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Examining Phage Infection Utilizing Mycobacterium Smegmatis

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A Novel Bacteriophage Tango

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EXAMINING PHAGE INFECTION UTILIZING MYCOBACTERIUM SMEGMATIS

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ABSTRACT

Bacteriophages, also known as phages, are viruses that are ubiquitous and survive and replicate within the host of the bacterial cell, Mycobacterium smegmatis. They are considered one of the most abundant organisms on earth (10^{31}). Structurally, they are 100-200nm in size and consist of a protein encapsulated head that contains DNA or RNA, a tail sheath and tail fibers. This research consisted of examining phage infection, by re-isolating a novel phage, Tango. Tango was originally isolated genetically in 2013 by a previous ISBT student, Anna Maccarrone. The phage was sent to genetic sequencing but two phages were discovered, instead of one, in clusters B and C. The genetic annotation was not able to be analyzed due to phage mutation and impurity. During spring 2015, this phage was re-visited following laboratory protocol specific to this experiment as well as incorporating my own ideas with the goal of re-isolating, purifying and extracting the genetic material using DNASE I and RNASE A. Genetic material extraction would also include electrophoresis, as well as spectrophotometric studies and future DNA sequencing.

INTRODUCTION

Mycobacterium smegmatis, strain mc^2 155, is a non-pathogenic bacterium that grows rapidly within 72 hours and is about 3.0 to 5.0 µm in diameter. It acts as a host for bacteriophages, which are viruses that are ubiquitous and include as many as 10^{31}different organisms since there are considered the most abundant organism on earth. The phages have the ability to infect and replicate within the host and will eventually lyse the bacteria. Temperate phages, especially lambda phages have the ability to enter into a dormant phase, where the genes are not expressed until it reverts to the lytic phase. During the lytic phase, the phage will inject its genetic material into its host and replicate itself until it consumes most to all of the bacteria, thus forming a plaque as evidence.

METHODOLOGY

For this research, I referred to the SEA-Phages lab manual for the protocol for most of this experiment. The procedures that was used to re-isolate Tango are included in this section. Fresh Mycobacterium smegmatis bacterial cultures was used every week (unless otherwise noted). Bacterial growth was allowed to grow between 3-5 days (72 hours max) on a shaking incubator at 37◦C at about 120rpm. All bacteria was tested via streak tests to prove purity. For the duration of this experiment, the bacteria was found pure with no contamination.

Isolating a Novel Phage using the Enrichment Culture Technique

For this protocol, the original soil sample was collected from Argentina located in South America 2013 and stored at 4◦C. It was revisited in spring 2015. The following media was used in order to isolate a novel phage using the enrichment culture technique.

Media

40mL sterile H_2O

5ml sterile 10X 7H9/glycerol broth

5ml AD Supplement

0.5mL of 100mM $CaCl_2$

5mL M. smegmatis culture (dated: 1/23/15-1/26/15)

The media listed was prepared by adding it to a sterile 250ml Erlenmeyer baffle flask with approximately ½ tsp of soil. It was incubated at 37°C on a shaking incubator for a minimum of 24 hours. After 24 hours, the enrichment culture was removed in order to prepare for the next step.[1]

Harvesting and Preparing the Enriched Sample

The next step was to remove the enrichment culture from the baffle flask after 24 hours and transfer it to a 50mL conical tube where it was set to spin in the centrifuge for 10 minutes at 3,000rpm (2,000xg). The reason was to be able to separate the particulate matter (i.e. soil, bacterial cells) from its liquid component. Once the components were separated, the supernatant was poured into another sterile 50ml conical tube to start the process of filter sterilizing it. Using aseptic technique a 5ml filter syringe was used with a 0.22µm filter several times and it was placed into one more 50ml conical tube and labeled 10^0. This is also known as the undiluted enrichment sample. From this point the 10^0 same can be stored at 4◦C or left at room temperature until the next step. I chose to leave my 10^0 sample at room temperature.

The next step involved diluting the enrichment sample using 10-fold dilutions (10^0 through 10^{-4}) which involved using setting up sterile micro centrifuge tubes labeled 0, -1, -2, -3 -4, negative control, positive control. The positive control in this experiment was inactive and was not used until 3/31/2015. In the tube labeled "0", I aseptically transferred about 200µl from the original 10^0 undiluted enrichment sample. In following micro centrifuge tube labeled -1 through -4, I pipetted about 90µl of sterile phage buffer to each tube. Proceeded to complete the serial dilutions by removing 10µl of sample from 10^0 and added it to 10^{-1},

[1] SEA-PHAGES Lab Manual. Part 1 Capture 8.2011

then vortexed it to ensure the contents were mixed thoroughly. Then removed 10µl from 10^{-1} and added it to 10^{-2}, vortexed, then removed 10µl from 10^{-2} and added it to 10^{-3}, vortexed. Removed 10µl from 10^{-3} and added it to 10^{-4}. Figure 1 shows the demonstration of how this is performed.

