Bacteriophages

Bacteriophages are viruses that attack bacteria. Each phage has a highly specific host and any given phage will act only on its own particular species or group of species of bacteria. Phages are commonly named in reference to their host and thus a phage that attacks staphylococcus is called staphylophage, while one that attacks *E. coli* is called a coliphage.

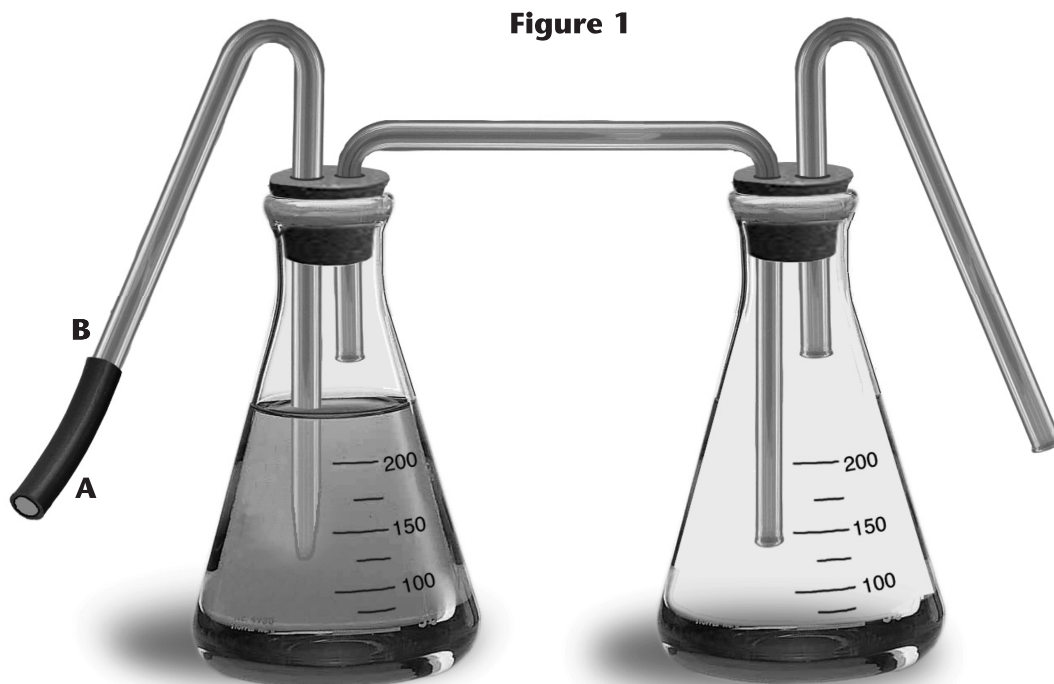
A typical phage is a particle close in size to that of a large molecule. It consists of a unit of nucleic acid encased in a protein coat. Like all viruses, they rely upon the host to provide the necessary activities and materials for their reproduction. A typical coliphage looks like a tadpole with an expanded head and elongated tail. When attacking the coliphage uses its tail to attach itself to the bacterium, and then like a syringe and needle it penetrates the cell wall and collapses its head, injecting DNA into the host.

The viral nucleic acid directs the metabolism of the bacterial host in the synthesis of new viral DNA and other materials for making a complete virus. After replication, the bacteriophages are released by a sudden rupture of the host cell wall (lysis) and is marked by a sudden decrease in turbidity of the bacterial suspension. The new viral particles are then free to infect other susceptible bacteria. The concentration of viral particles per milliliter of culture suspension is referred to as the titer.

Successful culture of the T-series phages requires a sensitive strain of bacteria, generally *E. coli* B. Proper sterile technique must be observed when culturing to insure success as well. For optimal growth of the phage, air should be gently bubbled through the medium. Growth can be achieved without bubbling by shaking the flasks at intervals; however, a lower titer will develop.

A bubbling apparatus may be made by using two 250 ml Erlenmeyer flasks, or bottles of similar size, fitted with two-hole stoppers. Bend 5 mm glass tubing as shown in figure 1. A small aquarium pump may serve as an air source and a three-way valve placed in the air supply line will serve as a convenient method of controlling the air flow. Flexible tubing (A) is used to connect the pump to the glass tubing of the bubbler. The air is filtered through bacteriological cotton inserted in the glass tubing (B) to create a sterile environment. The apparatus itself should be sterilized by covering the top portion with aluminum foil and then autoclaving it at 121°C for 15 minutes at 15 psi.

Figure 1



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Making the Media

1. Add 8 g of dehydrated tryptic soy broth and 5 g NaCl to 1 L cold distilled water. You will need to make 2 L, one for the agar and one for the soft agar.
2. Add 15 g of agar to 1 L of tryptic soy broth and bring to a boil, then autoclave at 121˚C for 15 minutes.
3. Lift the lid of the Petri dish just enough to allow the neck of the bottle in and pour about 20 ml of tryptic soy agar into the dish.
4. Cover and incubate overnight until the agar has set.
5. For soft agar add only 7 g to 1 L of tryptic soy broth and bring to a boil.
6. Divide the tryptic soy agar into 2 ml tubes to use as needed and then autoclave as described in step 2.

