The evolutionary ecology of phage mediated horizontal gene transfer

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A thesis submitted for the degree of
Doctor of Philosophy

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Abstract

The continuing rise of antibiotic resistance is threatening a return to a world where we are unable to treat the most minor of bacterial infections. Drug-resistant pathogenic bacteria are already killing hundreds of thousands of people and causing considerable economic damage around the world. These numbers will only increase unless steps are taken to curb the spread of resistance.

We need, therefore, to understand how resistance spreads and persists in populations of bacteria. One of the ways that resistance spreads is on plasmids, but it is not clear how these plasmids can persist in the absence of positive selection. It has been shown that conjugative plasmids can be maintained by experiencing episodes of horizontal gene transfer. However, numerous plasmids cannot transfer through conjugation, and how they persist is challenging to understand. Bacteriophages (which are the most numerous biological entity on the planet) could play a part in transferring plasmids horizontally.

My research aims to look at the transduction of an antibiotic resistance plasmid using both experimental and theoretical approaches. I start by looking at whether temperate or lytic phage could mobilize the plasmid and move it between bacteria. I then use a long term experiment to see whether phage can transfer and maintain a plasmid in a population of bacteria. I also examine modelling approaches to see the insights they can provide into how horizontal gene transfer and different aspects of transduction can allow plasmid persistence.

Bacteria-Bacteriophage interactions are complex and will be affected by several factors not considered here. However, my work shows that study of antibiotic resistance should significantly consider the effects of transduction, especially considering the large number of resistance genes that have already been found in the phageome.

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Abstract

The continuing rise of antibiotic resistance is threatening a return to a world where we are unable to treat the most minor of bacterial infections. Drug-resistant pathogenic bacteria are already killing hundreds of thousands of people and causing considerable economic damage around the world. These numbers will only increase unless steps are taken to curb the spread of resistance.

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List of Abbreviations

CF . . . . . Cystic Fibrosis

CFU . . . . . Colony Forming Unit

ds . . . . . Double Stranded

LB . . . . . Luria-Bertani

MIC . . . . . Minimum Inhibitory Concentration

M.O.I. . . . . . Multiplicity of Infection

PBS . . . . . Phosphate Buffered Saline

PFU . . . . . Plaque Forming Unit

ss . . . . . Single Stranded

TTE . . . . . Time To Extinction

WT . . . . . Wild-Type

1 Introduction

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The introduction of antibiotics in the 20th century represents one of the most significant breakthroughs in modern healthcare. Today, procedures such as routine surgeries, chemotherapy and life-saving transplants rely critically on the use of antibiotics[1]. However, while their use has helped humanity survive diseases that

were once untreatable, the continued overuse of antibiotics has dramatically increased the level and frequency of antibiotic resistance in microbial populations throughout the world[2]. The evolution of resistant bacteria could render some of the most widely used medicine in the world redundant. This would bring in a post-antibiotic world where medicine is, quite literally, taken back a hundred years[3]. There would be a decreased ability to treat properly common bacterial infections, increasing the risk of medical complications and fatalities[2]. The increasing global threat has captured the attention of policymakers such as the World Health Organization. They have stated that antimicrobial resistance represents one of the biggest threats to global health and security today[4]. It is predicted that by 2050, if nothing is done to curb the spread of resistance, 10 million people throughout the world will die as a result of drug resistance. This would be more than deaths attributed to diabetes and cancer combined[5].

1.1 Horizontal Gene Transfer and its mechanisms

Bacteria can evolve resistance not only by random mutations but also by acquiring new foreign DNA, and this results in genetic material other than from its parent cell. This transfer of DNA is known as horizontal gene transfer, and compositional analysis has shown that up to 90% of genes in γ-proteobacteria have been transferred horizontally[6]. Genomic analysis has shown how far horizontal gene transfer pervades the evolutionary dynamics in bacteria. There are closely related bacterial strains that differ greatly in their genomes, and only a minority of genes may be shared across a species[7].

Foreign DNA allows the bacteria to adapt to new environments without having to depend on mutations that are firstly rare, and not always beneficial, which may arise spontaneously from the population[8]. Horizontal gene transfer has had huge implications for bacteria, by introducing large amounts of genetic variation such as the gain of complex metabolic pathways[9]. It plays a central role in bacterial adaptation to environmental challenges, like the colonization of new environments, utilization of available carbon sources, and resistance to toxins[10].

Not all horizontal gene transfer is beneficial for the organism. These deleterious genes will, for the most part, eventually be lost from the genome in the same manner that non-beneficial mutations are lost. However, this is not necessarily true for mobile genetic elements, where high rates of horizontal gene transfer can allow parasitic mobile elements to be maintained in bacterial populations. In fact, using two species of bacteria from soil populations, it has been shown that recurrent transfer from a 'source' species can help to maintain low-cost elements in 'sink' species. This inter-species transfer can maintain the plasmid, counteracting against purifying selection[11].

Horizontal gene transfer that imposes a beneficial advantage has the potential to spread throughout a population of bacteria[12]. The real-world impact of horizontal gene transfer is amply demonstrated by the large-scale spread of antibiotic resistance throughout the world[13]. Horizontal gene transfer becomes even more significant as DNA may be transferred between bacteria of different taxa and even between phyla of bacteria[14].

Horizontal gene transfer has been traditionally thought to occur through three different mechanisms: transformation, conjugation and transduction. These mechanisms can be seen diagrammatically in Figure 1.1.

1.1.1 Natural Transformation

Transformation is the only kind of horizontal gene transfers that operates without the use of any type of mobile genetic elements or protection for the DNA from environmental damage. It is the uptake and integration of DNA that exists in the extracellular environment. This occurs when the bacteria use pili on their cell surface to pull in DNA that is close to them, allowing the uptake through their cell membrane[12]. Bacteria can release genetic material into their environment actively, or by ones that have died and been lysed (such as by phage). Additionally, it has been observed that bacteria can absorb the DNA of other bacteria that they have killed themselves[16]. Natural competence is induced in response to specific

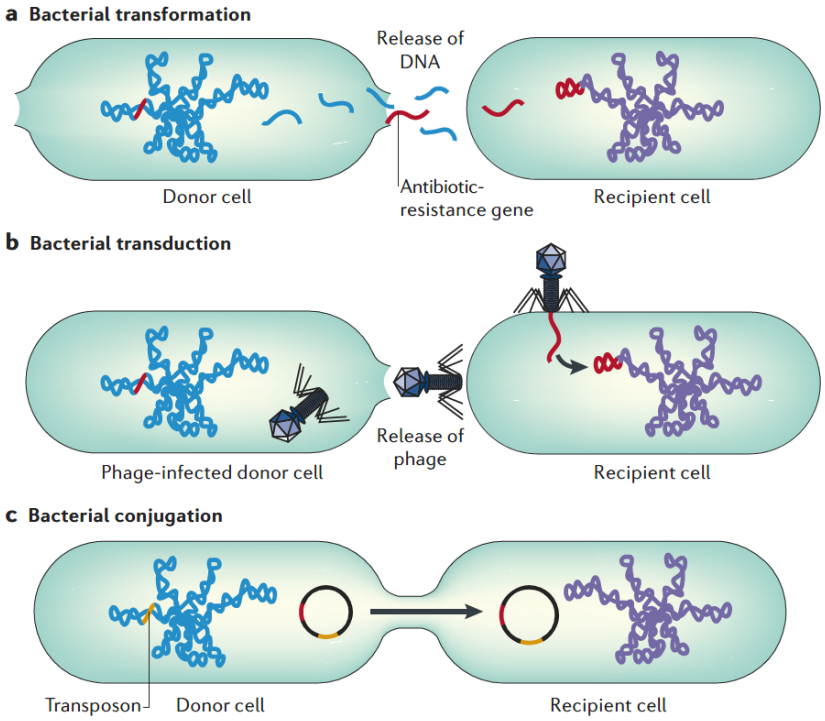


Figure 1.1: The three canonical forms of horizontal gene transfer: transformation, conjugation and transduction. Adapted from [15].

environmental cues that depend on the type of bacteria. It has also only been found to occur in some species of bacteria[17].

1.1.2 Conjugative Transfer

Conjugation takes place when the donor bacteria express a pilus that physically links the donor and recipient bacteria, and which DNA can pass through[12]. Genes that can be transferred through conjugation contains a sequence of bases called an origin of transfer that is recognized by the conjugative machinery of the bacteria[18].

Conjugation is most associated with plasmids because the conjugative transfer systems are most often found on plasmids. This ability to transfer themselves through conjugation allows plasmids to be spread quickly[12]. Plasmids may transfer themselves by conjugation by having an origin of transfer that is compatible with the cell (meaning they are mobilizable), or they may carry their own genes that enable themselves to be transferred through conjugation (conjugative plasmids)[19]. Conjugation was thought to be the dominant way that resistance plasmids travel,

however, recent genomic sequencing has shown up to 50% of them to be incapable of conjugation[20].