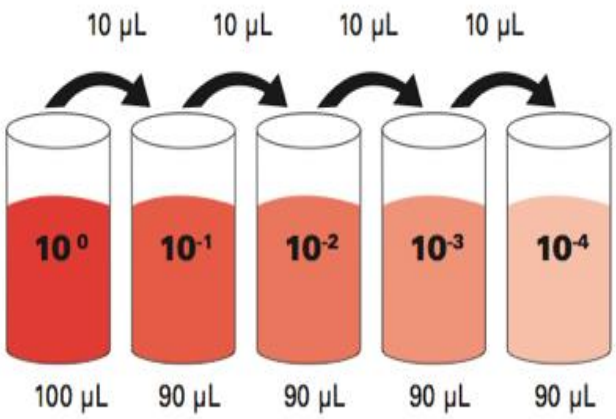


Figure 1: 10-fold Serial Dilution. Source: SEA-PHAGES Lab Manual, Part I Capture 9

Once the dilutions were completed. The next step included a plaque screening, where 50µl of each sample was placed in an aliquot of 0.5ml of Mycobacterium smegmatis, otherwise known as M.smeg. The tubes were labeled along with agar plates. The mixture was allowed to sit at room temperature between 15-30 minutes in order for phage infection to occur by infecting the bacteria. This was followed by adding 5ml of Top agar (TA) to each individual culture tube and poured immediately as an overlay. The plates were swirled for a few seconds and allowed to solidify (anywhere between 5 – 10 minutes), and incubated at 37◦C for a minimum of 24 hours to observe for possible plaque formations.

Serial Dilutions Purify the Phage: Using the Spot Test

This method was used in this experiment to make sure that the plaques that were observed on the plates were actually plaques which is visible by observation of a clear spot or a turbid, cloudy spot. The plaque morphology of the spot is indicative of the life cycle of the phage, if it is a plaque and not just a phenomena or air bubble.

The way that this procedure was done included pipetting about 5ml of top agar into 0.5ml M .smegmatis culture tube and pour directly onto an agar plate.

The plate should sit between 5-10 minutes to allow for the media to solidify. Next, I used a sterile micro centrifuge tube and added about 100µl of phage buffer followed by using a sterile wooden stick to swipe the surface of the chosen plaque. I swirled the stick into the phage buffer and then vortex it for thorough mixing. From there, I removed 5µl of sample and placed it on a designated area of the plate and allowed the spot to dry, which can and often did take more than 10 minutes to dry prior to inverting the plate and placing it into the incubator 37◦C for a minimum of 24 hours.

Following 24 hours, I checked on the status of the plates to observe for plaque formation. Results will be indicated in that part of this report.

Purify the Phage: The Phage Titer Assay

This assay was used to help determine the concentration of phage particles (pfu- plaque forming units) in a solution, in order to prepare for obtaining a high titer lysate. A titer is the number of plaque forming units on an agar plate per ml (pfu/ml) from the phage sample.

For this assay, I aseptically, took 100µl of phage buffer and added it to a sterile micro centrifuge tube. I picked a plaque from a spot test (2/11/15, "B")[which eventually led to a web plate, see results section, using a sterile wooden stick and placed it into the phage buffer. Swirled the stick and then vortexed it for thorough mixing. This procedure also required completing a 10-fold serial dilutions of 10^0 through 10^{-4} where I added 90µl of phage buffer, aseptically to the dilutions labeled 10^{-1} through 10^{-4}. From there, I proceeded to remove 10µl of sample from 10^0 and added it to 10^{-1} and vortex. I continued this method, also shown in Figure 1, until the dilutions were completed. The next step was to allow the mixture to sit at room temperature for 15-30 minutes and then proceed to add 5mL of Top Agar to each individual tube. This was followed by pouring the tubes onto agar plates as an overlay which was allowed to solidify between 5 to 10 minutes. Once solidified, the plates were inverted and placed into the 37°C incubator for a minimum of 24 hours. The next step was to check for plaques, preferably clear, lytic ones in order to count the pfu and observe for a web plate. Once this calculation has been calculated, it is considered your titer and no longer your assay.

Titer Calculation: Titer (pfu/mL) = (pfu/#µL) X (1000µl/mL) X dilution factor[2] (lab manual, Part 1 Tame 13, 2011)

Final Plaque Purification

For this procedure, the goal is isolate enough phage that is pure and not of a mixed population of other phages in order to complete the empirical test. First step was to harvest a phage lysate, from my chosen web plate which was about 13ml lysate, 10^{-4} from plate dated 3/23/15 and 6ml lysate (10^{-2}) from plate dated 3/26/15). Once the plate was chosen, I aseptically added 8ml of phage buffer and allowed it to sit in the fridge at 4°C overnight. The lysate labeled 3/27/15 was collected by inverting the plate to remove the lysate from the lid via a 5ml syringe and 0.22micron filter. It was then placed in a sterile 15ml tube and stored in the fridge. The other lysate that was collected, was also collected using the same method and also stored in the fridge until it was ready to be used.

The next step was to test the lysate by taking an agar plate and dividing it into ten sections. The reason was to perform a spot test on the phage lysate to test for purity and similar plaque morphology. The plate was then labeled **1**0^0 through 10^{-10}. I proceeded by adding 5ml of top agar to 0.5mL Msmeg as an overlay and allowed to solidify for a minimum of 10 minutes. In the interim, I removed about 100µl from my medium titer lysate (3/26/15) and completed serial dilutions by removing 10µl from my 10^0 lysate and added to 10-1

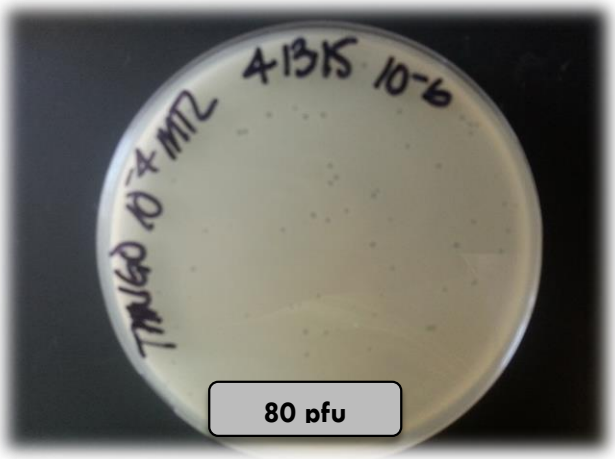
[2] SEA-PHAGES Resources Guide. Part 1 Tame 13, 2011.

and so on, up to 10-10 using sterile micro centrifuge tubes. Once complete, I removed 5µl from each tube and added to the designated area and also added 5µl of phage buffer as my negative control. The plates were allowed to dry for a minimum of 10 minutes, they were inverted and placed into the incubator at 37◦C for a minimum of 24 hours.

Prepare Empirical Testing of Phage Lysates

To prepare the dilutions there is a series of calculations that were used which included measuring the diameter of the plaque and the diameter of the plate in mm.[3]

Phage Titer (calculated from 10^{-6} plate dated 4/13/15)



This plate had 80pfu. Calculated the phage titer.

80 X 1000 X 1,000,000

=80 X 100,000

=8.0 x 10^6 pfu/ml or 8,000,000 pfu/ml

Radius (mm) = 0.5 X diameter= r

Plaque: 0.5mm X 1.0mm = 0.5mm
Plate: 0.5mm x 8.5mm= 4.25mm (or 42.5)

Area (mm^2) = πr^2

Plaque: π (0.5 mm)^2 = .7853981634
Plate: π (42.5)^2 = 5674.501731

Pfu (max web plate) = Area plate (mm^2) divided by Area plaque (mm^2)

5674.501731/.7853981634
= **7225.000001 or 7225.00 max web**

[3] SEA-PHAGES Resources Guide. Part 1 Tame 21.2011

Volume (ml) phage stock = pfu desired/pfu (per ml phage stock)

=3000/8,000,000= 3.74 X 10^{-4} ml or

= **0.000374ml or 0.0374 µl**

80pfu/50 µl X 1000/1 ml X 106 = 1.6 X10^{10} Titer

Titer: 1.6 X10^{10} pfu/ml

Vol (ml) = 7225/1.6 X10^{10} = 4.5 X 10^{-7} µl

(Recall, *7225* is from the max web calculation)

4.5 X 10^{-7} ml X 1000 µl/ 1ml = **4.5 X 10^{-4} µl/ 10** 10^{0}

Therefore,

.0045 µl = 4.5 X 10^{-3} µl 10^{-1} (amount too small)

.045 µl = 4.5 X 10^{-2}µl 10^{-2} (amount too small)

.45 µl = 4.5 X 10^{-1} µl 10^{-3} (amount too small)

4.5 µl = 4.5 X 10^{0} µl **10^{-4}** Chosen Titer

Therefore, 4.5 µl Tango (10^{-4}) and 95.5 µl phage buffer was used for the titer on the plates dates 4/15/15 (10^{-4}) to see if a series of web plates would be obtained.

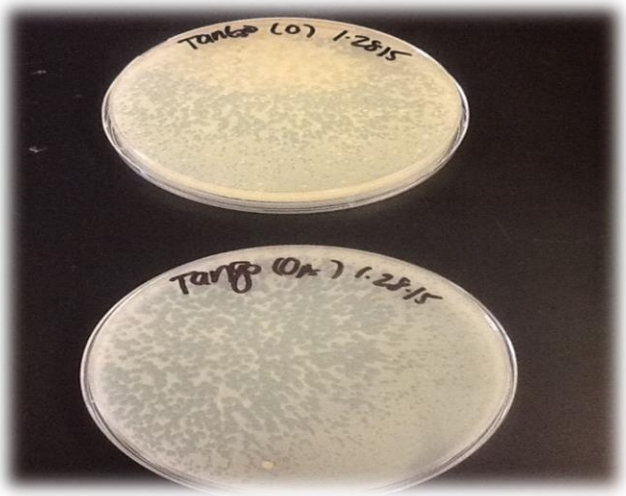
Innovative Research: A non-protocol approach

One of the interesting things about research, is to be willing to test the phenomena out to find out what it will do, which can lead to new understandings and discoveries. In fact, I did just that. A spot test from 10^{-2} was placed in the fridge after 48 hours in the incubator. The spot test remained uncontaminated and its life cycle stayed temperate and would not switch to lytic. What's interesting is that the plate became frozen and had yellow, transparent fluid (~ 11ml) in it, similar to the fluid found in a medium titer lysate. I decided to test it out, under the guidance of my instructor, Marsha Timmerman, M.S. and Dr. Nancy Jones, Ph.D. I serially diluted from 10^{0} to 10^{-5} (using the protocol found in Figure 1.

Another method that I chose to see if this phage would shift its life cycle was by alternating the sample amount in a serial dilutions set from 3/20/15. Meaning, the typical amount removed from an undiluted sample is about 10µl, I spent some time altering the amounts to 20µl, 50 µl, 75 µl, and 90 µl, which was all added to 90 µl of phage buffer (refer to Figure 1 for protocol) - all in an attempt to observe what the phage would do. The discovery of both of these experiments are found in the results section of this report.

A final attempt at innovation occurred when I tested the "A" and "B" portions from a spot test and serial diluted them to 10^{-4} using 90µl of phage buffer (10^{-1} through 10^{-4}). See phage titer assay for similar protocol. The phenomena for this portion is found in the results section.

RESULTS

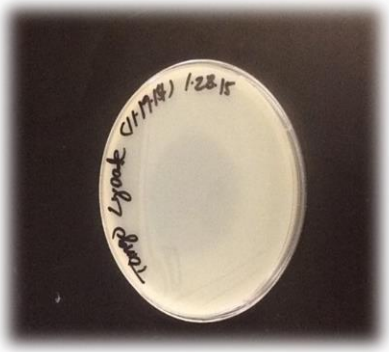


From this experiment, the results that Tango yielded was consistent to yield two different plaque morphologies- migrating lytic plaques (small in diameter) and temperate, circular plaques (medium in diameter).

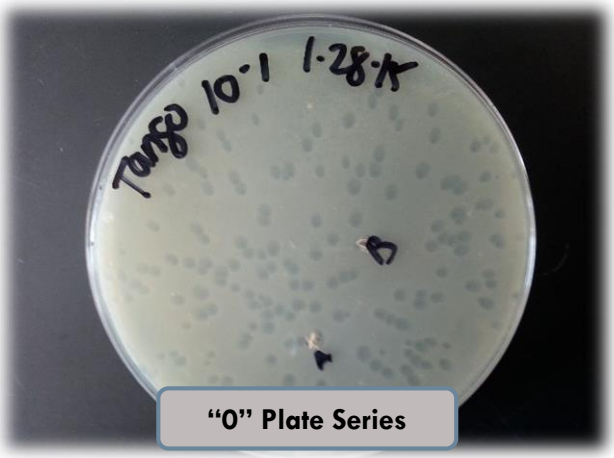
In the initial stages, the original enrichment sample from (10/31/13) was re-filtered and labeled "0A" and the new enrichment sample that was used -- labeled "0" (1/27/15-1/28/15). Both of these were plated using serial dilutions and both yielded similar results in plaque morphology. However there was cloudiness towards the end. The plaques are both lytic and migrating. The enrichment labeled "0" was used for the rest of the experiment (unless otherwise noted).

What this proves is that phage within the enrichment sample dated from 10/31/13 can survive and still be active for at least one year. In addition, the contents from the original soil sample also proved that phage can still be active in the soil despite changes in temperature.

The original high titer lysate (AM lysate) dated from 11/19/2013 was also tested and there was a bacterial lawn present. What was odd was a large, faint clearing, which could be indicative of a temperate phage. However, since I didn't see plaques similar to that found on plates "0" and "0A," I set it aside for further analysis. This lysate was retested on 3/27/15 by doing serial dilutions from 10^0 to 10^{-7}. Here are a few images from the dilutions, which prove that the lysate is still active after a year.