Procedure for Producing Coliphage Suspension of High Titer

1. Transfer 75 ml of tryptic soy broth aseptically to the first bottle of the bubbling apparatus.
2. Aerate the broth in the incubator for 24 hours at 37°C for a sterility check. If the broth is not clear after 24 hours the apparatus was not sterile.
3. Inoculate the sterile broth with 3-4 loopfuls of *E. coli* B or 0.5-1.0 ml of bacterial suspension prepared by washing a slant culture with 5 ml of tryptic soy broth. Lift the stopper from the bottle only far enough to facilitate inoculation.
4. Incubate approximately 2-3 hours until the broth is turbid, but not yet milky.
5. Pipet 0.5 ml of high titer coliphage stock into the broth.
6. Incubate until the broth clears. Bacterial lysis usually begins within 40 minutes and may be accompanied by foaming. Cultures will usually clear after $2^1/_2$ - 4 hours. A coliphage culture that does not clear within a reasonable length of time indicates that either contamination has occurred or a resistant strain of *E. coli* B has developed.
7. After the culture has cleared, remove it from the incubator, stopper it, and refrigerate it at 2-8°C overnight.
8. Centrifuge to remove cells and precipitated proteins.
9. Perform a sterile filtration. This should produce a clear, sterile coliphage stock. Unfiltered stock tends to be adsorbed onto the debris, and the resulting titer is reduced. T-series phages can also be sterilized by adding 1 ml of chloroform per 30 ml of broth, then shaking to saturate the solution with chloroform. After the chloroform has settled to the bottom, decant the supernatant into a sterile flask or bottle. The supernatant must then be aerated to evaporate residual chloroform.

Determination of Titer

A simple method of determining coliphage titer involves serial dilution of the coliphage stock followed by the double agar technique for plaque counts. The bacteria grow as tiny subsurface colonies forming a translucent layer over the agar plate, while the plaques appear as clear patches in the translucent layer of bacterial growth.

Materials Needed

6 Tryptic soy agar Petri dishes (20 ml each)
6 Soft tryptic soy agar tubes (2 ml each)
11 Tryptic soy broth tubes (9 ml each)
Agar slant culture of *E. coli* B

Procedure

1. Label the Petri dishes 10^{-6}-10^{-10}, leaving one blank. Allow them to warm to room temperature to insure a uniform pour of the soft agar.
2. Label the agar tubes the same way.
3. Label the broth tubes 10^{-1}-10^{-10}, leaving one blank. These will be used for a serial dilution.
4. Place the agar tubes into a beaker of boiling water and heat until the agar melts. Then transfer the tubes to a 46-50°C water bath until needed.
5. Using aseptic technique, pipet 1 ml of the phage stock into dilution tube 10^{-1}, mix thoroughly and transfer 1 ml from tube 10^{-1} to 10^{-2}. Repeat until you reach 10^{-10}, using a different sterile pipet for each dilution. Don't add any phage to the blank tube.
6. Prepare a broth suspension of *E. coli* B by adding 5 ml of tryptic soy broth to the slant culture and washing off the bacteria by gently shaking the tube. Pipet 2 drops of the suspension into each soft agar tube using a 1 ml pipet.
7. Pipet 1 ml of the blank tryptic soy broth to the blank soft agar tube containing *E. coli* B and pour it onto the corresponding Petri dish. Gently swirl the closed dish with a circular motion to form a uniform layer of soft agar. This will serve as your control and should have no plaques after incubation.
8. Using your dilution series tubes 10^{-6}-10^{-10}, remove 1 ml of phage stock and transfer it to the corresponding soft agar tube containing *E. coli* B. After mixing, immediately pour it onto the corresponding plate so the soft agar doesn't harden in the tube. Gently swirl the closed dish to distribute a uniform layer of agar.
9. After the plates have set, place them in an inverted position in the 37°C incubator for 12-24 hours. The titer can then be determined.

Discussion and Interpretation of Results

Theoretically, because of the even distribution of *E. coli* B and coliphage across the plate, a single coliphage will adsorb onto a single bacterium and lyse the bacterium as it reproduces. The liberated coliphage particles then infect surrounding host cells and repeats the cycle. Eventually this produces a clear area, or plaque, in the layer of bacteria. By picking a plate that has between 30 and 300 colonies, an accurate statistical estimate of phage titer can be made.

Example

The plate made from serial dilution tube 10^{-7} has 138 plaques. The titer, or number of infective particles/ml of stock suspension, is then equal to $138/10^{-7}$ or 138×10^{7} or 1.38×10^{9}.

A possible error in the titer can occur when more than one phage adsorbs to a bacterium or when two phages adsorb onto bacteria that are very close and give rise to one plaque when there should be two. This is the reason that it is essential to pick a plate of 30 to 300 colonies. The control plate should be used for comparison to avoid confusion.

Notes: