Exploring the Limits of F1 Phage Host Range

A Major Qualifying Project

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Abstract

Bacteriophages, viruses that exclusively infect bacteria, exhibit a startling range of traits despite their simple structure. We explored a subset of F1 bacteriophages (Tootsiepop, Piper2020, Seagreen) with a unique region in the left arm tail portion of the genome that we believed to confer expanded host range, as well as a right arm section that contains the immunity cassette and influences lysogenic activity. We did not observe expanded host range in our phages, but the phages did exhibit unique growth patterns that point to novel lysogenic activity.

Acknowledgements

Our research would not have been possible without the University of Pittsburg SEA-PHAGES culture archives for providing Seagreen and Piper2020. As well as the assistance of the Scarlet Shell lab (WPI) for providing *Mycobacterium smegmatis* MKD8. Finally we would like to thank Mike Buckholt for providing materials and support every step of the way and our advisor, JoAnn Whitefleet-Smith for her continued support, knowledge and guidance.

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Chapter 1: Introduction/Background

Introduction

Bacteriophages (Phages) are a type of virus that exclusively infect bacteria. Generally, each phage is extremely specialized for its host species, some only capable of infecting particular strains of a given bacterial species. Environments with a wider selection of host bacteria tend to produce phages with a broadened range of potential hosts, which are a unique find among the very selective viruses (Bielke et al., 2007). This paper explores a subcluster of F1 phages, all with a highly conserved unique region in the right-hand portion of the genome, likely including unique genes related to immunity, integration cassettes, and phage minor tail proteins. We hypothesized that this right-hand region, present in the phages Tootsiepop, Piper2020, and Seagreen, increases the host range of these phages.

Exploring the host range of a phage required cultivating a pure, high-quality lysate of phage and plating it on a bed of non-host bacteria. We ran into several challenges with contamination to our lysate, eventually forcing us to remove a phage, Cornucopia, from our methodology. Determining lysogeny involves several rounds of cultivation and purification, ending with a bacterial culture containing the integrated phage DNA, which can then be induced to return to a lytic cycle. The greatest challenge to this experiment was incidental contamination by non-host bacteria such as *Microbacterium foliorum*, which was startlingly persistent even in hostile environments. Unfortunately, due to lack of time we were unable to purify a lysogen sample.

Our time was split between lab work, research, and exploration of the genes necessary to produce unique host range and lysogeny, with the majority of time spent in the lab.

Background

There is currently extensive research being conducted on phages due to their extreme prevalence in nature and relative ease of cultivation. Despite their simplistic structure, phages are wildly divergent in genome complexity, resulting from high amounts of horizontal gene transfer between phages with shared bacterial hosts. Phage genetics is an exciting new frontier in genetic exploration, with each unidentified gene region having the potential to be unique to a subcluster or even a specific phage (Hatfull, 2008).

Our work was an outgrowth of the Seaphages soil exploration program, which discovered all six of the phages we used in our work. Three phages are part of an F1 subcluster, a subset of *Mycobacterium Smegmatis* phages believed to exhibit lysogenic characteristics and expanded host range due to the unique right-hand genome region that characterizes the subcluster.

One of our starting points for each of the phages we are working with was PhagesDB.org, a database of information about the discovery and characterization of assorted phages. We also used Phamerator.org, as shown in Figure 1, a bioinformatics tool capable of comparing phage genomes in a visual format. The similarities shown visually in Figure 1 are then represented in

Table 1 with exact percentages of similarity. We also used this source to look into the protein tails to support our research in host range and lysogeny.

1.1 F1 Subcluster

The subcluster our three experimental phages belong to shares a uniform left arm containing multiple minor tail proteins, as well as a unique and divergent right arm containing the immunity cassette. Similarities between the genomes of the three experimental phages can be found in Figure 1. The discovery locations and percent genomic similarity between the phage genomes can be found in Table 1.

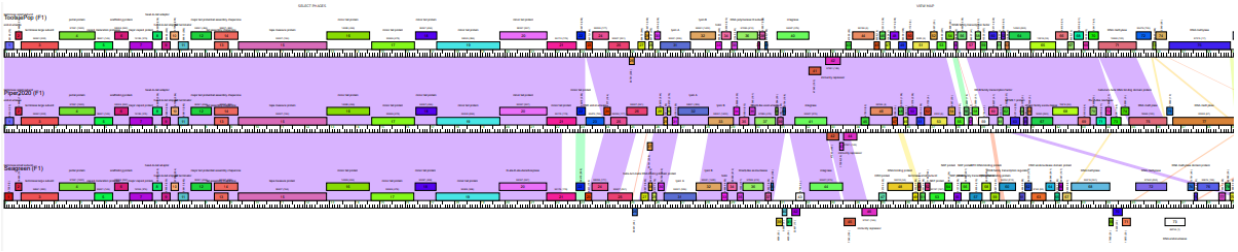

Figure 1: A Phamerator.org overview of gross genetic similarity across the F1 subcluster phages Purple indicates similarities between sequences, white indicates divergences. Note how the left-hand portion of the genome is identical between all three phages, whereas the right-hand portion begins to diverge (Cresawn et al., 2011).

Phage	Location found	% similarity to Tootsiepop
Tootsiepop	Charlton, Massachusetts, in a compost pile.	-
Piper2020	Melrose, Massachusetts, in the soil of a tomato plant.	96%
Seagreen	Durban, South Africa, in a flower bed.	91%

Table 1: Basic overview of F1 phage information Percent similarity sourced from PhagesDB.org using the Phamerator.org application. Location information from PhagesDB.org.

1.2 Non-Subcluster Phages

Three non-F1 phages were used as controls and comparisons for the subcluster phages. The first, CLAWZ, is a singleton *Gordonia rubripertincta* phage isolated in Worcester, Massachusetts. The second is LimaBean, a *M. foliorum* phage discovered in Worcester, Massachusetts, by a member of this research team. It exhibits robust growth and was one of the more robust phages we worked with. The last, Avle17, is a fairly standard A4 temperate *M. smegmatis* mc^2155 phage, isolated in Charlton, Massachusetts from a flowerbed.

1.3 Host Range

Phage host range is typically limited to one species or even one strain of bacteria that the phage grows well on. A phage that is growing well produces clear spots of lysis that can be picked and further researched. However, phages occasionally demonstrate the ability to infect multiple bacterial species within a family due to mutations in the tail genes that control phage interactions with the cell wall. Evidence of these host range-increasing mutations already exist in *Mycobacterium* phages, which have demonstrated host range expansion from non-pathogenic *M. smegmatis* strains to *M. tuberculosis* (Jacobs-Sera et al., 2012). As the F1 subclusters we are investigating are *Mycobacterium* phages, this demonstration of expanded host range is promising.

M. foliorum is the most universal host used in phage discovery which would make sense since it is isolated from different types of grasses. When studied for viability as a host, there were no traces of prophage or restriction-modification system which makes it a good candidate for being a host (Russell et al., 2019). In contrast, *G. rubripertincta* is discovered in seaweed (Arenskötter et al., 2004). *G. rubripertincta*. is ideal for detecting metals, such as iron, in samples (Schwabe et al., 2020). This host, as well as *M. smegmatis*, are known as temperate hosts. This seems to be where the similarities end when it comes to *G. rubripertincta* and *M. smegmatis*. The latter host is found in soil and water and its mutated strand named mc^2155, is altered to have no cell-clumping properties (Etienne et al., 2005).