1.1.3 Transduction

Transduction is mediated by viruses that are known as bacteriophages. Phages replicate independently of the cells that they are associated with. Phages can take segments of the genetic material of the host cell and package this into their capsid (protein coat), and inject this DNA into other cells, where it can either integrate into the genome or live as a plasmid[21]. The ability to transduce DNA from cell to cell is limited to larger dsDNA phages (50-100kb). Transduction operates using the following mechanism:

1. A phage attacks a cell by injecting its DNA into the cell.
2. The DNA gets expressed producing many copies of the phage.
3. The phage starts packaging up its DNA in its capsid, however, accidentally some cellular DNA gets packaged instead of the phage DNA.
4. The cell is then lysed releasing the phages into the extracellular environment.
5. The phage that contains the cellular DNA instead of the phage DNA then attaches to the cell and injecting that DNA into the cell.

1.2 Bacteriophages

Bacteriophages are the viral parasites of bacteria and are the most numerous form of life on the planet[22]. There are approximately 10^7 phages per millilitre of sea water[23], with a global population that is estimated at 10^{30}[24]. They are a critical part of bacterial ecology and are responsible for killing approximately 50% of all bacteria every 48 hours[25]. The ability of phages to reduce bacterial populations was recognized in the early 20th century. They been used to treat diseases such as dysentery and bubonic plague, in a medical procedure known as phage therapy[26].

Given the large diversity of phage species, there is considerable diversity in genome content. The genetic material can be encoded by either double or single-stranded DNA or RNA. Their genome sizes also vary greatly, from the 3.5 kb ssRNA genome of phage MS2 that can infect *E. coli* to the 500 kb dsDNA genome of phage G that infects *Bacillus*. They can code for things other than just phage replication, such as modified nucleotides to guard against restriction enzymes[27]. Currently, most phages that have been characterised belong to the *Caudovirales* order, which describes phages that have a dsDNA genome and are tailed. This order is further divided into the sub-orders: *Myoviridae*, *Siphoviridae* and *Podoviridae*[28].

Phage infects bacteria by adsorbing to the surface of the cell with their tail fibres. They recognise surface proteins like lipopolysaccharide (LPS) or type IV pili. After adsorption, they inject their DNA into the cell, and hijack the cellular machinery to reproduce. Once they have produced many copies of themselves, enzymes known as lysins are released which bursts the cell releasing the phages into the environment. When the number of free phages is plotted against time during the course of a bacterial infection, the different phases of the infection process can be observed as seen in Figure 1.2. During the first step, the phage adsorbs to the surface of bacteria, and the titre of free phage falls. The second step, known as the latent (or eclipse) period, the bacterial machinery is used to replicate and produce the viral progeny. The third step is known as the burst phase and the cell is burst to release the newly produced phage. The difference between the initial phage titre and the present phage titre is known as the burst size and this is the number of phages produced per single phage infection[27].

There are two different types of phage life cycles; lytic phages are virulent and replicate after they have injected their DNA into the bacterial cell. In contrast, temperate phages integrate themselves into the bacterial chromosome, lie dormant and replicate with the bacteria as a prophage[29]. Temperate phage can enter the lytic cycle at random, but they can also be induced once the bacteria are subject to DNA damaging agents (such as antibiotics) or once the SOS response has been

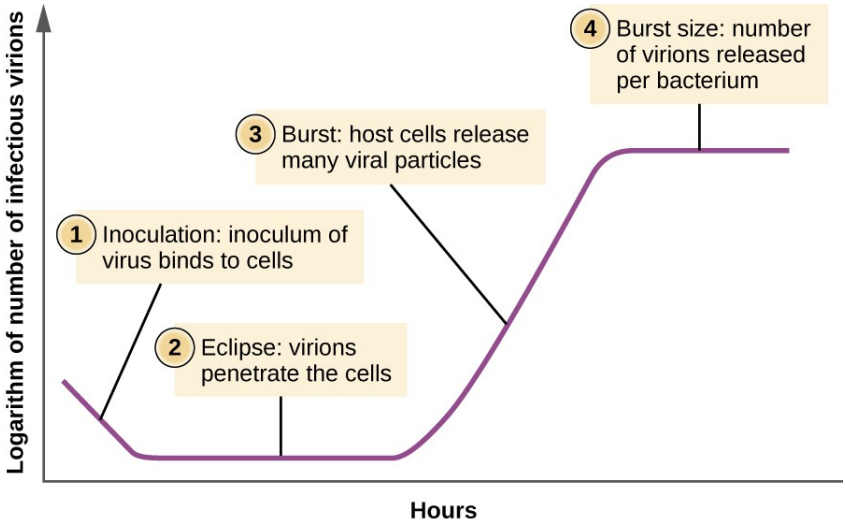


Figure 1.2: Idealised one-step growth during the course of a a phage infection. Adapted from [27].

activated[30]. The release of the temperate phage results in the death of the bacteria through lysis[31]. The two different life cycles of phage can be seen in Figure 1.3.

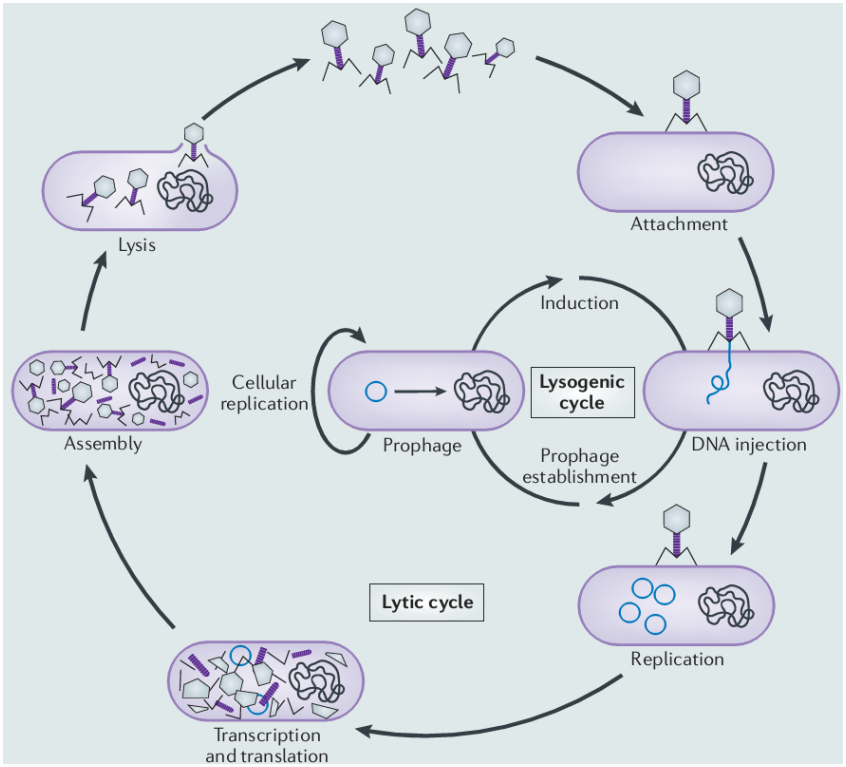


Figure 1.3: Visual representation of the different phage life cycles: lytic and lysogenic. Adapted from [27].

1.2.1 Phage Therapy

Phage therapy is the therapeutic use of bacteriophages to treat bacterial infections[32]. It dates back at least a century, and have been used in the USA until the 1990's[33]. Phage Therapy has been used continuously in the former Soviet Union but has been replaced by antibiotics in the west. This is largely explained by the inconsistent results on the efficacy and their specificity to certain bacterial species as opposed to high-spectrum antibiotics[34] . Phage therapy requires that the exact bacteria be identified and then *in vitro* testing of the phages that they are effective against the bacteria. Another impediment to the broader use of phage therapy is the lack of regulation around individualised therapies. Furthermore, it is difficult for the pharmaceutical industry to register intellectual property around specific phage preparations[35].

However, several recent developments show that phage therapy is coming back into vogue in other parts of the world. There are many advantages of using bacteriophages to treat bacterial infections as opposed to antibiotics. There is essentially an infinite diversity of phages, so if one does not work in treating an infection, another can be found relatively easily. Furthermore, if bacteria become resistant to the phage, then phage have the ability to evolve *in situ* to overcome this resistance[36].

