Comparative Immunity Analysis in *Mycobacterium smegmatis* and Mycobacteriophage¹

Author: Amy E. Schade

Faculty Mentor: Lee E. Hughes, Department of Biological Sciences, College of Arts and Sciences

Department and College Affiliation: Department of Biological Sciences, College of Arts and Sciences

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Bio:

Amy Schade is a rising Biology senior from Grapevine, Texas. As a part of UNT's nationally competitive debate team, Amy was awarded the title National Debate Scholar Summa Cum Laude. Amy participated in Howard Hughes Medical Institute's National Genomics Research Initiative as a freshman and UNT-HHMI's Undergraduate Research Program as a sophomore. This summer, Amy participated in HHMI's Exceptional Research Opportunities Program where she was enrolled in the University of Wisconsin-Madison's Integrated Biological Sciences Summer Research Program and worked with Prof. Paul Ahlquist who is a leading scientist on virus-host interactions. Amy hopes to go to graduate school to earn a doctorate and wishes to become a full-time researcher.

Abstract:

The purpose of this research is to expand the body of information related to mycobacteriophages and the relationship between those viruses and the bacteria that are immune to them. In this project the researchers used *Mycobacterium smegmatis* as the host bacteria as it is from the same genus as the organisms that cause tuberculosis and leprosy but is innocuous to humans. This research used mycobacteriophages isolated from the National Genomics Research Initiative classes at UNT from 2009 and 2010 which are members of a variety of clusters and subclusters. The lysogenic pathway was the focus of the project because it involves the insertion of the mycobacteriophage's DNA onto the bacterial chromosome. To isolate bacteria that are immune to certain mycobacteriophages, the bacteria were infected by the phage, and mesas were allowed to form. A mesa is a film of growth that occurs on top of a zone of clearing established by mycobacteriophages. A small amount was picked from the mesa and then streaked for isolation. After growing this colony into a large culture batch, immunity assays were performed which involved infecting the lysogen containing the phage against the 53 phages isolated from UNT. Some interesting immunity patterns were observed. The lysogen of the mycobacteriophage Adephagia, named Adephagia L1, is immune to the phage Lew as well as nine other phages. Lew L1 is immune to Adephagia and those same nine phages as well as the phage, Tootziepop. While their lysogens are immune to each other's phage, they do not share the immunity to Tootziepop. This means there was some change at the DNA level during the start of the lysogenic life pathway. Future research will focus on why immunity is or is not transferred. Many of the lysogens isolated were immune to only one phage which means they are likely very unique. Information from this project can help aid in the decision on which phage should be

sequenced next, as well as provide a means to examine how the phage genome is transferred into the bacteria.

Introduction

Bacteriophages are viruses that infect bacteria and are highly genetically diverse (1). Mycobacteriophages are viruses that infect bacteria in the genus *Mycobacterium* which includes *M. tuberculosis* and *M. leprae*. In this study, we used *M. smegmatis* because it is easy to work with in the laboratory due its short replication time and because it is innocuous to humans. *M. tuberculosis* has a very slow life cycle (24 hour doubling time) and *M. leprae* has never been shown to grow in vitro (2). Due to these problems and the high pathogenicity of the organisms, little research has been done on the genomic information of these bacteria. More information about the bacteriophage population can provide insights about how to manipulate *M. tuberculosis* and open up the possibility of phage therapy as an alternative to conventional antibiotics.

An emerging theme noted by virologists is the largely mosaic nature of the viruses in that short segments of genomic DNA are readily exchanged by horizontal exchange and are readily found in many different clusters and subclusters of the virus (1). The mosaic nature of the viruses poses some problems to the analysis of their evolutionary history due to the nonphylogenetic relationships which have been supported by comparative genomic studies (2). The molecular events thought to be causes for illegitimate recombination is still unknown but research seems to point to random recombination that result in a large amount of nonfunctional virus but also some viable progeny (2).

The mosaic nature of these viruses can be analyzed through genomic sequencing, but similarity can be tested experimentally through the isolation of bacteria resistant to some viruses and test them against others. We are particularly interested in the establishment of lysogeny by temperate bacteriophages and how that establishment results in the immunity of that prophage to other viruses similar to it. The mycobacteriophages we utilized in this study are all dsDNA viruses with icosahedral heads.

Materials and Methods

The phage tested with are from the National Genomics Research Initiative (now named Phage Hunters Advancing Genomics and Evolutionary Science) course taught at UNT during Fall of 2010, Fall

of 2011, and Summer of 2011. In total, the testing included 53 novel mycobacteriophage that infect *Mycobacterium smegmatis* mc²155. The comparative immunity analysis required a two step process.

First, we isolated a strain of the bacterium that was immune to particular phages. Phages which can insert into bacterial genomes are called temperate phages, and the bacteria containing them are referred to as lysogens. The naming process of each sub strain of bacteria is dependent on the phage from which they were isolated.

Second, we tested each of the phage isolated at UNT against the lysogen. This was done by plating a dilution of the phage against a plate of the lysogen. If the phage could not produce plaques (evidence of cell lysis) on the bacterial lawn, it was determined that the lysogen is immune to it.

