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Lysogeny and Use of Mycobacteriophage Pita2

Eleanor Behling

Dr. Neocles Leontis & Dr. Jill Zeilstra-Ryalls

May 2020

Abstract

The CDC has classified antibiotic resistance as the biggest health challenge of our era; every year 2 million lives are impacted and even lost due to resistant bacteria. Bacteriophages provide an alternative route to fighting infections that does not further the development of antibiotic resistance among bacterial species. A bacteriophage replicates inside a bacterial cell and then causes that cell to lyse, an event that kills the bacterial host. However, some phage can integrate their genomes into the host chromosome without causing lysis. The HHMI SEA-PHAGES program has generated a collection of bacteriophage that infect Actinobacteria species. Over 13,000 phages have been collected thus far, but fewer than 3,000 have been sequenced and genetically analyzed. The purpose of research into the lysogeny of discovered, but unsequenced, bacteriophage is to classify them by immunity range; that is, closely related bacteriophage are unable to infect the lysogenic host, while more distantly related phage are. Initially, a lysogen had to be isolated. For Pita2, a phage isolated and analyzed at Bowling Green State University, the host is *Mycobacterium smegmatis*. A purified lysogen of Pita2 was analyzed against bacteriophage with known DNA sequences to confirm that it is immune to infection by closely related phage. The lysogeny was then examined for its ability to identify close vs. more distantly related phage among a set unknown bacteriophage. Another virus, namely SARS-CoV2, prevented the completion of this study. However, it was possible to generate preliminary data that established the ability to use immunity as a means to classify newly isolated phage based upon the degree to which they can successfully infect the Pita2 lysogen.

Introduction

Bacteriophage, or phage, are viruses that infect bacteria. They belong to two broad types; lytic and temperate. Lytic phage infect and kill their host. Temperate phage can infect with host cell killing, but they can also integrate their DNA into the host's genome (Ofir & Sorek, 2017; *Figure 1*). The phage DNA-modified bacterium is called a lysogen. When phage are operating in this "stealth mode", the lytic pathway is turned off by a phage-encoded silencer gene product called the *cI* repressor protein. In addition to the suppression of the phage's own lytic pathway, the *cI* product can prevent infection by other related phage. This resistance to a secondary infection is known as superimmunity.

Phage have proven to be effective antibacterial agents, providing an increasingly important alternative to antibiotic therapies as bacterial resistance escalates. (Romero-Calle, 2019). Bacteriophage therapy was first pioneered in the treatment of dysentery by Felix d'Herelle as early as 1915. Since d'Herelle's initial excursion into medicinal use of bacteriophage, more work has been done in the categorization and discovery of new phage. To develop their use in therapies, it is important to continue investigating the biology of phage, which includes studying their relatedness. Based upon the principle of superimmunity, it should be possible to determine phage relatedness by examining the ability of phage to superinfect a lysogen. If the phage is closely related, infection should be blocked by superimmunity, if the phage is more distantly related, then newly infecting phage should be able to lyse the bacteria. (Mavrich, 2019). The goal of this project is to test the superimmunity hypothesis for the bacterium *Mycobacterium smegmatis* mc² 155 using newly isolated lysogens of the temperate bacteriophage Pita2.

Materials and Methods

Host and Media

The bacterial host used in the following described research was *Mycobacterium smegmatis* mc^2 155. The media used in isolation and immunity assays was L-agar (carbenicillin 50 μg/ml and cycloheximide 10 μg/m). When bacterial cells were plated on top of L-agar they were suspended in 3 mL of Top Agar (50 mL 7H9 media, 1 ml 100 mM of CaCl, and 50 ml top agar). Phage buffer used in these experiments was made from 10 mM Tris (pH 7.5), 10 mM $MgSO_4$, 68 mM NaCl, and 1 mM $CaCl_2$.

Isolation of Pita2

Pita2 was originally isolated in the Fall of 2017 by Natalie Wise. Protocols described in the HHMI Phage Discovery Guide were used. The phage was first isolated from an environmental soil sample using the direct isolation procedure. Pita2 phage was then purified through a series of dilutions and platings, and then amplified to obtain a high titer lysate. Phage DNA was extracted and sequenced. The phage genome was annotated by the SEA-PHAGES Spring 2018 cohort of students. Both the sequence and annotation were deposited in Genbank at the National Center for Biotechnology Information (Accession # MH576959). During the annotation process it was discovered that Pita2 has an immunity repressor gene. This gene confirmed that Pita2 is a phage that follows the temperate life cycle and thus is capable of forming a lysogen.

Lysogen Isolation

To isolate a lysogen of Pita2 serial dilutions were plated on a lawn of *Mycobacterium smegmatis* mc^2 155. Samples of bacterial mesas, areas of bacteria infected with phage, were used to streak plates for single *M. smegmatis* mc^2 155 colonies. These single colonies were potential lysogens. Following this streak-purification, a patch assay was performed to determine whether the candidate isolates were true lysogens. This assay involved streaking a bacterial mass first on a "Lysogen-only" L-agar plate and then through a lawn of *M. smegmatis* mc^2155 prepared on a second L-agar "Experimental" plate to form a small patch. Stable lysogens are those that grow on the Lysogen-only plate and produce a halo of lysis around the patched area on the Experimental plate. One such confirmed lysogen was then cultured and used in the immunity assay with other bacteriophage that were isolated by students in the SEA-PHAGES course.

Immunity Assay

Frozen stocks of phage lysates were used to test the bacteriophage isolated in Fall of 2018 by spotting serial dilutions onto both lawns of the wild type *M. smegmatis* mc^2155 bacteria and the stable Pita2 lysogen that were prepared on L-agar plates. These plates were incubated for 48 hours at 37 degrees Celsius. The plaques were then counted to compare titers on wild *M. smegmatis* mc^2 155 and the Pita2 lysogen.

PCR and Agarose Gel Electrophoresis

Polymerase Chain Reactions (PCR) were performed using primers purchased from Integrated DNA Technologies (Coralville, Iowa), "Ready-to-go" PCR tubes (GE Life Sciences; Pittsburgh, PA), and a MyCycler thermocycler (BioRad, Hercules, CA). The amplification protocols used for each reaction were STEP 1: 90°C for 1 min; STEP 2 (repeat 34 times): 90°C for 1 min, AT (Annealing Temperature corresponding to the melting temperature for the primers used in the

reaction) for 15 sec, 72°C for a sufficient amount of time to allow the DNA polymerase in the reaction to synthesize the full-length product; STEP 3: 72°C for 5 min; STEP 4: room temperature incubation.

Each reaction was analyzed using agarose gel electrophoresis. The PCR products were loaded into an agarose gel alongside a DNA ladder and positive control. These gels were run for 45 minutes before destaining and photographing.