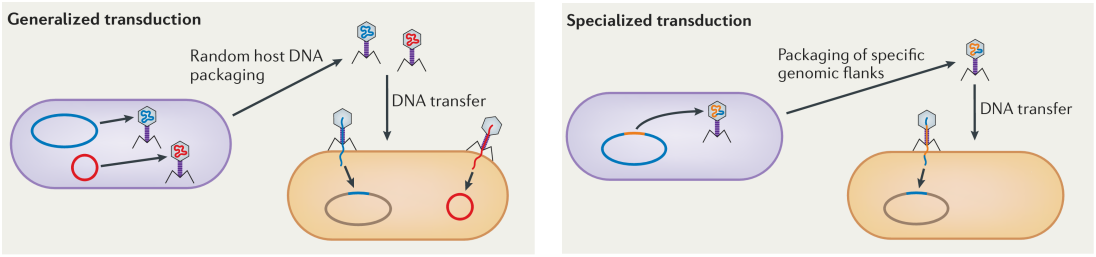
Currently, there are no phage therapy treatments approved for human use in either the European Union or United States[37]. However, there are signs that this may be changing. In 2014, the US National Institute of Allergy and Infectious Disease listed phage therapy as one of its seven ways of combating resistance[38]. In the United Kingdom, one controlled clinical trial of 24 patients looked at treating antibiotic resistant ear infections caused by *P.aeruginosa* showed considerable efficacy and safety[39]. Phagoburn, a clinical trial funded by the European Union and lasting four years from 2013 to 2017 looked at using cocktails of 12 bacteriophages to treat *P.aeruginosa* infections in 27 burn victims. However, the results have been uninspiring, with the phage treatment reducing bacterial load at a slower rate than the standard method of care[40]. One encouraging case is

one of a 15-year-old patient at Great Ormond Street Hospital who was suffering from an infection caused by *Mycobacterium abscessus*. The study used effective lytic phages that are able to treat the bacterial strain which were developed using genome engineering and forward genetics techniques. The patient was treated with a three-phage cocktail and made a full recovery[41]. In 2016, there was great public attention given to a patient who was infected with an antibiotic resistant strain of *Acinetobacter baumannii* in Egypt. The patient was brought back to the United States and the bacterial isolate was tested for susceptibility against a library of 104 *A.baumannii* phages. There were five phages that were found to be able to infect the bacteria, and the patient treated with a cocktail of those five phages. The patient made a full recovery[42]. Lastly, there is an increasing number of case reports of phage therapy being used to treat individual patients in both France and Belgium[43][44][45][46].

The dissemination of antibiotic resistance genes by bacteriophages used in phage therapy could be an unintended consequence of using phage therapy in the fight against resistant bacteria. There are arguments for using phage therapy as a complement to antibiotics[36], but the spread of resistance genes by phage could reduce the efficacy of such a treatment option.

1.2.2 Transduction and its mechanisms

Transduction is seen as a rare event, once every 10^7 to 10^9 phage infections[47] (although other studies have shown that this may be a low-end estimate by several orders of magnitude[48]), however the abundance of phage and bacteria results in transduction being responsible for a relatively large number of gene transfer events. It has been estimated that approximately 2×10^{16} transduction events happen globally, per second[49]. In fact, it has been estimated that up to 20% of all bacterial genomes have been acquired through phage-like elements[49]. Transduction can be divided into two separate sub-classes which can be seen diagrammatically in Figure 1.4. Specialised transduction is thought to be involved in the transfer of specific genes and is be mediated by *cos* phages. Generalised transduction can



(a) Generalised transduction can transfer any sequence of DNA using *pac* phages

(b) Specialised transduction can transfer specific sequences of DNA using *cos* phages

Figure 1.4: The two types of transduction: generalised and specialised. Adapted from [27]

transfer any sequence of DNA between bacteria and is reliant on *pac* phages. The two varieties of phage are differentiated by their method of packaging DNA. In *cos* phages, the *cos* site is recognized and the packaging begins before it is cleaved again at the second *cos* site. In *pac* phages, packaging starts with the cleavage at the *pac* site and then the DNA is cleaved again once the capsid has become full, which often results in more than one unit of the phage capsid being packaged. Transduction relies on the homology of bacterial DNA to these sites. This is why specialised transduction can only transfer specific genes because although there are *cos* site homologs, the probability that there are two *cos* site homologs within the distance that can be fitted into a capsid is rare. However, with generalised transduction, there is only one *pac* site homolog that is necessary[47]. The size of the DNA fragment that can be transferred by the phage is bounded by the size of the capsid. However, there are capsids upwards of a 100kb[50].

Recently, a couple of new novel transduction mechanisms have been identified (both in *Staphylococcus aureus*) that can play a substantial role in bacterial adaptation. In *auto-transduction*, populations of bacteria that contain temperate phage will have a small proportion that will express the prophage. This will lyse the bacteria and release those phages. These phages will then infect and kill the competing strains of bacteria that are susceptible to the phage. A small proportion of those phages will package DNA from the bacteria that they kill. They then go back and infect the original strain that expressed the prophage with the foreign

DNA. This results in the bacterial population containing the temperate phage to acquire antibiotic resistance genes at a very high rate[51].

Another phenomena, termed *lateral transduction*, occurs at frequencies far higher (approx. 1000 fold) than traditional transduction. The study that discovered it observed that *S.aureus* prophages are not excised from the bacterial genome until late in the life cycle. Therefore, the prophage phage starts amplifying themselves before excision (whilst still in the chromosome). This results in multiple integrated phage genomes which are alongside the nearby bacterial DNA. The resulting concatemers are packaged into viral capsid during which it is still integrated into the host chromosome. Each phage is only released when the capsid is full (*pac* site mediated transduction). In situ amplification boosts the phage replication and when excision finally happens, bacterial DNA several kilobases adjacent to the phage get packaged into the new phage and can infect new bacteria[52].

Another process involving the transfer of genes is gene transfer agents (GTAs). These are phage-like particles that are produced by bacteria that can enclose a random part of the host genome. However, these GTAs are limited in that they do not carry enough of the genes required for replication once they have infected new bacteria, so their next progression is hindered[53]. The horizontal gene transfer frequencies of GTAs are relatively high, with kanamycin resistance genes being transferred at orders of magnitude higher than transformation or traditional transduction[54].

1.2.3 Limits of transduction

There are many examples of broad host range conjugative plasmids[55][56][57] that can move resistance genes between very different bacteria. However, it is far more unlikely for the phage to move genes around across those high phylogenetic distances. The breadth of bacteria that a phage can to infect is known as its host range. It is generally believed that there is only a narrow range of closely related bacteria that can be infected by a single bacteriophage[58]. This is primarily due to the specificity of proteins on the host that the phage can bind to[59]. There are

examples of phages that can infect multiple hosts, but they pale in number to the phages that are genus-specific[60]. The host range is a limit of transduction, as DNA can only be transduced by a phage between species of bacteria that it can infect. However, there are examples of transduction occurring between different genera of bacteria such as from *Staphylococcus* to *Listeria*[61] and between *Sphaerotilus* and *Pseudomonas*[62]. In the broader picture of horizontal gene transfer, transduction can explain the movement of genes between different strains in the same species and in a minority of circumstances between different species. However, many resistance genes on pathogenic bacteria have come from phylogenetically very distantly related bacterial species[63]. This means that broad host range conjugative plasmids are more likely to be involved as opposed to transduction. Although, it may be that these conjugative plasmids are involved in the initial acquisition of resistance by the pathogenic species. However, transduction could play an essential role in disseminating the resistance genes between lineages of the pathogen.

The restriction modification system is used by bacteria to defend themselves against foreign DNA such as bacteriophages[64]. Typically, they consist of a pair of enzymes. One enzyme will methylate the DNA in a specific way, and the other enzyme will destroy the DNA that does not have that particular methylation tag[65]. The bacteria's own DNA will be methylated following replication, whereas invading DNA will not have those characteristic modifications and therefore be destroyed[66]. In fact, in some species of pathogenic bacteria such as *Staphylococcus aureus*, their phylogenies have deep branches that consist of groups of very divergent clones. What maintains these differences is restriction modification[67]. Restriction modification will be a limit to transduction because if genes packaged by the phage and infecting another bacteria are coming from distantly related bacteria, then the restriction modification system will destroy it. However, there exist several mechanisms that phages use to overcome restriction modification. The phage T7 expresses a protein that blocks DNA binding of the enzyme allowing the DNA to propagate[68]. Another mechanism is used by λ phage which uses the bacteria's own modification system to methylate its DNA so it will not be destroyed[69]. In

addition to a restriction modification, a recent study[70] found there to be several more antiphage defence mechanisms. These mechanisms need to be investigated further to understand their implications for transduction but could represent further limitations to the movement of genes by phage.

Another possible restriction to the phage mediated horizontal transfer of genes is superinfection exclusion. Superinfection exclusion is the ability of a virus that has already infected a bacterium to interfere with the infection of the same bacteria by a similar virus[71]. There is a lack of systematic studies of superinfection exclusion mechanisms. However, it has shown that there are diverse processes involved, which include surface modification and other defence mechanisms inside the cell[72]. Superinfection exclusion is particularly relevant for transduction by a temperate phage. As temperate phage resides in the genome of the bacteria, they exclude the bacteria from being infected by the same phage.

1.2.4 Transduction of antibiotic resistance genes

There is an increasing number of studies that provide evidence of the phage mediated transfer antibiotic resistance genes between bacteria.

