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Article

Comparing Virulence of Three T4 Bacteriophage Strains on Ampicillin-Resistant and Sensitive *E. coli* Bacteria

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SUMMARY

While bacteria have demonstrated antibiotic resistance since the advent of antibiotics, the threat that these resistant microbes pose has recently gained much more attention in scientific and public communities. Antibiotics work by disrupting functions and structures in bacteria that animal cells lack. Bacteria acquire resistance to these drugs through genetic mutations that allow them to sidestep the effects of the antibiotic. When this occurs, antibiotics may not be able to treat an illness caused by resistant bacteria. An alternative therapy is the use of bacteriophages, or viruses that infect bacteria.

In this experiment, we grew cultures of ampicillin-resistant and ampicillin-sensitive K12 strain *E. coli* and applied three strains of T4 bacteriophage, T4r+, T4r, and T4rIIA, to different cultures of the resistant and sensitive *E. coli*. After the bacteriophages had time to infect and lyse the bacteria, we determined the lysed percentage of each culture.

The results of this work showed that the wild type T4r+ caused the greatest amount of lysis of *E. coli*. There was not a significant difference in percentage lysed between cultures containing resistant and sensitive *E. coli* infected with the same strain of bacteriophage. These results suggest that the T4r+ bacteriophage may be the most effective in treating an *E. coli* infection, regardless of the ampicillin resistance of the *E. coli*.

INTRODUCTION

Over the last several years, bacterial resistance to antibiotics has risen as a critical threat to global health. In 2013, the Centers for Disease Control and Prevention (CDC) stated that the world had entered the "post-antibiotic era" and organizations such as the World Health Organization (WHO) have expressed concerns about the future of infectious disease treatment (1). In just the United States, 99,000 deaths are caused by hospital-acquired resistant infections annually, resulting in billions of dollars lost in the United States economy (2). Solutions to this growing problem of antibiotic resistance are an active area of research worldwide, and we explored the use of bacteriophages as an alternative germicide for antibiotic-resistant bacteria.

Antibiotics often work by targeting structures unique to bacteria that animal cells lack. Some of these features include bacterial membranes, bacterial DNA, and organelles used in bacterial protein synthesis, such as ribosomes (3). Each class of antibiotics targets a different component of bacterial cells by inhibiting or interfering with a function. For example, penicillin prevents bacteria from building cell walls. As a part of the beta-lactam class, penicillin works best on gram-positive bacteria, as it interferes with peptidoglycan production. In order to assemble their walls, bacteria link peptidoglycan molecules together with various proteins and lipids. The beta-lactam antibiotics interfere with this process. Without support from a cell wall, internal pressure causes the bacterial cell to lyse (3). In general, bacteria develop resistance to these antibiotics through genetic mutations that bypass the function of the drug. Mutations randomly occur in the DNA and can occasionally benefit the bacterial organism. If by chance the mutation helps the bacteria resist the effects of the antibiotic, then that bacteria will survive to pass on its genes, while sensitive bacteria will die (4). Genes can be transferred in several different ways, including binary fission and horizontal gene transfer. Binary fission is how a bacterial cell asexually reproduces to become two separate bacteria, thus the genetic information is passed from the parent cell to the two daughter cells (4). Horizontal gene transfer includes conjugation, transformation, and transduction. In conjugation, bacterial cells exchange nucleic acids through direct contact. Transformation is where a bacterial cell takes up extracellular DNA and incorporates this genetic material into its genome. In transduction, a bacteriophage will transfer genetic material from one bacterium to another. Through horizontal gene transfer, resistance can spread from one bacterium to a whole colony of bacteria (4). Therefore, when an ill patient takes antibiotics, if even one bacterium develops resistance and does not die, this resistance can spread and cause an entirely resistant infection. When this resistance occurs, alternative methods of treatment must be used.

