Potential Role of Bacteriophages in Facilitating the Horizontal Transfer of Plasmids

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A thesis submitted to the Department of Mathematics and Natural Sciences in partial fulfilment of the requirements for the degree of B.Sc. in Biotechnology

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Declaration

It is hereby declared that:

1. The thesis submitted is our own original work while completing our 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 that has been accepted or submitted, for any other degree or diploma at a university or other institution.
4. We have acknowledged all main sources of help.

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Abstract

Plasmids, small circular DNA molecules, are key players in bacterial ecology and evolution due to their role in horizontal gene transfer (HGT). This process allows bacteria to exchange genetic material, including antibiotic resistance genes (ARGs), across species and environments. The rapid spread of ARGs through HGT is a major contributor to the growing challenge of antibiotic resistance. This study focuses on the role of bacteriophages, or phages, in facilitating the horizontal transfer of plasmids. During viral replication, phages may accidentally incorporate fragments of host bacterial DNA, such as plasmids, into their capsids, a process known as viral mispackaging. When these phages infect new bacterial hosts, they can inadvertently introduce plasmid-borne genes, including ARGs, promoting the spread of resistance. The study explores this phenomenon in the context of transduction, where bacteriophages transfer genetic material between bacteria. If plasmids carrying ARGs are mispackaged and introduced into new hosts, this can accelerate the emergence of antibiotic-resistant strains. This has significant implications for public health, as the spread of resistance genes could outpace our ability to control infections with existing antibiotics. The findings highlight the need for further research into the role of bacteriophages in HGT and the dynamics of ARG dissemination. The study also emphasises the broader public health implications, underscoring the complexity of controlling antibiotic resistance. Phages could contribute to the spread of ARGs, adding a new layer to the already difficult challenge of managing antibiotic resistance in both clinical and ecological settings. To address this, there is a need for targeted interventions to mitigate the spread of resistance genes. Future research should focus on developing surveillance systems to track the movement of ARGs across environments and understanding the factors that drive their spread. Overall, this research highlights the crucial role of plasmids and bacteriophages in shaping bacterial populations.

Keywords: Bacteria, Bacteriophage, Horizontal gene transfer, Transduction, Viral packaging

Dedicated To

All of this is dedicated to our parents, friends, acquaintances and other well-wishers. We sincerely hope to make all of them proud.

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List of Abbreviations:

ARG - Antibiotic Resistance Gene

HGT - Horizontal Gene Transfer

CRISPR - Clustered Regularly Interspaced Short Palindromic Repeats

ESBL - Extended-Spectrum Beta-Lactamases

R-plasmid - Resistance Plasmid

Ori - Origin of Replication

MCS - Multiple Cloning Site

EDTA - Ethylenediaminetetraacetic Acid

SDS - Sodium Dodecyl Sulfate

LB - Luria-Bertani Broth

PEG - Polyethylene Glycol

EtBr - Ethidium Bromide

TBE - Tris/Borate/EDTA

DLA - Double Layer Agar assay

PFU - Plaque Forming Unit

Chapter – 1

Introduction

Antibiotic resistance is a growing problem in the modern world, something that is starting to become a bigger and bigger wall to innovation in the agricultural and clinical industries. The relentless increase in resistant bacterial infections, coupled with a dwindling pipeline of new antibiotics, presents a grave challenge to the treatment of common infectious diseases. Certain multidrug-resistant pathogens, such as methicillin-resistant *Staphylococcus aureus*, carbapenem-resistant *Pseudomonas*, and *Candida krusei*, have become prevalent in clinical settings and are only becoming more difficult to eradicate (*Multidrug-Resistant Organisms (MDRO)*, 2019). Bacteria can develop resistance to multiple types of drugs so quickly due to the horizontal gene transfer of plasmids containing antibiotic resistance genes. This can occur between different species of bacteria, which only worsens the problem. While conjugation and transformation are well recognized for their roles in horizontal gene transfer, transduction is the main mechanism that will be expanded upon further. This is mainly because it is a critical but less-explored area of study, especially concerning the spread of antibiotic resistance in bacterial populations.

Bacteriophages are viruses that target bacteria only. Like all other viruses, they infect cells by inserting their genetic material into the host, sometimes directly integrating with its genome, before hijacking the host's cellular machinery and replicating quickly. During this replication phase, mistakes may occur in packaging viral genetic material into the capsid. These errors can result in mispackaging bacterial DNA, including antibiotic resistance genes or plasmids, into the capsid, thus creating a transducing particle. When this infects a different cell, it inserts these mispackaged genes into the host, thus causing horizontal gene transfer. One point of contention when it comes to transduction is the way by which a plasmid may get mispackaged into a phage capsid. Transduction can thus potentially cause a rapid emergence of multidrug-resistant organisms.

The impact of phage-mediated gene transfer extends beyond natural environments, significantly affecting clinical settings where antibiotic resistance poses severe risks. In healthcare facilities, selective pressures from the heavy use of antibiotics create favourable conditions for the survival and proliferation of resistant bacteria (Kolář et al., 2001). Phages present in hospital wastewater,

biofilms on medical devices, and even in the gut microbiota of patients can act as vectors for the spread of resistance genes, challenging efforts to control infections and maintain the efficacy of antibiotic treatments. In intensive care units and other high-risk areas, the rapid transfer of antibiotic resistance genes through transduction may contribute to outbreaks of resistant infections, complicating treatment options and threatening patient outcomes.

This work aims to explore the role of bacteriophages in horizontal gene transfer of antibiotic resistance, focusing on plasmid mispackaging. The review will discuss the mechanisms by which phages facilitate the transfer of ARGs, analyse the factors that affect the likelihood of transduction events, and evaluate the implications of phage-mediated gene transfer in clinical settings. Additionally, the prospects of this study and any challenges it has faced will be discussed in detail. By investigating these topics, we seek to inform strategies for mitigating the spread of resistance and improving the management of bacterial infections in an era of increasing antibiotic resistance.

Chapter – 2

Contextual Overview

2.1 Bacteriophages and Bacterial Plasmids

Bacteriophages, commonly called phages, are some of the most abundant organisms in the biosphere (Clokie et al., 2011). Their presence can be seen in any environment in which bacteria are present, be it the soil, deep in the oceans, and even the guts of animals. Phages are viruses that specifically target bacteria. These play a massive role in regulating bacterial populations and microbial evolution and contribute to gene transfer through horizontal gene transfer (HGT).
Like any other virus, bacteriophages, too, are simply genetic material enclosed within a protein coat. However, phages exhibit a variety of shapes and sizes, often classified based on their morphology. The general structure of a phage includes the following components –

- **Genetic Material –** The viral genome is any one of the following: dsDNA (double-stranded DNA), ssDNA (single-stranded DNA), dsRNA (double-stranded RNA), or ssRNA (single-stranded RNA). Of all these, DNA phages, more specifically dsDNA phages, are the most common and make up the largest order of phages Caudovirales (Flores et al., 2024). The size of the phage genome varies greatly, ranging from a few thousand base pairs to well over 500,000 base pairs.
- **Head (Capsid) –** The head of the phage, composed of proteins, contains and protects the viral genome. It usually has an icosahedral shape.
- **Tail –** Many bacteriophages feature a tail structure that functions as a channel for injecting the viral genome into the host bacterium. The length and complexity of the tail can differ amongst phages; some, like Myoviridae, have long, contractile tails (Yap and Rossmann, 2014), while others, like Podoviridae, have short, non-contractile tails (Naureen et al., 2020).