Phageome studies involve taking the bacteriophage populations that exist in specific environments and isolating the DNA encapsulated by the capsid of the phage, and then sequencing that genetic material. One study[73] sequenced the phage populations in mice treated with antibiotics and found that there is an enrichment of phage-encoded genes that confer resistance via disparate mechanisms to the administered antibiotic, as well as genes that confer resistance to antibiotics unrelated to the administered antibiotic. Additionally, they showed that phages from the antibiotic treated mice provided naive microbiota with increased resistance. Another study analysed the phage packaged DNA from the faecal samples from healthy humans[74], and sequencing showed the presence of resistance genes bla_{TEM}, $bla_{CTX-M-1}$, $mecA$, $armA$, $qnrA$, and $qnrS$ in 77% of the 80 samples. Sequencing of the virome of the microbiota of patients who are suffering from cystic fibrosis (which includes, among others, *P.aeruginosa*), revealed an extensive

reservoir of antibiotic resistance genes[75]. They posited that this might explain the relatively fast adaptation of cystic fibrosis strains to their niches. Phage containing antibiotic resistance genes have been found in the environment. Sludge from wastewater treatment was sequenced and the phageome population was enriched with antibiotic resistance genes[76]. There have been examples of the sequenced phageome of pig faeces where up to 30% of the genes were coding for antibiotic resistance [77]. There are examples of phageomes drawn from fresh cut vegetables[78], river water[79], hospital waste water[80], all containing genes that code for antibiotic resistance. Furthermore, they are not bounded geographically, being found throughout the world.

However, the presence of antibiotic resistance genes in the virome of different environments should be viewed cautiously. Antibiotic resistance genes encapsulated by phage does not automatically imply transduction, as these phages could just be a biological dead end. Firstly, it is not a given that there are susceptible hosts that can be infected by these phages. Secondly, viewing transduction through this lens will automatically overestimate rates of transduction because most of the phages that contain resistance genes will decay (due to the short half-life of phage) without being to able to establish an infection. Moreover, a recent study[81] showed that the great abundance of resistance genes in other virome studies could be vastly overestimated by the presence of bacterial contamination.

However, examples of phage transducing resistance genes have been reported using in vitro studies. β-lactamase genes have been transduced by P1-like phages in *E.coli*[82] and by P22 phages in *Salmonella*[83]. Transduction of tetracycline resistance has been observed in both *Streptococcus*[84] and *Enterococcus*[85]. The temperate phage $W\beta$ is able to move fosfomycin resistance in *Bacillus anthracis*[86]. A conjugative plasmid that encoded resistance to imipenem, aztreonam and ceftazidime was moved between *P.aeruginosa* using phages AP-1 and AP-12[87].

Finally, on a more general level, it is difficult to understand the persistence of non-mobilizable antibiotic resistance plasmids without transduction. This has

already been shown in *S. aureus*, where there are very few conjugative plasmids. The other plasmids tend to move around using different forms of transduction[88].

1.2.5 Coevolutionary dynamics between bacteria and phage

Coevolution is defined as the process of reciprocal adaptation and counter-adaptation between ecologically interacting species[89]. Bacteriophage can be deadly towards bacterial populations [90], but bacteria can evolve resistance to the phage that is attacking it by *de novo* mutations and other mechanisms[91]. The most well-known of these is the CRISPR-Cas system in which phage DNA is recognized by the bacterial cell and then destroyed. CRISPR-Cas has been found in over 40% of bacteria[92]. There are other defence mechanisms such as restriction-modification, which destroys phage DNA once it enters the cell[93] and several more mechanisms that have been recently discovered[70]. Conversely, phages have evolved to overcome this resistance by bacteria. There are clear genomic indicators of anti-CRISPR systems[94] as well as anti-restriction modification systems[27]. Bacteria-phage systems have been observed to be very dynamic, and there is often a high rate of coevolution occurring, and sustained coevolution is common[91]. The sheer amount of bacterial and phage diversity is indicative of the magnitude of coevolutionary processes occurring[95]. Coevolution over time is a reasonably common trait and can be found in environmentally borne phage and bacteria. Local adaptation of bacteriophages to their bacterial hosts has been observed in soil[96], suggestive of widespread coevolution. The coevolutionary dynamics have been reasonably well characterized and are known to depend on the environmental conditions[91]. These dynamics have consequences in the applied realm as well because when phages are used as antimicrobials as in phage therapy, the ecological implications of coevolution need to be considered when examining their efficacy[97].

It is clear then that the coevolutionary dynamics between phage and bacteria are essential and that when examining phage mediated horizontal gene transfer, these dynamics need to be taken into account. Transduction can only occur between bacteria that are sensitive to the phage: a donor cell has to be sensitive

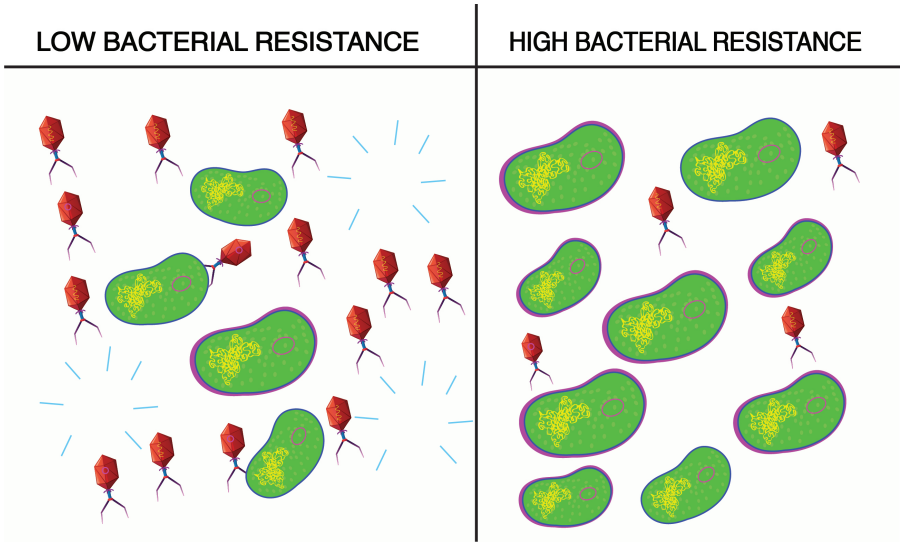


Figure 1.5: The dynamics of phage and bacteria with different levels of bacterial resistance. High bacterial resistance is indicated by a purple cell wall. In low bacterial resistance, transduction occurs because there is an abundance of sensitive donors and hosts. In high bacterial resistance, it is difficult for transduction to occur because there are no sensitive donors and hosts.

such that the phage can infect it and package up the bacterial DNA, and the recipient cell has to be sensitive such that it can be infected by the phage with the donor DNA. Therefore, how these resistances and sensitivities vary with time will significantly affect how transduction proceeds. How bacterial resistance to phage affects transduction can be seen diagrammatically in Figure 1.5. In low bacterial resistance, more phages that can take up the plasmid and infect other bacteria as opposed to high bacterial resistance.

The general concepts describing the coevolutionary dynamics bacteria and phage are between arms race and frequency dependent selection, and the variation of allele frequency with time in both of these types of dynamics are shown in Figure 1.6. Arms race dynamics occurs when are when a particular allele provides resistance to the phage, that allele will increase in frequency, before eventually leading to fixation. This will lead to a phage that can overcome that particular type of resistance increasing in frequency and eventually leading to fixation as well. This can be repeated with different resistance mechanisms, hence the arms race. Arms race dynamics will result in a larger resistance range in the bacteria versus a broader

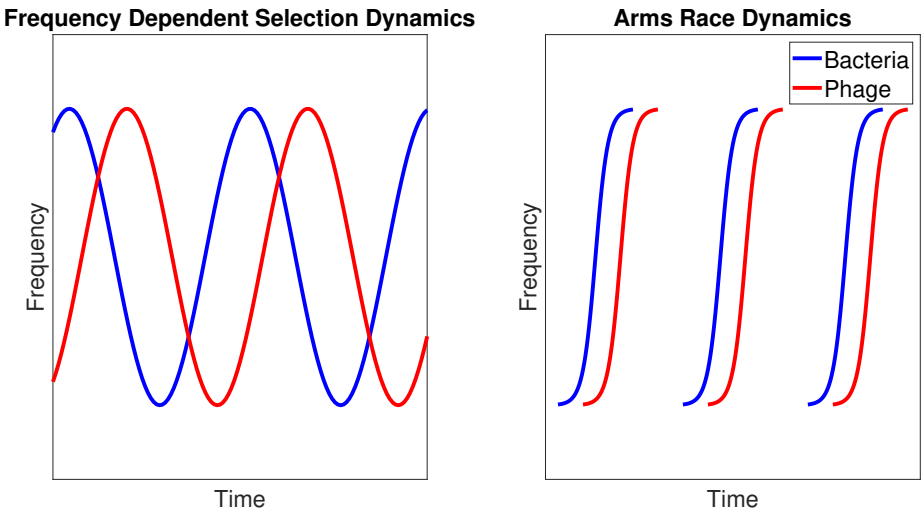


Figure 1.6: Plots showing the variation of allele frequency with time in the two different coevolutionary dynamics between bacteria and phage. Arms race dynamics leads to an increase in the range of phage genotypes that bacteria can resist, and bacterial genotypes that phages can infect. Frequency dependent selection dynamics leads to phages that can infect common bacterial genotypes. This selects for rare resistance alleles in bacteria, which then become common.

range of phage genotypes. Also, there is an increased number of sensitive host genotypes in the phage, allowing more bacteria to be infected. Therefore, the phage can still infect all past bacteria and the bacteria can resist all past phage[98]. Frequency dependent selection occurs when bacteria are infected by phage, and then a particular mutation that will provide resistance to that phage will rise in frequency. Consequently, the phage must evolve to overcome that resistance. Frequency dependent selection therefore favours bacteria that are resistant to the most common phage and also favours the phage that can infect the most common bacteria. This also means that over time the ranges of the infectivity of the phage and resistance of the bacteria are on average the same[99].