One alternative method is the use of bacteriophages to kill the bacteria causing the infection. Phages infect bacteria through six general steps of the lytic cycle (5). The first step, adsorption, is how a virion uses specific receptors on the host membrane or wall to attach to the cell. After adsorption, the virion must penetrate the cell. T4 bacteriophages complete this process by first using tail fibers to attach to the bacterium. Then using enzymes on its tail, the virion will create a hole in the wall or membrane. The virion's tail sheath contracts, causing the DNA to leave the protein coat and enter the host cell. The DNA then travels to the nucleus and is used as a template by the cell to create viral mRNA. This mRNA travels to the host cell's ribosomes and is used to make viral proteins. Once viral protein subunits, also known as capsomeres, form in the cell, they spontaneously self-assemble into a complete protein coat. Viral DNA is also replicated by the cell, and it combines with the protein coat to create a new intact virus.

	R T4r	S T4r	R T4r+	S T4r+	R T4rIIA	S T4rIIA
Plate 1	34	28	45	40	22	26
Plate 2	38	36	51	60	34	27
Plate 3	30	31	49	40	7.5	49

Table 1: Percentage of Resistant (R) and Sensitive (S) Bacteria Lysed for Each Trial of Each T4 Strain. T4r+ phage appears to have the highest percent lysis.

Finally, lysosome-like viral enzymes break down the cell wall and the host cell lyses as the new viruses leave the cell (5). Although bacteria can become resistant to phages through genetic mutations, phages can mutate in response. This coevolution allows phages to remain generally effective (5). Previous studies have also indicated that as bacteria develop resistance to phage infections, they may become more susceptible to antibiotic treatment, as the bacterial host must proverbially trade-off between resistant genes for antibiotics or phages (6).

In this experiment, we infected ampicillin-resistant and ampicillin-sensitive K12 strain *Escherichia coli* bacteria samples with three different strains of T4 bacteriophages. Ampicillin, an antibiotic of the beta-lactam class similar to penicillin, is often used in clinical settings to fight bacterial infections, and resistance to common drugs of the beta-lactam class is increasing in prevalence (6). By exposing the K12 *E. coli* cultures to ampicillin, the *E. coli* cultures developed ampicillin resistance and the surviving bacteria grew into their own cultures. The three strains of virus used were T4r+ wild type, T4rIIA, and T4r bacteriophage. These strains of T4 bacteriophages reproduce by the lytic cycle when infecting the *E. coli*. The "r" in T4r denotes a mutation that leads to rapid lysis of the bacterial host (7). In T4rIIA, mutations have occurred in the rII genes, specifically the A:5 section of these genes, that modify the proteins responsible for interacting with the host cell membrane (7). Previous experiments have indicated that the T4rIIA strain has reduced ability to infect K12 *E. coli* bacteria due to its modified proteins (8). The other mutant strain, T4r, differs from the wild type T4r+ in its size. T4r is physically larger than its wild type counterpart, and therefore diffuses through a bacterial culture more slowly (8). This slower diffusion rate of T4r would indicate that the wild type T4r+ may be most effective at infecting K12 *E. coli*.

The hypothesis of this research project was that the wild type T4r+ would cause the greatest percent lysis of the K12 *E. coli* bacteria, as compared to the T4r and the T4rIIA mutant strains. Additionally, we hypothesized that the antibiotic resistant bacteria would be more sensitive to bacteriophage infection. The results of the research demonstrate that the wild type viral strain caused the greatest amount of bacterial lysis and that there was not a significant difference in lysis between resistant and sensitive bacterial plates when infected with the same bacteriophage strain. These results indicate that the T4r+ phage may be the best of the three strains to treat an *E. coli* infection, and that ampicillin resistance may not affect T4 virulence.

RESULTS

To investigate the virulence of the bacteriophage strains, we added each strain to six petri dishes, three of which contained ampicillin-resistant *E. coli* and three of which contained sensitive *E. coli*. All of the petri dishes had bacterial confluent lawns. We calculated the percentage of the bacterial confluent lawn that lysed to determine which strain had caused the most lysis. For each of the resistant and sensitive petri dish sets, we used one petri dish as a negative control for lysis despite not being inoculated with bacteriophage. The wild type T4r+ bacteriophage demonstrated the highest percentages of lysis for both resistant and sensitive bacteria (**Table 1**). On average, the T4r+ strain had a higher percentage of lysis than the other strains (**Table 2**).

