

Isolation of Potential MS2 Bacteriophage Strains

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The prevalence of antibiotic-resistant bacteria enhances the need for identification and characterization of specific bacteriophages so pathogenic bacteria can be targeted specifically, as well as efficiently. The MS2 bacteriophage attaches to the pilus of the F+ Escherichia coli, infects, lyses, and kills the cell. Morphology variation of plaques was observed in preliminary experiments. Two plaque-type strains of the MS2 bacteriophage were isolated. The potential strains were characterized by size, by diffuse edge or defined edge, and as bordered or unbordered. Measurements of plaque morphologies were taken and a curve comparison plotted, displaying the variation among plaques of the separate morphologies. Overlap of curves indicated size variation to be a possible characteristic of the MS2 bacteriophage. Isolation of these two strains initiates future work towards the application of MS2 to pathogenic E. coli bacterium.

History

In 1915 Edward Twort, and in 1917 Felix d'Herelle, reported independently isolating a filterable entity capable of destroying bacteria culture. The destruction of the bacterial cell was noticeable at the appearance of plaques, which are clear spots formed in bacterial colonies during the rupturing of the cells. D'Herelle went further to categorize these filterable entities as bacteriophages, meaning eaters of bacteria. He also characterized a bacteriophage as a virus that multiplies in bacteria (1).

Introduction

The increasing prevalence of antibiotic-resistant, disease-causing bacteria enhances the need for isolation, identification, and characterization

of specific bacteriophages of pathogenic bacteria. Antibiotic resistance of bacteria is predominantly due to the misuse of antibiotics, seen when physicians inappropriately prescribe antibiotics to patients when an antibiotic treatment is not needed. Failure of a patient to adequately use a prescribed antibiotic during the duration of his or her illness also contributes to the rise of antibiotic resistance. Both cases demonstrate ways that bacteria become less susceptible to the antibiotic treatment by allowing the bacteria to generate offspring in subsequent generations that develop a stronger resistance to the antibiotic. This resistance is due to the minute population of bacteria that survive the antibiotic treatment reproducing with mutations beneficial to the survival of the bacteria in the antibiotic conditions. Antibiotics also are not bacteria-specific and thus can result in disruption in the balance and pH of normal flora, which are bacteria that are naturally found in our bodies. This disruption can lead to symptoms such as diarrhea and yeast infections (6, 7).

Presently, concerns related to drug-resistant bacteria and the side effects of antibiotics have led to the need for identification of innovative techniques to treat bacteria-associated illnesses. Bacteriophage therapy is one of the techniques of special interest as an alternative for antibiotic treatment. Bacteriophage therapy, known also as phage therapy, involves the use of a specific bacteriophage which is target-specific to a bacterial cell. The application of the phage to a bacterial cell occurs in five steps: adsorption, penetration, replication, maturation, and lysis. During adsorption, the phage attaches to the cell in order to inject its RNA (genetic material) into the cell. Penetration involves the actual injecting of the RNA. During replication, the viral genetic material takes over the host and uses the bacterial cell's own machinery for replication. The phage then matures into its infectious state (maturation) and is released in a process known as lysis. Lysis occurs when the phage particle releases a lytic enzyme that causes the cell wall to loosen, leaving it weak enough for the breakthrough of the matured phage (15). The introduction of bacteriophage therapy was seen in the early 1930s; however, because of the convenience of antibiotics, this technique was soon overturned (10). The contribution of phage identification in the field of bacteriophage therapy today will be used in possible applications to aid in defense against pathogenic bacteria that cause diseases such as anthrax, rheumatic fever, meningitis, and salmonella.