In order to best analyze the results of how the phage attacks the host, it is important to understand the characteristics of each host. As shown in Figure 2, there are three images that represent the difference in relationships between similar clusters and subclusters. The distance between each cluster represents the strength in distinction between them. The closer the clusters are to one another, the more similarities they have. Phage that have closer clusters are most likely to be grown on the same host than those that are spaced further apart.

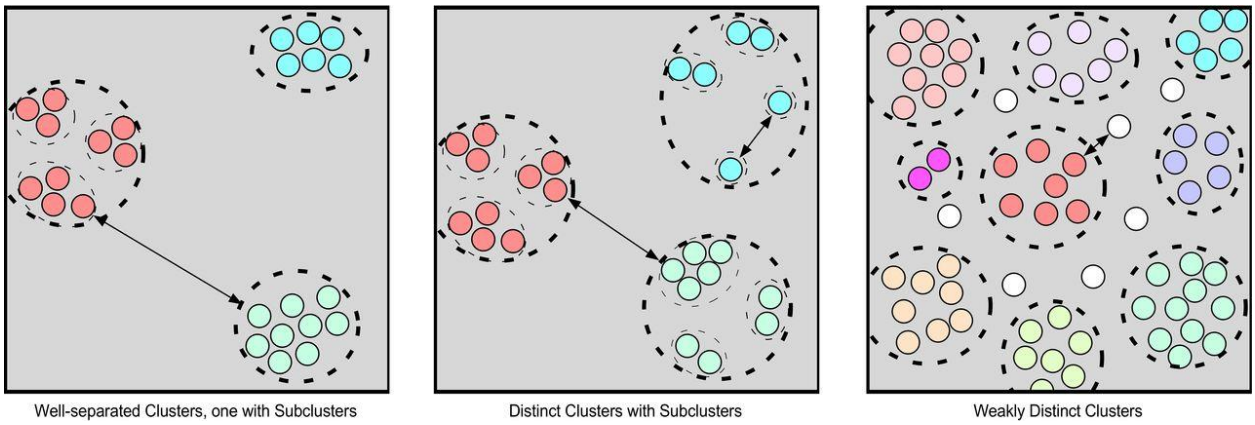

Figure 2: Relationship between clusters and subcluters (Pope et al., 2017) A series of illustrations that represent relationships between clusters and subclusters. The image on the left represents different clusters which are groups of each color, and subclusters within them, which are depicted as dashed circles within the larger cluster. The middle image shows the same clusters with additional subclusters with varying distances, while the right image shows clusters and subclusters that are near each other.

In areas with extreme diversity of host bacteria, such as compost piles and sewage, phages demonstrate broad host range as a survival adaptation. It becomes difficult to specialize with such a wealth of host bacteria, leading to favoring of broad-spectrum tail proteins that lend themselves to increased host range (Jensen et al., 1998). As all three of our F1 phages were found in areas of high biological activity - compost and plant soil - there was likely pressure to increase host range, potentially leading to the unique tail regions that characterize the subcluster, as seen on the right hand side of Figure 3. This sequence at the end of the last minor tail protein is highly similar in Tootsiepop and Piper2020, but diverges in Seagreen, demonstrating the unique tail region and supporting the hypothesis that the left-hand side of the genome may correlate to each phage's host range.

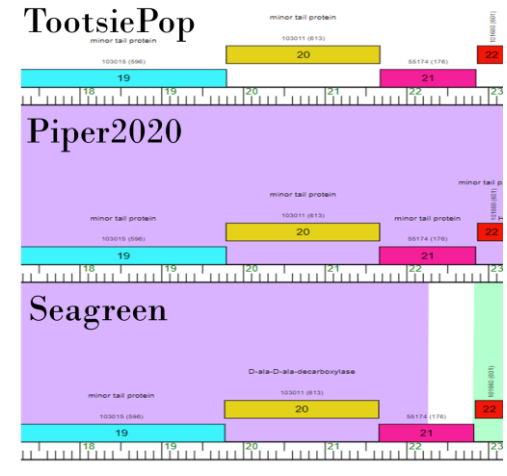

Figure 3: Side-by-side comparison of left-hand region Courtesy of Phamorator.org, this comparison shows the similarities (purple regions) of each phage.

Given the differences and specificity of each host's preferred phage, *M. foliorum* seems to be the most versatile host while *G. rubripertincta* will most likely have the most limited results. Given that most of our phage grow on *M. smegmatis*, there will be a lot to learn about the reactions to other hosts.

1.4 Lysogeny and how it can be used for future research

Another factor that is important to look into for our phage is the lysogeny of each sample. A lysogen can be defined as finding at least one prophage in a bacterial cell (Howard-Varona et al., 2017). This means that the phage integrates into the host instead of immediately lysing it, and further research is then needed as to how this affects the genetic response of the phage. Once integrated, the phage may remain inactive, or reactivate, lyse the cell, and return to a lytic life cycle. Figure 4 shows the immunity cassette for each studied phage. This region of the genome consists of an the integrase, which encodes an enzyme that allows integration of the phage DNA into a bacterial genome; the immunity repressor, which prevents transcription of an integrated phage genome, maintaining the phage as a lysogen; CRO, a regulatory gene that helps maintain lysogenic growth; the antirepressor, which represses CRO and helps return a lysogen to the lytic cycle; and excise, which removes the phage genome from the host genome upon returning to the lytic cycle (Hine, 2019).

Further research as to how each cassette works and what it reveals about each phage's lysogenic characteristics is important to understanding the stages of a particular phage's life cycle and the conditions under which it forms lysogens. The cassette contributes to the lysogen's properties and formation which we were unable to experimentally determine in our limited time-frame.

