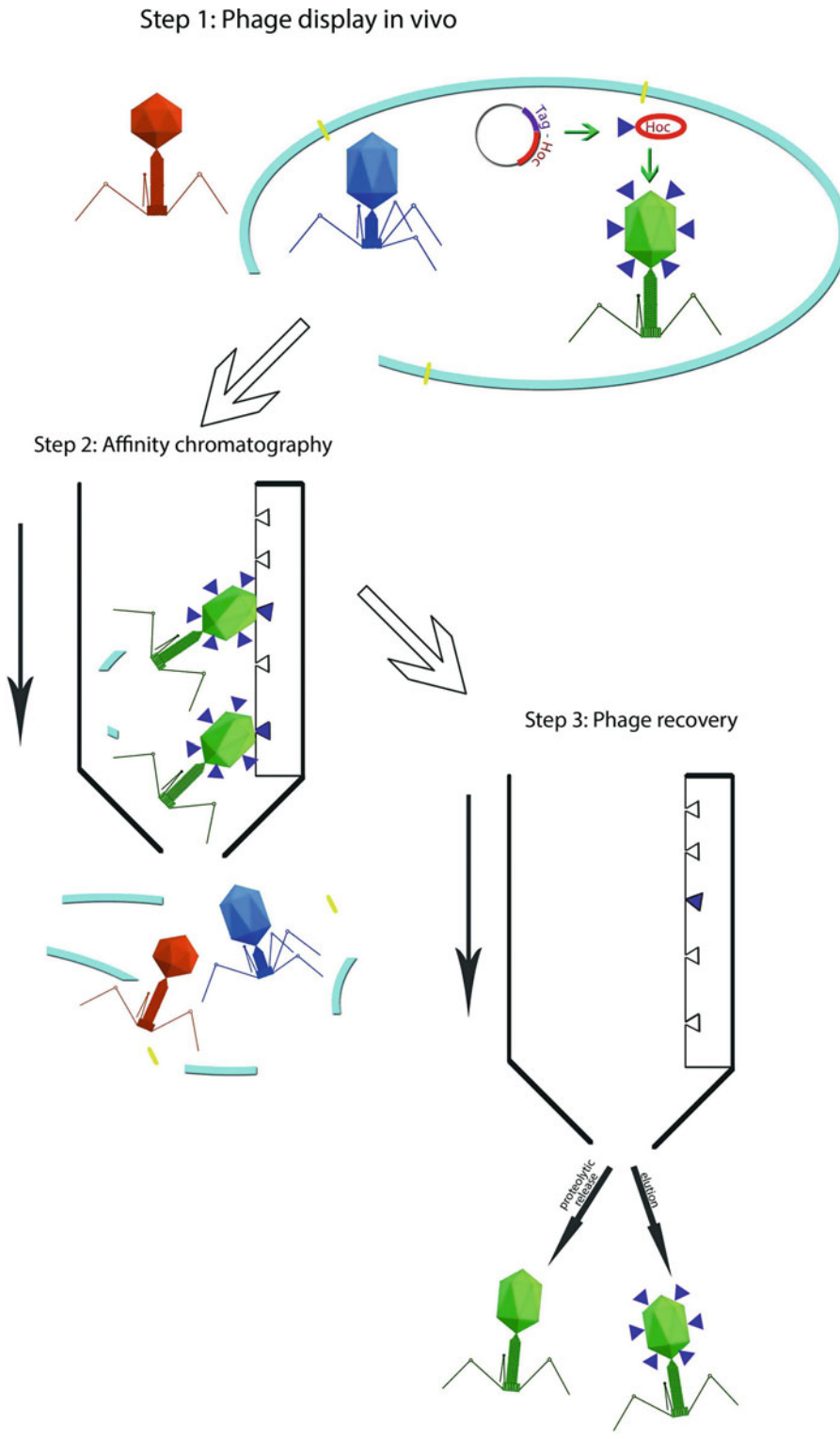

Fig. 1 Schematic diagram of the steps involved in affinity purification of phage [9]

and entire procedure can be done with a wild phage strain by *competitive phage display* [9]. In competitive phage display, users clone a structural phage gene (here: gpHoc) into an expression plasmid with the appropriate affinity tag for expression in the bacterial production strain. After infection of this strain by T4-like phages, we were able to recover a modified phage (a) from 1:1 mixtures with another phage [9], (b) from a solution with very low phage concentration (10 pfu/ml); and to purify phage lysates, typically to 5–100 endotoxin activity units per milliliter.

2 Materials

2.1 Phage Display Components

1. Expression vector containing *hoc* gene of T4 phage fused 5′-terminally to GST- or His-tag-coding sequence (*see* **Note 1**).
2. Selection antibiotic suitable for the expression vector used in the procedure (according to vector manufacturer's information).
3. Inducer of protein expression suitable for the expression vector used in the procedure (according to vector manufacturer's information, e.g., IPTG 0.5 mM).
4. Host bacteria: *Escherichia coli* expression strain sensitive to T4 phage (e.g., *E. coli* expression strain B834, Novagen; EMD Millipore Corporation; http://www.emdmillipore.com/) (*see* **Note 2**).
5. T4 bacteriophage in a liquid culture (*see* **Note 3**).
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: (1) selection antibiotic adequate for the expression vector used in the procedure, (2) agar 15 g/l for solid media in petri dishes.
7. Polyacrylamide gel electrophoresis of proteins: standard materials and reagents [10].
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.

2.2 Components for Affinity Chromatography

1. Standard affinity chromatography resins adequate to the affinity tag that is fused to the recombinant Hoc product: glutathione Sepharose for GST affinity tag, Ni-NTA agarose for 6-Histidine affinity tag.
2. Sodium phosphate buffer: 50 mM Na_2HPO_4, 300 mM NaCl, pH 7.5.

3. Sodium phosphate buffer with imidazole: 50 mM Na_2HPO_4, 300 mM NaCl, 500 mM, pH 7.5.
4. Glutathione buffer: 20 mM glutathione, 100 mM Tris, 200 mM NaCl, 0.1% Tween 20, pH 8.0.
5. Imidazole elution buffer: 500 mM imidazole, 50 mM Na_2HPO_4, 300 mM NaCl, 0.1% Tween 20, pH 7.5.
6. Endotoxin detection kit, e.g., EndoLISA (Hyglos GmbH; Bernried am Starnberger See, Germany; http://www.hyglos.de/en/products-services/products/endotoxin-detection/endolisar.html).

3 Methods

3.1 Competitive Phage Display In Vivo

1. Transform *E. coli* competent cells with an expression vector containing *hoc* gene of T4 phage fused 5′-terminally to GST- or His-tag-coding sequence; you may use either the Hanahan method [10], electroporation [10] or other. Plate the cells on LB medium with an appropriate selection antibiotic and culture in 37 °C overnight. This plate cannot be stored and should be used next day.
2. Prepare *initial cultures* for phage display. Inoculate a liquid culture of transformed bacteria in 10 ml LB medium with selection antibiotic from a single colony (*see* **Note 4**). When the culture reaches $OD_{600} = 0.5$ freeze immediately 0.5 ml portions of the culture in sterile glycerol (final concentration: 25%) in −80 °C.
3. Test one portion of the *initial cultures* for expression of recombined Hoc proteins. Culture the cell portion in 100–200 ml of LB medium with selection antibiotic up to OD600 = 0.5, collect a negative control sample of approx. 20 ml and save harvested cells in −20 °C. Induce expression in the remainder of the culture (according to the vector manufacturer's instructions, for example by adding IPTG up to 0.5 mM), and culture for further 3–12 h in 37 °C. Harvest the cells by centrifugation. Evaluate the expression of recombined Hoc proteins by SDS-PAGE [10], comparing control cell to the induced ones. Use this set of portions of transformed cells for the further procedure only when recombined Hoc protein is significantly overexpressed (*see* **Note 5**).
4. Prepare the culture of expression cells for phage display. Use one portion of *initial culture* and add it to 2.4 l of prewarmed to 37 °C LB medium with a selection antibiotic, put into six baffled flasks (400 ml each), and incubate with shaking in 37 °C until OD_{600} is 0.08–0.1 (*see* **Note 6**).

5. Induce recombinant protein expression in five flasks, the sixth serves as a negative control of expression (*see* **Note 7**). Add the expression inducer to final concentration equal to 1/10 of the concentration identified as the effective one (*see* **step 3**) (*see* **Note 8**), incubate with shaking in 37 °C for 1 h.
6. Infect four out of five induced flasks with the T4 phage; the fifth flask serves as a positive expression control (*see* **Note 7**). Add 10^6–10^7 pfu per 400 ml flask and incubate with shaking in 37 °C for 8 h. Clarify the lysate, centrifuge 4651 × *g* for 10 min, filter by sterile 0.22 μm filters. Sterile lysates can be stored in 4 °C for at least 3 months and used in portions (*see* **Note 9**).

3.2 Phage Purification by Affinity Chromatography

1. Mix 50 ml of filtered phage preparations lysates with 10 ml of the affinity resin; when you use (1) GST-Hoc fusion apply glutathione Sepharose, when (2) His-tag-Hoc fusion apply Ni-NTA agarose. Incubate overnight at 4 °C mixing gently.
2. Remove unbound fraction and wash the resin with (1) 5 l of sodium phosphate buffer for glutathione Sepharose, or (2) 5 l of sodium phosphate buffer with imidazole for Ni-NTA agarose (*see* **Note 10**).
3. Elute specifically bound phage particles from (1) glutathione Sepharose with glutathione buffer or (2) Ni-NTA with imidazole elution buffer. Three successive 15 ml elutions can be done (*see* **Note 11**).
4. Evaluate eluted phage preparations according to your needs: titrate by the two-layer method of Adams [11], test endotoxin content, or others.

4 Notes

1. Here we report a method employing competitive phage display on the wt T4 phage capsid by providing recombinant Hoc protein *in trans*. N-terminal fusions to Hoc have been demonstrated as effective in presentation of foreign elements on T4 phage capsid [9, 12–14]. However, other proteins can also be used after optimization the procedure. Furthermore, this method can be adapted for the isolation of other phages, provided an efficient expression system is available and a relevant phage gene can be fused to an affinity tag coding sequence for expression in the host bacterium.
2. Any bacterial strain that is intended for this work should be first tested for its sensitivity to T4 phage infection, for example, by dropping phage preparation dilution on a bacterial lawn plated

on solid LB medium. Phage-induced lysis should be clearly visible by individual plaques.

3. A wild type strain of T4 bacteriophage is appropriate for this procedure, due to *competitive phage display* which occurs inside the phage producing strain [9]. Briefly, phage particles are assembled in bacterial cytoplasm where there are both wild type proteins (expressed from the phage genome) and recombinant proteins (expressed from the expression vector) are present. Both types of proteins will be randomly incorporated to phage capsid. However, in the procedure one may use a defective phage that does not express Hoc proteins due to a mutation or deletion of the *hoc* gene, which allows for more effective incorporation of the recombinant proteins into capsid.
4. After transformation of *E. coli* with the expression vector it is useful to test a few colonies to select a most productive strain.
5. Effective expression of the recombinant protein in the host bacteria is of critical importance for the whole procedure. It is likely that you need to optimize the expression to individual conditions and reagents, including identification of most appropriate concentration of the inducer, temperature, and *E. coli* expression strain. It is very helpful to use baffled Erlenmeyer flasks and vigorous shaking to assure proper aeration, or to use a controlled aeration in a bioreactor.
6. Despite the fact that required OD600 $\leq$ 0.1 is relatively low, one need to make sure that bacteria are already in or are entering the logarithmic growth phase. Therefore duplication of OD_{600} value, for example, from 0.04 to 0.08 should not take longer than 30–40 min.
7. Comparison of recombinant Hoc production by induced and noninduced host bacteria can be visualized by SDS-PAGE [10] (*see* Subheading 3.1, **step 3**).
8. Concentration of the expression inducer (e.g., IPTG) in phage display culture can be reduced when phage burst from the culture is not satisfactory. Typically it ranges between 1/10 and 1/50 of concentration that allows for marked overexpression in the host bacteria.
9. Phage display-modified bacteriophage can be used directly for further purification (e.g., to remove endotoxins and other bacterial products), but it can also be used for other procedures, mixed with other phages and/or compounds and after that recovered by affinity chromatography (*see* Subheading 3.2).
10. This step can be optimized according to the user's needs. To obtain the highest purity of phage, the amount of washing buffer and the duration of this part of the procedure can be increased. If the requirements are less stringent, washing can be done faster using a smaller buffer amount.

11. Elution can also be optimized according to the user's needs. Phages can be eluted with one smaller amount of elution buffer to obtain higher phage titer. However, larger amounts of elution buffer and multiple elutions from the same resin allows for better overall recovery of the phage.

    Elution can be replaced by proteolytic release from the resin [9] if a protease cleavage site has been cloned between the affinity tag and the phage protein. Selected protease cannot destroy phage capsid; T4 phage is resistant for example to protease AcTEV (Life Technologies/Thermo Fisher Scientific), but this may not be the case for other phage particles.

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Chapter 8

Immobilization of Intact Phage and Phage-Derived Proteins for Detection and Biocontrol Purposes

Hany Anany, Luba Y. Brovko, Denis Arutyunov, Nilufar Poshtiban, Amit Singh, Upasana Singh, Michael Brook, Christine Szymanski, Stephane Evoy, and Mansel W. Griffiths

Abstract

The natural specificity of bacteriophages toward their hosts represents great potential for the development of platforms for the capture and detection of bacterial pathogens. Whole phage can carry reporter genes to alter the phenotype of the target pathogen. Phage can also act as staining agents or the progeny of the infection process can be detected. Alternatively, using phage components as probes offer advantages over whole phage particles, including smaller probe size and resilience to desiccation. Phage structures can be engineered for improved affinity, specificity, and binding properties. However, such concepts require the ability to anchor phage and phage-components onto mechanical supports such as beads or flat surfaces. The ability to orient the anchoring is desired in order to optimize binding efficiency. This chapter presents various methods that have been employed for the attachment of phage and phage components onto support structures such as beads, filters, and sensor surfaces.

Key words *Campylobacter jejuni* phage NCTC1267, ColorLok paper, Immobilization, Inkjet printing, Paramagnetic silica beads, Receptor-binding proteins

1 Introduction

The specificity and lytic activity of bacteriophages (phages) have led to consideration of their potential as biocontrol and detection tools for various bacterial pathogens. Immobilization of an intact whole phage particle or one of its components such as tail fibers, receptor-binding proteins (RBPs) and cell wall binding domain of phage endolysin (CBD), will broaden the application of phages and allow development of more phage-based technologies for rapid detection and control of pathogens [1]. Accordingly, phages were immobilized using different approaches [2]. For therapeutic and biocontrol purposes, phages have been immobilized on skim milk or whey protein [3], encapsulated and spun in polymer nanofibers [4],

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encapsulated in alginate and gelatine capsules [5–8], or immobilized on cellulose-based material [9].

On the other hand, phages immobilized on solid substrates have been used as capturing agents to detect target bacteria [1, 10, 11]. The binding between phage and its target bacterial cell can be monitored in real time using various sensors such as surface plasmon resonance (SPR) [12, 13] or magneto elastic biosensors [14–18]. Interestingly, RBP biosensors were proposed to simplify biosensor construction [19, 20]. Isolated RBPs have the ability to specifically bind to a certain domain on the bacterial cell wall and, as a result, can be used as a capturing and identification agent in the same way as antibodies [21].

Moreover, immobilized intact phage particles can be used not only to specifically bind but also to infect the target bacterium and cause its lysis and release of progeny phages. The progeny phages, which can be rapidly detected using any molecular based technique such as qPCR or isothermal DNA amplification techniques, can be used as an indication of the presence of the target bacterium [1]. In this context, the major advantage of using immobilized whole phage particles is the ability to enhance the sensitivity of the technique by removing the originally added immobilized phages enabling the detection of only the progeny, which are present in greater numbers than the target bacterium. Assuming that the same number of progeny phages is released from each infected bacterial cell, this assay is then semiquantitative. One of the major problems to this approach is ensuring the correct orientation of the immobilization of phages on the solid substrate, which should involve their heads being attached to the surface; leaving the tail fibers free and functional to access the phage receptors on the bacterial cell. With this in mind, several approaches for achieving this oriented immobilization have been proposed [2]. For instance, chemical or genetic modification of the capsid proteins to introduce affinity tags was suggested to directly orient phage particles during the immobilization step [20, 22–24]. A more simple and generic approach based on the charge difference and electrostatic interaction between the phage head and solid substrate was recently proposed [25]. It was claimed that the phage has a dipole nature with an overall negative charge on the head proteins and positively charged tail fiber proteins. As a result, phages will be attracted to positively charged surfaces by their heads, leaving the tail fibers accessible to the phage receptors on the bacterial cell.

In this chapter we describe immobilization techniques for use with intact phage particles and receptor-binding proteins (RBPs) on solid surfaces that have been successfully used to capture and detect different target bacteria. However, it should be mentioned that further studies are required to better understand the interaction between the immobilized phages (RBPs) and the supporting material, in particular their distribution and count per unit surface

area. Different tools can be used to have an estimate for the density of the immobilized phage particles such as scanning electron microscopy (SEM), atomic force microscopy (ATM) and evanescent wave light scattering.

2 Immobilization of Intact Whole Phage Particles

2.1 Immobilization of Whole Phage Particles on Paramagnetic Silica Beads

2.1.1 Materials

(a) S-NH2 paramagnetic silica beads (1 μm diameter) (Boca Raton, FL, USA) (PR-MAG00013-01).

(b) 3-aminopropyl triethoxylsilane (APTS).

(c) Tetrahydrofuran (THF).

(d) Centrifuge, sonicator, water bath, ZETA potential measurement instrument.

(e) Magnetic microfuge tube rack.

(f) Boekel Scientific Orbitron Rotator II (Boekel Industries Inc., Feasterville, PA, USA or Fisher Scientific).

2.1.2 Methods

Paramagnetic Silica Beads Modification

(a) Separate magnetic silica beads (100 mg) from the storage solution by centrifugation at 4000 × *g* for 2 min, and wash three times with 5 mL deionized water by multiple centrifugation steps, and then dry at 110 °C under nitrogen for 1 h.

(b) Redisperse the beads in tetrahydrofuran (THF) containing (3-aminopropyl triethoxylsilane (20% w/v, 5 mL) and the suspension sonicate at room temperature for 2 h.

(c) Centrifuge at 4000 × *g* for 2 min and then heat the mixture to 100°C under nitrogen for 1 h.

(d) Wash for five times with THF by centrifugation at 4000 × *g* for 2 min and then dry under vacuum for 10 h at 50 °C to remove residual THF.

(e) Measure the zeta potential of the modified silica beads at 25 °C with a ZETA PALS instrument (Brookhaven Instruments Corp., Holtsville, NY). Calculate the mean zeta potential for each batch of modified silica beads by suspending the beads in 2 mM NaCl (the zeta potential of beads should be around 18–20 mV).

(f) Store the dried APTS modified beads at 4 °C until used for phage immobilization.

Preparation of Phage

(a) Propagate phage with its host as described in Volume 1, Chapter 7.

(b) Purify the phage on a cesium chloride gradient as described in Volume 2 Chapter 13, then titrate and store at 4 °C.

Immobilization Step

(a) Resuspend the dry paramagnetic modified silica beads (PMSB) to a final concentration of 20 mg/mL and wash six times with magnetic microfuge tube rack, Dynal MPC-S (Life Technologies, Burlington, ON) using SM buffer without gelatin, (5.8 g NaCl, 4 g $MgSO_4.7H_2O$, 50 mL 1 M Tris-Cl pH 7.5, adjusted to pH 6.5 with HCl, final volume 1 L).

(b) Add 10 μL of purified phage (diluted to around to 10^6 PFU/mL) to 60 μL of washed PMSB beads and 930 μL of SM buffer to achieve a final concentration of around 10^5 PFU/mL of phage.

(c) Rotate the phage-beads mixture for 24 h at 4 °C using a Boekel Scientific Orbitron Rotator II.

(d) Wash the immobilized phage five times in phage buffer (0.74 g $CaCl_2$, 2.5 g $MgSO_4{\cdot}H_2O$, 0.05 g gelatin, 1 M Tris–HCl, pH 7.0 in a final volume of 1 L of double deionized water) to remove unbound phage particles from the beads using a magnetic microfuge tube rack and finally resuspended in 1 mL of phage buffer.

(e) To determine the infectivity of the beads, concentrate the phage-coated magnetic beads at the bottom of a microfuge tube using the magnetic particle separator, and spot them onto a fresh lawn of the host bacterium. A 30 μL aliquot of free phage (10^5pfu/mL) should also be spotted onto the same lawn of bacteria to gauge the infectivity of the phage-coated magnetic beads. The infectivity of the immobilized phage can be determined using a scale to indicate the extent of lysis, where: 5+ = complete lysis; 4+ ~75% lysis; 3+ ~50–75% lysis; 2+ ~25–50% lysis; 1+ < 25% lysis.

2.2 Inkjet Printing of Whole Phage Particles on Paper

2.2.1 Materials

(a) Triton X-100.

(b) Glycerol.

(c) Dimatix Materials Printer Cartridges (Hewlett-Packard, Mississauga, Canada).

(d) HP-certified multipurpose paper with ColorLok technology (Hewlett-Packard, Mississauga, Canada).

(e) Dimatix Materials Printer DMP 2800 (Fujifilm Dimatix Inc., Santa Clara, CA, USA; http://www.fujifilmusa.com/products/industrial_inkjet_printheads/index.html).

(f) Plastic container with barium chloride to store the printed paper.

2.2.2 Methods

(a) Propagate phage with its host as described in Volume 1, Chapter 7.

(b) Prepare the phage containing bioink by adding Triton X-100 (2 mM) and glycerol (30% v/v) to phage lysate (10^9

PFU/mL) and filter it through a 0.22 μm membrane filter. Prepare immediately before printing.

(c) Load the bioink into the Dimatix Materials Printer Cartridge using the provided syringe.

(d) Put the ColorLok paper in the Dimatix Materials Printer DMP 2800. Set the printer to a firing voltage of 40 V, firing frequency of 5 kHz, drop space of 20 μm, meniscus vacuum of 11.43 cm H_2O (in the water column), and nozzle cleaning cycles initiated every 120 s. Approximately 500 drops/cm will be deposited, each containing approximately 10 pL of bioink. A control bioink should be prepared replacing the bacteriophage with phage buffer in equivalent volumes.

(e) Allow paper to dry for 30 min before transferred to a humidity chamber (80–85% RH) at 25 °C, filled with barium chloride.

(f) The infectivity of the phage-printed paper can be determined by the overlay technique and/or in broth. The former approach is done by cutting the phage-printed paper into circles (around 2.5 cm diameter) and transferring these disks using sterile forceps to the surface of semisolid agar inoculated with the host bacterium. Incubate overnight at 37 °C and then visually examine zones of growth inhibition around the periphery of the paper containing phage. The infectivity of the phage printed on the paper can also be determined by incubating the phage containing paper disk (2.5 cm diameter) with 10 mL of the bacterial suspension in broth medium (around 10^3 CFU/mL) for 18–24 h at 37 °C. Count the bacteria after the incubation period and compare the count with a control tube that does not have the bioactive paper.

2.2.3 Notes

(a) A myovirus (rV5) isolated against *E. coli* O157:H7 [26], was used for the whole phage immobilization experiment on modified paramagnetic silica beads and ColorLok cationic paper. Optimization of the initial phage concentration is required for other phages.

(b) Based on our current results using whole phage immobilization protocols, it is recommended to use the immobilized phages on PMSB for the detection (capturing) of the target bacterium within 2 h after washing off excess phages. Storage of the immobilized phages for more than 24 h will affect the capture efficiency. On the other hand, the bioactive phage-based paper can be stored for 1 week at 4 °C in a container at 80–85% relative humidity.

2.3 Covalent Immobilization of Whole Phage onto Gold Layers

2.3.1 Approach#1: Activated Cysteamine Monolayers [10]

Materials

(a) Gold (Au)-coated Si substrates.
(b) Cysteamine hydrochloride (50 mM solution in deionized water).
(c) Solvents (acetone, isopropyl alcohol, ethanol, deionized water).
(d) Glutaraldehyde (2% solution v/v in deionized water).
(e) Tween-20 (0.05% solution v/v in deionized water).

Methods

(a) Si (100) substrates are sputter-coated with 20 nm thick layer of gold (Au) using 5 nm chrome as adhesion layer.
(b) The Au-coated Si substrates are sonicated sequentially with acetone, isopropyl alcohol, ethanol and finally deionized water for 5 min each, to clean the surface prior to functionalization.
(c) The washed surface is incubated with 50 mM solution of cysteamine hydrochloride at room temperature (25 °C) for 20 h on an orbital shaker to form a thiol-bound self-assembled monolayer (SAM) on the surface of the substrate.
(d) The cysteamine bound gold substrate is twice washed thoroughly with copious amounts of deionized water for 5 min each to remove any excess cysteamine.
(e) The terminal amine (NH_2-) of the surface bound cysteamine is activated by incubation with 2% solution (v/v) of glutaraldehyde for 1 h.
(f) The activated cysteamine-coated substrates are washed twice with deionized water and were immediately used for bacteriophage immobilization.
(g) The bacteriophages (10^{12} pfu/mL) suspended in SM buffer (pH–7.5) are immobilized on to the activated-cysteamine coated substrates by incubation for 20 h at room temperature (25 °C) on an orbital shaker.
(h) The phage-functionalized substrates are finally washed twice with 0.05% Tween-20 (v/v) in SM buffer followed by SM buffer alone to remove any loosely bound/unbound phages.
(i) An improved bacteriophage immobilization density can be achieved by carrying out the phage binding at higher temperature up to 40 °C.

2.3.2 Approach #2: One-Step Approach Using Dithiobis (Succinimidyl Propionate) (DTSP) [12, 27]

Materials

(a) Dithiobis (succinimidyl propionate) (DTSP) solution (2 mg/mL).
(b) Gold (Au)-coated Si substrates.
(c) Solvents (acetone, isopropyl alcohol, ethanol, deionized water).
(d) PBS (pH–7.4).
(e) Ethanolamine (10% v/v).

Methods

(a) Si (100) substrates are sputter-coated with a 20 nm thick layer of Au using 5 nm chrome as the adhesion layer.
(b) The Au-coated Si substrates are sonicated sequentially with acetone, isopropyl alcohol, ethanol, and finally deionized water for 5 min each, to clean the surface prior to functionalization.
(c) The washed surface is incubated with 2 mg mL^{-1} solution of DTSP for 30 m at room temperature (25 °C) on an orbital shaker to form a thiol-bound self-assembled monolayer (SAM) on the surface of the substrate.
(d) The DTSP-functionalized substrate is washed twice in acetone followed by deionized water to remove any excess DTSP and is used immediately for phage immobilization.
(e) The bacteriophages (10^{12} pfu/mL) suspended in SM buffer (pH–7.5) are immobilized on to the DTSP functionalized substrates by incubation for 20 h at room temperature (25 °C) on an orbital shaker.
(f) The phage-immobilized surface is washed extensively with PBS to remove any loosely bound/unbound phage.
(g) The unreacted succinimidyl group on the substrates is blocked using 10% solution of ethanolamine in water (v/v).

3 Immobilization of Phage-Derived Protein

3.1 Production and Purification of Phage Recombinant Binding Proteins

3.1.1 Campylobacter jejuni Phage NCTC1267 Gp047 Recombinant Receptor-Binding Protein [28]

Materials

(a) Sodium phosphate.
(b) Potassium phosphate.
(c) Sodium chloride.
(d) Potassium chloride.
(e) Dithiothreitol (DTT).
(f) Ethylenediaminetetraacetic acid (EDTA).
(g) Glutathione.
(h) PBS (phosphate-buffered saline, 1.8 mM KH_2PO_4, 10 mM Na_2HPO_4, pH 7.4, 2.7 mM KCl, 137 mM NaCl).
(i) PBS–DTT–EDTA buffer: PBS supplemented with 1 mM DTT and 5 mM EDTA.

(j) GA Elution buffer: PBS supplemented with 5 mM EDTA and 10 mM glutathione, pH 8.0–8.5.

(k) cOmplete Mini, EDTA-free protease inhibitor cocktail (Roche).

(l) LB medium (BD Biosciences) supplemented with 50 μg/mL of ampicillin.

(m) Ice.

(n) Standard pH-meter.

(o) Sonicator, e.g., Branson Sonifier 450 or a similar model.

(p) Standard low speed floor centrifuge, e.g., Beckman J2-21 or similar newer models.

(q) Standard microbiological shaker.

(r) Standard table-top laboratory shaker.

(s) 0.22 μm filter (Millipore).

(t) Glutathione-agarose beads (Sigma-Aldrich).

Production Methods

(a) Characterization and cloning of the gene encoding putative *Campylobacter* cell binding protein Gp047 from *C. jejuni* phage NCTC1267 were described previously [29].

(b) Transform the *E. coli* BL21 cells with the pGEX 6P-2 plasmid containing the phage gene.

(c) Grow the 2 L culture of bacterial cells at 30 °C to an OD_{600} of 0.5 using the LB medium with ampicillin.

(d) Induce the culture with 0.1 mM IPTG and incubate overnight at 30 °C with shaking at 250 rpm using the standard microbiological shaker.

(e) Harvest the cells by centrifugation using the conventional procedure.

Purification Methods

(a) Resuspend the cells in 100 mL of ice-cold PBS–DTT–EDTA buffer supplemented with the protease inhibitor cocktail.

(b) Disrupt the cells by sonication using conventional procedure.

(c) Remove the cell debris by centrifugation at 27,000× *g* for 30 min at 4 °C.

(d) Filter the supernatant through a 0.22 μm filter.

(e) Incubate the supernatant with the 10 mL of glutathione-agarose beads preequilibrated with the PBS–DTT–EDTA buffer. Should be done for 1 h at 4 °C with the gentle shaking.

(f) Wash the glutathione-agarose beads with the 50 mL of PBS–DTT–EDTA buffer containing the protease inhibitor cocktail and then with 200 mL of PBS–DTT–EDTA buffer.

(g) Elute the target protein by incubating the glutathione-agarose beads with 20 mL of the GA Elution buffer for 1 h at 4 °C with the gentle shaking.

(h) Dialyze the eluate against PBS (can be done overnight at 4 °C).

3.1.2 Production and Purification of Mycophage Minor Tail (Gp6) and Lysin (Gp10) Proteins [30]

Materials

(a) Sodium phosphate.

(b) Potassium phosphate.

(c) Sodium chloride.

(d) Potassium chloride.

(e) Dithiothreitol (DTT).

(f) Imidazole.

(g) Immobilized metal affinity chromatography (IMAC) buffer A: 50 mM sodium phosphate, pH 8.2, 1 mM DTT, 1 M NaCl, 30 mM imidazole.

(h) IMAC buffer B: 50 mM sodium phosphate, pH 8.2, 1 mM DTT, 1 M NaCl, 500 mM imidazole.

(i) PBS (phosphate-buffered saline, 1.8 mM KH_2PO_4, 10 mM Na_2HPO_4, pH 7.4, 2.7 mM KCl, 137 mM NaCl).

(j) cOmplete Mini, EDTA-free protease inhibitor cocktail (Hoffmann-La Roche Limited, Mississauga, ON, Canada; http://www.rochecanada.com/).

(k) LB medium (BD Biosciences, Mississauga, ON, Canada; http://www.bd.com/en-ca) supplemented with 25 μg/mL of kanamycin.

(l) Ice.

(m) Standard pH-meter.

(n) Sonicator, e.g., Branson Sonifier 450 or a similar model.

(o) Standard low speed floor centrifuge, e.g., Beckman J2-21 or similar more recent models.

(p) Standard microbiological shaker.

(q) 0.22 μm filter (Millipore).

(r) 1 mL HisTrap HP column (GE Healthcare, Little Chalfont, United Kingdom; http://www3.gehealthcare.com/en/global_gateway).