Results

Isolation of a Pita2 lysogen

A lysate of Pita2 was diluted and spotted on a lawn of *Mycobacterium smegmatis* mc² 155. A sterile toothpick was used to scrape the top layer of cells in the cloudy mesa area surrounding the plaques (both scrape sites were circled on the dilution plate indicated in *Figure 1* below). The scraped samples were streaked multiple times to purify the potential lysogen. *Figure 2* shows the patch assay that confirmed potential lysogen 'D' was a true lysogen of Pita2. On a lawn of the bacterial host the lysogen caused lysis, forming the plaque on the left plate in *Figure 2*; in contrast, the plate on the right shows normal bacterial growth, indicating the stability of the Pita2 lysogen.

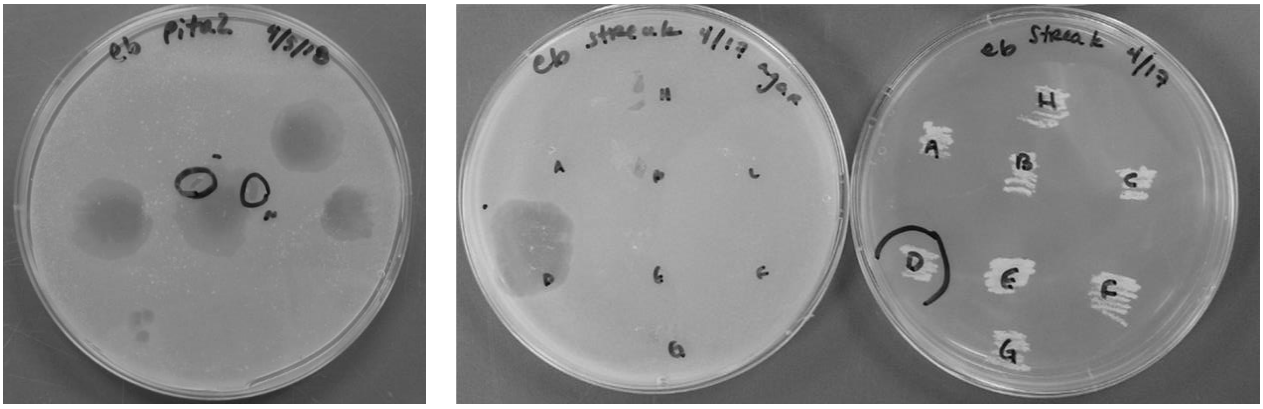


Figure 1. (Left) Pita2 dilutions spotted on Mycobacterium smegmatis mc² 155 with circled mesa sample sites. (Right) Successful patch assay indicating a stable lysogen on the spot labelled 'D'.

Superimmunity testing

A total of sixteen phage have been spot-tested against the Pita2 lysogen. These 16 were chosen because there is preliminary DNA sequence data available for all of them. However, the DNA sequences require deconvolution in order to assign a specific sequence to a specific phage. Until that time, only phage cluster representation among the sequences, and so the phage is known. Clusters are groups of bacteriophage that have highly similar genomes. A cluster can be further divided into subclusters. Pita2 is an A1 phage, and the expectation is that other A1 phage should be unable to infect the Pita2 lysogen. Table 1 lists the immunity assay results for the 16 experimental phage.

Table 1: Immunity test results for phage plated on both wild type (WT) M. smegmatis *and the* M. smegmatis *Pita2 lysogen.*

Phage (Cluster)	Plaques?	Titer on WT	Titer on Pita2	Fold Difference in Titer
Cabo (DOGEMS)	✓	5.00E-08	1.00E-08	5.0
Crysler (DOGEMS)	✓	3.00E-09	1.00E-10	30.0
Elohim (DOGEMS)	-	1.00E-07	-	-
Eugenia (B1-Lytic)	✓	2.00E-10	1.00E-10	2.0
Jerry Smith (DOGEMS)	-	7.00E-07	-	-
Joieb (S-Lytic)	✓	2.60E-09	2.00E-09	1.3
Joyride (DOGEMS)	-	4.00E-09	-	-
KekeDoULoveMe (DOGEMS)	✓	2.00E-10	1.00E-08	0.0
Kiddo (DOGEMS)	✓	1.00E-10	2.00E-09	0.1
Maliboo (DOGEMS)	✓	1.00E-10	2.00E-10	0.5
Maverix (DOGEMS)	✓	4.00E-10	3.00E-08	0.0
Pita2 (A1-Temperate)	✓	2.00E-07	3.00E-04	0.0
Pringar (DOGEMS/S-Lytic)	✓	2.00E-10	2.00E-09	0.1
Qbot (DOGEMS)	✓	1.20E-10	1.00E-10	1.2
Qrex (DOGEMS)	✓	5.00E-09	5.00E-09	1.0
Shalamarie (DOGEMS)	✓	1.80E-09	4.00E-10	4.5
Zilre (DOGEMS)	✓	6.00E-08	2.00E-08	3.0

The results of the initial immunity assay show the potential cluster alignments of these 16 phage in relation to the DOGEMS contigs results. In analyzing the fold difference between the titer of an experimental phage on the Pita2 lysogen (labelled Pita2 in Table 1) and the wild type *M. smegmatis* strain (labelled WT in Table 1) three distinct ranges can be deduced. The first and simplest to assign are phages Joyride, Jerry Smith, and Elohim. These three fall in the first range, they have absolutely no plaques present on the Pita2 lysogen. (Appendix) The nature of superinfection immunity requires a repressor protein encoded by Pita2 to bind to a specific sequence on the infecting phage DNA. This DNA sequence is likely present in Joyride, Jerry Smith, and Elohim as seen by their inability to infect and cause a lysis event on the lysogen. Because of this hypothesized genetic similarity, Joyride, Jerry Smith, and Elohim are potential members of Cluster A4. Among the DOGEMS contigs, one complete A4 genome is present. To determine which of these three potential A4 phage it belongs to, PCR is necessary.

The second range is those that have a fold difference in titer between 3.0 and 30.0. Three phage are in this group; Crysler, Cabo, Shalamarie, and Zilre. These phage are likely members of the G1 or J clusters which include only temperate phage.

The final range is the most ambiguous. Bacteriophage in clusters B, C, and S all follow the lytic life cycle. The phage in this group are Maverix, KekeDoULoveMe, Kiddo, Qbot, Qrex, and Pringar. They were assigned to this group because the range of difference in titer was between 0.0 and 2.0. Eugenia is a known cluster B1 bacteriophage that follows the lytic life cycle. There was a two-fold difference in the number of plaque forming units on Pita2 vs. the wild type. Eugenia was used as a benchmark for determining other potential members of the B1 cluster.

These six phage likely express the lytic life cycle because their titer was not significantly impacted by the presence of the temperate phage Pita2 within the host chromosome.