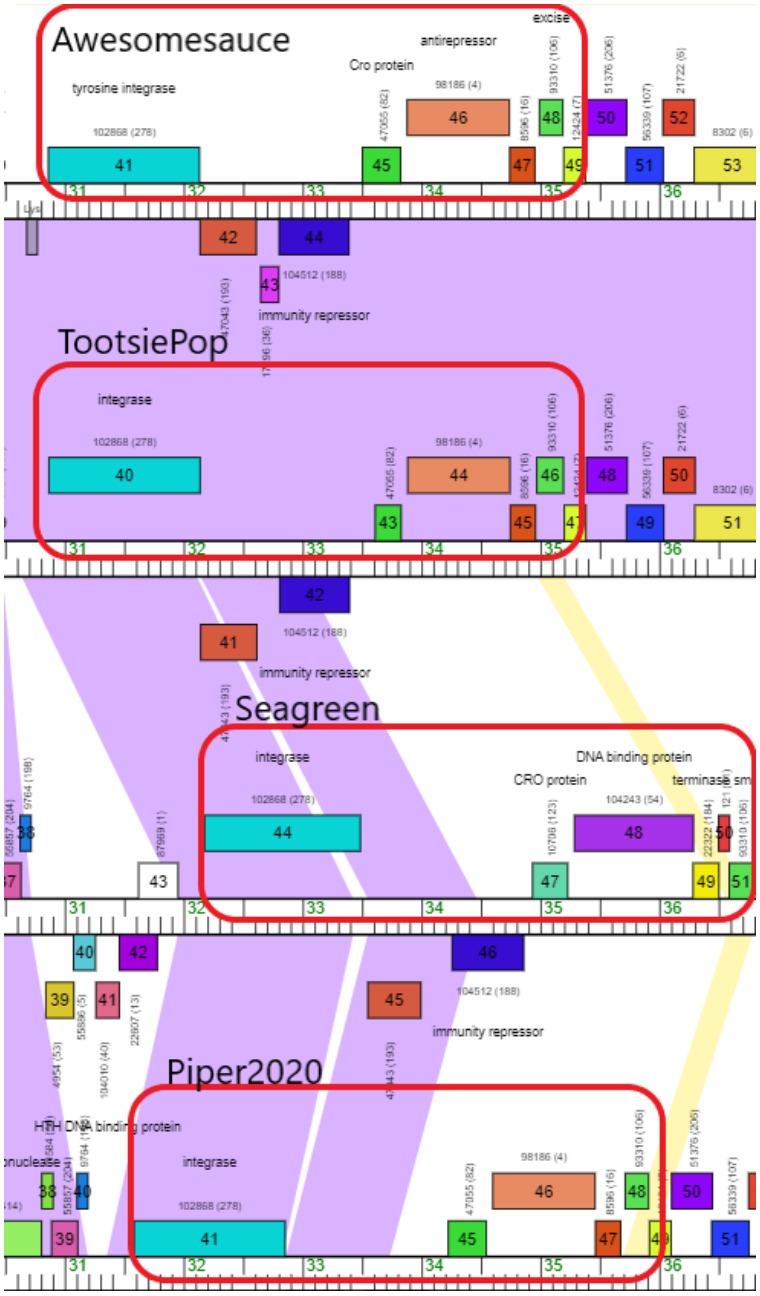

Figure 4: Immunity Cassette Regions Phamerator visualization of the immunity cassette regions of the genomes of Tootsiepop, Seagreen, Piper2020, and Awesomesauce as a fully annotated comparative phage.

Chapter 2: Methods & Materials

2.1 Bacterial Host Cultures

Five phages were used in this project. The phage LimaBean was grown on *M. folorium,* Clawz was grown on *G. rubripertincta*, and Tootsiepop, Piper2020, Seagreen, and Avle were grown on *M. smegmatis* mc^{2}155. *M. smegmatis* MKD8, *Enterococcus raffinosus*, *Staphylococcus epidermidis, Eschericha coli, Acinetobacter bayl,* and *Enterobacten aerogenes* were used as an additional host option, with no positive control phage.

Recipes for all agar and liquid media taken from the Seaphages Phage Discovery Instructor's Guide (Poxleitner et al., 2018). The *G. rubripertincta* and *M. folorium* hosts were cultured in PYCA liquid culture, and on PYCA plates. *M. smegmatis* mc^{2}155 and *M. smegmatis* MKD8 were grown in 7H9 liquid media and on LB plates with 7H9 top agar (1x); the recipes for both PYCA and 7H9/LB media can be found in the Appendix B of the Phage Discovery Instructor's guide (PDI). Both *M. smegmatis* mc^{2}155 and *M. smegmatis* MKD8 were initially cultured in Tween80-containing media for three days at 30 ℃, then moved to non-Tween media at a 1:1000 dilution and returned to the 30℃ incubator for another three days of growth before use as a host, per the host handling guidelines in Chapter 4 of the PDI.

LB plates and PYCA liquid media were stored at room temperature, while PYCA plates and 7H9 liquid media were stored at 4℃. Once autoclaved, both types of top agar were stored at room temperature in 30 mL aliquots. For use, tubes of top agar were microwaved until the agar was visibly melted and then placed in a boiling water bath until the agar came to a boil to ensure all agar was liquified. Calcium was then added to the tube if it had not been used before (33 µL added to PYCa and 135 µL added to 7H9). 3 mL of top agar was used for each plate. The recipe can be found in the PDI under Appendix B. One change that was made from the recipe is that the Ca was not added until the tube was actively being used.

Bottom agar was poured directly from autoclave bottles into plates in a horizontal flow hood, by estimate. Plates did not necessarily receive identical amounts of bottom agar.

To maintain healthy host cultures during the first three quarters of the project, each week 30 µL of the previous week's culture was moved to a fresh test tube containing 3 mL liquid media and 4-6 sterilized glass beads to discourage clumping. 16 x 150mm test tubes were used initially, before transitioning to 16 x 125mm test tubes at the halfway point of the experiment. The tubes were placed in the shaker at 30℃ and 250 rpm and allowed to grow back to confluence. In March, three quarters of the way through the project, protocols were changed such that each week 125 µL of each host culture was instead moved to a 125 mL baffle flask containing 6 mL of media (1:50 dilution), to encourage growth and reduce clumping.

To store hosts over extended periods, liquid cultures were streaked onto plates using a sterile metal loop, placed in the incubator for two or three days to allow growth, and then stored in the refrigerator at 4 ℃, adapted from Growing Bacteria from a Frozen Stock (Streak Plate) protocol in Appendix B of the Phage Discovery Student Guide (PDS) (*Phage Discovery Student*

Guide, n.d.). This created more stable colonies than the liquid cultures, allowing for long-term storage. Bacterial colonies were returned to liquid culture by picking from the plate using a sterile metal loop and suspension in 3 mL of liquid media.

Following their introduction to the project three quarters of the way through the year, the Tiny World alternate hosts *E. raffinosus*, *S. epidermidis*, *E. coli*, *A. bayl*, and *E. aerogenes* were revived from frozen stock using the Frozen Stock Streak Plate protocol in PDS Appendix B. After streaking on LB plates, individual colonies were picked with a sterile loop and placed in 7H9 liquid media, and were maintained each week according to the baffle flask protocol listed above.

2.2 Phage Maintenance

Initial phage particles were taken from storage at WPI or were kindly provided by the University of Pittsburgh archives.

Phage buffer for suspension and dilution of the phages was prepared according to the non-glycerol recipe Appendix B of the PDI.

Lysates for each phage were generated by combining 10 µL of phage solution with 250 µL of the appropriate bacterial host and plating the mixture as explained in PDS web plate protocol 7.1, then either picking a plaque as found in PDS protocol 5.4 and suspending it in buffer, or flooding the plate in buffer and filter-sterilizing the buffer that came off after absorbing phage for 2-4 hours, using protocol 6.3 in PDS.

High titer lysates were created by collecting lysate from web plates and diluting it using serial dilutions down to approximately 5×10^9 pfu/mL. High titer lysates are preferred for further experimentation, due to their increased stability stemming from the high number of phage particles present in solution.

The titer of each lysate was verified using dilution spot plates as per PDS protocol 6.4, wherein a bacterial lawn was laid down and then 10 µL each of a set of serial 1:10 dilutions of phage lysate were pipetted onto the lawn. These usually began with a sample of no dilution, then ranged from 10^{-1} to 10^{-7} dilutions. On each spot plate 10 µL of phage buffer was used as a negative control. We acknowledge that the preferred method for finding phage titers is using full plate titers, but we ran into difficulties identifying plaques on full plate titers and found that using spot plate titers worked best for our hosts.

2.3 Host Range

Host range for each phage was initially explored using cross-host spot plates, adapted from PDS protocol 5.6, wherein a lawn of bacteria was plated by combining 250 µL of bacteria with 3 mL of top agar, which was then poured onto the plate. Following that, 10 µL each of a set of serial 1:10 dilutions of phage lysate were pipetted onto the lawn, with 10 µL of phage buffer acting as a negative control and 10 µL of native phage acting as a positive control (LimaBean for *M. folorium*, Clawz for *G. rubripertincta*, Avle for the *M. smegmatis* mc^2155). The formation of plaques was taken as confirmation of infection.

We were also given access to several bacterial cultures for which there was no positive phage control. In those cases, 10 µL of a series of 1:10 serial dilutions of all six phage lysates were pipetted onto a lawn of each bacteria, with sterile phage buffer acting as a negative control. The formation of plaques in each lysate's designated area on the lawn was taken as confirmation of infection.

2.4 Genetic Annotation

In order to identify the underlying genetic causes of the phage host range behavior, we examined the auto-annotated genome of our three experimental phages (Tootsiepop, Piper2020, and Seagreen) on Phamerator.org. We compared nucleotide and amino acid sequences for several genes of interest in the immunity cassette using the PhagesDB.org Blast tool, and used the PhagesDB database to determine the cluster composition of the phams each gene of interest belonged to.

Chapter 3: Results

3.1 Lysate Characteristics

When plated on a lawn of an appropriate host, lytic phages form cleared areas of dead bacteria, called plaques; however, plaques of different phages demonstrate unique appearances and morphology. This section includes photographs of representative plaques of each variety of phage (Figures 3 and 4), to provide context for the platings on non-primary hosts. In each photo, dilutions of the featured phage from 10^{-1} to 10^{-7} (-1 to -7), as well as no dilution (N.D), were spotted onto its primary host.

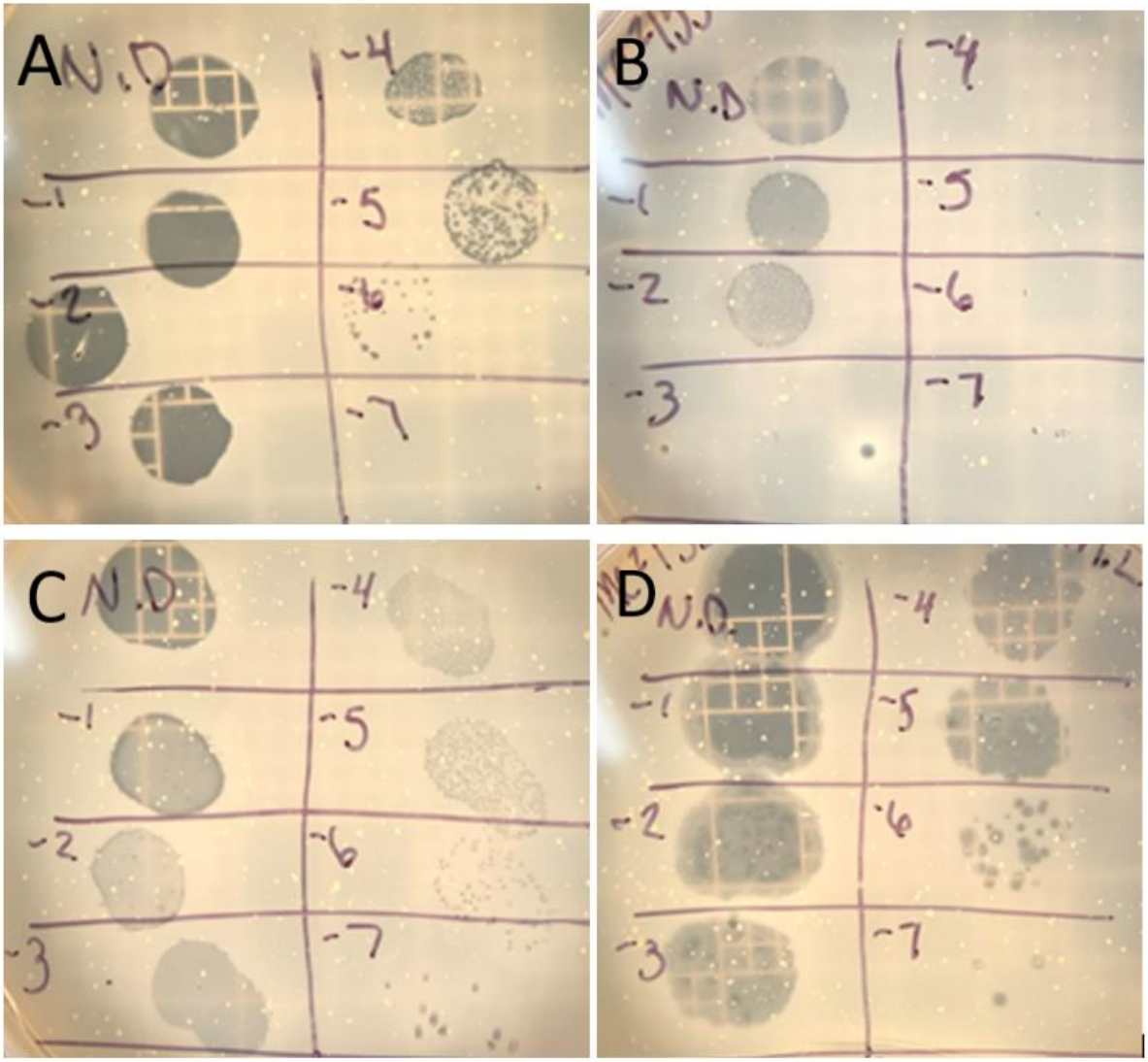

Figure 5: The experimental phages A. Tootsiepop, with best plaque definition seen at the -5 dilution. B. Piper2020, with best plaque definition seen at the -2 dilution. C. Seagreen, with best plaque definition seen at the -6 dilution. D. Avle, with best plaque definition seen at the -6 dilution. All four phages are plated on *M. smegmatis* mc^2155.

Figure 6: The positive control phages A. LimaBean, plated on *M. foliorum*, with best plaque definition seen at the -3 dilution. B. Clawz, plated on *G. rubripertincta*, with best plaque definition seen at the -5 dilution.

3.2 Host Range

No demonstration of expanded host range was found in any of the four experimental phages; plating at no dilution and from -1 to -7 showed no conclusive growth on *M. smegmatis* MKD8 (Figure 5), *G. rubripertincta* (Figure 6), and *M. foliorum* (Figure 7), or any of the Tiny Earth hosts: *A. bayl* (Figure 8), *E. aerogenes* (Figure 9), *E. coli* (Figure 10), or *S. epidermidis* (Figure 11). Due to the lack of expanded host ranges, our results lead us to believe that the unique right-hand portion of the genome has no correlation as to the phage's host range.

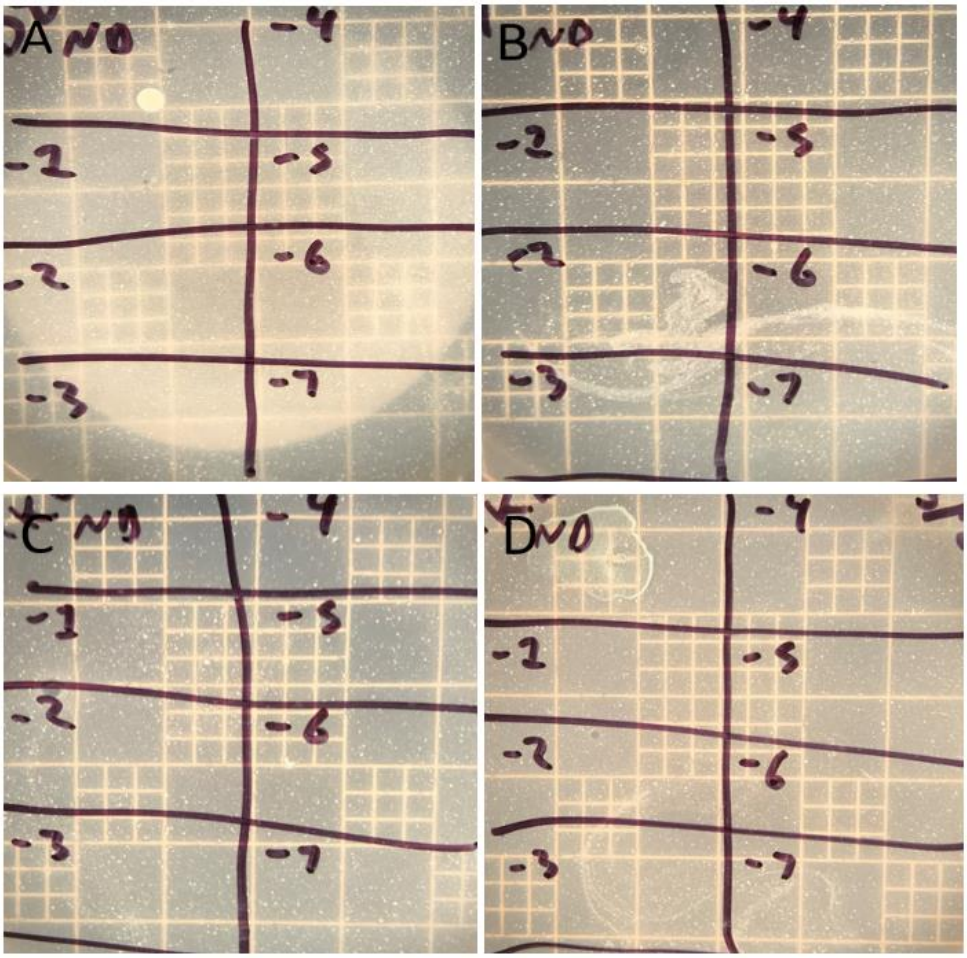

Figure 7: The four experimental phages spotted at dilution on *M. smegmatis* MKD8 No plaque growth is visible, though Avle (D) shows non-host bacterial growth similar to others found in various phage stocks, possibly indicating a lysogen carried with the phage lysate. A. Tootsiepop. B. Piper2020. C. Seagreen. D. Avle. *Note:* The *M. smegmatis* MKD8 strain did not have a positive control in these experiments..

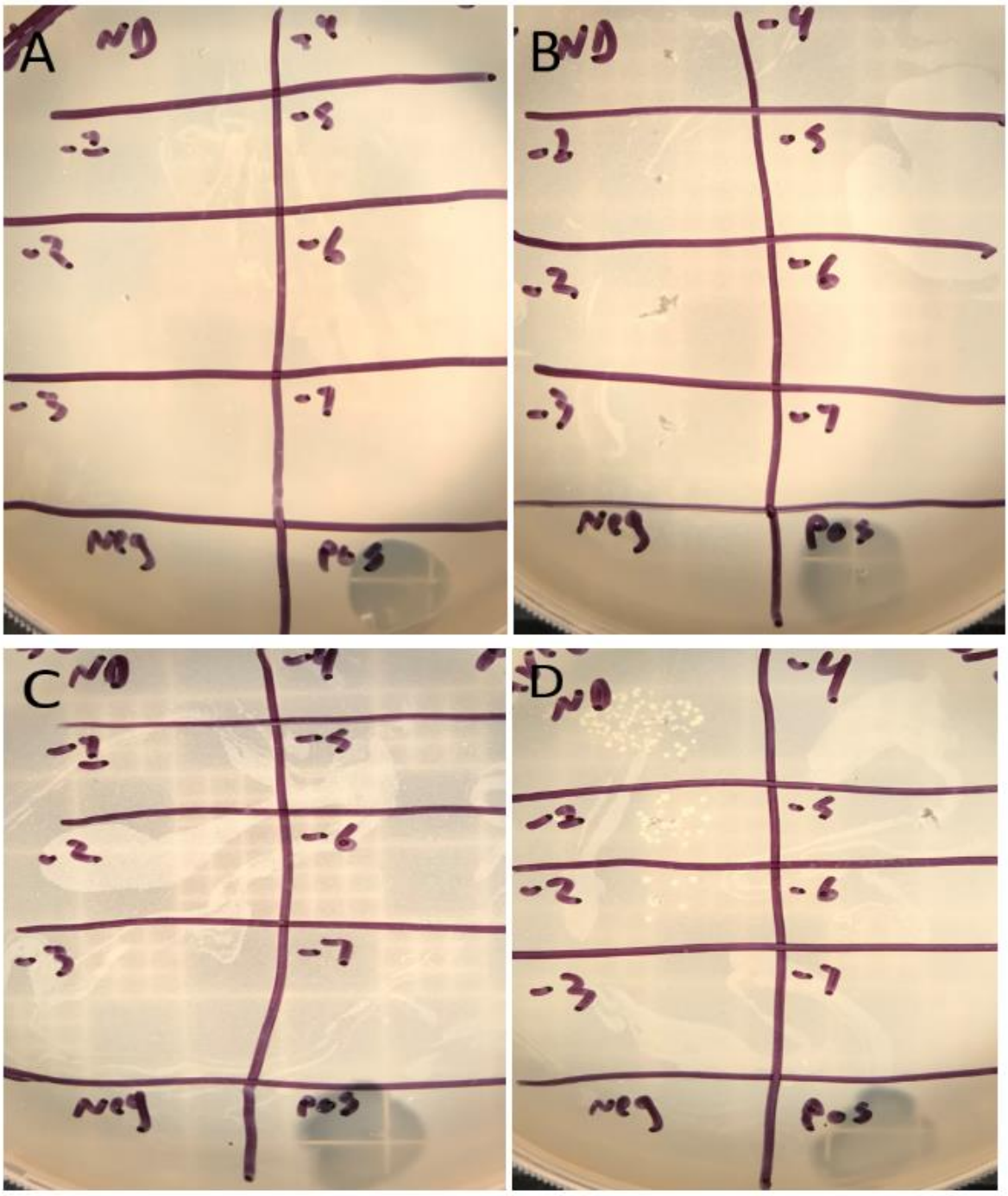

Figure 8: The four experimental phages spotted at dilution on *G. rubripertincta* Clawz acting as a positive control. No plaque growth is visible, though Avle (D) shows non-host bacterial growth similar to others found in various phage stocks, possibly indicating a lysogen carried with the phage lysate. A. Tootsiepop. B. Piper2020. C. Seagreen. D. Avle.