Production Methods

(a) Selection and cloning of gene(s) encoding putative mycobacteriophage cell binding protein(s) were described previously [30]. The appropriate gene was amplified directly from the lysate of the mycobacteriophage L5. Cloning procedures were performed using conventional methods;

(b) Transform the *E. coli* BL21(DE3) cells with the pET-30a(+) plasmid containing the phage gene;

(c) Grow 2 L culture of bacterial cells at 30 °C to an OD_{600} of 0.5 using the LB medium with kanamycin;

(d) Induce the culture with 0.2 mM IPTG and incubate overnight at room temperature with shaking at 250 rpm using the standard microbiological shaker;

(e) Harvest the cells by centrifugation using a conventional procedure.

Purification Methods

(a) Resuspend the *E. coli* cells in 100 mL of ice-cold IMAC buffer A with the protease inhibitor cocktail;

(b) Disrupt the cells by sonication using a conventional procedure;

(c) Remove the cell debris by centrifugation at 27,000× *g* for 30 min at 4 °C;

(d) Filter the supernatant through a 0.22 μm filter;

(e) Load the filtrate onto a 1 mL HisTrap HP column equilibrated with buffer A;

(f) Wash the column with 5 mL of buffer A with the protease inhibitor cocktail and then with 20 mL of buffer A;

(g) Elute the target protein with 2 mL of buffer B;

(h) Let the column stand for 10 min and then elute the rest of the protein with another 2 mL of buffer B;

(i) Combine the eluates and dialyze against PBS (can be done overnight at 4 °C).

3.1.3 Notes

(a) It was found that a number of genes cannot be amplified directly using the NCTC12673 *Campylobacter* phage lysate or the phenol–chloroform-purified DNA of this phage. Apparently, neither Taq nor Vent polymerase could use phage DNA as the substrate. It is hypothesized that this may be caused by the presence of the yet unknown modification(s) of the phage DNA. To overcome this obstacle the isothermal preamplification step was performed as described in ref. 29. Briefly, 40 mL of the NCTC12673 phage lysate (10^7pfu/mL) was treated with DNase I and RNase H (1 μg/mL each) to remove the host DNA and RNA. Phage DNA was subsequently extracted three times with phenol/chloroform solution followed by two chloroform extractions. DNA was then precipitated by isopropanol and dissolved in 100 μL of 10 mM Tris, pH 8.0. Then 5 μL of this phage DNA solution was used in the 50 μL preamplification reaction with phi29 DNA polymerase (Fermentas/Thermo Fisher Scientific) at 37 °C

overnight. Five μM (final concentration) exo-resistant random primers (Fermentas) were used in the reaction along with 10 U of Phi29, 1 U of yeast inorganic pyrophosphatase (Fermentas), and 0.5 mM of dNTPs (Roche). Five microliters of the resulted preamplified phage DNA was used in a standard 50 μL PCR reaction to amplify the gene 047 of the phage.

(b) All buffers should be prepared using MilliQ-grade water and prefiltered using the 0.22 μm filter. All the chemicals should be of molecular biology grade with at least 99% purity.

(c) All buffers should be chilled to 4 °C and kept at that temperature during the whole procedure, unless stated otherwise.

(d) The Gp047 protein samples can be stored at 4 °C in PBS for up to 18 months without the loss of the cell binding activity;

(e) It is recommended to use a glass filter or an empty chromatography column to wash the glutathione-agarose beads;

(f) It is important to check and adjust the pH of the GA Elution buffer to 8.0–8.5 before use.

(g) Determine the protein concentration by measuring the UV absorbance. The Christian-Warburg method should be used to determine the total protein concentration of the Gp10 preparation. Extinction coefficient at 280 nm was calculated for the His-tagged Gp6 by the ProtParam Tool (www.expasy.org) assuming all cysteine residues are in the reduced state and turned out to be 43,430 M^{-1} cm^{-1} with $A_{0.1\%}$ of 1.093;

(h) Disposable plastic syringes (e.g., BD Luer-Lok™) can be used for filtering the protein extract and washing the IMAC column although other conventional methods (e.g., peristaltic pump) can be used as well.

3.2 Immobilization Protocol

3.2.1 Immobilization of Campylobacter Phage Gp047 RBP onto Gold Layers [28]

Materials

(a) GST-GP047phage receptor binding proteins (RBPs).

(b) Phosphate buffered saline (PBS).

(c) 2 mg/mL glutathione.

(d) 0.05% Tween 20-PBS.

(e) 1 mg/mL bovine serum albumin (BSA).

(f) Acetone.

(g) Isopropyl alcohol (IPA).

(h) Ethanol.

(i) DI water.

(j) Sonicator.

(k) Orbital shaker.

(l) Eppendorf tube.

Methods

(a) The devices are washed sequentially in acetone, isopropyl alcohol, ethanol, and water for 5 min each to clean the surface prior to functionalization.

(b) The clean devices were incubated in 2 mg mL^{-1} solution of glutathione in PBS for 1 h on an orbital shaker at 1000 rpm.

(c) The GSH-SAM devices were washed twice in PBS for 5 min each to wash away the excess reagent from the surface.

(d) The functionalized devices were immersed in 5 μg mL^{-1} solution of GST-GP48 protein in PBS for 1 h on an orbital shaker at 1000 rpm followed by 5 min wash in 0.05% Tween-20 PBS and two 5 min washes in PBS.

(e) The 1 mg mL^{-1} solution of bovine serum albumin (BSA) was used to block any nonspecific binding.

3.2.2 Immobilization of Mycophage Gp10 Lysin onto Gold Layers [30]

Materials

(a) Gold substrate.

(b) Piranha cleaned silicon substrate.

(c) Acetone, isopropanol, ethanol, and MilliQ water.

(d) Cysteamine hydrochloride (Sigma-Aldrich).

(e) Glutaraldehyde (Sigma-Aldrich).

(f) Mycobacterium phage L5 lysin (Gp10) amplified using a suspension of mycobacteriophage L5.

(g) BupH phosphate buffered saline pack (PBS) (Pierce).

(h) Bovine serum albumin (BSA)Tween 20 (Sigma-Aldrich).

(i) Resazurin (Sigma-Aldrich).

(j) *Mycobacterium avium* subsp. *paratuberculosis* ATCC 19851, *Mycobacterium marinum ATCC* 927, *Mycobacterium smegmatis* mc^2155.

(k) Middlebrook 7H9 broth (BD Biosciences, USA) supplemented with an oleic acid-albumin-dextrose-catalase mixture (BD Biosciences, USA) and mycobactin J (Allied Monitor, USA).

(l) Branson Ultrasonics 1510 sonicator (40 kHz frequency, 80 W power),

(m) Hitachi S-4800/LEO 1430 Scanning Electron Microscope.

(n) Olympus 1X81 Fluorescence microscope equipped with FITC filter and Roper Scientific Cool Snaps HQ–CCD camera.

(o) Shaker incubator for bacterial culture.

Methods

Protein Immobilization

(a) Fabricate the piranha cleaned silicon substrates by sputtering 25 nm thick gold layer.

(b) Prior to surface modification, gold substrates should be washed by sonication in acetone, isopropanol, ethanol, and MilliQ water for 5 min each.

(c) Incubate the gold substrate overnight at 40 °C in 50 mM solution of cystamine hydrochloride.

(d) Modify the Cystamine self-assembled monolayer (SAM) substrates by 2% glutaraldehyde for 1 h. at room temperature, followed by washing twice in PBS.

(e) Incubate these modified substrates in 20 μg/mL solution of Mycophage lysin Gp10 in PBS for overnight at 60 °C in a temperature controlled water-bath. For negative control, incubate the modified substrate in PBS instead of protein.

(f) Expose the protein immobilized gold substrates to 1 mg/mL of bovine serum albumin for 30 min.

(g) Wash twice in PBS to remove unbound BSA.

Binding Assay

(a) Wash bacterial cells twice in PBS to remove the media.

(b) Expose the protein immobilized substrates to 10^9 cfu/mL of mycobacterial cells in PBS followed by incubation for 1 h at room temperature. *M. marinum* and *E. coli* cells were used to determine the specificity of the protein.

(c) Wash the immobilized surfaces in 0.05% of Tween 20 before analysis.

(d) For fluorescence microscopy, stain the bacterial cells with 50 μM resazurin for 20 min before exposure with protein-immobilized substrates.

(e) To record the fluoroscopic images, an Olympus IX81 microscope equipped with a FITC filter and a Roper Scientific CoolSnaps HQ CCD camera, can be used.

(f) Fix the samples with 2% glutaraldehyde for 2 h at room temperature followed by gradient of ethanol from 50% to 100% before SEM.

(g) Finally dry the samples by exposure to nitrogen.

(h) Record the SEM images by using a Hitachi S-4800/LEO 1430 microscope.

(i) Use ImageJ software (USA NIH) to analyze the microscopic images. Average numbers of the cells bound to the surface are indicated on the basis of the assessment of the cell number in the field of view and using eight gold-covered chips per test.

3.2.3 Immobilization of Campylobacter phage Gp047 Protein onto Beads [31]

Materials

(a) Dynabead M-280 Tosyl activated and/or Lyophilized Dynabead M-270 Epoxy beads
(b) 0.1 M Na-phosphate buffer.
(c) 3 M ammonium sulfate.
(d) GST-Gp48 phage RBPs.
(e) 0.1% (w/v) BSA.
(f) PBS.
(g) Glutathione.
(h) Magnetic rack.
(i) Eppendorf tube.
(j) Orbital shaker.

Methods

Preparation of Magnetic Beads

(a) Initially, 5 mg of lyophilized Dynabeads® M-270 Epoxy or 165 μL of Dynabeads® M-280 tosyl-activated beads were washed twice in 0.1 M Na-phosphate buffer (pH 7.4) for 10 min.
(b) The tubes containing washed beads were placed on a magnet for 1 min, and the supernatant was removed.
(c) The washed beads were resuspended in 100 μL of 0.1 M Na-phosphate buffer (pH 7.4) and 100 μL of 3 M ammonium sulfate (pH 7.4).

Note: The efficiency to capture and isolate bacteria from suspension can be examined in two different immobilization modes for receptor binding proteins: random coating and oriented immobilization.

Unoriented Immobilization of GST-GP48 RBPs onto Beads

(a) For random unoriented immobilization, add 100 μL of GST-Gp48 RBPs to the suspension containing prewashed beads and incubate the mixture overnight on an orbital shaker at 1000 rpm.
(b) Place the tube containing RBP-coated magnetic beads on a magnet and remove the supernatant.
(c) Wash the RBP coated magnetic beads four times in PBS-BSA buffer (PBS (pH 7.4) containing 0.1% (w/v) BSA) to block any uncoated surface.
(d) Resuspend the BSA blocked beads in 1 mL of PBS buffer (pH 7.4) and store at 4 °C.

Oriented Immobilization of GST-GP48 RBPs onto Beads

(a) For oriented immobilization, incubate the washed beads in 100 μL of 1 mg per mL solution of glutathione in PBS, and incubate overnight on an orbital shaker at 1000 rpm to form a self-assembled monolayer of glutathione (GSH SAM)).

(b) Wash the GSH SAM beads in PBS once and then place on a magnet and remove the supernatant. Incubate the GSH-SAM beads in 40 μg of GST-Gp48 RBPs in PBS for 1 h on an orbital shaker at 1000 rpm.

(c) Wash the RBP derivatized magnetic beads four times in PBS-BSA buffer to block the free surface.

(d) Resuspend the BSA blocked beads in 1 mL of PBS buffer (pH 7.4) and store at 4 °C until used for bacterial capture.

3.2.4 Immobilization of Mycophage Gp10 Lysin onto Tosyl-Activated Dynabeads® M-280 [32]

Materials

(a) M-280 Tosyl-activated Dynabeads® Life Technologies Inc. (USA).

(b) DynaMag 2 magnet Life Technologies Inc. (USA).

(c) Mycobacterium phage L5 lysin (Gp10) was amplified using a suspension of mycobacteriophage L5.

(d) BupH phosphate buffered saline pack (PBS) (Pierce).

(e) Bovine serum albumin (BSA).

(f) MilliQ water.

(g) Temperature-controlled water bath incubator.

Methods

(a) Prior to modification, Dynabeads® M-280 (100 mg/mL) should be washed twice with sterile PBS for 10 min.

(b) To separate the supernatant from the beads, the tubes should be placed on a DynaMag 2 magnet for 1 min.

(c) Resuspend the beads in 1 mL of sterile PBS.

(d) Incubate the cleaned beads with 100 μg/mL Mycophage lysin for 1 h at 37 °C followed by overnight incubation at room temperature.

(e) Further incubate the functionalized beads with 1 mg/mL BSA for 30 min to block the free surface and prevent nonspecific binding. All the incubations should be done with gentle shaking.

(f) Finally, wash the beads twice with sterile PBS to remove any unbound BSA.

(g) Since mycobacterial cells aggregate into clumps due to their hydrophobic cell surfaces, the sample to be tested should be sonicated for 5 min prior to incubation with the magnetic beads to get a homogeneous suspension and to prevent nonspecific deposition of aggregated cell masses onto the bead surfaces.

(h) Suspend the Mycophage lysin Gp10 functionalized beads in the sample, and incubate for 1 h at room temperature under gentle shaking.

(i) Separate the beads by incubating the samples on a magnetic separator for 5 min and wash twice with sterile PBS.

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Chapter 9

Peptidoglycan Hydrolytic Activity of Bacteriophage Lytic Proteins in Zymogram Analysis

Lorena Rodríguez-Rubio, David M. Donovan, Beatriz Martínez, Ana Rodríguez, and Pilar García

Abstract

Zymogram or zymography is an electrophoretic technique based on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), which enables visualization of enzymatically active protein species separated by molecular mass. The strategy is to perform SDS-PAGE on the proteins in question while including an opaque substrate of the enzyme embedded within the polyacrylamide gel. Here, we describe a zymogram protocol for phage lytic proteins (peptidoglycan hydrolases) using peptidoglycan (or whole cells) from a susceptible gram-positive bacterial species as substrate. Proteins are prepared and analyzed simultaneously on two separate gels: First, standard denaturing SDS-PAGE followed by conventional protein staining (e.g., Coomassie) is run to identify the migration pattern of the protein species in the sample; second, the zymogram gel in which either cells or peptidoglycan from a susceptible bacterium have embedded in the SDS gel matrix is performed. After electrophoresis, the SDS is removed from the zymogram gel, allowing the proteins (now separated by molecular mass) to assume an active conformation and ultimately digest the opaque substrate (yielding a nonopaque product). This results in a cleared spot in an otherwise opaque gel which corresponds to the location of an enzymatically active protein species. This assay can be used to qualitatively assay the enzymatic activity of endolysins from cell extracts, or to identify virion-associated peptidoglycan hydrolases in phage particles.

Key words Enzyme, Phage lytic proteins, SDS-PAGE, Hydrolytic activity

1 Introduction

Zymograms are generally used to detect both the hydrolytic activity of a peptidoglycan hydrolase on the basis of its substrate degradation as well as be used to identify enzymatically active species of proteins (contaminants) in putative purified protein preparations [1]. In theory, zymogram analysis can be performed for any enzyme acting on any biological substrate (e.g., LPS, DNA), and detection methods are based on the visualization of the reaction product or visual disappearance of the substrate [2]. Double-stranded bacteriophages encode peptidoglycan hydrolases (endolysins) to degrade

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the cell wall peptidoglycan and lyse the host bacteria to release the phage progeny at the last step of the lytic infection cycle. Some phages also encode structural lysins or virion-associated peptidoglycan hydrolases (VAPGHs) to degrade the peptidoglycan in the earliest stages of the infection cycle. Both types of proteins have an extremely high potential as antimicrobial alternatives to antibiotics in the fight against pathogenic bacteria, and thus, their study has increased recently in the face of worldwide increase in multidrug resistant pathogens [3, 4]. As substrate, live whole cells [5–9], autoclaved cells [10–19], freeze-dried cells [20–23] or crude peptidoglycan [24] can be used to identify peptidoglycan hydrolase activity. Usually, live whole cells are used to assess the hydrolytic potential of peptidoglycan hydrolases encoded by phages infecting gram-positive bacteria, while the other substrates are used for lytic proteins encoded by phages infecting both gram-positive or gram-negative bacteria. This technique provides advantages for the analysis of peptidoglycan hydrolases, e.g., proteins do not need to be purified, crude cell extracts containing the protein of interest can be used [6, 10, 12, 13, 18, 19, 21, 22, 25]. Related to this advantage, this technique allows detecting the presence and determining the molecular mass of structural peptidoglycan hydrolytic proteins in fully assembled virions, i.e., without cloning and overexpression [11, 14, 15, 26]. However, zymograms also have some inherent disadvantages including the need for correct refolding of the protein after SDS denaturation and electrophoresis. It is also helpful if the researcher is aware of the conditions required for high activity of the peptidoglycan hydrolase of interest (such as ionic strength, cations, and temperature), so that these can be included during the renaturation step.

Here, we describe a zymogram protocol to detect the catalytic activity of phage-encoded peptidoglycan hydrolases, using fresh cultured live bacterial cells embedded in the polyacrylamide gel (Fig. 1).

2 Materials

Prepare all solutions using ultrapure water (purifying deionized water to sensitivity of 18 MΩ cm at 25 °C) and store all reagents at room temperature (unless indicated otherwise).

2.1 For the Substrate (Bacterial Cells)

1. Appropriate medium for growing the target bacterium.
2. 50 mM sodium phosphate buffer pH 7: prepare 50 mM NaH_2PO_4 and 50 mM Na_2HPO_4. Add slowly the NaH_2PO_4 solution onto the Na_2HPO_4 solution until the desired pH is achieved.

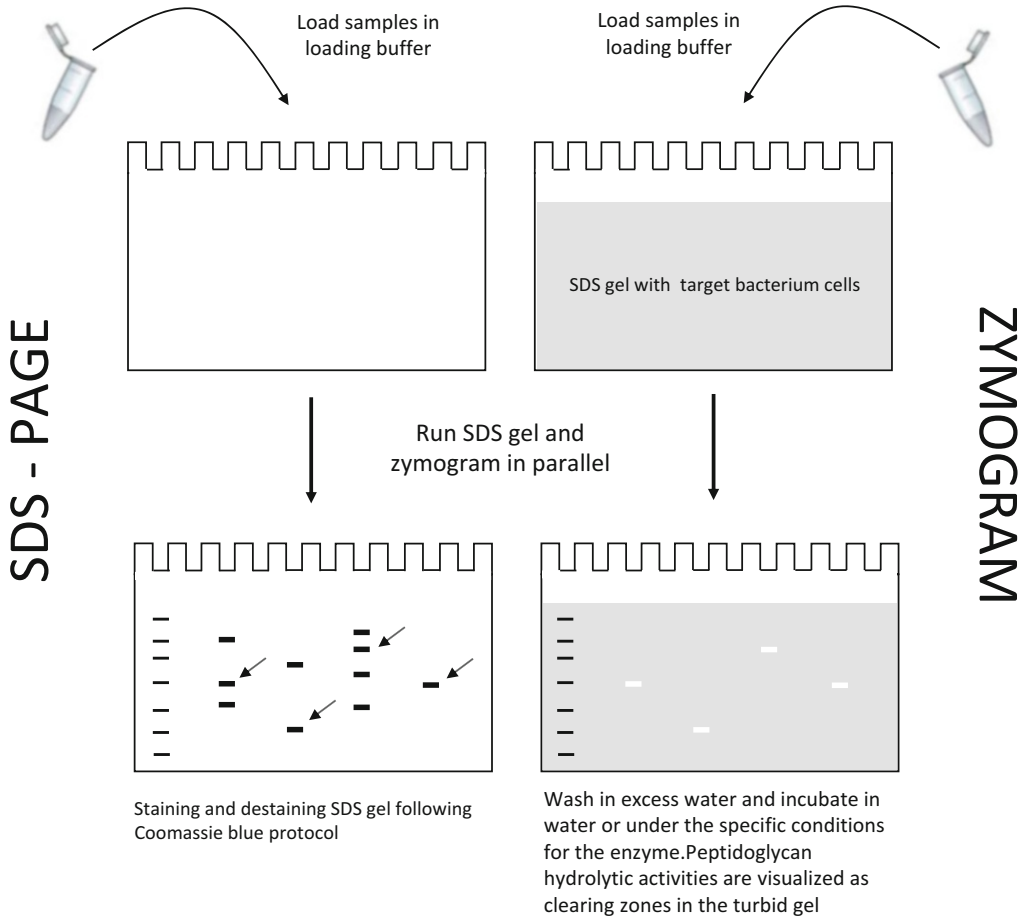

Fig. 1 Scheme of the steps to perform a zymogram analysis of phage-encoded lytic proteins using live whole cells embedded in the gel as substrate. Arrows in the SDS gel indicate the proteins in the mixture with peptidoglycan hydrolytic activity in the zymogram gel

2.2 SDS-PAGE Components

1. 1M Tris–HCl, pH 8.0.
2. 40% Acrylamide–Bis-acrylamide (37.5:1) (Bio-Rad Laboratories; http://www.bio-rad.com). Store at 4 °C.
3. 10% (w/v) sodium dodecyl sulfate (SDS).
4. 10% (w/v) ammonium persulfate (APS). Make aliquots and store at −20 °C (stable for >6 months) or prepare fresh.
5. TEMED (N,N,N′,N′-tetramethylethylenediamine). Store at 4 °C.
6. 4× Sample buffer: 0.2 M Tris–HCl, 0.4 M Dithiothreitol (DTT), 8% SDS, 40% (v/v) glycerol, 0.4% (w/v) bromophenol blue. Make aliquots and store at −20 °C (stable for up to 1 year).

7. 5× Electrophoresis buffer (per liter): 15 g Tris base, 72 g glycine. Store at 4 °C (up to 1 month).
8. Staining solution: 0.1% (w/v) Coomassie Brilliant Blue R-250, 7.5% acetic acid, 50% methanol. Store in a bottle wrapped with aluminum foil at room temperature.
9. Destaining solution: 10% acetic acid, 50% methanol.

3 Methods

3.1 Bacterial Cells Preparation

Grow 300 ml culture of bacterial strain of interest to mid-log phase (OD_{600} ~0.5) and separate cells from culture media by centrifugation. Add 300 μl of 50 mM sodium phosphate buffer pH 7 to the cell pellet (*see* **Note 1**). This will make a total volume of about 500–600 μl cells in buffer. Put cells on ice until they are added to the SDS gel mix (*see* **Note 2**).

3.2 SDS-PAGE and Zymogram

1. To perform a zymogram analysis, an SDS gel and a zymogram gel should be run in parallel. The Coomassie stained SDS PAGE will be a reference for the zones of clearing in the zymogram gel. Set up two sets of clean glass plates in the polyacrylamide mini-gel electrophoresis apparatus (e.g., Mini-PROTEAN® 3 Cell, Bio-Rad).
2. Prepare four 50 ml conical tubes to make the polyacrylamide gel mixtures.
3. Tube 1 contains the Gel Master Mix: 3.3 ml H_2O, 3.6 ml 1 M Tris–HCl pH 8.0, 5 ml 40% acrylamide–bis-acrylamide (37.5:1), 120 μl 10% (w/v) SDS.
4. Tube 2 contains the Running Gel (regular SDS gel): 5 ml Gel Master Mix, 600 μl 50 mM sodium phosphate buffer pH 7 (*see* **Note 3**), 70 μl 10% APS. Immediately before pipetting gel into glass plates, add 7 μl TEMED and swirl gently; then pipet the mixture into the glass plates (*see* **Note 4**). Gently overlay the running gel prior to polymerization with water or butanol to prevent contact with atmospheric oxygen and to provide an interface for a flat and level upper surface of the gel once polymerized.
5. Tube 3 contains the gel for the Zymogram: 5 ml Gel Master Mix, 600 μl bacterial cells in 50 mM sodium phosphate buffer pH 7, 70 μl 10% APS. The cell number should be sufficient to create an opaque solution. Immediately before pipetting the gel into a second set of glass plates, add 7 μl TEMED and swirl gently, then pipet the mixture into the zymogram glass plates. As with the SDS gel, gently overlay with water or butanol to prevent contact with atmospheric oxygen and permit leveling the running gel upper surface once polymerized.

6. Wait for gels to completely polymerize (about 10–20 min), then pour off water or butanol from the top of gels. If butanol is used, rinse with either water or running buffer and remove remaining liquid.
7. Tube 4 contains the Stacking Gel: 2 ml Gel Master Mix, 1.2 ml 1 M Tris–HCl pH 8.0, 2.8 ml H_2O, 70 µl 10% APS. Immediately before pipetting the gel into glass plates, add 7 µl TEMED and swirl gently, then pipet the mixture into the glass plates. Right after pipetting the gel into glass plates, place a 10-well gel comb without introducing air bubbles. Add more stacking gel if there are any air spaces. Be sure there are not bubbles under the comb or any gaps between the plates and the comb.
8. While waiting for stacking gel to polymerize, mix 30 µl of the protein sample with 10 µl of 4× Sample buffer (*see* **Note 5**) and just before loading, heat at 95 °C for 3 min (*see* **Note 6**).
9. Prepare the electrophoresis buffer by diluting 60 ml of 5× electrophoresis buffer in a final volume of 300 ml and adding 0.1% (w/v) SDS.
10. Remove combs, mount gels in the electrophoresis apparatus, add the electrophoresis buffer to the middle and bottom reservoirs, load 20 µl of each sample into each well, and the molecular weight marker (*see* **Note 7**) and run gels for about 1–1.5 h at 150 V.
11. Remove the gels from the electrophoresis apparatus and place them on a paper towel. Using a spatula pry the plates apart. Carefully place the SDS gel into the stain solution and the zymogram gel into deionized water (*see* **Note 8**) and shake gently. Add enough staining solution or water to cover the gels.

3.3 Staining/ Destaining SDS Gel

1. Incubate SDS gel in the staining solution for 30 min at room temperature with gentle shaking (*see* **Note 9**).
2. Pour off the staining solution (*see* **Note 10**). Add enough destaining solution to cover the gel (*see* **Note 11**) and incubate at least 1 h at room temperature with gentle shaking (*see* **Notes 9** and **12**).

3.4 Determining Peptidoglycan Hydrolase Activity in the Zymogram

1. After running the zymogram, place it into deionized water for 15 min to wash it, pour off water, add clean water and incubate at room temperature (*see* **Note 13**).
2. Monitor the time the zymogram gel spends in water for consistency from zymogram to zymogram in the timing of the photo documentation (*see* **Note 14**).
3. Depending on the level of enzyme activity, you might see clearing as early as 15 min (*see* **Note 15**).

4. Take pictures at appropriate time intervals, depending on enzyme activity (*see* **Note 16**).
5. By comparing the zymogram with the stained SDS gel, the molecular mass of the protein which is producing clearing in the turbid gel can be predicted.

4 Notes

1. The cell pellet can also be suspended in water.
2. Autoclaved cells, lyophilized cells or crude peptidoglycan (laboratory obtained or commercially available) can also be used. To prepare autoclaved cells, bacteria cells are harvested at exponential growth, washed three times with distilled water, suspended in distilled water (1/10 volume of the initial culture) and autoclaved.
3. The buffers in the zymogram and the SDS-PAGE should be identical so that migration of the proteins is not altered between the two gels. Use water in this step if cell pellet has been suspended in water.
4. Pipet the gel into the glass plates as fast as possible since polymerization starts immediately after adding TEMED.
5. You will be loading two gels, so the amount per sample should be twofold compared to a single SDS gel.
6. Do not heat samples for more than 3 min; there is the chance that some proteins could be irreversibly inactivated.
7. Before loading samples, wells should be rinsed to avoid any possible obstruction. Using a pipette and a tip, just pipet up and down the electrophoresis buffer in the top of each well. If there is any gel formation between the comb and the glass plates, it might be convenient to include some paper clips to keep tightly fitting the glass plates and the comb, while polymerizing.
8. If necessary, mark the orientation of the gel by cutting a corner from the bottom of the gel.
9. It has been reported that the staining and destaining times can be dramatically reduced and detection sensitivity significantly increased by application of enhanced heat (*see* [27]). For example, a 0.8 mm gel heated at 55 °C, 60 °C and 65 °C can be stained in 5, 2, and 1 min, respectively, and destained in 20, 15, and 8 min at the same temperatures, respectively.
10. The Coomassie stain solution can be recycled several times by filtering it.

11. Paper wipes or foam blocks can be placed in the destaining solution around the gel to help destaining since they will absorb the Coomassie blue dye. Avoid laying the wipes on the gel as this can cause uneven destaining.
12. Gels can be incubated from 1 h to overnight in the destaining solution. Stop when the level of destaining is sufficient for you.
13. You may need to repeat the wash step twice to completely remove the SDS and allow proteins to regain activity.
14. If you follow the protocol with autoclaved or lyophilized cells or crude peptidoglycan, transfer the gel to a refolding buffer containing 25 mM Tris–HCl pH 7.5 and 0.1% Triton X-100 after washing it the amounts may vary slightly depending on the authors (*see* [10–24]). Then incubate the gel between 30 min and 72 h at 37 °C. Zymograms are further stained with 0.1% (w/v) methylene blue and 0.01% (w/v) KOH for 1–2 h at room temperature (to stain the bacterial peptidoglycan) and destained with distilled water. The peptidoglycan hydrolase activity is detected as a clear zone against a dark blue background.
15. If there is no activity or very low activity after 1 h in water, replace water with an appropriate buffer that might enhance the activity of the candidate enzyme according to previous knowledge. Similarly, other agents can be added to the soaking buffer, e.g., 150 mM NaCl, to potentially enhance the enzyme activity. Addition of cations such as Ca^{2+} or Mg^{2+} may also help depending on the requirements of the protein. If the protein is not very active, incubation in water or under the specific conditions for the protein can also be continued overnight.
16. Pictures will be easier to take if zymogram is floating in water on a dark background.

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Chapter 10

Analyzing Phage–Host Protein–Protein Interactions Using *Strep*-tag® II Purifications

Jeroen De Smet, Hanne Hendrix, and An Van den Bossche

Abstract

After injecting their genome into the bacterial host cell, bacteriophages need to convert the host metabolism toward efficient phage production. For this, specific proteins have evolved which interact with key host proteins to inhibit, activate or redirect the function of these proteins. Since 70% of the currently annotated phage genes are hypothetical proteins of unknown function, the identification and characterization of these phage proteins involved in host–phage protein–protein interactions remains challenging. Here, we describe a method to identify phage proteins involved in host–phage protein–protein interactions using a combination of affinity purifications and mass spectrometry analyses. A bacterial strain is engineered in which a bacterial target protein is fused to a *Strep*-tag® II at the C-terminal end. This strain is infected with a specific bacteriophage, followed by an affinity purification of the tagged protein which allows the copurification of all bacterial and phage specific interacting proteins. After SDS-PAGE analysis and an in-gel trypsin digestion, the purified interacting proteins are identified by mass spectrometry analysis. The identification of phage proteins involved in interactions provides first hints toward the elucidation of the biological function of these proteins.

Key words Phage–host protein–protein interactions, Affinity purifications, Bacteriophage, *Pseudomonas aeruginosa*, Mass spectrometry

1 Introduction

Upon infection of a susceptible bacterial cell, bacteriophages hijack the molecular machinery of their host to obtain efficient production of phage progeny. Specific mechanisms have evolved to achieve this and protein–protein interactions (PPI) between phage proteins and key host proteins play a crucial role in this. These PPIs serve to inhibit, activate, or redirect the function of the bacterial interaction partner [1]. Consequently, a number of studies state that the examination of these phage–host PPIs can provide a powerful tool in the search for new antibacterial targets in drug discovery [2, 3]. However, only a limited number of host-phage PPIs have

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already been studied, most of them involving interactions of the RNA polymerase of the model organism *Escherichia coli* and proteins of its phages, although emphasis has been placed on *Pseudomonas aeruginosa* in recent years [4–6].

One of the main challenges in phage biology is the functional elucidation of the in silico annotated phage genes. Due to the progress in high-throughput sequencing techniques, there is an increasing gap between the number of annotated phage genes and their functional annotation. Currently, about 70% of the annotated phage genes are hypothetical genes of unknown function (NCBI Entrez database). Many of them are small, early expressed phage genes which are believed to be involved in the specific host-phage PPIs described above [1].

