CAN PHAGES BE CHEATED? - A STUDY ON THE INFLUENCE OF INDUCING MOLECULES ON BACTERIOPHAGE PROPAGATION AT LOW HOST CELL DENSITIES

By

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A thesis submitted to the Department of Mathematics and Natural Sciences in partial fulfillment of the requirements for the degree of
Master of Science in Biotechnology

Department of Mathematics and Natural Sciences
Brac University
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Declaration

It is hereby declared that

1. The thesis submitted is my own original work while completing degree at Brac University.
2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution.
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of Summer, 2008 has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Master of Science in Biotechnology on 30th June 2020.

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Ethics Statement

No human or animal model was used in this study.

Abstract

It has been reported *in vitro* that bacteriophages require a certain host cell density in order to propagate. However, previous studies have shown that in environment, especially in water bodies, bacteriophage infection takes place even when host cell densities are lower than the required threshold density observed *in vitro*. This leads to the indication that some factors must be in play that allows bacteriophages to propagate at such low host cell densities in the environment. In this study we investigated the effect of host cell densities on bacteriophage propagation in phage-host systems of *Vibrio* spp. and Shiga toxin-producing *Escherichia coli* (STEC) and determined the respective threshold densities. We found that phage propagation ceased at host cell densities beyond the threshold density but did initiate when supplemented with spent media of the respective phage and host cultures at the logarithmic phase of infection. Hence, we hypothesize that molecules produced by the host and/or phage have the ability to influence how bacteriophages take decision to infect its host cell, especially in the environment. Although we investigated the effect of these molecules in the phage-host system of *Vibrio* spp. only, similar studies with phage-host systems of different species may provide valuable insights to our understanding of phage-host interaction and therefore may contribute to the field of phage therapy.

Keywords: Bacteriophage, Propagation, Host cell density, *Vibrio* spp., *Escherichia coli*

Dedicated to my parents

Acknowledgement

The completion of my postgraduate thesis would not have been possible without the constant guidance of Almighty in every phase of my life.

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As I write the last paragraph, I have just realized that I have copied much of the words and phrases used here from the acknowledgement section of my undergraduate thesis! Seems like the “pro-procrastinator” in me has reached a new height! Maybe, life has changed much (or maybe it hasn’t) for many individuals mentioned in my undergraduate and postgraduate thesis book. Some have even left us for the life hereafter. But wherever we are or whatever we do, I hope we all become good human beings at the end of the day. I might have lost the will to write new words rather than just copying some of them, but may we never lose the will to become a better human being at any phase of our lives.

- A.B.M. Rezwanul Kabir
  June, 2020.

Table of contents:

List of Tables:

List of Figures:

Chapter 1: Introduction

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1.1 Background of the study:

Bacteriophages are the most abundant biological entity on Earth. They are obligate parasites and can infect bacteria (host). Likely so, bacteriophages are termed as "predators" and bacteria as "prey". This "predator–prey" relation is exploited by scientists to treat many infectious diseases (Maciejewska et al., 2018). However, prior to this, bacteriophages play a substantial role in nature and are a ubiquitous feature of prokaryotic existence (Clokie et al., 2011).

During infection, a phage binds to a bacterium and releases its genetic material into the host cell (Encyclopedia Britannica, 2020). After that a phage typically meets one of two lytic (virulent) or lysogenic (temperate) life cycles. However, in vitro studies have showed that host cells must reach a specific density before phage replication can take place. This particular host cell density, called the "critical density" or "replication threshold", is understood to be around 10^4 CFU/ml (Wiggins & Alexander, 1985). While it is still unclear why a certain critical density needs to be surpassed in order for new phage particles to be generated, one explanation could be that a certain metabolic state of the host is mandatory for the phage to undergo replication (Kasman et al., 2002).

Nevertheless, it is of interest to know why a certain metabolic state of the host is essential for phage population growth. To begin with, bacteria need to reach a certain metabolic state, with active expression of numerous genes, in order to maintain some crucial physiological activities such as biofilm formation, conjugation, motility, competence, antibiotic production, virulence, symbiosis, and many more (Miller & Bassler, 2001). Bacteriophages on the other hand take the advantage of this active metabolic state for their own predation (Laganenka et al., 2019). This is because a healthy bacterium at an active metabolic state would increase the probability of the production of new phage particles.

The concept of "critical density" can be compared to that of "activation energy (E_a)" of a chemical reaction. Activation energy can be defined as the minimum amount of energy needed for activating or energizing molecules or atoms to undergo a chemical reaction. Similarly, to undergo phage propagation, certain host cell density or "critical density" needs to be present. The comparison can be illustrated below in Figure 1.1.

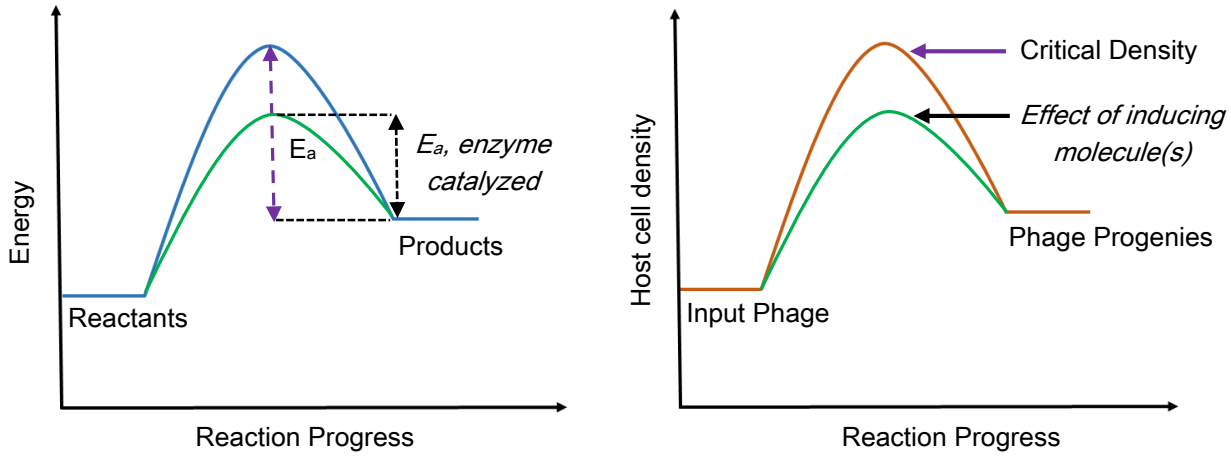

Figure 1.1. Comparison between activation energy and critical density.

While this activation energy needs to be surmounted for a reaction to take place, substances called catalysts can speed up a chemical reaction by lowering the activation energy while themselves being chemically unchanged at the end of the reaction. Thus, the chemical reaction can proceed without actually reaching the initial reaction barrier or the activation energy. This leads to our idea: whether there are molecule(s), which could have a similar effect like catalysts, capable of inducing phage propagation even at cell densities lower than the critical density? If yes, can these molecules be used to "trick" or "cheat" the bacteriophages into thinking that a high host cell density exists in the outside microenvironment so that they can undergo phage propagation even at low host cell densities and produce new phage progenies?

This investigation is thus important because in environment, especially in water bodies, bacteriophage infection has been reported to take place even when host cell densities are very low. Studies have shown that cyanophages effectively propagated in circumstances when marine cell density of *Synechococcus* spp. was as low as 10^3 CFU/ml (Suttle & Chan, 1994). Moreover, it was estimated that cyanophage propagation may still occur in marine environment at host cell densities of 10^2 CFU/ml (Waterbury & Valois, 1993). This leads to the indication that some factors must be in play that allows phages to propagate at such low host cell densities in the environment. Hence the idea of some molecules being responsible for such circumstances is highly probable and the results of this study can help us to better understand phage-host relation in environment.

1.2 Objective:

The main aim of this study is to observe how low host cell densities modulate bacteriophage infection decisions.

1.3 Specific Aims:

1. To observe the effect of *Vibrio* spp. cell density on the propagation of Vibriophage.
2. To observe the effect of Shiga toxin-producing *Escherichia coli* (STEC) cell density on the propagation of STEC-phage.
3. To determine the "critical density" of *Vibrio* spp. and STEC for the propagation of Vibriophage and STEC-phage respectively.
4. To observe the effect, in any, of inducing molecules on the propagation of Vibriophage at *Vibrio* spp. cell density beyond the "critical density".

Chapter 2: Literature Review

Chapter 2: Literature Review

2.1 Bacteriophages:

Bacteriophages are viruses that infect, replicate and multiply inside bacterial cells (Kasman & Porter, 2019). Phages are the most abundant biological entity on Earth and come in great diversity: in terms of host range, size, morphology, genomic organization and even the role they play in shaping up the biosphere (Simmonds & Aiewsakun, 2018; Hatfull & Hendrix, 2011; Doore & Fane, 2018). Likely so, bacteriophages are termed as "predators" and bacteria as "prey". This "predator–prey" relation is exploited by scientists to treat many infectious diseases (Maciejewska et al., 2018). However, prior to this, bacteriophages play a substantial role in nature and are a ubiquitous feature of prokaryotic existence (Clokie et al., 2011). Similar to other viruses, a specific phage usually infecst a single bacterial species, or sometimes even particular strains within a species (Kasman & Porter, 2019).

2.2 Life cycle of bacteriophage:

Bacteriophage replication has been broadly categorized into two replication methods: lytic and lysogenic. Interchange between these two methods is an important decision for bacteriophages as well as all the biological entities associated with them (Bohannan & Lenski, 2000; Weinbauer & Rassoulzadegan, 2004).

2.2.1 Lytic cycle:

During the lytic cycle of bacteriophage replication, the bacteriophage injects its genetic material into the host cell, which in turn allows the genetic material to replicate and thereby producing new phage particles. These particles then assemble and usually upon production of bacteriophage-

coded lysozymes, the bacterial peptidoglycan is broken causing lysis of the bacterial cells. The phage progenies then burst out of the cells, ready for reinfection (LibreTexts, 2016).

