Phage Isolation and Investigation

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Abstract

A novel bacteriophage, named phage #3, that infects several major strains of Pseudomonas aeruginosa has been discovered. The phage has a lambdoid morphology as seen by electron microscopy. Its nucleic acid is double stranded DNA and is 10-15 kb in length. Phage immunity tests have shown that the phage is temperate. The phage has been used to mediate transduction in the reconstruction of mutants. A pilot experiment has been developed to screen for mutant phages with a higher efficiency to mediate transduction.

INTRODUCTION AND BACKGROUND

A bacteriophage is a virus that infects bacteria and can either instantly kill a bacterial cell or integrate its DNA into the host bacterial chromosome (Madigan, et al,. 1997). If the phage DNA is integrated into the host, the phage can then stay within the bacteria causing no harm. This pathway is called the lysogenic cycle. On the other hand, the phage can also cause eventual lysis and death of the host after it reproduces inside the host and escapes with numerous progeny through the lytic cycle (Watson, et al., 1987). Figure 1 illustrates the differences between the lytic and lysogenic pathways. In very rare instances, phages make a mistake and can package the DNA from the bacterial cell instead of their own DNA when preparing to escape from the host in the lytic pathway. Then, when the phage infects a second bacterium later on, the DNA packaged by phage and the DNA from the second bacterium homologously recombine, or swap with one another. Thus, the phage has thereby moved DNA from one bacterium to another and none of the phage's own DNA is inserted in the second bacterium. This phenomenon is called transduction. This summer, I analyzed a bacteriophage I discovered, named phage #3, which is able to mediate transduction efficiently in *P. aeruginosa*.

The ability of the phage to mediate transduction in *P. aeruginosa* is important because the ability to move DNA around within different strains of *P. aeruginosa* allows various types of genetic analysis to be conducted. For example, this summer I used phage #3 to reconstruct mutants of *P. aeruginosa*. Mutants are bacteria that have phenotypes that are different from the wild-type bacteria. wild-type bacteria are bacteria genetically unaltered and represent a standard, or basis, to which various mutations are compared. Mutants are formed when mutations, or changes in the

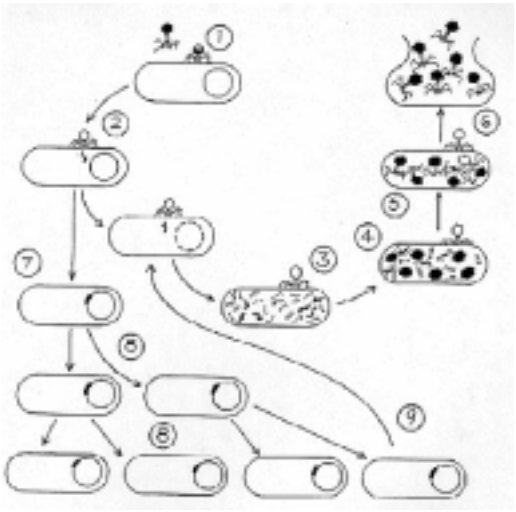


Figure 1. Lysogenic Cycle (pathway 1,7,8,9) versus Lytic Cycle (1,2,3,4,5,6)

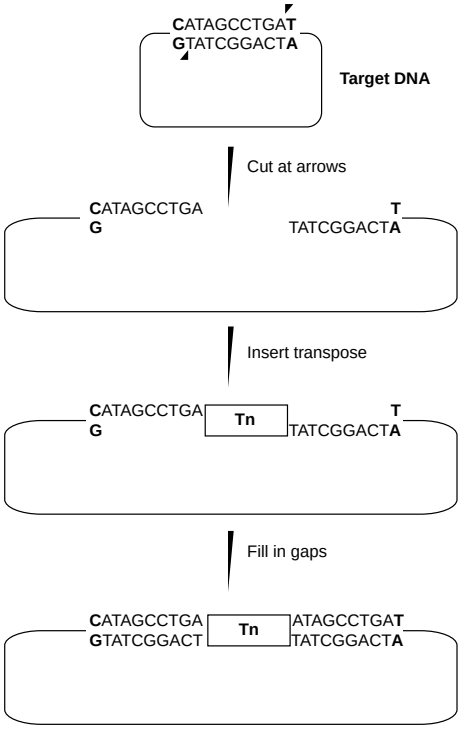


Figure 2. Transposon Insertion

DNA of the bacteria, cause genotypic differences from the wild-type bacteria that may cause the mutants to display a different phenotype.

