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V-phage a new pathway new protocol for the isolation of phage

Abstract

Recently it has been recognized that bacteriophages, the natural predators of bacteria can be used efficiently in modern biotechnology. They have been proposed as alternatives to antibiotics for many antibiotic resistant bacterial strains. Phages can be used as biocontrol agents in agriculture and petroleum industry. Moreover, phages are used as vehicles for vaccines both DNA and protein, for the detection of pathogenic bacterial strain, as a display system for many proteins and antibodies. Bacteriophages are diverse group of viruses which are easily manipulated and therefore they have potential uses in biotechnology, research, and therapeutics. The aim of this review article is to enable the wide range of researchers, scientists, and biotechnologist who are putting phages into practice, to accelerate the progress and development in the field of biotechnology (Hag, Chaudhry, Akhtar, Andleeb & Qadri, 2012). Bacteriophage research continues to break new ground in our understanding of the basic molecular mechanisms of gene action and biological structure. The abundance of research is growing so rapidly. The enrichment culture technique creates conditions that favor replication of specific bacterial phages. Phages are obligate intracellular parasites, and large numbers of a desired phage can be obtained by adding host bacteria and bacterial media to an environmental sample. By seeding the sample with host bacteria and using nutritional conditions optimized for bacterial growth, the phages that are specific to that bacterial species will infect the bacterial cells and replicate to higher concentrations. These phages can then be isolated at much higher frequency than with direct plating. This protocol is for isolation of phages specific to Mycobacteriumsmegmatis.[1]

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Introduction

Bacteriophages are the most abundant entities on earth. These bacterial viruses have genetic material in the form of either DNA or RNA, encapsulated by a protein coat. The capsid is attached to a tail which has fibers, used for attachments to receptors on bacterial cell surface. Most of the phages have polyhedral capsid except filamentous phages. Phages infect bacteria and can propagate in two possible ways; lytic life cycle and lysogenic life cycle. When phages multiply vegetative they kill their hosts and the life cycle is referred to as lytic life cycle. On the other hand, some phages known as temperate phages can grow vegetative and can integrate their genome into host chromosome replication, with the host for many generations. If induction to some harsh conditions like ultraviolet (UV) radiations occurs, then the prophase will escape via lysis of bacteria. After the discovery of bacteriophages in early 20th century many researchers thought about their (phages) potential of killing bacteria, which could undoubtedly make them possible therapeutic agents. But after World War II when antibiotics were discovered, this natural potential therapeutic agent got little attention and was only considered as a research tool for many years. Bacteriophages have contributed a lot to the field of molecular biology and biotechnology and are still playing its part. Many mysteries of molecular biology are solved by bacteriophages. Today when everything is much more advanced than ever before, bacteriophages are getting enormous amount of attention due to their potential to be used as antibacterial, phage display systems, and vehicles for vaccines delivery. They have also been used for diagnostic purposes (phage typing) as well.[2] In this research, protocol will be followed first as described. After the initial replication of phages, by following a predetermined technique, attempts to develop new pathways will be explored. In this portion of research, it is important to take precautions while collecting soil to ensure that the soil that is collected is not contaminated in any way. Aseptic techniques will help insure that the soil that has been collected remains free from contamination. In each of the steps precautions will be in place to insure the aseptic techniques are continued to be used throughout the experimental phase.

Objective

To collect a soil sample that is free from contamination. Isolation of phage and developing a new protocol for the isolation of phages.

Sea-Phage Protocol

Materials

1. 200-mL bottle or flask containing distilled water
2. Large bottles of disinfectant
3. Large Jar containing gauze pads and 90% alcohol
4. 15-mL conical tube (1)

