

Exercise 23-A

INTRODUCTION TO VIRUSES AND PHAGE TYPING

Introduction

Viruses are ultramicroscopic "life forms" that infect and reproduce within specific types of host cells. Individual virus particles or **virions**, are not cellular, but are more like macromolecular complexes, being composed primarily of a nucleic acid core (either RNA or DNA, but rarely both) surrounded by a layer of protein called a **capsid**. All viruses are obligate intracellular parasites or **hypotrophs** that must gain access to a host cell in order to reproduce. They are ubiquitous, and as a group probably infect every type of cell in existence.

Viruses that infect bacteria are called **bacteriophages** (Greek, phage = eat), meaning bacteria-eaters, and those able to infect *E. coli* cells are called **coliphages**. Some of these viruses, called **cytolytic** (cell lysing) bacteriophages cause the destruction of their host cells, while others called **temperate**, avirulent or asymptomatic bacteriophages can be incorporated into host cell chromosomes and reproduced along with host cells. When bacteria and **cytolytic** bacteriophages are cultured together on an agar plate, the destruction of the bacteria cells shows up as clear depressions or "windows" in the bacterial "lawn". These "windows" are known as **plaques**, and are readily visible to the naked eye. Their presence provides a clear indication of viral infection and the resulting cell death (lysis).

There are many different kinds of bacteriophages, but each one is quite host-specific, i.e., will tend to infect only one type of bacteria. For this reason, known types of bacteriophage may be used to identify specific types of bacteria in a procedure called **phage typing**. Phage typing is possible because the mechanisms binding viruses to host cell surfaces involve very specific molecular interactions between virion and host cell **receptors**. By mixing a known virus type with a number of different types of bacteria and growing these in separate lawn cultures, it is possible to identify the bacteria serving as the host strain. Only the correct host culture will develop plaques. Phage typing is a method sometimes used (along with serological typing) in the identification of unknown bacteria.

For this exercise students will use the **cytolytic coliphages** X174 and T2 and host strains of *E. coli* that are most susceptible to infection by these virus types. *E. coli* strain C is the optimum host for the coliphage X174, but can also be infected by the coliphage T2. *E. coli* strain B is the optimum host for the coliphage T2 and will not support the reproduction of X174. The plaques formed by these two virus types are distinctively different. The coliphage X174 causes the formation of plaques that are 3-4 mm in diameter and easily observed. The coliphage T2 causes the formation of plaques that are about 1mm in diameter and somewhat more difficult to see. During this exercise, host preference and plaque variation will be used to demonstrate a simple version of phage typing.

Materials:

Log phase cultures of *E. coli* (strain C and strain B), other cultures optional
Four tubes of top-agar
Four bottom-agar plates
100-1000 μ l (blue) and 10-100 μ l (yellow) digital pipettes and sterile tips
Coliphage X174 and coliphage T2 cultures (containing approximately 400 pfu/ml)

Procedure for Phage Typing: (Please work in groups of 2-3 people)

1. Obtain four tubes of top-agar and thoroughly liquefy the medium by placing the tubes into a beaker of boiling water (keep the tube caps on). Be careful to keep the water level in the beaker even with the level of agar in the tubes. This will prevent contamination, and will shorten the heating time required.
2. Place the tubes containing liquefied top-agar in the 45° C water bath provided.
3. Obtain four bottom-agar plates and label them A (θ X174 + *E. coli* #1), B (θ X174 + *E. coli* #2), C (θ T2 + *E. coli* #1), and D (θ T2 + *E. coli* #2). Add to this label the name of your group (initials are acceptable) and the date.

