Exercise 23-C
BACTERIOPHAGE REPRODUCTION AND PLAQUE FORMATION

Introduction

The reproductive cycle of a **cytolytic bacteriophage** called T2 begins with its **adsorption** onto a sensitive host cell. The phage (which is somewhat tadpole-shaped) attaches itself, tail first, to the cell wall of the host. The tail then undergoes a molecular restructuring allowing it to pierce the cell wall and membrane, thus forming a passage through which its nucleic acid core can enter the cell. Once inside the cell this nucleic acid loses any visible resemblance to the mature phage (virion) and may be referred to as a **vegetative phage** or as the viral **genome**. The phage nucleic acid (often with the help of a few viral enzymes entering along with it) reorganizes the synthetic machinery of the host cell and is rapidly reproduced. Next, protein is synthesized to form multiple capsid and tail portions. When the various protein components have been assembled, the genomes are "pulled" inside, giving rise to several hundred complete phage particles. The host cell then lyses, releasing these "daughter" virions into the environment. Thus is completed the lytic cycle of the bacterial virus. The entire process may take place within 20 minutes and can yield as many as two hundred virions per infected cell.

The period of time, from the adsorption of the phage to its host cell until the release of the progeny, may be referred to as the **latent period** or **burst time**. The number of phage particles released per cell at the end of this period is called the **burst size** or **burst number**. Unlike the reproduction of bacteria, the reproduction of bacterial viruses appears as a one-step procedure, with no increase in number until the end of the latent period, when the host cells lyse and a sharp rise in the number of phage particles occurs.

During this procedure a log phase culture of sensitive bacteria will be mixed with a phage suspension and incubated until adsorption occurs. The mixture will then be diluted, preventing further adsorption. Samples will be withdrawn and plaque counts made at frequent intervals. Using this information, and knowing the original bacterial count as well as the degree of dilution, it will be possible to calculate the average number of phage particles per bacterial cell at each time interval. A plot of this phage count versus time is an expression of the single-step growth response of a phage infection. From this curve one can also calculate the latent period (burst time) and the average burst size (burst number) for the virus type causing the infection.

Materials

Bacteriophage T2 and log phase broth cultures of their specific host bacteria (*Escherichia coli* strain B)
Top-agar - 18 tubes per student group
KCl broth (4.5mL each) in small tubes - 25 tubes per group
KCl broth (9.0mL each) in large tubes - 4 per class section
Trypticase soy broth (50mL) in a 150mL flask – one per class section
100-1000µL (blue) and 10-100µL (yellow) digital pipettes and tips
Bottom-agar plates - 18 per student group
Beakers (400 mL) - three per group
Dishpans with ice and plastic (50 tube) baskets
40% glucose solution - one tube per all lab sections
10% yeast extract - one tube per all lab sections
Note – The glucose and yeast extract are used by the instructor during the preparation of host cells.

Procedure:

Students should work in table groups (six person maximum), cooperate and share the workload in an organized manner. Each student will be expected to plot the results obtained and to complete the calculations required.

A. Preparation - These steps must be completed before the experiment can begin.

1. Obtain 18 tubes of top-agar, and heat them in three boiling water baths (seven tubes per beaker) until thoroughly melted (liquefied). Be careful to keep the water level in the beakers at the same level as the agar in the tubes (not above). This will prevent water from reaching the caps and entering the tubes (diluted top agar will not solidify properly and will no longer be sterile).

2. Label your liquefied top-agar tubes (group name, tape on top) and place them in the 45^{o} C water bath provided.

3. Obtain 25 tubes of KCl broth and label these in the following manner. These are dilution blanks and should be kept on ice. Place labeled tubes in 50-tube baskets in the ice baths provided.

   4^{-1}, 4^{-2}, 4^{-3}
   13^{-1}, 13^{-2}, 13^{-3}
   20^{-1}, 20^{-2}, 20^{-3}, 20^{-4}
   30^{-1}, 30^{-2}, 30^{-3}, 30^{-4}, 30^{-5}
   40^{-1}, 40^{-2}, 40^{-3}, 40^{-4}, 40^{-5}
   50^{-1}, 50^{-2}, 50^{-3}, 50^{-4}, 50^{-5}

4. Obtain one tube containing 9.0mL of KCl broth, label this with your group name, and place it into the 37^{o} C incubator. The viruses will complete their life cycle in this tube.