Bacteriophages can follow two major life cycles after infecting a bacterial cell: the lytic cycle and the lysogenic cycle. Some phages are obligately lytic, meaning they can only undergo the lytic cycle, while others, known as temperate phages, can switch between the two cycles.

On the exact opposite side of the spectrum, plasmids are small, circular, double-stranded DNA molecules that exist independently of the chromosomal DNA in bacteria. They are extrachromosomal elements that can replicate autonomously within the bacterial cell. Plasmids

are important for bacterial adaptation and evolution because they usually carry genes that offer selective advantages under specific environmental conditions, unlike chromosomal DNA, which is necessary for the fundamental survival of the bacterium. They vary greatly in size, with some being as small as 2,000 base pairs while others can reach over 1,000,000 base pairs.

Plasmids vary in their copy number within the cell. Some plasmids are maintained at a low copy number (less than 10 copies per cell), while others are present in high copy numbers (up to hundreds of copies per cell). The copy number is regulated by the plasmid's replication machinery and can influence the amount of gene product produced from plasmid-borne genes.

Similar to bacteriophages, plasmids are also a major driving force behind HGT, a process by which genetic material is exchanged between bacteria without the need for reproduction. HGT plays a critical role in bacterial evolution and adaptability. This occurs in three ways (Burmeister, 2015):

- **Conjugation –** Conjugative plasmids enable the transfer of plasmid DNA between bacterial cells through the process of conjugation. During this process, a donor bacterium forms a physical connection (pilus) with a recipient cell and transfers a copy of the plasmid. Conjugation can occur between bacteria of the same species or even across different species and genera, contributing to the spread of beneficial traits like antibiotic resistance.
- **Transformation –** In transformation, bacteria take up free plasmid DNA from the environment. This process can occur naturally in certain bacterial species or be induced artificially in laboratory settings. Once inside the cell, the plasmid can replicate and express its encoded genes, leading to new traits in the recipient bacterium.
- **Transduction –** This is the key HGT method that is being researched here. While transduction is typically associated with bacteriophages, plasmid DNA can also be transferred between bacteria via phage-mediated transduction. In some cases, phages inadvertently package plasmid DNA during their assembly and then transfer it to a new host bacterium during infection. This mechanism is less common than conjugation but still contributes to plasmid mobility.

One of the most significant roles of plasmids in bacterial evolution is their ability to carry and spread antibiotic resistance genes (ARGs) amongst populations. The acquisition of ARGs through plasmids allows bacteria to survive exposure to antibiotics, even those that are used in clinical and agricultural settings. This has led to the rise of multidrug-resistant bacterial strains, which pose serious challenges to public health.

2.2 Phage Life Cycle and Genetic Material Exchange

Bacteriophages (phages) replicate inside bacterial cells, following distinct pathways depending on the phage type. The relevant replication cycles are the lytic cycle and the lysogenic cycle, both of which show distinct ways in which genetic material can be transferred in and out of, and even throughout, a bacterial cell lineage.

- **Lytic Cycle:** In the lytic cycle, phages immediately take control of the host's cellular machinery to produce new phage particles. After the bacterial host has been filled with progeny virions, the host cell lyses, releasing the newly formed phages into the environment, where they can infect other bacterial cells. This process is highly efficient at producing large numbers of new phages but results in the death of the host.
- **Lysogenic Cycle:** In contrast, in the lysogenic cycle, certain phages can store their genetic material as a part of the bacterial genome, in a state called lysogeny. In this state, the phage DNA, called a prophage, is replicated passively as part of the bacterial chromosome. The prophage can remain dormant for extended periods, being passed on to daughter cells during bacterial division. Under certain environmental conditions, the prophage may reactivate and enter the lytic cycle, excising itself from the chromosome and initiating the production of new virions.

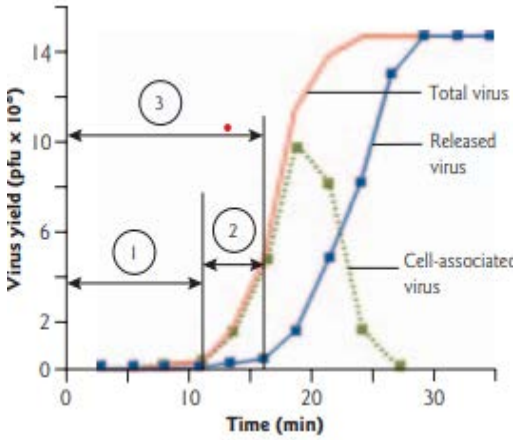

Figure 2.2.1: A one-step growth curve of bacteriophage λ following infection of susceptible bacteria (*Escherichia coli*). During the eclipse phase (1), the infectivity of the Cell-associated, infecting virus is lost as it uncoats; during the maturation phase (2) infectious virus is assembled inside cells (cell-associated virus), but not yet released; and the latent phase (3) measures the period before infectious virus is released from cells into the medium. Total virus is the sum of cell-associated virus and released virus. Cell-associated virus decreases as cells are lysed. This classic experiment shows that phages develop intracellularly

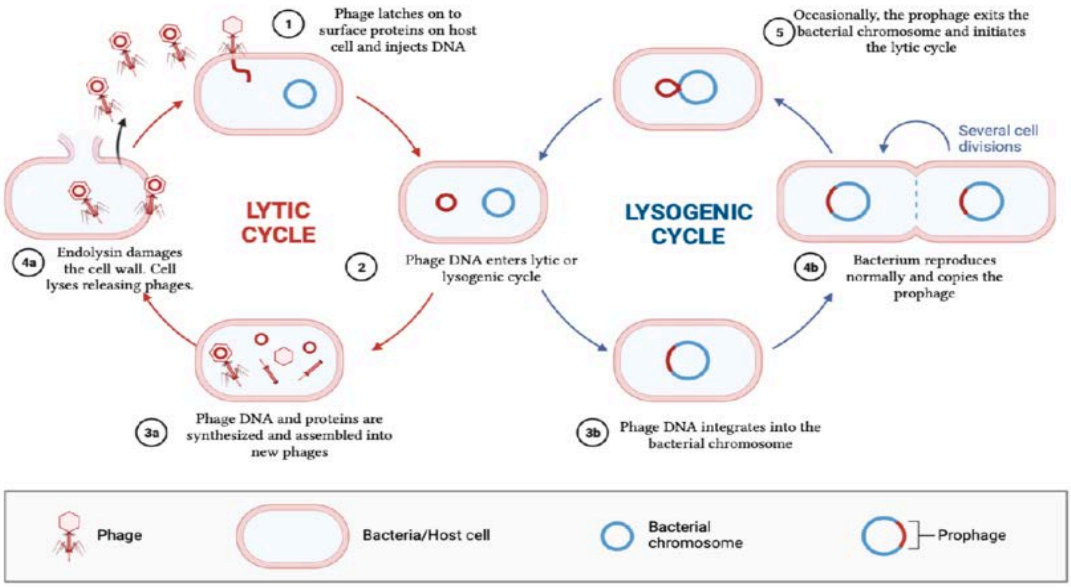

Figure 2.2.2: Lytic and lysogenic cycles. The figure above shows the reproduction process of a bacteriophage. The left side, bolded in red, is the lytic cycle. The right side, bolded in blue, is the lysogenic cycle. Adapted from (Alsobhi, 2021).