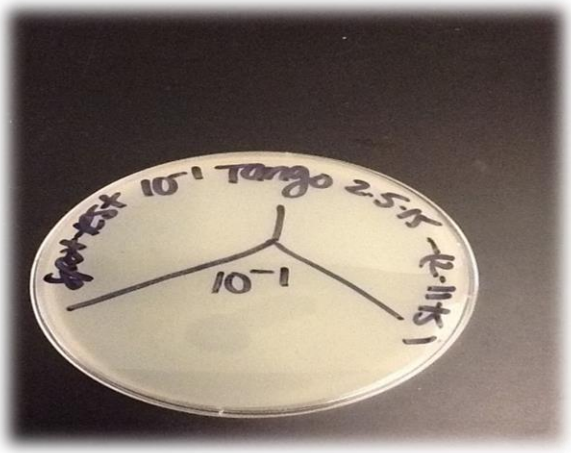


These are the result from the beginning of the spot test completed. The spot test was taken from the plate of labeled 10^{-1} from the "0" plate (although not noted). The plaques are clear and lytic.

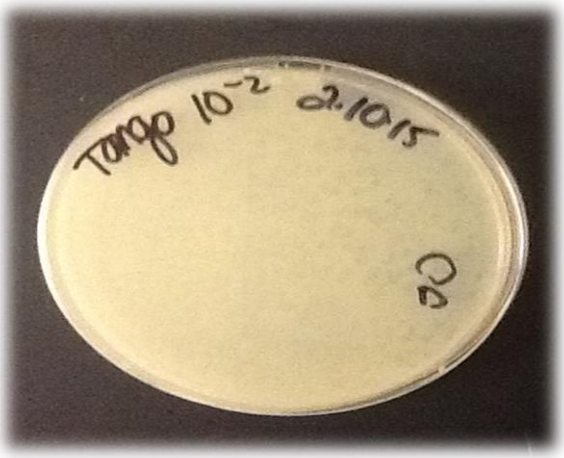


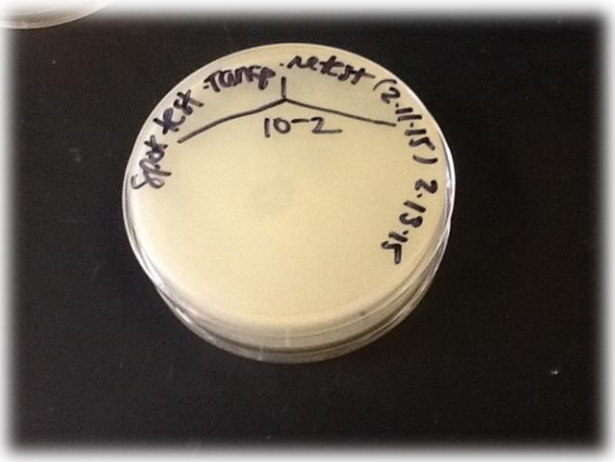
"0" Plate Series

These results are from "0A" plate series (10^{0} through 10^{-4}) which continued to produce turbid and faint plaques and most of the month of February 2015 was spent in an attempt to shift the life cycle from temperate (cloudy plaques) to lytic (clear plaques). So, a spot test was done to make sure these were plaques and were pure. The spot test 10^{-1} was taken from the plate 2/5/15 spent extra time in the incubator in hopes of triggering a life cycle switch and completed on 2/11/15.



These are the results from the "0" plate series (10^0 through 10^{-4}) which also produced turbid plaques. The plate on the left has two circles marked, I chose one of them and used the spot test method on the plate to the right. The results were similar, in terms of being turbid.



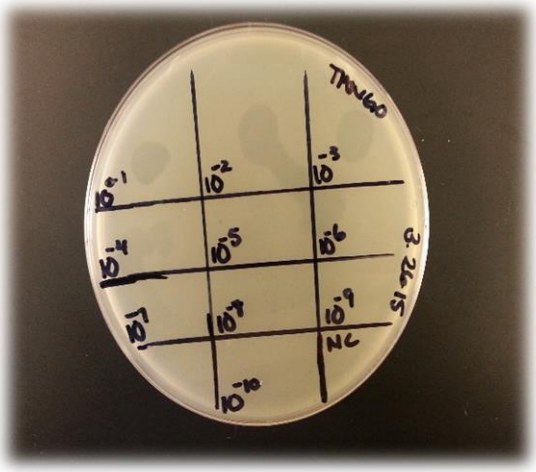


Revisiting the dilutions, discussed earlier in the Innovators Research section, there were various dilutions (20µl, 50 µl, 75 µl and 90 µl used in an attempt to see if Tango would produce enough plaques across a serial dilution set (10^0 -10^{-4}). I also tested bacteria that was not the freshest to observe how the phage would react.