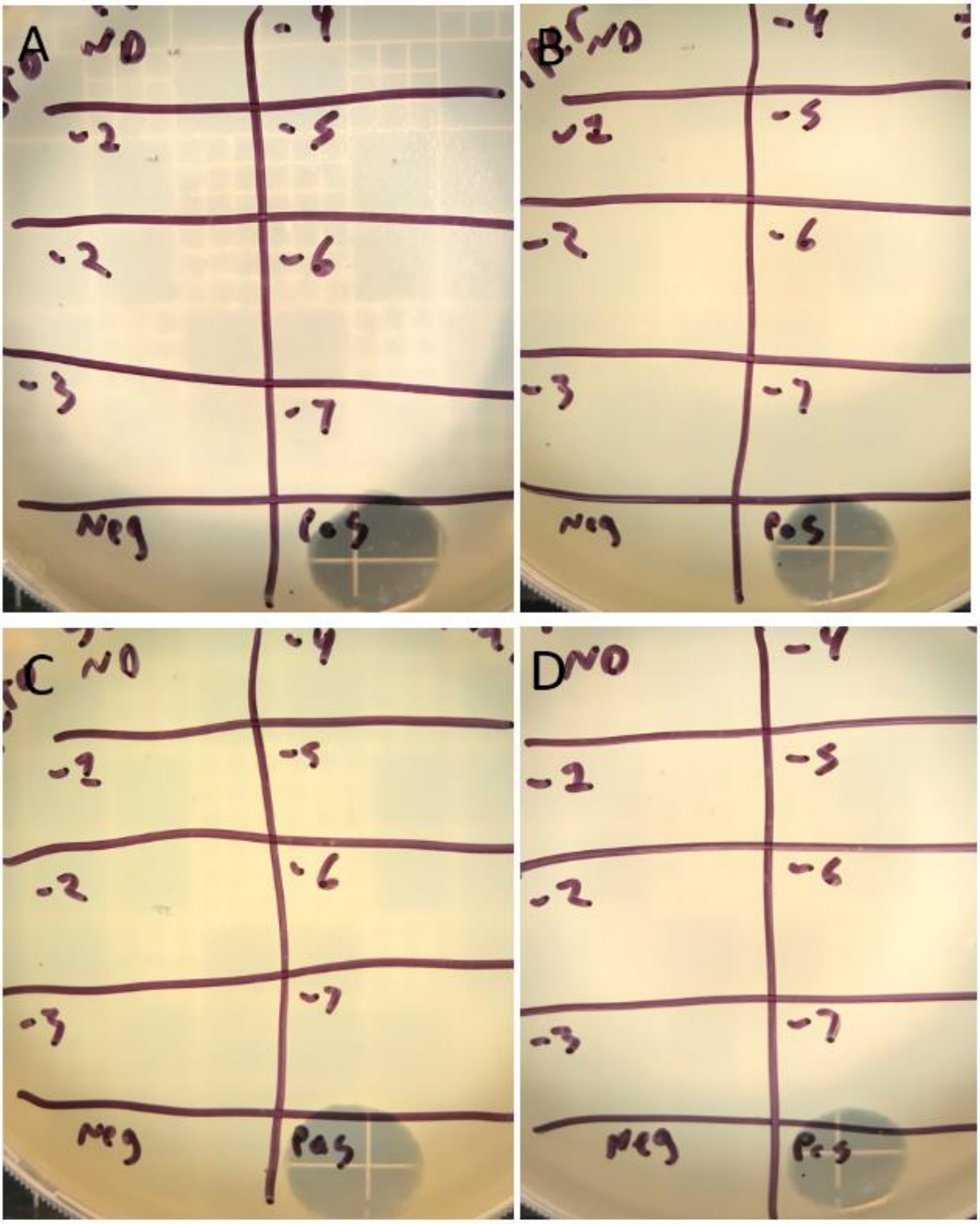

Figure 9: The four experimental phages spotted at dilution on *M. foliorum* LimaBean acting as a positive control. No plaque growth is visible. A. Tootsiepop. B. Piper2020. C. Seagreen. D. Avle.

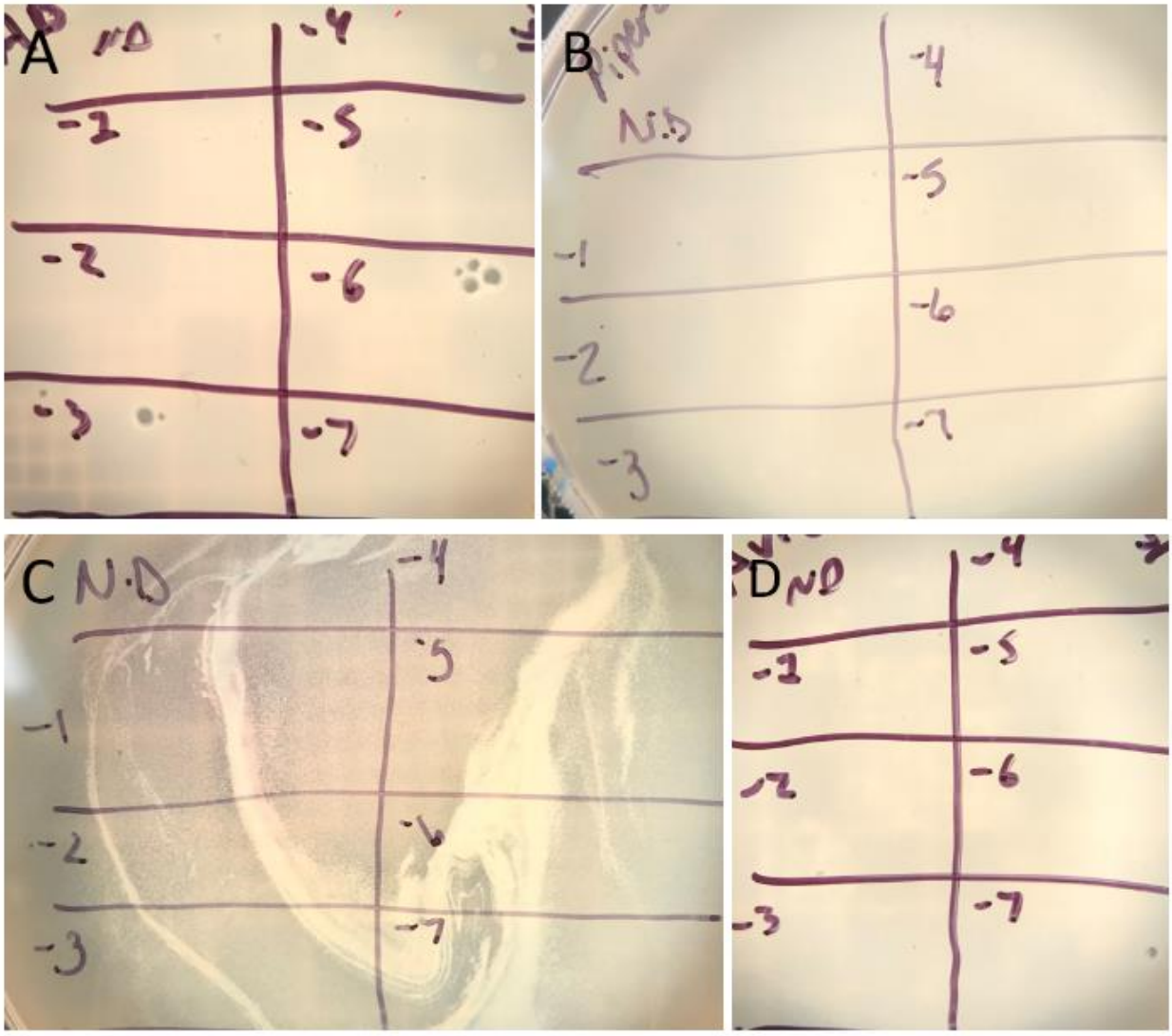

Figure 10: The four experimental phages spotted at dilution on *A. bayl* No plaque growth is visible. A. Tootsiepop. B. Piper2020. C. Seagreen. D. Avle.

Figure 11: The four experimental phages spotted at dilution on *E. aerogenes* No plaque growth is visible. A. Tootsiepop. B. Piper2020. C. Seagreen. D. Avle.

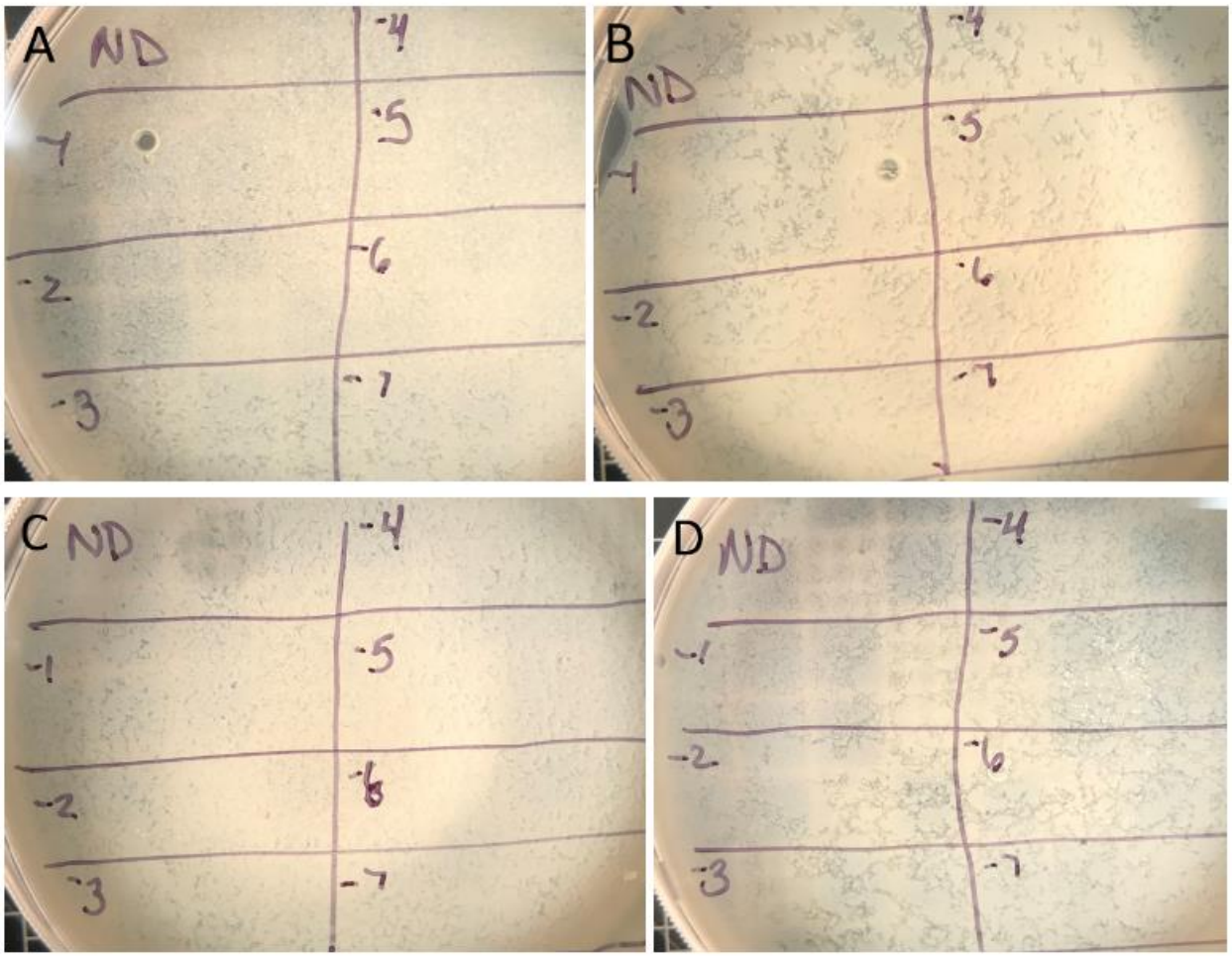

Figure 12: The four experimental phages spotted at dilution on *E. coli* No plaque growth is visible on Tootsiepop, Piper2020, or Avle, but there is mild, faded clearing in the undiluted region of the Seagreen plate. A. Tootsiepop. B. Piper2020. C. Seagreen. D. Avle.