Phages play a pivotal role in the movement of genetic material between bacteria, contributing to bacterial evolution and the spread of advantageous traits such as antibiotic resistance. As mentioned before, transduction is the process by which bacteriophages facilitate the transfer of bacterial DNA from one cell to another. This can occur when phages go through a process called a mispackaging event. This is when segments of bacterial DNA, including those from plasmids, are packaged into the phage capsid through errors in the viral DNA packaging or excision machinery. Normally, phage genomes contain specific packaging signals that direct the viral assembly machinery to load the viral genome into the capsid. However, in some cases, bacterial DNA sequences that resemble these signals can be mistakenly recognized, leading to the packaging of bacterial plasmid DNA or chromosomal fragments instead of or alongside phage DNA. These mispackaged phages are termed transducing particles, as they can transfer bacterial genes to other bacteria.

Once the transducing particle infects a new bacterium, the bacterial DNA can be integrated into the recipient's genome, potentially conferring new traits, such as antibiotic resistance. An experiment was carried out to prove these results. This was done by introducing phages to antibiotic-resistant strains of bacteria and later introducing those same phages to wild-type strains. A variety of phages were examined, infecting either *S. aureus*, *S. enterica* serovar Typhimurium, or *E. coli*. In these experiments, we monitored generalised transduction as the phage population used for infection was prepared by propagation on antibiotic-resistant cells rather than by induction of a lysogen. It was observed that lysogens of *S. aureus* carrying the generalised transducing phage could acquire DNA from non-lysogenic cells following spontaneous phage release in a process termed auto-transduction (Fillol-Salom et al., 2019).

Simply put, when a transducing particle infects a new host bacterium, it injects the bacterial genes into the new cell. These genes can be integrated into the recipient bacterium’s genome through homologous recombination, a process that allows foreign DNA to be incorporated into the host’s chromosome. Alternatively, if plasmid DNA is transferred, it can replicate independently within the new host, perpetuating the transferred genetic material. In this way, genes responsible for antibiotic resistance can spread rapidly through bacterial populations.

Before moving into any more detail, transduction, the result of these mispackaging events and one of the key drivers of bacterial evolution, must be explained. There are two major types of transductions –

- **Generalised Transduction:** Generalised transduction refers to the event of bacteriophage acquiring any portion of the host genome that occurs during the lytic cycle when the packaging machinery demonstrates reduced specificity in integrating DNA into phage particles (Valero-Rello et al., 2017). When bacterial DNA fragments are found in close proximity to the phage genome or when phage-induced lysis results in fragmentation of the host's DNA, errors may occur in the viral packaging machinery. Due to this error, phages can package any bacterial DNA, including plasmids, inside the capsid instead of viral DNA.
- **Specialised Transduction:** Specialised transduction is linked to the faulty prophage excision during the lysogenic cycle. Errors during the excision of a prophage from the bacterial chromosome to begin the lytic cycle might result in the simultaneous packaging of the nearest bacterial genes, particularly those on plasmids, alongside the phage DNA (Valero-Rello et al., 2017). As a result, the phage's integration site leads to the transduction of particular bacterial genes in the nearest proximity.

The implications of any form of transduction are vast. Phages serve as vectors for the horizontal transfer of a wide array of bacterial genes, including those for antibiotic resistance, virulence factors, and metabolic pathways (Pfeifer & Rocha, 2024). Among these, one of the most pressing consequences of phage-mediated gene transfer is the spread of antibiotic resistance genes. At the same time, phages contribute to the genetic diversity of bacterial populations by enabling the exchange of genetic material between distantly related bacteria through transduction. This genetic exchange can lead to new bacterial strains with enhanced pathogenicity or environmental resilience (González-Villalobos & Balcázar, 2022). Phage-mediated gene transfer exerts significant evolutionary pressure on bacterial populations. Bacteria that acquire advantageous genes through HGT may gain a selective edge, outcompeting other population members. This pressure can lead to the rapid proliferation of antibiotic-resistant strains, especially in environments where antibiotics are used extensively, such as hospitals or agricultural settings. It

significantly affects the human gut microbiome, as the abundance of tailed bacteriophages in the human gut suggests that transduction could be a significant mode of HGT in the gut (Borodovich et al., 2022).

Additionally, the constant threat of phage infection has driven bacteria to evolve various defence mechanisms, such as the CRISPR-Cas system, which allows bacteria to remember and destroy invading phage DNA. But of course, the continuous evolutionary race between bacteria and phages has also led to the evolution of phages with strategies to overcome these bacterial defences, further shaping the evolutionary landscape of bacterial populations.

2.3 More about Mispackaging Events in Phages

The assembly of bacteriophages is a highly regulated procedure that involves the formation of fresh virus particles within a bacterial host during the lytic cycle. An essential stage in phage assembly includes encapsulating the phage genome within capsids. This procedure is accomplished by identifying particular packaging signals on the phage genome. These signalling pathways direct the phage's packaging machinery to effectively insert viral DNA into the capsid (Rodríguez-Beltrán et al., 2021).

As mentioned previously, errors do occasionally occur in this process. Mispackaging of bacterial DNA into phage capsids can occur due to several factors, mainly during the assembly and DNA packaging phases. This inevitably leads to transduction, though how the errors occur often dictates how the transduction will take place. If an error arises during the lytic cycle, the transducing particles produced will lead to generalised transduction. Here, the phage mistakenly packages random fragments of bacterial DNA rather than its genome. When the mispackaged phage infects a new bacterial cell, it injects the bacterial DNA, which can then be incorporated into the recipient’s chromosome through recombination. This usually takes place when an error occurs in the viral packaging machinery. This causes the host chromosomal or plasmid DNA to be used as a substrate for DNA packaging into empty viral proheads instead of the proper viral DNA (Viret et al., 1991). The random nature of this process makes generalised transduction a potent mechanism for spreading diverse genetic material across bacterial populations. This

process is based on the theory that phages can carry and deliver plasmids into other bacteria during the next lytic cycle. A study on the frequency of mispackaging of *Prochlorococcus* DNA by cyanophage was theorised and concluded that generalised transduction was the dominant route of phage-mediated HGT in those bacteria (Laurenceau et al., 2020).

Consequently, errors in the lysogenic cycle led to the produced transducing particles causing specialised transduction instead. Specialised transduction occurs during the lysogenic cycle when a temperate phage excises itself from the bacterial chromosome to enter the lytic cycle. If the excision is not precise, the phage may carry adjacent bacterial genes and DNA. Unlike generalised transduction, specialised transduction only transfers specific bacterial genes near the prophage integration site. This process can lead to the transfer of important traits, such as resistance to antibiotics or metabolic adaptations, and has been observed in several bacterial species.

Numerous studies have documented mispackaging events where bacteriophages have transferred bacterial genetic material, including antibiotic-resistance genes, to new hosts. Recent studies have shown that phages obtained from clinical and environmental samples carry genes that provide resistance to antibiotics, including β-lactamases, tetracycline resistance genes (tetA), extended-spectrum-lactamases (ESBLs) genes along with fluoroquinolone resistance qnr genes that protect DNA gyrase and topoisomerase IV from fluoroquinolone binding (Lerminiaux & Cameron, 2019). The study demonstrates that recombination can be a key factor for forming a chimeric plasmid containing multiple drug-resistance genes. The study shows multiple plasmids containing one or two resistance genes recombining to form a chimeric or hybrid plasmid. Bacteria that obtained this plasmid became multidrug-resistant. Portions of these hybrid plasmids can be transduced among bacterial populations through bacteriophages in the mode of HGT (Rodríguez-Beltrán et al., 2021).

Another relevant example was the study of phage-transduced antibiotic resistance outbreak case studies, which included Shigella outbreaks due to the transfer of a plasmid-encoded azithromycin resistance gene in the United Kingdom (Baker et al., 2018). The low-frequency pathogenic

strains of *Shigella* acquired this specific plasmid, resulting in an infectious disease outbreak in the community.