DISCUSSION

Based on the data, the three different strains of T4 bacteriophage induced statistically significant different percentages of lysis in the host *E. coli* bacteria. We found that for both the resistant and sensitive bacteria, the T4r+ strain had the highest rate of lysis, while the T4rIIA strain had the lowest rate of lysis, with the T4r strain in between. This result supports the hypothesis that the T4r+ strain would cause the greatest amount of bacterial lysis. The results also showed that whether the bacteria were resistant or sensitive to ampicillin had no significant effect on phage susceptibility, disputing the hypothesis that the resistant bacteria would be more susceptible to phage infection. This result suggests that the mechanisms behind antibiotic resistance and phage resistance in *E. coli* bacteria are not the same.

The average lysis percentage for the wild-type T4r+ strain was 48%. This strain was the smallest in size of the three and did not have any mutations affecting its ability to interact with the cell wall of the bacteria (7, 8). Therefore, it was best suited to quickly spread, infect, and lyse the *E. coli* host cells. The T4rIIA strain of bacteriophage did have mutations that affected its adsorption capabilities (7). Since the bacteriophage was not as effective at attaching to the cell wall, the infection rate decreased. Thus, the lysis rate decreased as well, compared to the wild-type strain. The average lysis percentage for the T4rIIA strain was 28%. The T4r strain did not have these mutations, but was much larger than the T4r+ wild type (8). This larger size decreased the rate of diffusion for the bacteriophage through a bacterial culture. Therefore, the bacteriophage came into contact with less host bacteria, and did not infect as many cells as the wild type strain. The average lysis percentage for the T4r strain was 33%.

	T4r	T4r+	T4rIIA
R Average	34 ± 3.6[1]	48 ± 2.7[1]	21 ± 12[1]
S Average	32 ± 3.6[2]	47 ± 10.[2]	34 ± 12

Table 2: Average Percentage of Resistant (R) and Sensitive (S) Bacteria Lysed for each Strain of T4. T4r+ phage lyses *E. coli* most efficiently and is non-discriminate on ampicillin sensitivity.
[1] There was a statistically significant difference between the three resistant groups as determined by a one-way ANOVA ($F(2,15) = 20.632$, $p = 0.000049$ at $\alpha = 0.05$) and a post-hoc Tukey's Honest Significant Difference (HSD) test.
[2] There was a statistically significant difference between the T4r and T4r+ sensitive groups as determined by a one-way ANOVA ($F(2,15) = 4.600$, $p = 0.028$ at $\alpha = 0.05$) and a post-hoc Tukey's HSD test.

Although we took many steps to maintain constants and controls and ensure the accuracy of the data, there is always some level of error. We chose the drug ampicillin because it is a common choice to treat bacterial infections, and resistance can be common (6). However, the ampicillin-resistant bacteria in this experiment may not have truly been resistant to ampicillin. In all cultures of the resistant bacteria, a small percentage (less than 7%) of the bacteria did die, and we did not perform genetic analysis to check that the bacteria were in fact resistant. If this potential source of error occurred, assessing whether the reported lysis percentages for resistant bacteria were too high or too low would be difficult. Another source of possible error is if the cultures of bacteria were contaminated by something in the laboratory setting. The contamination may have killed some of the *E. coli*, leading the lysis percentages reported to be too high. There was a low chance of a contaminant in this experiment, as the negative controls showed no plaques, or clear-looking areas in the confluent lawn of bacteria where cells were lysed due to infection. A final potential source of error is if we did not properly dilute the bacteriophages of each strain. The original dilution of the T4rIIA strain was $1.0 \cdot 10^9$/mL, the T4r strain was $2.8 \cdot 10^9$/mL, and the T4r+ strain was $2.5 \cdot 10^9$/mL. In order to make these even dilutions, we added deionized water accordingly. If error was present in this process, the concentration of virus particles could have been different. If the liquids applied to the bacteria cultures had different concentrations of virus particles, the lysis percentages reported could have been anomalously low or high in some cases.

The results of this experiment indicated that the T4r+ strain was the most effective of the three tested strains in causing lysis of K12 *E. coli* bacteria. This indication could present pathways for further research into the most promising bacteriophages for phage therapy. To delve deeper into this topic, different classes of antibiotics could be used on the *E. coli* to examine their effect on the bacteriophage rate of lysis. Also, other strains of bacteriophage, rather than T4, could be used on various species of bacteria to determine their rate of lysis. Furthermore, research could be done into why the state of the bacteria being antibiotic resistant did not seem to affect susceptibility to phage infection.