5. Obtain 18 bottom-agar plates and label them (both top and bottom) with your group name and the following dilutions:

   $4\text{-}10^{-2}$, $4\text{-}10^{-3}$, $4\text{-}10^{-4}$
   $13\text{-}10^{-2}$, $13\text{-}10^{-3}$, $13\text{-}10^{-4}$
   $20\text{-}10^{-3}$, $20\text{-}10^{-4}$, $20\text{-}10^{-5}$
   $30\text{-}10^{-4}$, $30\text{-}10^{-5}$, $30\text{-}10^{-6}$
   $40\text{-}10^{-4}$, $40\text{-}10^{-5}$, $40\text{-}10^{-6}$
   $50\text{-}10^{-4}$, $50\text{-}10^{-5}$, $50\text{-}10^{-6}$

   After these plates have been properly labeled, stack them, agar side up on the desktop. You should place the plates in stacks ordered by their time intervals, with the plate labeled for the most dilute sample on the top of each stack. This will allow for maximum efficiency during the inoculation process.

B. Instructor Preparation - to be performed by the instructor during the lab period.

1. Make certain the students are ready to begin section "C" below, and then pipette 100µL of phage solution (5 x 10^{9} virions per mL) into 9.9mL of *E. coli* culture in the log phase of growth. Mix the phage and host cell suspension thoroughly by rolling the tube between the palms of your hands. **Note** - This step is considered to be time zero, the timing of this and the following steps is critical.

2. Allow the phage to adsorb to the host cells for three minutes and then dilute the culture by pipetting 500µL into 50mL of sterile trypticase soy broth. This dilution step should effectively separate the phage and host cells such that new infections are unlikely.

3. Pipette 1.0mL of this dilution into each of the 9.0mL broth tubes of the student groups.

C. Procedural steps to be completed by each student group.

NOTE – Use a 100-1000μL pipettes (blue tips) for 200μL and 500μL transfers, and 10-100μL pipettes (yellow tips) for 100μL transfers. **CLOSE LIDS ON TIP BOXES!**

1. Remove your growth tube from the 37^{o} C incubator and obtain 1.0mL of the bacteriophage/*Escherichia coli* mixture prepared by the instructor (step #3 above). Thoroughly mix the contents of this tube by rolling the tube between the palms of your hands.

2. Immediately remove 0.5mL (500μL) of broth from your growth tube and place it into the dilution tube labeled 4^{-1} in the ice bath. Return the growth tube to the incubator.

3. Prepare the appropriate dilutions for the four-minute time interval by pipetting 0.5mL (500μL) of liquid from the 4^{-1} tube into the tube labeled 4^{-2}. Mix the contents of this tube by rolling it between the palms of your hands. Then using a new pipette tip, transfer 0.5mL (500μl) of liquid from the 4^{-2} tube into the tube labeled 4^{-3}. Mix the contents of this tube as indicated above. After mixing the contents of each tube, return it to the ice bath immediately. The dilution tubes must be **kept on ice to "stop time".**

4. Separate the bottom-agar plates labeled 4-10^{-2}, 4-10^{-3}, and 4-10^{-4} from the stacks and place them agar side down on the desktop.

5. Obtain a flask or tube containing *E. coli* host cells in the log phase of growth.

6. Remove one tube of top-agar from the water bath and immediately add 200μL of the *E. coli* culture **AND** 100μL of infected cells from your four minute dilution tube labeled 4^{-3}.
   Note – Careful application of aseptic technique will save pipette tips.

7. Quickly mix the contents of this tube by rolling it between the palms of your hands and then carefully pour the top-agar over the agar surface of the appropriately labeled plate (4-10^{-4}). Tip the plate gently to be sure the molten agar covers the entire surface then return it to the desktop. Note that the dilution factor was increased by one negative exponent (10^{-3} dilution/ 10^{-4} plate). This is because only 0.1mL (100μL) of the infected culture was transferred to the bottom agar.

