Chapter 12

Visualization of Engineered M13 Phages Bound to Bacterial Targets by Transmission Electron Microscopy

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Abstract

The filamentous phage M13 is one of the most well-studied and characterized phages, particularly since it was introduced as a scaffold for phage display, a technique to express and evolve fusion proteins on the M13 phage's coat to study protein or peptide binding interactions. Since phages can be engineered or evolved to specifically bind to a variety of targets, engineered M13 phages have been explored for applications such as drug delivery, biosensing, and cancer therapy, among others. Specifically, with the rising challenge of antimicrobial resistance among bacteria, chimeric M13 phages have been explored both as detection and therapeutic agents due to the flexibility in tuning target specificity. Transmission electron microscopy (TEM) is a powerful tool enabling researchers to directly visualize and characterize binding of phages to bacterial surfaces. However, the filamentous phage structure poses a challenge for this technique, as the phages have similar morphology to bacterial structures such as pili. In order to differentiate between bacterial structures and the filamentous phages, here we describe a protocol to prepare TEM samples of engineered M13 phages bound to bacterial cells, in which the phage virions have been specifically labeled by decoration of the major capsid proteins with gold nanoparticles. This protocol enables clear visualization and unambiguous identification of attached filamentous phages within the context of bacterial cells expressing numerous pili.

Key words M13, Bacteriophage, Phage, Transmission electron microscopy, Filamentous phage, Virus

1 Introduction

Bacteriophages (or phages) are viruses that are specific to bacteria and harmless to humans [1]. Due to their biocompatibility and diagnostic and therapeutic potential, phages are increasingly studied and engineered for various applications such as drug delivery, biosensing, cancer therapy, and tissue regeneration [2, 3]. Phage capsids are generally composed of repetitions of a small number of capsid proteins [1, 4]. While they encompass a large diversity of morphologies, two major morphological classes are the tailed phages, which include an icosahedral head, and the filamentous, or rodlike, phages. One of the most well-characterized phages is

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M13, a filamentous phage that is about 1 μm in length and 6 nm in diameter. M13 has a single-stranded DNA genome packaged inside its capsid, which is composed of ~2700 copies of the major capsid protein pVIII (also called g8p), forming the bulk of the filamentous structure, and 3–5 copies of four minor capsid proteins, including the receptor-binding protein pIII (also called g3p) [5, 6]. M13 phage is a prominent tool in molecular biology as a system for phage display technology, in which protein variants can be rapidly selected, typically for binding activity. Major applications include display of a heterologous mutant library of antibody single-chain variable fragments (scFv) or display of a random library of peptides on the major (pVIII) or minor (pIII) capsid proteins [5]. The expression and selection of these proteins allows the discovery of M13-like phages engineered to bind to different targets. At the same time, since pVIII carries solvent-exposed carboxyl as well as primary amine groups, M13 phages have the potential for chemical surface modifications in order to achieve various functionalities [3].

An important application focus for phages is addressing antimicrobial resistance in bacteria, specifically for species that exhibit multidrug resistance and virulence. Prominent among these are *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa,* and *Enterobacter* spp., which are collectively known as the ESKAPE pathogens and are a rising global challenge that poses a great risk to public health [7]. As bacteria develop resistance to existing antibiotics, the rate of development of new therapeutics for multidrug-resistant bacterial infections has been relatively low due to lack of financial incentives [8]. Phages and phage-derived products are promising alternatives to antibiotics, potentially providing high specificity and biocompatibility as well as substantial room for innovation [9].

Prior work from multiple laboratories, including ours, has demonstrated the potential for engineering M13 phages to bind different bacterial host species. In particular, exchanging the original M13 receptor-binding protein with homologs from other filamentous phages (with specificity for other bacterial species) creates chimeric M13 phages that are able to bind to alternative bacteria with high specificity [10, 11]. Engineered phages could be conjugated to gold nanoparticles, enabling new methods for bacterial detection as well as photothermal therapy of bacterial infections in both in vitro and in vivo models [11–14]. A foundational method in developing such technologies is the ability to characterize the ability of engineered phage candidates to bind to different bacterial species.

