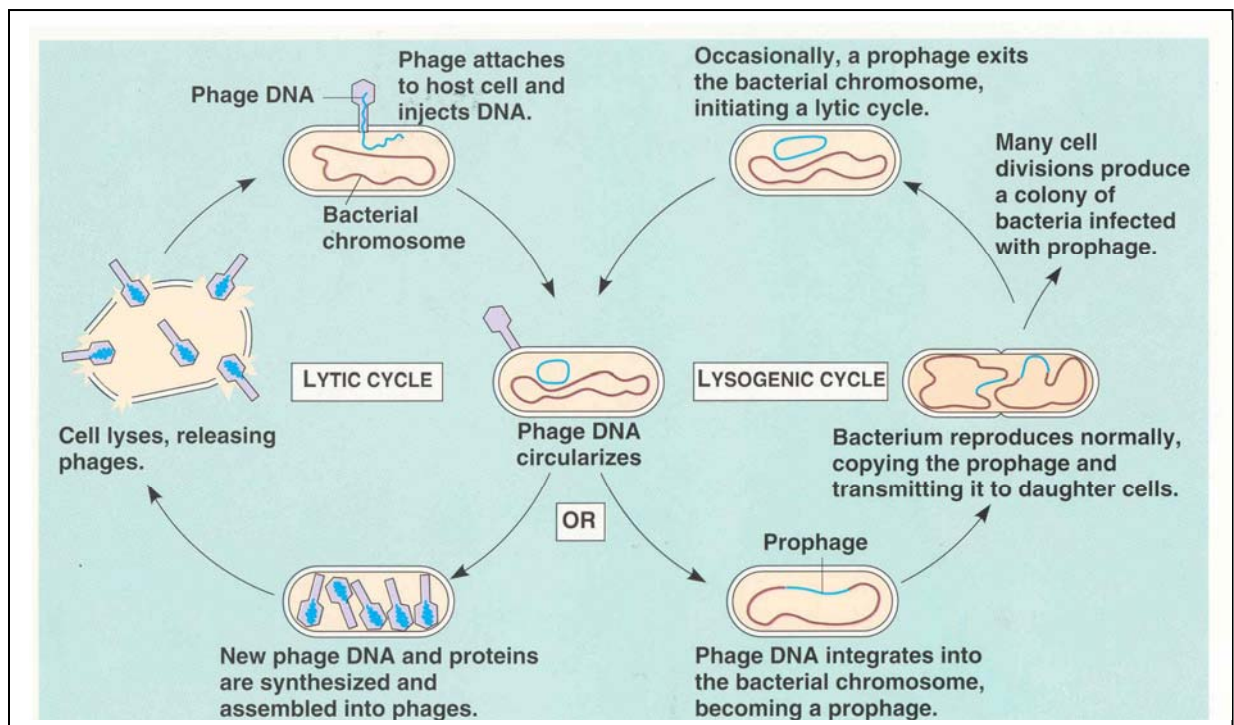


Lab #3 Isolation and Titration of Bacteriophage Lambda

Bacteriophage are small viruses that infect bacteria such as *E. coli*. Bacteriophage (also known simply as phage) are obligate intracellular parasites that cannot replicate on their own, but require a host cell to multiply. Phage infect their host by binding to the cell with their tail, drilling a hole in the cell wall and plasma membrane, and injecting their DNA into the cell.

Lambda phage is a temperate bacteriophage, meaning it can adopt one of 2 lifecycles; a lytic cycle or a lysogenic cycle. During the lytic cycle, lambda hijacks the synthetic machinery of the cell and forces the cell to produce more phage. Approximately 45 minutes post-infection, the bacterium lyses and about 100 new phage particles are released. Phage progeny infect neighboring cells and the cycle continues.

Alternatively, lambda can enter the lysogenic cycle, in which it integrates its genome into the host chromosome. The viral DNA is now called a prophage and the bacterial host is called a lysogen. As the lysogen replicates, the prophage is passively replicated and distributed to the progeny bacteria. If left unperturbed, lysogens may grow indefinitely without producing phage particles. However, if a lysogen is stressed with UV light, radiation or some other insult then the prophage will enter the lytic cycle, produce new phage particles and “jump ship”.



Inducing Lysogens to enter the Lytic Cycle

The lambda repressor (CI) is a DNA binding protein that binds to a regulatory region of the prophage DNA. The binding of CI to the prophage inhibits gene expression required for the lytic cycle, and thus maintains the lysogenic cycle of a lysogen. Environmental conditions that destroy CI result in a shift from the lysogenic to the lytic cycle.

Today we will work with a mutant form of lambda phage called CI₈₅₇. CI₈₅₇ is a temperature-sensitive (ts) mutant in which the lambda repressor protein (CI) is functional at the permissive temperature of 32°C, but non-functional at the non-permissive temperature of 40°C. You will be given a culture of a CI₈₅₇ lysogen grown at 32°C. You will induce the lysogen to enter the lytic cycle by shifting the temperature of the culture from 32°C to 40°C. The higher temperature will alter the conformation of the mutant CI protein, causing CI to dissociate from the prophage DNA. In the absence of functional CI, the lytic genes are expressed, new phage are produced and the E. coli host cells lyse.

How will we know if we have successfully induced a lytic lifecycle on the lysogens? If we see the bacterial culture go from turbid (cloudy) to clear then we know that the phage have replicated and lysed their host cells. This may be seen with the naked eye, but we will also assess this change by monitoring OD₆₅₀ of the culture before and 1 hour after induction. Recall that a concentrated culture of live cells has a high OD₆₅₀, but that the OD₆₅₀ of the culture will decrease if cell concentration drops due to cell lysis.

Procedure:

1. Calibrate the spectrophotometer
 - a. Set the wavelength control knob to 650 nm.
 - b. Set the filter to the 600-950 nm range.
 - c. With the sample chamber empty and the lid closed, adjust the 0%T knob (left knob) until the scale reads zero Transmittance (far left zero line).
 - d. Push the mode button to change to absorbance.
 - e. Obtain 3 ml of lambda broth into a small glass test tube; this is your blank. Place the blank into the sample chamber and close the lid. Adjust the 100%T/OA knob (right knob) until the scale reads zero absorbance (far right zero line). The spectrophotometer is now calibrated.
2. Obtain a flask of CI₈₅₇ lambda lysogen from the 32°C water bath.
3. Place 3 ml of the culture in a glass test tube and place the tube in the spectrophotometer.
4. Measure the absorbance (OD₆₅₀) of the culture and note it here: _____
5. Move the culture to the 40°C water bath and incubate for 1 hour.
6. After 1 hour, measure the OD₆₅₀ of the culture and note it here: _____
7. If there is no obvious evidence of cell lysis then return the culture to the incubator and continue shaking until lysis is evident
8. Save the lambda/E.coli culture for the next experiment.

Question:

Was there a change in OD₆₅₀ of the culture after the shift in temperature? Was there visual evidence of lysis? Provide a rationale for the result.

Determination of Phage Titer

When phage are mixed with an excess of bacteria and plated on nutrient agar, the bacteria grow rapidly to form a lawn, phage infect bacteria, phage produce viral progeny, the host cell lyses and progeny phage infect neighboring cells. The cycle of infection, phage replication and host lysis creates a zone of cell death called a plaque. Each plaque is derived from a single infecting phage particle. When a small number of phage are plated with a large number of bacteria, the plaques are well spread out and can easily be distinguished one from another.

In today's experiment, the class will be infecting *E. coli* bacteria with lambda CI₈₅₇ phage and performing a plaque assay. This technique allows us to determine the concentration of phage (also known as plaque forming units, or pfu) in the phage culture you isolated in the previous induction experiment. The process is summarized as follows: Serial dilutions of the phage stock are produced, and then each is mixed with an equal amount of *E. coli*. Phage are given time to adsorb to and infect *E. coli* cells. Melted soft agar is added to each tube, and the mixture is poured onto agar plates. Plates are incubated at 37°C until plaques appear (12-24 hours). Each plaque contains virus progeny derived from a single infecting phage. After counting plaques, we can use dilution factors to determine the concentration of phage in the original culture.