5. 10-mL culture tube (1)
6. 10-mL pipette (two for each soil sample)
7. 5-mL pipette (14 + one for each soil sample)
8. Micro-centrifuge tubes (7)
9. Micro-pipette tips (100-microliter)
10. Bunsen burner
11. Pipettes
12. Micro-pipettes (100 or 200 microlitre)
13. Labeling pen
14. Sterilization filters
15. 1.0mL syringes
16. 10mL phage buffer (PB) (add 1 mM CaCl2)
17. 5mL of M. smegmatisculture (7, I glass culture tubes with lids and a rack)
18. 40mL of top agar (TA) (add 1 mM CaCl2)
19. 5mL 10x7H9/glycerol broth
20. 0.5mL specific bacterial phages of 100 mM CaCl2
21. 5mL of AD supplement
22. Agar plates 100 mm (total 7)
23. Sterile conical tube with cap
24. vortexes
25. Incubator
26. Centrifuge
27. Microwave oven

Soil collection

Collect soil sample

i. Label conical tube

ii. Place soil into the tube

iii. Make sure to mark and record collection site. A navigation unit may be used to record latitude and longitude.

In the laboratory prepare enrichment culture by:

i. Using a clean spoon add 1 gram of soil from the collected soil, and put into Erlenmeyer flask.

ii. Using aseptic techniques add:

a. 40mL of sterile H2O (use sterile pipette).

b. 5mL of sterile 10x 7H9/glycerol broth.

c. 5mL of AD solution.

d. 0.5mL of 100mL CaCl2

iii. Add M. smegmatis culture to flask.

Incubate the flask at 27°C for 24hours. While in the incubator place on shaker for the entire time.

Preparing the soil sample

Centrifuge the enriched culture

1. After 24hours transfer the contents of the Erlenmeyer flask to small conical tube.
2. Make sure to balance the tube and spin at 3,000rpm for 20minutes.

Prepare a phage filtrate

1. Pour the liquid from the centrifuged soil sample into a small conical tube.
2. Attach filter unit, and aseptically cap the tube. Label correctly. This will be 100 undiluted soil sample. May store at 40°C.

Dilutions of enriched soil sample 100-10-4 using serial 10-fold dilutions

1. Arrange micro-centrifuge tubes in rack and carefully label with proper dilutions.
2. Add 90 microlitre of Phage Buffer to each of the four tubes.
3. Label one micro-centrifuge tube 0 and aseptically transfer 100 microlitres of enriched soil sample into this tube.
4. Add 10 microlitres of 100 soil sample to the tube marked 10-1 and vortex well (5 min). This tube is the 10-1 (or 1:10) dilution.
5. Add 10 microlitres of the 10-1 soil sample to the tube marked 10-2 and vortex well (5 min).
6. Continue each of the dilutions until the tube marked 10-4 is complete.

A positive control is obtained of the Phage Buffer from a previously prepared sample.

A negative control of filtered 1.0mL of Phage Buffer into tube marked micro-centrifuge.

Plaque Screening

Add 50 microlitres of each sample (including controls) to 0/5mL of M. smegmatis.Assemble the following items

a. Culture tubes containing 0.5mL of an M. smegmatis culture (total 7).

b. A 100 microlitres or 200 microlitres micro-pipette.

c. Micro-pipette tips.

d. Positive and negative control tubes.

e. Phage enrichment tubes (100-10-4).

For each of the samples including the positive and negative control.

1. Label the culture tubes containing 0.5mL of M. smegmatis with the same markings of the micro-centrifuge tubes.
2. Using a micro-pipette:

a. Dispense 50 microlitres of each sample into the appropriate culture tube.

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b. For the negative control, add 50 microlitres of filtered-sterilized phage buffer to the phage-negative control tube.

c. For the positive control, add 50 microlitres of the prepared phage solution to the phage-positive control tube.

Add top agar and plate each soil samples and each control

i. Label each agar plates (7) with sample and control information plus the date and initials.

ii. Heat top agar until melt down at approximately 55°C. Remove from bath.

iii. For each soil sample, and each control:

iv. Use sterile 5-mL pipette, aseptically transfer 4.5mL of top agar to the culture tube.

v. Immediately pull the mixture back up into the pipette. Let mixture run down and pull it back up several times.

vi. Transfer the mixture to the properly marked plate and discard the used pipette.

vii. Swirl the plate gently to spread the top agar evenly over the entire surface of agar plate.

viii. Cover and let plates stand undisturbed for 20minutes, allowing top agar to completely solidify.

