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The Bacteriophage “fabiolayun”

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Introduction

A virus is an infectious particle that consists of genes packed in a protein coat. A virus is not considered a living organism because it lacks the structures and metabolic machinery of cells. Thus, a virus cannot reproduce or carry out metabolic activities outside of a host cell (Reece *et al*., 2011). In order to reproduce, a virus must utilize the host cell’s replication mechanism by infecting and inserting viral DNAs, carried in capsids, into the host cells. A capsid is the protein shell enclosing the viral genome, and the shape of a capsid varies depending on the type of virus (Reece *et al*., 2011). It is observed that many of the most complex capsids are found in bacteriophages, a specific type of viruses that infect bacteria as their host cells (Reece *et al*., 2011). The typical morphology of a bacteriophage includes a hexagonal head, a tail, and tail fibers.

The more rapidly reproduction occurs, the quicker a change in gene expression will appear: evolution is a result of a change in gene expression of a population. The rapid reproduction cycle and relatively simple genomes of viruses often promote speciation of viruses. Even a small change in genetic variation can lead to a significant change in appearance and behavior within a rapid reproduction cycle. According to Morris *et al*. (2008), in mycobacteriophages, bacteriophages that target *Mycobacterium* as their hosts, it is observed that the gene order (genome organization) can be repeated or arranged in a different order from one species to another. Once a new species of bacteriophages is captured and observed, an independent experiment can be used in order to distinguish unique traits of the phage.

Recently, the study of bacteriophage has been receiving great attention in research. The reasons include genetics, epidemiology, and therapeutics (Asai *et al*., 2013). Often, phages are used as tools to move DNA around for cloning, mutation, and other laboratory techniques (Asai *et al*., 2013). The study of bacteriophage is not just about the comparison of the phages or identification of a new phage. Scientists also study biodiversity by observing and analyzing bacteriophages in order to understand environmental patterns of evolution (Asai *et al*., 2013). Bacteriophages are a perfect fit for studying environmental patterns of evolution due to their ubiquity in nature and rapid reproduction cycle. In addition, it is possible that bacteriophages could be used to kill specific antibiotic-resistant bacteria that cause disease (Asai *et al*., 2013). For instance, *Mycobacterium tuberculosis* causes tuberculosis (TB), a deadly disease that attacks the lungs. Within past 10 years, *M. tuberculosis* has become resistant to anti-tuberculosis agents (Gillespie, 2014). In order to continue the research on *M. tuberculosis*, *Mycobacterium smegmatis* is frequently used at the lab because *M. smegmatis* is believed to be a distant cousin of *M. tuberculosis*, genetically (Flores *et al*., 2005). Also, *M. smegmatis* is generally considered a non-pathogenic microorganism (Reyrat & Kahn, 2001).

Studies in the past strongly suggested that one of the bacteriophages’ characteristics is calcium-dependency. Over a long time, it has been believed that a calcium ion is required for bacteriophages to infect bacteria (Shafia & Thompson, 1964). According to Lanni (1960), it is possible for a bacteriophage to have two phases: calcium-dependent and calcium-independents phases during its lifespan. Insertion of phage DNA material into the host cell is highly affected by divalent ions, and the inhibition of infection can be controlled by calcium ions (Shafia &

Thompson, 1964). In this particular experiment, there were two major objectives: discovering a new phage and understanding its profound significance. A new phage from the soil sample was studied, analyzed, archived, and reported to the University of Pittsburgh. Furthermore, an independent experiment was conducted in order to determine if the newly found phage, named "fabiolayun" is calcium-dependent when infecting bacteria, and if not, what other metal divalent ions could replace calcium ions. There were three different types of divalent ions that were used in the phage buffer and top agar solution: Mg^{2+}, Ca^{2+}, and Mn^{2+}. Throughout the entire procedure, the host organism was *M. smegmatis*.