2.2.2 Lysogenic cycle:

Similar to the lytic cycle, the bacteriophage adheres to and injects its genetic material into the host cell. However, in the lysogenic cycle, the phage does not kill the host cell but instead uses it as a shelter where it exists in a dormant state, i.e it causes no harm to the host cell but rather quietly uses its resources. The phage genetic material becomes part of the bacterial genome and is termed as a prophage or is left free as a plasmid and becomes latent within the bacterium (Ackermann, 2012). This can sometimes bring some changes in the phenotype, such as increase in the pathogenicity, of the bacteria (Figueroa-Bossi et al., 2001). As the host cell divide, the phage genetic material also undergoes replication. However, upon different conditions, a shift from lysogenic cycle to lytic cycle may take place and when it does, it results in the lysis of the host cell (Oppenheim et al., 2005).

However, recent studies have shown that small molecules are produced by phages and are used in a similar manner to Quorum Sensing to decide between the transitions from lysogenic to lytic cycle (Erez et al., 2017).

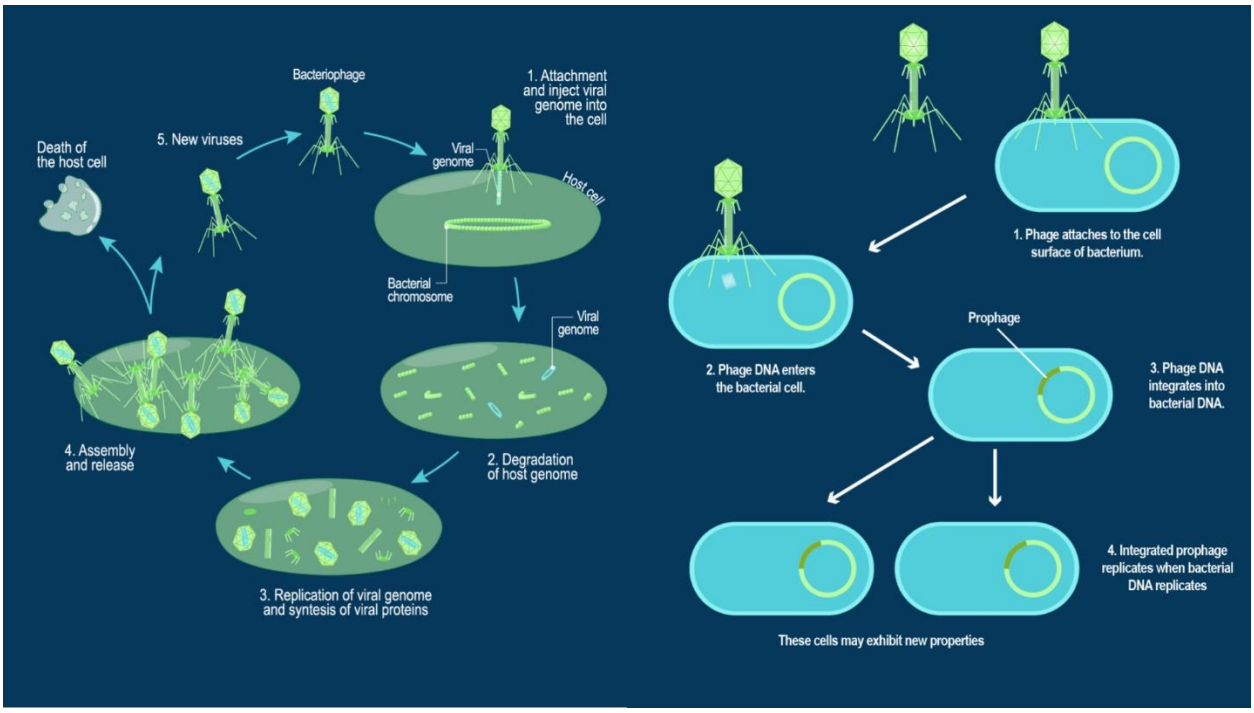

Figure 2.1. Stages of the lytic and lysogenic cycle
(Retrieved from: https://www.technologynetworks.com/immunology/articles/lytic-vs-lysogenic-understanding-bacteriophage-life-cycles-308094).

2.3 Lytic-Lysogeny Decision:

Lytic viruses infect the host cells, produces phage progenies and lyse the host cells to allow these phage progenies to initiate reinfection. On the contrary, temperate phages are able to integrate their genome into the host genome and remain in a dormant stage. The strategy of lysogeny has been observe to take place in surroundings that comprises of spatial structure, oscillating population dynamics, or periodic environmental collapse (Wahl et al., 2019).

It is interesting to wonder how bacteriophages decide to enter lytic stage or stay in lysogeny post-infection into a host cell. An important factor that drives the lytic-lysogeny decision is the multiplicity of infection (MOI) or simply a measure of the number of bacteriophages infecting per host cell (Maloy, 2003). This kind of circumstances usually favours the lysogenic cycle because this strategy would help the phages to avoid wiping out the host cells (Brüssow & Hendrix, 2002). Therefore, bacteriophages that decide to stay in lysogeny have a greater chance of surviving since they are less likely to run out of host cells to infect (Abedon, n.d). Also, this can be regarded as a survival strategy that has been promoted through natural selection since a host cell that already has a prophage incorporated into it is less likely to be infected with the same type of phage (Dimmock, 2016).

In nature other factors that that drive the lytic-lysogeny decisions include media availability, temperature, cell size and rate of phage adsorption (Kourilsky & Knapp, 1974). While in laboratory, DNA-damaging agents (like UV radiation and chemicals) can determine the lytic-lysogeny decisions & Jobling, 1996; Reece et al., 2011).

2.4 Mechanisms that direct the lytic-lysogenic decision:

A wide variety of mechanism that direct the lytic-lysogenic decisions of a phage have been accounted in different studies. Some of them have been explained here.

2.4.1 The bacteriophage λ regulatory network:

It has been found that in bacteriophage λ the levels of the lambda protein CII found with the host cell has a particular influence on the lytic-lysogeny decision. When the level of the lambda protein CII is high, the production of CI repressor is less and thus this results in a shift to the lytic cycle. On the other hand, when the level of the lambda protein CII is low, the production of CI repressor is high, preferring the phage to move to the lysogenic stage (Atsumi & Little, 2006; Shao et al., 2018).

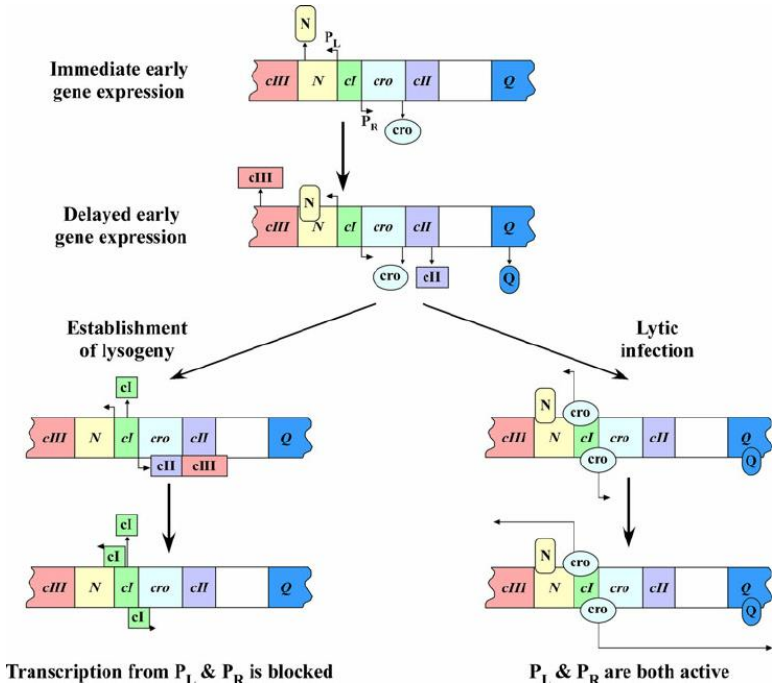

Figure 2.2. The mechanism involving the switch between the lytic cycle and lysogeny of phage lambda

(Golais et al., 2012. Retrieved from: https://www.researchgate.net/figure/Lytic-cycle-and-lysogeny-of-phage-lambda-N-protein-antiterminator-switches-the_fig2_230884901).

2.4.2 Use of feedback loops by "decision-making network" in response to DNA replication:

Changes in the environment that surrounds the host cell can initiate a variety of cellular responses. One such response may come in the form of the levels of different rates of DNA replication in different cells thereby affecting the level of production of many proteins. Studies have shown that lambda phage can respond to fluctuations in protein production by bifurcating into different cellular pathways (Arkin et al., 1998). The lambda "decision-making network" makes use of feedback loops to control cI expression and thus decides between lytic-lysogenic cycles (Shao et al., 2018).