Primer Design

The goal of the primers designed here was to "deconvolute" the DNA sequences of the phage. This deconvolution is done using Polymerase Chain Reaction (PCR). PCR primers corresponding to each phage cluster represented in the DNA pool were designed according to an analysis of the tail protein sequences of all sequenced phage in that cluster. The experimental clusters present in the 2018 DNA pool are A4, B1, C1, G1, J, and S. Table 2 lists the resulting primers, which are specific for phage in each cluster. These primers conform to parameters that are required for PCR and generate products that are easily visualized by agarose gel electrophoresis.

Table 2: Primer sequences designed to uniquely identify phage DNA belonging to clusters represented among the 2018 sequenced phage.

Cluster	Forward Sequence (5'-3')	Reverse Sequence (5'-3')	Product Length (bp)	Annealing Temp. (°C)
A4	CAT GCC CGA GTT CGG CT	GAT CCA GCT CCC GGT GGA CGA CT	333	64
B	GGC TCA TGA CCG CCG ATG	CGA GGC GGT GTC CTC CC	876	61
C1	AAC AAG CAG TAC GAC GTG TCC	GCG GTG CAC TGC TTC AT	710	57
G1	CCG GCG GTC TCT ACA TCG C	GCG TTC TTC GAG GAC AAG GCG	353	61
J	CAA CCT GGC CGA CCC CGC	TGG AAG TTC CGC GTC AAG GCC AC	794	65
S	CGC TGG AGG CAA CCG G	CCC TGA ACT GCC AGG CC	460	60

Deconvolution Results

First, to test the validity of the primers Joieb was run with the S-set of primers. Joieb was sequenced separately from the DOGEMS phage and is in the S cluster. The S-primers successfully amplified the major tail region of Joieb's DNA, making Joieb the perfect positive control for the other deconvolution PCR results. Figure 2 is a picture of the gel showing Joieb PCR product.

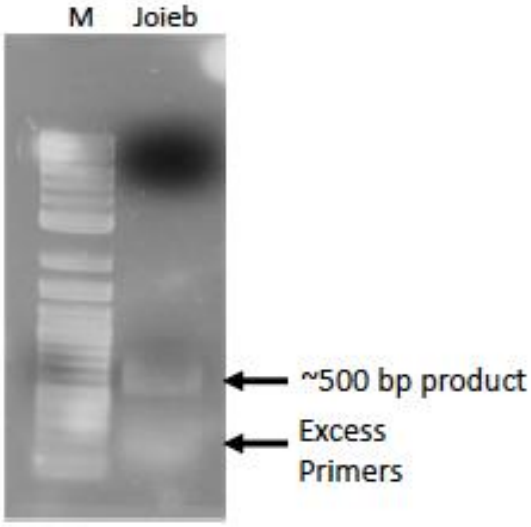


Figure 3. Image of agarose gel with PCR product from Joieb DNA and the Cluster S-specific-primers (see Table 1 for primer sequences). M is the "2 log" DNA molecular size marker (NEB).

After confirming that the primers designed for cluster S successfully identified Joieb (the individually sequenced cluster S phage), experimental trials began. Figure 4 is an image of the results of the first experimental PCR trial. This trial was done by the 2018 SEA-PHAGES class in DNA of all of the sequenced phage were used as template in separate amplification reactions using the S primers.

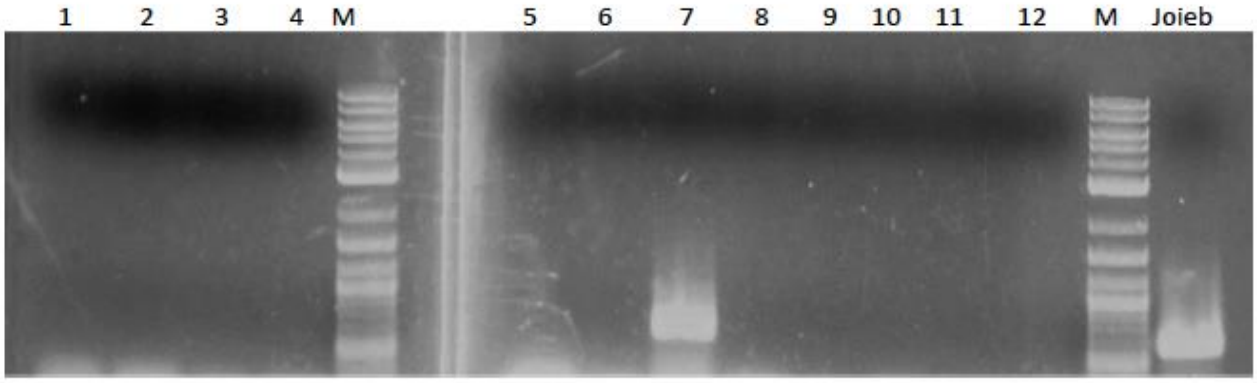


Figure 4. PCR confirming that Pringar is an S cluster phage. The template DNA samples used are as follows: Lane 1: Bali, lane 2: Elohim, lane 3: Maverix, lane 4: Crysler, lane 5: Qrex, lane 6: Shalamarie, lane 7: Pringar, lane 8: Kiddo, lane 9: Qbot, lane 10: KekeDoULoveMe, lane 11: Joyride, and lane 12: Zilre. Pringar DNA was the only template that was amplified with the S primers other than the positive Joieb control. M: "2 log" DNA molecular size marker (NEB).

Pringar is the only S phage present in the DOGEMS group. (Fig. 4). Following this identification, the G primers were used in PCR of the remaining phage. The expected product, a DNA sequence with a length of 353 base pairs, was not observed. As seen in both figures below, the only wells showing any DNA were the two wells with a standard, and the Joieb positive control.

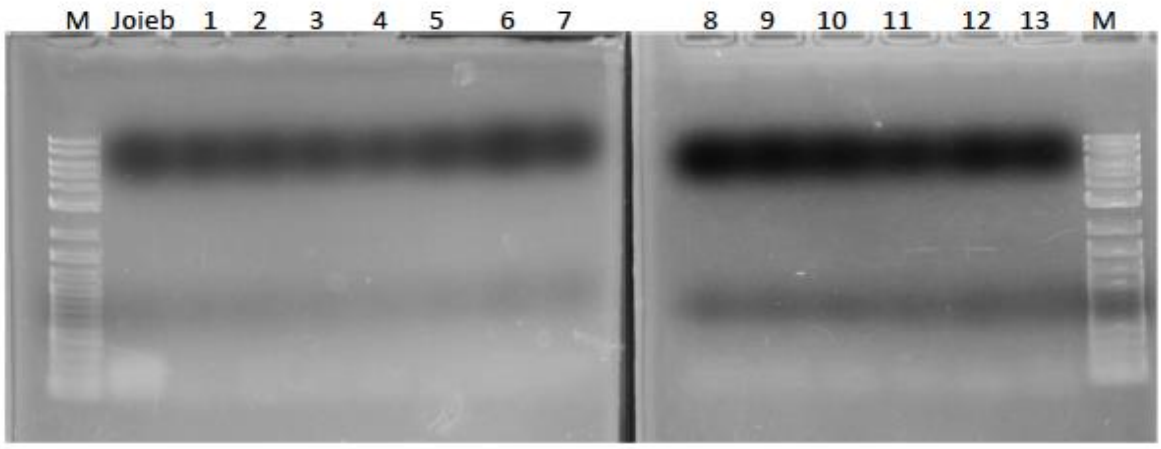


Figure 5. Image of agarose gel with PCR products from amplification of phage DNA with G primers. Joieb DNA is the positive control. Lanes are as follows: Lane 1: Cabo, lane 2: Crysler, lane 3: Elohim, lane 4: Jerry Smith, lane 5: Joyride, lane 6: KekeDoULoveMe, lane 7: Kiddo, lane 8: Maliboo, lane 9: Maverix, lane 10: Qbot, lane 11: Qrex, lane 12: Shalamarie, and lane 13: Zilre. M: "2 log" DNA molecular size marker (NEB).