Oral treatment of broilers with bacteriophages to reduce the concentration of *Salmonella enteritidis* in caecal contents was conducted at the Brazilian Agricultural Research Corporation by Fiorentin, Vieira, and Barioni (4). Bacteriophages isolated from free-range chickens were tested as a therapeutic agent in treating *S. enteritidis* by orally administering the phage to the one-day-old broilers and measuring the reduced concentra-

tion of phage in the caecal content. Five days after treatment the bacteriophage-treated group showed reduction of 3.5 orders of magnitude in comparison to the group of broilers that were left untreated. Samples collected 10, 15, 20, and 25 days after the original treatment showed steady reduction of the bacterial colonies in the caecal content.

Tanji and colleagues reported the therapeutic use of phage for controlling *Escherichia coli* 0157:H7, which is associated with hemorrhagic colitis (13). Their research was based on three phages that were isolated from waste water and animal feces. Named SP15, SP21, and SP22, all three phages showed strong virulence against *E. coli* 0157:H7. In order to determine the effects of the phage on *E. coli* infection, mice were orally infected and then treated with the mixture of phage types SP15, SP21, and SP22. The phage concentration in the feces was monitored for 9 days. As the dose of phage increased, there was a decrease in the content of *E. coli* bacteria found in the feces.

In the study reported here, the MS2 bacteriophage was used to infect the nonpathogenic bacterial strain, *E. coli* 15597 in order to form plaques. Preliminary research conducted in Dr. Alvin L. Winters' laboratory at The University of Alabama indicated that the MS2 bacteriophage caused lysis with variation in plaque morphology (Fig. 1). Plaque types ranged in size, with small plaques categorized as being under 2mm, medium plaques being ~2mm, and large plaques being larger than 2mm. Variation also occurred in the plaque borders, with there being diffuse and defined edges. The diffuse edges were characterized as not having a distinct circular border as seen with the defined edge plaques. These observations suggested that there were possible isolate strains of the MS2 bacteriophage to be identified. Once isolated, these strains were to be characterized and applied to a particular *E. coli* strain, as seen in the case of the SP bacteriophage noted in the study conducted at the Tokyo Institute of Technology by Yasunori Tanji and colleagues.

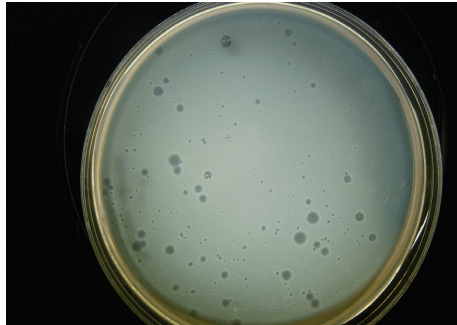


Figure 1
Various plaque morphologies obtained from preliminary research conducted in Winters' laboratory.

Material and Methods

Escherichia coli

E. coli bacterium isolate strain 15597, also known as C3000, was used during experimentation. This is a nonpathogenic bacterium isolated from fecal matter and found in the gastrointestinal tract of mammals. The bacterium is grown well on ATCC medium. ATCC medium is nutrient broth, in gelatin state termed agar, that provides all the essentials necessary for maximum bacterial growth. This medium consists of 10 g/L of tryptone, 1 g/L of yeast extract, 8 g/L NaCl, 10mL of 10% glucose, 2 mL of 1 M CaCl₂, 1 mL of thiamine, 15 g of agar (if needed) and 1 liter of distilled water. The *E. coli* bacterium is cultivated at a temperature of 37°C either in a shaking water bath for broth (liquid medium) or in an incubator for growth on an agar plate. Excess *E. coli* was kept frozen at -80°C until use.

Bacteriophage

MS2 bacteriophage is a small RNA virus, approximately 26 nm in diameter. This bacteriophage is part of the Leviviridae family and is not harmful to humans, plants, or animals (2). The *E. coli* bacterial cell is its only host. The MS2 bacteriophage was the first bacteriophage to be completely sequenced; it consists of only 3,569 bases (12). Its RNA encodes for only four proteins: the A maturation protein, replicase protein, coat protein, and the lysis protein. It is used in experimentation as a surrogate for pathogenic viruses, and because of its simple structure, the MS2 bacteriophage is used in molecular biology to determine protein-protein interaction and RNA-protein interaction (1, 11, 12, 14). Obtained from the America Type Culture Collection, this bacteriophage is icosahedral in structure, that is, a twenty-sided structure. The MS2 virus infects this *E. coli* strain by attaching to the sex pilus (used in bacterial conjugation) and injecting its RNA contents into the cell. The infection results in ten thousands of matured infectious viral particles which then diffuse out and repeat the infection process. This area of infection results in a plaque (a clear spot in the bacterial lawn overlay). These plaques are of various morphologies, varying in size and border shape.

Procedures

Previous studies in the Winters' laboratory provided the various plaque morphologies, which were present on one cultivation. To isolate each plaque type a process of plaque purification was implemented through

plaque assays, which are the plating of the virus for growth, and dilutions. To obtain the bacteriophage particles from the plaque, a wire (inoculating loop) was used. The inoculating loop was touched down into the center of the plaque (picking), gathering an ample amount of phage particles. Suspension of the particles was onset by placing the inoculating loop in 1 mL of broth. Ten to the minus two (10^{-2}) dilutions for each morphology were completed in series until a countable plate (that is, a plaque count of 300 or less) was reached. Dilutions were done by pipeting $10\mu\text{L}$ out of the broth containing the bacteriophage and dispensing it into $990\mu\text{L}$ of broth creating the first 10^{-2} dilution. Bacteriophage concentration was diluted sufficiently down to the 10^{-8} dilution in order to obtain a countable plate.

Cultivation of *E. coli* bacteria was performed by first streaking the bacteria across an ATCC medium nutrient agar (LB Amp) plate and incubating for colony growth. A bacterial colony was picked in a method analogous to plaque picking and placed in a flask with 5mL of broth. The bacteria-broth mixture was allowed to shake overnight in a 37°C shaking water bath for a period of 6-8 hours for optimum growth.

Once the bacteria had reached bacterial growth peak, one micro liter (μL) of the broth-bacteria mixture was placed inside a tube containing 3mL of soft agar (50% nutrient agar and 50% nutrient broth) along with $100\mu\text{L}$ of the dilution, mixed gently, and poured over the agar plate. The plate was incubated in the 37°C incubator for 8-12 hours. This plaque purification process was repeated until results of identical morphology throughout the plate were seen. To confirm isolation of the plaque, three subsequent plaque assays were consecutively repeated to verify they would give the same morphology at each trial.

Plaque size photography was taken of each plaque isolate. The digital image allowed for calculation of plaque size. By placement of a specific-sized steel ball, with diameter of exactly 2mm, next to the plaque being measured, accurate measurement was extrapolated relative to the known steel ball diameter (Fig. 2). Recordings of each isolated plaque size and morphology were noted.

Results

Two isolate strains were obtained. A strain causing a clear defined border plaque in comparison to another strain causing a haloed diffuse bordered plaque were independently isolated (Fig. 2). Observation of the plaque morphology indicated this significant difference although the plaque purification process was meant to isolate morphological strains by size. Standardization of the soft agar medium, used for the bacterial lawn in the plaque assays, became a variable which interfered with the isolation

of morphologies of plaques by size. Equal amounts of measured ingredients were used for each batch of soft agar made, but unnoticeable human error contributed to the slight variation that significantly affected the gel strength of each subsequent batch.