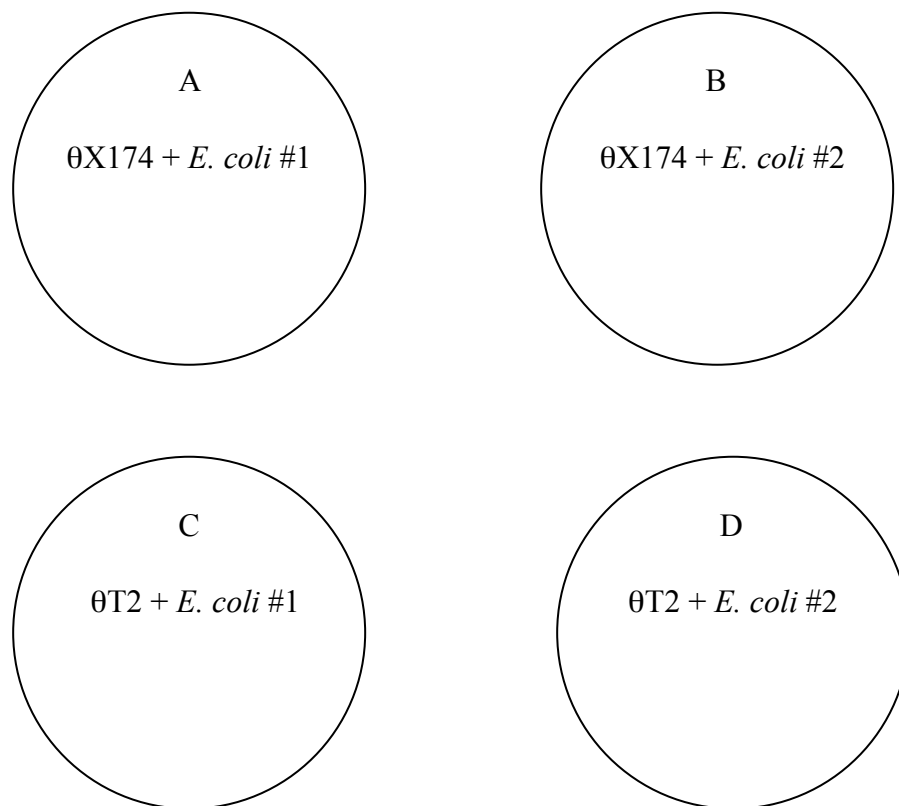


Fig. 23A.1 - Phage typing plates with labels. Writing out complete labels on both sides of the plate will assist both preparation and data collection.

4. Obtain one log phase sample of each of the two different types of bacteria provided. These samples are labeled *E. coli* #1 and *E. coli* #2.
5. Obtain one tube each containing coliphage X174 (θ X174) and coliphage T2 (θ T2).
6. Remove **one** top-agar tube from the water bath and add 100 μ l of the coliphage X174. To this same tube add 200 μ l of *E. coli* strain #1.

7. Mix the bacteria and virus combination by rolling the tube between the palms of your hands and then quickly pour it over the surface of the bottom-agar in the plate labeled A. Gently tip the plate in a circular motion until the top-agar covers the entire surface, and then allow the plate to rest on the desktop until the agar solidifies.
8. Remove a **second** top-agar tube from the water bath and add 100µl of the coliphage X174. To this same tube add 200µl of *E. coli* strain #2.
9. Mix the bacteria and virus combination by rolling the tube between the palms of your hands and then quickly pour it over the surface of the bottom-agar in the plate labeled B. Gently tip the plate in a circular motion until the top-agar covers the entire surface, and then allow the plate to rest on the desktop until the agar solidifies.
10. Remove a **third** top-agar tube from the water bath and add 100µl of the coliphage T2. To this same tube add 200µl of *E. coli* strain #1.
11. Mix the bacteria and virus combination by rolling the tube between the palms of your hands and then quickly pour it over the surface of the bottom-agar in the plate labeled C. Gently tip the plate in a circular motion until the top-agar covers the entire surface, and then allow the plate to rest on the desktop until the agar solidifies.
12. Remove the **fourth** top-agar tube from the water bath and add 100µl of the coliphage T2. To this same tube add 200µl of *E. coli* strain #2.
13. Mix the bacteria and virus combination by rolling the tube between the palms of your hands and then quickly pour it over the surface of the bottom-agar in the plate labeled D. Gently tip the plate in a circular motion until the top-agar covers the entire surface, and then allow the plate to rest on the desktop until the agar solidifies.
14. When the agar in these plates has solidified, tape the plates together in a stack, invert them and place them in the 37° C incubator until the next laboratory session.
15. After incubation, observe the plates and look for plaques. Using the information provided in the introduction, determine which of the plates contain *E. coli* strain C and which plates contain *E. coli* strain B.