MATERIALS AND METHODS

Petri Dish Preparation

To create each petri dish, 8g of LB nutrient agar powder (Bio-Rad) were first measured out on a spring scale and added to an Erlenmeyer flask with 200mL of deionized water in a manner that prevented clumps. The flask was covered with cling wrap with a small hole punched in the top and was placed in a microwave. The flask was heated in intervals to prevent the broth from bubbling over until the broth appeared clear. After the broth had cooled to about 60°C, a medium-sized petri dish was filled about half way and the broth was allowed to cool and congeal. Once solid, the plate was inverted and dried overnight.

Ampicillin-Resistant Bacteria Preparation

To create the ampicillin-resistant *E. coli* bacteria, 2 g of LB broth powder (BD Difco) were measured out on a spring scale and added to a flask with 100mL of deionized water in a manner that prevented clumps. The flask was covered with cling wrap with a hole and was heated in intervals in a microwave until the broth appeared clear. After the broth had cooled to 60°C, the broth was evenly poured into 4 separate test tubes, about 8 mL each. For each test tube, an inoculation loop was used to swab a small amount of *E. coli* from the original dish (Carolina Biological Supply Company) and was shaken inside the tube to loosen the bacteria. The caps were loosely put on and the test tubes were incubated for 24 hours at 37°C. Using a micropipette, the liquid from the test tubes was transferred to 16 microcentrifuge tubes and was spun at 1200 rpm for 3 minutes. Then, the bacterial pellet in each microcentrifuge tube was resuspended and all of the broth was combined. Using a micropipette, 500µL of broth was dispensed onto a petri dish and spread around via gentle shaking. Three ampicillin antibiotic disks (Carolina Biological Supply Company) were placed in the petri dish, equidistant from each other and the edges of the dish. Each disk had an ampicillin concentration of approximately 0.012 mg/mL, or about 10 mcg. After the liquid had solidified, the petri dish was inverted and incubated at 37°C for 24 hours. After the bacteria had grown, an inoculation loop was used to swab bacteria from the edge of the zone of inhibition. The same steps were repeated three times with the *E. coli* from the zone of inhibition to help ensure the bacteria were resistant to the antibiotic.

Exposing Bacteria to Bacteriophages

For the main part of the experiment, an ampicillin-resistant bacterial broth and an ampicillin-sensitive bacterial broth were created using the method for ampicillin-resistant bacterial broth preparation as listed above (using K12 *E. coli* from the original Carolina Biological Supply Company dish for the sensitive broth). Then, 10 petri dishes received 500µL of resistant broth and 10 petri dishes received 500µL of sensitive broth. The petri dish was shaken gently in circles on the lab bench in order to spread the broth evenly across the whole dish. Once dried and inverted, the petri dishes were incubated at 37°C for 24 hours.

To prepare the bacteriophages, the T4r, T4r+, and T4rIIA supply stocks (Carolina Biological Supply Company) were diluted with deionized water to create equal concentrations of $1.0 \cdot 10^9$/mL for each strain. One milliliter of each phage strain was dispensed on each of three resistant and three sensitive petri dishes. The last two petri dishes were left with no bacteriophage. After the plates had dried and been inverted, all of the plates were incubated at 37°C for 24 hours.

Data Recording

To record the data, a ruler was used to make a grid of 0.5 cm by 0.5 cm squares on the petri dishes. Using these squares, the area of the zones of inhibition was found for each petri dish and divided by the total area of the petri dish to determine a percentage.

Statistical Tests

To test the differences in percent lysis between viral strains, a one-way ANOVA Test was carried out at a confidence level of 0.95 by inputting the percent lysis for each plate within each strain. If the results were significant, a Tukey's Honest Significant Difference (HSD) Test was performed to determine where the significant differences were. The same process was carried out to compare differences between percent lysis of the same strain on resistant versus sensitive plates. To carry out these calculations, the Social Science Statistics website was used for the ANOVA Test (9), and the iCalcu website was used for the HSD Test (10).

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