In our lab, one of the main ways that we induce mutations is through transposon insertions, which is the introduction of foreign DNA into bacterial DNA. Figure 2 shows that through inserting a novel segment of DNA in the bacterial genome, one has the potential to interrupt a bacterial gene (Madigan, et al., 1997). As a result, the gene will be neither transcribed nor translated properly, and will not yield a functional protein, causing disruptions in cellular processes. After inserting a transposon, one can test for phenotypic differences in the altered bacteria against the wild-type. For instance, after randomly inserting a transposon in *P. aeruginosa*, one might perform a biofilm assay, which tests for the formation of biofilms. If altered bacteria are unable to form biofilms but the wild-type bacteria can, then the altered bacteria is said to be a biofilm mutant. More tests are conducted to see if there are other phenotypic changes that have been caused by the transposon insertion. Thus, one characterizes the mutant bacteria by its various phenotypic differences from the wild-type.

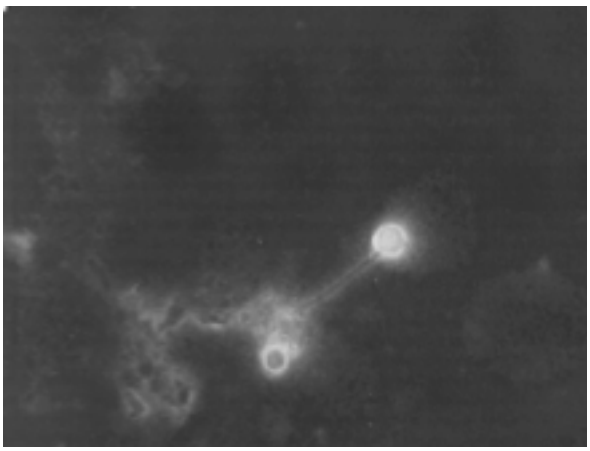


Figure 3. 100,000 X Magnification. Phage #3.

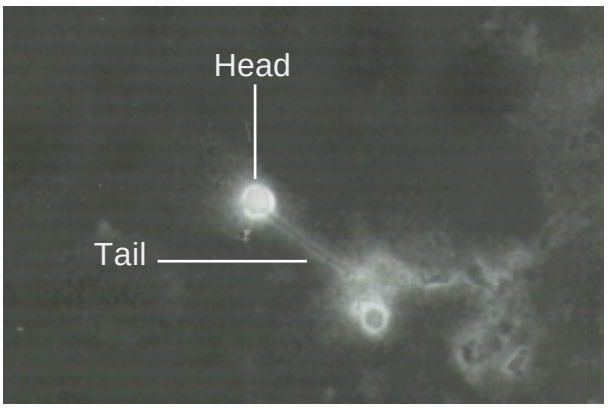


Figure 4. 100,000 X Magnification. Phage #3.

Biofilms are bacterial communities that grow on different surfaces such as catheter lines, surgical implants, contact lenses, the lungs of patients with cystic fibrosis, industrial and drinking water pipelines, and on the surfaces of plant roots (O'Toole & Kolter, 1998a). The phage is an excellent tool in verifying our hypotheses about which genes are a part of biofilm development.