Materials and Methods

Collecting environmental sample, preparing the enrichment culture, and harvesting the enriched sample

One gram of the soil sample was mixed with pre-made enrichment solution (50 mL of 1X7H9/glycerol broth, 5 mL of AD supplement, 500 μL of 100 mM $CaCl_2$, 40 mL of H_2O, and 5 mL of late stationary phase *M. smegmatis* culture) in the Erlenmeyer flask. The soil sample was shaken at 220 rpm for 48 hours at 37 °C and centrifuged at 3,000 rpm for 10 minutes to pellet particulate matter, including most of the bacterial cells. The supernatant was filtered by a syringe and a filter (0.22 μm) -- this was the undiluted enrichment sample.

The dilution technique

As directed by the lab manual by Asai *et al*. (2013), four diluted phage samples were made by adding 10 μL of previous dilution sample to 90 μL of the phage buffer until it reached $1{:}10^4$ (positive control=an aliquot of phage; negative control=the phage buffer).

Plaque screening with the plating technique

With the plating technique (Asai *et al*., 2013), 50 μL of each dilution, including controls, was separately added to 0.5mL of *M. smegmatis*. The mixtures were vortexed and allowed to infect for 20 minutes. Lastly, each sample was mixed with 4.5 mL of top agar and was poured into a petri dish, appropriately labeled. The plates were incubated for 24 hours in a 37 °C incubator.

Capturing to taming

After two rounds of plaque screening, no plaque was observed. In case there were just too many plaques, for the second round, the purifying step was preceded by selecting a random spot on the plate that was incubated. However, no plaque was observed after the purification; two plaques were then borrowed from Madison Ochs and named as FY1 and FY2.

Purifying the phage

According to the plaque streak protocol (Asai *et al*., 2013), the phages were purified by three streaks per a plate. For each streak, a new sterile stick was used to streak an adjacent un-streaked area, approximately one third of the total area. The third streak was the most diluted and marked. After streaking, the mixture of 4.5 mL top agar solution and 0.5 mL of aliquot of *M. smegmatis* was poured from the most dilute area to the most concentrated area (Asai *et al*., 2013). The plates were incubated for 24 hours in a 37 °C incubator. This procedure was repeated at least three times.

The phage-titer assay

After one phage was selected, with the dilution technique (Asai *et al*., 2013), the titer of the phage was calculated according to the formula in the manual (Asai *et al*., 2013), which involved counting the number of plaques on a plate within a range from 20 to 200 and finding the appropriate dilution.

The web-plate technique: making a Medium-Titer-Lysate (MTL) plate

The estimated amount of dilution sample (original estimation) was calculated in order to make a web plate based on the phage-titer assay (Asai *et al*., 2013). The calculation involved setting up a ratio between the area of dish and the area of plaque. As directed by the manual (Asai *et al*., 2013), four more estimates were calculated based on the original estimation: ten times above, five times above, five times below, and ten times below of the original estimation. The five estimated dilutions were plated according to the plating technique (Asai *et al*., 2013). The plates were incubated for 24 hours in a 37 °C incubator and checked every 24 hours.

The lysate technique

Out of the five plates, the selected MTL plate was flooded with 8 mL of the phage buffer for about four hours. The lysate was filtered by a 0.20 μm filter. The dilution technique (Asai *et al*., 2013) was used to reach $1{:}10^{10}$. The MTL titer was calculated (Asai *et al*., 2013). Each dilution of 5 μL was used (negative control= 5 μL of the phage buffer).

Making ten High-Titer-Lysate (HTL) plates

By using the MTL lysate, a HTL plate was made using the web-plate technique as guided by the manual (Asai *et al*., 2013). The procedure was repeated nine more times in order to produce nine more HTL plates.

The High-Titer-Lysate (HTL)

With the HTL plates, the HTL was made using the lysate technique (Asai *et al*., 2013). The dilution technique (Asai *et al*., 2013) was used to reach $1{:}10^{12}$. The HTL titer was calculated (Asai *et al*., 2013). Each dilution of 5 μL was used (negative control= 5 μL of the phage buffer).

Analyzing phage using electron microscopy

By using the electron microscopy procedure in the manual (Asai *et al*., 2013), a PELCO tab and a fresh grid were used: 10 μL of the HTL was absorbed by the grid, washed away twice with water (10 μL), and stained with 10 μL of 1.0% uranyl acetate. Each step required a two-minute waiting period. The grid was taken to the electron microscopy facility.