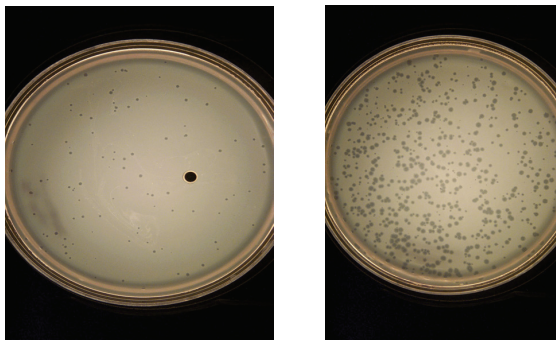


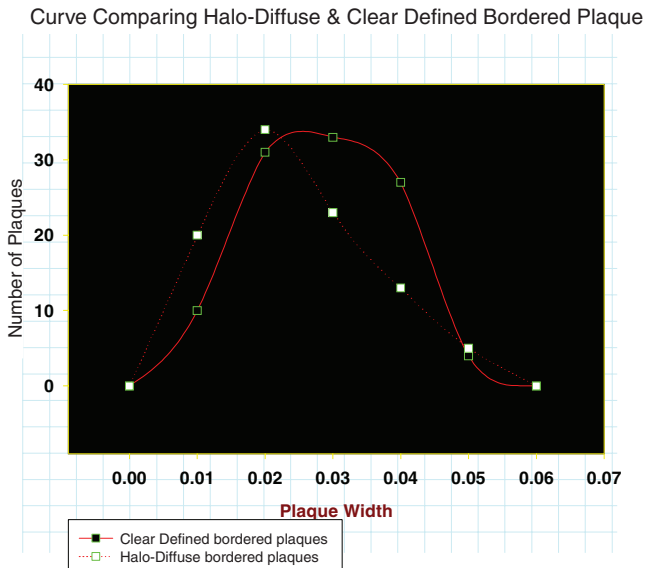
Figure 2
Digital images of both isolate strain plaques, the clear defined plaques (left) and halo border plaques (right). The image on the left also shows the steel ball used for measuring plaque size.

Initially, the method used to determine isolation was the use of the comparison of plaque sizes (small, medium, and large) with the 2mm scale. This scale was later disregarded, however, because of the wide range of sizes observed for each plate with the majority of plaques close to being an isolate strain. The determination of plaque morphology isolation thus became visual comparison between plates. The first isolate noted was the tiny plaque, and tiny plaques did tend to fall along the range of approximately 1mm or less. Medium plaque morphology was the next strain for attempted isolation; however, there was a constant overlap of plaque sizes between the denoted medium plaque and the isolated tiny plaque for each plaque assay. Larger plaques, with width of 2+mm, were noticed. These plaques were purified for isolation of their strain.

Due to the varying gel strength in the soft agar medium, the isolates initially identified as tiny plaque isolates were re-plated using the same soft agar batch that was currently being used for the isolation of the large plaque, to ensure consistency of pore size. The burst size (width of plaque) of the tiny plaques increased to approximately the same size as the large plaques observed for the plaque purification processes. Disregarding the increase in burst size of the tiny plaque, it was noted that the morphology of all plaques seen on the plate was clear and had defined circular borders. This trait was observed from the tiny plaque plates prior to the re-plating of the plaques for medium consistency. Through a series of plaque

purification processes for the large plaques, the haloed border became a consistent appearance for each plaque despite the burst size. To ensure strain isolation for this haloed plaque, three consecutive plaque purification processes were repeated and each assay resulted with the same haloed bordered plaque.

Further characterization of both isolate strains, the clear defined and the halo bordered plaques, were recorded by measurement data of plaque widths. One hundred plaques for each morphology were measured, recorded, and plotted onto a graph to create a curve comparison between the two strains. The expected Gaussian curve was not obtained, but there was an overlap in plaque width between the two plaque types, as shown below.



Discussion

The extensive use of the MS2 bacteriophage as a surrogate virus in many studies as well as its being a system for the study of molecular biological function create a need for understanding the characteristics of the MS2 bacteriophage, itself. This study was specifically designed to isolate plaque morphology-varying strains of the MS2 bacteriophage. Because plaque morphology is specific to a virus-host interaction complex, observation of varying plaque morphologies resulting from the pure strain of MS2 infecting the C3000 strain *Escherichia coli* indicated numerous strains of the MS2 bacteriophage. Due to the lack of information reported

in the literature identifying any strains of the MS2 bacteriophage, experimentation to isolate these strains was implemented.