Questions:

1. What is a bacteriophage; a coliphage; a virion; and a plaque?
2. What is phage typing, and why can viruses be used to identify specific types of bacteria?
3. How do the plaques formed by the coliphage X174 differ from those formed by the coliphage T2?
4. Which of the bacteria strains tested was a better host for the coliphage X174, and which was a better host for the coliphage T2? How did you know?
5. Which of the four plates contain *E. coli* strain C and which plates contained *E. coli* strain B? How did you know?

SOME REPRESENTATIVE VIRUS PARTICLES

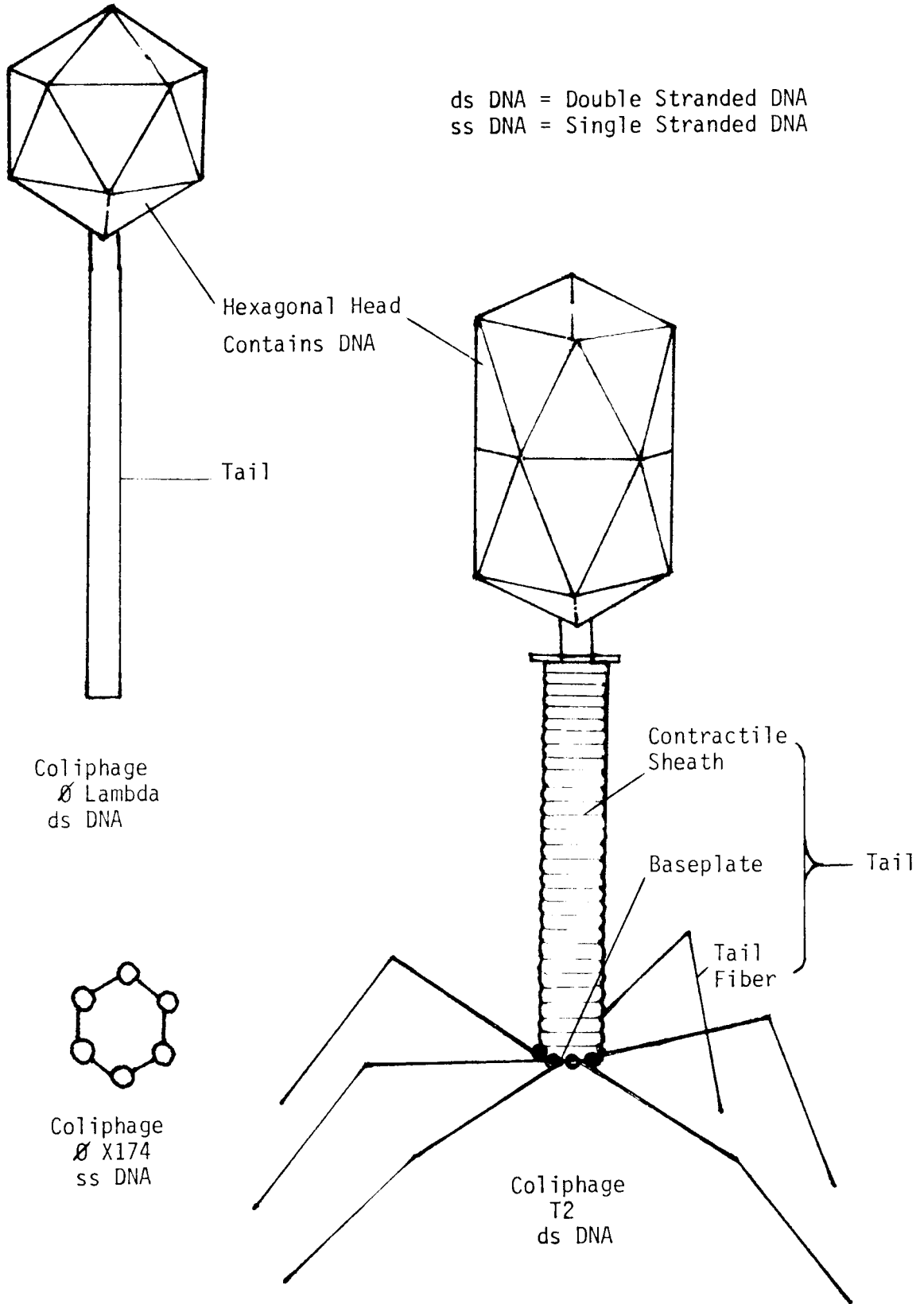


Fig. 23A.2 Illustrations of Bacteriophage Structure and Parts

Name _____

Lab Section _____

WORKSHEET
Exercise 23A
Phage Typing

Goals: _____

Materials & Methods:

The phage typing procedure was followed.