Isolating and purifying phage genomic DNA

DNA from the phage was isolated using the Promega Wizard DNA Cleanup kit (10 mL of the HTL, 40 μL of nuclease mix, and 6 mL of the phage precipitate) as directed by the manual (Asai *et al*., 2013). In order to lyse the phage heads and release the DNA, precipitated phage pellets were re-suspended in the kit’s Clean Up Resin. The DNA was purified through two small columns from the kit: the unbounded material to the columns was washed away with 2 mL of 80% of isopropanol. The DNA was centrifuged for five minutes at 14,000 rpm and eluted with water at 80 °C. After one minute of spinning, the DNA was combined into a single tube and analyzed by a NanoDrop spectrophotometer. DNA was stored at 4°C.

Restricting and analyzing phage genomic DNA

The DNA was digested using five restriction enzymes (Asai *et al*., 2013) as it is shown below (see Table 1). After the digestion, 4 μL of viscous tracking dye was added to each of the enzyme solutions. With the direction of a T.A., the gel electrophorese was run for at least three hours (Voltage = less than 100 millivolt; 70~80 milliamp). Lastly, by measuring the distance migrated, the generated fragments were calculated in a spreadsheet according to the formula in the lab manual by Asai *et al*. (2013).

Table 1. Digesting the phage genomic DNA

Enzyme used	Cutsmart buffer (μL)	DNA (μL)	BSA (μL)	Buffer #2 (μL)	Buffer #3 (μL)	*Eco* Buffer (μL)	Enzyme (μL)	dH_2O (μL)	Total tube volume (μL)
Control	2	2	.	.	.	.	.	16	20
*Bam*HI	.	2	2	.	2	.	0.5 of *Bam*HI	13.5	20
*Cla*I	2	2	.	.	.	.	0.5 of *Cla*I	15.5	20
*Eco*RI	.	2	2	.	.	2	0.5 of *Eco*RI	13.5	20
*Hae*III	2	2	.	.	.	.	0.5 of *Hae*III	15.5	20
*Hind*III	.	2	2	2	.	.	0.5 of *Hind*III	13.5	20

Experimental procedure: observing calcium-dependency of the phage

To each 0.5 mL of calcium-free *M. smegmatis* culture, 5 μL of 100 mM each ion solution (Ca^{2+}, Mg^{2+}, and Mn^{2+}) was added (negative control =0.5 mL of calcium-free *M. smegmatis* culture). The *M. smegmatis* cultures were allowed to infect for at least for 20 minutes. Each 100 mM ion solution of 7.2 μL was added to 0.72 mL of calcium-free phage buffer (negative control = 0.72 mL of calcium-free buffer) and mixed. With the plating technique (Asai *et al*., 2013), four plates, divided into 10 equal areas, were plated with top agar that contained appropriate ions and *M. smegmatis* culture (45 μL of each ion solution was added to 4.5 mL top agar). Each ion solution (10 μL) was added in 1 mL of premade HTL, separately, in order to make $1{:}10^0$, containing a certain ion (negative control = 1 mL of calcium-free HTL). With the dilution technique (Asai *et al*., 2013), the HTL was diluted to reach $1{:}10^8$. As if performing the titer-assay, 5 μL of each appropriate ion dilution was plated on the appropriately labeled area, according to the wheel method (Asai *et al*., 2013). The negative control was 5 μL of the phage buffer, containing appropriate ions for each plate. The plates were incubated for 48 hours at 37 °C.

Achieving and reporting the phage

In each sterile tube, 4.7 mL of HTL was archived with 300 μL of DMSO to prevent the formation of ice crystals. Two tubes in total were sent to the University of Pittsburgh. In addition, the phage was reported to the online Mycobacteriophage Database.

Results

Plaque screening & borrowing plaques

There were no plaques observed from the original soil sample after two attempts. As a result, two plaques, FY1 and FY2, were borrowed from Madison Ochs. The plaques were from the soil sample collected at the GPS coordinate of 41.747676 (latitude) and -72.692240 (longitude).