Transmission electron microscopy (TEM) is a powerful tool enabling researchers to directly visualize bacterial or phage samples at nanometer scale, using accelerated electron beams to interact with samples loaded on Formvar/carbon-coated mesh grids to obtain microscopy images [15]. TEM could be used to verify and

characterize the attachment of a newly engineered phage to specific bacterial cells. However, the filamentous phage structure poses a challenge in interpreting these images, due to the difficulty in differentiating between bacterial structures, particularly pili, and filamentous phages, which both have long, filamentous morphologies with roughly similar dimensions (Fig. 1). Below, we describe a method to unambiguously identify the phages in a mixture of cells and phages using TEM after staining with uranyl acetate. The phages are differentiated from any bacterial cell structures through labeling with a primary antibody against the major coat protein (p. VIII) and a secondary antibody loaded with gold nanoparticles (Figs. 2 and 3).

2 Materials

If not otherwise specified, all reagents should be molecular biology grade. All solutions, media, and buffers should be prepared with ultrapure water, filtered through 0.22-μm pore size filters and equilibrated to room temperature prior to use.

1. Orbital shaker.
2. Benchtop centrifuge.
3. Spectrophotometer.
4. 50-mL volume Eppendorf tubes.
5. 1.5-mL volume Eppendorf tubes.
6. Reverse action tweezers.
7. Kimwipes.
8. Pipettes.
9. Pipette tips.
10. Petri dishes.
11. Formvar/Carbon 200 Mesh, Ni Grid (Electron Microscopy Sciences, SKU:FCF200-Ni-50).
12. TEM sample grid holder.
13. 1 × phosphate-buffered saline (PBS).
14. Washing solution: 1% w/v bovine serum albumin (BSA) in 1 × PBS buffer.
15. Blocking solution: 0.5% w/v gelatin solution in 1 × PBS buffer.
16. Primary antibody (mouse anti-M13 g8p IgG) working solution: 1:100 dilution from stock (Thermo Fisher Scientific, catalog number: MA1-06604) in 1 × PBS buffer.
17. Secondary antibody (donkey anti-mouse IgG preadsorbed to gold nanoparticles [6 nm dia.]) working solution: 1:20