Phage infections were set up as follows:

	<i>E. coli</i> strain	Bacteriophage
Plate A		
Plate B		
Plate C		
Plate D		

Medium used: _____

Incubation Temperature: _____ Duration of Incubation: _____

Data & Results:

	Plaques (yes or no?)	Relative Size of Plaques (large or small?)
Plate A		
Plate B		
Plate C		
Plate D		

Additional Notes: _____

Conclusions:

Based on your data, did you use the correct phage type for each plate? _____
Explain. _____

Based on your data, which *E.coli* strain (#1 or #2) is *E. coli* strain C? _____ Explain. _____

Exercise 23-B

ISOLATION AND PURIFICATION OF COLIPHAGES FROM THE ENVIRONMENT

Introduction

As explained in an earlier exercise, bacteriophages that infect *Escherichia coli* are referred to as coliphages and occur in a variety of forms. Many of these, e.g., the T- even and T- odd phages, X174, Bacteriophage Lambda, and M13 are currently used in research. Various coliphages differ in terms of their size, structure, nucleic acid composition and the exact mechanisms by which they interact with their host; however, all cause the formation of plaques, so can be recognized when grown with bacteria in a lawn culture. We will use this aspect of bacteriophages to isolate them from the environment.

Although bacteriophages are common in the environment, they do not usually occur in large numbers. They are obligate intracellular parasites, so they are most likely to be found in habitats that support the growth of their specific hosts. In order to successfully isolate coliphages, it is essential to select an environment having a relatively high population of *E. coli*. Raw sewage is rich in coliform bacteria, and is therefore a convenient source. Raw sewage can be filtered to remove the bacteria present, and can then be used as a source of bacteriophage; however, raw sewage is likely to contain human viruses as well. To avoid possible infection with human viruses, raw sewage can be replaced by “simulated” sewage, i.e., a combination of virus types obtained from commercial sources.

Not all strains of *E. coli* are equally susceptible to phage infection. Many have restriction/modification systems (enzymes) that interfere with phage development. Since *E. coli* strain C has no restriction/modification system, it can serve as host to many different virus types. The number and variety of plaques that can be obtained with *E. coli* strain C is therefore greater than with other bacteria. Students may use either *E. coli* strain C or an *E. coli* strain obtained from the environment as the host type for this phage isolation.

Materials:

Filtered sewage (Procedure A) or plates containing *E. coli* and “simulated” sewage (Procedure B)
Log phase cultures of *E. coli* (strain C or otherwise)
Log phase cultures of other bacteria (used only if phage typing is being performed)
Top-agar in small tubes and bottom-agar in plates
Sterile test tubes (small) and caps
100-1000 μ l (blue) and 10-100 μ l (yellow) digital pipettes and sterile tips
KCl broth (1 ml aliquots in small screw-top tubes)
KCl broth (4.5 ml aliquots in small snap-cap culture tubes)

Procedure A – Infecting *E. coli* with Environmental Phage: (work in pairs or groups of three)

1. Obtain a 2.0 ml sample of filtered sewage, label it (masking tape on top) and place it into the 45^o C water bath provided.
2. Obtain three tubes of top-agar, label them 0.1ml, 0.5ml and 1.0ml, and then place them into a boiling water bath. Match the water level with the agar level, so water does not get inside the tubes. When the agar is thoroughly liquefied, transfer the tubes to the 45^o C water bath provided.