Purification of the phage

After the fourth trial of purification of the phage, many isolated phages were observed for both FY1 and FY2. There were four attempts in total.

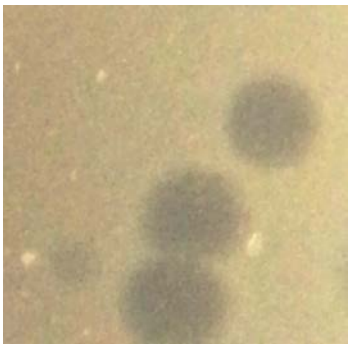

Figure 1. Plaques in MTL plate
The plaques exhibited bulls-eye morphology. The average diameter was about 1.8mm after 48 hours of incubation. In later stages of incubation, the diameter of plaques increased, but the number of plaques did not increase.

Plaque morphology (FY1) & growth characteristics

The average diameter of the plaques was approximately 1.8mm after 48 hours of incubation. The biggest diameter was 2.2mm, and the smallest diameter was 1.0mm. The shape was circular; the edge of the plaques was very smooth. The plaques had bulls-eye morphology, a turbid center surrounded by a ring of clearing. The plaques appeared during the earlier stage of the infection: the turbidity was low before 24 hours. However, after 24 hours, turbidity started increasing in a bulls-eye pattern, which became more turbid with an increase in distance from the center. There was no change in the number of plaques that appeared in later stages of incubation: only the diameter increased with age.

Phage morphology

The total length of the phage was about 311nm. The phage had a median-sized hexagonal head and a long tail (Figure 2). The ratio between the length of the head to the length of the tail was approximately 1:3. The approximate length of the head was 76nm and the approximate length of the tail was 235nm (Figure 2). The head was almost black, and the end of the tail was triangular. In the electron microscopy, no tail fibers were observed. The overall shape resembles a lollipop.

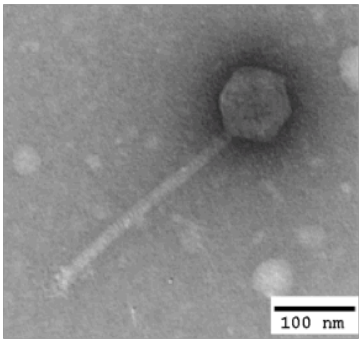

Figure 2. A picture of the phage "fabiolayun" from the electron microscopy. Cal: 0.994036 nm/pix. HV=80.0kV. Direct Mag: 88000x. Trinity EM Facility.

Titer values

The phage-titer assay was 1.1×10^8 pfu/mL (plaque-forming units per milliliter). The medium-titer-lysate had the titer value of 5.4×10^9 pfu/mL. The high-titer-lysate had the titer value of 4.0×10^{10} pfu/mL.

Quantified data of phage genomic DNA

The DNA yield was 266.9 ng/nL. The ratio between the A260 10nm path and the A280 10nm path was 1.87. The ratio between the A260 10nm path to the wavelength of 230 nm path was 0.42.