Phage #3 is also useful for reconstructing mutants. This means that a test is performed to see if a particular transposon insertion in the mutant bacterium is the true cause of the observed phenotype. Transposon insertions are convenient in that they contain antibiotic resistance markers. For example, if it is thought that a transposon insertion (carrying tetracycline [Tc] resistance) inserted in a certain gene prevents a biofilm from forming, then through transduction with phage #3, the interrupted gene can be transferred from the mutant to wild-type bacterial cells. By growing the transduced cells on medium with the antibiotic tetracycline, we can select only for those cells that received the gene encoding resistance to the antibiotic and, consequently, the interrupted gene. Then the resistant cells can be tested in a biofilm assay. If the cells do not form biofilms, then it is very likely that the transposon insertion suspected for causing the inability to form biofilms is indeed the cause of the observed phenotype. If one neglects to perform this test, one can not be certain that the transposon insertion is truly the cause of the observed phenotype since a random mutation could yield the same phenotype.

Before the biofilm assay is performed, one must check to see if the reconstructed transductants are immune to further phage infection. To review, in rare instances, phages may package a bacteria's DNA instead of its own. Antibiotic resistance could be conferred to different bacterial transductants by homologous recombination of the transposons. These transductants are susceptible to further phage infection because the DNA of the transduced bacteria do not carry phage DNA, which confer immunity to phage infection. Instead, these transductants carry their original

bacterial DNA and the DNA from the bacteria the phages first infected. These transductants with no phage DNA are ideal for further experimentation because phage #3 can still be used in genetic analysis of the transductants. However, when a phage packages its own phage DNA instead of a bacterium's DNA, it can insert its own DNA into a second bacterium's DNA. Should this occur, the second bacterium is no longer susceptible to phage infection because it already has phage DNA inserted into its own DNA (Watson, et al., 1987). For example, some of the transductants that grow on tetracycline will have been infected once by a phage carrying the transposon for tetracycline resistance and have undergone homologous recombination, but then they also will have been subsequently infected by a wild-type phage that inserted its own DNA into the host. These cells that were infected twice are not useful because they are immune to further phage infection.

While testing for biofilm formation is one example of an observable phenotype, testing for the phenotype involving detachment was also executed. Wild-type *P. aeruginosa* bacteria normally form biofilms after 24 hours of growth. By 48 hours, however, the bacteria detach and cease to be a part of the biofilm. The mutants I reconstructed were 814, 821, 824, and 829, and these are all det mutants, meaning they do not detach after 48 hours.

The phage was investigated through electron microscopy and restriction digests. Four reconstructions were then performed using the phage. A pilot experiment to induce mutations in the phage in the hopes of improving its ability to mediate transduction was also developed.

METHODS

Strains. Pseudomonas clinical isolates were obtained from the Dartmouth Hitchcock Medical Center and grown on blood agar. After starving the bacteria by growing them in LB liquid media for 24-48 hours in the 37°C incubator, the phages were first isolated by centrifugation for 5 minutes. The supernatant was then decanted and saved. Four drops of chloroform were added to the solution to kill any residual bacteria.

Media. Luria-Bertani (LB) plates and liquid media were used to grow cultures of bacterial strains.

Electron Microscopy. 300M Cu grids were prepared on 0.4% formvar. The grids were negatively stained using 40µl 2% uranyl acetate per grid. The grids were first placed on 20µl of phage diluted to 108 PFU/ml for 4 minutes. Trials where LB and water were the diluting agents were performed. After placing the grids on the sample, the grids were rinsed in distilled water, dried, and then placed on the uranyl acetate for 2 minutes (Harris, 1997). Observations were made using the Electron Microscope JEM 100CX model at 100,000X magnification.

Reconstruction of Mutants. To reconstruct a mutant, one must first make lysates and then perform a transduction.

Making Lysates. As an example, the mutant 814 is TcR and is also unable to detatch from surfaces after forming biofilms. 100µl phage #3 (1011 PFU/ml) grown on *P. aeruginosa* were added to 100µl of 814 with 1µl CaCl2 (final concentration = 1mM). Two sets of the above phage and cell mixtures were made. The controls were 100µl phage only and 100µl cells only, both containing 1µl CaCl2. After 15 minutes, the solutions were added each to 3.5mL of LBTop agar and plated on LB plates and grown in the 37°C incubator overnight. The bacteria plus phage mixtures were scraped into a new tube and LB was used to wash off any remaining material on the plates. The tube was placed on a shaker for 5 hours and then centrifuged, keeping the supernatant. Chloroform was added to the supernatant, and a phage titer assay was performed.