3. Obtain three plates of bottom-agar and label them 0.1 ml, 0.5 ml, and 1.0 ml.
4. Obtain one tube of *E. coli* broth culture. If you are using your own isolate, you should already have it on hand.
5. Using a clean sterile pipette, transfer the appropriate amount of warmed filtered sewage (0.1, 0.5 or 1.0 ml) into each of the top-agar tubes.
6. Remove each top-agar tube (**one at a time**) from the water bath, and add 200 μ L of the bacteria culture being used. Mix the tube contents by rolling the tube between the palms of your hands and then pour it over the surface of the agar in the appropriately labeled plate. Tip the plate gently to insure that the top-agar covers the entire surface. **Do not remove your top-agar tubes from the water bath until just before you are ready to use them** since cooled agar will solidify and will tend to form lumps when poured onto the bottom agar surface.
7. Allow the plates to remain undisturbed on the desktop until the top-agar has solidified completely. Once this has occurred, tape the plates together in a stack, invert them and place them into the 37 $^{\circ}$ C incubator until the next lab session.
8. Remove your plates from the incubator and observe the number and variety of plaques present. Count the number of each plaque type readily distinguished and determine the approximate number of **plaque forming units** (PFU) per ml. of sewage used. Note that some plaques may appear turbid due to bacteria growing within them at a low density. These turbid plaques are the result of **lysogeny** (infection with a temperate phage) and the bacteria growing within them are carrying **prophages** (viral DNA that has entered the host chromosome).

Procedure B – Purification of Selected Coliphage (work in pairs or groups of three)

Infection of an *E. coli* lawn culture with several different types of coliphage will usually result in the formation of many different-looking plaques. Plaques can vary considerably in size and appear more or less clear depending upon the degree of cell lysis present. If the plaques on a plate are separated from one another, it is possible to obtain a pure sample (one type of virus) by transferring virions from the center of a single plaque to a tube of sterile medium. By making a serial dilution of the sample and plating various concentrations with new, susceptible *E. coli* cells, it is possible to determine the number of PFU per ml in the pure culture, and how many PFU exist on the surface of a wire needle after it has been stabbed into the center of a plaque.

Procedure for Purification: (Students should work in pairs or groups of three)

1. Obtain one tube containing 1ml of sterile KCl broth, four tubes containing 4.5ml of sterile KCl broth, three tubes of top-agar and three bottom-agar plates. Label the 4.5ml KCl broth tubes to indicate degree of dilution (10^{-1} , 10^{-2} , 10^{-3} and 10^{-4}). Label the bottom-agar plates 10^{-3} , 10^{-4} and 10^{-5} .
2. Thoroughly liquefy the top-agar by placing the tubes in a beaker of boiling water (as described in the exercise above), and then place them into the 45 $^{\circ}$ C water bath provided. Be careful to prevent beaker water from entering the tubes (match the water level in the beaker with the agar level inside the tubes).

3. Select a plate containing well-isolated plaques. Determine which of the plaques you wish to use for your purification procedure and mark its location on the underside of the plate with a glass marker.
4. Flame-sterilize and cool an inoculating needle and then stab it into the center of the plaque selected. Be careful not to touch any of the surrounding bacteria. Transfer this phage sample to the 1ml KCl broth by plunging the needle into the liquid and agitating it for a few seconds to dislodge the phage particles adhering to the needle.

Stab the same plaque more than once, but not more than four times. Be sure to dislodge the phage particles into the sterile KCl broth after each stab. **Record the number of times you stab the plaque.**

5. Prepare a serial dilution of your phage sample as follows:
 - a) Transfer 500 μ l (0.5ml) of the phage sample into the tube labeled 10^{-1} . Mix the suspension by forcing the liquid into and out of the pipette tip several times or by rolling the tube vigorously between the palms of your hands.
 - b) Transfer 500 μ l (0.5ml) of liquid from the tube labeled 10^{-1} into the tube labeled 10^{-2} , and mix the tube contents well as described above.
 - c) Transfer 500 μ l (0.5ml) of liquid from the tube labeled 10^{-2} into the tube labeled 10^{-3} , and mix the tube contents well as described above.
 - d) Transfer 500 μ l (0.5ml) of liquid from the tube labeled 10^{-3} into the tube labeled 10^{-4} , and mix the tube contents well as described above.