The methodology of the isolation of each strain was simple. It consisted of numerous series of dilutions and plaque assays. However, variables such as the diffusion rate of the viral particles, the culturing of the bacteria, and the gel strength of the soft agar medium prolonged the isolation process.

MS2 is one of the smallest bacteriophages, being approximately 26 nm in diameter. According to early experimental studies, the diffusion rate of smaller particles is quite rapid in comparison to the diffusion rate of larger particles through various media. After the required 8-12 hour incubation period, the tens of thousands of new infectious virus particles continue to diffuse past the area of infection. These new particles diffuse into areas where there are other concentrations of virus particles with strains that encode for another specific morphological plaque. During the plaque purification process, when the plaque of a specific morphology is picked, numerous particles other than the expected morphology-causing particle are also picked. Therefore, even during the incubation period there is still possible contamination of the visible plaques with particles containing other phage strains (8).

The bacteria culture also affected the isolation process. Bacteria grow at an exponential rate with the optimum period of growth being 6-8 hours. After this 6-8 hour period, there is resulting cell death. This cell death affects the burst size of the plaques by reducing the number of viable bacterial cells that the bacteriophage can infect. At any particular time in the 6-8 hour period, there was a variation in the amount of viable cells present. Therefore, there was always slight variation in burst size among plaque plates. The variation of burst size caused by the bacteria culture made it difficult to isolate plaque morphology by size.

Gel strength, or pore size, of the soft agar used for the bacterial lawn in the plaque assays also affected the burst size of the plaques. The stronger the gel strength, the smaller the pore size, resulting in a smaller plaque. The lower the gel strength, the larger the pore size, resulting in a larger plaque. This indicates that the burst size of the plaque is not independent of the gel strength, so resulting plaque size could be due to the strain of virus or the gel strength. Large batches (1000mL) of soft agar were made at one time, and the entire batch would have one gel strength. When that particular batch was depleted, the subsequent batch would have different gel strength. Methods of careful measurement were carried out in an attempt to standardize each batch, but, seemingly, the slightest variation would significantly affect the gel strength. This made it impossible to isolate plaque morphology in regards to size (8).

Once the two strains, clear defined and halo bordered, were isolated, the data were analyzed by comparing the plaque width of each morphology. Initially the Gaussian curve was to be used to confirm isolation when isolation by size was under examination. If size isolation were possible, each isolated size morphology would have a plaque size range of its own with little or no overlap among the other strains. When a non-Gaussian curve was still implemented in the comparison of the two plaque types, an overlap was seen with these two plaque strains, which may indicate that the MS2 bacteriophage is not isolatable by size. This may be due to the fact that mutation rates for RNA viruses, such as MS2, are extremely high. They are approximately 300-fold higher than the normal DNA virus mutation rates (2, 3). This is close to the rate of one mutation per replication. With each of the ten thousand new infectious viral particles repeating the infection process, the mutation rates of RNA viruses will remain high. In these mutation cases with RNA viruses, in comparison to the DNA viruses, these mutations are most likely due to the fact that the RNA polymerase is not as specific as the DNA polymerase; alternatively, it could be possible that size variation is a common characteristic of the MS2 bacteriophage.

In future work, electron microscopy will be conducted in order to obtain a visual image to verify whether the bacteriophage causing the plaques are, indeed, the MS2 virus particles. After characteristics have been recorded, the experimentation of applying each phage strain to various *Escherichia coli* types and application in an array of media can then be put into effect. Application of bacteriophage specific strains on various *E. coli* strains will indicate the effectiveness of that particular strain to a particular bacterium. Experimentation with various media will demonstrate how plaque size may vary among nutrient agar and broth.

In conclusion, isolation of these two strains of the MS2 bacteriophage indicates that isolation of a frequent-mutating RNA virus is possible and that other strains can be potentially isolated. With the identification of these new strains, application to pathogenic bacteria, seen in similar experimentation, will soon be possible.

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