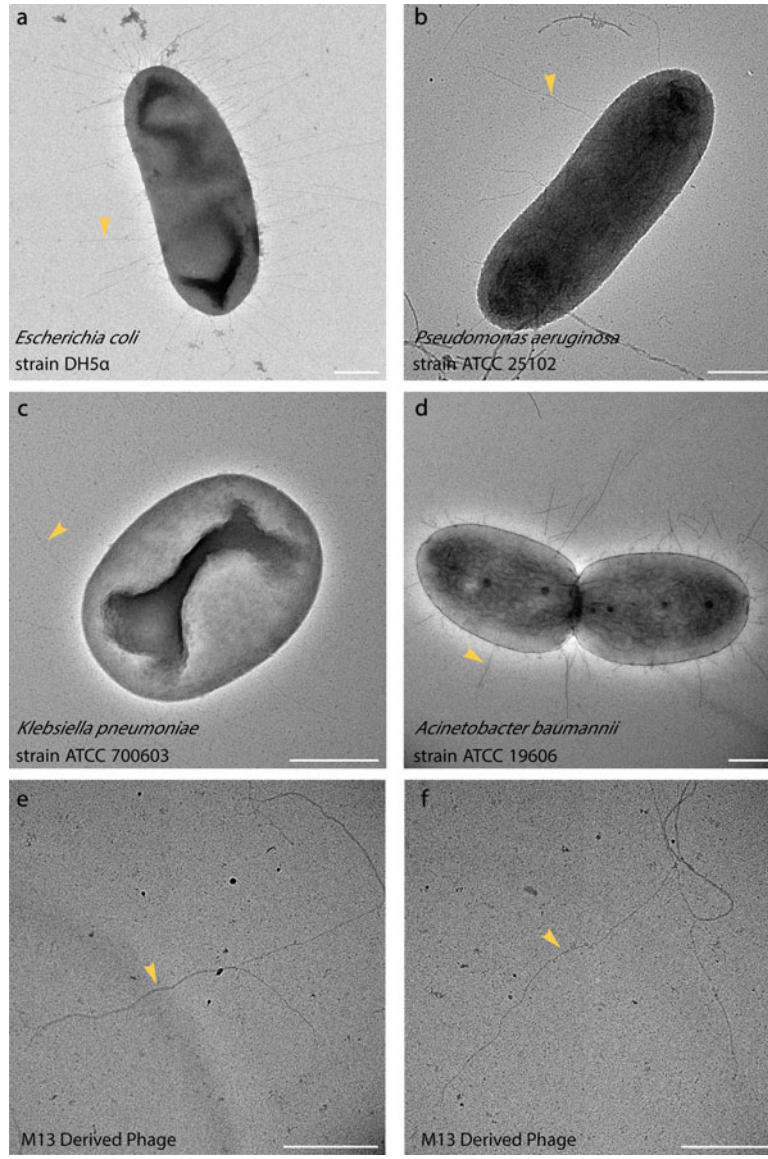

Fig. 1 TEM images of bacterial strains and M13-derived phages stained with 1% uranyl acetate. Pili can be observed in standard strains of (**a**) *Escherichia coli,* (**b**) *Pseudomonas aeruginosa,* (**c**) *Klebsiella pneumoniae,* and (**d**) *Acinetobacter baumannii*. Images of M13-derived phages (**e**, **f**) fixed on Formvar/carbon nickel grids demonstrate the morphological similarity between bacterial pili and M13-derived phages. All images have a scale bar of 500 nm. The yellow arrows indicate examples of pili on bacterial surfaces (**a**–**d**) or phages (**e**–**f**)

dilution from stock (Abcam, catalog number: ab105276) in 1 × PBS buffer.

18. 1% w/v uranyl acetate staining solution (*see* **Note 1**).
19. Purified chimeric M13 phage sample in × PBS buffer.

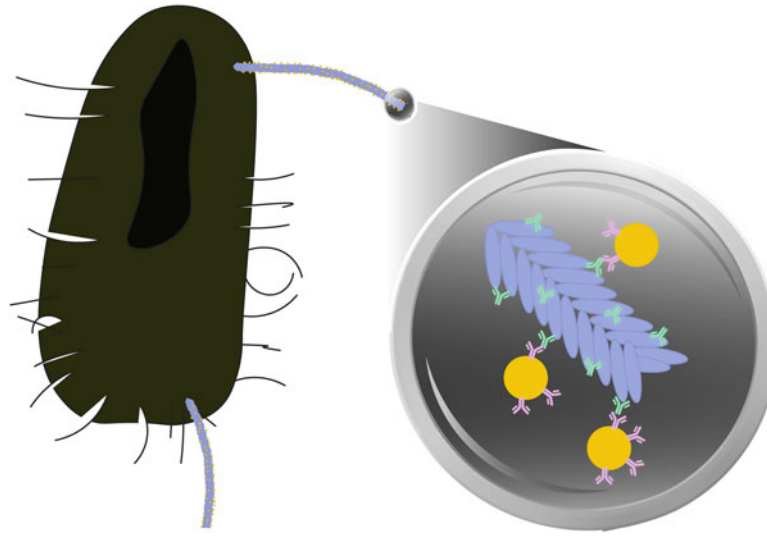

Fig. 2 Strategy for specific labelling of M13-derived phages with bacterial cells using antibody-bound gold nanoparticles. The phage coat is composed of repetitions of major capsid protein pVIII (purple) and is shown bound to a bacterial cell (dark oval) bearing pili (black curved lines). The phages are labeled with 6 nm gold nanoparticles through a 2-step procedure. The phage-cell sample is first incubated with mouse anti-pVIII primary antibodies (light green) and then incubated with 6 nm gold nanoparticles (yellow) preadsorbed with donkey anti-mouse secondary antibodies (pink)

20. Target bacterial cells on culture plates.
21. Luria–Bertani (LB) broth or other appropriate growth media.

3 Methods

All procedures should be done at room temperature unless specified otherwise. All bacteria should be spun down at 5000 rpm for 5 min using a benchtop centrifuge.

1. Grow an overnight culture from the appropriate bacterial species (*see* **Note 2**).
2. Subculture 100 μL of the overnight culture of bacterial cells into 5 mL fresh media and grow to log phase (*see* **Note 3**).
3. Spin down bacterial cells, remove supernatant of spent media, wash once with 5 mL of 1 × PBS buffer, and then resuspend bacterial cell pellet in 5 mL of 1 × PBS buffer (*see* **Note 4**).
4. Pipet 1 mL of cell suspension into a 1.5-mL Eppendorf tube, mix with appropriate amount of phage sample, and allow to bind for 30 min at room temperature (*see* **Note 5**).