Currently, there is only indirect data indicating that transduction occurs in hospitals. Bacteriophages extracted from hospital-acquired methicillin-resistant *S. aureus* infections were observed to efficiently transduce antibiotic-resistance genes to susceptible bacteria in the laboratory (Stanczak-Mrozek et al., 2015). In the same way, tetracycline and penicillin resistance genes could potentially be transduced among hospital isolates of *S. aureus* (Mašlanˇová et al., 2016). Thus, bacteriophage-mediated transfer of antibiotic resistance in laboratory settings may significantly contribute to the establishment and persistence of such resistance in bacterial populations through transduction (Lerminiaux & Cameron, 2019). Lerminiaux & Cameron also argue that in Gram-negative bacteria, transduction has been documented for transferring several antimicrobial resistance genes, including extended-spectrum β-lactamase genes, from *Pseudomonas* hospital isolates to other *Pseudomonas* strains in laboratory settings. In the same way, β-lactamase genes can be transduced among *Acinetobacter* strains in a laboratory setting (Krahn et al., 2016).

More contemporary research revealed that more than 70% of hospital stool samples tested positive for bacteriophages containing ARGs, and hospital wastewater was polluted with bacteriophages containing ARGs (Lerminiaux & Cameron, 2019). Antibiotic treatment could facilitate the transmission of ARGs since antibiotic-treated mice exhibited a higher prevalence of bacteriophages that carry ARGs within their intestines compared to their non-antibiotic-treated counterparts (Modi et al., 2013). Therefore, both chromosomal and plasmid-borne ARGs could be transferred via transduction.

Some more laboratory studies of viral mispackaging and transduction include –

1. In 2022, Pfeifer et al. demonstrated the phages packaging mechanism of plasmid DNA into capsids in laboratory settings. The successful transduction of antibiotic resistance genes via phage-plasmids indicated how bacteriophage can transfer the resistance genes among widely non related hosts. For example, the study used different strains of P1 phages, temperate bacteriophages that can transduce large plasmids carrying ARGs (Pfeifer et al., 2022). Such

observation illustrates the capacity of phages to facilitate the rapid transfer of antibiotic resistance genes in natural environments (Pfeifer et al., 2022).

2. Another recent study has demonstrated that genes carrying plasmids undergo evolutionary processes that are fundamentally distinct from those chromosomes carrying genes (Rodríguez-Beltrán et al., 2021). Evidence indicates that a few bacteriophages can carry DNA segments exceeding 100 kb, adequate for plasmid transfer (Ochman et al., 2000). Bacteriophages successfully transduced a 5667 bp plasmid containing tetracycline and aminoglycoside resistance genes amongst *S. aureus* strains in a laboratory setting (Zeman et al., 2017).
3. In 2014, Matilla and Salmond demonstrated that bacteriophages were capable of transducing a 5620 bp plasmid containing a kanamycin resistance gene between *Serratia* and *Kluyvera* species, however at a reduced frequency compared to the transduction of chromosomal resistance genes. Also, in laboratory settings, tetracycline and chloramphenicol resistance genes on 27 kb plasmids were transduced by bacteriophage between *Actinobacillus* sp (Willi et al., 1997).
4. Leclerc et al. (2022) conducted a study in which the evolution of multidrug-resistant bacteria was theorised to be caused by both the antibiotic and phage targeting the bacterial species. Here, it was found that when the concentrations of antibiotics and phages were high, the bacteria were suppressed properly. On the other hand, introducing a small concentration of phages after applying the antibiotic drove ARG evolution, and multidrug-resistant bacteria started proliferating. This all occurred due to transduction.

Recent environmental research has also demonstrated that phage populations significantly affect the propagation of bacterial genes in different ecosystems, including soil, wastewater, and oceans. Particularly, maritime environments have been demonstrated to host phages that carry genes for antibiotic resistance, indicating that phage-mediated HGT is a prevalent event across multiple bacterial communities. Similarly, in 2020, Laurenceau et al. demonstrated the differences in the mispackaging rates of the cyanophage families and their role in the evolution of a cyanobacterium named *Prochlorococcus* through horizontal gene transfer. The study

emphasised the genetic evolution of the coexisting populations and subpopulations of *Prochlorococcus* driven by the phage-mediated HGT.

2.4 Plasmids and Antibiotic Resistance Genes

Plasmids are small, circular, double-stranded DNA molecules distinct from the chromosomal DNA of bacteria. Unlike the bacterial chromosome, plasmids are extrachromosomal and can replicate independently within the host cell. These genetic elements are often categorised based on their functions, and one of the most significant types is the R-plasmid (resistance plasmid), which carries genes that provide bacteria with resistance to antibiotics. Plasmids typically range in size from a few kilobases to hundreds of kilobases and often contain genes that confer selective advantages to their bacterial hosts. Plasmids are equipped with an origin of replication (Ori), which allows them to replicate independently of the bacterial chromosome, ensuring they are passed on during cell division. Many plasmids are conjugative, meaning they carry the necessary genes to facilitate their own transfer between bacterial cells via conjugation, a process wherein two bacteria physically connect through a structure called a pilus, allowing the transfer of genetic material. Some plasmids can also be mobilised through other means, such as transformation or transduction, expanding their capacity to spread through bacterial populations.

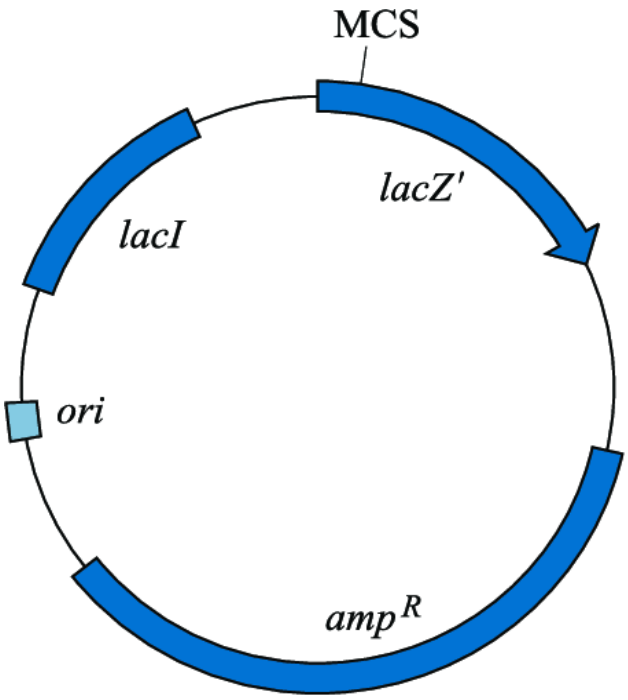

Figure 2.4.1: Generalised pUC plasmid. It contains an ampicillin resistance gene (amp R), part of a beta-galactosidase gene (lacZ'), the Lac repressor gene (lacI) and an origin of replication (ori). The multiple cloning site (MCS) is located in the beta-galactosidase gene.

Plasmids are a major vehicle for disseminating ARGs, enabling bacteria to survive exposure to antibiotics. The acquisition of genes needed to elaborate the various mechanisms is greatly aided by a variety of promiscuous gene transfer systems, one of which is bacterial conjugative plasmids (Bennett, 2009). These resistance genes encode proteins that neutralise antibiotics, reduce drug uptake, or actively pump the drug out of the cell. Plasmid-encoded resistance genes can be spread within bacterial populations and across species through HGT. R-plasmids are commonly found in clinical pathogens, such as *Escherichia coli*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*, and are a major cause of hospital-acquired infections. Additionally, plasmids carrying antibiotic resistance genes have been isolated from environmental bacteria, particularly in areas exposed to high levels of antibiotics, such as agricultural sites, wastewater treatment plants, and aquatic environments.