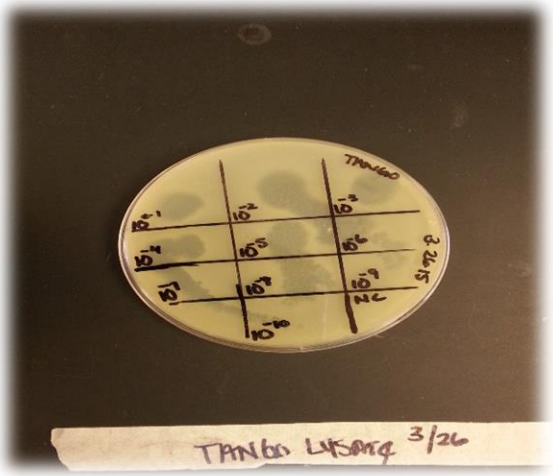
The results that was observed was a bacterial lawn with no plaque morphology and no contamination. This prompted some further experimentation to identify what would work since Tango started to not produce plaques.

Since the spot tests from A and B were lytic initially, they began to look a little turbid as indicated on the plate below (far left). They were placed in the fridge, wrapped in Para film. They were re-visited in March 2015 and were found to be frozen with liquid within them. I chose not to use the liquid from the plate with "A" and "B" but collected the fluid on 3/26/15 from the plate below, 10^{-2} to test it out—The same plate that was shown in the methodology section of this report.

The fluid from the frozen plate on the previous page dated 2/11/15 (10^{-2}) was filtered and serially diluted from10^{0} through 10^{-4} and a web plate was obtained. The web plate was flooded with phage buffer and the contents collected were labeled (6ml MTL lysate 10^{-2} 3/26/15). The plate below indicates the results from using the spot test method to check for purity. Below is the result from serially diluting the lysate from 10^{0} through 10^{-10} followed by using the spot test method. As a result, the plaques are lytic and round except for some overgrowth onto other areas of the plate which occurred between 24-48hours, where the plaques are more prominent on the photo on the right. It can also be noted that there are individual plaques on 10^{-6} and 10^{-9}. A negative control was also done which produced a nice bacterial lawn and no signs of contamination.

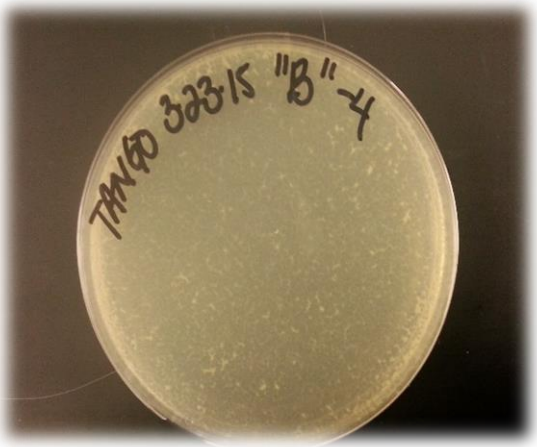


~24 hours

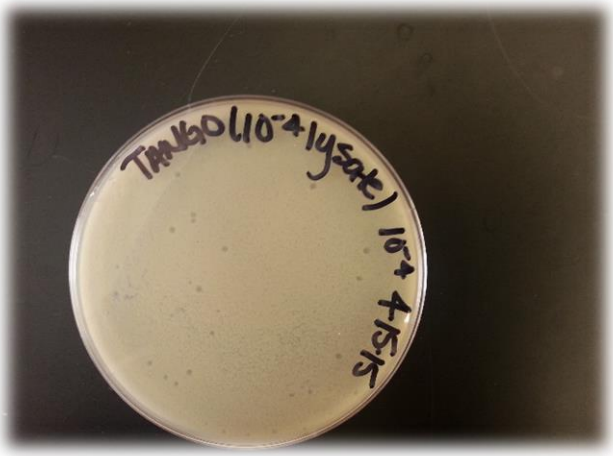


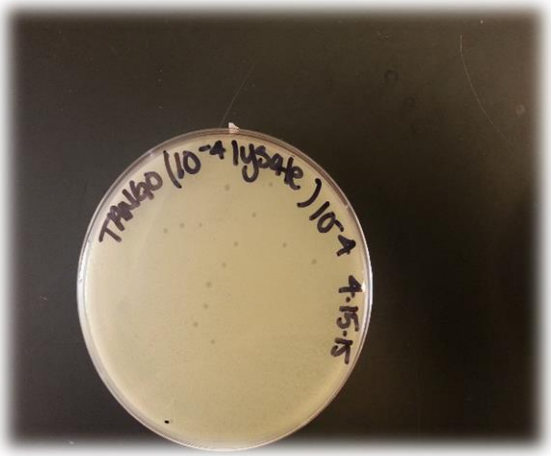
~48 hours

Here are the results from several web plates that Tango was able to produce. The plate on the left was flooded using phage buffer and medium titer lysate was collected (10^{-4}) from a spot test that was serially diluted from 10^{0} through 10^{-4}.