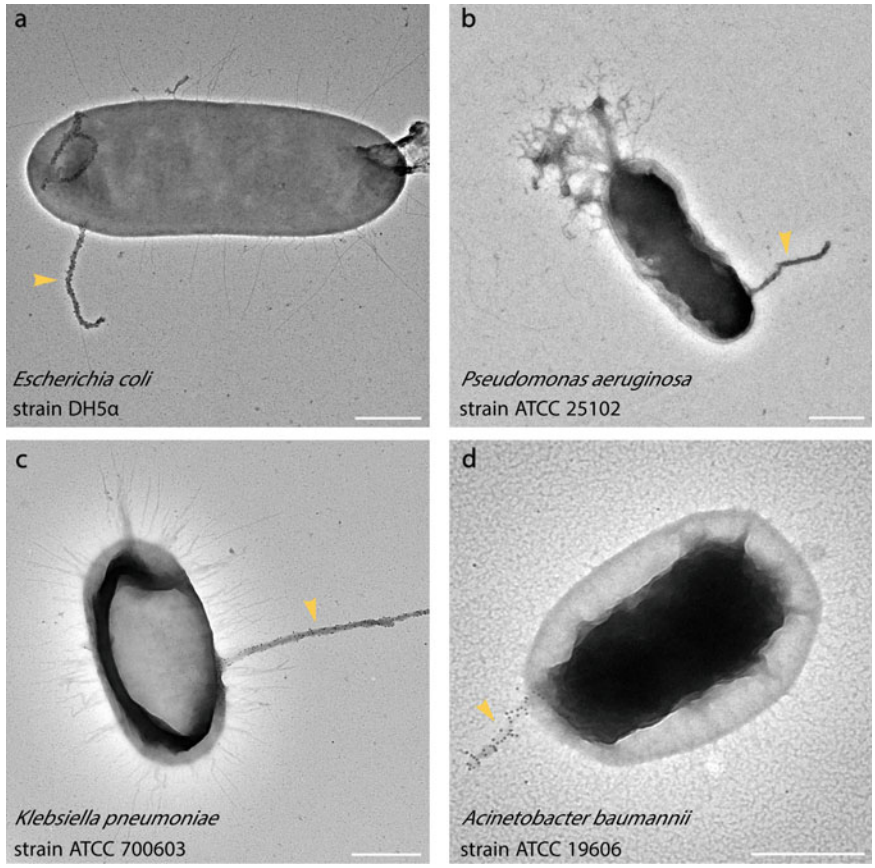

Fig. 3 TEM images of gold-labelled phages bound to cells stained by 1% uranyl acetate. All images show M13-derived phages expressing various receptor-binding proteins attached to the surface of bacterial cells. The cell–phage mixtures were fixed on formvar/carbon nickel grids. All images have a scale bar of 500 nm. The bacterial species are (**a**) *Escherichia coli,* (**b**) *Pseudomonas aeruginosa,* (**c**) *Klebsiella pneumoniae,* and (**d**) *Acinetobacter baumannii*. The yellow arrow in each image points to the phage labeled by the 2-step strategy illustrated in Fig. 2. Note that the width of the phage structure is increased compared to unlabeled phages, due to the bound antibodies and nanoparticles. Bacterial pili can be readily differentiated from the phages due to their lack of labeling and are prominently observed in (**a**) and (**c**)

5. Spin down bacteria (possibly with bound phage), remove supernatant containing unbound phage, and wash once with 1 mL of 1 × PBS buffer (*see* **Note 6**).
6. Resuspend cell pellet in 1 × PBS to obtain a final concentration with optical density at 600 nm (OD600) between 2 and 3.
7. Retrieve a TEM mesh grid with reverse action tweezers and position tweezers on the benchtop while holding, with the shiny side of the grid facing upward (*see* **Note 7**).
8. Load an 8 μL droplet of resuspended bacteria (with potentially bound phage) sample on the shiny side of the grid and incubate for 2 min.