One such method of HGT that plasmids can be transferred by is transduction, where phages inadvertently package and transfer plasmid DNA during their replication cycle. The interaction between plasmids and phages is a significant factor in the rapid spread of antibiotic resistance genes across bacterial communities.

Phages occasionally mispackage bacterial DNA, including plasmids, during viral assembly. When this happens, a plasmid carrying antibiotic-resistance genes can be transferred to another bacterial host via transduction. This process can occur in both generalised and specialised transduction, depending on the type of phage and the circumstances of mispackaging (Fillol-Salom et al., 2019).

Certain plasmids may also contain sequences that increase the likelihood of being recognized by the phage packaging machinery. Some plasmids have phage-related elements, such as packaging or recombination sites, that facilitate their packaging into phage particles (Pfeifer & Rocha, 2024). This makes phages potent vectors for plasmid-mediated resistance gene transfer. Numerous studies have also shown that phages isolated from clinical and environmental sources can carry plasmids with resistance genes. For instance, phages carrying blaCTX-M or blaNDM-1 genes have been implicated in transferring β-lactamase genes between Gram-negative bacteria in hospital settings (Mazumder et al., 2020). Environmental studies have also demonstrated the transfer of tetracycline and sulfonamide resistance genes via phage-mediated transduction in bacterial populations found in wastewater.

The interaction between plasmids and phages is a key driver of HGT and a significant factor in the evolution of antibiotic-resistant bacterial populations. The consequences of this interaction are far-reaching, affecting both clinical and environmental ecosystems. In the environment, especially in areas exposed to antibiotic residues (e.g., from agricultural runoff or sewage), plasmid-phage interactions facilitate the spread of resistance genes among bacterial populations (Manyi-Loh et al., 2018). These genes can move between environmental reservoirs and human pathogens, raising concerns about the global spread of resistance. Interestingly, the movement of antibiotic resistance genes between human, animal, and environmental bacteria aligns with the

One Health concept (WHO, 2022), which recognizes that the health of people, animals, and ecosystems is interconnected. Phage-plasmid interactions contribute to this cycle by promoting gene flow across different sectors.

2.5 Challenges and Future Prospects

Plasmid-phage influences are critical in bacterial HGT and evolution, yet many questions remain unanswered. Mispackaging events are likewise still mysterious events in a way, as there remain several critical gaps in our knowledge about them. One of the key challenges is determining the frequency with which plasmids are mispackaged by phages and transferred between bacterial hosts. For example, it is unclear why some phages are more prone to mispackaging bacterial DNA than others and how environmental pressures, such as antibiotic exposure, affect these rates. This information is critical for assessing the risk of phage-mediated resistance spread in both clinical and environmental settings. Besides that, much of the current research is based on in vitro studies. Understanding the dynamics of plasmid-phage interactions in natural environments or within the complex microbiomes of the human body is still an area that requires further investigation. Since phages are used for therapeutic purposes to treat bacterial infections, there are concerns regarding unintended plasmid transfer. Another area of interest is understanding how stable plasmids are once transferred via transduction. Factors that influence whether a plasmid can establish itself and replicate in the recipient cell remain poorly understood, especially in cases where the recipient is not naturally competent to maintain the plasmid (Mell & Redfield, 2014).

Another area that warrants further investigation is the host range of mispackaged phages and their ability to transduce genes between distantly related bacterial species. Understanding transduction efficiency across diverse bacterial hosts could illuminate the potential for antibiotic resistance genes to spread in mixed bacterial communities. Given the increasing interest in using phages as alternatives to antibiotics, assessing the risks of mispackaging during therapeutic phage use is crucial. Mispackaging could inadvertently transfer harmful genes, such as antibiotic resistance or virulence factors, complicating efforts to treat bacterial infections with phages.

Developing quantitative models and experimental techniques to measure the rate of plasmid mispackaging in different phage systems will be essential for future research. This will help determine how often plasmid DNA is transferred via phages in laboratory and natural settings. Environmental microbiology studies should continue to investigate the role of phages in transferring resistance genes between bacteria in soil, water, and human-associated microbiomes. Particular attention should be paid to environments where antibiotic use is high, as these are hotspots for developing and spreading resistance (Kunhikannan et al., 2021).

Phage therapy holds promise as an alternative to antibiotics, but ensuring the safety and specificity of therapeutic phages is critical. Researchers must carefully assess the risk of plasmid mobilisation and mispackaging in phages used for therapy to avoid potentially worsening the antibiotic resistance crisis.

Chapter – 3

Materials and Methods

3.1 Materials

3.1.1 Containers –

1. Petri dishes
2. microcentrifuge tubes
3. falcon tubes (15 ml/50 ml)
4. conical flasks
5. glass beakers

3.1.2 Equipment –

1. Micropipette (100-1000 µl
2. 20-200 µl
3. 10-100 µl)
4. 37°C incubator
5. 37°C shaker incubator
6. spectrophotometer
7. gel electrophoresis apparatus
8. thermal cycler
9. centrifuge
10. vortex mixer
11. heat block
12. UV light

3.1.3 Growth Media –

1. Luria-Bertani broth (ready-made)
2. Luria-Bertani agar (LB Broth + 1.5% agar)
3. Luria-Bertani soft agar (LB Broth + 0.6% agar)
4. MacConkey agar (ready-made).

3.1.4 Reagents –

A. **For differential and selective media preparation –**

i. Ampicillin
ii. Arabinose

B. For plasmid isolation –

i. Alkaline lysis solutions:
ii. Solution I (pH 8) - 1M Tris-HCl, 0.5M EDTA, 1M glucose in distilled water
iii. Solution II - 10N NaOH, 1% SDS in distilled water
iv. Solution III (pH 5.2) - 5M sodium acetate, glacial acetic acid in distilled water
v. Ethanol
vi. Phenol: Chloroform: Isoamyl alcohol (25:24:1, v/v)
vii. Chloroform: Isoamyl alcohol (24:1)
viii. Tris-EDTA buffer (pH 8)

C. For PEG Precipitation –

i. 10% PEG
ii. 1M NaCl

D. For competent cell preparation –

i. 0.1M $CaCl_2$ solution
ii. 15% glycerol + $CaCl_2$

E. For gel electrophoresis –

i. 1% agarose gel
ii. 1% TBE buffer
iii. EtBr

F. For phage enrichment and DNA isolation –

i. SM buffer (pH 7.5)
ii. Proteinase K

iii. Proteinase K buffer - (18 µl 0.5M EDTA + 15 µl 10% SDS)
iv. Digestion Enzyme Mix (DNase + RNase)
v. Phenol:Chloroform:Isoamyl Alcohol (25:24:1)
vi. Absolute ethanol

3.2 Methodology:

3.2.1 - *Escherichia coli* bacteria isolation

- A piece of raw chicken meat weighing 20g was collected and ground on a mortar-pastel using normal saline, amounting to 12ml. The liquid solution was collected into a falcon tube.
- 1 ml of the liquid solution was taken for dilution (up to 10^{-5}) and was dropped onto MacConkey agar plates, and the spread plate technique was used to lawn the surface.
- The plates were incubated for 18-24 hours, and results were observed. The dark pink (indication of E. coli) single colonies that grew on the plates were taken for subculture onto Luria-Bertani agar (LA) plates. These bacterial strains were used to enrich bacteriophage.