It should be noted that these exact models represent the absolute extremes of the spectrum of possible dynamics. Empirical systems that have been studied have shown evidence for both of these types of dynamics[100][101]. Furthermore, there have been examples of populations which display characteristics of both dynamics within the same population. Coevolution between *P.fluorescens* and Φ2 were dominated by arms race dynamics for the first 200-250 generations and then

dominated by frequency dependent selection after that[102]. Interestingly however, when the same bacteria and phage were grown in different environmental conditions, the dynamics were completely different. In soil, they displayed frequency dependent selection from the start[103]. Coevolution assays of six different lytic phages with *P.aeruginosa* found both arms race and frequency dependent selection occurring dependent on the phage. The mechanistic basis for which kind of dynamics are favoured are currently unproven but was argued that how a phage adsorbs on to the bacteria determines which kind of dynamics it favours[101]. Coevolution also has implications for plasmid dynamics because it has been shown to limit responses to non-phage related selection[104]. This means that compensatory mutations which can ameliorate the costs of plasmid carriage are less likely to occur in the presence of phage predation.

1.3 Plasmids and the plasmid paradox

Plasmids are autonomously replicating genetic elements. They are smaller than the genome, and in general, they do not contain the genes required for basic cellular activity[21]. Usually, plasmids are circular and double stranded. However, non-circular plasmids have been observed[21]. If they enter the cell as a double stranded element they do not need to be integrated into the genome to become established[12]. In most cases, a plasmid will contain essential genes that benefit the host under specific conditions (such as resistance genes), genes that help the plasmid to the detriment of the host cell (such as genes that code for conjugation), selfish genetic parasites (such as toxin-antitoxin systems), and other genes without known function[21].

While plasmids can have a diverse array of accessory functions, they play a central role in the spread of antibiotic resistance. This phenomenon is considered an unavoidable consequence of the (over)use of antibiotics[21]. Additionally, a broad array of resistance genes for most types of antibiotics can be exchanged in a single event because resistance genes are usually concentrated in integrons and transposons on plasmids. However, these plasmids may also carry other genes such

as toxins, virulence factors and structures for the colonization of animal or plant hosts. Bacterial sequencing has shown that plasmids play a large role in the diversity of closely related bacterial strains. The difference between a harmless commensal bacteria and a deadly pathogen may simply be the presence of a plasmid, as is the case with anthrax (although this is a virulence plasmid)[105]. Similarly, most of the virulence factors in *Shigella flexneri* are encoded on a single 210kb plasmid[106].

The stability of plasmids has been shown to depend on three factors: the rate of segregational loss, the fitness cost of carriage imposed by the plasmid, and the rate of horizontal gene transfer[107]. One of the fundamental questions that my work attempts to answer is whether bacteriophages could be an answer to the plasmid paradox, which poses the question as to how plasmids can persist in the long term. Plasmids often pose a fitness cost and will be lost in the absence of positive selection for the genes which the plasmid contains. In conditions which do not favour plasmid carriage, then the plasmid can be viewed simply as a parasite that decreases the fitness of the bacteria. However, supposing that the genes on the plasmid are useful, then this can also lead to selection against the plasmid. If the genes are useful, then they will tend to move to the chromosome, leaving the plasmid to be purely parasitic. It has been theorised that episodes of horizontal gene transfer could be an answer to the plasmid paradox, and high rates of horizontal transfer can allow plasmid persistence even as parasites that reduce bacterial fitness[8]. A recent study[108] has shown experimentally in *E.coli* that conjugative plasmids that encode antibiotic resistance genes can persist through very high rates of conjugation, even in the absence of positive selection. Another study[109] looked at the rates of conjugative transfer of a mercury resistance plasmid with and without positive selection. The plasmid reached fixation in the absence of positive selection, and this was found to be primarily through conjugation. However, other analyses have shown that rates of horizontal transfer in most cases are too low to maintain plasmids[110].

Further complicating the picture is that recent genomic sequencing of plasmids has shown that only 50% of them contain the genes necessary for conjugation[18], meaning that for the other half of plasmids, transduction and transformation are

the only avenues that they may move horizontally through. Therefore, if plasmids can be stably maintained and transferred between bacteria by phages this represents a possible solution to the plasmid paradox. However, one study[111] study that runs contrary to this idea showed that bacteriophages limit the conditions by which conjugative plasmids can exist by increasing their loss under weak selection conditions. The study used theoretical and experimental approaches to explore the idea of the effect of bacteriophage driven selection on the dynamics of conjugative plasmids. They used the soil bacterium *Pseudomonas fluorescens* SBW25 together with a mercury resistance plasmid and the lytic phage SBW25γ2 and passaged using serial transfer for 130 generations. It was shown that phages reduced plasmid persistence under weak or no selection for mercury resistance, thereby accelerating their loss. This suggests that rather than a solution to the plasmid paradox, lytic phage amplifies the plasmid paradox because horizontal gene transfer is less probable to occur due to phage predation.

1.3.1 Mathematical models of plasmid dynamics

Theoretical approaches and mathematical models have long been used to explore how costly plasmids may exist. The situations under which plasmids may persist have been called the "existence conditions". Stewart and Levin examined this first in 1977, where they studied at plasmid persistence in the context of conjugative transfer[112]. They used a population dynamics model and investigated the parameter space in population growth, conjugation rate and segregation rate using biologically realistic numbers in order to find the existence conditions. They analysed this for both continuous (chemostat) and discrete (serial transfer) bacterial populations. The model showed a large set of existence conditions and found that a plasmid can be stably maintained only when the cell density and conjugational transfer rate are large enough to overcome segregational loss and selection against the plasmid. This model was further expanded upon by Macken, Levin, and Waldstätter in 1994. This model extended the original and partitioned the total bacterial population into plasmid bearing and plasmid free sub-populations[113]. They used this model to show that

the establishment of a population of plasmids may depend on them being introduced initially at a high enough number. Moreover, they do show that it is possible for plasmids that impose a cost to become established. However, in opposition to that is a another model by Bergstrom, Lipsitch, and Levin in 2000. They initially used empirical studies that posit that plasmids are not conjugatively transmitted at high enough rate to maintain themselves. They modelled what happened to plasmids using a population dynamics approach, and found that plasmids cannot persist indefinitely solely by carrying genes that are beneficial to the host. Rather these beneficial genes will, in the long term, move to the chromosome of adaptive mutants. They do go on to show that plasmids can be maintained if they can transfer local adaptation genes on to newly arrived bacteria that bear evolutionary advantages or, if they can transfer the genes intermittently between non-competing strains. However, it should be noted that this model explicitly makes it impossible for the plasmid to be maintained through conjugation, even at maximum bacterial density. In fact, another study by Lili et al. in 2007 took this model and expanded it[114]. They found that the parameter space required for plasmid maintenance is far less strict than had been found by Bergstrom et al. Furthermore, they found that parasitic plasmids may persist through conjugation. They described persistence as oscillations between the plasmid bearing and plasmidless bacteria.

While these models have described conjugative plasmids quite extensively, none have looked at the existence conditions for non-conjugative plasmids, and how other types of horizontal gene transfer can maintain plasmids. Therefore, these models are not largely relevant to studying transduction (and its effect on plasmid persistence). One study that did look at the existence conditions of strictly non-mobilizable plasmids was done by Peña-Miller et al. They used a population dynamics approach and found that it is very challenging for these plasmids to persist in the long term[20]. This model posited that the only way that costly plasmids may persist in the long term is for them to continually experience some form of (unspecified) horizontal gene transfer. Another study used theoretical approaches to examine experiments which showed the effect of phage predation on a conjugative mercury

resistance plasmid[111]. Harrison et al. showed with experimental data that phages reduced the existence conditions for the plasmid. They supplemented this with a model that used ordinary differential equations to show that the phage drive plasmid dynamics through the ecological effects that they have on bacteria, and they ultimately limit the existence conditions by killing off donors and receptors needed for horizontal gene transfer. However, in the presence of phage and no selective pressure they found bistability in the system (plasmid fixation and plasmid loss), meaning temporary maintenance of the plasmid followed by rapid loss. To explore this, they used an individual based model which showed that conjugative plasmids could hitchhike with chromosomal mutations providing resistance to phage. These recurrent sweeps of resistance mutations drive the temporary maintenance of the plasmid followed by the its loss.

1.4 *Pseudomonas aeruginosa* as a model organism

Pseudomonas aeruginosa is an opportunistic nosocomial pathogen that has gained increasing clinical relevance where it is a leading cause of chronic pulmonary conditions such as cystic fibrosis (CF)[115]. Moreover, the health burden also comes from *Pseudomonas* ventilator acquired pneumonia which has a mortality rate of close to 40%[116].