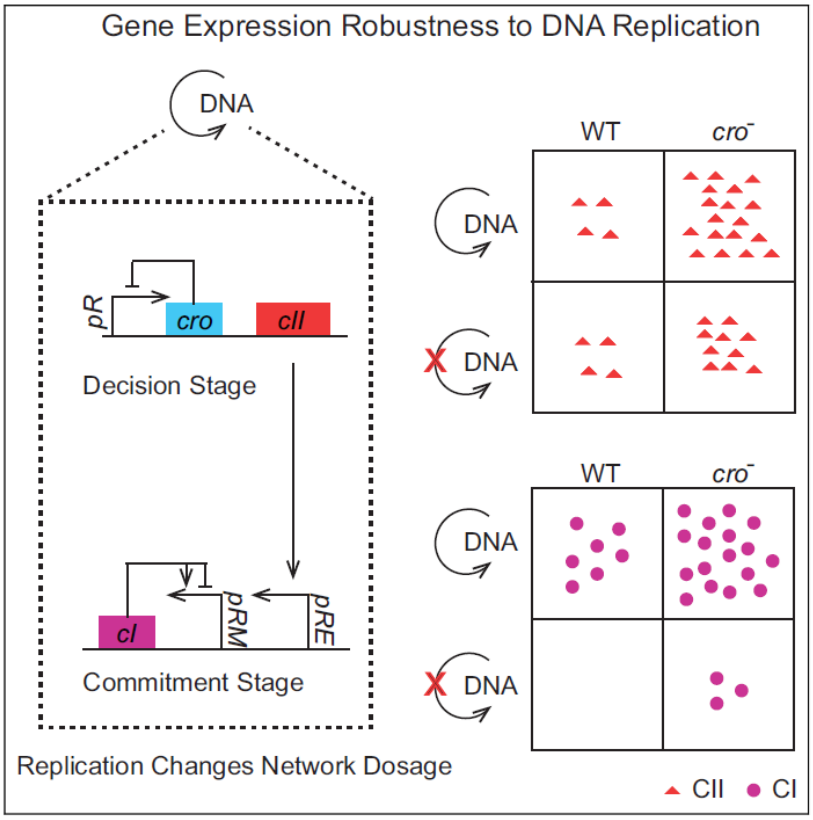

Figure 2.3. Use of feedback loops by "decision-making network" in response to DNA replication (Shao et al, 2018).

2.4.3 Phage-encoded peptides to establish communication:

While the aforementioned mechanisms are largely seen to be in action in lambda phages, studies have shown that phages of the SPbeta group encodes a communication peptide that helps the progeny phages to decide between lytic-lysogeny decisions in the succeeding infection (Erez et al. 2017). The study showed that lysogenic cycle is preferred when the concentration of the communication peptide is high.

Erez et al. (2017) proposed that at the first infection the phage genes *aimR* and *aimP* are expressed. AimR, as a dimer, activates AimX expression which in turn prevents lysogeny and leads the phage to undergo the lytic cycle. After several succession of infections, the concentration of the peptide increases and upon infection of a phage, the expression of AimX is deactivated and thus the lysogenic is induced.

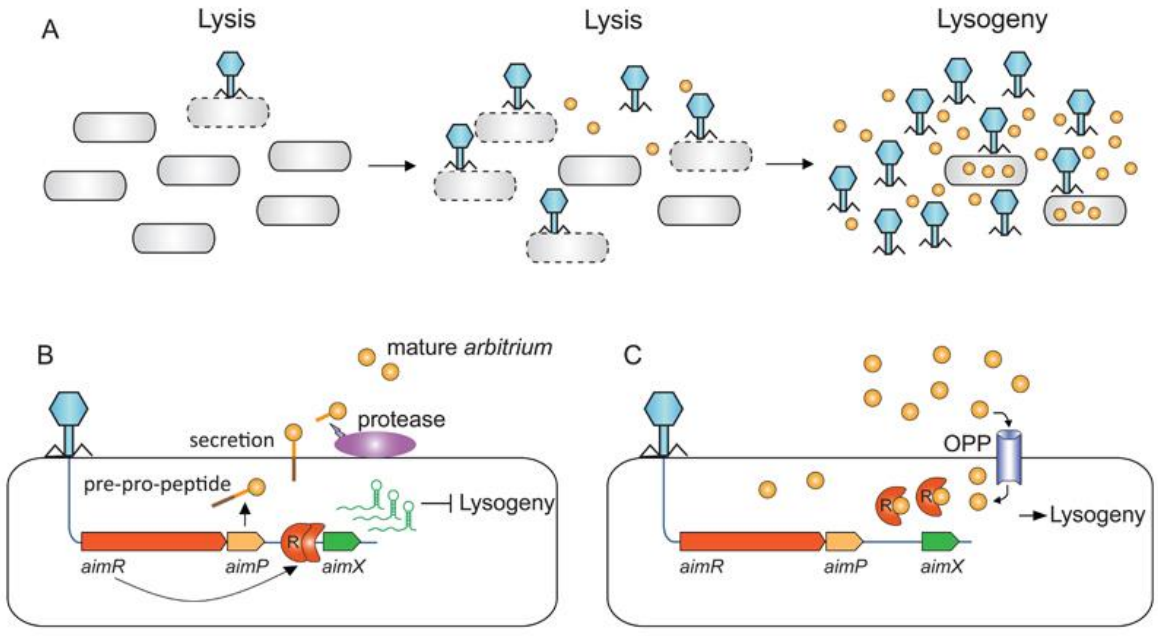

Figure 2.4. Model showing how phage-encoded peptide directs lysis-lysogeny decisions (Erez et al., 2017).

In another study, it has been reported by Silpe & Bassler (2019) that based on host cell densities, bacteriophages can shift between lytic and lysogenic phase by encoding a quorum-sensing receptor that identifies an autoinducer produced by the host. Silpe & Bassler (2019) also stated that the phage protein Qtip isolates and inactivates the cI repressor thus favouring the shift to the lytic stage.

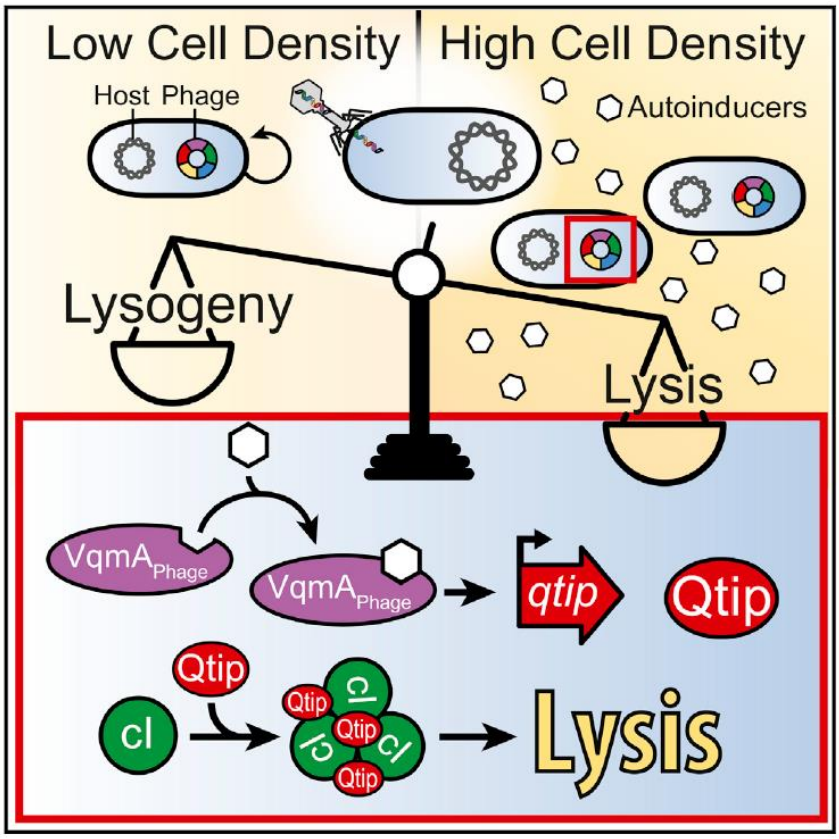

Figure 2.5. Phage-encoded receptor guiding the lytic-lysogeny decisions
(Silpe & Bassler, 2019).

2.5 "Critical Density" or "Replication Threshold":

In order to survive, bacteriophages must have a significant supply of host cells inside which they can replicate. Studies have showed that host cells must reach a specific density before phage replication can take place. This particular host cell density, called the "critical density" or "replication threshold", is understood to be around 10^4 CFU/ml (Wiggins & Alexander, 1985). While it is still unclear why a certain critical density needs to be surpassed in order for new phage particles to be generated, one explanation could be that a certain metabolic state of the host is mandatory for the phage to undergo replication (Kasman et al., 2002). Abedon (2008) compared the concept of this "critical" bacterial density to that of the "critical mass of a radioactive material". In his comparison. Adedon (2008) showed that similar to the need of the "critical mass of a radioactive material" for its "radioactive chain reaction" by "radioactive-decay-initiating neutrons", the is a requirement of the "critical" or threshold host cell density for continuous phage propagation by "infection-initiating phage virions" or input phage.

2.6 Quorum Sensing (QS):

Quorum Sensing (QS) is a mechanism through which bacteria can communicate among themselves through regulation of gene expression in compliance with population density via the help of signaling molecules (Encyclopedia Britannica, 2020). Bacteria uses QS for a variety of purposes including biofilm formation, bioluminescence, competence, swarming, sporulation, motility and for virulence (Kaur et al., 2018). For decades scientists wondered how bacteria can communicate among themselves to collectively thrive as a whole. The first concepts of QS arose in the late 1960s and early 1970s when it was showed that extracellular molecues were required for the genetic competence in *Streptococcus pneumonia* (Tomasz, 1965) and luminescence in marine bacteria (Nealson et al., 1970; Greenberg, 1979). The identification of the genes that

regulated luminescence (lux) of *Vibrio fischeri* by Engebrecht & Nealson (1983) and later tracing back of QS in *Vibrio fischeri* to a molecule called 3OC6-HSL by Eberhard, et al. (1981) were vital studies that had shone light in the understanding of QS. Over the years, advancements in biological science and technology as a whole has enabled scientists to discover much about QS: the different mechanisms and the wide array of signaling molecules that are involved. The advancements have reached such heights that scientists are now considering to use anti-QS molecules to prevent biofilm formation and thus disrupting the virulence of pathogenic bacteria (Jiang et al. 2019). Using anti-QS molecules can pave the way for alternative treatment strategies of pathogenic bacteria in a world where antibiotic resistance has emerged as a global issue.

2.7 Autoinducers in Quorum Sensing:

Autoinducers are small signaling molecules that take part in cell-to-cell communications in bacteria (eLife, 2017). The production and detection of autoinducers help the bacteria in various aforementioned physiological processes (Waters & Bassler, 2005). These autoinducers maybe hydrophobic molecules such as acyl-homoserine lactone (AHL) or larger peptide-based molecules and they act in different manner. AHL control the expression of particular genes by binding to transcription factors while peptide autoinducers stimulate more complicated signaling pathways that include bacterial kinases (Courses.lumenlearning.com, 2020).