Discussion

The SEA-PHAGES program continues to isolate hundreds of new bacteriophage every year; relatively few of these will be sequenced or annotated. Characterization of bacteriophage is critical to the development of effective methods of phage therapy, it provides information about superinfection, host range, and life cycle. The immunity repressor gene of Pita2 was identified during annotation of its genome, and the presence of this gene allowed for this project to proceed. The ability of the phage DNA to integrate into the host chromosome gave us the opportunity to test for superinfection by other bacteriophage. Those that were closely genetically related to Pita2 were hypothesized to be unable to infect the Pita2 lysogen. Conversely, those bacteriophage that proved capable of infecting the lysogen would likely be more distantly related or unrelated to Pita2.

Pringar was hypothesized to be a member of clusters B, C, or S based upon the superimmunity assays. Using PCR and the cluster S primers, Pringar was determined to be a cluster S phage. Therefore, the ability to use a Pita2 lysogen as a tool in cluster assignments of unsequenced phage was confirmed.

The primers listed in Table 2 were designed based upon the major tail sequences of multiple bacteriophage from each represented cluster, except for cluster B. The cluster B phage did not have a large enough major tail gene, and as such the minor tail protein sequence was used in

designing the B primers. These primers will make it possible to resolve future sequencing results involving pools of phage.

In order to complete the project, multiple addition amplifications were planned; however, another virus, SARS-CoV2 postponed them. Nevertheless, the usefulness of the Pita2 has already been demonstrated, and can be used to characterize future newly isolated phage.

Appendix

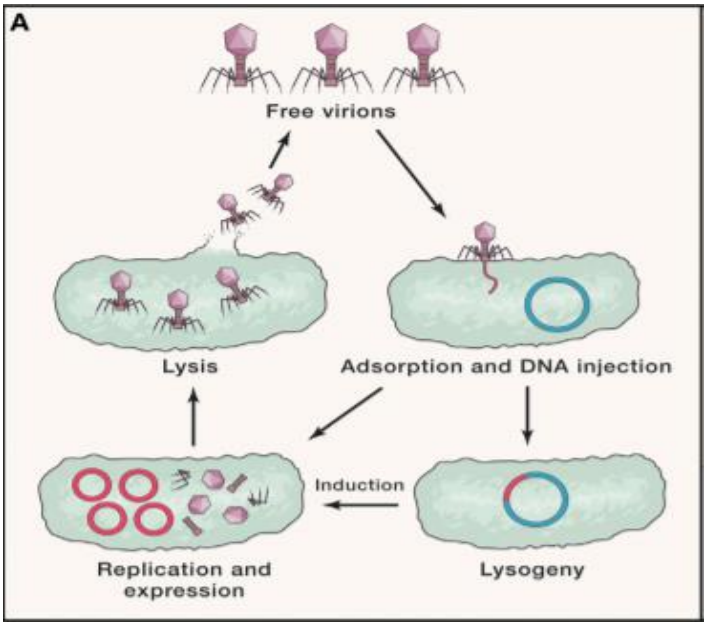
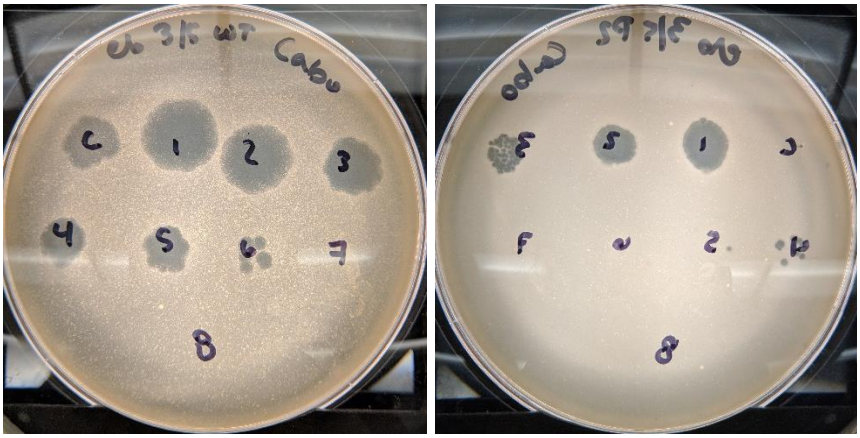


Figure 6 (left). Lifestyle options of temperate bacteriophage from Contemporary Phage Biology Ofir and Sorek.

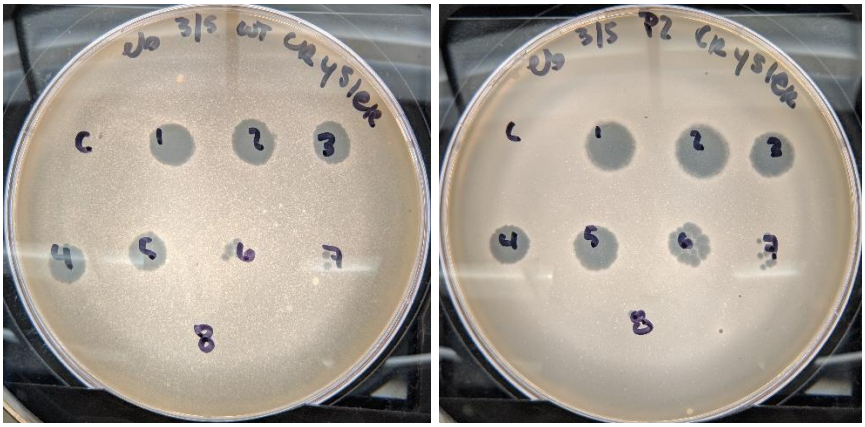
Bowling Green 2018 DOGEMS Notes

Contig 1	Prob complete	Cluster C1
Contig 2	Poss complete	Cluster J
Contig 3	Poss complete	Cluster S (not JoieB)
Contig 4	Complete	Cluster A4
Contig 5	Poss complete	Cluster J (same genome as contig 2)
Contig 6	Incomplete/mixed	Cluster B1
Contig 7	Incomplete/mixed	Cluster B1
Contig 8	Incomplete/mixed	Cluster B1
Contig 9	Incomplete/mixed	Cluster B1
Contig 10	Incomplete/mixed	Cluster B1
Contig 20	Incomplete/mixed	Cluster G1