The reference strain PA01 is the one that is most commonly used in laboratory research, and it was originally sampled from a burn would victim in Australia in 1954[117]. PA01 was the first *P.aeruginosa* genome to be completely annotated [118]. It is a gram-negative bacteria with large genome (6.3 Mb) containing over 5500 genes and it is thought that this results in its ability to survive in a wide variety of environments such as soil, water, insects, and animals. It can live in many forms such as colonies, planktonic cells, and biofilms[115].

P. aeruginosa is naturally resistant to many commonly used antibiotics, which is mainly caused by its outer membrane not being greatly permeable to antibiotics. It contains efflux pumps which provide an additional layer of resistance[119]. The

genome of *P.aeruginosa* also encodes a β-lactamase that can provide resistance to β-lactams[120]. Furthermore, the accessory genome (in the form of plasmids, integrative and conjugative elements) provides extensive resistance to antibiotics[121].

PA01 has been extensively characterized and been used widely throughout microbiology. A large body of work has been devoted to identifying the phenotypic and genotypic features. PA01 is not naturally competent so the only ways in which it can receive DNA horizontally is through either conjugation or transduction[122]. Additionally, in *P.aeruginosa*, conjugative plasmids are relatively rare within the scope of the accessory genome[123]. Therefore, PA01 is an ideal strain to be used for studying transduction.

1.4.1 Temperate phages in Pseudomonas aeruginosa

There is significant diversity in *P.aeruginosa* temperate phages. It has been reported that there are at least 60 different temperate phages[124], and the majority of isolates are thought to contain at least one prophage[125]. *P. aeruginosa* phages are considered a reservoir for genetic diversity. A study of 18 diverse P. aeruginosa phages showed that 82% of the predicted *P. aeruginosa* phage proteome is not yet known[126]. However, it has been found that temperate phages may play a role in interstrain differences in virulence, and as contributors in biofilm development.

Whole-genome sequencing of *P. aeruginosa* strain Liverpool Epidemic Strain (LES) revealed six prophage clusters. Mutations in genes from three of these prophage clusters reduced virulence[127]. ΦCTX is another temperate phage that increases virulence in a number of infection models by encoding for a pore-forming toxin[128]. Temperate bacteriophages can also alter other aspects of *P. aeruginosa* biology. The presence of Pf4 mediates the appearance of small colony variants, which is associated with poor lung function in CF patients[129]. Many *P. aeruginosa* strains contain two different prophages, that have evolutionarily specialized as narrow-spectrum antibacterial bacteriocins known as R (evolved from P2 phage) and F (evolved from lambda phage) type pyocins[130]. Background pyocin production is low, but can be stimulated by DNA damaging agents in a similar way to prophage

Table 1.1: Predicted temperate phages in PA01 using PhiSpy and their locations in the genome

Predicted Prophage	Location in Genome (bp)
PA0pp.1	620666 - 717153
PA2pp.2	2379310 - 2400280
PA2pp.3	3070366 - 3119183

induction[131]. Temperate phages have also been found to be able to drive and mediate adaptive evolution in *P.aeruginosa*. The experiments tested CF infections with and without a set of three temperate phages in order to experimentally test their role in pathogen evolution. They used temperate phages which were active in CF infections. They found that the bacteria in the treatment with temperate phages showed more parallel evolution and faster selective sweeps than the bacteria without phages. This showed that phages allow quicker evolution of bacterial pathogens and contribute in their adaptation to the host environment and possible treatments[132].

In order to find which prophages are present in the experimental strain PA01, the computer program PhiSpy was used. PhiSpy is an algorithm used to find prophages in bacterial genomes[133]. The algorithm was used on the PA01 sequence on the NCBI database (NC-002516[118]) and it resulted in three predicted phages. These phages along with their locations in the genome are shown in Table 1.1.

1.5 The model plasmid pNUK73

The model plasmid primarily used in this work is pNUK73 (5.13 kB), which was originally constructed from another plasmid pNI10. pNI10 was originally developed in *Pseudomonas fulva*. pNUK73 is a naturally occurring plasmid and received prominence because of its use as a shuttle vector between *E.coli* and *Pseudomonas*[134].

pNUK73 does not belong to any known incompatibility group. It contains three genes: a *rep* replication gene, an aminoglycoside 3'-phosphotransferase gene conferring resistance to aminoglycoside antibiotics such as kanamycin and neomycin, and a *lacZ* reporter gene. Notably, both the resistance gene and the replication

protein are naturally occurring, and the only modification is the insertion of a lacZ reporter. It exists mainly as a monomer and the average copy number per cell is 11 in PA01[135]. Importantly for the work conducted here, it lacks the genes necessary for mobilising itself via conjugation, meaning that its only way of moving horizontally is via transduction or transformation. It has been shown in previous studies that pNUK73 can impose a relatively large fitness cost. *P. aeruginosa* PA01 containing pNUK73 showed approximately 20% decrease in fitness compared to wild-type PA01. However, in regards to conferring resistance to neomycin, it produces approximately 20 fold increase in minimum inhibitory concentration for the plasmid bearing species as compared to the wild type[135]. The schematic representation is shown in Figure 1.7.

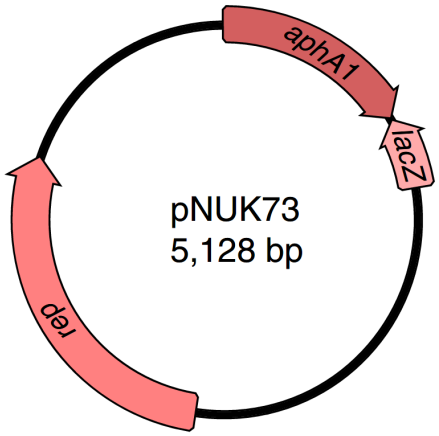


Figure 1.7: Visual representation of the model plasmid pNUK73. The reading frames for genes are shown as arrows, with the direction of transcription indicated by the arrowhead. Adapted from [135].

1.6 Antibiotics

Antibiotics are compounds that are harmful to bacteria by either having an inhibitory or killing effect on them. They encompass a wide array of drugs that are categorised based on their mechanism of action as well as whether they are bactericidal or bacteriostatic. Bactericidal antibiotics are those where exposure leads to bacterial death and bacteriostatic antibiotics are those where exposure limits growth but the

bacteria are still living[136]. In general, most current antibiotics work by exploiting bacterial pathways inhibiting cell wall, protein, RNA or DNA synthesis[137].

1.6.1 Neomycin

Neomycin is of the class of aminoglycoside antibiotics[138]. Aminoglycosides are broadly bactericidal[137], and they operate via the inhibition of protein synthesis in the cell[139]. The mechanism of action can be seen in Figure 1.8. Aminoglycosides bind to the 16s rRNA component of the 30s ribosomal subunit. The interaction between the 16s rRNA component can make a change in the conformation of the complex created between an mRNA codon and its tRNA at the ribosome. This induces tRNA discrepancies which cause the incorporation of incorrect amino acids into a new protein. The binding of the drug to the ribosome does not immediately stop translation. Instead, induces incorrect translation by the addition of the wrong amino acids into the extending peptide strands[140]. This mistranslation means that the newly synthesised protein does not fold as intended and the incorporation of those proteins into the cellular envelope weakens the integrity of the cellular structure. This lets in further amounts of the drug, before finally killing the cell[137]. Aminoglycosides are still occasionally used to treat *Pseudomonas* infections, however they are not a front-line class of drug for the treatment of *P.aeruginosa* infections[141].

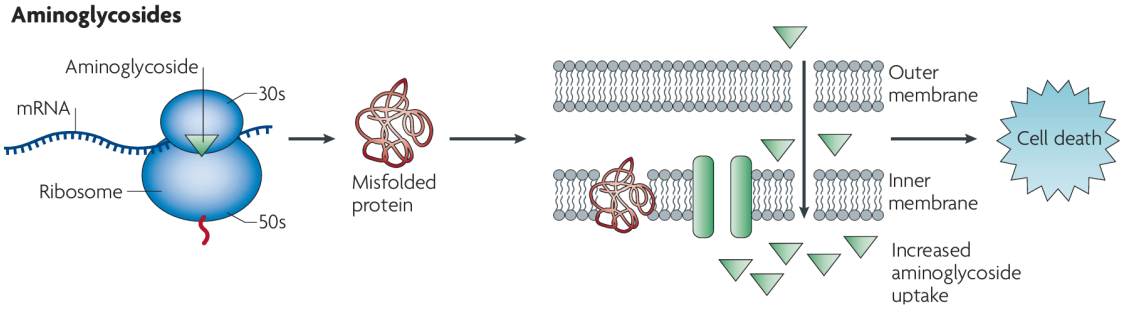


Figure 1.8: Mechanism of action of aminoglycoside antibiotics such as neomycin. When aminoglycosides bind to the 30s subunit of the ribosome, they cause the wrong amino acids to be incorporated into the protein. The misfolded membrane proteins cause more drug to be taken up by the cell and eventual death of the cell. Adapted from [137].