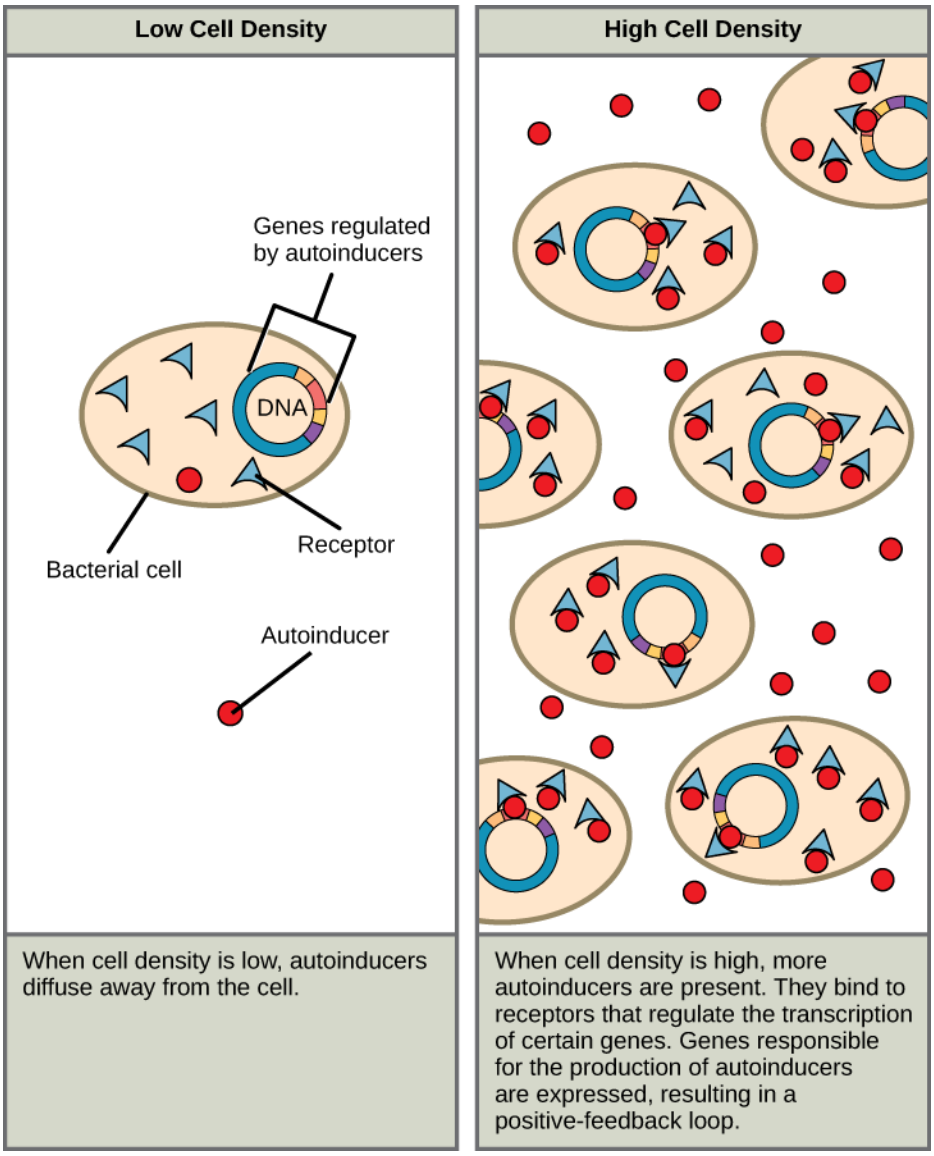

Figure 2.6. Autoinducers facilitating bacteria in Quorum Sensing (QS)
(Retrieved from: https://courses.lumenlearning.com/wm-biology1/chapter/reading-signaling-in-bacteria/).

Chapter 3: Materials and Methods

Chapter 3: Materials and Methods

3.1 Growth of *Vibrio* spp and Shiga toxin-producing *Escherichia coli* (STEC):

Previously isolated *Vibrio* spp and Shiga toxin-producing Escherichia coli (STEC) were used as hosts in the study. Their identify was reconfirmed by growing *Vibrio* spp. and STEC on Thiosulfate-Citrate-Bile Salts-Sucrose (TCBS) Agar and Sorbitol Macconkey AGAR (SMAC) for *Vibrio* spp and STEC respectively. Upon reconfirmation, the hosts were sub-cultured on Luria Bertani Agar (LA) on regular basis.

3.2 Bacteriophage enrichment:

Previously isolated Vibriophage and STEC-phage were needed to be enriched and a concentrated working stock was requited to be maintained throughout the study. For phage enrichment, few colonies of over-night host cultures were added to 9ml of freshly prepared Luria Bertani broth (LB) and allowed to incubate for 2 hours at 37^{o}C by placing inside shaker incubator.

After 2 hours, 1ml of phage solution was added to the 9ml liquid young culture and again kept inside the shaker incubator at 37^{o}C for 4 hours. This allowed the phage to infect the host and amplify in number. After 4 hours, the liquid culture containing both host cells and phages were centrifuged at 13000 rpm for 5 minutes to deposit the host cells. The supernatant was collected and syringe filtered through 0.22-micron syringe filter to exclude the remaining host cells. The resultant solution (stock) contained phages at high concentration and was stored at 4^{o}C.

3.3 Phage titer determination:

Throughout the study, it was essential to quantify the number of phages present inside a solution. Whether to determine the titer of stock or other solutions, we have carried out the technique of Soft Agar Plaque Assay.

For this, a single colony of the over-night host culture was added to a vial containing 3ml LB and then placed inside the shaker incubator at 37ºC for 2 hours. After 2 hours, 300µl of young host culture was added to 0.7% soft agar (soft agar contains 0.8% Bactoagar, along with Nutrient broth) followed by the addition of 10µl of phage solution of a certain dilution (phage solutions were diluted beforehand in order to get accurate phage titer). The resulting mixture was poured evenly on top of LA and was allowed to solidify. The plates were then kept at 37ºC and incubated over-night. Upon over-night incubation, plaques were counted and recorded. The phage titer was determined by simple back calculation.

3.4 Determining the effect of bacterial concentration on phage propagation:

The effect of bacterial concentration on the propagation of bacteriophages was investigated. Descending concentrations of host cells were infected with same amount of phages and incubated for 2 hours and resulting phage titer was measured.

The bacteria *Vibrio* cholera WT-333 of approximately 10^6 in number was allowed to grow up to early logarithmic phase (young culture) by incubating for about 2 hours. The young culture was then serially diluted 10 times using 1.9ml of LB in vials. Then 20µl of vibriophage (JSF35) solution was added to each of the dilutions respectively. The resulting mixture was the allowed to grow in shaking incubator at 37ºC for 2 hours. After the incubation period, the resulting solution was made free of bacteria by centrifugation at 13000rpm and syringe filtered through 0.22-micron syringe

filter to exclude the remaining bacterial cells. Each dilution was then serially diluted up to 8 times. 20µl of phage solution from each dilution was taken from each dilutions and soft agar plaque assay was performed as mentioned in section 3.3. Over-night incubation was followed at 37^{o}C and plaque count was recorded. The number of phages propagated was then found out by carrying out back calculation.

The exact protocol was carried out for the STECphage-STEC setup as well.

3.5 Preparation of Spent Media (soup):

Prerequisite to determining the effect of spent media on phage propagation, it was necessary to prepare the spent media. Spent media is basically the liquid culture media after a culturing a biological entity for a fix period of time. The spent media used in the study should've contained host cells, molecules secreted by the host cells and components of the original culture media. Spent media after 2 hours of incubation with phage-host was used since we predicted that if any molecule(s) with such effect are likely to be produced at early growth phase of the host and/or phage.

To prepare the spent media, few colonies of over-night *Vibrio* spp. cultures were added to 7.5ml of freshly prepared Luria Bertani broth (LB) and allowed to incubate for 2 hours at 37^{o}C by placing inside shaker incubator. Afterwards, 2.5µl of vibriophage solution was added, mixed and further incubated for 2 hours.

After 2 hours, the solution was made free of bacteria by centrifugation at 13000rpm and syringe filtered through 0.22-micron syringe filter to exclude the remaining bacterial cells. The remaining was thus the requited spent media.

3.6 Determining the effect of spent media on phage propagation:

In order to determine the effect of spent media on phage propagation, supplements of spent media and input phage was added to a low density *Vibrio* spp.. Following incubation, aforementioned soft agar plaque assay was performed and phage count was determined. Controls were setup where freshly prepared LB media was supplemented instead of spent media.

Liquid young cultures of *Vibrio* spp. were diluted till dilution factor 10^{-5}. Then, 2.7ml of spent media, 300µl of bacteria at dilution factor 10^{-5} (thus maintaining the working threshold of dilution factor 10^{-6}) and 100µl of input phage was mixed together and incubated for 2 hours. Two more replicates were also prepared similarly. Controls were prepared containing freshly prepared LB broth instead of spent media. Aforementioned soft agar plaque assay was then performed on certain phage dilutions and the number of plaque forming unit (PFU) was calculated and recorded.

Chapter 4: Results

Chapter 4: Results

4.1. Effect of host cell density on phage propagation:

4.1.1. Effect of *Vibrio* spp. cell density on the propagation of Vibriophage:

The effect of bacterial concentration on the propagation of vibriophages was investigated. Descending concentrations of *Vibrio* spp. were infected with same amount of vibriophages and incubated for two hours and resulting phage titer was measured.

Table 4.1. Variation in phage account after incubation in different concentrations of *Vibrio* spp..