8. Repeat steps #6 and #7 for each of the dilution blanks in the ice bath. Note that if you start with the most dilute sample in each set (time interval), you can use the same pipette tip for all samples within that time interval. **You must change tips between time intervals.**

9. Allow the top-agar to solidify without disturbing the plates. If the plates are moved before the agar hardens, the surfaces will be lumpy and it will be difficult to count the plaques that form.

10. At each additional time interval, (13, 20, 30, 40, and 50 minutes from time zero) obtain samples from your growth tube (in the incubator) and prepare the appropriate dilutions as indicated in step #3 above. Note that you will be required to prepare more dilutions for the longer time intervals. As long as the dilution samples are kept on ice, the timing of other steps is less critical. Use the time between sampling to prepare top-agar tubes and pour plates as indicated in steps #4-8. Be careful to use the appropriate dilution tubes and bottom-agar plates for each time interval (see chart below), and use a new sterile pipette tip for each dilution step.

The dilution **plates** to be prepared at each time interval are as follows:

Time (minutes from time zero)	Final Dilutions On Plates		
4 minutes	10^{-2}	10^{-3}	10^{-4}
13 minutes	10^{-2}	10^{-3}	10^{-4}
20 minutes	10^{-3}	10^{-4}	10^{-5}
30 minutes	10^{-4}	10^{-5}	10^{-6}
40 minutes	10^{-4}	10^{-5}	10^{-6}
50 minutes	10^{-4}	10^{-5}	10^{-6}

11. When the agar has solidified in all plates, stack them in three groups of six, tape them together, and place them into the 37^{o} C incubator (agar side up). Allow the plates to incubate until the next lab session.

12. Count the number of plaques visible on each plate containing between 30-300 plaques. For plates containing a very large number of plaques you may divide the plate into sections, count the number of plaques per section and multiply by the number of sections. This will allow you to obtain a fairly accurate record of the number of plaques present.

13. Multiply the plaque number times the dilution factor (expressed as a positive exponent) to determine the total number of plaque forming units (PFU) present per mL of infected culture. For example, if the 13 minute 10^{-3} dilution plate contains 85 plaques, the number of PFU may be determined by multiplying 85 times 10^{3}. The number of PFU present at 13 minutes (as indicated by this plate) is 8.5×10^{4} or 85,000. Record your results for each plate at each time interval. (**Note** - In this assay system, any free virion or infected cell will serve as a PFU; the character of the PFUs changes over time.)

14. If you have more than one countable plate at each time interval, determine the average number of PFU present at that time interval. (Remember that the original phage/*E. coli* mixture was diluted 1:1000 by the instructor before you began your dilutions.)

15. Using the 4-cycle semi-log paper provided, plot the number of PFU as a function of time. Use this graph to determine the **burst size** and **latent period** for the virus being studied.

Questions:

1. How do viruses reproduce themselves within host cells?

2. What is the latent period (burst time) as described in this exercise and how is it determined?

3. What is the burst size (burst number) as described in this exercise, and how is it determined?

4. According to the data obtained by your group, what is the approximate latent period and burst size for the coliphage T2 when grown in *E. coli* strain B cells?

NOTE – For ease of plaque counting, your instructor may substitute the coliphage X174 and *E. coli* strain C when preparing materials for this exercise. The θX174 genome enters host cells by means of a DNA-pilot protein (H protein) but the life cycle is similar to that of T2.

Name_____ **Lab Section**_____

WORKSHEET
Exercise 23C
Bacteriophage Reproduction and Plaque Formation

Goals: _____
_____
_____

Materials & Methods:
The Bacteriophage Reproduction procedure was followed.

Bacteriophage used: _____ *E. coli* strain infected: _____

Data & Results:

	Number of Plaques	Dilution Factor	PFU/ml
4 min.			
13 min.			
20 min.			
30 min.			
40 min.			
50 min.			

Plot your data on semilog paper (next page) as demonstrated by your instructor.

Conclusions:

What was the average initial PFU/ml for this infection? _____

What was the average maximum PFU/ml for this infection? _____

What was the burst time for this infection? _____ Explain what this means. _____
_____

What was the burst size for this infection? _____ Explain what this means. _____
_____