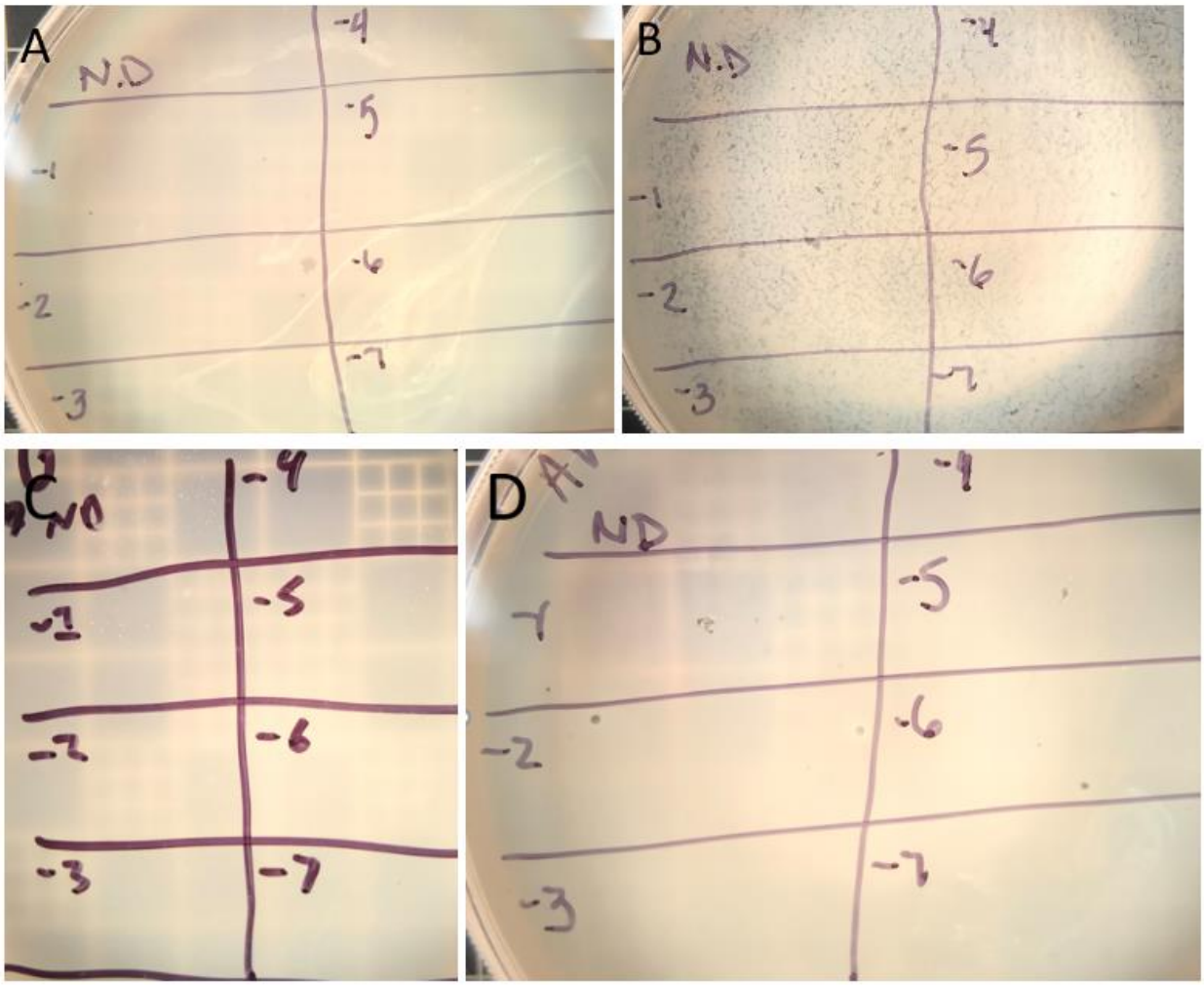

Figure 13: The four experimental phages spotted at dilution on *S. epidermidis* No plaque growth is visible. A. Tootsiepop. B. Piper2020. C. Seagreen. D. Avle.

High concentrations of phage have the capability to kill bacteria through sheer quantity, creating false plaques. As, besides Seagreen on *E. coli*, even undiluted phage lysate showed no clearing on any other host than the primary host *M. smegmatis* mc^2155, none of the other two F1 phages or Avle seem to demonstrate expanded host range to these hosts. Seagreen presents a possibility of expanded host range, or simply an example of the high-concentration phenomenon.

3.3 F1 Phage Immunity Cassette

The three experimental F1 phages (Tootsiepop, Piper2020, and Seagreen) share a unique right-hand genome region. The genes in this region include the immunity cassette and various other machinery associated with lysogen assimilation. The locations of each phage's version of these genes are listed in Table 2. The immunity cassette genes of interest are integrase, the immunity repressor, CRO and excise.

	Awesomesauce (fully annotated comparative phage genome)	Tootsiepop	Piper2020	Seagreen
Integrase	Gene 41 (annotated)	Gene 40 (annotated)	Gene 41 (annotated)	Gene 44 (annotated)
Immunity repressor	Gene 44 (annotated)	Gene 42 (annotated)	Gene 44 (annotated)	Gene 46 (annotated)
CRO	Gene 45 (annotated)	Gene 43 (possible match)	Gene 45 (possible match)	Gene 47 (annotated)
Antirepressor	Gene 46 (annotated)	Gene 44 (likely match)	Gene 46 (likely match)	Gene 48 (likely match, annotated as DNA binding protein)
Excise	Gene 48 (annotated)	Gene 46 (likely match)	Gene 48 (likely match)	Gene 51 (likely match, annotated as terminase small subunit)

Table 2: The genetic breakdown of each phage The table shows location of each gene of interest in the genomes of the experimental phages Tootsiepop, Piper2020, and Seagreen, as well as a comparative phage, Awesomesauce. Genes with definitive annotations are indicated, as well as likely but unannotated or mislabeled genes.

Nucleotide and amino acid similarities between these genes are listed in Table 3. The E value similarities were determined by blasting the sequences of relevant Awesomesauce genes against the PhagesDB.org database using the PhagesDB.org nucleotide and protein blast tools. In cases where the PhagesDB.org blast report did not include Seagreen, due to the uniqueness of Seagreen's CRO and antirepressor sequences, the relevant gene sequence and gene product from Seagreen were blasted against the same gene from Awesomesauce using the NCBI nucleotide and protein blast functions.

The pham of each gene and the clusters within that pham, according to PhagesDB.org, are listed in Table 4.

	Tootsiepop	Piper2020	Seagreen
Integrase	0.0/0.0	0.0/0.0	0.0/0.0
Immunity repressor	0.0/e-109	0.0/5e-98	1e-47/3e-25
CRO	0.0/6e-35	0.0/1e-55	NSS/NSS
Antirepressor	0.0/e-160	0.0/e-160	NSS/1e-06
Excise	e-108/4e-32	e-108/4e-32	0.58/5e-24

Table 3: Nucleotide and protein similarities between notable genes The table shows E value similarity to Awesomesauce of the nucleotide and amino acid sequences of similar genes across Tootsiepop, Piper2020, and Seagreen. Arranged as nucleotide similarity/amino acid similarity. NSS stands for No Significant Similarities.

	Tootsiepop and Piper2020	Seagreen
Integrase	102868: AY, BV, BW, BX, CQ, CY, DD, DL, DN, F, FA, K, L, T (278)	102868: AY, BV, BW, BX, CQ, CY, DD, DL, DN, F, FA, K, L, T (278)
Immunity repressor	104512: F (188)	104512: F (188)
CRO	47055: AD, CV, CY, CZ, DN, F, I (82)	10706: DN,F (123)
Antirepressor	98186: F(4)	104243: F(54)
Excise	93310: F(106)	93310: F(106)

Table 4: Gene pham clusters The pham each gene belongs to, as the phage clusters and number of genes in the pham. Arranged as Pham #: Clusters (number of genes).

Chapter 4: Conclusions & Future Work

The lack of host range diversity demonstrated that the unique portions in the tail region and the right-hand arm of the subcluster's genome did not increase the number of hosts each phage was capable of binding to. Given this unsupported hypothesis, further research could be conducted regarding the similarities and differences of the subcluster's genome to related phages to determine the true impact of the unique right-hand region.

One way to further this research is to look into lysogeny and the immunity cassette. F1 phages possess an immunity cassette, made up of the integrase, immunity repressor, CRO, antirepressor, and excise, making them capable of forming lysogens. However, in the course of our experimentation we failed to produce lysogens. We witnessed some growth that could potentially have been lysogens, but presenting in a very unusual form and carried with the phage lysate.

When run through Phamorator.org, there are minimal similarities between the immunity cassettes of Awesomesauce, Seagreen, TootsiePop, and Piper2020 (as shown in figure 4). Understanding the similarities and divergences of the F1 subcluster's immunity cassette can help further investigate the lysogeny of each phage. This opens the doors to learning much more about the infection process into different types of bacteria.

Further experimentation should explore the characteristics of the F1 cluster's unusual lysogen activity, and the unique gene sequences that produce it.

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