Input Phage (PFU)/ml	Dilution factor	Phage count (PFU/ml)		
		Test 1	Test 2	Test 3
$1.5x10^6$	Raw	$1.6x10^9$	$2.0x10^9$	$1.5x10^9$
	10^{-1}	$5.9x10^9$	$4.0x10^8$	$4.0x10^8$
	10^{-2}	$4.0x10^8$	$2.0x10^8$	$3.0x10^8$
	10^{-3}	$3.0x10^8$	$2.0x10^8$	$2.5x10^8$
	10^{-4}	$2.0x10^8$	$1.0x10^8$	$1.2x10^8$
	10^{-5}	$8.0x10^5$	$4.0x10^5$	$1.0x10^6$
	10^{-6}	$2.4x10^5$	$3.0x10^5$	$6.0x10^5$
	10^{-7}	$8.0x10^3$	$2.6x10^3$	$1.4x10^4$
	10^{-8}	$6.0x10^2$	$1.0x10^3$	$2.0x10^3$
	10^{-9}	$8.0x10^2$	$1.0x10^2$	$2.5x10^3$
	10^{-10}	$4.0x10^1$	$6.0x10^1$	$1.0x10^2$

Using the results of the plaque assay, the phage count in each of the bacterial dilutions upon the 2 hour incubation period was calculated in PFU/ml. Using the values of three tests, three exponential graphs were constructed. It was found that all three curves generated were quite uniform.

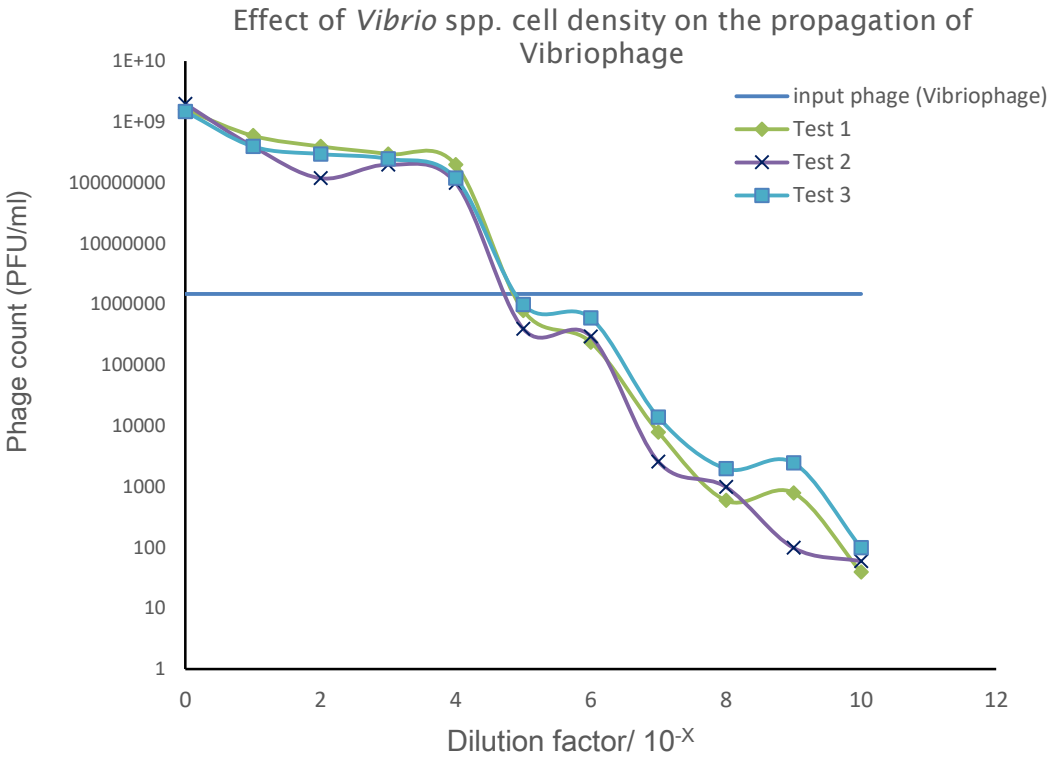

Figure 4.1. Effect of *Vibrio* spp. cell density on the propagation of Vibriophage, shown as individual tests.

Since the three curves were quite uniform, the mean PFU/ml from each bacterial dilutions was calculated and error bars were measured using the standard deviation. The three curves were then combined to form one curve that showed the combined effect of bacterial concentration on phage propagation.

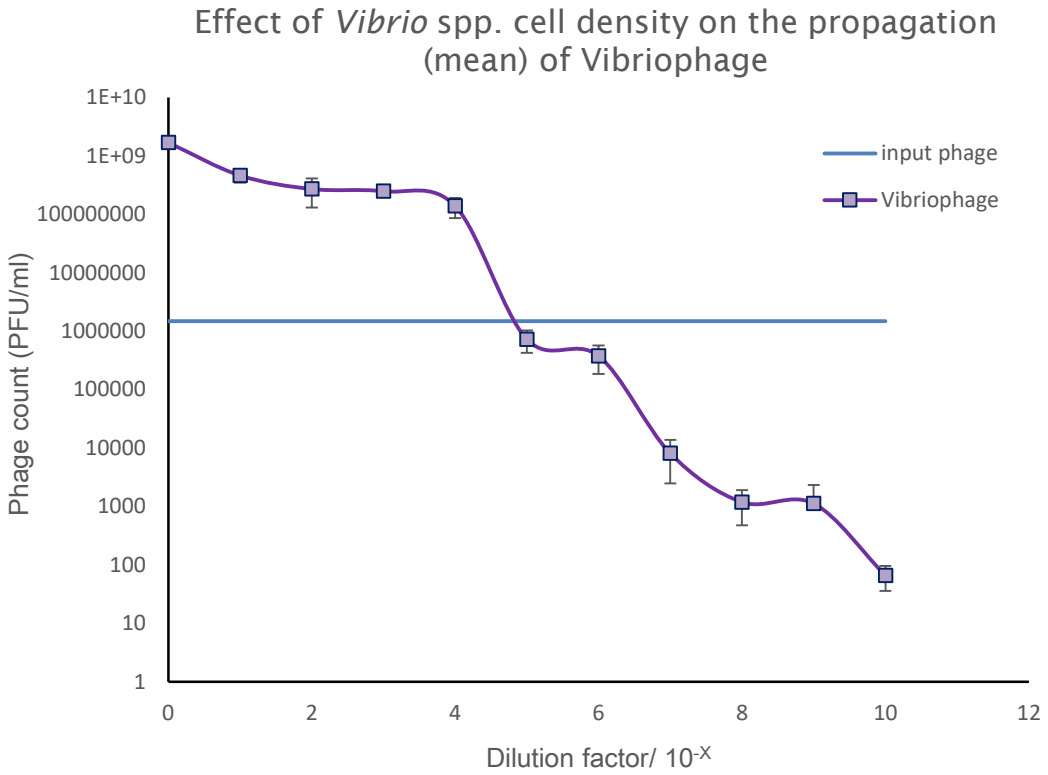

Figure 4.2. Effect of *Vibrio* spp. cell density on the propagation (mean) of Vibriophage.

According to the graph, it can be observed that as the concentration of bacteria decreased, the number of vibriophage propagated also decreased exponentially. The decrease was gradual from highest bacterial concentration till around dilution factor of 10^{-4}, after which a sharp decrease in the number of phage was observed. From highest bacterial concentration till dilution factor of 10^{-4}, mean percentage change from input phage to the final number of phage increased to 113233%, 31011%, 18122%, 16567% and 9233% respectively.

The line for input phage coincided with the graph at around dilution factor 10^{-5}. At this point, there was no further propagation of phage, i.e. the total phage number was equal to the input phage that was added at the beginning of the incubation period. Beyond this dilution factor, the number of phage was seen to drop exponentially. From bacterial dilution factor 10^{-5} till dilution factor 10^{-10}, mean percentage change from input phage to the final number of phage decreased to 51%, 75%, 99% and then eventually dropping off to about 100% for the last 3 dilution factors.

4.1.2. Effect of Shiga toxin-producing *Escherichia coli* (STEC) cell density on the propagation of STEC-phage.*:*

After a trend was observed between bacterial concentration and phage propagation between Vibrio and vibriophage, it was essential to observe whether similar phage-host relationship can be found in a different species of bacteria.

Hence, for this purpose Shiga toxin-producing *Escherichia coli* (STEC) was chosen as host and STEC-phage as the corresponding bacteriophage.

The experimental setup for the previous experiment (vibrio-vibriophage) was kept unchanged but this time, STEC & STEC-phage was used as the host and phage respectively.

Table 4.2. Variation in phage account after incubation in different concentrations of STEC.

Input Phage (PFU/ml)	Dilution factor	Phage count (PFU/ml)	
		Test 1	Test 2
$1.5x10^6$	Raw	$9.0x10^9$	$7.5x10^9$
	10^{-1}	$4.2x10^8$	$5.5x10^8$
	10^{-2}	$7.5x10^7$	$3.0x10^8$
	10^{-3}	$2.1x10^8$	$9.0x10^7$
	10^{-4}	$9.0x10^6$	$2.3x10^7$
	10^{-5}	$1.2x10^6$	$9.0x10^5$
	10^{-6}	$1.5x10^6$	$1.1x10^6$
	10^{-7}	$7.5x10^5$	$9.0x10^5$
	10^{-8}	$1.2x10^6$	$4.5x10^5$
	10^{-9}	$4.5x10^5$	$5.5x10^5$
	10^{-10}	$4.5x10^5$	$7.5x10^5$

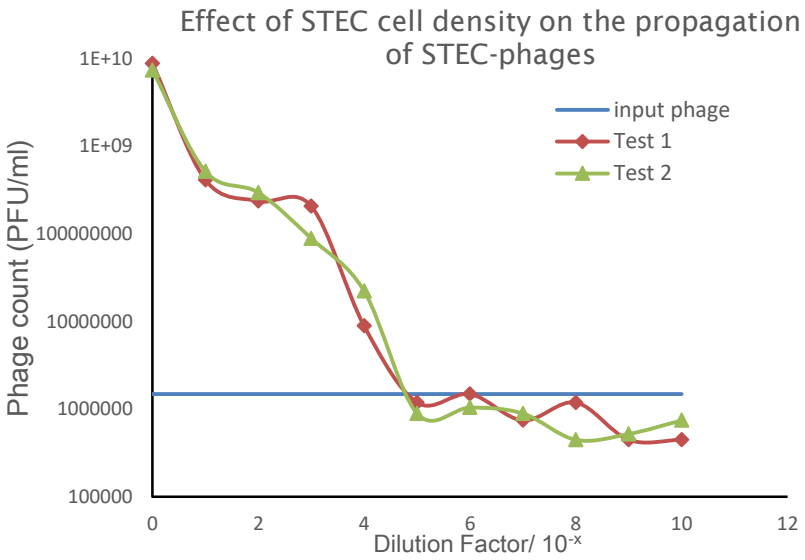

Figure 4.3. Effect of STEC cell density on the propagation of STEC-phage, shown as individual tests.