Here, we describe a technique to identify and study host-phage PPIs by using affinity purifications coupled to mass spectrometry analyses. Key proteins of the host, in this case the opportunistic pathogen *Pseudomonas aeruginosa* [7, 8], are used as bait to pull-down interacting host and phage proteins. An advantage of using PPI techniques to study phage proteins is that their bacterial interaction partner provides a first direct clue toward the function of the phage protein. Indeed, it has become clear that proteins mediate their biological function through PPIs [9]. Moreover, the use of affinity purifications offers a wide screen for both direct and indirect interactions, in contrast to binary PPI techniques such as the yeast two-hybrid system [10]. To distinguish true and false positive interactions, we emphasise that secondary and complementary PPI assays need to be performed once an interaction is identified. However, when setting up a large-scale analysis involving several target host proteins and bacteriophages, the discrepancy between true and false hits is tremendously facilitated [11].

An overview of the different steps of the protocol is illustrated in Fig. 1. Once a target protein of the host is selected for analysis, a mutated *P. aeruginosa* strain is engineered which carries a genomic introduced affinity tag fused to its *C*-terminus. We selected the eight amino acid long *Strep*-tag® II, which is based on the streptavidin-biotin system [12]. Because of the small size, there is a very low chance of interference with the protein folding and function, and the protein complex can be purified in a one-step protocol [13]. The strain is constructed by "in vivo homologous recombination," using the λ Red recombination system [14] and a cassette containing the homologous fragments, a *Strep*-tag® II and a gentamicin resistance gene (*see* Fig. 2) (*see* Subheading 2.1). Prior to affinity purification, the viability of the strain and its sensitivity to phage infection are verified and compared to the wild type strain. Moreover, the detectability of the tagged protein is investigated (*see* Subheading 2.2). Subsequently, the strain is infected with a *P. aeruginosa* specific phage, the infection is stopped in the early stage of infection and an affinity purification is performed (*see*

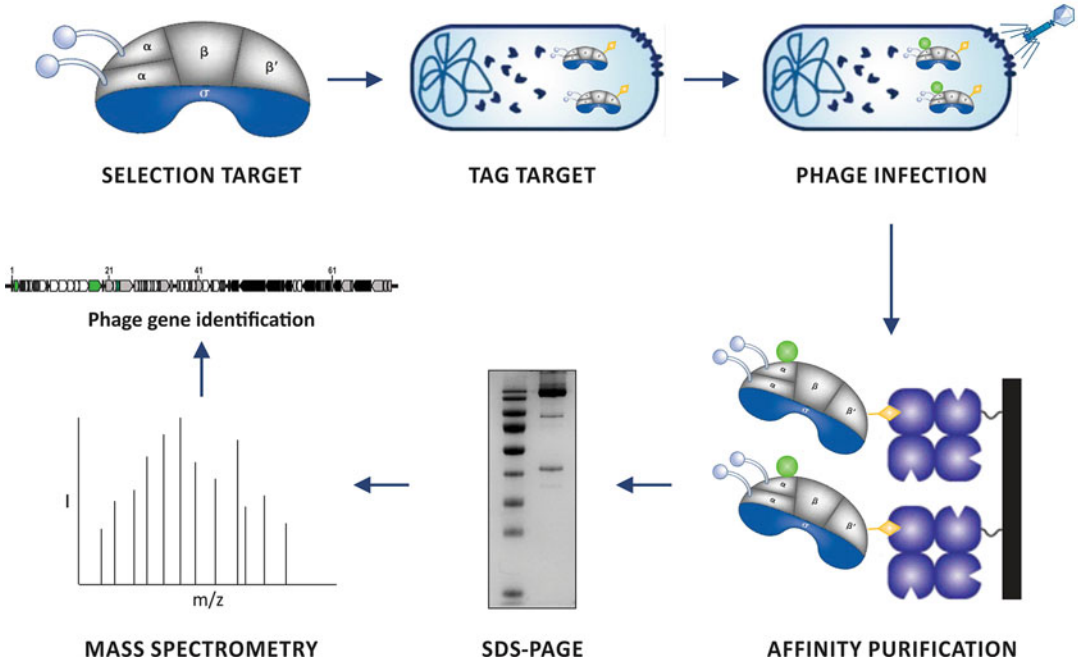

Fig. 1 Overview of the protocol used for the identification of interacting phage proteins. After selecting a bacterial target protein, a *Strep*-tag® II is fused at the C-terminal end of this target using in vivo homologous recombination. In the next step, the recombinant strain is infected with a specific bacteriophage and the infection cycle is stopped in the early phase of infection. The cells are lysed and an affinity purification is performed to purify the target protein and all interacting proteins. The eluted protein samples are loaded on SDS-PAGE. Finally, the samples are subject to an in-gel trypsin digestion and the resulting peptides are analysed by mass spectrometry, to identify interacting phage proteins

Subheading 2.3). The eluted fractions are subject to SDS-PAGE analysis (*see* Subheading 2.4), after which an in-gel trypsin digestion is performed and the samples are sent for mass spectrometry analyses to identify all purified proteins (*see* Subheading 2.5). By screening against a database that contains all host proteins and all "stop-to-stop" protein sequences in all six reading frames of the used phages, biases toward annotated genes are avoided and new, previously unannotated proteins might be identified (proteogenomics) [11, 15].

2 Materials

Prepare all solutions with ultrapure water and use analytical grade reagents. Prepare and store all reagents at room temperature (unless stated otherwise).

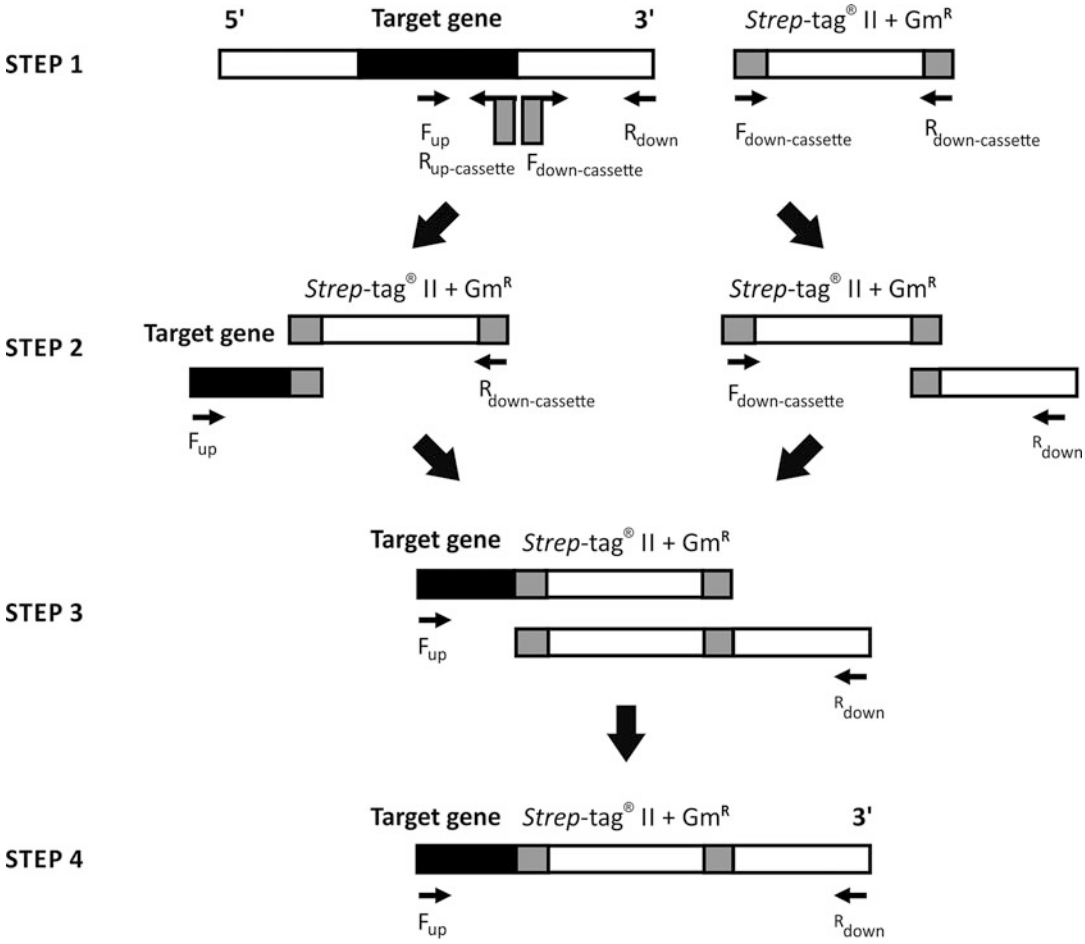

Fig. 2 Steps to produce the DNA construct for homologous recombination. In **step 1**, the separate fragments are amplified (C-terminal part of target gene without the stop codon, cassette containing *Strep*-tag® II + Gm^R gene and the 3′ region of the gene). In **step 2**, the two fragments are allowed to fuse and primers are added to get the two constructs which share the Gm^R cassette. In **step 3**, the fragments obtained in **step 2** are fused and primers are added. In **step 4**, the full DNA fragment is amplified using the outer primers

2.1 Construction of a C*-Terminal Strep-tag® II Fusion Protein in P. aeruginosa PAO1*

2.1.1 Construction of the Strep-tag® II Construct

1. 1 ng–1 μg/μl *P. aeruginosa* PAO1 genome (template).
2. 1 ng–1 μg/μl plasmid coding for gentamycin resistance (template).
3. DNA polymerase enzyme with corresponding PCR buffer (commercially available).
4. 10 mM dNTP solution.
5. Primers for sequencing: 5 μM working solution.
6. Primers for construct design: 20 μM working solution.
7. GeneJet PCR Purification kit (Thermo Fisher Scientific; https://www.thermofisher.com/).
8. GeneJet Gel Extraction kit (Thermo Fisher Scientific).

9. TOPO TA cloning kit for sequencing (Thermo Fisher Scientific).
10. Agarose.
11. Ethidium bromide (50 μg/ml).
12. 6× Loading buffer: 40% (w/v) sucrose, 0.1% (w/v) bromophenol blue.
13. TAE running buffer: 40 mM Tris (pH 7.2), 0.5 mM sodium acetate and 50 mM ethylene-diaminetetraacetic acid (EDTA).
14. DNA size concentration ladder (e.g., GeneRuler DNA ladder mix, Thermo Fisher Scientific).
15. PCR machine (T3000 Thermocycler, Biometra; http://www.biosciences.ie/biometra).

2.1.2 In Vivo Recombination

1. *P. aeruginosa* PAO1 strain containing the pUC18-RedS plasmid (coding for the lambda-Red recombination proteins [14]).
2. Autoclaved Lysogeny Broth (LB): 1% (w/v) Bacto tryptone, 1% (w/v) NaCl, 0.5% (w/v) yeast extract.
3. Autoclaved LB solid: 1% (w/v) Bacto tryptone, 1% (w/v) NaCl, 0.5% (w/v) yeast extract, 1.5% (w/v) agar.
4. 1000× stock of carbenicillin (200 mg/ml).
5. 1000× stock of gentamycin (30 mg/ml).
6. 20% (w/v) L-arabinose.
7. 300 mM sucrose.
8. 100% glycerol.
9. Primers for sequencing: 5 μM working solution.
10. DNA polymerase enzyme with corresponding PCR buffer.
11. 10 mM dNTP solution.
12. GeneJet PCR purification kit (Thermo Fisher Scientific).
13. Spectrophotometer (LKB Novaspec® II, Pharmacia Biotech; http://www3.gehealthcare.com/).
14. Electroporator (Bio-Rad Pulser) and 0.2 cm electroporation cuvettes (Bio-Rad laboratories).
15. PCR machine (T3000 Thermocycler, Biometra).

2.2 Verification of the Constructed Strains

2.2.1 Effect on the Bacterial Viability and the Infectivity of the Phage

1. *P. aeruginosa* PAO1 strain containing the *target*::*Strep*II fusion (made in Subheading 3.1) and the wild type *P. aeruginosa* PAO1 strain.
2. Pure stock of selected phage (>10^{10} PFU/ml), stored in phage buffer at 4 °C (*see* **Note 1**).
3. Autoclaved LB.
4. Autoclaved LB solid.

5. Autoclaved LB soft: 1% (w/v) Bacto tryptone, 1% (w/v) NaCl, 0.5% (w/v) yeast extract, 0.7% (w/v) agar.
6. Phage buffer: 10 mM Tris (pH 7.5), 100 mM $MgSO_4$, 150 mM NaCl.
7. Spectrophotometer (LKB Novaspec® II, Pharmacia Biotech).

2.2.2 The Production of the Strep-tag® II-Fused Protein

1. *P. aeruginosa* PAO1 *target*::*Strep*II strain and the wild type *P. aeruginosa* PAO1 strain.
2. 50 ml autoclaved LB containing 30 μg/ml gentamicin (using a 1000× stock of 30 mg/ml).
3. Filtered (0.22 μm) TE buffer: 50 mM Tris (pH 8.0), 2 mM EDTA.
4. Cooled transfer buffer: 25 mM Tris, 192 mM Glycine, 20% (v/v) Ethanol (*see* **Note 2**).
5. PBST buffer: 140 mM NaCl, 10 mM KCl, 10 mM Na_2PO4, 1.8 mM KH_2PO_4, 0.1% (v/v) Tween, pH 7.5 (*see* **Note 3**).
6. Blocking solution: PBST + 5% (w/v) Powder milk.
7. Ultrapure H_2O.
8. A protein carrying a *Strep*-tag® II (positive control).
9. Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare).
10. Hen Egg White Lysozyme (HEWL, Sigma Aldrich; https://www.sigmaaldrich.com/).
11. Pefabloc® SC (4-(2-aminoethyl)-benzene-sulfonyl fluoride, aebsf, aminoethyl-benzene-sulfonyl fluoride, 4-2-, proteinase k inhibitor; https://www.sigmaaldrich.com/).
12. Benzonase® nuclease (EMD Millipore Corporation; http://www.emdmillipore.com/).
13. Prestained reference ladder (e.g., the PageRuler Prestained Protein Ladder (Thermo Fisher Scientific)).
14. Monoclonal anti-*Strep*-tag® II antibodies conjugated to horseradish peroxidase (HRP, IBA).
15. Whatman paper (Sigma-Aldrich).
16. Transparant paper.
17. Nitrocellulose membrane (Hybond-C Extra, Ge Healthcare).
18. Amersham Hyperfilm ECL (18 × 24 cm) (GE Healthcare).
19. Hypercassette Blue Std Depth 18 × 24 cm (GE Healthcare).
20. Mini Trans-Blot® Cell (Bio Rad): Gel Holder Cassette, Foam Pads, Trans-Blot Central Core, Bio-Ice Cooling Unit and Mini-PROTEAN® Tetra Cell Systems (electrophoresis chamber).

21. Sonicator (Sonics Ultra cell; https://www.sonics.com/).
22. Heating block (95 °C).
23. WT17 mini tumbling table (Biometra).

2.3 Affinity Purifications

1. *P. aeruginosa* PAO1 *target*::*Strep*II strain and the wild type *P. aeruginosa* PAO1 strain.
2. 600 ml autoclaved LB containing 30 μg/ml gentamicin.
3. Pure stock of selected phage (>10^{10} PFU/ml), stored in phage buffer at 4 °C (*see* **Note 1**).
4. Resuspension buffer: 10 mM Tris (pH 8.0), 150 mM NaCl, 0.1% (v/v) NP-40 (*see* **Note 4**).
5. Wash buffer: 100 mM Tris (pH 8.0), 150 mM NaCl, 1 mM EDTA, or *Strep*-tag® Washing Buffer (IBA) (*see* **Note 4**).
6. Elution buffer: 100 mM Tris (pH 8.0), 150 mM NaCl, 1 mM EDTA, 2.5 mM desthiobiotin, or dilute 10× *Strep*-tag® II Elution (Buffer E, IBA).
7. Regeneration buffer: dilute 10× *Strep*-tag® Regeneration Buffer (IBA).
8. Hen Egg White Lysozyme (HEWL, Sigma Aldrich).
9. Pefabloc® SC (Merck).
10. Benzonase® nuclease (EMD Millipore Corporation).
11. 10× BugBuster® Protein extraction reagent (http://www.emdmillipore.com/).
12. *Strep*-Tactin® Sepharose beads (Sigma-Aldrich).
13. A 10 ml Bio-Rad Poly-Prep® Chromatography column (Bio-Rad Laboratories; http://www.bio-rad.com/).
14. An ice-cold collection tube (300–600 ml) (stored at −80 °C, at the start of the procedure).
15. An icy water bath.

2.4 SDS-PAGE

1. SDS-PAGE 4× loading buffer: 200 mM Tris (pH 6.8), 8 mM EDTA, 40% (v/v) glycerol, 4% (w/v) SDS, 0.4% (w/v) bromophenol blue.
2. Separation gel 12%: Tris–SDS buffer pH 8.8 (1.5 mM Tris (pH 8.8), 0.4% (w/v) SDS), 12% (v/v) 37.5:1 acrylamide–bisacrylamide gel, 0.01% (v/v) APS (ammonium persulfate), 0.001% (v/v) TEMED (N,N,N′,N′ tetramethylethylenediamine).
3. Stacking gel 4%: Tris–SDS buffer pH 6.8 (1.5 mM Tris (pH 6.8), 0.4% (w/v) SDS), 4% (v/v) acrylamide–bisacrylamide gel, 0.01% (v/v) APS, 0.001% (v/v) TEMED.

4. Running buffer: 25 mM Tris (pH 8.3), 192 mM glycine, 0.1% (w/v) SDS.
5. Isopropanol.
6. Standard reagents for Coomassie blue staining (e.g., GelCode Blue Safe (Thermo Fisher Scientific) or the more sensitive Imperial Protein Stain (Thermo Fisher Scientific)).
7. Heating block (95 °C).
8. Standard 1D-gel electrophoresis unit (e.g., Mini-PROTEAN® Tetra Cell Systems, BioRad).

2.5 Mass Spectrometry Analyses

1. 133 mM NH_4HCO_3: 1.05 g NH_4HCO_3/100 ml ultrapure H_2O.
2. 100 mM NH_4HCO_3: 0.79 g NH_4HCO_3/100 ml ultrapure H_2O.
3. 50 mM NH_4HCO_3: 25 ml 100 mM NH_4HCO_3 + 25 ml ultrapure H_2O.
4. 20 mM NH_4HCO_3: 10 ml 100 mM NH_4HCO_3 + 40 ml ultrapure H_2O.
5. 55 mM iodoacetamide (IAA) in 100 mM NH_4HCO_3: 0.01 g IAA/ml 100 mM NH_4HCO_3 (prepare shortly before use) (*see* **Note 5**).
6. 10 mM dithiothreitol (DTT) in 100 mM NH_4HCO_3: 0.0015 g DTT/ml 100 mM NH_4HCO_3 (prepare shortly before use).
7. 50 mM acetic acid: dilute 286 µl acetic acid in ultrapure H_2O to 100 ml.
8. Trypsin Gold (Promega): 20 µg lyophilized trypsin/ml 50 mM acetic acid (store at −80 °C).
9. Trypsin digestion buffer: 150 µl trypsin (20 µg/ml) + 90 µl 133 mM NH_4HCO_3 (prepare shortly before use).
10. 5% formic acid in 50% acetonitrile.
11. 25 mM NH_4HCO_3 in 50% acetonitrile: 195.65 mg NH_4HCO_3/100 ml ultrapure H_2O + 100 ml acetonitrile.
12. Vacuum centrifuge (Martin Christ Gefriertrocknungsanlagen GmbH; Osterode am Harz, Germany; https://www.martinchrist.de/).
13. Oven at 37 °C.
14. Sonicator bath (Branson 2210, https://www.bransonic.com/).
15. Water bath at 56 °C.
16. Icy water bath.

17. Mass spectrometry facility: Group of Prof. Jean-Paul Noben, Biomedical Research Institute and Transnational University Limburg, Hasselt University, 3950 Diepenbeek, Belgium.
Equipment: Easy-nLC 1000 liquid chromatograph (Thermo Fisher Scientific) on-line coupled to a mass calibrated LTQ-Orbitrap Velos Pro (Thermo Fisher Scientific) via a Nanospray Flex ion source (Thermo Fisher Scientific) using sleeved 30 μm ID stainless steel emitters
Software: Proteome Discoverer software v.1.3 (Thermo Fisher Scientific) with build-in Sequest and interfaced with an in-house Mascot v.2.4 server (Matrix Science).

3 Methods

3.1 Construction of a C-Terminal Strep-tag® II Fusion Protein in P. aeruginosa PAO1

3.1.1 Construction of the Strep-tag® II Construct

Once a target protein of *P. aeruginosa* is selected, a DNA construct which will be used for homologous recombination is made using an "overlap-extension" PCR. This construct contains the C-terminal part of the target protein fused to a *Strep*-tag® II, followed by a gentamicin resistance (Gm^R) gene and the fragment downstream of the target gene. The principle for constructing this *Strep*-tag® II-containing DNA fragment is shown in Fig. 2.

1. Design the six primers which are needed to generate the three fragments (*see* **Note 6**). These fragments are a cassette, which contains the *Strep*-tag® II sequence at the 5′ end followed by the Gm^R gene, the 3′ part (approx. 300 bp) of the target gene and the downstream 300 bp.
2. Amplify the three fragments using standard PCR. Use the *P. aeruginosa* PAO1 genome as template for the 3′ fragment of the gene and the downstream fragment. Use a vector containing the Gm^R gene as template for the cassette.
3. Check the correct size of all three fragments by gel electrophoresis and purify the product with a PCR purification kit (*see* **Note 7**).
4. Combine 20 ng of the cassette with an equimolar amount of the 3′ part of the gene. Use this mixture as the template for an "overlap extension" PCR without adding primers. After 5 cycles, add the primers (F_{up} and $R_{down\text{-}cassette}$) to amplify this fragment and perform a standard PCR for 30 cycles (*see* **Note 8**).
5. Combine 20 ng of the cassette with an equimolar amount of the downstream fragment and perform an "overlap extension" PCR as described in **step 4**. In this case, the primer couple $F_{down\text{-}cassette}$ and R_{down} is used to amplify the fragment.
6. Repeat **step 3** for both fragments.

7. Combine 20 ng of both fragments and use this mixture as the template for an "overlap extension" PCR. After 5 cycles, add the primers F_{up} and R_{down} to amplify the entire construct.
8. Repeat **step 3** for this construct.
9. Use two primers situated in the middle of the cassette to sequence the construct and check if the annealing sites between the fragments contain no mutations (*see* **Note 9**).
10. If the construct is mutation free, clone the fragment (approx. 100 ng) into the pCR4-TOPO vector using the "TOPO TA cloning kit for sequencing" following the instruction manual.
11. Check the entire construct for mutations by sequence analysis (*see* **Note 10**).
12. Use 10–20 ng of the correct plasmid as a template for a standard PCR to gain sufficient amounts of the DNA construct (*see* **Note 11**).
13. Repeat **step 3**.

3.1.2 In Vivo Recombination

Once the DNA construct is ready it has to be transformed to *P. aeruginosa*. First, fresh electrocompetent *P. aeruginosa* cells are prepared using the method by Choi et al. [16]. Next, the construct is transformed to these cells using electroporation, after which homologous recombination occurs and the correct mutants are selected.

1. Inoculate 6 ml of LB/Cb^{200} with 120 µl overnight culture of *P. aeruginosa* PAO1 cells containing the pUC18-RedS plasmid.
2. At an optical density at 600 nm (OD_{600nm}) of 0.4, add 60 µl of 300 mM L-arabinose (final concentration of 0.2%) to induce the Red operon.
3. Divide the culture over four Eppendorf tubes (1.5 ml/tube) after 2.5 h of induction.
4. Spin the cultures (2 min, 13,000 rpm (16,000 × *g*)).
5. Remove the supernatant and dissolve the pellet in 1 ml of 300 mM sucrose (*see* **Note 12**).
6. Repeat subsequently **step 4**, **step 5** and **step 4**.
7. Remove the supernatant and collect the pellets into one tube by dissolving them in a final volume of 100 µl 300 mM sucrose (*see* **Note 13**).
8. Mix the 100 µl cells with 500–1000 ng of the construct and transfer them to a 0.2 cm electroporation cuvette (*see* **Note 14**).
9. Electroporate the cells with a pulse of 2.5 kV (*see* **Note 15**).
10. Add 500 µl of preheated (37 °C) LB to the electroporation cuvette and transfer the entire volume to a glass tube.

11. Shake the cells for 2 h at 37 °C to allow recombination to occur.
12. Plate 50 µl, 200 µl and the rest of the cells on selective medium (LB/Gm^{30}) and incubate overnight at 37 °C.
13. Pick the colonies, dissolve each of them in a small volume LB and streak them on LB /Gm^{30}. Incubate a second time overnight at 37 °C.
14. Pick a few single colonies and dissolve them in 100 µl LB/Gm^{30}. Perform a PCR on 5 µl of the culture and use DNA gel electrophoresis to confirm the correct insertion of the construct. Use primers that are situated 100 bp upstream and downstream of the insert to ensure insertion at the correct location in the genome (*see* **Note 16**).
15. Purify the PCR product and check the sequence for mutations by DNA sequencing analysis.
16. If the sequence is correct, store a 20% glycerol stock of the strain at −80 °C.

3.2 Verification of the Constructed Strains

3.2.1 Effect on the Bacterial Viability and the Infectivity of the Phage

Once a correct strain is constructed, the effect of the insert on the viability of the bacterial cells and the infectivity by *P. aeruginosa*-specific phages is tested. Neither of these parameters should experience an effect compared to a wild type *P. aeruginosa* strain. First the viability of the mutant is analysed.

1. Inoculate 4 ml of LB/Gm^{30} with 40 µl of an overnight culture of *P. aeruginosa* PAO1 *target*::*Strep*II and 4 ml of LB with 40 µl of an overnight culture of the wild type *P. aeruginosa* PAO1 strain.
2. Measure the OD_{600nm} every 20 min during 5 h, for both cultures.
3. Plot the OD_{600nm} in function of time and compare both curves. No differences should be present.

   Next, the effect on phage infection has to be investigated. For this, the "efficiency of plating" (EOP) is determined, using the double-agar method.
4. Mix 4 ml LB Soft with 200 µl of an overnight culture of *P. aeruginosa* PAO1 *target*::S*trep*II and 100 µl of a dilution of the phage (*see* **Note 17**).
5. Pour the mix on top of an LB agar plate (*see* **Note 18**).
6. Repeat **steps 4** and **5** by using the wild type *P. aeruginosa* PAO1 strain.
7. Incubate the plates overnight at 37 °C.
8. Count the number of plaques formed and determine the "plaque forming units" (PFU)/ml. Calculate the EOP as the ratio of the PFU/ml on the constructed strain to the PFU/ml on the wild type strain. The EOP should approximately be 1.

3.2.2 Production of the Strep-tag® II-Fused Protein

After verifying the viability and infectivity of the engineered strain, the presence of the tagged protein under physiological conditions is investigated (*see* **Note 19**). Therefore, a Western blot is performed on the cell lysate of the constructed cells (without phage infection). If the protein is produced, a signal should be detected when using monoclonal anti-*Strep*-tag® II antibodies which target the *Strep*-tag® II.

1. Inoculate 50 ml of LB/Gm^{30} in a 200 ml flask with 1 ml of an overnight culture of the engineered *P. aeruginosa* strain *target*::*Strep*II. As a negative control, inoculate 50 ml LB with an overnight culture of wild type *P. aeruginosa* PAO1 cells and follow the same procedure.
2. Grow the cells at 37 °C to an OD_{600nm} of 0.3 (*see* **Note 20**), transfer them to a 50 ml-tube and collect the cells by centrifugation (30 min, 4600 × *g*, 4 °C).
3. Discard the supernatant, dissolve the cell pellet in 500 µl TE buffer and transfer the sample to a 1.5 ml Eppendorf tube.
4. To lyse the cells, the sample is first subjected to one freeze-thaw cycle (*see* **Note 21**).
5. Subsequently, incubate the sample for 15 min at room temperature while gently agitating, after the addition of 10 µl of 5 mg/ml HEWL, 10 µl of 100 mM Pefabloc® SC, and 1 µl Benzonase® nuclease.
6. Sonicate the sample 8 times 5 s (amplitude 40%) and add 166 µl 4× loading buffer.
7. Boil the sample for 5 min at 95 °C (*see* **Note 22**).
8. Load 15–20 µl of the sample on a polyacrylamide gel and subject it to SDS-PAGE as described in 'Subheading 3.4 (*see* **Note 23**). Load 5 µl of the prestained reference ladder next to the sample (*see* **Note 24**). As a negative control, load 15–20 µl of the cell lysate of the wild type cells. As a positive control, load a fraction of a protein carrying a *Strep*-tag® II.
9. Prepare a Western blot "sandwich": Soak the foam pads in transfer buffer and put them on each side of the holder. Soak two Whatman papers (size of the foam pads) in transfer buffer and put them on each side of the holder. Soak the nitrocellulose membrane (size of the gel) in transfer buffer and put it on the side which will be connected with the positive pool of the power source. Soak the gel in transfer buffer and put it on the negative side of the holder. Close the holder (*see* **Note 25**–**27**).
10. Place the holder in a tank filled with cooled transfer buffer and run an electrical field of 100 V (350 mA) over it during 1 h–1 h 30 min (*see* **Notes 28** and **29**).

11. Place the membrane in a small box (approximately the size of the membrane) with the protein side facing up and block it by incubating the membrane with 50 ml blocking solution for 1 h at room temperature, while gently shaking.
12. Discard the blocking solution and rinse the membrane with PBST to remove the residues of blocking solution.
13. Incubate the membrane at room temperature for 1 h with 10 ml PBST to which 2 μl monoclonal anti-*Strep*-tag® II antibodies was added (1:5000 dilution), while gently agitating (*see* **Note 30**).
14. Discard the solution and wash the membrane by incubating it three times with 10 ml PBST for 3 min while agitating and rinse the membrane with water.
15. Mix detection solutions A and B to a 1:1 ratio and drop 2 ml onto the protein side of the membrane (*see* **Note 31**). Incubate for 2 min at room temperature.
16. Drain off the detection solution and dry the membrane by gently shaking (*see* **Note 32**).
17. Place the membrane between to transparent papers and place it in the audioradiography cassette with the protein side of the membrane facing up.
18. Bring the cassette to the dark room, place a sheet of X-ray film on the membrane (*see* **Note 33**) and close the cassette.
19. Allow exposure of the chemiluminescent reaction on the film (3–20 min) (*see* **Note 34**).
20. Develop the film by placing it in developing solution while gently shaking.
21. Once a good signal is visible, rinse the film with water and place the film in fixation solution until the film becomes completely transparent.
22. Rinse the film with water, place it in a rack and let it dry.
23. Repeat **steps 18–22** two to three times to optimize the results.

3.3 Affinity Purifications

To search for protein–protein interactions between bacteria and their phages, the target proteins are purified by affinity purification. Therefore, the engineered strains are infected with phages and the infection cycle is stopped at the early stage of infection, since it is believed that most host-phage protein-protein interactions occur at this stage [1, 11]. To not disturb the interactions, a mild lysis of the cells is performed, followed by a pull-down of the target protein/complex and all its interaction partners.

1. Inoculate 600 ml LB/Gm^{30} in a 2 l flask with 8 ml of an overnight culture of the engineered *P. aeruginosa* strain *target*::*Strep*II.

2. Grow the cells at 37 °C to an OD_{600nm} of 0.3, infect them with a *P. aeruginosa* specific phage (MOI 5-10) and incubate at 37 °C (*see* **Note 35**).
3. Stop infection at the early stage of infection by chilling the culture in an icy water bath during 5–10 min (*see* **Note 36**).
4. Transfer the culture to an ice-cold tube and spin the culture (4600 × *g*, 45 min, 4 °C) (*see* **Note 37**).
5. Discard the supernatant and resuspend the cell pellet in 8 ml resuspension buffer supplemented with 100 µl of 100 mM Pefabloc® SC and 500 µl of 20 mg/ml HEWL (*see* **Note 21**).
6. Subject the sample to one freeze-thaw cycle.
7. Add 10 µl Benzonase® nuclease and 800 µl 10× BugBuster® Protein extraction reagent and incubate the sample for 10–20 min at room temperature while gently agitating (*see* **Note 38**).
8. Spin the sample in ice-cold Eppendorf tubes (30 min, 16,000 × *g*, 4 °C), collect the supernatant and place on ice.
9. Prepare the affinity purification column: Add 1 ml *Strep*-Tactin® Sepharose beads to a 10 ml Bio-Rad Poly-Prep® Chromatography column. Wash the beads two times with 2 ml of wash buffer (*see* **Note 39**).
10. Load the supernatant on the column, collect the flow through (FT) and store at 4 °C.
11. Wash the beads five times with 1 ml wash buffer. Collect the wash fractions in separate Eppendorf tubes (W1-5) and store at 4 °C.
12. Elute the proteins with elution buffer in six fractions of 500 µl. Collect the elution fractions in separate Eppendorf tubes (E1-6) and store at 4 °C.
13. Regenerate the column by adding three times 5 ml of regeneration buffer and twice 4 ml of wash buffer. Close the column, add 2 ml wash buffer and store the column at 4 °C.
14. Concentrate the elution fractions by ultrafiltration (Amicon Ultra-0.5 ml Centrifugal filter, 3 kDa).