Following this procedure, the 4 best E. coli samples were selected and named Ec4.4, Ec5.1, Ec12.2.1, and Ec12.3, and the numbers were used to indicate the strains.

3.2.2 *Escherichia coli* Bacteriophage isolation –

- The liquid solution, collected from the ground meat and the collected water sample, was centrifuged 3-4 times at 13500 rpm for 15-20 mins and then filtered using 0.22u filters.
- The sub-cultured bacterial strains, isolated from raw meat, were inoculated into a falcon tube containing 10 ml of Luria Bertani broth (LB) and incubated on a shaker incubator for 2h to make a young culture. 1ml of that filtered liquid solution was added to the young culture and again incubated on a shaker incubator overnight for phage enrichment.
- The enriched solution was centrifuged at 13500 rpm for 15 minutes, filtered using a 0.22-micron filter, and kept in the fridge at 4°C.
- A DLA test was conducted to ensure the presence of bacteriophage in the enriched solution.

- Formed plaques containing phages were observed and collected from there.

Following the above process, 4 phages were isolated, of which 3 phages were from environmental water, and 1 was from raw meat. The phages were named EcP4.4, EcP5.1, EcP12.2.1, EcP12.3(m), where 'm' indicates the phage collected from meat.

3.2.3 Plasmid DNA isolation

- Active cultures of *E. coli* DH5α cells containing the pGLO plasmid were taken and grown overnight in LB broth containing ampicillin and arabinose.
- These cells underwent alkaline lysis as per standard lab protocol to isolate the aforementioned plasmid.
- Gel electrophoresis was performed to verify whether the plasmids were successfully isolated.

3.2.4 Competent cell preparation

- Wild-type *E. coli* cells were grown overnight in LB broth at 37°C.
- 1 ml of those cells were then inoculated in LB broth and incubated until the optical density at 600 nm was recorded at 0.4.
- These cells were made competent through CaCl2 treatment per standard lab protocol.

3.2.5. Transformation of competent cells

- The competent cells were induced to uptake the isolated plasmids through the heat-shock transformation method as per standard lab protocol.

- Transformed colonies were plated on LB agar media containing ampicillin and arabinose. The transformation was verified using UV light.

3.2.6 Bacteriophage enrichment and DLA assay

Collected bacteriophages were enriched using the untransformed wild-type *E. coli* cells. According to standard laboratory protocol, a DLA assay was conducted to verify if enrichment was done successfully.

3.2.7 Infection of transformed cells with bacteriophage and phage lysate retrieval

- The phages were serially diluted and introduced to the transformed bacterial cells in LB broth before being plated. This was allowed to incubate overnight at 37°C before the plates were observed for confluent lysis.
- PEG-NaCl solution was added to these plates to extract the phages present.
- This mixture was centrifuged and filtered to remove superfluous bacterial cells or debris. Therefore, the phage lysate was obtained.

3.2.8 Bacteriophage Purification - PEG Precipitation

Polyethylene glycol (PEG) precipitation was performed. It enabled adequate phage titer, which was required for phage-DNA extraction.

- 1 volume of phage high titer lysate and PEG at a ratio of 1:2 precipitant: lysate was added.
- Solution was incubated at 4°C overnight. Then, the solution was centrifuged for 30 minutes at 4°C at 10000 rpm.
- After centrifugation, obtained pellets were collected and preserved in the SM buffer.

3.2.9 Bacteriophage DNA isolation and Gel electrophoresis

- Digestion enzyme mix was added to the phage lysate and incubated for an hour at 37°C.
- Proteinase K was added, and the mixture was incubated for an hour at 37°C.
- After this, standard lab protocol was maintained for any phenol:chloroform-based DNA isolation method. The obtained phage DNA pellets were preserved in a buffer.
- Similarly, an alkaline-based DNA extraction method was performed with the test bacteriophage strains to obtain plasmid DNA pellets, if any.

- Finally, the pellets that were obtained were used in gel electrophoresis to check whether any mispackaging event had occurred.

Chapter – 4

Results

The purpose of the experiment was to evaluate and understand the bacteriophage mispackaging efficiency of host bacterial plasmid. The initial steps of the experiment included -

4.1 Bacteria & Bacteriophage Isolation

Bacteria, as well as their associated bacteriophages, were isolated from the natural environment, such as water and meat samples collected from various sources. Obtained bacteria and bacteriophage strains were enriched.

As a lactose fermenting bacteria, *E. coli* produces acidic byproducts that lowers pH in MacConkey agar media, leading to the appearance of pink colonies.

Figure 4.1.1: Collected *E. coli* on MacConkey Agar Plates

4.2 Bacteriophage Collection and Enrichment

Spot test was conducted to find the appropriate bacteriophage strains. Appearance of clearing circular dot areas throughout the plates indicated test *E. coli* cells were susceptible to bacteriophage infection. Such clearing circular areas suggests that infected bacteria were lysed and progeny phages were released which infected adjacent bacterial cells. The decline in metabolism of test bacterial cells stopped this circular motion chain reaction, resulting in the appearance of clear circular areas on plates. Bacteriophage isolated from wild type *E. coli* strains and standard phage enrichment procedure was followed.