The experiment was repeated twice and upon getting two similar graphs, and resultant graph was constructed using mean PFU/ml from each bacterial dilutions and error bars were calculated using standard deviations.

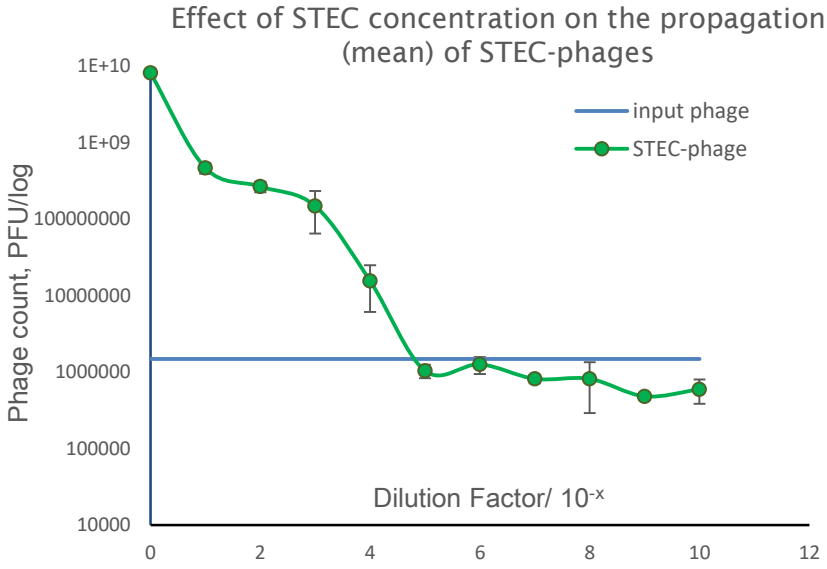

Figure 4.4. Effect of STEC cell density on the propagation (mean) of STEC-phage.

From the graph for STEC and STEC-phage indicated that similar trend was also observed as in the case for vibro and vibriophage. Here, we also observe that as the bacterial concentration decreased, the total number of phage propagated also decreased. However, unlike the previous experiment with vibrio-vibriophage setup, the STEC-STECphage setup yielded a significant increase in the number of phage propagated for highest bacterial concentration. From bacterial dilution factor of 10^{-1} till dilution factor of 10^{-4}, mean percentage change from input phage to the final number of phage increased to 31400%, 17900%, 9900% and 950% respectively.

Also in line with the vibrio-vibriophage system, the line for input phage coincided with the graph at around dilution factor 10^{-5} indicating that there was no further propagation of phage at this point. Beyond this dilution factor, the number of phage was seen to drop exponentially. However, unlike the vibrio-vibriophage system, in STEC-STECphage system, the mean percentage decrease in the number of phage was substantially lower, with as low as 15% decrease in dilution factor of 10^{-6} and as high as 68% in dilution factor of 10^{-9}.

4.2. Determination of a working bacterial concentration:

Now that the effect of the bacterial dilution on phage propagation was studied in two different model and species, vibrio-vibriophage setup and the STEC-STECphage setup, the next step of our study was to determine a working bacterial dilution beyond which no new phage propagation compared to the input phage would be obtained. To determine this bacterial dilution, the resultant graphs of both the setup was put together.

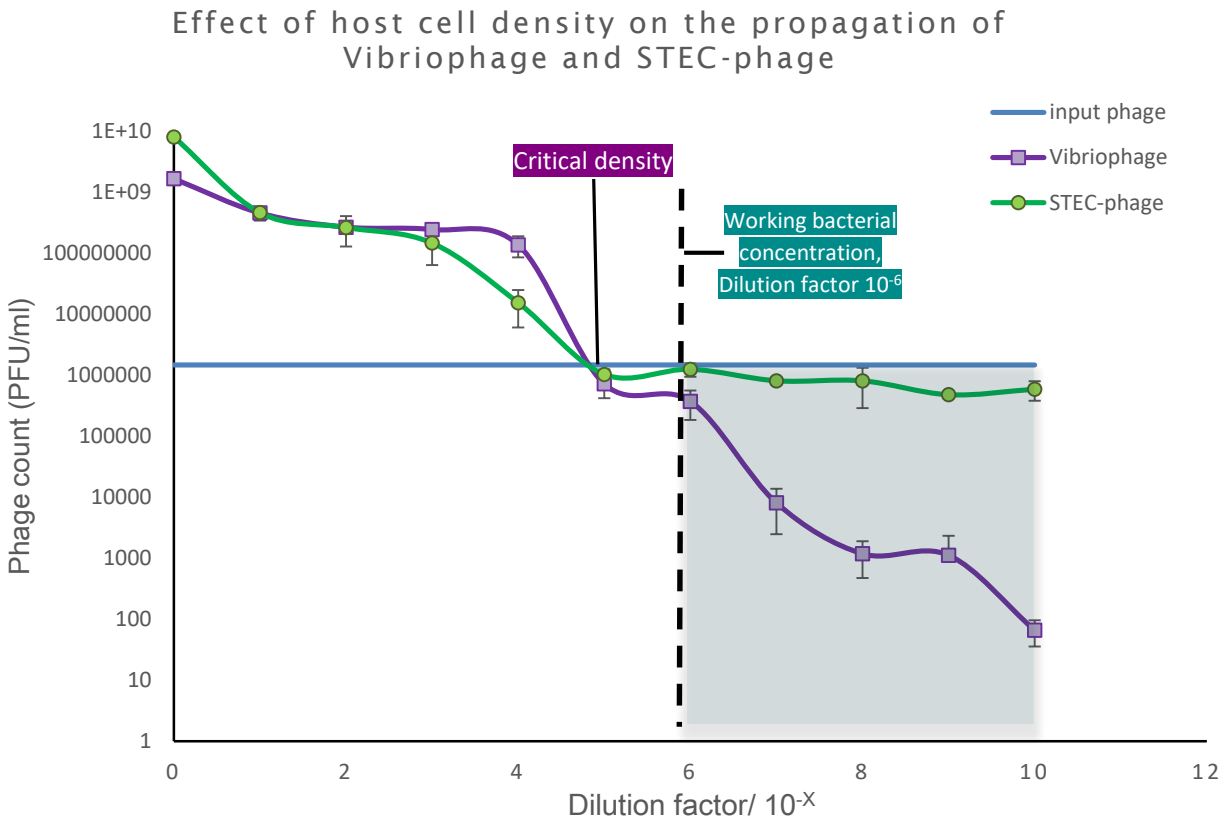

Figure 4.5. Graph showing the critical density and working bacterial concentration.

From the graph, it was observed that this threshold bacterial concentration for the both setups was around bacterial dilution factor 10^{-5}.However, keeping into account errors that might have been incorporated in the results, we decided to choose dilution factor of 10^{-6} as our working bacterial concentration. This is because at this dilution factor, we would be certain that provided a particular number of phage input, no new propagation of phage particles would take place.

4.3. Effect of spent media on vibriophage propagation:

By now in our study, we know how bacterial concentration affects phage propagation in phage-host system of two different species. Moreover, we also found out a threshold bacterial concentration at which no new phage propagation would take place if a certain amount of phage is provided as input.

The next step of our study was to observe whether spent media (soup) at the early stage of infection (around 2 hours) containing host and phage could have any effect in inducing phage propagation to occur at the threshold bacterial concentration.

In order to study this effect, spent media free of any host cell was collected. A certain ratio (9:1) of spent media to host culture of threshold bacterial concentration was mixed together. 2 more replicates of this was made to ensure a total number of 3 observations (n=3). A control, containing Luria Bertani broth instead of the spent media, was also prepared. Afterwards, a certain amount of input phage was added to all the setups and then put on shaking incubator at 37^{o}C for 2 hours. After the incubation period, the solutions were made free of any bacterial cells and then serially diluted. Plaque assay was then performed on certain dilutions and the number of plaque forming unit (PFU) was calculated and the results were recorded in PFU/ml.

Table 4.3. Variation in vibriophage count after treatment with spent media.

		Phage count (PFU/ml)		
		Test 1	**Test 2**	**Test 3**
Stock (Input phage)		$2.5x10^6$	$2.5x10^6$	$2.5x10^6$
Spent media (Soup)		$2.6x10^6$	$8.5x10^6$	$3.3x10^6$
Soup+Stock		$5.1x10^6$	$1.1x10^7$	$5.8x10^6$
Control		$2.4x10^5$	$1.2x10^6$	$1.4x10^6$
Sample (Soup+Bacteria+input phage)	**Replicate 1**	$1.2x10^7$	$2.2x10^7$	$8.3x10^6$
	Replicate 2	$1.2x10^7$	$2.1x10^7$	$9.8x10^6$
	Replicate 3	$1.0x10^7$	$2.5x10^7$	Count Discarded/Anomaly
	Mean	$1.2x10^7$	$2.3x10^7$	$9.1x10^6$

The results were then used to plot bar graphs for the individual tests to show the comparative differences between the phage counts.