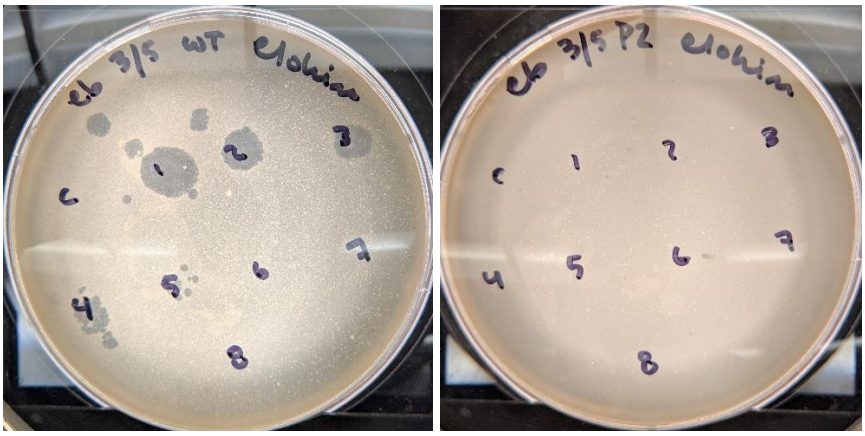
Figure 7. DOGEMS results from the Bowling Green SEAPHAGES 2018 cohort indicating which clusters were potentially present in the DNA pool.



Figures 8a (left) and 8b (right). Dilutions of phage Cabo on both the WT host and Pita2 lysogen.



Figures 9a (left) and 9b (right). Dilutions of phage Crysler on both the WT and Pita2 lysogen.



Figures 10a (left) and 10b (right). Dilutions of Elohim on WT and Pita2 lysogen. No plaques were observed when dilutions were spotted on the Pita2 lysogen.

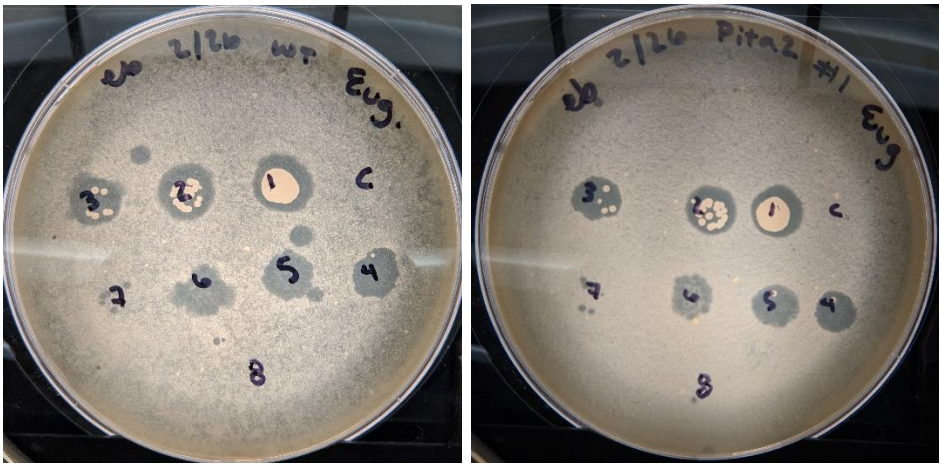


Figure 11a (left) and 11b (right). Eugenia, a cluster B phage on both WT and Pita2 lysogen.

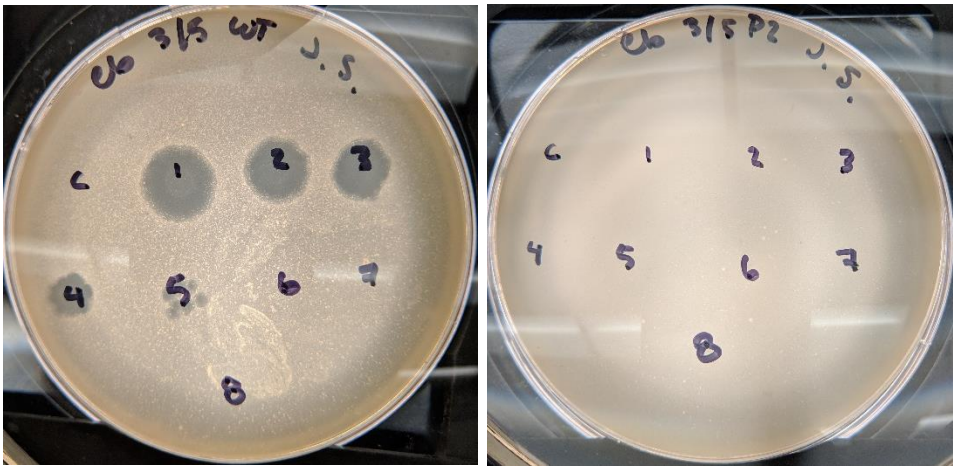


Figure 12a (left) and 12b (right). DOGEMS phage Jerry Smith on WT and Pita2 lysogen. No plaques were observed on the Pita2 lysogen.

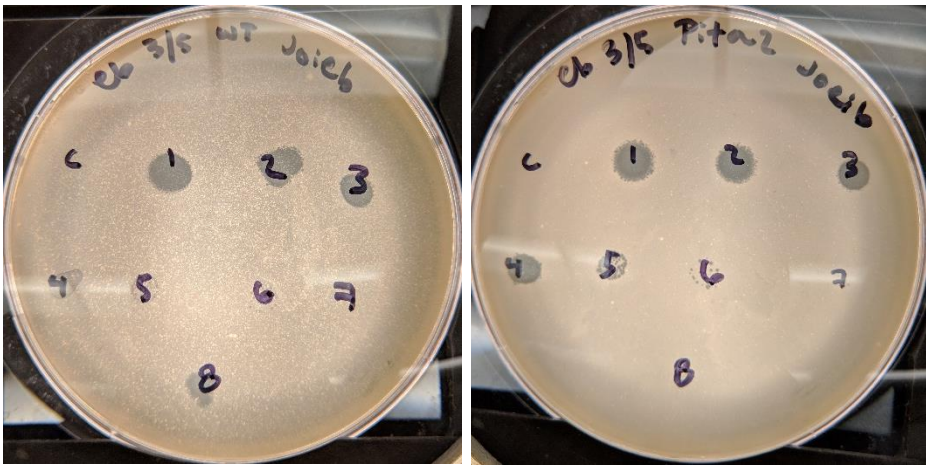


Figure 13a (left) and 13b (right). S phage Joieb on WT and Pita2 lysogen.