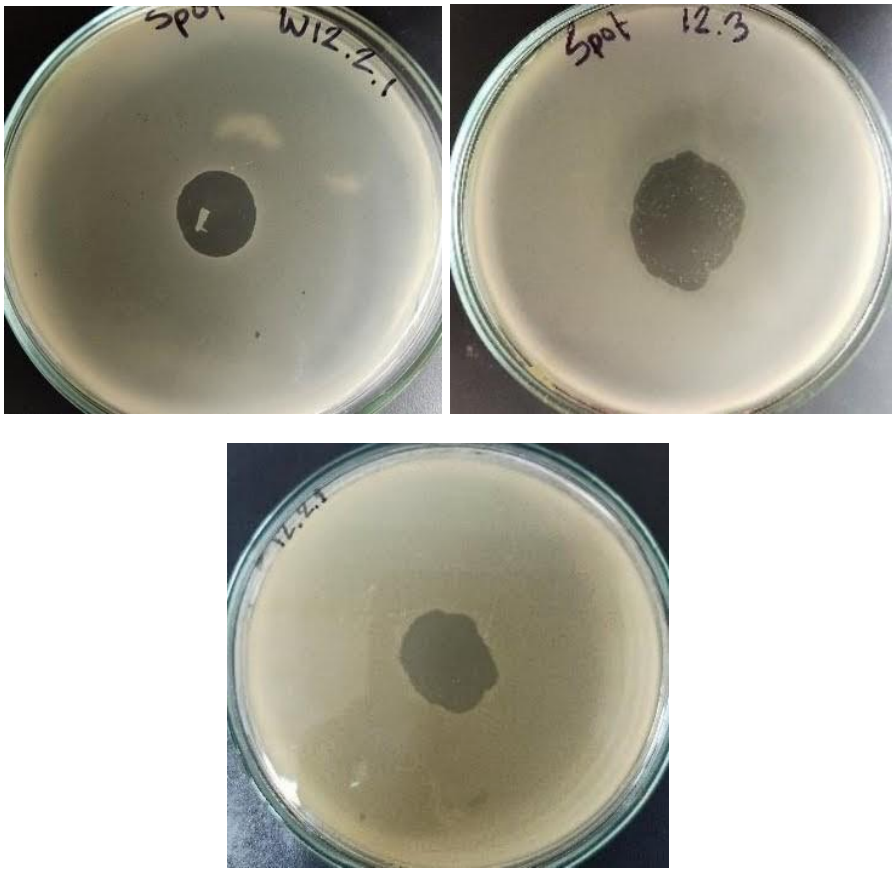

Figure 4.2.1: Spot Test for Appropriate Bacteriophage Collection

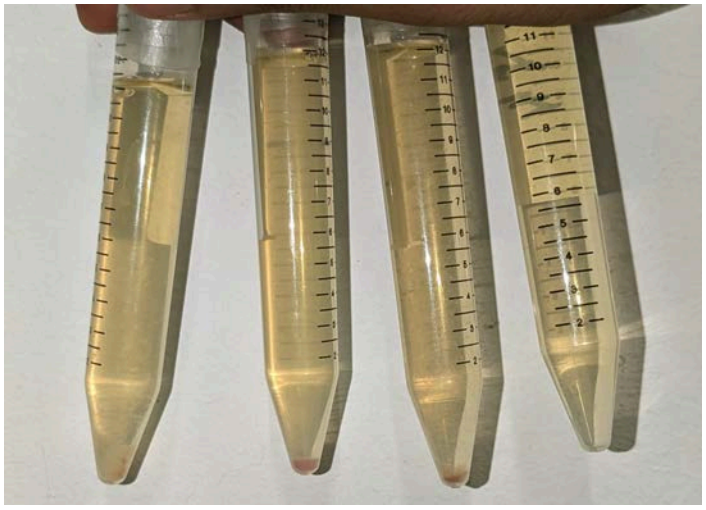

Figure 4.2.2: Enriched bacteriophage strains

4.3 Bacteriophage Infection Efficacy (DLA Method)

Bacteriophage infection susceptibility towards test competent *E. coli* cells was evaluated through the DLA method. PFU count was taken. Different bacteriophages showed different levels of susceptibility.

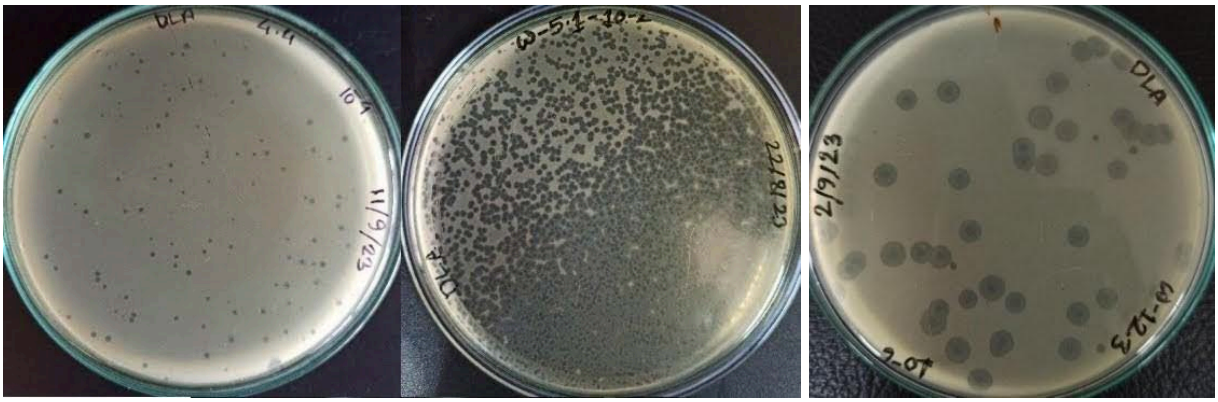

Figure 4.3.1: DLA of the acquired bacterial strains for phage collection

4.4 pGLO Plasmid Isolation

pGLO containing plasmid was isolated from pre-existing transformed *E. coli* DH5α using the traditional alkaline lysis method of plasmid DNA extraction.

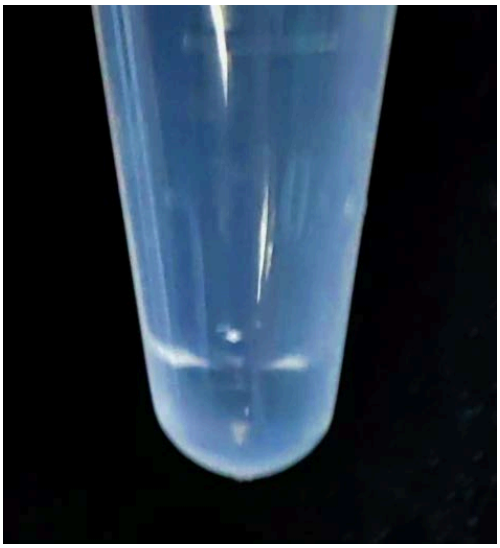

Figure 4.4.1: Extracted pGLO plasmid from *E. coli* DH5α strain

4.5 Competent Cell Preparation and Transformation of pGLO Plasmid*:*

Test *E. coli* cells that lack natural competence and transformation ability were subjected to $CaCl_2$ treatment that created pores in the cell membrane. Ca^{2+} ions bind with phosphate of DNA and decrease electrostatic repulsion. Thereafter, heat-shock treatment allowed the membrane to become more porous and facilitated the uptaking of pGLO plasmid DNA by test bacterial cells. Later the transformed cells were spread plated which provided satisfactory glow under UV light.

Figure 4.5.1: Transformed *E. coli* Cells (Spread & Streak Plates)

The remaining steps of the experiment included bacteriophage enrichment, infecting the transformed cells, collecting the phage after incubation, DNA extraction of those phages and gel electrophoresis methods.

4.6 PEG Precipitation

PEG precipitation was performed for the purification of phage populations as a preparation step for phage DNA extraction method. Combined action of a hygroscopic substance (polyethylene glycol) and co-precipitant sodium chloride bound with water molecules, resulted in precipitation of phages that was collected after centrifugation and resuspended in buffer solution.

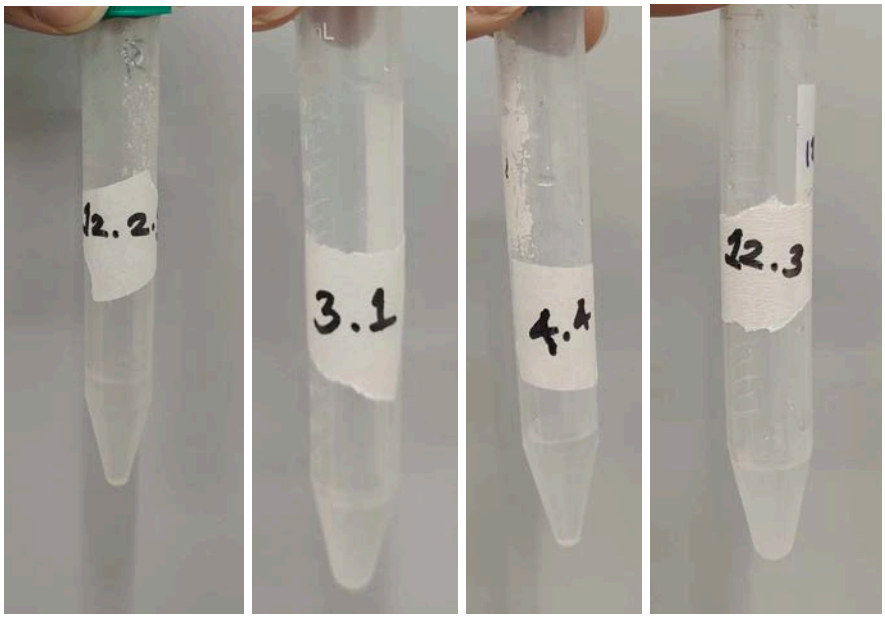

Figure 4.6.1: PEG Precipitated Phages in SM Buffer

4.7 Phage-DNA Extraction Method

Digestion enzyme-based phenol-chloroform DNA extraction method was performed to extract phage DNA pellets. Obtained pellets were preserved in the TE buffer (pH 7.6) for the further steps.