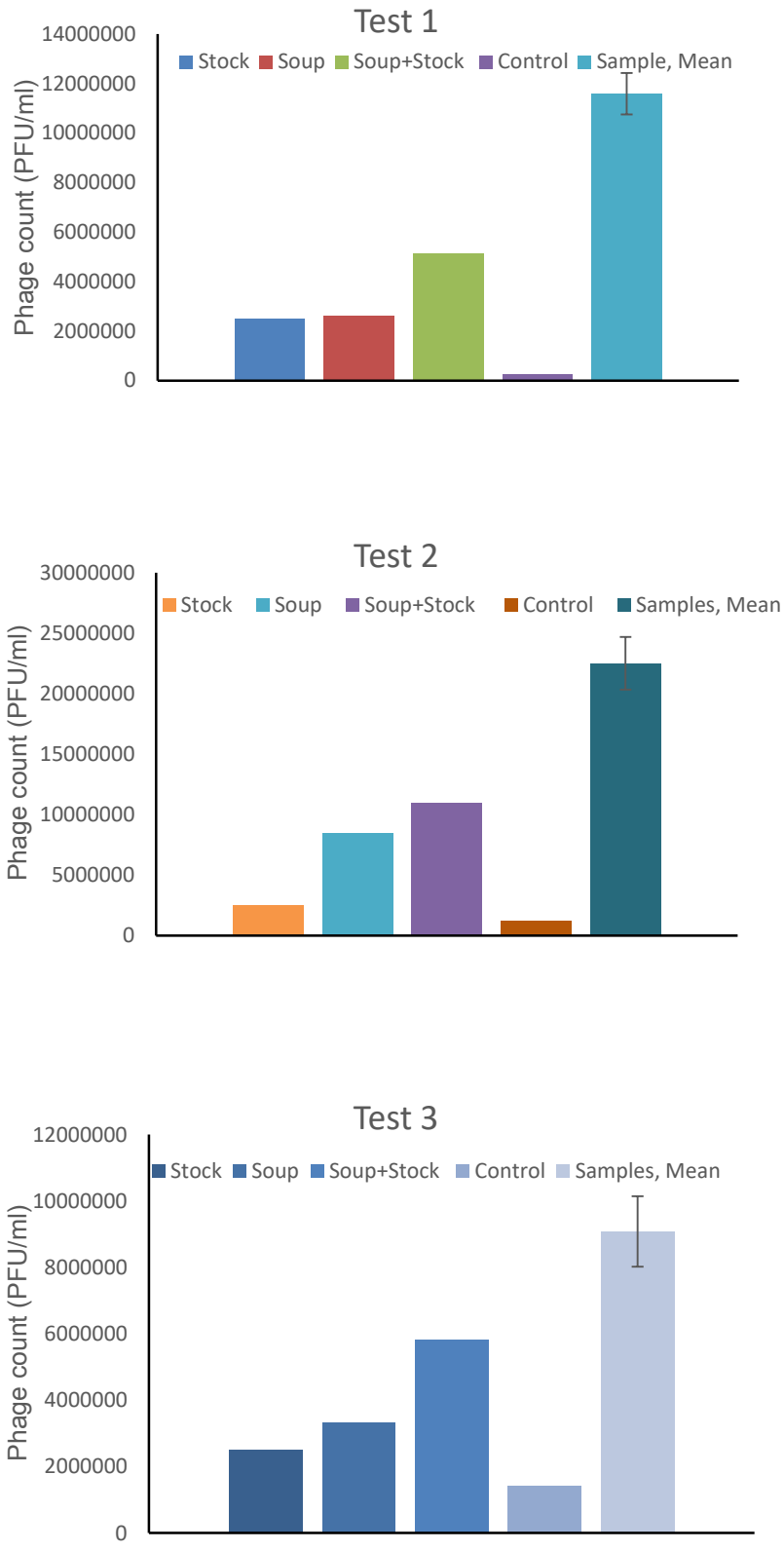

Figure 4.6. Effect of spent media on vibriophage propagation, shown as individual tests.

From the three graphs, it was observed that with similar phage input, phage count increased significantly in the samples where with spent media was incorporated. In contrast to these, phage count decreased substantially in control which had no supplement of spent media.

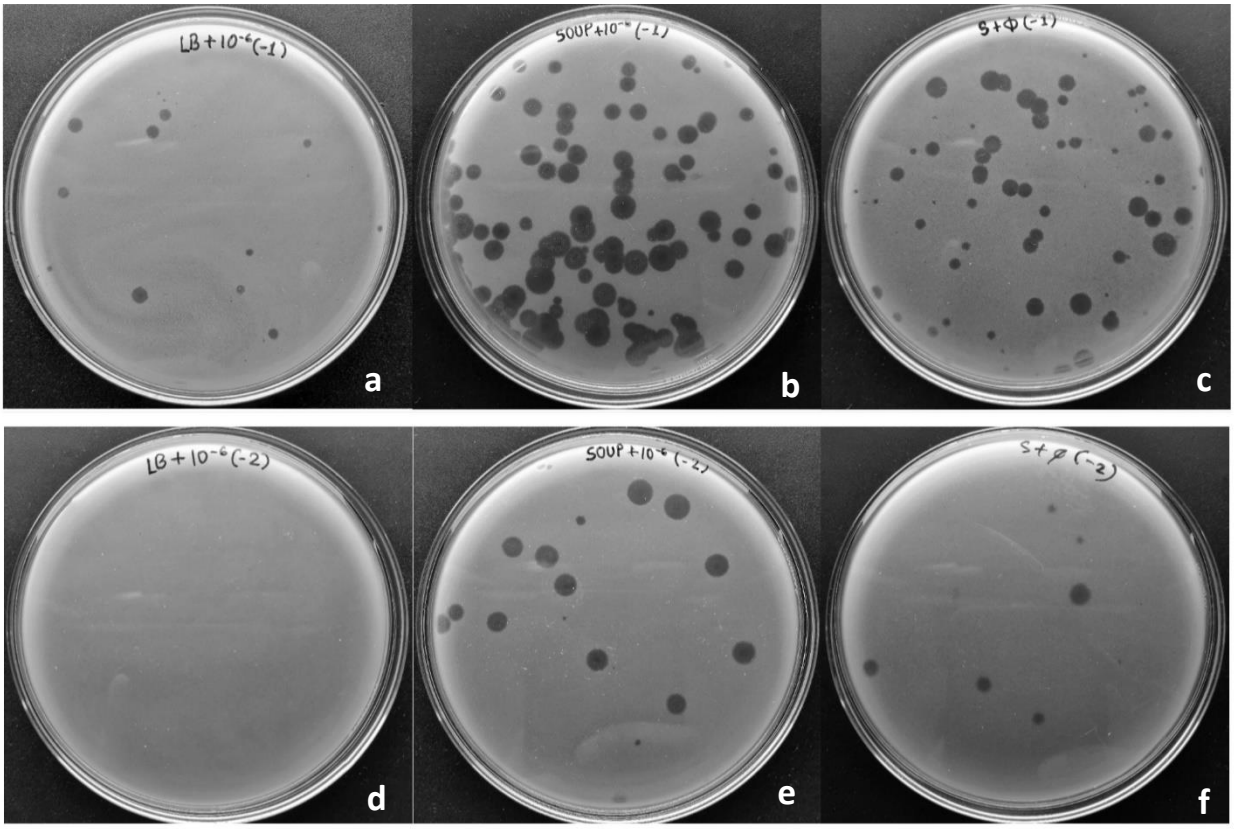

Figure 4.7. Plaques produced by vibriophage on a lawn of *Vibrio* spp.
(a) and (d): Control, containing LB media, bacteria (at dilution factor 10^{-6}) and input phage.
(b) and (e): Samples, containing spent media, bacteria (at dilution factor 10^{-6}) and input phage.
(c) and (f): Total page count of spent media and input phage.

In order to get a clearer picture, the percentage change in phage count before and after the end of incubation period in control and samples were graphed. It must be noted that the spent media also had a phage count of its own. Therefore, the phage count of the spent media was also taken into account while calculating the total phage count in samples before incubation period.

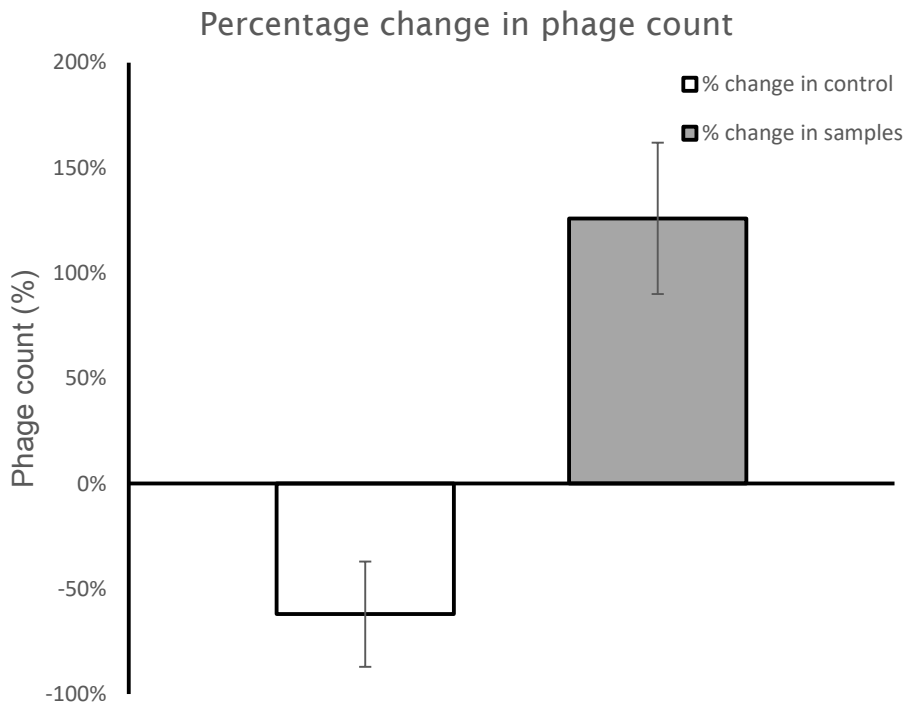

Figure 4.8. Effect of spent media on the propagation of vibriophages shown as a percentage change in phage count.