Isolation of Lysogens

The isolation of lysogens is achieved through creating a spread plate of the control strain of *M. smegmatis* into a bacterial lawn. Then, a spot test was performed in which a serial dilution to 10^{-4} was created and then placed onto the bacterial lawn in 5 μ g spots. The infection of the phage to this strain was certain because the isolation process to get the phage was done with this strain.

The infection process of lysogenic cycle is different than that of lytic cycle, and that is also apparent through the morphology of the plaques created. While lytic phages create very clear plaques, the turbidity of plaques from temperate phages varies. Additionally, many times they will create what is called a “mesa.” A mesa is very cloudy center spot surrounded by a clear area within a plaque. This overgrowth is indicative of the majority of the bacteria being lysed, and then some bacteria was selected for and able to grow. Because of this, samples were taken of the mesa and streaked to obtain isolated colonies of bacteria. The bacteria in the mesas are assumed to be immune to the phage since they are able to grow in the presence of it.

Because of the nature of the plaques needed, many phages that have been isolated were not good candidates for isolating lysogens. If the phage does not have the capability to use the lysogenic pathway and only the lytic, then it is not possible to obtain bacteria immune to it this way. Figure 1 shows an example of these spread plates with plaques A and C not having mesas, but plaque B having a great mesa.

Testing and Verifying Lysogens

After isolating a putative lysogen, it was verified to be a lysogen. This test is quite simple because it requires only creating a larger culture stock of the putative lysogen, and then infecting it with the phage on which the mesa was created. If the putative lysogen is infected by that phage, then it fails. Many putative lysogens failed. This may be due to the mesa including some of the normal strain of *M. smegmatis* or that the phage switches from the lysogenic to lytic life cycles quickly and for unknown reasons.

After the lysogen has been verified, it is tested against all of the other phage that have been isolated at UNT. Fifty-three phages are tested against each lysogen. A numbering system was created to facilitate the process. Figure 2 shows the testing of a lysogen of Tootziepop versus five different viruses.

The testing process is simple in that a serial dilution is created of the phage, and then plated on a bacterial lawn of the lysogen in increasing dilutions to 10^{-4} . This is done because sometimes false positive results occur at the 10^0 position. Even if the lysogen is immune to the phage, the sheer amount of viral particles in the area can cause cell lysis. To determine that the phage can infect the bacteria, the entire dilution was tested. If the phage can infect the bacteria, the lysogen is not immune to that phage.

Results and Discussion

I analyzed this immunity across the lysogenic phage present at UNT and looked for patterns. Many times, immunity is not transferred between the lysogens of similar phage. Many lysogens were isolated. Figure 3 contains a compiled list of the information we have gathered of the lysogens and the phage to which they are immune. We took particular notice of the interaction of immunity between the four phage—Adephagia, Lew, Tootziepop, and WootieJr.

In the case of Dale, Jaurez, and Moses, their lysogens were only immune to the phage spot test from which they were isolated. This means they are quite unique and further experimentation is needed. Sequencing of their genomes will be helpful in placing them into a cluster of other like phage.

Figure 3 summarizes the immunity of the four lysogens isolated from the phage Adephagia, Lew, Tootziepop, and WootieJr. It was originally thought that if the lysogens were immune to certain phage, it

was because they had strong homology to each other and thus were not able to infect the prophage. If Lew's lysogen was immune to the Tootziepop virus, then Tootziepop's lysogen should be immune to Lew; however, that was not the case. Tootziepop's lysogen is infected by Lew.

Along that same line, although Adephagia L1 and Lew L1 are immune to both the Adephagia virus and the Lew virus as shown in Figure 4, they are not also immune to the same other viruses. Specifically, Lew is immune to both Mojo and Tootziepop while Adephagia is infected by both. Interestingly, all four lysogens are immune to Bruce1.

Conclusion

Further research must be done to understand the way in which immunity is transferred. Genomic experimentation can provide insight as to how the phage chromosome is incorporated into the bacterial chromosome and how that influences future infection by another similar or distinct phage.

References

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Figure 1: Spot Test to Isolate Lysogens

A: No mesa. B: Good mesa. C: No mesa.

Figure 2: Plating of Phages against Tootziepop L1

Figure 3. Phages to which Lysogen Is Immune

Lysogen	Phage they are immune to
Adephagia L1	Adephagia, Biscuits, Bruce1, Chempanal, Cornelius, Einahpets, James KC, Lew, Moxley, Trash
Dale L2	Dale
Jaurez L1	Jaurez
Legion L1	Q88, TeenyTiny, Legion
Lew L1	Adephagia, Biscuits, Bruce1, Chempanal, Cornelius, Einahpets, JamesKC, Lew, Mojo, Moxley, Tootziepop, Trash
Moses L5	Moses
Tootziepop L1	AAA, Biscuits, Bruce1, Cehmpanal, Cornelius, Dam777, Locksmitj, Mojo, Timshel, Tootziepop, WootieJr
WootieJr L1	Bruce1, Dam777, Timshel, WootieJr

Figure 4: Plating of Phage 9 (Moses), 22 (Adephagia), and 35 (Lew) against Adephagia L1

Note the immunity to Lew and Adephagia phage.