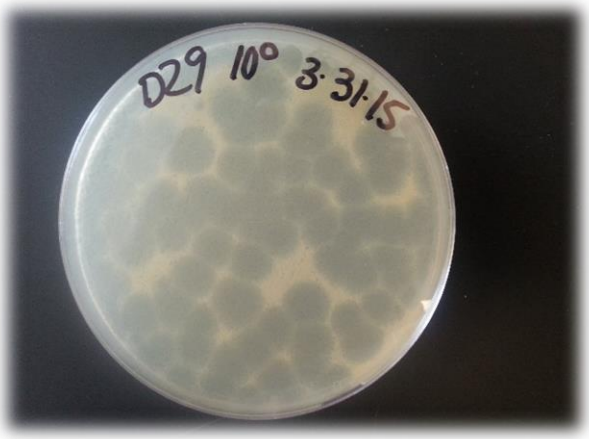


Referring back to the titer that was calculated earlier. The results are shown from a five plate infection which yielded similar results to the plates indicated below—included two plates out of the five plate infection. Unfortunately, the titer did not produce enough plaques for a web plate. Therefore a high titer lysate was not able to be obtained as of yet.





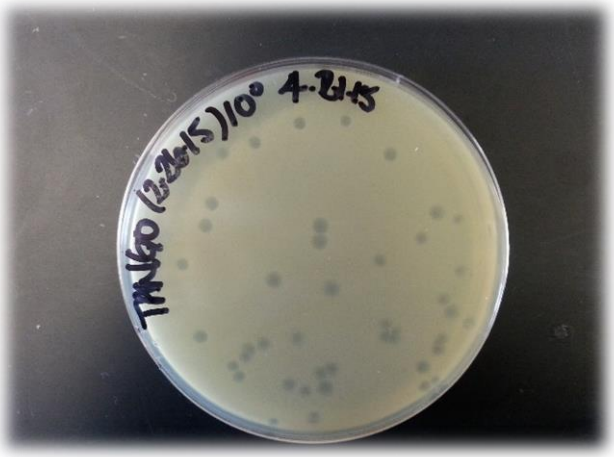
In terms of results from the controls that were completed during the experiment. From several of the controls that were used during the experiment, here is an example of expected results. On the right, is an image of a typical negative control that yielded good results — a bacterial lawn and no contamination. The positive control (aka D29)[4] is a phage but was initially inactive and was plated on 3/31/2015, which produced large lytic plaques from 10^0 (in pure form) but the results remained intermittent and not consistent.

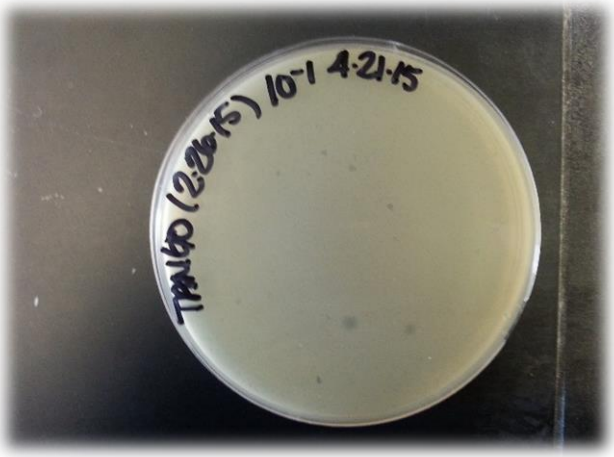


[4] The Actinobacteriophage Database | Phage D29. (n.d.). http://phagesdb.org/phages/D29

On a separate note, Tango has been consistent with producing migrating plaques that were initially lytic from both samples of "0" and "0A" but then there was a shift and they started producing turbid, circular plaques. It was understood from the beginning that there were different clusters (B and C). Revisiting the spot tests from earlier, the plaques began to produce lytic, migrating morphologies which stayed consistent from experiments conducted on both lysates 10^{-4} as well as 10^{-2}. However, from using Innovator's Research, there is a very high probability that the clusters have been separated.