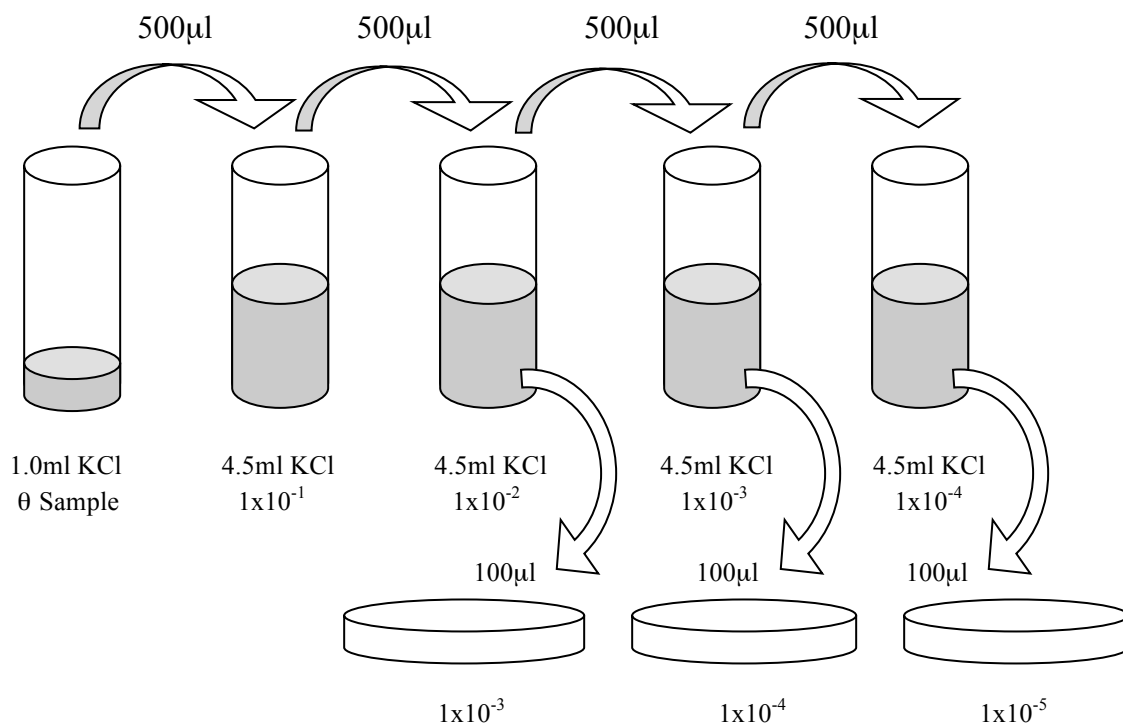


Fig. 23B.1 - Preparing a serial dilution of bacteriophage.

6. Determine the purity and density of your phage sample by plating it on the bottom-agar as indicated in the diagram and described below.

- a) Obtain one tube of *E. coli* C broth culture. If you are using your own isolate, you should already have it on hand.
- b) Remove **one** top-agar tube from the water bath, and add 200 μ L of *E. coli* culture. Then add 100 μ l of liquid from the dilution tube labeled 10^{-4} and mix the tube contents well as described above. Quickly pour the contents of this tube over the surface of the bottom-agar in the plate labeled 10^{-5} and tip the plate gently to spread the top-agar over the entire surface.
- c) Remove a **second** top-agar tube from the water bath, and add 200 μ L of *E. coli* C. Then add 100 μ l of liquid from the dilution tube labeled 10^{-3} and mix the tube contents well as described above. Quickly pour the contents of this tube over the surface of the bottom-agar in the plate labeled 10^{-4} and tip the plate gently to spread the top-agar over the entire surface.
- d) Remove a **third** top-agar tube from the water bath, and add 200 μ L of *E. coli* C. Then add 100 μ l of liquid from the dilution tube labeled 10^{-2} and mix the tube contents well as described above. Quickly pour the contents of this tube over the surface of the bottom-agar in the plate labeled 10^{-3} and tip the plate gently to spread the top-agar over the entire surface.

Note - If you begin with the most dilute phage sample and work backward as described, it is not necessary to change pipette tips between dilution samples.