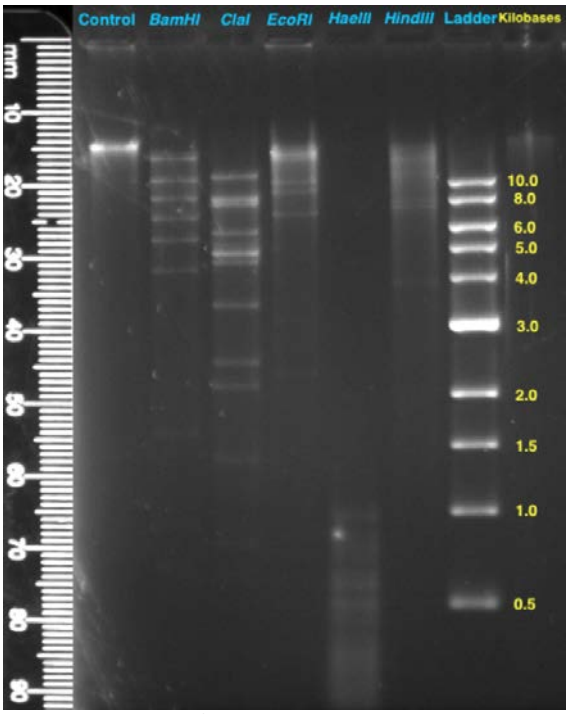

Figure 3. The picture of agarose gel electrophoresis. The calculated total fragment lengths by the enzymes *Bam*HI, *Cla*I, *Eco*RI, and *Hind*III were 44461.508, 55841.083, 48998.208, and 48907.741 base pairs, respectively. The enzyme *Hae*III left too many individual bands, so it was impossible to calculate the total fragment length.

DNA digests patterns and analysis of phage genomic DNA

The enzymes *Bam*HI, *Cla*I, and *Eco*RI digested the DNA quite successfully. The calculated total fragment lengths by the enzyme *Bam*HI, *Cla*I, and *Eco*RI were 44461.508, 55841.083, and 48998.208 base pairs, respectively (Figure 3). The enzyme *Hind*III exhibited less clear bands, and its calculated total fragment length was 48907.714 base pairs (Figure 3). The enzyme *Hae*III cut the DNA into many individual fragments; therefore, the total fragment length could not be calculated.

Average plaque diameter for each divalent ion

With the sample size value of 20, the observed average diameters of plaque in no ion, Ca^{2+}, Mg^{2+}, and Mn^{2+} ion solutions were 0.62 mm, 1.255 mm, 0.6275 mm, and 0.975 mm, respectively. There was a significant difference in number of plaques observed between no ion present and Ca^{2+} present, between no ion present and Mn^{2+} present, between Ca^{2+} present and Mg^{2+} present, and between Mg^{2+} present and Mn^{2+} present ($P< 0.01$; see Figure 4). There was no significant difference in number of plaques observed between no ion present and Mg^{2+} present ($P=0.94$; see Figure 4) and between Ca^{2+} present and Mn^{2+} present ($P=0.12$; see Figure 4).

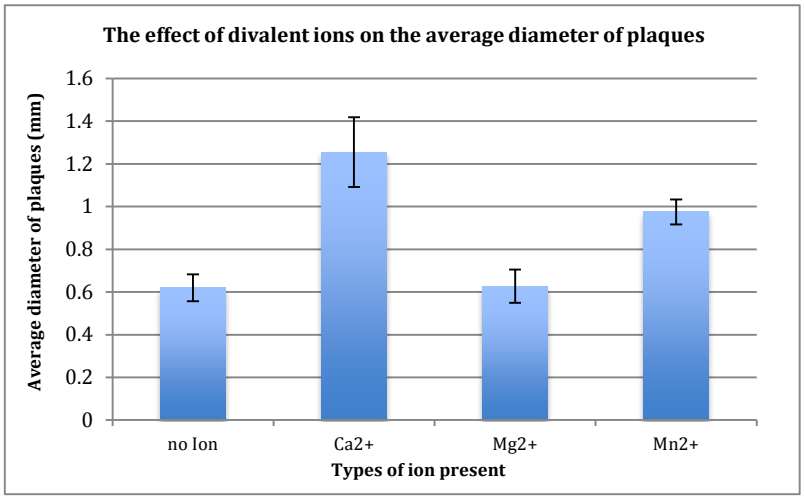

Figure 4. The graph of the effect of divalent ions on the average diameter of plaques
The average diameter of randomly selected 20 plaques in each divalent ion present solution was calculated. There were significant differences among two-group-comparison ($P<0.01$) except between no ion present and Mg^{2+} present and between Ca^{2+} present and Mn^{2+} present ($P>0.05$), according to T-test.

The number of the plaques observed for each divalent ion

After 48 hours of incubation, in the $1:10^8$ dilutions, only one plaque was observed when no divalent ion was present. With Ca^{2+} and Mg^{2+} ions, there were two plaques observed. With Mn^{2+}, three plaques were observed (see Figure 5). In the $1:10^7$ dilutions with no ion, Ca^{2+}, Mg^{2+}, and Mn^{2+} ions, 18, 27, 30, and 31 plaques were observed, respectively (see Figure 5). The data

was consistent that the smallest number of plaques observed was when there was no ion present and the largest number of plaques observed was when Mn^{2+} was present (see Figure 5). There was only one attempt.