These are the results from a serial dilution set that was plated from 10^{0} to 10^{-7} on 4/21/15 from a plate (2/26/15) that is not able to be located but in this case, only 10^{0} and 10^{-1} produced lytic plaques, circular in shape yet different from the initial "0" and "0A" plaques. It is most likely evident that the clusters have a high probability of being separated based on plaque morphology but genetic sequencing would have to be done in order to finalize this.





DISCUSSION

Throughout this research, it was discovered that this bacteriophage, Tango has been able to survive from the original enrichment sample as well as from its high titer lysate that was originally isolated in November 2013 and tested for thirteen weeks—between January- April 2015. It was discovered that Tango has two clusters, which is indicative of two phages that needed to be separated. The phage morphology difference indicated this, as the initial serial dilution proved Tango to produce lytic, migrating plaques and as the experiment continued, circular, turbid plaques were produced. There was also the challenge of having Tango to stay consistent with its serial dilution plaque morphology. There were many incidences in February where there were either turbid plaques that were produced or no plaques produced. Other incidences of lytic plaques, were mostly prominent on the spot tests (following the initial serial dilutions from 1/28/15)

The chance arose to be innovative when the spot tests became frozen when stored in the fridge. It was odd that the fluid was not clear but was transparent and yellow in color. I decided to test it and received the confirmation from my professor. The fluid removed was filtered, serial diluted and plated. It produced consistent plaques as well as a web plate. That was the turning point of the experiment and Tango was able to produce abundant plaques. Another lysate was also collected from the spot test "B" which was serially diluted and produced a web plate on (10^{-4}) in 3/2015. The phage titer was calculated in April 2015 but did not produce enough plaques to infect the five plate series, which was needed to eventually produce a high titer lysate and obtain the DNA analysis.

In addition, there is strong probability that the phage morphology has been separated from its combined clusters, B and C by proof of plaque morphologies. It has been observed that the plaques were in the lytic life cycle and their morphology changed from migrating, oblong plaques to circular plaques. This strongly suggest that the clusters have been separated.

Working with a phage that underwent numerous mutations in this experiment was very challenging. It was hard to figure out what to expect from day to day. Observing Tango's plaque morphologies was an indicator for co-evolution/mutations and whether the phage was multiplying to produce abundant plaques, remaining dormant or had a slow growth rate. Factors in observing the growth rate was highly attributable to the growth of the bacteria, Mycobacterium Smegmatis, which was made fresh every week. The only time fresh bacteria was not used, was in the case of altering the dilution amounts, which was for experimental purposes only—to see what the phage would do. It became evident that there were no plaque morphologies; only a bacterial lawn.

During this research, I could have incorporated a streak test protocol in addition to the spot test to further observe for either purity or contamination. Contamination was very minimal in this experiment. Other factors that was a concern was the positive control. The positive control, aka D29 is a phage that is used via protocol. The control was inactive for most of the experiment, which yielded results on 3/31/2015 but still was not consistent. Lastly, it was unfortunate that I was not able to perform the DNA Analysis and spectrophotometer readings for Tango since I needed a high titer lysate but in the future, I have hopes of re-visiting and continuing to work on this phage.

CONCLUSION

Tango is a novel bacteriophage that was successful in remaining active since 2013. It was able to produce both lytic and temperate plaques as a result of its life cycle. Phages are able to produce plaques that are visible on agar plates as a result of phage infection. Phage infection occurs when the phage finds a receptor, i.e. protein on a bacterial cell and attaches itself—this is called adsorption. Once attachment occurs, the process is irreversible and the phage is able to inject its genetic material from its capsid head, through its hollow tube and into the host (bacterial cell). From that point, the phage will continue to make replicas of itself, until the bacterial cell bursts (lyse) and the process continues, which is evident from the plaque morphologies, which will either be clear. On the other hand, if the phage enters the temperate (lysogenic) life cycle. This is when the phage enters into a dormant phage because the DNA from the phage will combine with the bacterial chromosome which gets passes on to its daughter cells and becomes a prophage via lysogeny. However, during this phage, with the right conditions, the lytic cycle can be triggered and the phage will begin to produce clear plaques.[5] This is what occurred in this experiment that made it such a discovery was having Tango change from a temperate phage, although not initially the case, to a lytic phage from temperature change. This was evident from the frozen plate.

This research has resulted in several discoveries while working with Tango.

1) The high titer lysate that was collected in 11/2013 is proved to still be effective and active as indicated by clear plaques on agar plates dated 1/28/2015. Proving that lysate can survive over a year
2) Phage can shift from a temperate (dormant) life cycle to a lytic life cycle with the right or adverse conditions (i.e. temperature change)
3) Phage separation is possible through purification via spot tests and serial dilutions to separate the different plaque morphologies
4) Phage mutations and co-evolution are inevitable and can lead to future research in phage therapy and disease.

[5] SEA-PHAGES Resources Guide. Part 1 Capture 4. 2011

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