Neomycin was used in the experiments described in this work because pNUK73, the non-conjugative plasmid contains aminoglycoside 3'-phosphotransferase gene conferring resistance to aminoglycoside antibiotics like neomycin. Previous studies[123] have characterized the MIC of the WT and strains containing the plasmid. The presence of the plasmid causes the MIC of neomycin to change from 4 µg/ml to 80 µg/ml, a 20-fold increase. This allows the identification of cells that have the plasmid by plating samples on agar plates containing a high concentration of neomycin.

1.6.2 Rifampicin

Rifampicin is of the rifamycin class of antibiotic[142]. Rifampcin is an incredibly powerful antibiotic and works for against a wide range of gram-positive bacterial infections such as tuberculosis, meningitis, Legionnaires' diseases and leprosy[143]. The mechanism of action can be seen in Figure 1.9. Rifampicin operates by having a high affinity to binding to *rpoB* which encodes for the β-subunit of RNA polymerase, which is where the newly transcribed RNA originates[144]. The β-subunit is found within the channel within the DNA-RNAP complex which is formed during transcription[145]. The *rpoB* pocket is known to be highly conserved, and when Rifampicin binds to this, the RNA molecule cannot progress past two nucleotides of transcription as it is sterically blocked from doing so[146]. Rifampicin is considered to be bactericidal in gram-positive bacteria and bacteriostatic in gram-negative bacteria. This difference is thought to be attributed to drug uptake rates, and not the affinity to RNAP[147].

Rifampicin was used in the experiments described in this work because a strain (PA01:rpoBH531C) contains a costless chromosomal mutation that provides resistance to rifampicin. Rifampicin resistance is nearly always due to a genetic change in the β subunit of bacterial RNA polymerase[149]. Therefore, the resistance is a stable chromosomal resistance marker. The rifampicin resistant strain that was used carries a pair of mutations in the same codon (531) that give an amino acid substitution that could not be achieved by a single mutation (H to C). This is very important, as sequencing rpoB allows distinguishing spontaneous rifampicin

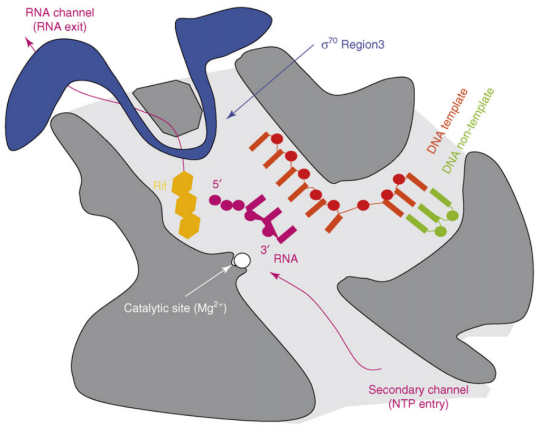


Figure 1.9: Mechanism of action of rifampicin. When rifampicin binds to the *rpoB*, transcription is blocked from proceeding. Adapted from [148].

resistant mutants (with a single mutation) from the strain that was used in this experiment. Previous studies[150] have characterized the MIC of the WT and strains containing the chromosomal resistance mutation.

1.7 Thesis Outline

In the second chapter, I look at using both temperate and lytic phage as possible carriers to move pNUK73 between *P.aeruginosa*. I hypothesise that the prophage activation in the presence of antibiotic allows the plasmid to be packaged up and moved between bacteria. I isolate temperate phage that has been expressed at different concentrations of Neomycin and then use that temperate phage to infect bacteria without the plasmid and see if there is any change in resistance. While I find that there is no change in resistance, the DNA enclosed within the capsids is extracted and the plasmid is found using PCR. I then use six environmentally sourced lytic bacteriophages to observe if they take up and transfer the plasmid. To do this I infect plasmid bearing bacteria with the each of the phages and then extract the phages and allow them to infect the bacteria without the plasmid and measure if there are any changes in resistance. Three phages that showed an increase in resistance, meaning that they were able to transduce the plasmid.

In the third chapter, I use the best performing phage from my earlier work, LUZ19 and looks at its role plasmid maintenance over four days, compared to a

no phage control, low dose antibiotics and both antibiotics and phage. I use a bacterial strain that has a stable, costless chromosomal resistance to rifampicin together with my plasmid bearing strain and the phage to see if there are double resistant bacteria that arise from the transfer of the plasmid. I use sequencing techniques to find that the phage can transfer the plasmid to the other strain. I also find evidence of coevolution occurring between the bacteria and phage. However, I do not see evidence that the evolution of resistance by bacteria has a negative impact on transduction, contrary to the initial hypothesis.

My fourth chapter looks at modelling the processes experimentally examined in the previous chapters. I first look at a simple model that stresses the importance of general horizontal gene transfer in the persistence of a plasmid in a broad ecological context. The second model looks specifically at transduction and attempts to computationally replicate the experiments done in the third chapter. It uses an ordinary differential equation approach to modelling the ecology of two populations which represent bacteria with and without the plasmid and the phage mediated transfer between them. This allows me to explore the critical parameters and ecological dynamics that play the most significant roles in transduction. The third model uses an agent-based modelling approach to test and verify the hypothesis that a phage with a lower burst size and a longer latent period is better at transducing the plasmid than a phage with the opposite characteristics.

2

Transduction Assays using Temperate and Lytic Phage

2.1 Abstract

Plasmids play a considerable role in the dissemination of antibiotic resistance, but it is not yet clear how they can be stably maintained in the absence of positive selection. It has been shown that episodes of conjugation could maintain plasmids, but it is not clear how small non-conjugative plasmids can persist in the long term. Transduction is a form of horizontal gene transfer mediated by bacteriophages. Here, I examine how pNUK73, a small plasmid that confers resistance to neomycin could be moved between *Pseudomonas aeruginosa*. I first test temperate phage that exists in the genome of the bacteria along with the plasmid. The phages are induced in plasmid bearing bacteria with varying concentrations of neomycin. These phages are isolated and then used to infect plasmidless bacteria to test whether they are able to transfer the plasmid. The phage was not able to transfer the plasmid, but the plasmid was found to have been packaged in their capsid. I then test six lytic phages (three from *podoviridae* and three from *myoviridae*) and use them to infect plasmid bearing bacteria. After the infection is complete, I isolate the phage and use them to infect plasmidless bacteria to see if they can transfer the plasmid. I

find that three of them (LUZ19, LUZ7 and 14/1) can transfer the plasmid with higher than previously reported transduction frequencies.

2.2 Introduction

Antibiotics represent one of the most vital medical innovations of the last century. However, the emergence of large scale antibiotic resistance poses one of the most significant threats to modern society; causing issues for spheres of life such as healthcare systems, food security, and the global economy[151]. Even at present, it is estimated that 25,000 people a year die as a result of drug-resistant infections in Europe alone, and the yearly economic impact of resistance is estimated at €1.5 billion[152].

While bacteria can acquire *de novo* mutations that allow them to become resistant to antibiotics, another way that they may become resistant is through horizontal gene transfer of resistance genes[80]. Therefore, in order to understand the dissemination of resistance it is vital to understand the dynamics of horizontal transfer. One form of horizontal gene transfer is transduction, which is the transferring of genetic material by bacteriophages[21]. A large proportion of resistance plasmids are non-conjugative[18] and many large non-conjugative plasmids have active partitioning systems (mechanisms that ensure the inheritance of plasmids during cell division) or toxin-antitoxin systems that help to maintain the plasmids. However, many small plasmids lack these additional mechanisms for stability. Although selection for the genes encoded by the plasmids can help maintain these small plasmids in the short-term, for these plasmids to be maintained in the long term must require some form of horizontal gene transfer such as transduction.

Moreover, bacteriophages represent the most numerous biological entity of the planet, with a global population that has been estimated at 10^{30}[24]. This could be a hidden reservoir of antibiotic resistance genes and there have been several phageome (all of the DNA packaged inside phage capsids) studies in different environments that have shown the presence of antibiotic resistance genes[73][74]. There have been an estimated 2×10^{16} transduction events occurring per second

throughout the world[49]. Therefore, transduction could represent a sizable force in the dissemination of antibiotic resistance.

In this chapter, I explore the role that phages can play in moving a highly unstable small antibiotic resistance plasmid between bacteria. The model system I am using is the opportunistic pathogen *Pseudomonas aeruginosa* PA01 and plasmid pNUK73 which confers resistance to aminoglycoside antibiotics such as neomycin. *P.aeruginosa* is not naturally competent[122] meaning that transformation of the plasmid is not possible. Additionally, pNUK73 lacks the genetic material to be able to transfer itself via conjugation[135]. Therefore, the only conceivable way for the plasmid to move between bacteria is via transduction. Moreover, it is challenging to understand the persistence of non-mobilizable antibiotic resistance plasmids without transduction. This is true in *Staphylococcus aureus* which has very few conjugative plasmids. The other plasmids tend to move around using different forms of transduction[88].