3.4 SDS-PAGE

The eluted fractions are subsequently subjected to SDS-PAGE. The one-dimensional separation of the proteins present in the samples, allows a first analysis of the composition of the eluted fractions. Moreover, gel electrophoresis removes low molecular weight impurities, including detergents and buffer components, which are often not compatible with downstream mass spectrometry analysis.

1. Prepare a 12% SDS-PAGE gel: Pour the separation gel mixture between the two glace plates of the Mini-PROTEAN® Tetra Cell Systems (*see* **Note 40**). Add a small layer of isopropanol

and wait until the gel has solidified. Remove the isopropanol and pour the stacking gel mixture (*see* **Note 40**). Place the comb insight and wait until the gel has solidified.

2. Place the gel in the holder and subsequently place the holder in the tank. Fill the tank with running buffer.
3. Suspend aliquots of 10–15 µl proteins in SDS-PAGE 4× loading buffer and denature by heating them at 95 °C for 5 min.
4. Load the protein samples in the wells (after removal of the comb) and run an electric field of 200 V until the electrophoresis front reaches the bottom of the gel (*see* **Note 41**).
5. Remove the gel from the glace plates, place it in a box and wash the gel with water for 15–30 min.
6. Stain the SDS-PAGE gel for 0.5–2 h with a MS-compatible standard Coomassie stain like GelCode Blue Safe Stain (Thermo Fisher Scientific).
7. Wash the gel overnight with water, to reduce the background stain.

3.5 Mass Spectrometry Analyses

Lastly, the composition of the samples has to be identified. Therefore, gel pieces are sliced from the SDS-PAGE gel and subjected to an in-gel trypsin digestion. Afterward, the obtained peptides are analysed by LC-MS/MS analyses. During the experiment, gloves must be worn at all times, and contact with skin, hair, and clothes should be avoided (*see* **Note 42**). Moreover, keratin-free materials should be used.

1. Excise protein bands (8–13 spots in total) from the gel using a 1000 µl micropipette after widening the opening of the tip with a scalpel (*see* **Note 43**–**45**).
2. Transfer each gel piece into a separate 1.5 ml Eppendorf tube.
3. Remove the residual water, submerge each gel piece in 100 µl 25 mM NH_4HCO_3 in 50% acetonitrile, and incubate for 10 min at room temperature.
4. Remove the liquid, and repeat **step 3** until the Coomassie blue is completely removed from the gel pieces (approx. 3 times).
5. Dry the pieces in a vacuum centrifuge for 10–15 min at 40 °C (*see* **Note 46**).
6. Submerge the pieces in 30 µl 10 mM DTT in 100 mM NH_4HCO_3 to reduce all disulfide bonds (reduction) and incubate for 1 h at 56 °C.
7. Cool the samples to room temperature and remove the liquid.
8. Submerge the pieces in 30 µl 55 mM IAA in 100 mM NH_4HCO_3 to modify cysteine residues and prevent reformation of disulfide bonds (alkylation).

9. Incubate for 45 min in the dark, shake every 10 min using a vortex. Remove the liquid afterward.
10. Add 100 µl 100 mM NH_4HCO_3, incubate for 10 min, and remove the liquid (hydration).
11. Add 100 µl acetonitrile, incubate for 10 min, and remove the liquid (dehydration).
12. Repeat **steps 10** and **11**.
13. Dry the pieces in a vacuum centrifuge for 10–15 min at 40 °C.
14. Submerge the pieces in 10 µl trypsin digestion buffer, and incubate for 45 min in an icy water bath.
15. Add 30 µl 50 mM NH_4HCO_3, and incubate overnight at 37 °C.
16. Collect the supernatants containing the tryptic peptides in new Eppendorf tubes, one for each gel piece.
17. Submerge the pieces in 20 µl 20 mM NH_4HCO_3, sonicate 20 min in a sonicator bath and collect the supernatants in the corresponding tubes (*see* **Note 47**).
18. Submerge the pieces in 50 µl 5% formic acid in 50% acetonitrile, sonicate 20 min in a sonicator bath and collect the supernatants in the corresponding tubes (*see* **Note 47**).
19. Repeat **step 18**.
20. Store the collected supernatants at −20 °C until mass spectrometry analyses can be performed (*see* **Note 48**).
21. To identify the peptides present in the samples, send the samples to a mass spectrometry facility for analysis. In this case, an Easy-nLC 1000 liquid chromatograph (Thermo Fisher Scientific), which is on-line coupled to a mass calibrated LTQ-Orbitrap Velos Pro (Thermo Fisher Scientific), is used at the Biomedical Research Institute and Transnational University Limburg (Hasselt University, Belgium) [17].
22. RAW data are analyzed with the Proteome Discoverer software version 1.3 (Thermo Fisher Scientific) with build-in Sequest and interfaced with an in-house Mascot v.2.4 server (Matrix Science). MS/MS spectra were searched against a database containing all *P. aeruginosa* PAO1 proteins and all "stop-to-stop" protein sequences in all six frames of all phages.

4 Notes

1. The phage stock does not need to be ultrapure (e.g., cesium chloride centrifugation); instead a PEG-precipitated stock can be used.

2. The transfer buffer is stored at 4 °C and can be reused a few times.
3. A 10× PBS buffer without Tween can be made and stored at room temperature. Tween is freshly added to the PBS at the moment of use.
4. The buffer is stored at 4 °C.
5. Light sensitive: store in a dark place.
6. In total, six different primers have to be developed (*see* Fig. 2). Two primers ($F_{down\text{-}cassette}$ and $R_{down\text{-}cassette}$) to amplify the fragment containing the *Strep*-tag® II-tag and the Gm^R gene. $F_{down\text{-}cassette}$ starts with the *Strep*-tag® II-tag sequence followed by a stop codon. $R_{down\text{-}cassette}$ comprises the C-terminal part of the Gm^R gene. Two primers (F_{up} and $R_{up\text{-}cassette}$) to amplify the C-terminal part of the target gene (approx. 300 bp). $R_{up\text{-}cassette}$ contains the C-terminal sequence of the target gene (without stop codon!) fused to the 5′-region of the fragment containing the *Strep*-tag® II-tag and the Gm^R gene. Likewise, both fragments will share 18–25 bp homology. Equally, two primers ($F_{down\text{-}cassette}$ and R_{down}) are designed to amplify the region downstream of the target gene (approx. 300 bp). In this case, $F_{down\text{-}cassette}$ shares 18–25 bp homology with the 3′-region of the fragment containing the *Strep*-tag® II-tag and the Gm^R gene.
7. If there are multiple bands, use the gel excision kit to select the fragment of the correct size.
8. For the "overlap extension" PCR it is important to use equimolar amounts of the fragments that you want to fuse, which corresponds to an equal number of DNA molecules. Therefore, you have to take into account the size of the fragments. For example, 10 ng of a 1000 bp fragment is equimolar to 1 ng of a 100 bp fragment.
9. Design and use primers which are situated in the Gm^R cassette and located at a distance of 200–300 bp to the overlap regions.
10. Use all six primers used for the design of the construct to ensure full sequence coverage of the construct.
11. It is sometimes necessary to repeat the amplification to get sufficient amounts of construct, which is around 1 μg of DNA.
12. Be careful when removing the supernatant, since the pellet loses its consistency after the multiple washing steps.
13. First dissolve pellet of one tube in 100 μl 300 mM sucrose and then transfer solution to second tube.
14. Never add more than 3 μl of DNA construct due to the risk of arcing caused by high salt concentrations. When the

concentration of the DNA construct is to low, first apply an ethanol precipitation on the sample to increase the final concentration.

15. The time constant should be between 4.8–5.1 ms. Increasing the number of washing steps can increase this value.
16. If the PCR on the cells fails, it is recommended to first perform a genome extraction on an overnight culture of the cells. The attained genomic DNA can then be used as template which will yield better results.
17. The dilution of the phages should contain about 100 of PFU/ml. This amount depends on the size of the plaques formed, since the plaques formed on the plate should be countable. If necessary, several dilutions can be plated.
18. Ensure that the soft agar forms an even layer on top of the plate, which helps for the counting of plaques.
19. The detectability of the tagged proteins is verified under the same conditions that will be used for the affinity purifications.
20. An OD_{600nm} of 0.3 was used, since this is the OD_{600nm} at which the cells will be infected with the phages prior to the affinity purifications in Subheading 3.3.
21. At this point, the cells can be stored at −80 °C.
22. At this point, the sample can be stored at −20 °C.
23. Depending on the size of the target protein, the percentage of the gel might be adjusted: 8% for high molecular weight proteins, to 15% for low molecular weight proteins.
24. A prestained ladder is chosen, since it will be visible on the membrane after blotting. Likewise, the ladder can be used to verify that the proteins are successfully transferred to the membrane during the Western blot.
25. During the Western blot procedure it is important to wear gloves.
26. It is advised to cut one corner of the membrane to recognize the front and back side after transfer of the proteins.
27. After closing the "sandwich," air bubbles between the gel and the membrane should be removed by rolling a rod over it.
28. To keep the temperature low, the tank can be placed on ice or a Cooling Unit can be placed inside the tank.
29. The time of transfer depends on the size of the protein. For proteins with a high molecular weight, a longer transfer time is needed. The transfer can be verified by the presence of the prestained ladder.

30. The dilution of antibody that should be used and time of incubation depends on the brand and the antibody, and might be optimized prior to the experiment.
31. The detection solutions should always be kept on ice.
32. To take the membrane, a pincer should be used.
33. Cut one corner of the membrane and place it in the same direction as the corner of the membrane.
34. The time of exposure should be optimized each time. Therefore, 2 or 3 films can be used.
35. The "multiplicity of infection" (MOI) is the ratio of the amount of the phages (PFU) versus the amount of bacterial cells (CFU, "colony forming units") at the time point of infection. The desired MOI should be optimized for each phage prior to the large-scale affinity purification. Therefore, the amount of bacterial cells (CFU/ml) 5 min after phage infection should be reduced to less than 5% of the CFU/ml before infection, to accomplish a successful and synchronic infection.
36. The early stage of phage infection is estimated approximately as 1/3e of the length of the infection cycle.
37. To ensure that the infection cycle is stopped in the early phage of infection, it is very important that the sample stays cool until the cells are lysed.
38. The cells are lysed when the sample turns from a turbid to a clear solution.
39. All buffers should have the same temperature as the column to avoid the formation of air bubbles. To be sure, the purification can be done at 4 °C, however, the procedure can also be performed on the bench at room temperature.
40. APS and TEMED are added just before pouring the gel. Invert a few times to obtain a well-mixed solution.
41. Take care that the electrophoresis front (potentially containing small phage proteins) does not run off the gel.
42. To avoid keratin contamination, gloves and keratin-free materials should be used. Moreover, it might help to perform all manipulations under a hood.
43. If the protein bands are not clearly visibly, it might help to collect all elution fractions in which proteins are present, concentrate them by ultrafiltration and perform a new SDS-PAGE analysis prior to the in-gel digestion.
44. Take a picture before and after isolation of the protein bands to visualize the localisation of the picked spots on the SDS-PAGE gel.

45. As an alternative to manually slice the gel, a number of automated spot pickers are available as well.
46. Dry gel pieces become white and loosen from the wall of the Eppendorf tube. The dried gel pieces can be stored at −20 °C for a few months until further analysis is performed.
47. All supernatants originating from the same gel piece are collected in one Eppendorf tube.
48. Do not discard the extracted gel pieces, but store the gel pieces at −20 °C until MS analyses are performed. If the digestion fails, it can be repeated with the same gel pieces [18].

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Chapter 11

Techniques to Assess Phage–Biofilm Interaction

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Abstract

Biofilms are ubiquitous in nature found on nearly every type of living and inert surface. They basically consist of microorganisms attached to surfaces and surrounded by a self-produced matrix of extracellular polymeric substances. Phages have proven to be successful in controlling biofilms. Here, we describe methods to characterize phage–biofilm interactions, specifically to assess biofilm biomass and to visualize the biofilm structure, discriminating infected cells using targeted molecular probes.

Key words Bacteriophage, Bacteria, Biofilm, Control, Microscopy

1 Introduction

Biofilms are an important bacterial survival strategy and are reported frequently as a major virulence factor in pathogenic bacteria. Biofilms are surface-associated bacterial communities encased in a hydrated extracellular matrix which helps maintenance of the complex heterogeneous structure, which confer physical protection against external stresses [1]. Biofilms have great clinical relevance, and are responsible for numerous pathologies as those associated with the use of medical devices [2–4].

The growing interest in bacteriophages (phages), as biofilm control agents relies on the fact that lytic phages are capable of penetrating the three dimensional structure of the biofilm and killing biofilm associated cells, that are difficult to target by conventional antibiotic [5–7].

There are not many direct visualization methodologies to assess phage–host interactions in biofilms. The use nucleic acid mimics (peptide nucleic acid (PNA) and locked nucleic acid (LNA)) with fluorescence in situ hybridization (FISH) can be a good option for the detection of microbial cells within a biofilm. The FISH technique is based in molecular probes to target a specific sequence within a cell [8]. Recently, studies based on microscopy using these

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probes allowed the understanding of interspecies interactions, and also spatial distribution of species in polymicrobial communities [9, 10]. The use of selective probes associated with FISH is clearly a state-of-the-art technology in biofilm research, showing many advantages. Indeed, they can be applied directly in naturally occurring biofilms, maintaining the structure of the biofilm. A large number of fluorescent labels can be attached to the probes, allowing multiplex experiments to be performed without a need of altering the microorganisms and their behavior during the experiment. Furthermore this technology can be exploited to characterize phage/biofilm interaction since it is possible to target phage mRNA during replication inside their hosts making infected cells fluorescent.

In this chapter, protocols to characterize phage–biofilm interaction are presented focusing not only in biofilm formation and biomass characterizations (Subheading 3.1), but also assessment of phage efficacy after biofilm infection/control experiments (Subheading 3.2). Finally, a microscopy protocol (Subheading 3.6) using specific probes that has become an excellent tool for the study of phage–host interactions by allowing the analysis of different aspects of these communities, is detailed. The microscopy techniques described can be adapted to different conditions of biofilm formation (e.g., materials support, media used, medium replacement and incubation times) as well as the different treatments with phage (MOI, single phage, phage cocktails and combination phage with antibiotics).

2 Materials

2.1 Biofilm Formation

1. 24-well microplate(s) (*see* **Note 1**).
2. Sterile culture medium (*see* **Note 1**).
3. Overnight grown bacteria. Transfer a loopful of the host bacterium to 100 mL Erlenmeyer containing 25 mL of sterile culture medium and incubate 16 h at the proper host growth temperature.
4. Microplate reader (600 nm filter).
5. Sterile cell scrapers.
6. Incubator with rocker or orbital incubator.
7. Sterile saline solution (0.9% wt./vol. NaCl).
8. Sonication bath.

2.2 Biofilm Control with Bacteriophages

1. 24-well microplates containing biofilms.
2. Bacteriophage.
3. Sterile culture medium.

4. Sterile cell scrapers.
5. Sonication bath.
6. Incubator with rocker or orbital incubator.

2.3 Bacteriophage Titration

1. Sterile SM Buffer (5.8 g l^{-1} NaCl, 2 g l^{-1} $MgSO_4.7H_2O$, 50 mL l^{-1} 1 M Tris–HCl pH 7.5).
2. 96-well microplates.
3. Sterile molten top-agar *(MTA)*.
4. Petri plate containing a thin layer of agar with appropriate growth media.
5. Overnight grown bacteria.
6. Static incubator.

2.4 Biofilm Cell Enumeration

1. Agar plates with appropriate growth media (20 plates).
2. 96-well microplates.
3. Sterile saline solution: 0.9% NaCl.
4. Static incubator.

2.5 Biomass Quantification by the Crystal Violet Assay

1. Biofilms of the bacteria of interest.
2. Sterile saline solution: 0.9% NaCl.
3. Methanol.
4. Crystal violet solution 1% (vol./vol.) prepared in water.
5. Acetic acid 33% (vol./vol.) prepared in water.
6. Sterile deionized water.
7. Microplate reader (570 nm filter).

2.6 Biofilm Fixation for Microscopy Techniques

1. Biofilms of the bacteria of interest prepared in coupons (*see* **Note 1**).
2. Methanol 100% (v/v).
3. Tissue paper.
4. Phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, at pH 7.2).
5. Paraformaldehyde 4% (vol./vol.) prepared in PBS.
6. Ethanol 50% (vol./vol.) prepared in water.
7. Probe: Dissolve the original probe aliquot (lyophilized) in 10% acetonitrile and 1% trifluoroacetic acid at a final concentration of 100 μM. Prepare a probe stock solution at 4 μM, adding 40 μL of the original solution to 960 μL of ultrapure water. Use the stock solution to prepare a probe working solution at 200 nM, in hybridization solution (10% dextran sulfate, 10 mM NaCl, 30% formamide, 0.1% sodium pyrophosphate, 0.2% polyvinylpyrrolidone (Sigma-Aldrich), 0.2% (wt./vol.)

Ficol, 5 mM disodium EDTA, 0.1% Triton X-100, 50 mM Tris–HCl (pH 7.5)). Protect aliquots from light exposure.

8. DAPI (4,6-diamidino-2-phenylindole): Prepare a stock solution of DAPI (Life Technologies/Thermo Fisher Scientific, USA; https://www.thermofisher.com/) at 5 mg/mL in distilled water. Prepare a working solution at 100 μg mL^{-1} in PBS or deionized water.
9. Sterile distilled water.
10. Washing solution (5 mM Tris base, 15 mM NaCl, and 1% Triton X-100, (pH 10)).
11. Petri dishes wrapped in aluminum foil and with moist absorbent paper inside.
12. Coverslip.
13. Static incubator.
14. Coplin jar.
15. Immersion oil.

3 Methods

Carry out all procedures at room temperature, unless otherwise specified.

3.1 Biofilm Formation

Biofilms samples are prepared in sterile culture medium and washed with the appropriate buffer, after which they can be directly stained in the adhesion support or stained after a fixation step to evaluate biofilms structure, or for a quantitative approach, after sonicated in the buffer, determine the amounts of bacteria present in biofilms (CFU) and also measure plaque-forming unit (PFU) counts in biofilm infected.

1. Add to a 24-well microplate 1 mL of sterile culture medium.
2. Add 10 μL of a bacterial culture grown overnight diluted to an $O.D._{600}$ of 1.0.
3. Incubate the plate in an incubator at appropriate temperature conditions and under agitation (120 rpm) during the desired period of time (e.g., 24 h–7 days). For periods longer than 24 h replace the media (remove all media by pipetting and add 1 mL of fresh medium) to remove planktonic bacteria and enhance biofilm formation.
4. At the end of the desired biofilm formation period, remove all media and planktonic bacteria by pipetting.
5. Wash the wells twice with 1 mL of saline solution.

6. Resuspend in 1 mL of saline solution.
7. Use a cell scraper to scrape the biofilm from the well surface.
8. Put the 24-well microplate on a sonication bath for 5 min (*see* **Note 2**).
9. Quantify the viable cells present in the biofilms as described below (Subheading 3.4) (*see* **Note 3**).

3.2 Biofilm Control with Bacteriophages

After quantifying the numbers of viable cells in the biofilms, in at least three independent experiments were performed in triplicate, the efficacy of bacteriophages for biofilm control can be evaluated. To maintain the infection parameters identical between the experiments, a constant initial multiplicity of infection (MOI) must be used. MOI is calculated according to the number of bacteriophages per number of viable host cells and for instance, an MOI of 1 represents that there is one bacteriophage to each host cell (*see* **Note 4**).

1. After biofilm formation and washing (Subheading 3.1), add 950 μL of sterile media and 50 μL of bacteriophage at a proper concentration to ensure the desired constant multiplicity of infection (MOI) (*see* **Note 5**).
2. Incubate the plate in an incubator with an orbital shaker, at the proper temperature during at least 4 h (*see* **Note 6**).
3. Take samples to quantify the numbers of bacteriophages and viable cells (*see* Subheadings 3.3 and 3.4), respectively, present in the planktonic stage (*see* **Note 7**).
4. Remove the spent media and wash twice with saline solution, to remove unattached bacteria and phages.
5. Add 1 mL of fresh saline solution.
6. Use a cell scraper to scrape the biofilm from the surface.
7. Put the 24-well microplate on a sonication bath for 5 min.
8. Take samples to quantify the numbers of bacteriophages and viable cells (*see* Subheadings 3.3 and 3.4), respectively, present in the biofilm.

3.3 Bacteriophage Titration

During infection, bacteriophages can be found in both planktonic and biofilms and should be quantified.

1. Prepare successive serial dilutions (1:10) in SM buffer of the bacteriophage solutions (add 20 μL bacteriophage solution and 180 μL of SM buffer to a 96-well microplate).
2. Add to a test tube 100 μL of diluted bacteriophage solution, 100 μL of overnight grown bacteria, and 3–5 mL of MTA (47 °C) and tap gently.
3. Pour the mixture into a petri plate containing a thin layer of agar with medium and swirl carefully.

4. Let the plates dry for 1–2 min.
5. Incubate inverted overnight under optimal temperature conditions.
6. Count the bacteriophage plaques in the dilution which resulted in 20–200 plaques.
7. Determine the titer of triplicate preparations according to Eq. 1.

$$\text{Bacteriophage titer (PFU per mL)} = \frac{\text{No. of plaques} \times \text{Dilution factor}}{\text{Volume of phage sample (mL)}} \quad (1)$$

3.4 Biofilm Cell Enumeration

1. In 96-well plates, serially dilute the bacterial samples in sterile saline solution (20 μL of sample in 180 μL of saline solution).
2. Add a drop of 20 μL of sample on placed in Petri plate containing solid medium (*see* **Note 8**).
3. Allow the drop to dry completely.
4. Incubate overnight at the proper growth temperature for 16–18 h.
5. Count the colonies formed in the drop of the dilution with 3–30 colonies.
6. Calculate the number of viable cells using Eq. 2.

$$\text{No. of viable cells (CFU per mL)} = \frac{\text{No. of colonies} \times \text{Dilution factor}}{\text{Volume of sample (mL)}} \quad (2)$$

3.5 Biomass Quantification by the Crystal Violet Assay

1. After biofilm washing step (Subheading 3.1) add methanol (1 mL) to each well and allow fixation of biofilms to occur for 15 min.
2. Remove the methanol and allow the wells to dry at room temperature for about 20 min.
3. Add 1 mL of 1% crystal violet to each well and incubate for 5 min at room temperature without shaking.
4. Remove the excess of crystal violet with tap water.
5. Wash the wells twice with 1 mL of deionized water and allow the wells to dry at room temperature.
6. Solubilize the dye crystals formed inside the cell by adding 1 mL of 33% acetic acid to each well.
7. Read the absorbance at 570 nm, using 33% acetic acid as blank.

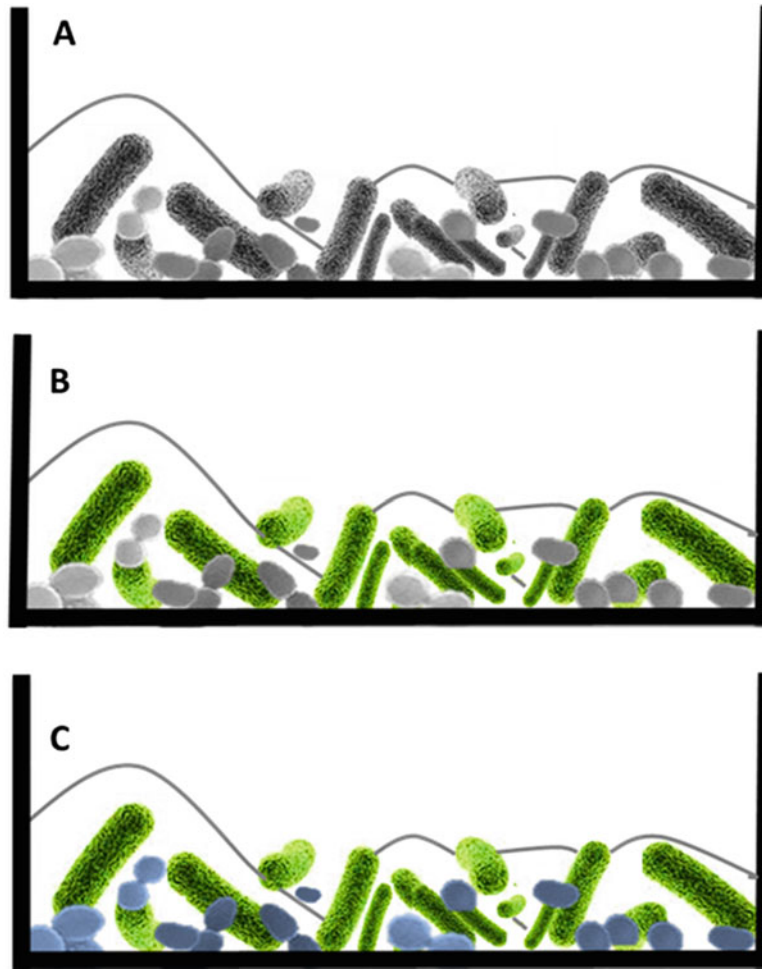

Fig. 1 Staining biofilm. (**a**) Biofilm fixation; (**b**) PNA FISH hybridization; (**c**) DAPI staining

3.6 Biofilm Fixation for Cell Microscopy Techniques

After biofilms formed and washed (*see* Subheadings 3.1 and 3.2), the biofilm is fixed directly on the coupon and using PNA FISH (specific stains) and DAPI staining (nonspecific stain) to assess the biofilm spatial organization and the species distribution.

3.6.1 Hybridization Procedure in the Adhesion Substrata

1. After washing the biofilms with the saline solution (*see* Subheadings 3.1 and 3.2), apply a volume of methanol sufficient to cover the entire surface (in this case add 1 mL in each well) (Fig. 1a).
2. Incubate at room temperature during 15 min (*see* **Note 9**).
3. Remove excess of methanol with a paper towel, allow to air dry.
4. Apply enough paraformaldehyde, to cover the surface and let it soak for 10 min. Remove paraformaldehyde excess as described above.

5. Cover with ethanol, leave for 10 min and remove ethanol excess and leave to air-dry.
6. Place coupons in petri dishes previously wrapped in aluminum foil and with moist absorbent paper inside.

3.6.2 PNA FISH Hybridization and DAPI Staining

1. After fixation of the biofilm samples (*see* Subheading 3.6.1), apply 20–40 μL of probe working solution in the well, cover with a coverslip, and incubate for 90 min at the proper hybridization temperature in Petri dishes previously wrapped in aluminum foil and with moist absorbent paper inside (Fig. 1b) (*see* **Note 10**).
2. Fill a Coplin jar with washing solution and place in the hybridization chamber, together with the coupons, to heat.
3. Remove coupons from the Petri dishes, remove the coverslip and immerse coupons in the wash solution. Incubate for 30 min.
4. Remove coupons from the Coplin jar and allow to air dry (in the dark).
5. Apply 100 μL of DAPI, and incubate for 10 min in dark (Fig. 1c) (*see* **Note 11**).
6. Wash the samples with PBS, drain excess buffer, leave to air-dry.
7. Place the immersion oil and coverslip, and observe by fluorescence (fluorescence microscopy and/or confocal microscopy) using an appropriate filter for the fluorochrome coupled to the probe (*see* **Notes 12** and **13**).

4 Notes

1. Biofilms can also be formed on a variety of different substrates. In this case, coupons of the different materials (stainless steel, glass, rubber, silicone, acrylic, etc.) can be cut and placed on the wells. The use of coupons allows the observation, in a simple and direct way, of the biofilm structure by microscopy, since the coupons can be easily removed from the wells. For biofilm formation, microplates can be used (e.g., 6-well, 12-well, 24-well, 48-well, and even 96-well microplates). There are different types of media for growing different types of cells.
2. The time for sonication should be optimized to promote the complete removal of all the biofilm-encased cells without causing lysis (e.g., CFU counts vs sonication time and biomass determination by crystal violet should be performed).

3. It is necessary to quantify the number of viable cells in order to allow the researcher to use a constant MOI throughout all the infection assays.
4. The MOIs commonly used vary between 0.1 and 1000 but other bacteriophage–host ratios can be tested.
5. In case of control experiments, add 950 μL of sterile media and 50 μL of SM buffer.
6. According to the results from different authors, the maximum cell lysis is obtained after 4–5 h and after this point there can be observed an increase of cell growth due to the present of phage resistant phenotypes.
7. The infection of biofilms with bacteriophages results often in the release of cell clusters to the planktonic phase and therefore this should be assessed.
8. Alternatively, to facilitate counting of the colonies after placing the drop in a Petri plate containing solid medium, tilt the plate 45 °C allowing the drops to run down the plate.
9. This step is very important to avoid biofilm detachment during the hybridization process. For nonspecific dyes (e.g., DAPI) that can easily diffuse through the membrane and cell wall, a preliminary fixation step is not necessary. Also, a preliminary fixation step for assessment of viability is not recommended. When it comes to specific staining such as FISH, a preliminary fixation step is necessary to permeabilize the cells through the opening of pores in the cell wall.
10. The hybridization time may be lower, depending on the efficiency of the probe used.
11. Whenever a multiplex approach is desired (contra-staining, distinguishing two or more groups of microorganisms simultaneously), DAPI staining must be the last to be held and should begin staining immediately after the drying step (*see* Subheading 3.5, **step 1**). In case not want a posterior staining of the FISH procedure, must immediately pass to step. The use of DAPI must be handled with care due to their carcinogenic properties, and volume added should be adjusted depending on the size of the adhesion support.
12. The coupons can be stored in the dark for a maximum of 24 h before microscopy in petri dishes previously wrapped in aluminum foil and with moist absorbent paper inside.
13. The immersion oil used should contain an antifade reagent that prevents bleaching of the sample (phenomenon of photochemical destruction of a fluorochrome).

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Chapter 12

Screening for Growth-Inhibitory ORFans in *Pseudomonas aeruginosa*-Infecting Bacteriophages

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Abstract

Like all viruses, bacteriophages heavily depend on their host's physiology for reproduction. Therefore, phages have evolved numerous proteins that influence the host metabolism to facilitate the infection process. Some of these proteins strongly perturb the host cell, ultimately leading to cell death. These growth-inhibitory phage proteins presumably target key metabolic processes, which may provide a basis for innovative phage-derived antibacterials. Unfortunately, most of these proteins are the so-called ORFans, since they have no known function or sequence homology to any other gene. We here describe a screening method for the identification of growth-inhibitory ORFans of bacteriophages infecting gram-negative bacteria (e.g., *Pseudomonas aeruginosa*), using the pUC18-mini-Tn7T-Lac vector system, which allows for stable single-copy integration of the phage ORFans in the *Pseudomonas* genome under the control of an IPTG-inducible promoter. Furthermore, we describe a method to examine the effect of the phage proteins in different hosts, using different vector copy numbers. Finally, we explain how to investigate the effect of ORFan expression on the host morphology using time-lapse microscopy.

Key words Bacteriophage, *Pseudomonas aeruginosa*, Gateway cloning, Chromosomal integration, Spot test, Bio-screen, Time-lapse microscopy

1 Introduction

Strictly lytic bacteriophages rely heavily on the bacterial metabolism for their propagation. From the start of infection, phages must establish a favorable environment for replication and counter several bacterial defense mechanisms. Therefore, phages have evolved an incredible number of highly diverse proteins that either inhibit or adapt bacterial metabolic processes to their own benefit [1]. Although not all of these interactions are detrimental to the host cell, many of them do lead to cell-cycle arrest or even host lethality. As such, a novel source of antibacterials might originate from mining the thousands of available sequenced phage genomes and can reveal functional insights into phage biology.