9. Cut a piece of Parafilm with appropriate size and make 4 droplets of 500 μL washing solution on Parafilm for each sample. If not specified, all volumes of droplets for washing should be 500 μL in this protocol (*see* **Note 8**).
10. Using reverse action tweezers, move the sample grid to the surface of the first washing solution droplet with shiny side facing downward in contact with the washing solution. Wash sample by pipetting the droplet up and down for 50 times using a 200-μL pipette (*see* **Note 9**).
11. Move the sample grid to each of the other three washing solution droplets in order and repeat the washing step on each droplet.
12. In a Petri dish, make a droplet of 100 μL of blocking solution, transfer the sample grid to the surface of the droplet, and incubate for 1 h at room temperature (*see* **Note 10**).
13. Transfer the sample grid to a new droplet of washing solution and wash once.
14. In a Petri dish, make a droplet of 100 μL of primary antibody working solution, transfer the sample grid to the surface of the droplet with shiny side in contact with liquid surface, and incubate for 1 h at room temperature.
15. Repeat **steps 9–12** to wash and block.
16. In a Petri dish, make a droplet of 100 μL of secondary antibody (coated gold nanoparticles) working solution, transfer the sample grid to the surface of the droplet, and incubate for 1 h at room temperature (*see* **Note 10**).
17. Wash sample grid with ultrapure water droplets for five times.
18. Hold sample grid by its rim using reverse action tweezers with shiny side facing upward, add 8 μL of uranyl acetate staining solution to grid surface, and incubate for 1.5 min (*see* **Note 11**).
19. Dry sample by dabbing the grid gently on a Kimwipe tissue.
20. Store sample in a sample holder until ready to examine under transmission electron microscope (*see* **Note 12**).

4 Notes

1. Uranyl acetate is radioactive. Follow standard practice for radioactive material in the laboratory when handling. After resuspending powder in ultrapure water, fully dissolve the solid to make the 1% w/v solution, filter, and store solution at 4°C for short-term usage.

2. Sterility measures should be in place including using appropriate antibiotics to ensure quality of samples.
3. Assuming bacteria growing in log phase is ideal for phage attachment (e.g., for receptor expression), check culture optical density over time consistently to ensure bacteria growth is in log phase prior to proceeding to the next steps. This step may be modified if a different growth phase is preferred.
4. Washing of cells is performed by fully resuspending the cell pellet through careful pipetting up and down or vortexing at low speed. Then spin down again and remove supernatant. Be careful not to create too much shear stress during the wash step which may damage the cell surface.
5. Mix 10–100 times excess bacteriophages to bacteria (or other ratio as desired) to ensure sufficient binding.
6. Wash step done similarly as in **Note 4**. Avoid excessive stress to prevent cell damage.
7. Lay a piece of Kimwipe tissue on the benchtop to prevent the TEM mesh grid from dropping directly onto the benchtop. All handling of the grid with reverse action tweezers is done above Kimwipe tissues. In all steps involving transferring of the mesh grid, gently hold the rim of the grid without touching the inner mesh using reverse action tweezers, since the sharp tip of the tweezers may break the Formvar/carbon film. Avoid bending or other physical damage to the grid during transfers.
8. The size of Parafilm should be large enough to hold 4 of the 500 μL water droplets for each sample. In general, 4 grids of Parafilm in length should be enough for each sample. To make Parafilm stick to the surface of the benchtop without moving during the downstream wash steps, spray water onto the benchtop and push the water with one side of the Parafilm until it sticks to the benchtop due to water surface tension. Move the Parafilm around horizontally to ensure Parafilm is stuck to the surface uniformly, especially by 4 corners. To make the droplet, pipette 500 μL of the solution onto parafilm. Keep droplets well-separated to prevent them from combining together.
9. During the washing steps, it is important to make sure the grid floats on top of the liquid droplet without sinking to the bottom. If that happens, drying the sample with a piece of Kimwipe could potentially make the grid float again. Pipette up and down gently in the liquid droplet to avoid formation of air bubbles.
10. Keep the Petri dish covered with its lid during all incubation steps to reduce drying of the droplet. To prevent denaturing of the antibodies, only prepare primary antibody working

solution and secondary antibody (coated with gold nanoparticles) working solution prior to the step when they are required. It is not necessary to filter after diluting from stock solutions.

11. Do not stain samples with uranyl acetate solution longer than 4 min since this will overstain the cells.
12. The sample should be stable at room temperature for no less than 2 weeks.

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