7. Allow the plates to remain undisturbed on the desktop until the top-agar has solidified completely. Once this has occurred, tape the plates together in a stack, invert them and place them into the 37° C incubator until the next lab session.
8. After incubation, observe the plaques present on your phage purification plates. If they are all similar in appearance, you were probably successful in purifying your phage sample. Count the number of plaques present and determine the number of plaque-forming units (PFU) present per ml of your original phage sample. This will tell you how many infective phage particles were transferred from the plaque to the saline on your needle.

Note – If you stabbed the plaque more than once, you must divide the number of PFU/ml by the number of stabs to obtain an accurate representation of the number of phage particles transferred with each stab.

Questions:

1. How many different types of plaques were visible on your phage isolation plate? How many different types of virus appear able to infect *E. coli* strain C?
2. What is lysogeny? Did any of your bacteria become lysogenic? What is the condition of the viruses within cells that have undergone lysogeny?
3. How many virions were transferred by stabbing a plaque with a wire needle?

Name _____

Lab Section _____

WORKSHEET
Exercise 23B
Isolation and Purification of Coliphages from the Environment

Goals: _____

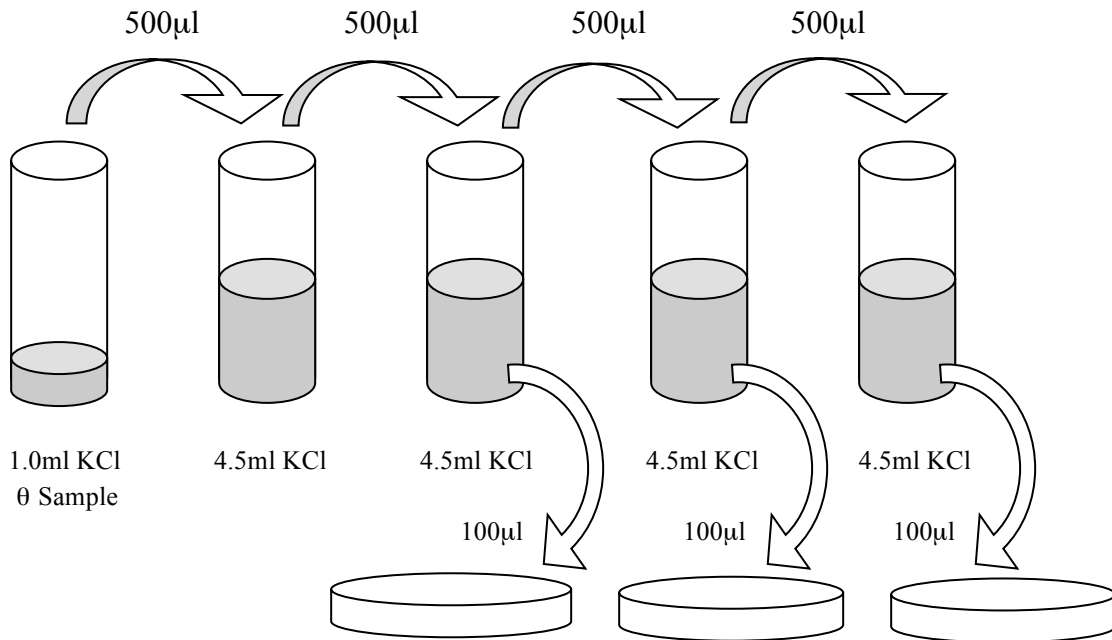
Materials & Methods:

The purification procedure was followed.

Description of plaque chosen (large or small?): _____

Number of stabs: _____

Complete the diagram and table to represent the serial dilution you performed.



	Tube 1	Tube 2	Tube 3	Tube 4	Plate 1	Plate 2	Plate 3
Individual Dilution Factor							
Total Dilution Factor							

E. coli strain infected: _____ Medium used: _____

Incubation Temperature: _____ Duration of Incubation: _____

Data & Results:

	Plate 1	Plate 2	Plate 3
Number of Plaques			

Conclusions:

How many phage particles were present in the original 1 mL of KCl broth? _____

You should have observed the most number of plaques on the first plate and the least number of plaques on the last plate. Did you? _____ What do your observations of plaque number tell you about the success of your dilution technique? Did your technique improve since the last time you tried (Ex. #13)? _____