Performing Transductions. 100µl phage grown on 814 was then added to 100µl *P. aeruginosa* plus 1µl $CaCl_2$. Two sets of the phage and cell mixtures were made, and phage only and cell only controls were prepared as described above. After waiting 15 minutes, the mixtures were poured onto Tc plates and allowed to incubate at 37°C for 48 hours. Colonies that appeared on the Tc plates were retested for TcR by picking the colonies and placing them on fresh Tc plates.

Phage Titer. Serial dilutions were prepared of phage and 10µl of dilutions were spotted onto a *P. aeruginosa* overlay. An overlay is formed by adding 100µl *P. aeruginosa* to 3.5mL LBTop agar and pouring onto LB plates. Counting the number of plaques on the plate after plate was incubated at 37°C for 24 hours yields the number of plaque forming units, or individual phages.

Transductant Immunity. To select for phage sensitive transductants, a vertical line of phage grown on *P. aeruginosa* was pipetted onto Tc plates. The transductants were then picked from a toothpick and streaked horizontally from the left end of the plate, through the line of phage, to the right end of the plate. Those cells that showed lysis and therefore yielded thinner lines after passing through the phage were kept.

Detachment Assay. 100µl of a mixture of M63, 20% Arginine (20µl/ml), and MgSO4 (1µl/ml), were added to each well in two 96 well plates. Then a frogger (multi-prong device) was used to transfer the reconstructed transductants into the 96 well plates, and the first plate was allowed to incubate at 37°C for 24 hours, the second plate for 48 hours. After this incubation period, a biofilm assay was performed.

Biofilm Assay. The 96 well plate was vigorously shaken to remove cells. The plate was then rinsed in clean distilled water and 125µl crystal violet were added to each well. Ten minutes later, the crystal violet was vigorously shaken off, and the plate was rinsed twice. If purple rings were present

around the well, then the cells formed biofilms. (O'Toole & Kolter, 1998b)

Isolation of Phage DNA. I started with 0.5ml of phage (1011 PFU/ml). SDS was added to 0.5% and proteinase K was added to 0.05mg/ml. Eppendorf tubes were heated for 15-30 minutes in a 65°C water bath. A 1:1 phenol:chloroform solution extraction was performed 4 times, and the extraction was allowed to proceed for approximately 5 minutes after adding the phenol/chloroform. The mixtures were centrifuged at high speed and the aqueous phase was kept. Equal volumes of the phenol/chloroform solution and phage were used in each extraction. Next, NaoAc was added to 0.3M and 3 volumes of 100% cold ethanol was added. The mixture was then allowed to incubate at -20°C for 30 minutes. Then the mixture was centrifuged and the supernatant was discarded. Three volumes of 70% ethanol were added to the phage DNA, the mixture was centrifuged, and the supernatant was discarded. This 70% ethanol wash was performed 3 times and then the DNA was dried in a speedvac for 10 minutes. The DNA was resuspended in distilled water.

Restriction Digest. 6.5µl of distilled water, 2µl of phage DNA, 1µl of restriction enzyme buffer, and 0.5µl restriction enzymes were combined and allowed to incubate usually for 3 hours. Controls of uncut phage DNA and a λBstEII marker were used on each gel.

Gel Electrophoresis. 2µl of agarose loading dye was added to the restriction digests. 1% agarose gels were used for the gel electrophoresis.

Pilot Experiment. Cultures of 821 (TcR) and *P. aeruginosa* were grown in LB overnight. Various dilutions of page #3 grown on *P. aeruginosa* were added to 100µl 82l with 1 µl CaCl2 (1mM final concentration). After waiting 15 minutes, the solution was added to 3.5mL of LBTop Agar and poured onto LB plates. 24 hours later, 96 visible plaques were picked using a pipet and each placed into a 96 well dish with the wells already containing 100µl of M63. The next day, 100µl of 372 (Gentamycin resistant [GmR]) were added to 1/2 of the wells in the 96 well dish and 15 minutes were allowed to pass for the transduction to occur. The other 1/2 of the wells were left as controls, containing only phage grown on 821. Half of a second 96 well dish contained 372 only as a control. Using a frogger, the contents of the 96 well plates were transferred onto TcGm plates.