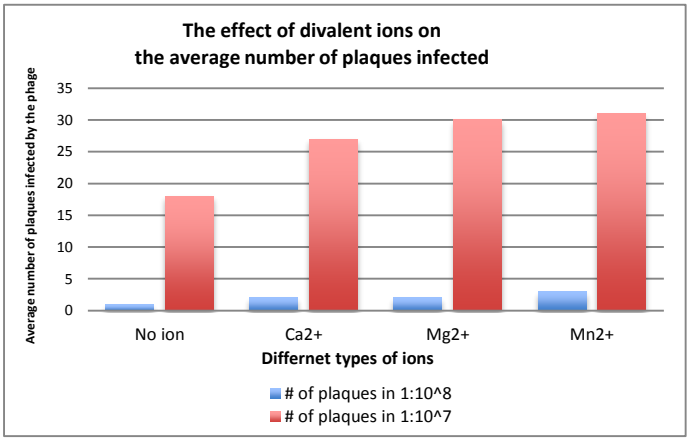

Figure 5. The graph of the effect of each divalent ion on the average number of plaques infected. The blue color represents the average number of plaques in each divalent ion solution when the dilution factor was $1:10^8$. The red color represents the average number of plaques in each divalent ion solution when the dilution factor $1:10^7$.

Discussion

Incomplete capturing

For the first round, the soil sample was collected in Illinois and utilized in 72 hours. After 24 hours of incubation, no plaque was screened. In fact, with one exception, the entire class did not get successful results. The second round involved collecting the soil sample on the campus, Trinity College, in Connecticut. Even though there were still four people who did not screen the plaques, including myself, the overall class result was quite successful. Because of the tight schedule, people who did not screen the plaques had to borrow plaques: this incomplete capturing has not been clearly answered. One possibility is there could have been so many phages in the plate that the entire *M. smegmatis* cells were infected by phages after 24 hours of incubation. However, this possibility still does not answer why there was no plaque observed during the second round. The purification step would have exhibited plaques if there were just an infinite number of plaques during the plaque screening, but it did not. It was clear that the enrichment procedure had no error (The vast majority could screen the plaque). Thus, it can be reasonably inferred that there was no viable phage in the soil sample during the second round.

Residual calcium ions in the experiment: calcium-dependency of the phage

It is true that there were residual calcium ions in the undiluted solutions for each ion. The HTL used in the experiment was made by the phage buffer containing $CaCl_2$. However, the effect of the residual calcium on the results of the experiment was negligible to some extent as the undiluted solutions were diluted to reach $1:10^8$.

The effect of the absence of divalent ions on the phage infectivity and phage growth

The phage growth rate and phage infectivity were at the lowest when there was no divalent ion present (Figure 4; Figure 5). This could be explained by a dual role of divalent ions in the early latent period of phages, including penetration and phage synthesis mechanisms (Shafia & Thompson, 1964). According to Puck (1953), the first step of recognition involves electrostatic forces that are generated by cations of the medium (Rountree, 1955). The electrostatic forces permit reversible attachment of phage to cell surface before the penetration (Rountree, 1955). After the attachment, the phage particle goes through a short period in which it

cannot be activated again if the divalent cations are removed from the medium (Rountree, 1955). It is believed that the abortive separation of phage protein and DNA at the cell surface and the inability of the DNA to penetrate when divalent cations are not present in the medium (Rountree, 1955). Furthermore, in order to replicate its DNA, the DNA polymerase of the host cell requires a divalent cation for catalysis (Tabor & Richardson, 1989). Thus, it can be concluded that the low phage growth rate and phage infectivity without the presence of divalent ions were due to the lack of electrostatic forces that enable attachment, the inability of DNA to penetrate the host cell membrane without the divalent ions, and the absence of catalysis for the DNA polymerase of the host cell, provided by the divalent cations.