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Since not all of these protein–protein interactions are targeted toward crucial bacterial proteins and thus have detrimental effects on the host cell, we hypothesize that phage proteins which are growth-inhibitory to their host when individually expressed, show the most promise in tackling crucial metabolic pathways. Therefore, the pUC18-mini-Tn*7*T-Lac vector system is used to screen phage proteins for their effect on *P. aeruginosa* growth. This *E. coli—P. aeruginosa* shuttle system permits stable single-copy integration of the phage genes in the *Pseudomonas* genome under the control of an IPTG-inducible promoter [2]. Single-copy expression is preferred over high-copy expression to reduce the number of false positive results due to unnaturally high amounts of recombinant protein in the host cell and growth retardation associated to recombinant expression. Moreover, the expression cassette encodes the $lacI^q$ gene to limit leaky expression [3], which is preferable when screening for growth inhibitory proteins. A gentamicin resistance cassette allows for selection in *P. aeruginosa*. However, it is also stably maintained in the absence of selection. Insertion of the expression cassette does not influence expression of the neighboring *P. aeruginosa* genes [2]. By identification of their host target and exploring their mode of action, these toxic phage ORFans could ultimately lead to the development of novel antibiotics in the near future and reveal functional information [4].

To make the screening for indispensable phage–host interactions more feasible, some rational criteria can be used to reduce the number of proteins of interest. Although bacteriophage–host interactions are involved in practically all stages of the infection cycle, most interactions are hypothesized to take place during the early stages [1]. Apart from the selection for early-expressed phage proteins, additional criteria for an ORFan to be selected may include that (1) it has to be smaller than 250 amino acids (a survey of 52 described phage–host interactions revealed that 90% concerns phage proteins of this size [1]), (2) no functional prediction, e.g., DNA metabolism or structural protein, is known (3), it does not have an obvious predicted toxic effect (e.g., nuclease, lysins) and that (4) the location is predicted to be in the cytoplasm.

This chapter describes a method to screen for phage ORFans having an inhibitory effect on their host's cell growth. Although *Pseudomonas aeruginosa* PAO1 is used as the target organism, this method can readily be adapted to screen for antibacterial proteins against other pathogenic bacteria for which phages are available. The only prerequisite is the availability of compatible expression vectors. We first describe the cloning of the ORFans as single copy expression cassettes integrated in the *Pseudomonas aeruginosa* genome using Gateway cloning (ThermoFisher Scientific, Waltham, Massachusetts, USA). Moreover, the identification of inhibitory phage ORFans is described. Furthermore, the effect of the

phage proteins on different hosts, using different vector copy numbers is explained. Finally, the effect of ORFan expression on the host cell morphology is investigated using time-lapse microscopy, which can reveal different phenotypes, e.g., filamentation, delayed growth, or growth arrest of *P. aeruginosa*.

2 Materials

2.1 Cloning of the Phage ORFan

1. Chemically competent *E. coli* cells, e.g., One Shot TOP10 chemically competent *E. coli* (ThermoFisher Scientific, Waltham, Massachusetts, USA).
2. pUC18-mini-Tn*7*T-Lac vector and the Gateway Vector Conversion System (ThermoFisher Scientific).
3. LB medium: 10 g tryptone, 5 g yeast extract, 10 g NaCl, (15 g agar). Bring up to 1 L with demineralized water and autoclave. Cool down to room temperature before adding antibiotics.
4. Antibiotics: 1000× stock of kanamycin sulfate (50 mg/ml; final concentration for selection: Km^{50} = 50 μg/ml) and 1000× stock of ampicillin (100 mg/ml; final concentration for selection: Amp^{100} = 100 μg/ml).
5. Ultrapure water.
6. Mineral oil.
7. Glycerol.
8. Agarose.
9. Phage DNA as PCR template.
10. Primers:

    ORFan primers

    pENTR_F: GCGGCCGCCTTGTTTAAC

    pENTR_R: GTCGGCGCGCCCACCCTT

    pUC18-mini-Tn*7*T-LAC_F: CGGTTCTGGCAAATATTCTGA

    pUC18-mini-Tn*7*T-LAC_R: GGAGGGGTGGAAATGGAGTT.
11. High fidelity DNA polymerase.
12. Taq DNA polymerase.
13. pENTR/SD/D-TOPO cloning kit (ThermoFisher Scientific).
14. 10 mM dNTP solution.
15. DNA marker.
16. Plasmid miniprep kit.
17. LR Clonase Enzyme mix (ThermoFisher Scientific).
18. Proteinase K.

19. 0.5 ml PCR tubes.
20. 10 mm petri dishes.
21. Sterile toothpicks.
22. 96-well PCR plates.
23. 96-well microtiter plates.
24. Micropipettes (single channel and multichannel).
25. PCR thermocycler (both for single tubes and 96-well plates).
26. 37 °C incubator.
27. DNA electrophoresis equipment.
28. Temerature controlled water bath.

2.2 Analysis of the Toxicity in Pseudomonas aeruginosa and Escherichia coli

1. *P. aeruginosa* PAO1 [5] strain.
2. pTNS2 vector [2].
3. pHERD20T vector [6] and the Gateway Vector Conversion System (ThermoFisher Scientific).
4. Autoclaved LB medium both liquid and solid.
5. Antibiotics: 1000× stock of gentamicin (30 mg/ml; final concentration for selection: Gm^{30} = 30 μg/ml), and 1000× stock of carbenicillin (200 mg/ml; final concentration for selection: Cb^{200} = 200 μg/ml).
6. 1000× stock of Isopropyl β-D-1-thiogalactopyranoside (IPTG) (1 M; final concentration for induction: 1 mM).
7. Ultrapure water.
8. Sucrose (final concentration: 300 mM).
9. Mineral oil.
10. Glycerol.
11. Agarose.
12. Primers:

    RBS_F: 5′-TAAGAAGGAGCCCTTCAC-3′

    GlmS_up: 5′-GTGCGACTGCTGGAGCTGAA-3′

    Tn7T_R: 5′-CACAGCATAACTGGACTGATTTC-3′

    GlmS_down: 5′-GCTCTCGCCGATCCTCTACA-3′

    pHERD20T_F: 5′-ATCGCAACTCTCTACTGTTTCT-3′

    pHERD20T_R: 5′-TGCAAGGCGATTAAGTTGGGT-3′.
13. Taq DNA polymerase.
14. 10 mM dNTP solution.
15. DNA marker.
16. 1.5 and 2 ml Eppendorf tubes.
17. 10 mm petri dishes.

18. Sterile toothpicks.
19. 96-well PCR plates.
20. 96-well microtiter plates.
21. Micropipettes (single channel and multichannel).
22. PCR thermocycler (for 96-well plates).
23. 37 °C incubator.
24. Electroporator, e.g., Gene Pulser Xcell™ (Bio-Rad, Hercules, California, USA).
25. Electroporation cuvettes with a 2 mm gap (Bio-Rad).
26. DNA electrophoresis equipment.
27. Microcentrifuge.
28. Bioscreen C (Growth Curves USA, Piscataway, New Jersey, USA).
29. Honeycomb plates (Growth Curves, USA).

2.3 Live Cell Time Lapse Microscopy

1. Autoclaved LB medium (liquid and agar).
2. 1000× stock of IPTG (1 M; final concentration for induction: 1 mM).
3. 1000× stock of gentamicin (30 mg/ml; final concentration for selection: $Gm^{30} = 30$ μg/ml).
4. Agarose (Eurogentec, Liège, Belgium).
5. 50 ml falcon.
6. Gene Frames (1.7 × 2.8 cm) (ThermoFisher Scientific).
7. Coverslips (ThermoFisher Scientific).
8. Microscope glass slides (Rogo Sampaic, Wissous, France).
9. A temperature-controlled (Okolab, Ottaviano, Italy) Ti-Eclipse inverted microscope (Nikon, Champigny-sur-Marne, France) with a 60x objective, a Ti-CT-E motorized condenser, and a CoolSnap HQ2 FireWire CCD-camera.
10. Software: NIS-elements AR software (Nikon); Open source software Fiji (i.e., ImageJ; https://fiji.sc/).

3 Methods

3.1 Cloning of the Phage ORFan

Because of the usually large numbers of ORFans and the need for several vector systems containing the same phage genes, we have used the Gateway cloning system from ThermoFisher Scientific (*see* **Note 1**). This system is based on exploiting the enzymes from bacteriophage λ, utilized by this temperate phage for integration and excision of the phage genome to and from a well-defined location in the bacterial genome with the use of specific recognition sites (attachment (att) sites) [7].

3.1.1 Construction of the Phage ORFan Constructs in the pENTR Vector

The ORFan genes are first amplified from start to stop codon using a high fidelity DNA polymerase and adding an extra 5′-CACC overhang (*see* **Note 2**). Subsequently, they are cloned in the pENTR/SD/D-TOPO vector using the directional pENTR/SD/D-TOPO Kit.

1. To construct an entry clone for each ORFan, always mix 0.5 μl PCR product (*see* **Note 3**) with 0.25 μl pENTR/SD/D-TOPO vector (*see* **Note 4**) and 1 μl of the provided salt solution in a final volume of 6 μl.
2. Incubate the mixture for 15 min at 22 °C to ligate the PCR products in the vector (*see* **Note 5**).
3. Transform the whole ligation mixture (6 μl) to chemically competent *E. coli* cells. Plate the complete cell volume on one LB plate containing Km^{50} (*see* **Note 6**).
4. To check the transformants using colony PCR, pick up 4 colonies per construct in 100 μl LB/Km^{50}. This can be done in a 96-well microtiter plate (*see* **Note 7**).
5. Grow them for 2 h at 37 °C.
6. Transfer 2.5 μl of each cell suspension to a 96-well PCR plate using a multichannel pipette and add the following components to each well (*see* **Note 8**): 0.05 μl (0.25 U) DreamTaq DNA polymerase, 0.5 μl 20 μM pENTR_F primer, 0.5 μl 20 μM pENTR_R primer, 2.5 μl 10× DreamTaq DNA polymerase Green buffer, 0.5 μl 10 mM dNTP mix, and 18.5 μl ultrapure water (total volume of 25 μl). Mix everything by pipetting up and down and spin down the mixture by centrifugation. Finally add one drop of mineral oil on top of each well (*see* **Note 9**).
7. Run the following PCR program in a heated PCR block (lid temperature set at 99 °C) (*see* **Note 10**): 5 min at 95 °C; 30 cycles of 30 s at 95 °C, 30 s at 54 °C and 1 min 30 s at 72 °C; 5 min at 72 °C; hold at 12 °C.
8. Once the program is running, prepare a temporary −20 °C cell stock of the picked colonies by adding 60 μl LB and 40 μl 100% (v/v) glycerol to the remaining cell suspension. Also prepare a 1% agarose gel for DNA electrophoresis.
9. After the PCR, directly run 10 μl of the PCR product on the solidified gel. Also add to each row of samples a DNA marker (e.g., GeneRuler DNA Ladder Mix from ThermoFisher Scientific). The expected length is the length of your ORFan gene plus 57 bp derived from the vector backbone.
10. For each ORFan, select one correct transformant and inoculate this clone from the −20 °C cell stock in 4 ml LB/km^{50}. Grow overnight (*see* **Note 11**).

11. The next day, perform a plasmid miniprep of the pENTR/SD/D-TOPO_ORFan constructs using a standard miniprep kit.
12. Verify the plasmid by DNA sequencing (*see* **Note 12**). The expected sequence from forward to reverse primer is 5′-GCG GCCGCCTTGTTTAACTTTAAGAAGGAGCCCTTCACC–ORFan–(AA)GGGTGG GCGCGCCGAC -3′.

3.1.2 Gateway Subcloning to pUC18-Mini-Tn7T-LAC-GW

To express early phage proteins in *P. aeruginosa* PAO1, all phage genes are transferred to the *E. coli—P. aeruginosa* shuttle expression vector pUC18-mini-Tn7T-Lac [2, 3], which was first made Gateway compatible using the "Gateway Vector Conversion System" following the instructions provided in the kit.

1. Prepare the following reaction mixture for each phage gene: 150 ng entry clone, 150 ng pUC18-mini-Tn7T-LAC-GW destination vector, and 1 μl LR Clonase Enzyme mix in a final volume of 5 μl (*see* **Note 13**).
2. Incubate for 2 h at 25 °C.
3. Inactivate the enzyme mix for 10 min at 37 °C by adding 0.5 μl (1 μg) proteinase K solution (ThermoFisher Scientific).
4. Transform the entire ligation mixture (5 μl) to chemically competent *E. coli* cells. Plate the complete cell volume on one LB plate containing Amp^{100} (*see* **note 14**).
5. To again verify the transformants using colony PCR, follow **steps 4–10** of Subheading 3.1.1, but replace the Km^{50} by Amp^{100}. For the PCR program, change the primers to pUC18-mini-Tn7T-LAC_F and R. Using these primers, the expected length of the PCR product is the length of the ORFan gene plus 443 bp.
6. Perform a plasmid miniprep of the pUC18-mini-Tn7T-LAC_ORFan constructs. The construct should again be verified by DNA sequencing (e.g., Sanger sequencing). The expected sequence from forward to reverse primer is 5′-CGG TTCTGGCAAATATTCTGAAATGAGCTGTTGACAATTAA TCATCGGCTCGTATAATGTGTGGAATTGTGAGCGGATA ACAATTTCACACAGGAAACAGAATTCGAGCTCCTCACT AGTGGATCCCCCATCAAACAAGTTTGTACAAAAAAGCA GGCTCCGCGGCCGCCTTGTTTAACTTTAAGAAGGAGC CCTTCACC – ORFan – AAGGGTGGGCGCGCCGACCC AGCTTTCTTGTACAAAGTGGTTCGATGGGCTGCAGGAA TTCCTCGAGA AGCTTGGGCCCGGTACCTCGCGAAGGC CTTGCAGGCCAACCAGATAAGTGAAATCTAGTTCCAAA CTATTTTGTCATTTTTAATTTTCGTATTAGCTTACGACG CTACACCCAGTTCCCATCTATTTTGTCACTCTTCCCTA AATAATCCTTAAAAACTCCATTTCCACCCCTCC-3′.

3.2 Analysis of the Toxicity in Pseudomonas aeruginosa and Escherichia coli

3.2.1 Chromosomal Integration into the P. aeruginosa Genome

Stable integration into the *P. aeruginosa* PAO1 genome is achieved by cotransformation of the pUC18-mini-Tn*7*T-Lac_ORFan construct and the helper plasmid pTNS2 by electroporation to *P. aeruginosa* PAO1. The helper plasmid (a suicide plasmid in *Pseudomonas*) encodes the Tn*7*T site-specific transposition pathway, which facilitates the insertion of the ORFan gene between *P. aeruginosa* PAO1 genes PA5548 and PA5549, respectively encoding a transporter protein and the glucosamine–fructose-6-phosphate aminotransferase GlmS (Fig. 1). The in vivo phage protein expression is under the control of an IPTG-inducible tac promoter [2]. Although this method is described for *P. aeruginosa* PAO1, it can also be applied to other bacteria, as long as the strain contains a Tn*7* attachment site (attTn*7*) downstream of a *glmS* gene [2] (*see* **Note 15**).

1. For each ORFan, prepare an overnight culture of the wild-type PAO1 strain in 4 ml LB medium.
2. The next day, follow the "10 min method to prepare electrocompetent *P. aeruginosa* cells" described by Choi et al. [8] to

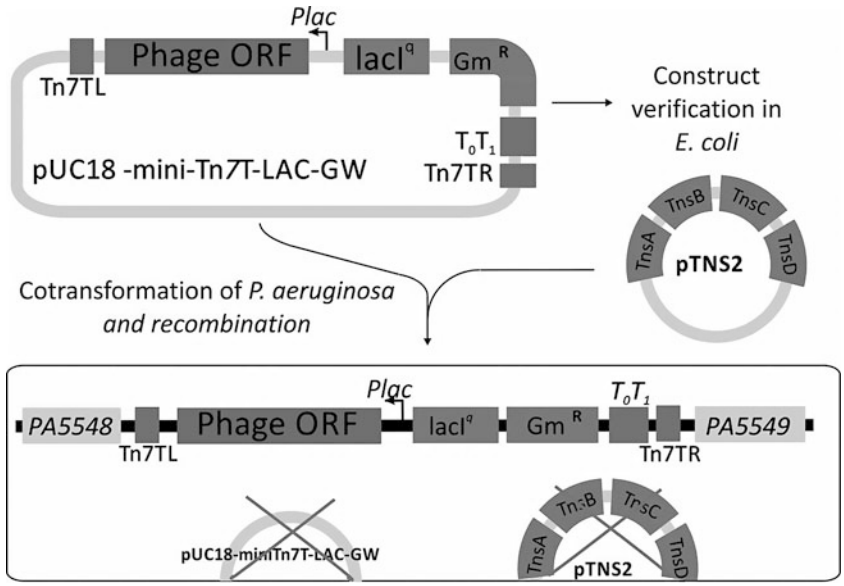

Fig. 1 The pUC18-mini-Tn*7*T-LAC-GW vector system allows for single-copy expression of phage proteins in *P. aeruginosa*. Cotransformation of a pUC18-mini-Tn*7*T-LAC-GW construct with pTNS2 (encoding TnsABCD which mediates a site-specific Tn*7* transposition pathway) to *P. aeruginosa* allows for specific integration of the expression cassette (from Tn*7*TL to Tn*7*TR) between *P. aeruginosa* genes *PA5548* and *PA5549*. As pUC18-mini-Tn*7*T-LAC and pTNS2 do not contain an *ori* for *P. aeruginosa*, these plasmids are lost during cell division. The expression cassette from left to right contains the phage gene behind a *lac* promoter, the *lacI*^{q} repressor which prevents basal expression from the *lac* promoter, a gentamicin resistance gene *aacC1* (Gm^{R}) and the transcriptional terminators T_0 and T_1 to prevent undesired readthrough from chromosomal promoters into cloned sequences

make the cells electrocompetent. Briefly, divide per ORFan 3 ml overnight culture in two 2 ml eppendorf tubes and spin the cells down for 2 min at 12,100 × *g*. Discard the supernatant, wash the cells in 1 ml 300 mM sucrose and centrifuge for 2 min at 18,000 × *g*. Repeat the washing step in 1 ml 300 mM sucrose twice. Finally, resuspend both pellets together in 100 µl 300 mM sucrose (*see* **Note 16**).

3. Immediately continue with adding 300 ng pUC18-mini-Tn7T-LAC_ORFan plasmid and 500 ng pTNS2 plasmid to the cell mixture. Repeat this step for each ORFan.
4. Incubate for 5 min at room temperature.
5. Transfer the mixture to a cold electroporation 0.2 cm cuvette. Set up the electroporator to 25 µF, 200 Ω, and 2.5 kV (*see* **Note 17**), place the cuvette in the track and simultaneously push both red buttons until you hear a high tone.
6. Quickly add 450 µl of liquid LB medium to the cells and transfer the content of the cuvette into an 1.5 ml Eppendorf tube.
7. Repeat the last two steps for every ORFan.
8. Incubate for 1.5–2 h at 37 °C while shaking.
9. Plate the complete cell volume on 2–3 LB plates containing Gm^{30} (*see* **Note 18**).
10. Incubate the plates for 20–24 h at 37 °C.
11. To confirm the presence of the expression construct in the *P. aeruginosa* genome, two PCR reactions per colony are performed, which respectively check the integration on the PA5548 side (primer couple GlmS_up—RBS_F) and on the PA5549 side (primer couple GlmS_down—Tn7T_R). The former primer couple should result in a fragment of the length of the ORFan gene plus 330 bp, the latter primer couple in a fragment of 272 bp (Fig. 1). To do this, pick up four transformants per ORFan in 100 µl LB/Gm^{30}.
12. Perform two PCR reactions per colony using the primer couples as described in **step 11** and following the **steps 5–9** of Subheading 3.1.1. Each reaction mixture consists of 5 µl cell suspension, 1.5 µl of 20 µM F_primer, 1.5 µl of 20 µM R_primer, 0.15 µl DreamTaq DNA polymerase, 2.5 µl 10X DreamTaq DNA polymerase Green buffer, 0.5 µl 10 mM dNTP mix, and 14 µl ultrapure water. Use the following PCR program: 10 min at 95 °C; 30 cycles of 45 s at 95 °C, 30 s at 54 °C, and 2 min at 72 °C; 15 min at 72 °C; hold at 12 °C. This can be done in a 96-well PCR plate (*see* **Notes 19** and **20**).
13. Make a 20% glycerol stock of each strain with correct genomic insertion (one per ORFan) and store at −80 °C.

3.2.2 Single Copy Expression of the Phage Protein in P. aeruginosa

Once the correct *P. aeruginosa* strain is generated, the impact of the phage ORFan on the bacterial growth can be tested. Both the growth on solid medium and liquid medium is examined, as they give complementary information about the phenotypical effect caused by the phage protein. Both experiments are described with nutritionally rich LB medium. However, it is hypothesized that certain phage–host interactions may only be crucial under specific physiological conditions (where the target is present and active). Therefore, similar experiments can be done on well-defined minimal growth medium for *Pseudomonas* [9] and on artificial sputum medium [10], which mimics the sputum of a cystic fibrosis patient where *P. aeruginosa* infections are common. First, a spot test on solid LB medium is performed.

1. Prepare three overnight cultures of each mutant strain in a 96-well microtiter plate. Per well, inoculate a small volume of 20% glycerol stock (from **step 13** of Subheading 3.2.1.) in 150 μl of LB/Gm^{30} (*see* **Note 21**). Incubate the plate overnight at 37 °C while shaking.
2. Make a 100-fold dilution series (10^0, 10^{-2}, 10^{-4}, 10^{-6}) in LB/Gm^{30} of each independent overnight culture in a 96-well microtiter plate using a multichannel pipette.
3. Spot per dilution series in parallel 2 μl sample on LB/Gm^{30} solid medium with and without IPTG (*see* **Note 22**) using a multichannel pipette (*see* **Note 23**). Repeat this step for each mutant strain.
4. Incubate both plates overnight at 37 °C.
5. Compare the growth of the mutant *P. aeruginosa* strains with and without induction of expression of the phage protein. A growth-inhibitory phage ORFan shows reduced bacterial growth on solid medium with IPTG (*see* **Note 24**).

   Next, the effect on growth in liquid medium is investigated. In this setup, nutrients are more easily accessible, which may give different results. Moreover, a growth curve can give a first hint toward functional prediction. For example, a growth curve similar to the growth curve of a wild-type *P. aeruginosa* strain may indicate filamentous growth. Growth curves of the individual strains are determined via a Bioscreen (*see* **Note 25**).
6. Prepare overnight cultures similar to **step 1**.
7. Prepare a dilution of 1:100 in fresh LB/Gm^{30} for each culture both with and without (control) IPTG to obtain a final volume of 100 μl using a multichannel pipette. Transfer the cell dilutions to a Honeycomb microplate (10 × 10 well plate) (*see* **Note 26**).

8. Follow the OD_{600nm} in function of the time for 10 h in intervals of 30 min using a Bioscreen C™ Analyzer (*see* **Note 27**).
9. Compare the growth curves of the *P. aeruginosa* strains with and without induction of expression of the phage protein.

3.2.3 High Level In Vivo Expression of the Phage Protein in P. aeruginosa and E. coli

Although single-copy expression is preferred, high-copy expression may also be of interest, as the abundance of a phage protein during infection is usually unknown. However, it should be noted that high-copy expression can lead to false-positive results due to expression-associated toxicity. For a high level in vivo expression of phage proteins in *P. aeruginosa*, the multicopy *E. coli-P. aeruginosa* shuttle vector pHERD20T [5] is used, which was first made Gateway compatible using the "Gateway Vector Conversion System" following the instructions provided in the kit (*see* **Note 28**). This allows for an efficient transfer of the phage ORFan gene from the already available pENTR/SD/D-TOPO_ORFan plasmid (Subheading 3.1.1) into the Gateway-compatible pHERD20T vector.

1. To construct the pHERD20T_ORFan, follow **steps 1–6** of Subheading 3.1.2. For the PCR program, use the primers pHERD20T_F and pHERD20T_R, which results in a PCR product with the length of the ORFan gene plus 338 bp. The expected sequence from forward to reverse primer is 5′-ATCG CAACTCTCTACTGTTTCTCCATACCCGTTTTTTTGGGC TAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACA TACCCATGGGATCTGATAAGAATTCGAGCTCGGTACCC ATCACAAGTTTGTACAAAAAAGCAGGCTCCGCGGCCG CCTTGTTTAACTTTAAGAAGGAGCCCTTCACC – ORFan – AAGGGTGGGCGCG CCGACCCAGCTTTCTTGTACAA AGTTGGTGGGGATCCTCTAGAGTCGACCTGCAGGCAT GCAAGCTTGGCACTGGCCGTCGTTTTACAACGTCGTG ACTGGGAAAACCCTGGCGTTACCCAACTTAATCGCCTT GCA-3′.
2. The analysis of the toxicity is similar to **steps 1–9** of Subheading 3.2.2. However, always use Cb^{200} instead of Gm^{30} and induce the phage ORFan expression with 0.2% (w/v) arabinose, as the phage gene is under control of an arabinose inducible p_{BAD} promoter.

Furthermore, to study potential conservation of the bacterial target for these phage proteins between gram-negative bacteria, the constructed pUC18-mini-Tn7T-Lac and pHERD20T can also be tested in *E. coli*, in which both vectors are present in high copy number. The analysis is analogous to **steps 1–9** of Subheading 3.2.2.

3.3 Live Cell Time Lapse Microscopy

To visualize the exerted effect of the toxic phage proteins on cell morphology and growth, time lapse microscopy is performed. To this end, the bacteria are transferred to a solid matrix of medium ensuring sufficient supply of nutrients to the cells during the experiment. Moreover, due to the discontinuity of this matrix, enough oxygen is present in the reservoir to warrant aerobic conditions. These solid LB medium pads are made using a protocol also described by de Jong et al. [11]. This article features an instructive video of the protocol. The data acquired during time lapse microscopy is analyzed with Open source software Fiji.

1. Prepare an overnight culture of each mutant strain which contains a growth-inhibitory ORFan.
2. The next day, start with preparing the agarose pads. First, remove the plastic foil from one side of the gene frame and carefully attach this end to a clean microscope glass slide. The glue assures fixation of the gene frame on the microscope glass slide.
3. Take a 50 ml falcon containing 75 mg of agarose and add 5 ml of LB.
4. Boil the mixture in the microwave and assure complete dissolution of the agarose.
5. Add 5 μl of 1 M IPTG (1 mM) together with 5 μl Gm^{30} to the falcon and vortex briefly to homogenize the mixture.
6. Transfer 500 μl of the LB–agarose mixture inside the attached gene frame.
7. Mount a clean coverslip on top of the warm medium and gently press the four corners to assure formation of a planar agarose pad.
8. Allow the LB-agarose to solidify; these pads can be kept at 4 °C up to a day if necessary (*see* **Note 29**).
9. Prewarm the pad at 37 °C, preferably at least 2 h before the start of the experiment.
10. Cut squares of approximately 5 × 5 mm out of the pad with a sterile scalpel. Repeat **step 1** to make a new microscope glass slide with gene frame and transfer to the latter the cut squares with the tip of a scalpel (*see* **Note 30**). Multiple pads can be positioned next to each other inside one 1.7 × 2.8 cm gene frame (*see* **Note 31**).
11. Dilute the overnight culture 1:100 in LB medium to obtain an end concentration of approximately 1.10^7 CFU/ml. This dilution prevents bacteria being positioned to close to each other, thus decreasing the risk of nutritional deprivation during growth under the microscope.

12. Vortex the bacterial dilution briefly to assure the individual cells are well spread on the pad and do not clump together.
13. Transfer 2 μl of the diluted cell culture to an LB-agarose square and allow the drop to dry for typically 1–2 min.
14. Remove the top sticker of the gene frame and mount a coverslip onto the slide (*see* **Note 32**).
15. Spread a drop of oil on the coverslip and position the microscope glass slide above the objective. For each pad, a number of positions can be selected and monitored.
16. The images are acquired every 10 min during a time period of 5 h using NIS elements AR software.
17. Create time lapse movies from the performed experiment using open source software Fiji, an image processing program distributed by ImageJ.
18. Open the file in Fiji and select the series of frames you wish to show in a time lapse movie.
19. Add Time stamper via "Image" > "Stacks" > "Time stamper" and adjust the time interval corresponding to the microscopy setting. Add a scale bar via "Analyze" > "Tools" > "Scale bar" (*see* **Note 33**).
20. Save the file in .AVI format.

4 Notes

1. If only a few ORFans need to be screened for inhibition, they can also directly be cloned in the pUC18-mini-Tn7T-Lac vector. However, by comparing non-Gateway- and Gateway-cloned ORFans, we found that adding the 5′ untranslated region provided by the pENTR/SD/D-TOPO vector (including at least the Shine–Dalgarno sequence and a translational enhancer; 5′-TTAACTTTAAGAAGGAGCCCTTCACC-3′; preferably the whole extra sequence since only then, the same inhibitory levels can be observed) is required for a correct protein expression. This can also be done by adding this extra sequence as a tail to the forward primer.
2. The 5′-CACC overhang is needed for directional cloning.
3. Prepare the reaction mixture on ice. Also keep the vector tube always on ice. To obtain the highest cloning efficiency, use a 0.5:1–2:1 molar ratio of PCR product–TOPO vector. The molecular weight of the vector is approximately 2601 bp × 649 g/Mol = 1688.049 ng/pmol. In the cloning reaction, 0.25 μl of a 15 ng/μl solution is used, which means 3.75 ng or 0.0022215 pmol TOPO vector. Consequently, use 0.001–0.004 pmol of the PCR product in the cloning reaction. Using 0.5 μl usually gives a good number of transformants.

4. For difficult to clone proteins, the amount of vector in this reaction can be increased to 1 μl as suggested by the manufacturer.
5. This incubation time can be shortened to up to 5 min. We had the best results with 15 min.
6. If higher amounts of TOPO vector are used, the transformation mixture should be plated on two plates to assure formation of single colonies.
7. For difficult-to-clone ORFans, more colonies can be screened.
8. A master mix should be made for the total number of colonies that are screened. Keep the enzyme on ice and also prepare the master mix on ice.
9. This can easily be done using a 1 ml tip. Fill the whole tip and gently push the pipette to add one drop to each well. Special adhesive tapes could also be used.
10. The extension time should be the length of the longest ORFan plus 57 bp. DreamTaq polymerase has an average amplification rate of 1 kb/min. Hence, using an extension time of 1 min 30 s, or even 1 min, should be sufficient for most ORFans.
11. In most cases, selecting a single transformant (having a PCR product at the correct length) for plasmid isolation and verification by DNA sequencing is sufficient.
12. This DNA sequencing can be outsourced, for example, to Eurofins Genomics. When including the stop codon TGA or TAA, we often observe deletion of 1 or 2 adenines at the vector 3′ side (AAGGGTGGG). This does not give any problems for further analysis.
13. For difficult to clone ORFans, the amount of enzyme mix can be increased to obtain more transformants. Prepare the mix on ice.
14. Plating the whole mix on one plate usually provides single colonies. If this is not the case, plate the mix on more plates.
15. Integration of an growth-inhibitory phage ORFan into the clinical *P. aeruginosa* strain PA14 and the industrial fermentation strain *Pseudomonas putida* KT2442 is already successfully tested.
16. Always freshly prepare the electrocompetent *P. aeruginosa* cells on the day of use. Since *P. aeruginosa* cells form biofilms, it is recommended to perform the experiment in the morning (max. 16 h of incubation).
17. A cold cuvette is used to avoid arcing. It is also recommended to wear gloves and to not touch the aluminum electrode plates. The cuvettes can be reused by washing them with 70% ethanol and storing them at −20 °C.