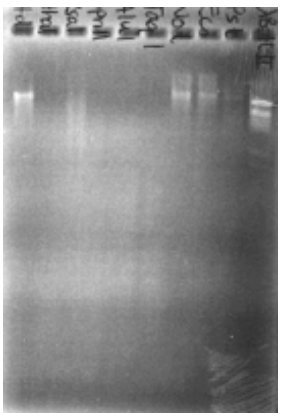
RESULTS:

The four goals for this project were to: 1) perform electron microscopy on the phage to discover its morphology and estimate phage #3's size, 2) use restriction digests to estimate the length of the phage DNA, 3) use the phage to reconstruct mutant strains, and 4) to isolate mutant phages that are able to mediate transduction at a higher efficiency.

Electron Microscopy. Phage #3 appears to be an icosahedral lambdoid phage with a tail approximately 110nm in length with a head approximately 50nm in diameter. Figures 3 and 4 show phage #3 grown on *P. aeruginosa*. These pictures were taken with Electron Microscope JEM 100CX at the Rippel Electron Microscopy Facility, Dartmouth College.

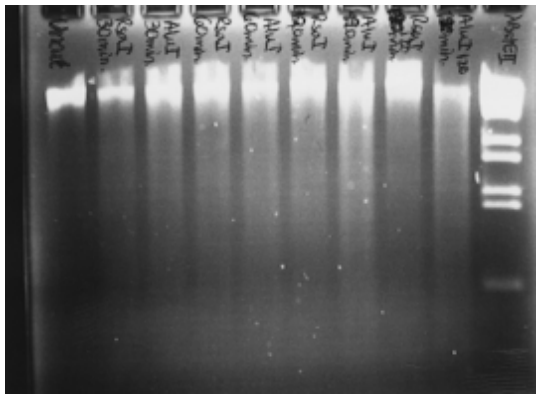
Analysis of Phage Nucleic Acid. The goal of phage DNA digestions was to determine the size of the phage DNA. I had hoped that using various restriction enzymes would produce distinct bands that would be helpful in calculating the phage DNA length. According to gel electrophoresis the phage has DNA of length between 10 kilobase pairs and 15 kilobase pairs. The phage DNA is double-stranded and not single stranded, as shown by digestion of phage DNA with different restriction enzymes. The following restriction enzymes recognize 6 base pair sequences except for the restriction enzyme TaqI, which recognizes 4 base pair sites: BamHI, NotI, PstI, EcoRI, TaqI and HindIII. Initially, all of these enzymes except TaqI did not appear to cut the phage DNA, since distinct bands were not visible in the gel. No bands were visible in the lane where TaqI was used in the digestion, indicating that TaqI completely digested the DNA. Lane #6 on Gel #11, Figure 5, shows that TaqI completely digested the phage DNA.

TaqI has a 4 base pair recognition sequence and can digest single-stranded DNA. Since TaqI digested the phage DNA, either the DNA is single-stranded or the phage DNA is in some way protected from digestion by BamHI, NotI, PstI, EcoRI, and HindIII. In order to find out if the DNA is single stranded or not, RsaI and HhaI were tested. These restriction enzymes recognize 4 base pair restriction sites and can cut single-stranded DNA. I also tested AluI and DpnII, which recognize 4 base pair restriction sites, but do not cut single-stranded



Legend for Figure 5, Gel #11:

Lane	Contents
1	Uncut DNA
2	HhaI
3	RsaI
4	DpnII
5	AluI
6	TaqI
7	Not
8	Eco
9	Pst
10	λBstEII



Legend for Figure 6, Gel #13:

Lane	Contents
1	Uncut DNA
2	RsaI after 30 min.
3	AluI after 30 min.
4	RsaI after 60 min.
5	AluI after 60 min.
6	RsaI after 90 min.
7	AluI after 90 min.
8	RsaI after 120 min.
9	AluI after 120 min.
10	λBstEII

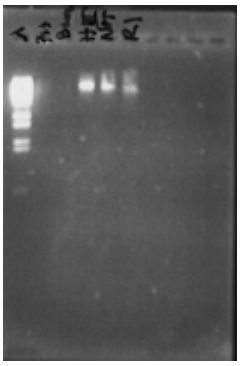


Figure 7: Gel #14:

Legend for Figure 7

Lane	Contents
1	lBstEII
2	PstI
3	BamH
4	HindIII
5	NotI
6	EcoRI

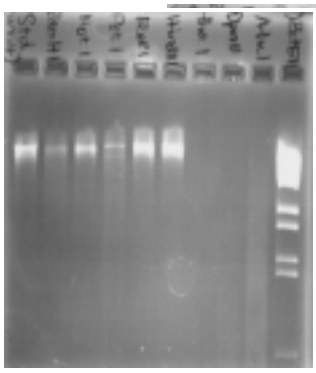


Figure 8: Gel #12:

Legend for Figure 8

Lane	Contents
1	Uncut DNA
2	BamHI
3	NotI
4	PstI
5	EcoRI
6	HindIII
7	HhaI
8	DpnII
9	AluI
10	λBstEII

DNA. All four of these restriction enzymes cut the phage DNA and showed no clear bands. Thus, the phage DNA should be double-stranded.

I also tried 30 minute, 60 minute, 90 minute, and 120 minute digests with RsaI and AluI (see Figure 6, Gel #13). Since these both digested the DNA, I wanted to see if a partial digest might yield bands and eliminate the possibility that I was digesting the DNA for too long a time period. These digests did not yield distinct bands either.

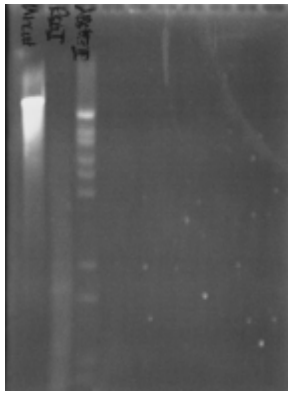
In order to see if the DNA I was using was not clean enough and therefore uncuttable, I performed the phage DNA isolation twice. After still seeing no bands and having run 10 different gels, we decided to ask for advice from outside sources. New England BioLabs support said that many bacteriophages have protected DNA and are not able to be cut by many restriction enzymes. Ward et al. (1993) asserts that the DNA of many bacteriophages contain modified DNA that may cause the restriction enzymes to be unable to digest the phage DNA.

More recent digests have shown that BamHI and PstI may be cutting because the bands from these digests are not as bright and thick as the uncut DNA and the other digests involving 6 base pair restriction enzymes (see Figure 7, Gel #14). Lanes #2 and #3 show PstI and BamHI, respectively, cutting the phage DNA. Gel #12, Figure 8, illustrates how the 4 base pair cutters AluI, DpnII, and HhaI completely digested the phage DNA. However, even though the DNA was cut, no distinct bands were visible and we are not sure why.

I also want to try running the gel for a longer period of time and at 50mV instead of 100mV. The first time I tried this approach, the λBstEII bands were much more distinct and separate rather than melded together as one bright thick band (Figure 9, Gel #15). Thus, I may need to run the gels for a longer time frame to allow bands, to separate that might otherwise be very close to one another and indistinguishable. This hypothesis is currently being thoroughly tested.

Because the uncut phage DNA travels even a shorter distance along the gel than the heaviest size marker (8,454 bases) on the λBstEII, I estimate the size of the phage DNA to be in the range of 10-15 kilo-base pairs (Figure 9, Gel #15, compare uncut DNA in lane #1 with λBstEII DNA in lane #3).