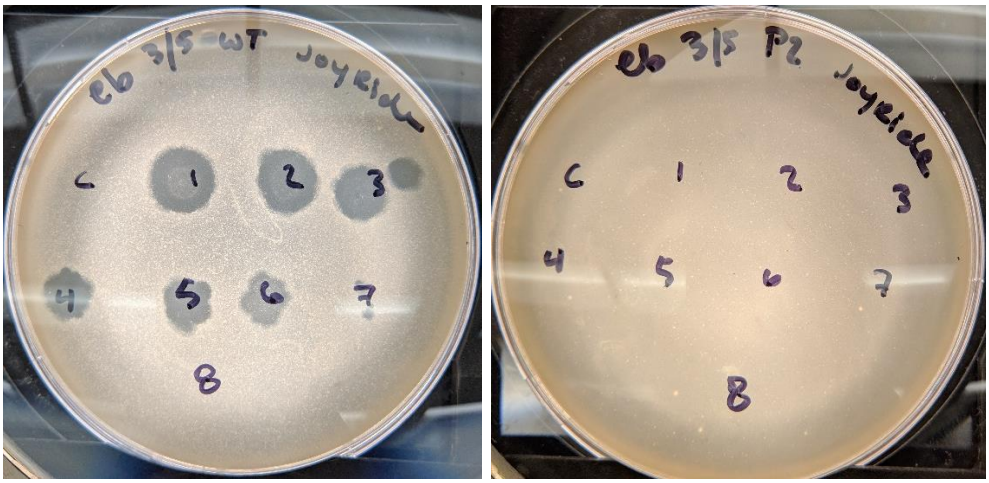


Figure 14a (left) and 14b (right). Joyride on WT and Pita2 lysogen. No plaques were observed when dilutions were plated on the lysogen.

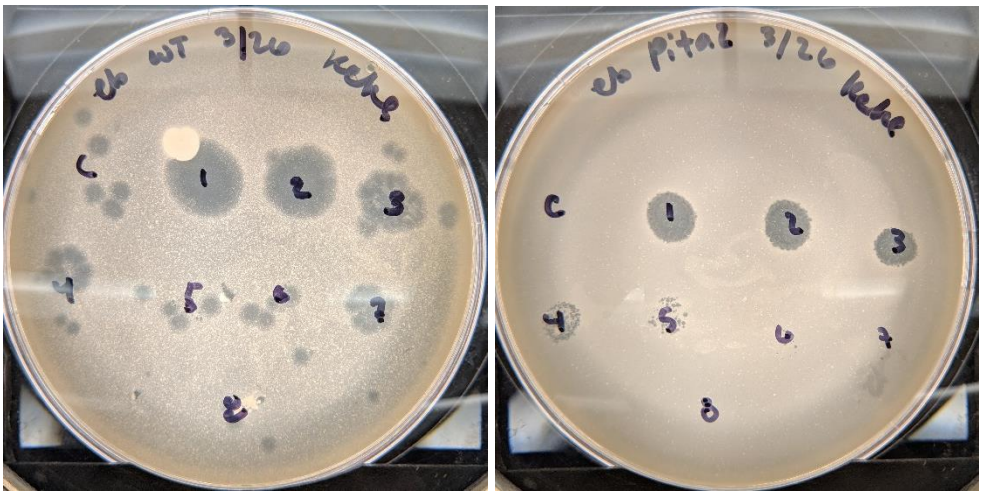


Figure 15a (left) and 15b (right). KekeDoULoveMe on WT and Pita2 lysogen.

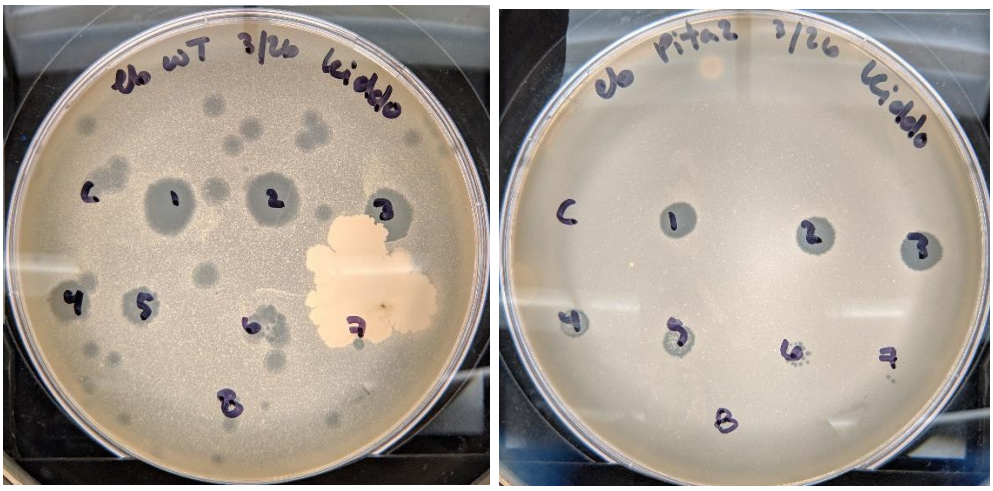
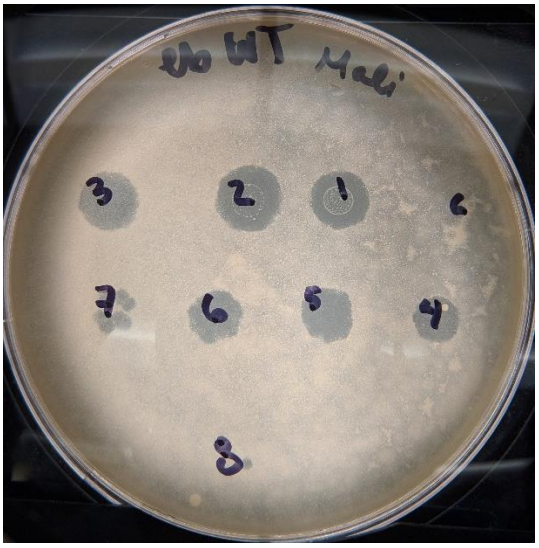


Figure 16a (left) and 16b (right). Kiddo on WT and Pita2 lysogen.