18. The transformation mixture should be plated on two or three plates to assure proper drying of the volume.
19. To ensure proper lysis of the *P. aeruginosa* cells, the 96-well PCR plate can be placed in a microwave for 5 s at 1000 W.
20. Although sequencing confirmation is not necessary if both fragments have the expected length, this can be done for extra verification. The expected sequence from RBS_F to GlmS_up is TAAGAAGGAGCCCTTCACC – ORFan – AAG GGTGGGCGCGCCGACCCAGCTTTCTTG TACAAAGTG GTTCGATGGGCTGCAGSGAATTCCTCGAGAAGCTTGG GCCCGGTACCTCGCGAAGGCCTTGCAGGCCAACCAG ATAAGTGAAATCTAGTTCCAAACTATTTTGTCATTTTTA ATTTTCGTATTAGCTTACGACGCTACACCCAGTTCCCA TCTATTTTGTCACTCTTCCCTAAATAATCCTTAAAAACT CCATTTCCACCCCTCCCAGTTCCCAACTATTTTGTCCG CCCACAAGCCGGGGCAGGCATGCGGCCCCGGCGCTC GCTGTCAATCGCGCAACGGCAGCGCTTCGTTGCTCCT GCGGCTGGCGAACCAGTCCAGCACGGTGAACCAGGC GCCGATGCCCAGGCCGATGCCCAATACCCACAGGGT GGC GGGCGGCCCC TTCAGCTCCAGCAGTCGCAC.
21. To check the experimental set-up, it is recommended to include a negative and positive control. As a negative control, a *P. aeruginosa* strain encoding an empty pUC18-mini-Tn7T-Lac expression cassette may be used. In addition, a *P. aeruginosa* strain with a known growth-inhibitory phage ORFan is used as a positive control.
22. It is important to first spot on medium without IPTG and then with IPTG, since contact of the pipette tip with the medium can transfer traces of IPTG.
23. For more precise pipetting, use Finntip® Flex pipette tips (ThermoFisher Scientific).
24. Leaky expression of the p_{tac} promoter can cause bacterial growth inhibition without induction of the expression of the phage protein.
25. If only a small number of ORFans are tested, this step can be done in glass culture tubes, following the OD_{600nm} of 4 ml cell cultures with a Novaspec®II spectrophotometer (Pharmacia).
26. This step can also be done in a 96-well microtiter plate spectrophotometer. However, condensation and evaporation during such experiment often interferes with the OD readings or the lid has to be removed before measuring.
27. The measuring time and the interval time can be adapted.
28. Other *E. coli—P. aeruginosa* shuttle vectors can also be used, for example pME6032.

29. The pads can be kept at 4 °C in a petri dish surrounded by Parafilm to prevent drying out of the medium. However, keeping the pads longer than a day is not recommended.
30. These pads can contain different concentrations of inducing agents.
31. Make sure the pads are separated by approximately 3–4 mm to avoid touching of the pads when the coverslip is applied. This would result in diffusion of cells, antibiotics and inducing agents from one pad to another.
32. The coverslip will make contact with the agar pad. Do not apply pressure in the middle of the coverslip, as this could lead to disruption of the pads.
33. Make sure that the time scale is set at the correct pixels/micron value as this is a microscope-dependent feature.

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Chapter 13

Use of Greater Wax Moth Larvae (*Galleria mellonella*) as an Alternative Animal Infection Model for Analysis of Bacterial Pathogenesis

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Abstract

Alternative infection models of bacterial pathogenesis are useful because they reproduce some of the disease characteristics observed in higher animals. Insect models are especially useful for modeling bacterial infections, as they are inexpensive, generally less labor-intensive, and more ethically acceptable than experimentation on higher organisms. Similar to animals, insects have been shown to possess innate immune systems that respond to pathogenic bacteria.

Key words *Galleria mellonella*, Insects, Infection model, Wax worm, Pathogenesis, Larvae

1 Introduction

Larvae of the greater wax moth (or honeycomb moth) *Galleria mellonella* are off-white caterpillars with black feet and small black or brown heads (Fig. 1). In their natural habitat, "wax worms" live as nest parasites in bee colonies, and eat honeycombs or beeswax, which gives them their name. Although they do not attack bees directly, beekeepers consider them pests because they damage beehives and may spread honeybee diseases. Commercially, wax worms are raised as food for reptilian pets, fish and birds. Wax worms are also used extensively as an alternative infection model for the study of bacterial pathogens such as *Pseudomonas aeruginosa* [1–3], the *Burkholderia cepacia* complex [4–6], *Bacillus cereus* [7], *Francisella tularensis* [8], *Legionella pneumophila* [9], *Listeria monocytogenes* [10], *Mycobacterium* strains [11, 12], *Proteus mirabilis* [13], and), *Salmonella* Typhimurium [14], *Staphylococcus aureus* [15], and *Vibrio parahaemolyticus* [16], as well as several fungal pathogens [17–22].

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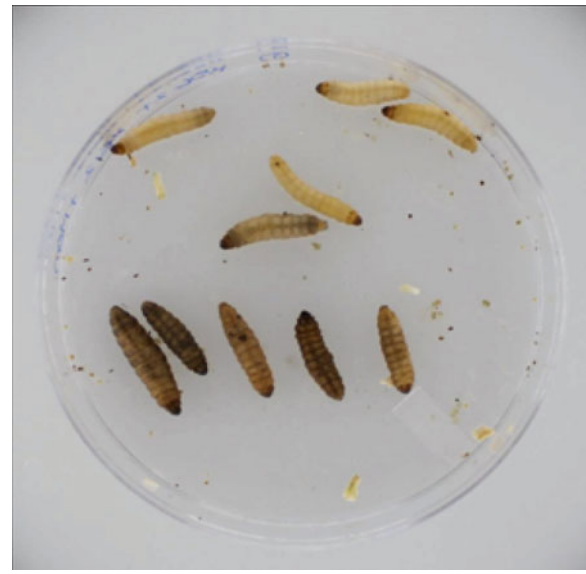

Fig. 1 *Galleria mellonella* larvae in a 10 cm diameter petri dish. For three of the larvae (between 2 and 4 o'clock on the petri dish), the wax worms are unresponsive to touch, and have turned dark brown due to melanization 48 h following infection with a lethal dose of pathogenic bacteria

The innate immune systems of insects such as *G. mellonella* share a high degree of structural and functional homology to the innate immune systems of mammals [23, 24]. Although the immune systems of insects may display innate memory [25, 26] but not clonal selection mechanisms, they can offer resistance to microbial infections [27]. This immune defense involves both cellular and humoral defenses [24]. The humoral immune response of insects consists of the processes of melanization (Fig. 1), hemolymph clotting, production of numerous types of cytokines [28], and pattern recognition molecules [29] and the synthesis of a number of potent antimicrobial peptides. The cellular reactions which are initiated and regulated like those of mammals [30] include phagocytosis, nodulization, and large-scale encapsulation. Analysis of insect responses to bacterial pathogens therefore can provide an accurate reproduction of the reaction observed in higher organisms [24, 31]. A correlation exists between the virulence of pathogens in wax worms and mice has been established [2, 26, 32]. Moreover, temperature-associated changes in bacterial virulence can be addressed in the wax worm infection model because *G. mellonella* larvae can be maintained at temperatures up to 37 °C. Finally, alternative insect infection models such as *G. mellonella* larvae provide comparable data to that of higher organisms, but are more cost-effective, less labor-intensive, and more ethically acceptable. Therefore, for several reasons, *G. mellonella* larvae serve as an attractive alternative infection model.

There are several instances where the wax moth larvae infection model has also been employed to test the efficacy of bacteriophage activity against pathogenic bacteria in vivo. In these experiments, phages are observed to rescue of the wax worms challenged with lethal doses of pathogenic bacteria. Unlike some seedling- or plant-infection models that have been observed to inactivate or reduce the activity of applied phages [27, 34], the wax worm infection model produces negligible phage inactivation, and provides a therapeutic treatment environment more similar to that observed in higher organisms such as mice. Examples of different pathogenic bacteria being successfully treated by bacteriophage application in the wax worm infection model include the *Burkholderia cepacia* complex [5, 34–36], *Clostridioides difficile* [37], *Cronobacter sakazakii* [38], *Klebsiella pneumoniae* [39], and *Pseudomonas aeruginosa* [40–46].

2 Materials

1. Purchase sufficient *G. mellonella* larvae from a commercial supplier (e.g., Carolina Biological Supply Company (www.carolina.com/; *see* **Note 1**), Knutson's Live Bait (www.knutsonlivebait.com/wax_worms.html; *see* **Note 2**), Recorp Inc. (www.recorpinc.com) UK Waxworms Limited (www.ukwaxworms.co.uk). Within Europe, Vivara offers a varied collection through local suppliers (http://www.vivara.com/).

   It is important to use a supplier that can deliver high-quality larvae. Wax worm larvae that are exposed to extremely high- or low-temperatures during shipping cause large variability in experimental outcomes. Wax worm larvae are typically shipped at fifth or sixth instar stage (approximately between 2 and 3 cm in length) and are suitable for use immediately. Larvae can be stored at room temperature or 4 °C for up to 2 weeks in wood chips and do not require food. If stored at 4 °C, the wax worms should be kept at room temperature for several hours before they are utilized. Healthy larvae are uniformly cream-colored with no areas of dark discoloration, and can rapidly right themselves if turned over. Discard any larvae showing signs of melanization (brown colored or blotchy) or pupation.
2. A 10-μl Hamilton syringe (Reno, NV) (available through Sigma-Aldrich) is required to inject approximately 5 μl of inoculum into the wax worm larvae. Alternative syringes can be utilized, including disposable or repeating syringes (*see* **Note 3**).
3. For bacteria, overnight cultures are pelleted and resuspended in 10 mM $MgSO_4$. For bacteria that are resistant, supplement this solution ~1.0 mg/ml ampicillin (or other antibiotic) as a

prophylactic to prevent infection with normal resident bacteria present on the larval surface. Unless otherwise specified, all reagents can be obtained from Sigma-Aldrich Canada (Oakville, ON), and all media can be obtained from Difco Laboratories (Detroit, MI) (*see* **Note 4**).

3 Methods

3.1 Infection of G. mellonella Larvae

1. Prepare a container for injecting larva by placing a circle of 10 cm filter paper in the bottom of a 10 cm petri dish. Using blunt tipped tweezers, place ten healthy wax worm larvae of similar size into the petri dish.
2. A 10-μl Hamilton syringe is used to inject 5-μl aliquots into the *G. mellonella* larvae via the hindmost left proleg or anterior most prothoracic leg at its base. Between injections, rinse the syringe by aspirating several volumes of 70% ethanol, followed by rinses with sterile dH_20. Following injection, larvae are placed in a static incubator in the dark at 30 °C, the optimum temperature for wax worm growth and development [4]. After the injection, inspect the larvae to ensure that they begin to crawl and are not bleeding (a clear yellowish fluid), leaking gut contents (a brownish color) or body fat (a white wax tissue). Similarly, inspect the larvae a few hours postinfection to ensure that the infection does not cause symptoms within the first 8 h.
3. For 50% lethal dose (LD50) experiments, a series of tenfold serial dilutions containing from 10^6 to 0 bacteria in 10 mM $MgSO_4$ (plus prophylactic antibiotic) are injected into the *G. mellonella* larvae. Control larvae are injected with 5 μl of only 10 mM $MgSO_4$ (plus prophylactic antibiotic) in order to measure any potentially lethal effects of the physical injection process. Ten larvae are injected at each dilution, and larvae are scored as dead or alive 48 and 72 h postinfection at 30 °C.
4. Larvae are considered dead when they display no movement, or inability to right themselves, in response to shaking of the petri dish or touch of the face plate with a pipette tip. Melanization or pigmentation usually indicates a strong immune reaction to the infection (*see* **Note 5**). For each bacterial strain, data from three independent experiments are combined, and LD_{50}s are calculated using the Systat computer program [24]. Briefly, Systat (San Jose, CA) fits a curve to the infection data in the following form: $Y = [A + (1 - A)]/[1 + \exp(B - G \times \ln X)]$, where Y is the fraction of larvae killed by the infection, A is the number of larvae killed by control injections, X is the number of bacteria injected, and B and G are Systat-generated variable parameters designed to best fit the curve to the data points. For linear relationships between X and Y, we used a linear regression model using the Systat computer program to determine the LD_{50}.

3.2 Additional Experimental Protocols

1. For time-to-death experiments, live versus dead larvae are monitored every 6–12 h postinfection. *G. mellonella* larvae are injected with serially diluted bacteria as before and monitored for their survival over a 72-h period. Three independent trials are conducted consisting of 10 worms per bacterial concentration for each bacterial strain. No more than one control larva should die in any given trial. In instances where greater than one control larva die, the data from infected larvae should not be not used (*see* **Notes 6** and **7**).
2. To monitor bacterial loads in larval hemolymph over time, larvae are injected with between 500 and 800 colony-forming units (CFU). For more virulent bacteria, this number of CFU can be reduced. For the zero time point, larvae are infected and allowed to sit for 20 min before their hemolymph is collected. Equal volumes of hemolymph are collected from five living worms at each time point and combined into a microcentrifuge tube, serially diluted, and plated onto agar for quantification. Three groups of five larvae are used for each time point in order to quantify bacterial loads. To extract the hemolymph, place the petri dish containing the wax worms on ice, until no movement of the larvae is observed. With a scalpel, make an incision between two larval segments near the larva tail, and squeeze the hemolymph into a microfuge tube. Each larva produces approximately 15–50 μl of hemolymph.
3. During hemolymph extraction it is easy to accidentally disrupt the wax moth larval gut, resulting in sample contamination. To reduce the chance of contamination, cut the larvae nearest the tail and away from the gut. To prevent the hemolymph from coagulating and turning brown, the hemolymph must be processed within 10 min of collection. Autoclave and dispose of wax worm larvae according to local safety rules (*see* **Notes 8** and **9**).

3.3 Analyses of Bacterial Virulence Mutants

1. Randomly or site-specifically mutagenized bacteria can be screened in wax worm larvae for the loss of virulence factors that contribute to wax worm death. Bacteria containing mutations to genes encoding virulence factors will permit the survival of infected *G. mellonella* larvae, whereas wild-type or parental bacterial cells will kill the infected larvae.
2. In these experiments, it is important to measure the loss of bacterial virulence relative to that of wild-type controls. Bacterial cells are grown to a specific concentration, based on optical density at 600 nm, washed and resuspended in 10 mM $MgSO_4$, and after appropriate dilution in 10 mM $MgSO_4$, injected into the wax worm larvae. Worms that undergo melanization and death over the same time frame as the worms injected with wild-type bacteria positive controls, possess mutations in the bacteria that do not affect bacterial virulence. However, worms

that do not melanize and die, harbor bacteria that possess mutations that reduce or eliminate bacterial virulence. Isolation of this bacterium's site of mutation can determine the gene encoding the virulence factor involved in wax worm pathogenesis.

3. Alternatively, if the bacteria to be studied are randomly mutagenized with a transposon that carries an outward facing promoter, transcriptional fusions can be produced with genes beside the transposons site of insertion, and increased expression of a virulence factor gene will result in a decreased time to wax worm death relative to control worms injected with the wild type bacteria. In such an experimental arrangement, "pools" of 5–10 different bacterial mutants can be combined and injected into one worm, greatly reducing the number of worms utilized, and the time required to screen a library of bacterial mutants.

4 Notes

1. Carolina Biological ships third–fourth instar larvae, but their weight varies which can have an impact on immunity; therefore, must be fed.
2. Knudson's ships second–third instar larvae in woodchips with oatmeal, but they are not sure whether growth occurs.
3. In the case of the latter, 32 gauge needles are ideal; limiting back bleeding. Injection at the base of the prothoracic (front) legs will also limit bleeding.
4. Additional prophylactic activity may involve washing the larvae with 70% ethanol for 30 s, and drying the insects on layers of autoclaved absorbent filter paper.
5. Penetration of the gut resulting in release of gastric fluid enzymes may elicit similar symptoms.
6. This protocol was used to investigate the pathogenesis of *Campylobacter jejuni* strains by Joanne MacKinnon and Andrew M. Kropinski (National Microbiology Laboratory @ Guelph, personal communication). This bacterium did not display virulence toward *G. mellonella* presumably due to significant differences in the optimal growth temperatures of the two organisms.
7. One of the salient problems with using *G. mellonella* larvae in such studies is variation in qualitative and quantitative responses. Herein we address ways to abrogate these problems. Larval responses to signaling molecules including hormones has established strain-specific differences in magnitude of response [43] which can be limited by ensuring supplier

consistency in insect strain or by raising a colony yourself. The latter has the advantage of ensuring larval quality (e.g., health status, stress control, and immune capacity) and is usually less costly compared with intermittent purchases.

8. To limit variation physiological variation within a strain a given larval instar (stage of larva development) is essential. Although growth is affected by larval diet [44] on traditional grain-glycerol media [45, 46], up to six instars are consistently achieved. The specific instar is based on measuring the dark head capsule of the larva [47]. However, even within a given instar we have determined that immunological differences exist due to differences in the number and types of blood cells, 200 mg larva (sixth instar) has a lessened response compared to the same instar larvae weighing 250 mg. These problems may be avoided by using larvae weighing 250 mg thus ensuring the same instar and physiological status and precluding sex and stage-specific immunological attributes [48].
9. Incubation temperature influences blood chemistry [49] and hemocyte activity [50]. This can be abrogated by incubating 200 mg cold-stored larvae on a diet known to support good growth until they achieve 250 mg weight. At this point the basic cellular and humoral responses to bacteria from previously chilled larvae will be physiologically comparable to non-chilled 250 mg larvae.

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Chapter 14

Use of a Silkworm Larva Model in Phage Therapy Experiments

Jumpei Uchiyama, Iyo Takemura-Uchiyama, and Shigenobu Matsuzaki

Abstract

Antibiotic-resistant bacteria can cause intractable infections in humans and animals, with damaging effects to health care and economics. Phage therapy is considered a possible alternative to chemotherapy for treating infections, but still requires laborious in vivo experiments before its introduction into society and its further development. Recently, silkworm larvae have been recognized as highly convenient and useful model animals, and an alternative to higher animals. We describe the procedure for experimental phage therapy to treat *Staphylococcus aureus* infections in silkworm larvae.

Key words Animal model, Phage purification, Phage therapy, Silkworm larvae, *Staphylococcus aureus*

1 Introduction

Drug-resistant bacteria (e.g., methicillin-resistant *Staphylococcus aureus* and multiple-drug resistant *Pseudomonas aeruginosa*) can cause intractable infections in humans and industrial animals, and the spread of these infections has resulted in severe deleterious effects to health care and economics in recent years [1, 2]. Thus, alternatives to chemotherapy are being investigated to address the problems caused by drug-resistant bacteria.

Phage therapy has a long history in Eastern Europe since the first phage was discovered by Félix d'Herelle, but it has received little attention in Western countries for many years. However, phage therapy has recently been revitalized and it is now being reevaluated as a possible alternative or complement to the treatment of infectious diseases using chemotherapy [1, 2].

As in the case of the development of chemotherapeutic agents, efficient in vivo experimental techniques are required to evaluate phage therapies. To reduce the time, labor, and costs of animal experiments, invertebrates (e.g., nematodes and insects) have been proposed as alternatives to vertebrate animal models [3–6]. At present, several invertebrate models are available that have been

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used in phage therapy experiments [7–10]. In particular, silkworm (*Bombyx mori*) larvae have been proposed as a useful model for studying antimicrobial agents [10–13]. We here describe a procedure for phage therapy experiments in which silkworm larvae are used as a model for in vivo evaluating the therapeutic effects and safety of a therapeutic phage to combat *S. aureus* infections.

2 Materials

2.1 Purification of Phage by Iodixanol Density-Gradient Ultracentrifugation

1. *S. aureus* and *S. aureus* phage.
2. Tryptic soy broth.
3. Polyethylene glycol 6000 and NaCl.
4. Sterilized TM buffer: 10 mM Tris–HCl [pH 7.2], 5 mM $MgCl_2$; sterilized saline.
5. DNase I (10 mg/mL as the stock solution) and RNase A (10 mg/mL as the stock solution).
6. Sterilized 0.45 μm syringe filter and 1 mL sterilized disposable plastic syringe.
7. Cooled centrifuge, and rotor and tubes for it.
8. Iodixanol for ultracentrifugation: iodixanol (OptiPrep™, Alere Technologies AS, Oslo, Norway) is diluted to 40%, 35%, and 30% with sterilized saline (*see* **Note 1**).
9. Ultracentrifuge, and associated rotor and tubes.

2.2 Preparation of Silkworm Larva Infection Model and Phage Therapy Experiment

1. *S. aureus*, and purified phage suspension (prepared in Subheading 3.1).
2. Tryptic soy broth and sterilized saline.
3. 40% iodixanol (diluted with sterilized saline).
4. HIMC: heart infusion broth supplemented with 50 mM $MgCl_2$ and 50 mM $CaCl_2$.
5. Silkworm (Hu·Yo × Tukuba·Ne) larvae (*see* **Note 2**).
6. Silkworm larvae housing cage: disposable plastic food containers and KimWipes (*see* **Note 3**).
7. Artificial diet for silkworm larvae (e.g., Silkmate 2S; Nihon Nosan Kogyo, Tokyo, Japan).
8. Antibiotic-free artificial diet for silkworm larvae (e.g., Silkmate; Katakura Kogyo, Tokyo, Japan).
9. 32-gauge disposable needle (e.g., No. 32 Dentronics Needle, Dentronics, Tokyo, Japan) and 1 mL sterilized disposable plastic syringe.

10. Densitometer or colorimeter, and Petroff-Hausser counting chamber (Hausser Scientific, http://www.hausserscientific.com/).
11. Incubator (27 °C).

3 Methods

3.1 Preparation of Purified Phage

1. The *S. aureus* phage is cultured on *S. aureus* in 300 mL of tryptic soy broth. After the bacteria are lysed, the bacteria–phage suspension is poured into 250 mL tubes and centrifuged (10,000 × *g*, 10 min, 4 °C). After removing the bacteria and debris by centrifugation, the granulated polyethylene glycol 6000 and solid NaCl are added directly to the phage lysate to be 10% and 0.5 M, respectively. It is then mixed using a magnetic stirring bar and stirrer. The mixture is centrifuged (10,000 × *g*, 40 min, 4 °C), and the supernatant is removed thoroughly. The phage pellet is then suspended in 2.3 mL of sterilized TM buffer supplemented with 50 μg/mL of DNase I and RNase A in a 15 mL tube. After incubating at 37 °C for 60 min with shaking, the crude phage sample is transferred into 1.5 mL microtubes, which are then centrifuged (20,000 × *g*, 30 s) to remove any debris. Approximately 1 mL of the supernatant is collected from each microtube.
2. The crude phage sample is subjected to purification by iodixanol density-gradient ultracentrifugation. A discontinuous iodixanol gradient is constructed manually by layering 1 mL of the iodixanol solutions from the highest to the lowest concentrations (40%, 35%, and 30%) starting at the bottom of a clean ultracentrifuge tube (Fig. 1). A 2 mL suspension of the phage is placed on top of the discontinuous iodixanol gradient and centrifuged (200,000 × *g*, 2 h, 4 °C). Next, after collecting the phage band, another discontinuous iodixanol gradient is constructed by sandwiching 1 mL of the purified phage (middle) between 1 mL of 40% iodixanol solution (bottom) and 1 mL of 30% iodixanol solution (top) in a clean ultracentrifuge tube. A 2 mL volume of saline is added on top of the discontinuous gradient. The tube is then centrifuged (200,000 × *g*, 2 h, 4 °C) (Fig. 1). The phage band is collected and filtered using a sterilized 0.45 μm syringe filter and a 1 mL sterilized disposable plastic syringe. The purified phage is stored at 4 °C until use (*see* **Note 4**). The concentration of the purified phage is measured (in plaque-forming units) using a plaque assay, immediately before the animal experiments (*see* **Note 5**) [14].

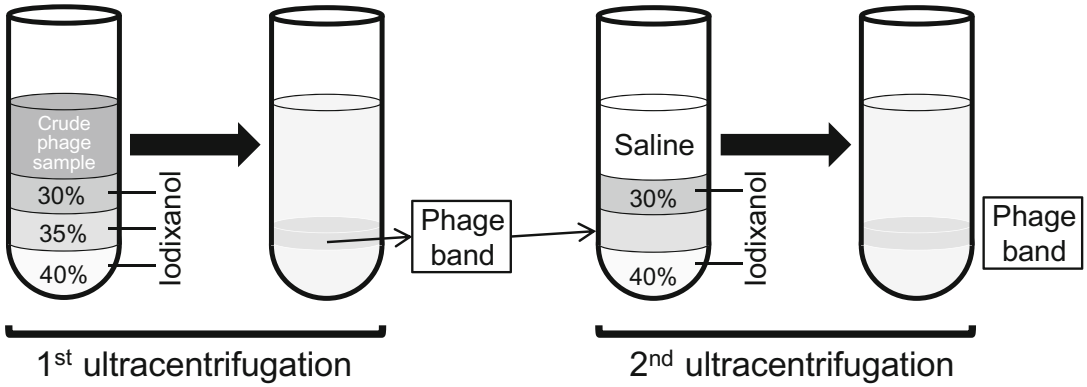

Fig. 1 Iodixanol density-gradient ultracentrifugation procedure. First, 1 mL each of 40%, 35%, and 30% iodixanol, and 2 mL of the phage solution are layered from the bottom of the tube. After the first ultracentrifugation step, the bacterial debris in the upper phase of the tube is removed and the phage band is collected. The phage suspension is then sandwiched between 40% and 30% iodixanol to obtain a discontinuous iodixanol gradient. After mounting 2 mL of saline on it, the phage suspension is then subjected to a second ultracentrifugation step

3.2 Silkworm Larvae Experiment

3.2.1 Preparation for Phage Therapy Experiments Using Silkworm Larvae

1. Silkworm larvae in the larval housing cage are maintained at 27 °C in an incubator (Fig. 2a). On the final day of the fourth instar, silkworm larvae are fed for 1 day with antibiotic-free artificial food (*see* **Note 6**). On the following day (i.e., the first day of the fifth instar), the silkworm larvae are used in the experiments.
2. The safety of the phage itself and the vehicles, i.e., iodixanol and HIMC, is examined. 0.05 mL of the phage suspension, 40% iodixanol, and HIMC are administered into the hemolymph via the dorsal surface of the silkworm larvae using a 32-gauge disposable needle and a 1 mL sterilized disposable plastic syringe (Fig. 2b) (*see* **Note 7**). The silkworm larvae are then kept in a new clean silkworm larva housing cage without feeding. The survival and behavior of the larvae are recorded daily for 1 week. If lethality is not observed after the administration of any of the samples into the silkworm larvae, the phage therapy experiment can be performed.

3.2.2 Phage Therapy Experiment Using Silkworm Larvae

1. The bacteria are cultured in tryptic soy broth until the mid-log phase and are then washed three times with saline and suspended in sterilized saline. Bacterial suspensions are prepared at various concentrations using a densitometer/colorimeter and a Petroff-Hausser counting chamber (*see* **Note 8**). 0.05 mL of each bacterial suspension is injected into the hemolymph via the dorsal surface of the silkworm larva (*see* **Note 7**) (Fig. 2b). The infected silkworm larvae are kept in new clean larval housing cages without feeding. The lethality and

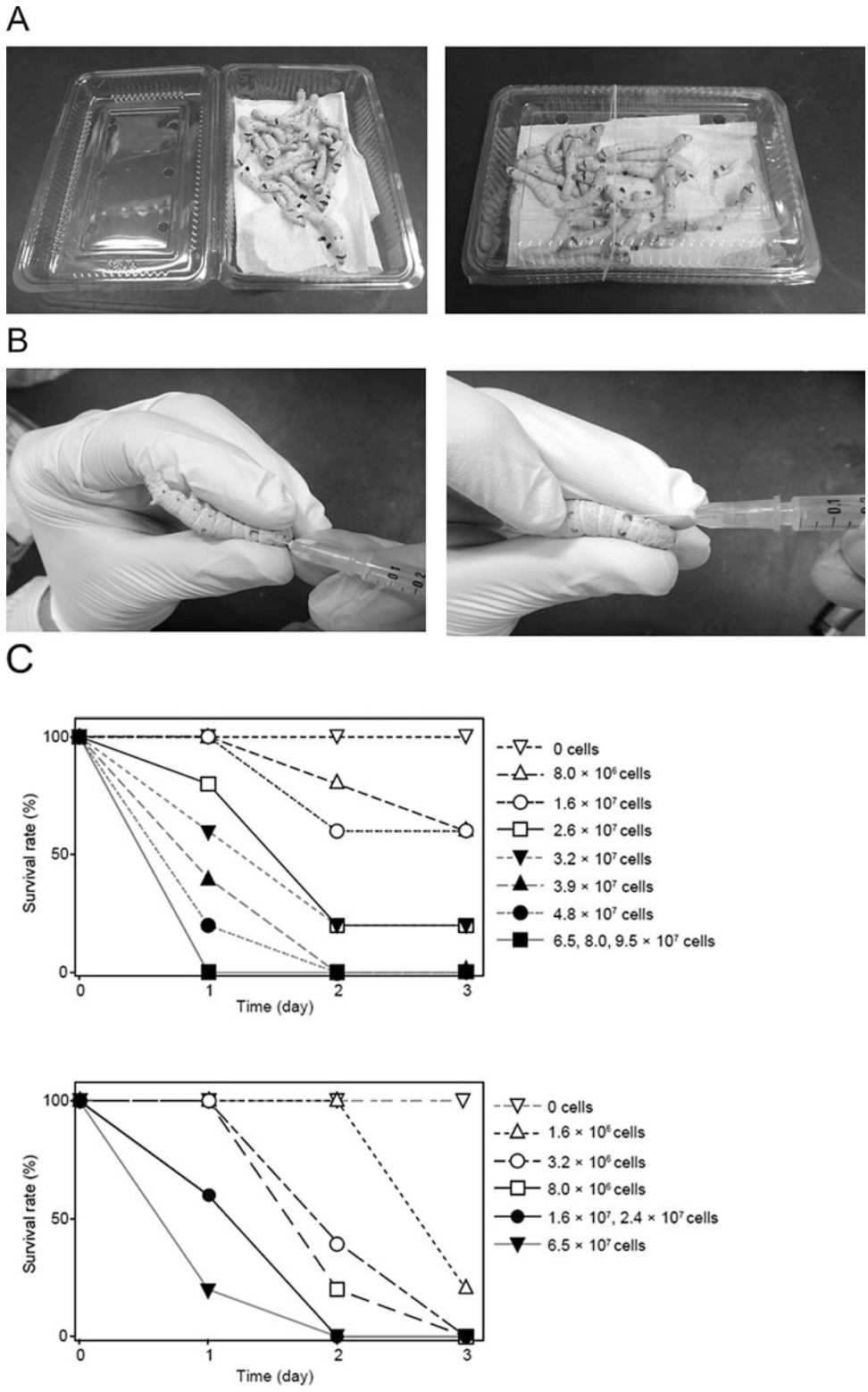

Fig. 2 (**a**) Silkworm larva housing cage. The lid is opened (left) and closed with a rubber band (right). (**b**) Injection procedure in silkworm larvae. The silkworm larva is held with the fingers (left) and the needle is

behavior are recorded daily. The minimal bacterial concentration that obtains 100% lethality on day two is selected for use in further experiments (e.g., Fig. 2c) (*see* **Note 9**).

2. The purified phage is diluted in HIMC (e.g., the multiplicity of infection [MOI] relative to the bacterial inoculation dose). Next, 0.05 mL of the phage suspension or control solution (i.e., HIMC) is injected into the hemolymph of each infected silkworm larva via the opposite side of the dorsal surface using a 32-gauge disposable needle and a 1 mL sterilized disposable plastic syringe (Fig. 3a) (*see* **Note 7**). The silkworm larvae are kept in new clean silkworm larva housing cages without feeding. Lethality and behavior are recorded daily for a specific experimental period (e.g., Fig. 3b) (*see* **Note 10**).