Incubate the plates at 37°C

a. Invert plates after the top agar has solidified quickly but gently.

b. Place all plates into the incubator at 37°C.

c. Check for plaques after 24hours.

After 24hours the plates were removed from the incubator and examined. Plaques were discovered on several plates. The procedure was repeated several times with the same results. Single individual plaques were isolated.

Several successful attempts were made to isolate individual plaques: It was discussed and determined, due to the difficulty in understanding the directions of Sea phage protocol, a different pathway needed to be developed.

New Pathway for Isolation of Bacteria Phage

a. Being careful to follow aseptic techniques, 100 microlitres of phage buffer was dripped over individual plaques, being careful not to upset each individual plaque.

b. Allow the phage buffer to remain standing over each plaque for 20minutes.

c. Place the plate covered into incubator at 37˚C.

d. Allow plate to remain in incubator for 20minutes, allowing phage to attach to the M. Smegmatis.

e. Carefully remove plates from incubator.

Labeling Agar Plates (containing antibiotic)

A. Carefully label each plate

B. Name

C. Date

D. Time

E. Number of the individual plaque.

Collection of isolated plaques using aseptic techniques

1. Using micro-pipette, draw up the phage buffer that was placed over the individual plaque. Pay special attention not to touch the agar and only draw up the phage buffer and not agar.
2. Using aseptic techniques place 200mL of M. smegmatis into micro-centrifuge tubes along with the plaque and phage buffer solution previously collected.
3. Vortex the collected plaque samples
4. Using aseptic techniques while using micropipette to transfer individual plaque in 10mL glass culture tube.
5. After liquidating the previously prepared top agar, allow top agar to cool to 55°C.
6. Using aseptic techniques and Working quickly (so top agar does not reset) measure 5mL of top agar using pipette.
7. Release some of the agar in to the 10mL and immediately draw the mixture of top agar and plaque sample back up again.
8. Using aseptic technique, remove top of agar plate. Carefully allow the mixture of top agar and plaque preparation to flow from the pipette.
9. Place top back on plate and swirl gently. Allow plate to sit 20minutes.

Incubate the plates at 37°C for 24hours. Read and record results. Place samples back into the incubator for another 24hours.

After 24hours remove the samples from the incubator. Read and record results.

Discussion

After the 48hours the soil samples plates were removed from the incubator. Each plate was carefully examined and the results of each plate were recorded. The samples from the original three attempts, stemming from the Sea phage protocol, each showed plaques development. The 100, 10-1,10-3 plates each showed the plaques. The procedure was repeated three times and each time there was growth.

The procedures for the Sea Phage protocol were difficult to understand, it was discussed that new protocols should be worked on. With the assistance of the Biology Department there were discussions on ways to develop another protocol. This protocol we determined would be called V-Phage.

There were three attempts with the new V-phage protocol, of which two of the three produced plaques. The plaques were produced from varying pre-existing soil samples. Care was taken to develop these plaques into individual plaques and was unsuccessful in further development. More research needs to be done in further developing this new protocol.

Conclusion

The V-Phage research has given the scientific community an alternate pathway for the isolation of Phage. The V-Phage pathway was designed as an alternative to the Sea-Phage pathway created by The

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Howard Hughes Medical Institute. With the assistance of the biology department the researchers have created an alternative protocol. This protocol was developed within the laboratory to provide a less complex procedure, for elementary students. Details given above give a glimpse of the range in which phage can be isolated in the field of biotechnology and medical science. Phage applications range from the diagnosis of disease, through phage typing, prevention, to treatment. There is hope that phages will be found to be useful to humans through the fields of medicine.

There are concerns about the use of phages, that include the safety and efficiency issues, as well as immune response to the administered phages. Growth and purification of the phages are also additional issues that need to be addressed. Due to the rapid progression of science in the fields of biotechnology and molecular biology, it is of great importance that phages present in the biosphere could answer many questions in the science community that scientist are having.

Author's note

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None.

Conflict of interest

The author declares no conflict of interest.

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