Reconstructing Mutants. I used phage #3 to reconstruct the mutants 814, 821, 824, and 829. Phage #3 successfully mediated transduction producing numerous transductants. Efforts to conduct detachment assays on the reconstructed transductants were unsuccessful for reasons we have yet to determine in the lab. For some reason, the *P. aeruginosa* wild-type bacteria are not detaching after 48 hours, as they normally should. We suspect a problem with M63 media might be causing these odd results.



Legend for Figure 9, Gel #15

Lane	Contents
1	Uncut DNA
2	RsaI
3	λBstEII

Pilot Study. In terms of finding mutant phages that have a higher rate of transduction, we have developed a pilot project which we are currently testing on a small scale to determine its feasibility. The pilot project showed promising results for the first two trials. No colonies grew on either control or on the plates containing potential transductants. Seeing no colonies on the potential transductant plates was not surprising because the rate of transduction is extremely low. After performing these experiments many times in the fall, I hope to find a few colonies that grow on the TcGn plates. If colonies grow on the TcGn plates, then the phage successfully mediated transduction. Based on the number of colonies on the plate, we can backtrack and determine the number of plaque forming units in the well from which the phage was located, eventually calculating how efficient the phage mediates transduction.

Is Phage #3 a Temperate Phage? There is strong evidence to support that phage #3 is a temperate phage, meaning that it can exist as a prophage in infected cells rarely causing lysis. In other words, the phage can integrate itself into the genome of a bacterium without causing the death of the bacterial cell. Phages that integrate their DNA in the bacterial DNA are in the lysogenic cycle and can enter the lytic cycle at any time causing the lysis of the bacteria. When testing for phage immunity, those transductants that showed no cell lysis after passing through the phage were hosts to phages and consequently immune to further infection. Notice in the following phage immunity test (Figure10) how the line of bacterial cells in the first two rows becomes thinner towards the middle, where the phage is located. This alteration is lysis due to infection by the phage. Meanwhile, the bottom two rows appear unaltered and are immune to phages, so they are not lysed when a phage infects them.

The immunity of these transductants to phage infection indicates that the phage has the ability to integrate itself into bacterial DNA. In order to buttress this hypothesis, I will be performing a Southern blot on transductants that are immune to further phage infection and transductants that are not immune to further infection. By probing with phage DNA, we can see if the immune transductants have integrated phage DNA into their own endogenous genome.

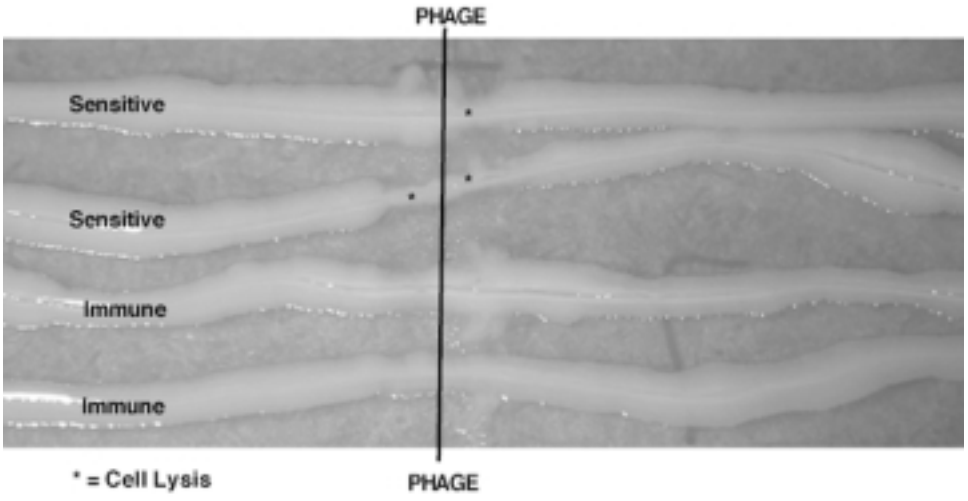


Figure 10. Phage Immunity Test

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