4 Notes

1. Phage purification by iodixanol density-gradient ultracentrifugation is strongly recommended because iodixanol is safe for living organisms. In our experience, if phages are purified by conventional CsCl density-gradient ultracentrifugation, the purified phages still contain CsCl residues even after dialysis, which is toxic to the silkworm larvae. Thus, iodixanol is safer than CsCl for the silkworm larvae. Moreover, the iodixanol should be diluted with sterilized saline on a clean bench to avoid unexpected contamination.
2. The silkworm larva hatches from an egg, molts four times, and then produces a cocoon. Each developmental stage of the larvae is known as an "instar." For example, the fourth instar indicates that the silkworm larva has molted three times. Silkworm strains can be purchased at any larval instar (e.g., Hu·Yo × Tukuba·Ne at the fourth instar) from a silkworm breeding company (e.g., Ehime Sanshu [Ehime, Japan]). If it is not possible to access commercially available silkworm larvae, eggs can be purchased from a company and reared in the laboratory. They should be housed in an incubator at 27 °C and antibiotic-containing artificial food is fed to the silkworm larvae after they hatch.

Fig. 2 (continued) inserted into the dorsal vessel via the dorsal surface of the silkworm larva, where a maximum of 0.05 mL is injected slowly into the hemolymph (right). (**c**) Results of the silkworm larva infection experiments with *S. aureus* strains SA27 (top) and SA14 (bottom). *S. aureus* strain SA27 was more sensitive to phage S25-4 than SA14. The bacterial dose, i.e., the minimal bacterial concentration that obtained 100% lethality on day two, was selected for further experiments, i.e., 3.9×10^7 and 1.6×10^7 cells of strains SA27 and SA14, respectively. These figures are drawn based on the supplementary Tables S2 and S3 of reference 10

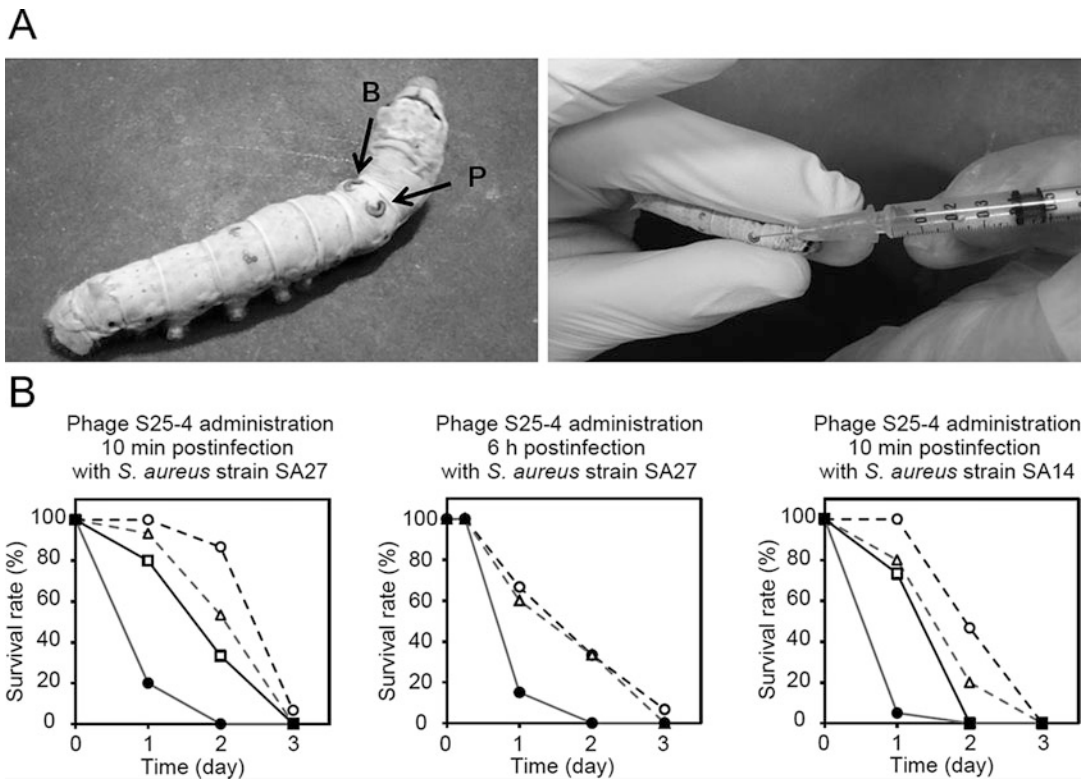

Fig. 3 Phage therapy experiments using the staphylococcal silkworm larva infection model with phage S25-4. (**a**) Procedure for phage administration into silkworm larvae. Phage S25-4 is classified in the family *Myoviridae*, genus *Kayvirus* [15, 16]. Photograph of the silkworm larvae (left). The arrows with "B" and "P" indicate the sites of bacterial inoculation and of phage administration respectively. After the silkworm larvae are infected, as shown in Fig. 2b, the phage is administered via the opposite side of the silkworm larva's dorsal surface relative to the site inoculated with bacteria (right). (**b**) Results of the phage therapy experiments using *S. aureus* phage S24-4. Silkworm larvae were infected with *S. aureus* strain SA27 (left and middle columns) or SA14 (right column). Then, phage was administered at 10 min (left and right columns) or 6 h after infection (middle column). The survival rates of the silkworm larvae were recorded daily. Five silkworm larvae were tested in each treatment group. The phage therapy experiments were performed in triplicate (15 silkworm larvae in total) and negative control experiments were performed in quadruplicate (20 silkworm larvae in total). The survival rates were calculated by the total number of surviving silkworm larvae in the replicate experiments. Symbols in the left and right columns: ○, MOI of 1; △, MOI of 10^{-2}; □, MOI of 10^{-4}; ●, HIMC-treated control. Symbols in the middle column: ○, MOI of 1; △, MOI of 10^{-1}; ●, HIMC-treated control. According to Fisher's exact test, the survival rates of the phage-treated (MOI of 1) and HIMC-treated groups in all the panels differed significantly on day two ($P < 0.01$). The phage-administered groups exhibited longer life-prolonging effects than the HIMC-administered groups

3. To prepare the silkworm larva housing cages, air vents must be made in the lids of disposable plastic containers. For example, holes can be made in the lid using a punch press or a burned inoculation loop. KimWipes are placed at the bottom of the disposable plastic container. When housing silkworm larvae in

disposable plastic containers, the lids should be firmly closed to prevent them from escaping (e.g., by wrapping rubber bands around the plastic container, or by stapling the lid on the plastic container).

4. Each phage exhibits differences in stability, particularly after purification. Thus, it is recommended that the changes in the concentration of the purified phage should be measured for a specific period (e.g., one to 3 months) to facilitate the further animal experiments.
5. The double-layered agar method is used. The top and bottom agars are tryptic soy broth containing 0.5% and 1.5%, respectively.
6. Artificial food containing antibiotics is fed to protect the silkworm larvae from bacterial infections during sericulture. However, the silkworm larvae must be fed on antibiotic-free artificial food for 1 day to eliminate the antibiotics from the larvae. The artificial diets with and without antibiotics can be purchased from companies such as Nihon Nosan Kogyo (Tokyo, Japan) and Katakura Kogyo (Tokyo, Japan), respectively.
7. The injection procedure needs to be performed carefully and consistently. The thin needle should be inserted into the dorsal vessel at an angle and only a tiny volume of the solution (i.e., 0.05 mL) is slowly injected. Some mistakes may occur during the injection procedure. For example, the needle may be mistakenly inserted into the midgut over the dorsal vessel, preventing the phage from being injected into the hemolymph. During the injection, the needle makes a hole in the surface of the silkworm larva and excessive bleeding may occur, which can significantly affect the health of the silkworm larva.
8. The bacterial concentration can be calculated from the turbidity using a turbidimeter (e.g., a Klett-Summerson photoelectric colorimeter (Thomas Scientific) or a spectrophotometer). The turbidity corresponds to the bacterial concentration, which can be measured using a Petroff-Hausser counting chamber.
9. Since the silkworm larvae are not fed, the experiments must be completed within a few days. As shown in Fig. 2b, the minimal bacterial concentration that obtains 100% lethality on day two is selected for further experiments.
10. Silkworm larvae are different from vertebrates (e.g., in terms of their circulatory system and immune systems); thus, life-prolonging effects are used as an index when evaluating the effects of the phage.

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Chapter 15

Exploitation of *Drosophila* Infection Models to Evaluate Antibacterial Efficacy of Phages

Hye-Jeong Jang, Hee-Won Bae, and You-Hee Cho

Abstract

Nonmammalian infection models have been exploited to understand the various aspects of host-pathogen interactions and also provided innovative research platforms for identification of virulence factors, screening for antimicrobial hits, and evaluation of antimicroial efficacy. Here we describe a relatively straightforward protocol to assess the antibacterial efficacy of bacteriophages (phages) toward the opportunistic human pathogen, *Pseudomonas aeruginosa*, based on the systemic infection model using the fruit fly, *Drosophila melanogaster*. Since phages, unlike antibacterial chemicals, can be easily and sensitively enumerated by simple assays, it is also possible to address the pharmacokinetic properties of administered phages even in this small-scale infection model.

Key words Small-scale, Infection model, *Drosophila melanogaster*, *Pseudomonas aeruginosa*, Phage, Antibacterial efficacy, Pharmacokinetics

1 Introduction

Common virulence and defense mechanisms in host–pathogen interactions are largely based on the conservation of the molecular determinants in bacterial virulence as well as in innate immunity to bacterial pathogens. Over the past decade, various infection models have been developed and exploited based on a number of traits that meet the need between genetic tractability and similarity to mammals. One of the extensively studied infection model hosts, the fruit fly, *Drosophila melanogaster* is experimentally well defined with a relatively well-characterized innate immune system in response to bacterial infections [1, 2].

Drosophila defends itself against infecting bacteria by recruiting both humoral and cellular components of the immune response [2], which are activated primarily by two parallel signaling pathways: Toll and Imd pathways [3]. The Toll pathway is activated primarily upon infections by gram-positive bacteria, and results in activation of the Rel family transcription factors, Dorsal and Dif,

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whereas the Imd pathway is activated preponderantly by gram-negative bacteria, and activates the third Rel family transactivator, Relish. Activation of those Rel family transactivators in *Drosophila* triggers the synthesis of appropriate antimicrobial peptides (AMPs) [2, 3].

Both Toll and Imd pathways exhibit remarkable similarities to the Toll-like receptor and tumor necrosis factor alpha pathways in mammals, respectively. This feature and the genetic tractability of *Drosophila* make it one of the best nonmammalian host animals to model human–pathogen interactions, as extensively demonstrated by its use for analyzing *Pseudomonas aeruginosa* (PA) pathogenesis [4–7]. PA is an opportunistic human pathogen capable of experimental acute infection when it is injected into the *Drosophila* dorsal thorax by simple pricking. The injected bacteria are capable of invasive proliferation, resulting in systemic spread, which provokes the activation of *Drosophila* immune system throughout the whole body [8]. To assess and validate the bioactivity of antibacterials using the *Drosophila* systemic infection model, antibacterials including antibiotic compounds and therapeutic phages can be preferably delivered by feeding, i.e., simply transferring starved flies into the fly media containing appropriate amount of the antibacterials. The combination of bacterial infection and antibacterial administration routes has been successfully exploited to evaluate the antibacterial efficacy of the therapeutic phages in PA [9–11]. As well, we were able to measure the time-course distribution of phages in the fly body, which is the basic pharmacokinetic property of phages in *Drosophila*. Based on these, we suggest this simple protocol to reliably measure the antibacterial efficacy of various PA phages.

2 Materials

Prepare all media and solutions using sterilized water and reagents. Autoclave is required for all the supplies except for cornmeal media. Prepare and store all reagents at room temperature (unless otherwise indicated). Follow the waste disposal regulations when disposing waste materials and the biosafety guidelines when using bacterial cultures as described elsewhere [12].

2.1 Fly Maintenance

1. Fly stocks: Store live fly strains at 25 °C in cornmeal media. These can be obtained from: Carolina Biological Supply Company (http://www.carolina.com/), UC San Diego Drosophila Stock Center (https://stockcenter.ucsd.edu/info/welcome.php), Bloomington Drosophila Stock Center at Indiana University (https://bdsc.indiana.edu/), or Ward's Science (https://www.wardsci.com/).

2. Cornmeal media: 0.93% agar, 6.24% dry yeast, 4.08% corn meal, 8.62% dextrose, 0.1% Tegosept (Methyl 4-hydroxybenzoate, *Drosophila* antifungal agent, USBiological) 0.45% (vol/vol) propionic acid.
3. Sucrose media: 5% sucrose, 1% agar, 0.1% Tegosept.

2.2 Phage Preparation

1. Phage stocks: Store phage strains at −80 °C in a 2:1 mixture of phage solution and 60% glycerol or at 4 °C in phage buffer (*see* **Note 1**).
2. Phage buffer: 10 mM $MgSO_4$, 10 mM Tris (pH 7.6), 1 mM EDTA.
3. Top agar: 0.7% Bacto agar.

2.3 Bacterial Culture

1. PA stocks: Store bacterial strains at −80 °C in a 2:1 mixture of LB culture broth and 60% glycerol.
2. LB broth: 1% tryptone, 0.5% yeast extract, 1% NaCl.
3. LB agar plate: 1% tryptone, 0.5% yeast extract, 1% NaCl, 2% Bacto agar.
4. Cetrimide agar plate: 4.53% cetrimide agar (Difco), 1% glycerol.

2.4 Fly Infection

1. Fly equipments with fly pad for CO_2 anesthesia.
2. Sterilized 0.4-mm tungsten needle (*see* **Note 2**).
3. Phosphate buffered saline (PBS; 1×) solution: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4.

3 Methods

Carry out all procedures at room temperature, unless otherwise specified.

3.1 Fly Preparation

1. Grow the fly strain on cornmeal media (*see* **Note 3**).
2. Collect newly hatched female flies and keep them to the age of 5–7 days at 25 °C (*see* **Note 4**).
3. Perform the experiments using 15–30 flies per group.

3.2 Phage Preparation

1. Prepare 1 mL of sucrose media in fly vials.
2. Overlay the media with less than 100 μL of phage solution containing 10^7–10^{10} plaque-forming units (PFUs) of phages (*see* **Note 5**).
3. Air-dry the phage-containing sucrose media for 1 h under laminar flow.

3.3 Bacterial Preparation

1. Streak frozen glycerol stock of PA onto a fresh LB agar plate and incubate overnight (~14 h) at 37 °C (*see* **Note 6**).
2. Inoculate single colonies from the plates into culture tubes containing 3 mL LB broth and incubate overnight at 37 °C.
3. Subculture by diluting the culture in 3 mL LB so that the optical density at 600 nm (OD_{600}) is 0.05 and incubate the culture at 37 °C until the OD_{600} reaches 2.7–3.0, which corresponds to ~10^9 colony-forming unit (CFU)/mL (*see* **Note 7**).
4. Centrifuge 1 mL of the culture aliquot for 2 min at 6000 × *g* and discard the supernatant.
5. Wash once using 1 mL of PBS and then centrifuge as in **step 4**.
6. Resuspend the bacteria in 1 mL of PBS and prepare the bacterial suspension with OD_{600} of 0.03 (i.e., ~10^7 CFU/mL) by serially diluting the cells in PBS (*see* **Note 8**).

3.4 Measurement of Pharmacokinetics

1. Transfer the flies into an empty fly vial for starvation for 3 h.
2. After 3-h starvation, transfer the flies into a sucrose media containing appropriate amount of phages and store the vials at 25 °C for 12 h (*see* **Note 5**).
3. Transfer the fed flies into a fresh sucrose media without phages.
4. Remove 3–6 flies from the vial at every 12 h and homogenize each fly individually in 100 μL of phage buffer using a plastic pestle (*see* **Note 9**).
5. Determine the phage titer in the homogenates by measuring the PFUs. Two assays (spotting and plaquing) are generally performed (*see* **Note 10**) (Fig. 1).

3.5 Systemic Infection

1. Anesthetize the flies with CO_2 and place them as a group on the fly pad.
2. Dip the sterilized 0.4-mm tungsten needle into PBS-diluted bacterial suspension with the OD_{600} of 0.03 (*see* **Note 11**).
3. Prick the dorsal thorax by inserting the tip of the needle into the thorax (*see* **Note 12**) (Fig. 2).
4. Repeat **steps 2–3** until all flies in the group have been infected.
5. Transfer the infected flies to the sucrose media with or without phages and incubate the vials at 25 °C.

3.6 Assessment of Antibacterial Efficacy

Antibacterial efficacy can be assessed conventionally by determining the survival rate of the flies (A) or the bacterial proliferation within the fly tissues (B).

(A) *Survival rate determination*

1. Count the number of living flies. Flies begin to die around 24 h postinfection (*see* **Note 13**).

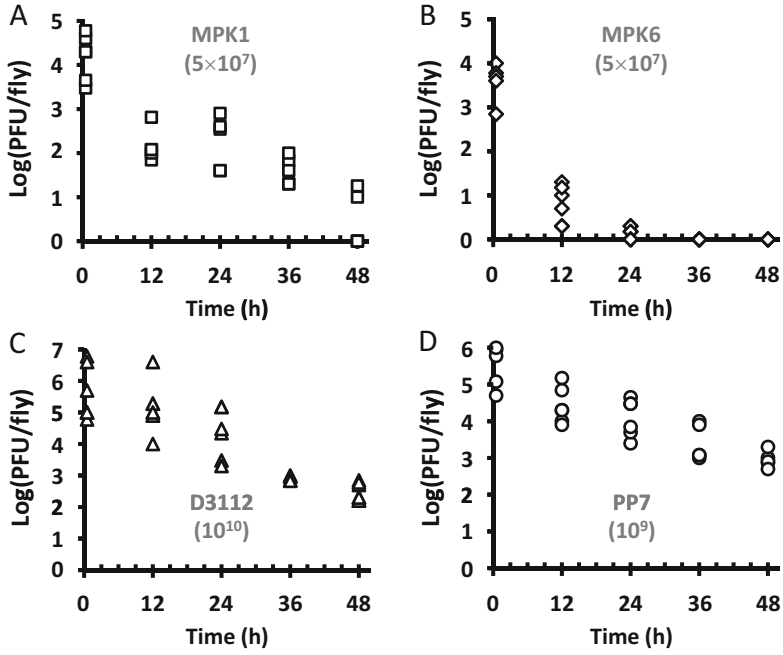

Fig. 1 Pharmacokinetics of PA phages. Phage samples (in 50 μL of PBS) of MPK1 (**a**; myophage, square), MPK6 (**b**; podophage, diamond), D3112 (**c**; siphophage, triangle), and PP7 (**d**; leviphage, circle) were overlaid on the surface of the 1-mL sucrose media. Groups of flies ($n = 5$) were collected at 0.5, 12, 24, 36, and 48 h and their homogenates were removed to measure the PFU per fly, which is shown in a log scale. The amounts of phages administered in the sucrose media are designated

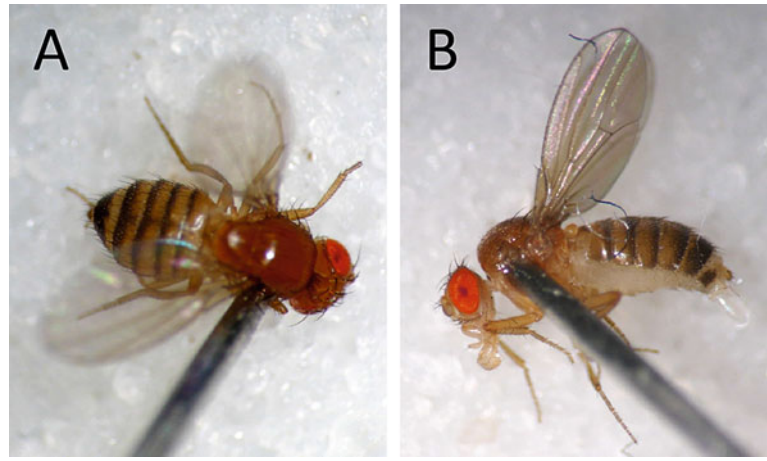

Fig. 2 Fly systemic infection. The flies are softly pricked (less than 0.2 mm deep) with a 0.4-mm tungsten needle that has been slightly dipped into the bacterial suspension. The dorsolateral thorax is punctured with the tip of the needle. (**a**) Top view; (**b**) side view

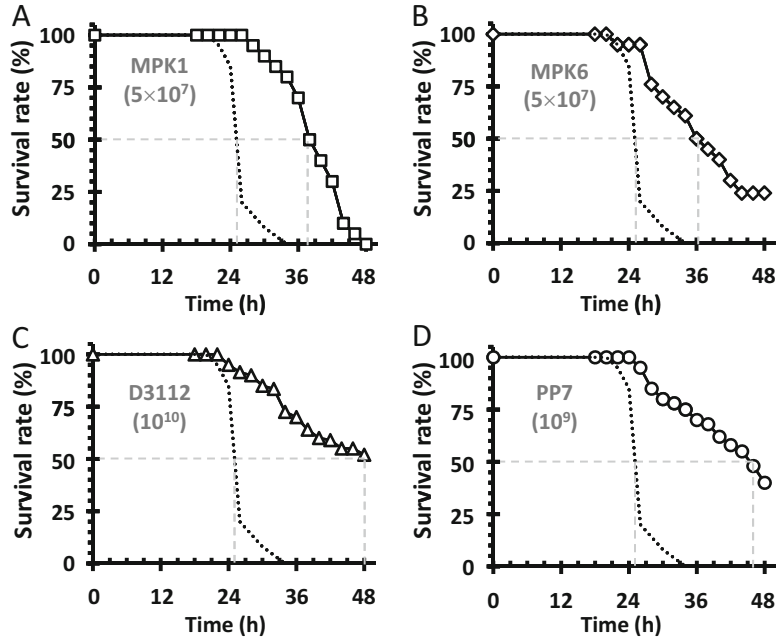

Fig. 3 Evaluation of antibacterial efficacy using PA phages. PAO1-infected flies ($n = 150$) were transferred to new medium overlaid with nothing (dotted line) or phage samples of MPK1 (**a**), MPK6 (**b**), D3112 (**c**), and PP7 (**d**) as described in Fig. 1. The amounts of phages administered in the 1-mL sucrose media are designated. The grey dotted lines represent the time required to reach 50% mortality. The significance has been verified based on logrank tests with all the *p* values below 0.001

2. Monitor the fly death up to 60 h, although the 100% mortality has been achieved around 48 h postinfection.
3. Determine the survival percentage as a function of time.
4. Carry out the independent experiments more than five times.
5. Determine the statistical significance of the survival difference between the groups, based on Kaplan–Meier logrank tests (*see* **Note 14**) (Fig. 3).

(B) *Bacteremia determination*

1. Remove six flies from the vials at various time points and homogenize each fly individually in 100 μL of LB broth using a plastic pestle (*see* **Note 9**).
2. Plate serial dilutions of the fly homogenates on cetrimide agar plates and incubate the plates for 18 h at 37 °C.
3. Count the CFUs to enumerate the bacterial load in the homogenates.
4. Carry out the independent experiments more than five times.

4 Notes

1. The phage titer will last at least a week at 4 °C.
2. It gives good results as well to use a 0.1-mm tungsten needle.
3. Either Oregon R or Canton S can be generally used as the wild type strain of *Drosophila*. Flies can be contaminated with various microbes, in which extra treatments should be made for decontamination.
4. Using female flies gives more consistent results in the pricking experiment than using male flies.
5. Phage stocks are generally 10^{11}–10^{12} PFU/mL. The highest phage titer in sucrose media is practically 10^{10} PFU/mL, which is generally sufficient to show antibacterial activity without toxic effects. We have generally used ~10^{8} PFU/mL for myophages and podophages, 10^{9} PFU/mL for leviphages, and 10^{10} PFU/mL for temperate siphophages [9–11] (Figs. 1 and 3). The appropriate amount of phages should be carefully optimized, since the PK properties are significantly dependent on the amount used in the feeding in the 1-mL sucrose media.
6. We have been using the PAO1 strain due to its susceptibility to wide range of isolated phages.
7. The doubling time may vary, depending on the strains used. For PAO1, this doubling time takes about 5 h.
8. **Steps 4–6** can be simplified by omitting the washing steps: take 10 μL of the culture and transfer it to 990 μL of PBS. PBS can be replaced with 10 mM $MgSO_4$ for PA strains.
9. We usually make observations at 0.5, 12, 24, 36, and 48 h postinfection.
10. For spotting assay, droplets (3 μL) of the serially diluted homogenates are spotted onto an LB medium layered with 50 μL of PAO1 cells at the OD_{600} of 1.0. The plates are incubated at 37 °C for 16–24 h. For plaque assay, aliquots (10 μL) of the serially diluted homogenates that may contain about 10^{2} PFU phages are mixed with 10^{7} CFU of PAO1 cells at the OD_{600} of 1.0 and resuspended in 100 μL of phage buffer. After 10-min incubation at 37 °C, 3 mL of top agar is added and the mixture is plated onto preequilibrated LB agar plates. Plaques were visualized after 16–24 h of incubation at 37 °C.
11. This needs to be done to prevent bacterial sedimentation during the fly infection procedures.
12. By this way, we effectively introduce 50–200 bacteria locally in the fly body.
13. We generally monitor ~every 3 h postinfection. Once the mortality commences, 1-h interval would be better.

14. Exclude the flies that die before 12-h postinfection, since this "initial" death is likely a result of mechanical injury or septic shock. The percentage of the "initial" death should not exceed 5%, otherwise the survival rate can be confounded.

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Chapter 16

Duckweed (*Lemna minor*) and Alfalfa (*Medicago sativa*) as Bacterial Infection Model Systems

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Abstract

Alternative animal host models of bacterial infection have been developed which reproduce some of the disease conditions observed in higher animals. Analogously, plants are useful for modeling bacterial pathogenesis, in some cases revealing broadly conserved infection mechanisms. Similar to animals, plants have been shown to possess innate immune systems that respond to invading viruses, bacteria, and fungi. Plant infection models often yield results faster, are more convenient, and less expensive than many animal infection models. Here, we describe the use of two different plant-based infection models for the discovery of virulence genes and factors involved in bacterial pathogenesis.

Key words Bacteria, Pathogenesis, Infection models, Virulence, Virulence factors, Duckweed, Alfalfa

1 Introduction

A number of animal models, such as for mice and rats [1–5], have been adapted for bacterial infection studies. In addition, several alternative animal infection models have been developed, including *Galleria mellonella* (greater wax moth) larvae [6], *Drosophila melanogaster* (common fruit fly) [7], *Caenorhabditis elegans* (nematode) [8], and *Danio rerio* (zebrafish) embryos [9], applying protocols which have been outlined in this volume. These models reproduce some of the disease conditions observed in higher animals. However, plants are also gaining recognition for their usefulness in modeling bacterial pathogenesis, in some cases revealing broadly conserved infection mechanisms [10–13]. Similar to animals, plants have been shown to possess innate immune systems that respond to invading viruses, bacteria, and fungi [14, 15] with the production of oxidative bursts, antimicrobial peptides, and secondary metabolites [16, 17]. These responses parallel some of the most important defenses that invasive bacteria encounter in animal hosts. For bacterial pathogens that are able to infect an expansive range of hosts, plants represent inexpensive and easily

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manipulated infection models for the exploration of virulence factors, the infection process, and the progressions through which relatively benign bacteria evolve into pathogens. For bacterial pathogens able to infect a wide range of hosts, such as *Pseudomonas aeruginosa*, the utilization of multiple infection models has led to the identification of several shared universal virulence factors [18]. However, this multi-host approach has also demonstrated that many common bacterial virulence factors may not always be produced during the infection of specific hosts [19], although some of these expressional differences may relate to the location of the infection on the plant, with noted differences existing between the roots, stems, and leaves [20–22]. Several excellent plant models have been used previously to study bacterial pathogenesis, including onion [23], tomato [11], lettuce [24], *Arabidopsis thaliana* [25–27], and a pea colonization model [28]. However, we will focus this article on two of the most utilized and useful plant infection models currently in use today, the alfalfa seedling [21, 29] and duckweed [30, 31] infection models.

Alfalfa (*Medicago sativa*) is a perennial flowering legume of the pea family Fabaceae cultivated as an important forage crop worldwide. With a deep root system, it is resilient to drought, each plant normally living 4–8 years, with a slow-growing seedling stage. An alternative model of infection has been developed in which alfalfa seedlings are germinated overnight, a leaf wounded with a needle, and then inoculated with dilute suspensions of bacteria. Infected alfalfa seedlings show disease symptoms within 6 days, and, therefore, this alternative host model is faster, more convenient, and less expensive than many animal infection models. The alfalfa seedling alternative infection model has been used to streamline the discovery of genes encoding new virulence determinants [21, 29].

Common duckweed (*Lemna minor*, family Lemnaceae) is one of the smallest known flowering plants and can be found growing on the surfaces of freshwater bodies throughout the world. A monocotyledon plant, it can reproduce both sexually through fertilization and asexually by budding. The latter strategy provides a means by which to generate a large clonal population from a single plant. By eliminating the genetic variability common to current plant and animal infection models, the duckweed model allows the infection process to be studied with greater reproducibility. Axenic or sterile cultures of duckweed are easily obtained, thereby permitting the examination of an isolated, bipartite bacterial infection process. The high-throughput potential of the duckweed model has been demonstrated through a virulence factor screen of a *Burkholderia cenocepacia* mutant library, resulting in the identification of several novel putative virulence factors [31].

The use of phages to control pathogenic bacteria directly in plant infection models has been tested in several examples. Under in vivo conditions, phages applied to plant infection models have

met with limited success [32]. In some instances, there can be present endogenous plant-derived inhibitory compounds that inactivate phages inside of plants and plant tissues, such that direct phage treatment may be less effective in plant models than other types of infection models. Nevertheless, surface or soil biocontrol of plant pathogens and plant growth promotion has been observed [33–40], and can be facilitated by phage or phage cocktail applications. This suggests that phage treatment of bacteria peripherally associated with the roots or leaves can be successfully used in agricultural settings, against certain types of bacterial infections of plants. Bacterial species that have been successfully treated with phages in biocontrol-type models include *Dickeya dadantii* [33], *Erwinia amylovora* [34], *Pseudomonas syringae* [35, 36], *Ralstonia solanacearum* [37, 38], *Salmonella oranienburg* [39], and *Xylella fastidiosa* [40].

2 Materials—Alfalfa

1. Alfalfa seeds (variety 57Q77) can be obtained from Pioneer Hi-Bred International, Inc. (Johnston, Iowa, USA; http://www.pioneer.com/).
2. Alfalfa plant growth and infection is performed on water agar plates (dH_20 water solidified with 1% Difco Bacto agar and 1% Difco Noble agar). Ingredients can be purchased from Difco Laboratories (Detroit, MI).
3. Other reagents relating to bacterial growth can be obtained from Difco Laboratories. Overnight bacterial cultures are grown aerobically in 2 mL of Luria-Bertani broth in 15-mL conical tubes (VWR International, Radnor, PA, USA; https://www.vwr.com/) for 18 h at 30 °C with shaking at 225 rpm.

3 Methods—Alfalfa

3.1 Seedling Germination

1. To disinfect and accelerate germination, seeds are immersed in concentrated sulfuric acid (approximately 10 mL for 300 seeds) for 20 min and then washed with 500 mL of sterile distilled water (dH_2O) four times.
2. Alfalfa seeds are then covered with 60 mL of sterile dH_2O in a 125-mL Erlenmeyer flask and incubated at 32 °C with shaking for 6–8 h to enhance uniform germination.
3. The seeds are rinsed twice with 60 mL of sterile dH_2O and incubated overnight in 60 mL of sterile dH_2O at 32 °C with shaking.
4. The following day, sterile forceps are used to place the seedlings (10 per plate) on the surface of water agar (deionized water solubilized with 1% Difco Bacto Agar and 1% Difco Noble agar).

5. Within 1 h one leaf of each seedling is wounded by piercing the leaf with a 20-gauge needle.
6. Immediately after the leaves are wounded, the seedlings are surface-inoculated with 10 μL aliquots of diluted bacterial cells.
7. The bacterial cultures used for the inoculum were serially diluted in 0.85% NaCl, and dilution aliquots were plated onto LB agar plates for quantitation.
8. Petri dishes containing seedlings are sealed with Parafilm in order to maintain a high level of humidity, and incubated in a 37 °C warm room under a desk lamp with an average of 8–12 h of artificial light per day at an intensity of 640 lx. Approximately 20 seedlings are used per bacterial inoculum.
9. At 7 days postinfection, the seedlings are visually monitored and scored for disease symptoms, in order of increasing severity of disease symptoms: (1) chlorosis, which is a yellowing of leaf tissue due to a lack of chlorophyll, (2) stunting of root growth, (3) lack of root hairs, and (4) necrosis, evident by brown areas on the seedling.