Procedure:

1. In the previous experiment you induced the lytic cycle in the CI₈₅₇ lysogen. Now that culture flask contains a large concentration of phage particles. We will determine the concentration (Titer) of phage by plaque assay.
Label a 1.5 ml microcentrifuge tube as lambda and with your initials. Pipet 1 ml of your CI₈₅₇ culture into the tube. Centrifuge the tube at 13,000 RPM for 1 minute. Label a new microcentrifuge tube as lambda. Decant the supernatant into the new microcentrifuge tube. Discard the tube with the pellet. The supernatant is your phage stock. It contains a high concentration of phage.
2. Obtain 8 Lambda plates. Label them 1-8 and with your initials and the date (label on the bottom toward the outside edge). Place them upside down in the 37°C incubator to warm.
3. Label 8 glass culture tubes as 1-8 and with your initials. Aseptically add 0.3 ml of a log phase *E. coli* culture (strain C600) to each tube. Obtain the *E. coli* from the instructor.
4. Make seven 10-fold serial dilutions of your phage stock
 - Label 7 microcentrifuge tubes as 1-7. Add 180 µl of SM broth to each tube
 - Add 20 µl of your CI₈₅₇ phage stock to tube #1, then mix
 - Using a fresh pipet tip, transfer 20 µl from tube #1 to tube #2 and mix
 - Using a fresh pipet tip, transfer 20 µl from tube #2 to tube #3 and mix
 - Using a fresh pipet tip, transfer 20 µl from tube #3 to tube #4 and mix
 - Using a fresh pipet tip, transfer 20 µl from tube #4 to tube #5 and mix
 - Using a fresh pipet tip, transfer 20 µl from tube #5 to tube #6 and mix
 - Using a fresh pipet tip, transfer 20 µl from tube #6 to tube #7 and mix

5. Add 100 μ l of the first phage dilution to tube #1 of E. coli. Add 100 μ l of the second phage dilution to tube #2 of E. coli, and so on.
6. Incubate the E.coli/phage mixtures at room temperature for 20 minutes (phage adsorb to cells and inject their DNA at this point).
7. Take your tubes and Lambda plates to the **pouring station** at the back of the room. Get the bottle of top agar out of the 55⁰C incubator. You need to work quickly at this point so the top agar does not solidify before you and others have a chance to use it.
8. Aseptically add 2.5 ml of top agar to tube #1 (use a p10ml micropipetor), vortex lightly to mix, and pour the contents of the tube onto plate #1. Quickly spread the top agar over the entire plate by tilting the plate (as demonstrated by the instructor).
9. Put the top agar back into the 55⁰C incubator as soon as you are done with it.
10. Allow the top agar on your plates to solidify (15 minutes).
11. Place the plates in a 37⁰C incubator upside down. Plaques will appear after 12 hours. The instructor will put your plates in the refrigerator at 4⁰C until next week.
12. Count plaques the following lab period. Choose a plate that contains more than 25, but less than 250 plaques. Count the number of plaques on this plate and enter the value below on the appropriate line.

Plate # _____ Number of plaques _____

13. Use this data and the dilution factors to calculate the titer (concentration) of phage in the original stock. Enter the value below as plaque forming units per ml (pfu/ml) and show your calculations.

Titer of original phage stock _____ pfu/ml

Calculations

Questions:

1. What are bacteriophage?
2. Briefly describe the events of Lambda's lytic cycle.
3. What is a prophage?
4. What is a plaque?
5. What do we mean by the term temperature-sensitive mutation? How does this type of mutation differ from other types of mutations with respect to expression of the mutant phenotype?
6. Why does the CI_{857} lysogen enter the lytic cycle when the temperature is raised to 40°C ?
7. You wish to determine the titer (pfu/ml) of phage in a stock culture. You do 4 10-fold serial dilutions of the phage. You combine 0.1 ml of each phage dilution with 0.3 ml of *E. coli* and plate the mixture on agar plates. The next day you observe 100 plaques on the most dilute plate. What is the titer of phage in the original stock culture? Show your calculations.

Notes