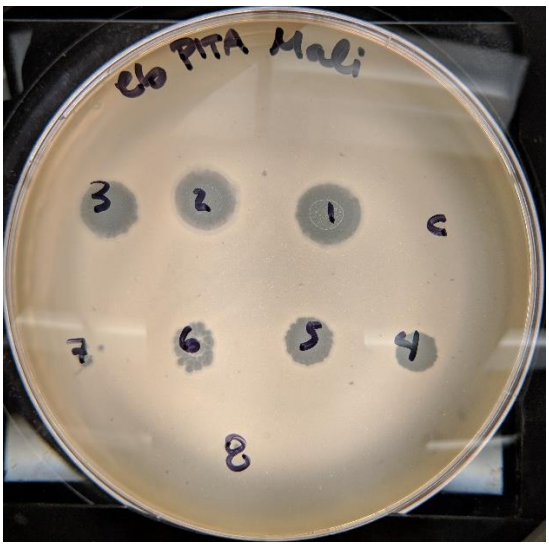
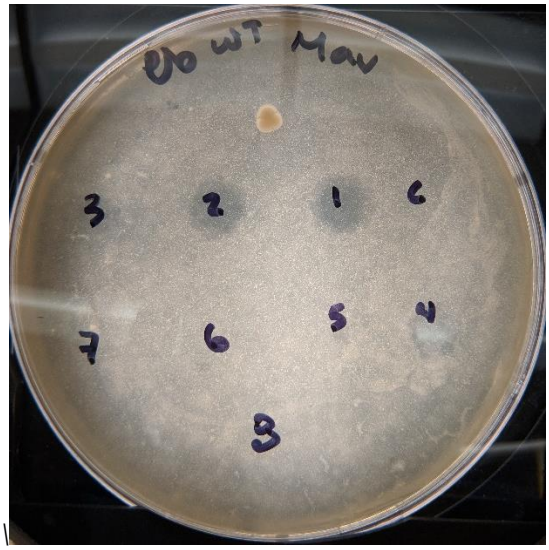


Figure 17a (left) and 17b (right). Maliboo on WT and Pita2 lysogen.



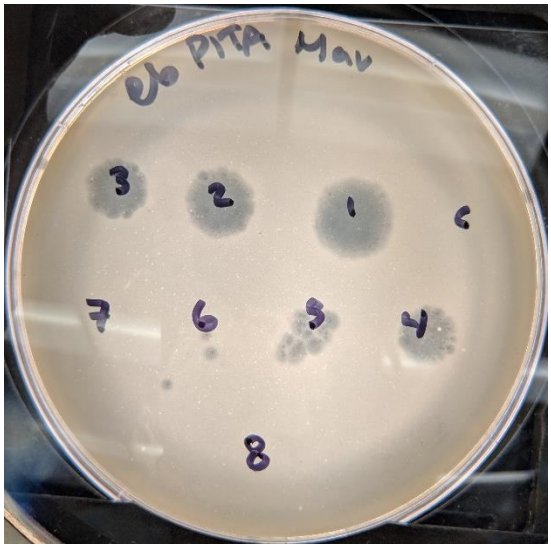


Figure 18a (left) and 18b (right). Maverix on WT and Pita2 lysogen.

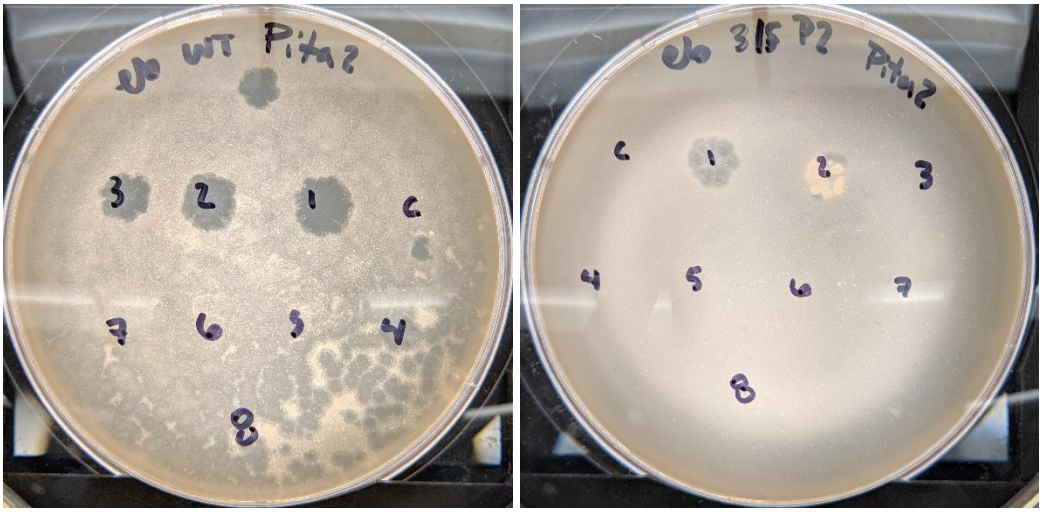


Figure 19a (left) and 19b (right). Pita2 phage plated on WT and Pita2 lysogen.

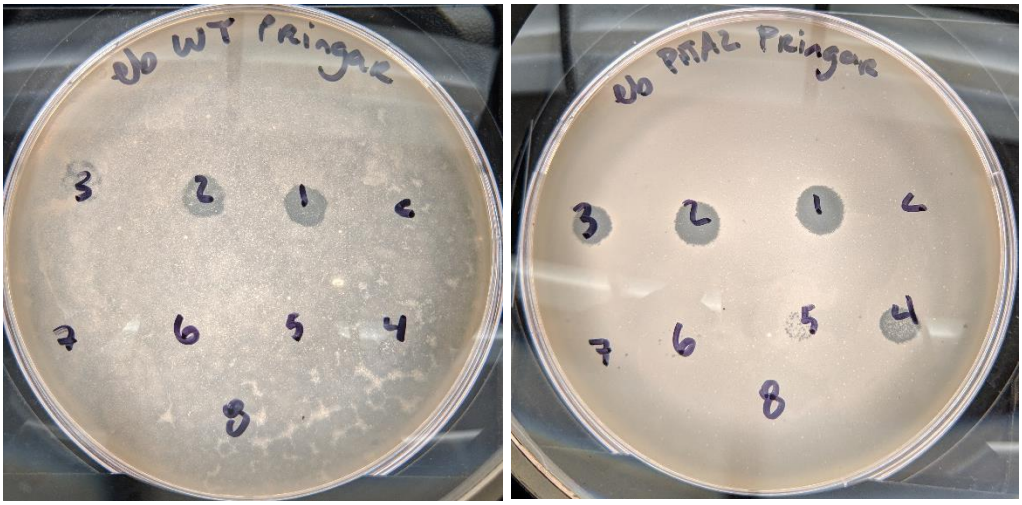


Figure 20a (left) and 20b (right). Pringar, an S phage, on WT and Pita2 lysogen.