3.2 Recovery of Bacteria from Infected Alfalfa Seedlings

1. For each bacterial strain, five to ten infected seedlings are homogenized in a Kontes tissue grinder (Thermo Fisher Scientific) in 1 mL of 0.85% NaCl.
2. The resulting suspension is serially diluted in 0.85% NaCl and plated onto LB agar to determine the number of CFU per seedling.
3. Seedlings with disease symptoms are randomly selected for bacterial quantitation except for seedlings inoculated with strains that do not cause symptoms of disease (*see* **Notes 1** and **2**) .
4. Analysis of variance (ANOVA) and linear regression can be performed with INSTAT software (GraphPad Software, San Diego, CA, USA; https://www.graphpad.com/).

4 Materials—Duckweed

1. Duckweed plants can be obtained from any number of sources including botanical gardens, commercial greenhouses, or academic botany departments. Once obtained, duckweed can be propagated continuously in nonsterile water at little additional cost.
2. For bacterial infection experimentation, plants are grown statically in 24-well plates at 30 °C in sterile Schenk-Hildebrandt medium supplemented with 1% w/v sucrose (SHS) from Sigma-Aldrich Canada (Oakville, ON; https://www.sigmaaldrich.com/). Plants should be maintained under a

light–dark cycle of 18/6 h to promote asexual reproduction by division, prior to bacterial infection. Under these conditions, duckweed plants will produce two to three generations per week.

3. All other reagents relating to bacterial growth can be obtained from Difco Laboratories (Detroit, MI). Overnight bacterial cultures are grown aerobically in 2 mL of Lysogeny broth (LB) in 15-mL conical tubes (VWR International, Radnor, PA) for 18 h at 30 °C with shaking at 225 rpm.
4. For selection of specific markers, antibiotics can be obtained from Sigma-Aldrich Canada (Oakville, ON). Antibiotics are included in the bacterial media at typical concentrations depending upon the bacterial strains used: for *E. coli*, for example, tetracycline (Tc) can be used at 10 μg/mL, and trimethoprim (Tp) at 100 μg/mL.

5 Methods—Duckweed

5.1 Plant Surface Sterilization

1. For axenic growth, submerge the duckweed plants in 10% (v/v) bleach for 10 s using a sterile inoculating loop.
2. Transfer the plants to 70% (v/v) ethanol and submerge for 10 s as above.
3. Finally, transfer the plants into an excess of sterile Schenk-Hildebrandt medium supplemented with 1% w/v sucrose (SHS) to recover. The plants should be rinsed in this media until no trace of bleach and ethanol remains.

5.2 Plant Infections

1. To separately infect each individual plant, fill each well of a 96-well plate with 180 μL of SHS and one duckweed plant comprising 2–3 fronds (large-divided leafs; e.g., an intermediate stage of growth).
2. To begin the duckweed plant infection, centrifuge 1 mL of an overnight bacterial culture for 5 min at 5000 × *g*, and gently resuspend the cell pellet in 1 mL SHS to wash the cells.
3. Repeat the centrifugation of the bacterial cells as above, and again, gently resuspend the cells in a final volume of 1 mL SHS. For bacterial species exhibiting relatively high lethality, dilution in SHS to an appropriate concentration can be made in order to best observe the infectious process.
4. Starting at the left side of the 96-well plate, tenfold serial dilutions are made across the plate: 20 μL of the final washed bacterial cell suspension is added to the 180 μL in the first column of eight wells, using a multichannel micropipette (e.g., Eppendorf Research Plus micropipette, Hamburg,

Germany). Dilutions across the plate should produce a final volume of 180 μL in each well.

5. Infection plates are typically wrapped in cellophane to reduce evaporation of liquid from wells and placed at 30 °C in the dark.
6. Surviving plants can be counted at 24 h, 48 h, or 96 h, depending upon the lethality of the bacterial strain used. Plants are identified as "alive" when more than 10% of the plant remains green after the chosen timepoint, and plants that display >90% loss of green pigmentation are considered dead. In our experience, individual plants began to show signs of morbidity at high bacterial doses by 24 h, with bacterial infections reaching completion by 96 h (4 days). After this time, surviving plants tend to persist, having resisted the initial infection.
7. Several independent trials should be performed consisting of 4–8 replicate infections serially diluted five times, and separate overnight bacterial cultures should be grown for each trial.
8. To enumerate the total number of bacterial cells in each well, 10 μL from each dilution well can be spotted onto LB agar plates using a Research Plus multichannel micropipettor (Eppendorf) following cell dilution, and incubate at 37 °C overnight. Wild-type nonattenuated bacteria should be tested in parallel as controls.
9. Statistical analysis of the LD50 values represent the average of replicates ±standard error, and LD50 values among the strains can be compared using Student's *t*-tests. LD50 values for duckweed infections can be determined according to the protocol described by Randhawa [41], with LD50 values derived from each independent trial combined to produce an average and standard error.

6 Notes

1. For all bacterial strains tested in the alfalfa model, the number of bacteria recovered was at least tenfold higher than the inoculum, indicating that all bacteria were able to grow on alfalfa. For bacteria unable to cause disease in alfalfa seedlings, it may be due to their inability to grow on the seedlings.
2. Different bacteria occasionally show differing effects in alfalfa. For example, *P. aeruginosa* infections are localized to the wounded leaves and create greater tissue maceration, whereas some members of the *Burkholderia cepacia* complex do not require leaf wounding, and are more damaging to the seedlings at 37 °C rather than 30 °C.

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Chapter 17

Use of a Chicken Embryo Lethality Assay to Assess the Efficacy of Phage Therapy

Angélina Trotereau and Catherine Schouler

Abstract

To combat infectious diseases induced by antibiotic-resistant bacteria in human and animals, phage therapy has regained attention by the scientific community. Before phages can be widely accepted as therapeutics in the same way as antibiotics, convincing detailed applied experimental evidence must be available. The embryonated chicken egg model has been used to study the virulence of many pathogens. We describe here a procedure to test the efficacy of phage therapy to treat colibacillosis using a chicken embryo lethality assay, this being potentially applied to others bacterial infection.

Key words Animal model, Eggs, Phage therapy, *Gallus gallus*, *Escherichia coli*

1 Introduction

In recent years there has been a high resurgence of pathologies caused by *Escherichia coli* in poultry farms around the world, resulting in avian colibacillosis becoming the leading bacterial pathology in the poultry industry [1]. Avian pathogenic *E. coli* (APEC) can induce a huge diversity of extra intestinal syndromes including infection of yolk sac resulting in death of chicken embryo [2, 3]. Bacteria may be acquired in ovo if the hen has salpingitis or via fecal contamination of eggs at laying [4]. Moreover, since APEC strains are phylogenetically related to human *E. coli* pathogenic strains, the transmission of APEC to humans is another concern that is gaining attention [5–7]. Colibacillosis can generally be controlled using antimicrobials yet the increase in antimicrobial resistance has become problematic due to frequent use of antibiotics, which promotes multiple drug resistance. Indeed, in 2015 in France, the proportion of multiresistant strains was 5.3% in hens/chickens and 2.7% in turkeys (https://www.resapath.anses.fr/resapath_uploadfiles/files/Documents/2016_RESAPATH%20Rapport%20Annuel_GB.pdf). This has prompted an urgent search for alternative treatments, including

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phage therapy [8]. Several studies have been performed to assess the efficacy of phage therapy to prevent or to cure avian colibacillosis. It has been shown that the injection of 10^4 phage R provided sufficient protection after the injection of 10^6 *E. coli* strain MW (O18:K1) intramuscularly and protection with 10^8 phage R following intracranial injection of *E. coli* strain MW [9]. Coliphages SPR02 and DAF6 are able to propagate on an APEC strain of serotype O2. The administration of these two phages prior to or after inoculation of the APEC strain to chickens by the respiratory route protected the animals. When the phage administration was carried out 24 h after inoculation of the APEC strain, a relatively high level of protection was observed, whereas the animals were poorly protected if the phages were administered 48 h after inoculation of the APEC strain [10, 11].

Before conducting controlled clinical trials (targeted animals or human) that are essential to settle the efficacy of phage therapy, evaluating it in relevant models that are straightforward to implement like the live avian embryo, is essential.

Chicken embryos can be used as a model for many bacterial infections including *Escherichia coli*, *Yersinia enterocolitica*, *Clostridium perfringens*, *Enterococcus cecorum*, *Enterococcus faecalis*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Salmonella enterica*, and *Francisella tularensis* [12–20]. In addition, the avian embryo lethality assay was also found to be suitable to discriminate between virulent and avirulent isolates, making it a powerful model [15, 21, 22]. Indeed, chicken amniotic fluid contains many antibacterial components involved in embryo protection and defense [23]. Moreover, avian embryos are currently not regulated by legislation as animal experiments in many countries. Accordingly, assessing the efficacy of phage therapy in such animal model is more ethically acceptable and is more relevant that in vitro studies.

We here describe a procedure for in vivo evaluating the therapeutic effects and safety of a coliphage to combat *E. coli* infection using embryonated eggs as a model.

2 Materials

1. *Escherichia coli* and purified coliphage suspension.
2. Eleven-day-old SPF embryonated eggs.
3. Lysogeny broth (LB) [24].
4. 70° ethanol.
5. Sterile/apyrogenic Dulbecco's Phosphate-Buffered Saline (DPBS).
6. Sterile/apyrogenic saline.
7. 1 mL sterilized disposable plastic syringe.

8. 18G (1.2 × 40 mm) sterile needles.
9. 25G (0.5 × 16 mm) sterile needles.
10. Cooled microcentrifuge, rotor, and tubes.
11. Portable Egg Candling Light Candler Tester.
12. Egg incubator or classical laboratory incubator.
13. Adhesive tape.
14. Spectrophotometer.
15. Incubator with shaking (37 °C).

3 Methods

3.1 Egg Incubation Conditions

1. Obtain specific pathogen-free (SPF) embryonated avian eggs from a suitable company.
2. Upon arrival, place eggs in an egg incubator with an automatic egg turner to rotate eggs regularly (Fig. 1, *see* **Note 1**).
3. Incubate eggs at 37.8 °C and 45% humidity with the egg air space up (large end up).

Fig. 1 Example of an egg incubator

3.2 Egg Candling

1. Use a light egg candler to check eggs for infertility by candling after about 7 or 8 days of incubation.
2. Remove the eggs from the incubator and place them in a dark room.
3. Hold the large end of each egg one at a time against the candler.
4. Observe the egg to determine if it is fertile or infertile (**Notes 2** and **3**).
5. Discard eggs that are unfertilized, and return the viable eggs to the incubator. Do not leave eggs outside of the incubator for more than 30 min.
6. On day 11, again candle the eggs and make a pencil mark about 2 mm from the end of the air sac.

3.3 Embryonated Egg Infection

1. Grow the bacteria in 5 mL of lysogeny broth (LB) overnight at 37 °C with shaking (180 rpm). Briefly centrifuge 1.5 mL of the overnight culture, then suspend the bacterial pellet in 1.5 mL of sterile/apyrogenic DPBS. Measure OD at 600 nm of 1/10 dilution of the bacterial suspension to adjust the bacterial concentration of the inoculum to 10^3 cfu/mL (*see* **Note 4**).
2. Prepare a phage suspension in sterile/apyrogenic saline at 2×10^4 pfu/mL.
3. Wash the eggshell around the pencil mark with 70% ethanol.
4. With an 18G (1.2 × 40 mm) sterile needle, punch a small hole in the shell at the pencil mark without piercing the shell membrane.
5. Draw up the bacterial inoculum into a sterile 1 mL syringe and attach a 25G (0.5 × 16 mm) needle.
6. Carefully insert the syringe with needle at a 45° angle in the hole than inject 100 μL into the allantoic cavity.
7. Seal the hole with a small piece of adhesive tape.
8. Inoculate 20 eggs with bacteria, and 10 eggs with 100 μL of sterile/apyrogenic DPBS.
9. Place the eggs back into the egg incubator with the air space pointed up.
10. Two hours after the bacterial inoculation, inject, as the same way as for the bacteria, 100 μL of phage solution into ten eggs (as a control) and into ten eggs that have been previously inoculated with bacteria.
11. Place the eggs back in the egg incubator.
12. Daily candle the eggs to monitor mortality up to 6 days (*see* **Notes 5** and **6**, Figs. 2 and 3).
13. Present the data of survival as Kaplan–Meier curves and analyze them using the log-rank test [25].

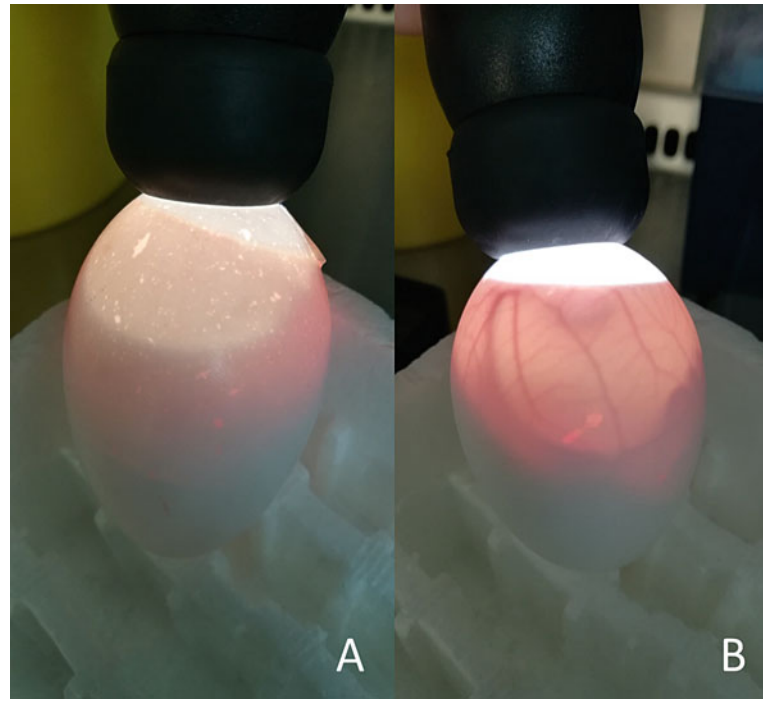

Fig. 2 Examination of embryo lethality by eggs candling. (**a**) Dead embryo. (**b**) Live embryo

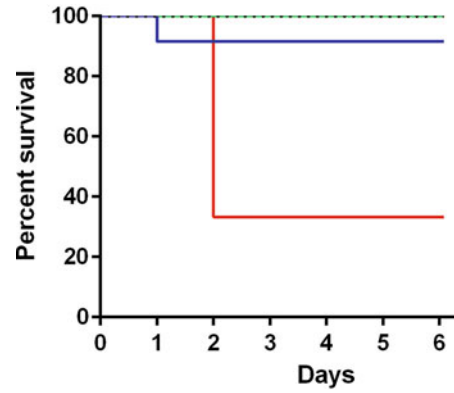

Fig. 3 Kaplan–Meier survival curves of embryonated chicken eggs infected with 100 cfu *E. coli* strain BEN5202 (a virulent avian strain of serogroup O2:K1) alone (red curve) and with the addition of 2000 pfu of coliphage ESCO5 [26] 2 h after the inoculation of *E. coli* (green curve). As controls, ten eggs have been inoculated with 2000 pfu of phage ESCO5 (blue curve) or 100 μL of DPBS (dashed red curve). Percent survival is shown on the *y*-axis, and days post infection are indicated on the *x*-axis. Results indicated that the addition of phage ESCO5 allowed 100% of chicken embryos to survive an infection by the avian pathogenic strain BEN5202, in contrast to the control, which gave a survival rate of 30%. However, it should be noted that one embryo died 1 day after the injection of the phage alone

4 Notes

1. Optimal egg incubation conditions would be obtained using a specific incubator. However, eggs could be incubated in a classical laboratory oven with some tips. A dish of water should be placed in the oven to increase the hygrometry. Manually rotate eggs at least twice a day.
2. Thin blood vessels leading to a bean-shaped embryo should be clearly visible. Unfertilized eggs will appear as a small blood spot with a visible egg yolk.
3. Do not leave eggs outside of the incubator for more than 30 min.
4. As each phage exhibits differences in stability phage involving that titers could decline over time prior to application, it is recommended to freshly determine the phage concentration of the parental solution.
5. The absence of movement and the breakdown of the blood vessels are characteristics of embryo mortality.
6. It is also possible to monitor bacterial and phage multiplication by sampling 100 μL of allantoic fluid without inducing death of embryo.

Acknowledgments

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Chapter 18

Quantitating Phage Efficacy in Ready-To-Eat Meats

Andrew Chibeu and Sampathkumar Balamurugan

Abstract

Bacteriophages are being applied in biocontrol of bacterial pathogens in foods and food processing environments. There is need for the development of standardized protocols to quantify the effectiveness of phage preparations in reducing food-borne pathogens on foods. Here, we present a procedure for the verification of the effectiveness of a phage preparation in reducing *Listeria monocytogenes* on ready-to-eat (RTE) meats. The protocol is designed taking into account real-world scenarios and avoiding common errors reported in previous phage decontamination assays.

Key words Bacteriophage, Biocontrol, *Listeria monocytogenes*, Ready-to-eat meat, LISTEX™ P100

1 Introduction

Bacteriophage use in the biocontrol of food-borne pathogens on food has been successfully applied in poultry, beef, fish, cheese, sprouts, melons, and other foods [1–7]. Previous studies have shown that successful phage-based pathogen intervention in food greatly depends on the chemical composition of the food and its specific matrix [3]. In ready-to-eat (RTE) products, protocols for the application of phages and their target bacteria require individual optimization, also taking into account the type of food matrix [3, 7, 8].

We here demonstrate how the efficacy of a bacteriophage preparation can be tested in reducing a food-borne pathogen on RTE meat. In an experiment to assess the efficacy of a commercial anti-*Listeria* phage preparation in reducing *Listeria monocytogenes* on RTE roast beef and cooked turkey, the key features of the experimental procedure that lack in previous phage studies are: (1) A *L. monocytogenes* four-strain cocktail is added at concentrations which mimic the contamination levels that occur in real-life scenarios; (2) Bacterial viable counts are determined following removal of unbound phages from stomached rinses prior to direct plating for *L. monocytogenes* in order to avoid overestimation of phage killing

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effect; (3) The rate of phage and host application are presented per unit area; (4) The phage decontamination study is performed at recommended storage temperature of the RTE meat (4 °C) in comparison to abusive temperature (10 °C).

2 Materials

2.1 Personal Protective Equipment

1. Disposable gloves.
2. Lab coat.
3. Disposable solid-front gown.
4. Biological safety cabinet (BSC).
5. Appropriate footwear.

2.2 Equipment

1. Refrigerators set at 4 °C and 10 °C.
2. Incubator and shaking incubator set at 37 °C.
3. Styrofoam meat trays (Dyne-A-Pak Inc., Laval, QC Canada).
4. 8″ × 6″ commercial barrier bags [oxygen transmission rate: 40–50 cc/m^2 24 h^{-1}; (Winpak Ltd., Winnipeg, MB, Canada)].
5. Chamber machine C 200 (MULTIVAC AGI, Knud Simonsen Industries Ltd., Rexdale, ON, Canada).
6. Sterile disposable spreaders (Arben Bioscience Inc., Rochester, NY, USA; Catalogue number KG-5P; http://www.arbenbio.com/).
7. Pipettor and pipettes.
8. Micropipettors and pipette tips.
9. Meat core cutter (Custom made to cut RTE meat slices into 10 cm^2 cores; alternatively can use a biscuit cutter e.g. Endurance®, RSVP International, Inc. Seattle, WA, USA; https://www.rsvp-intl.com/).
10. Steel plate work surfaces precooled to 4 °C (Custom made).
11. Stomacher® 80 *micro*Biomaster lab blender (Seward Laboratory Systems, Inc. Bohemia, NY, USA; http://seward.co.uk/) and Stomacher® 80 bags.
12. Benchtop centrifuge with swinging bucket rotor (Eppendorf 5804 R; Westbury, NY, USA).
13. Sealable Tupperware® containers.
14. Autoclavable plastic Nalgene™ buckets (Thermo Fisher Scientific Inc., Waltham, MA, USA).
15. 0.45 μm syringe filter.
16. 10 mL sterile, disposable syringe.
17. Spectrophotometer.

18. Disposable cuvettes with lids.
19. Water baths for tempering media (42 °C and 50 °C).
20. Vortex.

2.3 Reagents

1. LISTEX™ P100 (Micreos Food Safety B.V. Wageningen, Netherlands; https://www.phageguard.com/) (*see* **Note 4.1**).
2. *Listeria monocytogenes* cocktail (must consist of serotypes 1/2a, 1/2b, and 4b) (*see* **Note 4.3**).
3. Phosphate buffered saline (PBS; 100 mM NaCl, 20 mM Na_2HPO_4, pH 7.4).
4. Tryptic Soy Broth (TSB; BD Biosciences, San Jose, CA, USA) prepared according to manufacturer's directions.
5. Sliced, ready-to-eat meat (roast beef or cooked turkey).
6. 5 M HCl.
7. Tryptic Soy Agar (TSA; BD Biosciences, San Jose, CA, USA) prepared according to manufacturer's directions.
8. Oxford Agar (EMD Chemicals Inc., Gibbstown, NJ, USA; http://www.emdmillipore.com/) prepared according to manufacturer's directions.
9. Virucidal solution, freshly prepared (See ref. [9] in Volume 3 on use of virucides in phage decontamination studies).
10. SM buffer (10 mM NaCl, 10 mM $MgSO_4$, 50 mM Tris–HCl, pH 7.5).
11. Distilled deionized (Nanopure®) water.

3 Methods

3.1 Ready-To-Eat Meat Sample Preparation

1. Obtain freshly sliced meat products direct from the processing facility and store in tightly sealed Tupperware® containers at 4 °C until ready to use.
2. Place fresh, refrigerated samples on the precooled steel block work surfaces (wrapped with clean aluminum foil and refrigerated to 4 °C).
   (a) Samples should be kept on a precooled steel plate throughout.
   (b) Replace the plate with chilled one if the temperature increases noticeably during the previous steps.
3. Using autoclave sterilized meat core cutter or stainless steel cookie cutters, cut 162 uniform slices of meat with 10 cm^2 top-surface area.
4. Discard the remaining meat remnants in a biohazards waste bag.

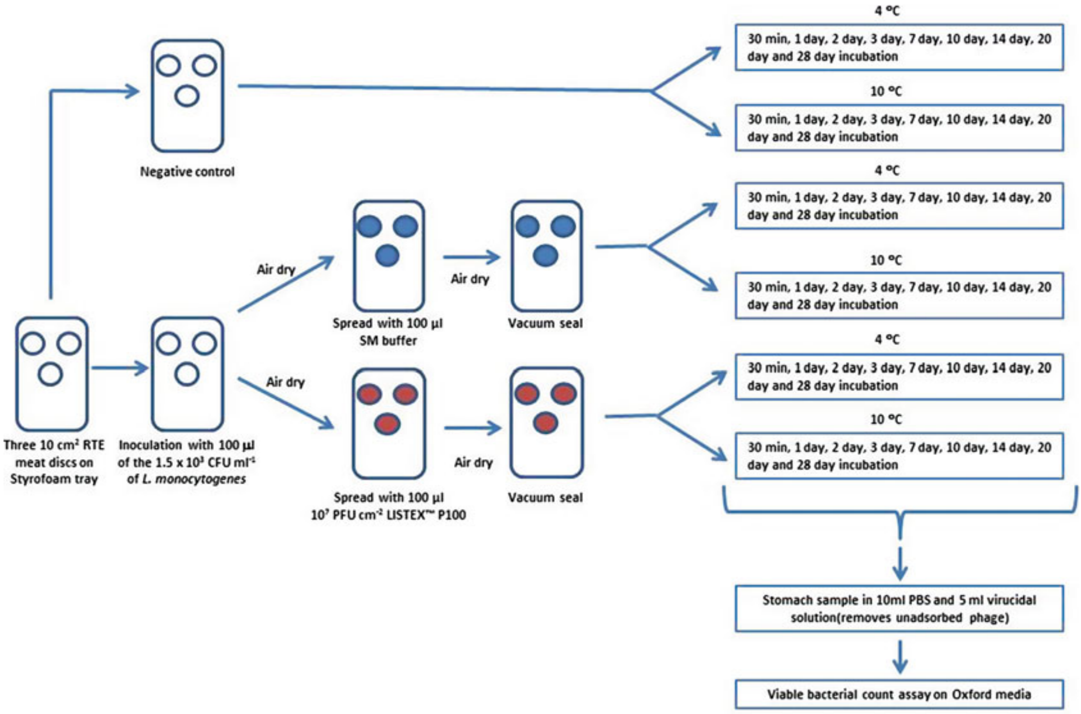

Fig. 1 Example of a sample preparation flow chart

5. Return all sliced meat samples to the Tupperware® container at 4 °C when not in use.
6. Repeat **steps 1–5** for each processed meat-type to be used (roast beef and cooked turkey). For each treatment, there are 9 storage times, "t", (t = 30 min; 1 day; 2 days; 3 days; 7 days; 10 days; 14 days; 20 days and; 28 days) and 2 storage temperatures (4 °C and 10 °C).

3.2 Preparation of Negative Controls

1. Place three 10 cm^2 meat slices individually on Styrofoam trays in the BSC and put in individual 8″ × 6″ commercial barrier bags (Fig. 1).
2. Vacuum seal 18 of the bags using the MULTIVAC chamber machine.
3. Store 9 of the vacuum sealed triplicate sample trays at 4 °C and label them "negative control 4 °C" and: 30 min; 1 day; 2 days; 3 days; 7 days; 10 days; 14 days; 20 days; and 28 days (shelf life).
4. Store the remaining nine vacuum sealed triplicate sample trays at 10 °C and label them "negative control 10 °C" and: 30 min; 1 day; 2 days; 3 days; 7 days; 10 days; 14 days; 20 days; and 28 days (shelf life) (Fig. 1).

3.3 Inoculating Samples with *L. monocytogenes*

1. Place three 10 cm^2 meat slices individually on Styrofoam trays (Fig. 1) in the BSC, inoculate 108 sliced 10 cm^2 meat slices from Subheading 3.1, **step 3** (36 sets of triplicate samples on Styrofoam trays) by spreading 100 μL of the 1.5 x 10^3 CFU/mL of *L. monocytogenes* inoculum on one side of the slice.
2. Air-dry the inoculum for 15 min in BSC to allow binding of the bacterium to the meat surface.
3. Take 18 triplicate sets of inoculated meat samples on Styrofoam trays from previous step and spread with 100 μL SM buffer and allow to air dry for 15 min and then put in individual 8″ × 6″ commercial barrier bags.
4. Vacuum seal 18 of the triplicate sample trays using the MULTIVAC chamber machine.
5. Label nine vacuum sealed triplicate sample trays "L. mono 4 °C" and: 30 min; 1 day; 2 days; 3 days; 7 days; 10 days; 14 days; 20 days; and 28 days (shelf life).
6. Store these samples at 4 °C.
7. Label nine vacuum sealed triplicate sample trays "L. mono 10 °C" and: 30 min; 1 day; 2 days; 3 days; 7 days; 10 days; 14 days; 20 days; and 28 days (shelf life).
8. Store these samples at 10 °C.

3.4 Inoculating Samples with Phage

1. Determine the volume of phage dilution to be spread over the meat slice to ensure application of 10^7 PFU/cm^2.
   (a) If phage was accurately diluted to ~10^9 PFU/mL, the expected plating volume will be 100 μL.
2. Take the remaining 18 triplicate sets of inoculated meat samples on Styrofoam trays from Subheading 3.3, **step 1** and spread the appropriate volume of phage preparation on the same surface as the *L. monocytogenes* inoculation.
3. Vacuum seal 18 of the triplicate sample trays using the MULTIVAC chamber machine.
4. Label nine sets of triplicate sample trays "L. mono + phage 4 °C" and: 30 min; 1 day; 2 days; 3 days; 7 days; 10 days; 14 days; 20 days; and 28 days (shelf life).
5. Store these samples at 4 °C.
6. Label nine sets of triplicate sample trays "L. mono + phage 10 °C" and: 30 min; 1 day; 2 days; 3 days; 7 days; 10 days; 14 days; 20 days; and 28 days (shelf life).
7. Store these samples at 10 °C.

3.5 Enumerating Viable Bacteria in the Samples

1. Use dissecting scissors to aseptically open the vacuum sealed meat samples.
2. Using sterile forceps aseptically transfer each meat sample to an appropriately labeled Stomacher® 80 bag.
   (a) Double-bag each sample to minimize the risk of infectious material leaking from the bags.
3. Using a sterile pipette, add 10 mL of sterile PBS to the bag.
4. Using a sterile pipette, add 5 mL virucidal solution to the bag to inactivate the remaining phage on the samples.
5. Place the bag in an autoclavable Nalgene™ bucket.
6. Repeat **steps 1–5** for all samples.
7. Place the bag into the Stomacher® lab blender, taking care to leave the top 3–4 in. of the bag above the paddles.
8. Blend the sample for 2 min (use a timer) at medium setting.
9. Transfer the bag containing the homogenized sample to another autoclavable Nalgene™ bucket.
10. Repeat **steps 7–9** for all samples.
11. Serially dilute the homogenate, tenfold, in sterile PBS to yield 1000 μL each of 10^{-1} and 10^{-2} dilutions.
12. For each sample plate 100 μl of the10^{-1} and 10^{-2} dilutions on 90 mm Oxford agar plates in triplicate.
13. If no colonies are observed on any of the plates, plate 1000 μL of undiluted homogenate (spread plate four 250 μL aliquots of the undiluted homogenate on four 90 mm Oxford agar plate).
14. Incubate the plates for 48 h at 37 °C and enumerate typical *Listeria* colonies.
15. *L. monocytogenes* appears on Oxford Agar as green colonies surrounded by a black halo.

4 Notes

4.1 Phage Preparation

Fresh LISTEX™ P100 should be prepared and employed in the amount recommended by the manufacturer. Phage stock should be serially diluted in sterile SM buffer to a working stock of 2×10^9 PFU/mL. Standard soft agar overlay method can be employed to confirm the phage titers. Titration plates must be incubated at 30 °C. Plated volumes should be adjusted to ensure plating of 10^7 PFU/cm^2.

4.2 Handling of Listeria monocytogenes: Aseptic Precautions

1. All manipulations of pathogen will be performed in a BSC.
2. All disposable plastic ware will be disposed in the autoclave waste bucket in the hood, and autoclave sterilized prior to disposal.

3. All glassware will be decontaminated by autoclaving prior to washing and reuse.
4. All work areas, and laboratory equipment used should be labeled with signs indicating the use of *Listeria monocytogenes*.

4.3 *Listeria monocytogenes* Inoculum Preparation

1. Using a sterile, disposable inoculating loop, transfer a single colony of *Listeria monocytogenes* from a fresh plate (not more than 3 days old) to a labeled culture tube containing 5 mL of tryptic soy broth (TSB).
2. Incubate for 24 h at 37 °C with shaking, at 160 rpm, to obtain a concentration of approximately 10^9 CFU/mL (equivalent to an optical density at 600 nm [OD600] ~1.2).
3. Confirm the optical density, OD, by transferring 600–1000 μL of culture to a cuvette and measuring absorbance at $\lambda = 600$ nm.
4. Transfer the remaining culture to a sterile centrifuge tube.
5. Harvest the cells by centrifuging at 7000 × *g* for 10 min.
6. Use a sterile pipette to aspirate the supernatant from the tube.
7. Resuspend the cell pellet in 5 mL of PBS.
8. Repeat **steps 5–7** in Section 4.3 twice to wash the cells twice.
9. Prepare 10 mL of serial tenfold dilutions of the *L. monocytogenes* cell suspensions, in sterile PBS, to obtain the desired cell concentrations (The target counts on spiked food are 10^3 CFU/cm^2).
10. Mix equal volumes (e.g., 10 mL) of prepared *L. monocytogenes* cell suspensions of isolates belonging to serotypes 1/2a, 1/2b and 4b and one representative outbreak strain.

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