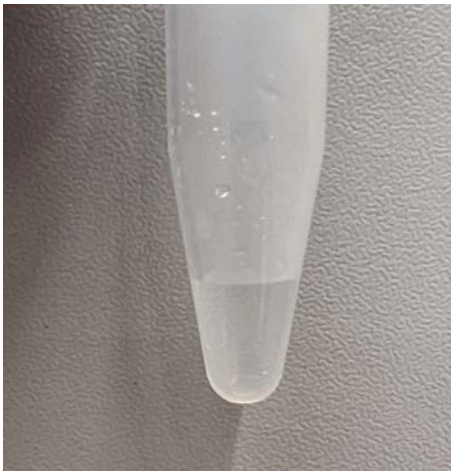

Figure 4.7.1: Extracted DNA Pellets suspended in buffer

4.8 Gel Electrophoresis Method

Gel Electrophoresis was performed with the obtained DNA pellets. A 10kb reference ladder was used for a comparison with the obtained result. However, all the wells provided either smearish or no clear DNA bands at all. Well 1 is the ladder, and Well 10 & 11 are extracted plasmids for reference. Wells 4, 7 & 8 look similar but they have multiple debris lines, as well as these being quite hazy. Wells 12 & 13 are hazy with no site of clear lines. Well 2 & 8 have a large amount of smudge for the whole length, which does not direct to anything other than debris mixed with DNA, and possibly plasmid. Wells 2 & 4 has a very blurred and incomplete line at around 7.5kb, although the length does not represent the size of the whole plasmid, which is at 2.7kb. there is no way to confirm the presence of plasmid for sure.

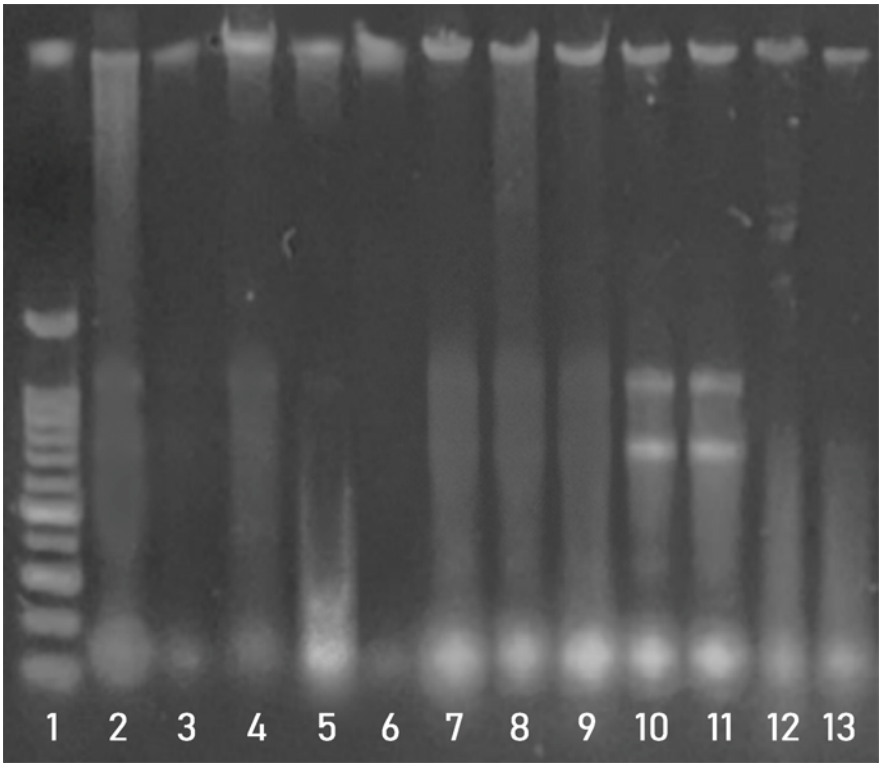

Figure 4.8.1: Gel electrophoresis of genomic materials extracted from phages

Since, no visible bands of the plasmid according to the references in the gel electrophoresis were found, that indicates the event of mispackaging of plasmid from the successfully transformed *E. coli* cells did not occur within the test bacteriophages strains under this experiment condition.

Chapter – 5

Discussion

The experiment aimed to investigate whether plasmid DNA could be mistakenly packaged into phage capsids during bacteriophage replication, a phenomenon that has significant implications for the horizontal transfer of antibiotic resistance genes among bacterial populations. Although the findings did not provide conclusive evidence of such mispackaging, several critical factors emerged that warrant further exploration.

One of the primary challenges encountered was the qualitative assessment of phage DNA and plasmids using gel electrophoresis. The small size of plasmid DNA presents difficulties in visualisation, particularly when attempting to differentiate between plasmid fragments and potential background noise in the gel. Even with a proper DNA ladder, the presence of plasmid DNA remained ambiguous, suggesting that more sensitive detection methods are necessary.

The limitations of gel electrophoresis are well-documented; small fragments may not migrate distinctly, appearing as debris rather than identifiable bands. This phenomenon is particularly pertinent when considering that only a small portion of the plasmid, potentially a non-coding region, might be mispackaged into the phage capsid. Such small fragments would likely evade detection under standard conditions, thereby obscuring potential evidence of mispackaging events.

Another significant factor influencing the results is the inherent size difference between phage genomes and plasmids. Phage capsids have a limited capacity for genetic material; if the plasmid exceeds this capacity, it may not be fully packaged into the capsid. For instance, studies have shown that phages can package DNA segments that are approximately 40 kb long, which aligns with many mobilizable plasmids. If a plasmid is significantly larger than the phage genome, it may lead to an absence of transducing particles capable of mediating gene transfer.

Moreover, even if mispackaging occurs, detecting it poses challenges due to the vast difference in the number of potential transducing particles compared to regular phages. The low frequency of mispackaged plasmids would make them difficult to visualise within a gel matrix. Additionally, variations in plasmid structure—such as linear, supercoiled, or circular forms – further complicate detection efforts. The lack of complete sequencing of the plasmid also hinders accurate identification and characterization of any mispackaged fragments.

Given these challenges, future experiments should consider employing more sophisticated techniques for detecting phage-plasmid interactions. For example, quantitative PCR (qPCR) could be utilised to identify low-frequency mispackaged plasmid fragments with greater sensitivity than traditional gel electrophoresis methods. Additionally, optimising experimental conditions by using specialised bacteriophage strains known to facilitate phage-mediated horizontal gene transfer could enhance detection capabilities.

It is also critical to explore the role of phage-plasmids – elements that function as both phages and plasmids – in mediating antibiotic resistance gene transfer. Recent findings (Pfeifer & Rocha, 2024) indicate that these entities can carry a diverse array of antibiotic resistance genes and facilitate their spread across bacterial populations without requiring direct cell-to-cell contact typically necessary for conjugation.

In conclusion, while this experiment did not yield definitive evidence of plasmid mispackaging into phage capsids, it underscored the complexities involved in studying phage-mediated horizontal gene transfer. Enhanced detection techniques and a deeper understanding of phage-plasmid dynamics are essential for elucidating their roles in antibiotic resistance transmission among bacteria. Further investigations are warranted to fully comprehend these interactions and their implications for public health.

Chapter – 6

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