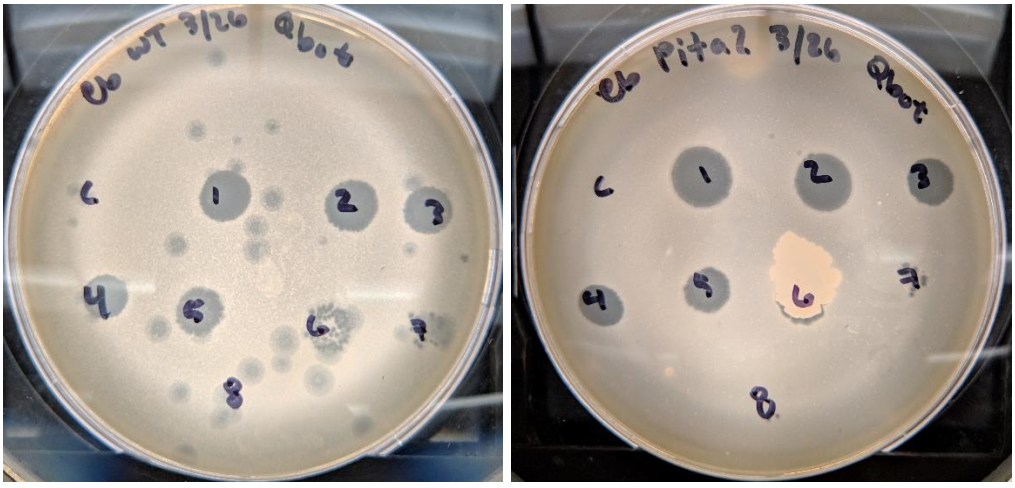


Figure 21a (left) and 21b (right). Qbot on WT and Pita2 lysogen.

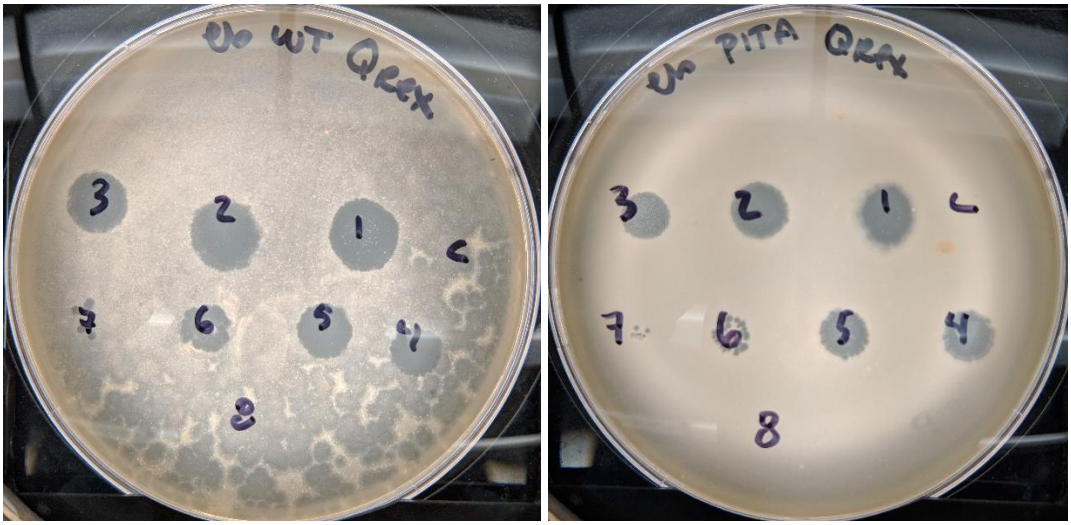


Figure 22a (left) and 22b (right). Qrex on WT and Pita2 lysogen.

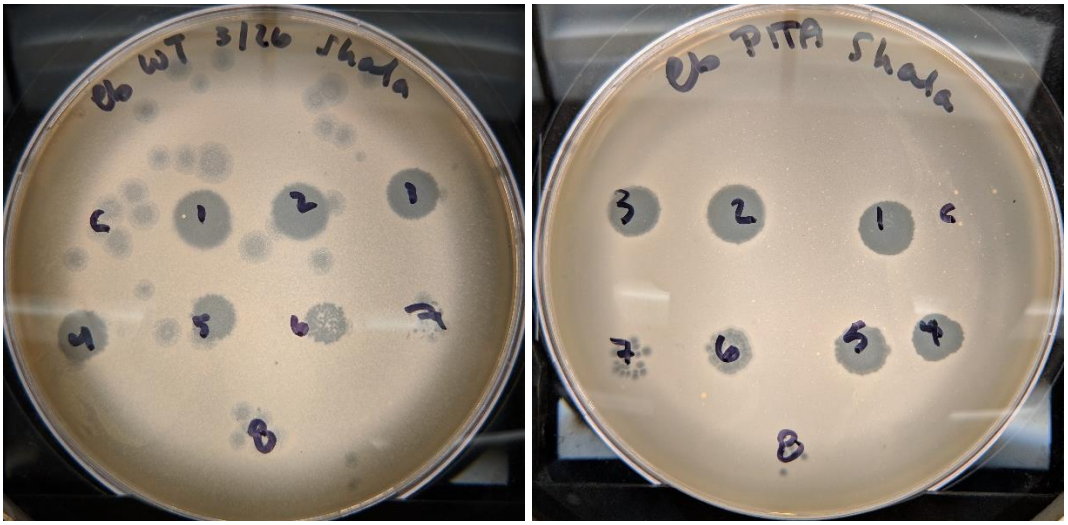


Figure 23a (left) and 23b (right). Shalamarie on WT and Pita2 lysogen

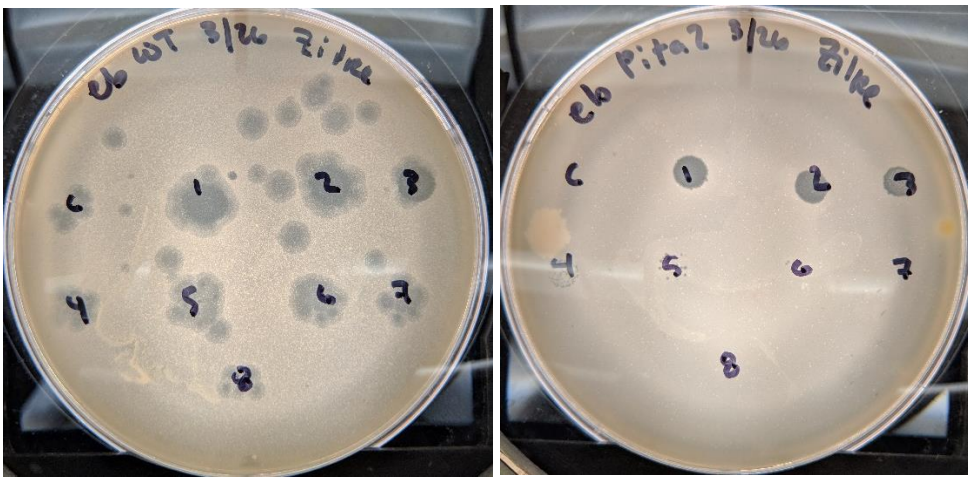


Figure 24a (left) and 24b (right). Zilre on both WT and Pita2 lysogen.

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