From the graph, it was observed that phage count decreased significantly in control. The mean decrease in control was noted to be 62%. This result was in line with the first phase of our study where we determined dilution factor of 10^{-6} as the threshold bacterial concentration that doesn't allow new phage particles to propagate. When this threshold bacterial concentration was supplemented with LB broth and input phage, the phage count dropped as shown in the previous graphs.

On the other hand, samples which were supplemented with spent media and input phage along with host at the threshold bacterial concentration, phage count was observed to increase significantly with a mean increase of 126%. Although, phage count was not supposed to increase at this threshold bacterial concentration, supplements of spent media caused the phage particles to propagate. Moreover, the lower bound value of this increase is 90%, meaning that the phage count in samples was almost double than that of the count before the incubation period. This made us conclude that “something” must have been present in the spent media that had induced the phage particles present in samples to proceed to infection even at the threshold bacterial concentration.

Chapter 5: Discussion

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In the study, we found that host cell density has a significant role in the way bacteriophages take decision to infect their host cells. We also found that some molecules might aid bacteriophages to infect their host cells even in situations where host cell population is very low.

In the first phase of our study, we observed a non-linear decrease in phage propagation with subsequent linear decrease in host cell density in two different species, *Vibrio* spp and *E. coli*. New phage progenies were not produced until a certain host cell density was reached. This particular host cell density, called the "critical density" or "replication threshold", is understood to be around 10^4 CFU/ml (Wiggins & Alexander, 1985). Although according to our results, the critical density was found to be around 10^3 CFU/ml (dilution factor of 10^{-5}), taking into account that initial bacterial count in the logarithmic phase was in the region of 10^8. While it is still unclear why a certain critical density needs to be surpassed in order for new phage particles to be generated, one explanation could be that a certain metabolic state of the host is mandatory for the phage to undergo replication (Kasman et al., 2002). However, other factors might also contribute to this phenomena.

So what really happens to the phage-host system in solution from highest host cell density till we reach the point critical density? According to our results, we found that the number of phage progenies after incubation reached its maximum at highest host cell densities and decreased in a non-linear trend till the critical density was reached at which there was no change in the number of phage particles before and after the incubation period. This implies that at high cell densities, especially till cell densities of around 10^5 CFU/ml, there were plenty of host cells in the liquid media for the input phage particles to undergo propagation.

A number of reasons can account for the huge increase in phage number till critical density was reached. Firstly, phage count is significantly high at high cell densities simply because phage can locate bacteria quicker, i.e., a very short search time (Abedon, 2004). At high cell densities, the generation time and optimal lysis time of a phage is also shorter and thus a substantial number of phage progenies are produced (Shao & Wang, 2003; Wang et al., 1996). Although, our results have shown that the number of phage particles decreased with each dilution, this drop in phage count was steady and a noticeable quantity of new progenies were produced. This might refer that the Multiplicity of Infection (MOI), stated as the ratio of infectious virions added per cell in a culture, was enough to produce a steady supply of new phage progenies. Although in our study, this steady production of new phage particles was observed to be slightly different among phage-host system of *Vibrio* spp and *E. coli*. Production of new phage progenies was observed to more sturdy in Vibriophage-*vibrio* system than in STECphage-STEC counterpart.

However, it is equally logical to wonder what happens to phage propagation at host cell densities lower than this critical density? In our study, we found that the number of phage particles was seen to drop off significantly beyond the critical density when compared to the input phage, with this reduction in phage count being more evident in Vibriophage-*vibrio* system than in STECphage-STEC counterpart which might be due to differences in the adsorption rate and stability between the two phages. There could be a number of reasons that can account for this overall drop in phage count. Firstly, in contrast to as discussed in the case of high cell density, the generation time of a phage is considerably higher in low cell density (Shao & Wang, 2003; Wang et al., 1996). This implies that within a fixed time period, phage progenies will be much higher in high cell density and vice versa. However, this does not explain why a reduction in phage count compared to the input phage was observed in our study. Although, it is reasonable to argue that phage particles can

degrade over time having failed to find enough bacteria to adsorb to and hence replicate, but this still does not answer why such a significant decrease in phage number can be accounted for since the incubation period was only 2 hours and that too at a very tolerable temperature of 37^{o}C. This is where the fundamental hypothesis of our study approaches: whether phages can assess their host microenvironment and decide whether to proceed to the lytic phase or stay in lysogeny that would benefit them and their progenies for the long run. And in the case where they decide to stay in lysogeny, can phages be "tricked" to proceed to the lytic phase by the introduction of molecules or substances that would otherwise make them "believe" that a high host cell density exists in the surrounding microenvironment.

Hence, in the second phase of our study, we wanted to observe if some molecule(s), which might be produced by the host and/or phage, can induce bacteriophages to undergo lytic phase and produce new phage progenies even in low host cell densities where this phenomenon is not supposed to take place *in vitro*. For our study, we needed to choose a certain host cell density that would be beyond the critical density yet contain a certain number of host cells to promote, if it eventually occurs, lysis. Therefore, we selected host cell density of around 10^3 CFU/ml as our working host cell density. We added supplements of spent media to a low density *Vibrio* spp. culture and controls were prepared containing freshly prepared LB broth instead of spent media and kept the remaining setup similar to the first phase of the study.

Our results showed that the Vibriophage-Vibro system, at *Vibrio* spp. cell density beyond the critical density, produced significant number of new phage progenies when supplemented with spent media. Meanwhile in its counterparts, controls supplemented with LB broth, phage count was observed to decrease considerably. This indicated the presence of molecules in the spent media

capable of inducing the vibriophages to proceed to the lytic phase “believing” that a high *Vibrio* spp cell density existed in the surrounding microenvironment.

Although, in the course of our study it was not possible to determine the type let alone the identity of these “inducing molecules”, previous studies by Silpe & Bassler (2019) have shown that a host-produced autoinducer and a phage-encoded receptor might be responsible for this induction. In their study, Silpe & Bassler (2019) showed that in high host cell densities, where the host produces autoinducers as part of quorum sensing, phage encodes a receptor that in turn binds to the autoinducers, thus helping the phage to undergo lysis. Therefore, we believe that similar phenomenon might have taken place where autoinducers present in the spent media have caused the phages to encode similar receptors and hence lysis occurred. However, further studies need to be conducted in order to test this phenomenon.

Chapter 6: Conclusion and Future directions

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In this study, we wanted to seek answers to a number of research questions related to how phage replication and crucial lytic-lysogeny decisions are influenced by host cell densities. The results of this study can provide great insights to the understanding of phage-host relationship that can help direct future research in the field.

Through methodical and quantitative analysis of phage counts, we have shown in this study how phage replication may depend on varying host cell densities. We have managed to compare this dependency in two phage-host system of two different species of bacteria. This can have huge implications in the industrial production of bacteriophages and in the prevention of pathogenic bacteria, especially in the treatment against enteric pathogens like *Vibrio cholera* and Shiga toxin-producing *Escherichia coli* (STEC). Moreover, this understanding of phage-host relation might also shine light into the understanding of viruses that infect eukaryotes.

Results in the second phase of our study showed that molecules, possibly autoinducers, may guide phages in their lytic-lysogeny decisions in situations where host population is very low in the surrounding microenvironment, especially in water bodies. Also analysis of these molecule(s), the metabolic pathways through which they are produced and the genes that play important functions in their production might help us to unravel vital evolutionary information which can aid us comprehend how viruses have evolved over time. Moreover, these results may add information to the current knowledge of Quorum Sensing (QS) and communications between the host as well as its phages. When coupled with the existing tools of phage therapy, a better understanding of the action and importance of these molecules may help broaden the strategies that maybe be developed

in this promising new field. As previously stated, this might also in turn help us in the understanding of viruses that infect eukaryotes.

However, in our study we could not determine the type or identity of these inducing molecules and therefore, it is imperative that we extend our work. Since we suspect that these inducing molecules might be autoinducers, we have already planned to deploy several mutant strains of *Vibrio* spp. that overproduces, do not produce or partially produce autoinducers and notice if similar results are obtained.

Despite observing the effect of host cell concentration on phage propagation in two phage-host system, the influence of some molecules on lytic-lysogeny decision was only studied in vibriophage-vibrio system. This also opens up the scope of future research that maybe be carried out to further strengthen our findings. Although not presented in this thesis work, we have made several attempts to establish two different vibriophage-*vibrio* systems comprising of two host-specific vibriophage of similar genome size to further strength our findings. However, little success was obtained in establishing such vibriophage-*vibrio* counterparts. Nevertheless, we plan to continue our search for such combinations.

Altogether, the study has provided useful information in the knowledge of phage-host relationship and in turn may contribute to the field of phage therapy which has long been considered as noteworthy ammunition in our never-ending battle against pathogenic bacteria.

We hope that the study will at least make a miniscule contribution in our efforts to understanding the most abundant biological entity on earth (or beyond, who knows!): viruses.

Chapter 7: References

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Appendix – I

Instruments used:

Autoclave	Wisd Laboratory Instruments Made in Korea
Electronic Balance	Model: WTB 200 RADWAG Wagi ELEktroniczne
Incubator	Model: DSI 3000 Digisystem Laboratory Instruments Inc. Made in Taiwan
Microcentrifuge	Model: MC-12 Benchmark Scientific
Refrigerated microcentrifuge	Model: ScanSpeed 1730R Labogene
Shaking Incubator	Model: JSSI-1000C JS RESEARCH INC. Made in Rep. of Korea
Spectrophotometer	Model: UVmini-1240 UV-VIS spectrophotometer SHIMADZU Corp
Syringe filter	MS® MCE Syringe Filter Membrane Solutions, LLC
Vortex Mixer	Model: VM-2000 Digisystem Laboratory Instruments Inc. Made in Taiwan
Water Bath WiseBath®	Wisd Laboratory Instruments DAIHAN Scientific Co., Made in Korea