Relatively high phage growth rate with calcium ions – the effect of divalent ions on the average diameter of plaques

Among calcium, magnesium, and manganese, calcium had the most significant effect on the phage growth rate (Figure 4). The quantity of the average diameter of plaques signifies the rate of phage replication and of the lysing process. The faster the phage induces lyses of the host cells, the bigger the plaques are observed. As a result, the average diameter of plaques represents the phage growth rate in unit per time. The absence of calcium ions causes abrupt cell lysis because the host outer membrane is not stabilized by divalent cations (Cvirkaitė-Krupovič *et al.*, 2010). Calcium ions are especially vital at stabilizing the host cells' outer membranes (Cvirkaitė-Krupovič *et al.*, 2010). In addition, calcium ions enable some phages, which are unable to lyse the host cells due to the presence of citrate or oxalate, to lyse (Adams, 2014). Bacteria that adsorb phages in the presence of citrate or oxalate cannot lyse, liberate the phages, and multiply (Adams, 2014). However, this inhibition of lysis can be resolved in the presence of calcium ions (Adams, 2014). Hence it can be concluded that the stabilization of *M. smegmatis* or potentially resolved inhibition of lysis by calcium ions could have resulted in a faster rate of lysing process, which was essentially observed as the growth rate of the phage.

Relatively low phage infectivity with calcium ions – the effect of divalent ions on the number of plaques observed

Among calcium, magnesium, and manganese, the phage did not have the highest number of plaques observed with calcium ions (Figure 5). The phage exhibited the highest infectivity with manganese: it was consistent that the highest number of plaques observed was with manganese in both $1{:}10^8$ and $1{:}10^7$ dilutions (Figure 5). The number of plaques observed decreased in the following order: manganese, magnesium, and calcium (Figure 5). The numbers of plaques observed are equivalent to the number of *M. smegmatis* cells being infected by the phage.

One possibility is that manganese and magnesium have a more critical role during the DNA replication than calcium ions do. Perhaps manganese and magnesium affect the phage gene regulation, such as encoding the viral enzyme *lysin*, by aiding the expression of the DNA. In fact, DNA polymerases can utilize manganese in substitution for magnesium or other divalent metal ions as the catalysis (Tabor & Richardson, 1989). While it is traditionally believed that magnesium results in the highest activity as DNA polymerase catalysis, particular DNA polymerases (T7 DNA polymerase and DNA polymerase I) could more efficiently encode DNA when manganese was substituted for magnesium (Tabor & Richardson, 1989). Tucker (1961) also supports that magnesium functions not only as an adsorption cofactor in the early stage of infection but also at some late stage in phage development.

Another possibility is that *M. smegmatis* requires calcium ions to sufficiently grow, and the types of divalent cations, present in the medium, do not affect the phage’s infectivity as long as a divalent ion is provided. According to Norgard and Imaeda (1978), calcium ions were essential for the transformation of *M. smegmatis*; other divalent cations, including magnesium, manganese, and zinc ions, could not replace calcium ions in the transformation of *M. smegmatis*. Among many divalent cations, only magnesium could stimulate the transformation process when calcium ions were present in the medium (Norgard & Imaeda, 1978). Thus, it is valid to speculate that the low infectivity of the phage with calcium ions could be due to the absence of calcium ions’ negative impact on the *M. smegmatis* to sufficiently grow and to increase its population – if there is limited growth of bacteria cells in the plate, there will be a limited number of infected bacteria cells by the phage.

Overall, it was determined that the phage “fabiolayun” was calcium-dependent in terms of growth rate, as it has been traditionally believed. However, it was not calcium-dependent in terms of viral infectivity. This calcium-independency could be explained with two possible reasons: it could be the phage’s unique property or it could be due to the physiological factor involved in the transformation of *M. smegmatis* (Norgard & Imaeda, 1978). The unresolved question could be answered by conducting an experiment. The future experiment will require repeating the exact same procedure with a different phage. If the results are consistent with this experiment, then the effect of calcium ions is *M. smegmatis* specific. If the results are not consistent with this experiment, then the effect of calcium ions is the phage “fabiolayun” specific.

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