I describe two different experiments where I look at using two different varieties of bacteriophage to see whether they can, and to what degree do they move the plasmid pNUK73. This plasmid provides resistance to neomycin in *P.aeruginosa* PA01. Temperate phages are integrated into the in the genome of the bacteria, and in the presence of environmental stressors (such as antibiotics) they can become excised and enter the lytic cycle. Figure 2.1 shows a microscope image of PA01/pNUK73 grown on agar containing 0.25 $\times$ MIC neomycin. The red spots indicate plaques where prophages have been excised and lysed the bacteria. This shows that the presence of the antibiotic neomycin activates the SOS response, which in turn activates the temperate phage. Temperate phage moving bacterial genes around has precedent in *S. aureus*, where there is a family of mobile genetic elements that are called phage-inducible chromosomal islands (PICI)[47]. These are approx. 15 kb elements that usually code for virulence factors and are essentially parasitic on the temperate phage that move them around. Furthermore, as the temperate phage is SOS-induced, treatment by antibiotics can promote the spread of PICI[153]. It has also been observed that these phage can move between bacterial species[61].

Examples where prophage package up chromosomal pathogenicity elements has been observed in *Enterococcus faecalis*, where phages were found that contained platelet binding proteins[154]. However, similar phenomena in *P.aeruginosa* and the movement of plasmids have not been explored. In order to do so, I use varying concentrations of neomycin to stress the bacteria that contain the plasmid such their prophages are excised, lysing the bacteria. These temperate phages are then isolated and tested for the presence of the plasmid using PCR. Once the presence of the plasmid has been confirmed then the temperate phage is used to infect bacteria without the plasmid and then tested for any changes in the level of resistance.

The second experiment looks at lytic phages which are found in the environment and adsorb on to bacteria that have receptors that they have an affinity for, infect and kill those bacteria. There have been several studies on the phageome of different environments being enriched with antibiotic resistance genes. A study of phage DNA from the faecal samples of humans showed the presence of resistance genes[74]. Another study looked at the phageome of the microbiota of patients suffering from cystic fibrosis and found an extensive reservoir of resistance genes[75]. Further, one experiment took phage populations from the microbiota mice treated with antibiotics and used them to treat naive microbiota. The naive microbiota showed increased resistance[73]. However, the presence of antibiotic resistance genes does not imply transduction as these phages could be a biological dead end. It is not guaranteed that the phage will infect susceptible hosts, even if they exist.

Moreover, due to the short half-life of phage, viewing transduction through this lens will automatically overestimate transduction rates. There have been some studies that attempt to perform in vitro transduction, such as a conjugative resistance plasmid that was transferred between *P.aeruginosa*[87], and where β-lactamase genes have been transduced in *Escherichia coli*. However, a small non-mobilizable antibiotic resistance plasmid has not been observed to be transduced. Therefore, in order to observe transduction of antibiotic resistance plasmid in real time, I use six species of lytic phage (three from *podoviridae* and three from *myoviridae*) and use them to infect bacteria which contain the plasmid.

After infection, this phage is then isolated and then the used to infect bacteria without the plasmid, and then sampled to see whether there are any changes to the level of resistance.

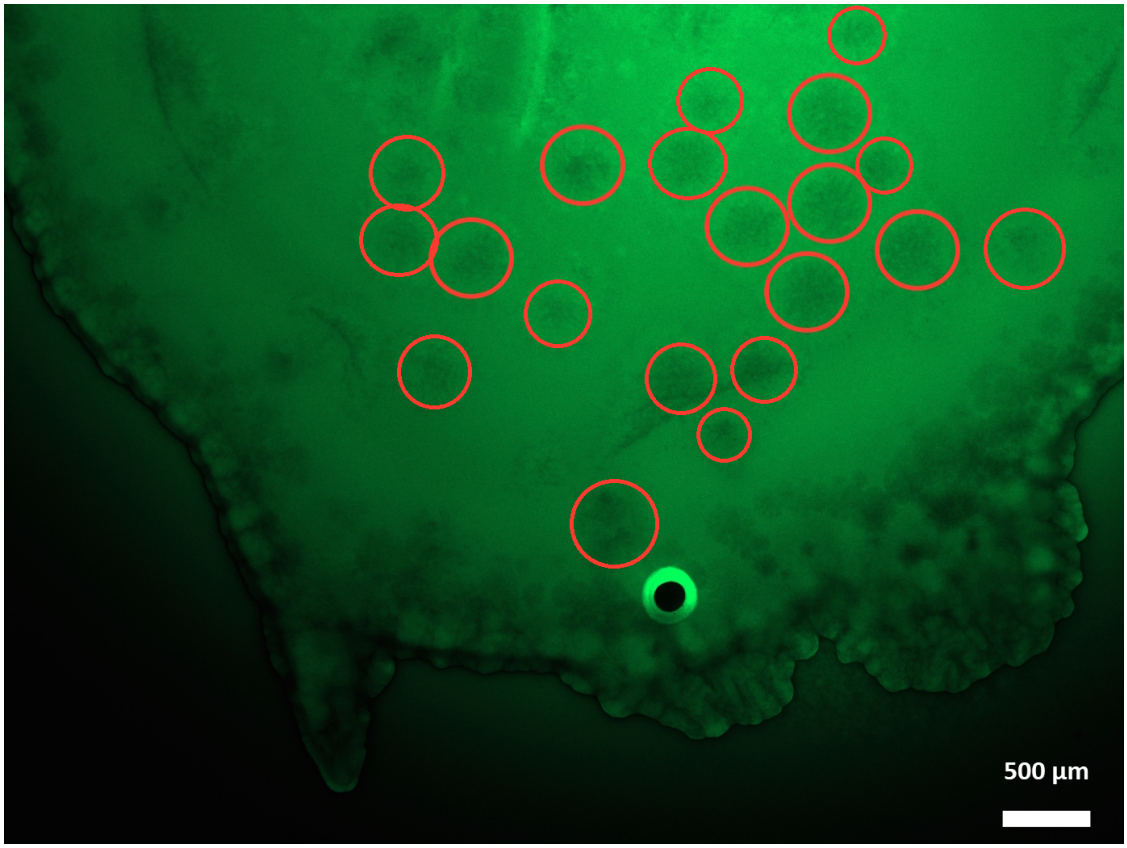


Figure 2.1: Fluorescent microscope image of PA01/pNUK73 grown on LB Miller agar containing 40 mg/L neomycin where the red circles show plaques caused by prophage excision

2.3 Materials and methods: Temperate phage

2.3.1 Isolating bacterial colonies and culture growth conditions

The first step was to obtain populations of PA01 containing the plasmid in which the temperate phage could be induced and excised. Single colonies of *P.aeruginosa* PA01/pNUK73 isolated from frozen stocks by plating on to a Luria Bertani (LB) Miller agar plate (15g/L agar) and by incubating at 37°C for 24 h. Four replicate populations were established for each of the five antibiotic concentration treatments.

Bacteria were cultured in 14 mL Falcon tubes containing 3 mL LB Miller broth incubated at 37°C and 225 rpm continuous shaking for 24 h.

2.3.2 Temperate phage induction

There is broad diversity of temperate phage which are present in different strains of *P. aeruginosa*[121]. PhiSpy[133] is an algorithm used to find prophage in bacterial chromosomes and it was used on the PA01 sequence on the NCBI database (NC-002516[118]). PhiSpy found three predicted phages, these phages along with their locations in the genome are shown in Table 2.1.

Table 2.1: Predicted temperate phages in PA01 using PhiSpy and their locations in the genome

Predicted Prophage	Location in Genome (bp)
PA0pp.1	620666 - 717153
PA2pp.2	2379310 - 2400280
PA2pp.3	3070366 - 3119183

In order to induce the temperate phage, the overnight cultures were diluted 100 fold and varying quantities of Neomycin were added at the concentrations tested which were 0, 20, 40, 80, 160 mg/L (which is 0, 0.25, 0.5, 1, 2 × MIC respectively). The cultures were incubated at 37°C and 225 rpm continuous shaking for 24 h.

2.3.3 Temperate phage purification

The temperate phage must then be isolated from the bacteria. After 24 h, 1 mL of the overnight cultures was added to 100 μL of chloroform and vortexed. These samples were then centrifuged at 13300 rpm for 2 minutes. Then the cell-free supernatant was syringed through a 0.22 μm filter to remove any remaining bacterial matter. The phage lysate was plated on LB miller agar to verify that there were no remaining viable bacteria. These stocks were stored at 4°C. It is not possible to measure their density because they will not be able to infect PA01 due to superinfection exclusion. Therefore, bacteria cannot be lysed and plaques that can be used to measure density will not form.

2.3.4 Establishing replicate populations of plasmidless bacteria

Populations of plasmidless bacteria which will be used to be infected by the phage must be grown. Single colonies of *P.aeruginosa* PA01 were isolated from frozen stocks by plating on to a LB Miller agar plate (15g/L agar) and by incubating at 37°C for 24 h. Bacteria were cultured in 14 mL Falcon tubes containing 3 mL LB Miller broth incubated at 37°C and 225 rpm continuous shaking for 24 h. These bacteria are chromosomally identical to the plasmid bearing strain, and will therefore contain the exact same prophages. The bacterial density of each culture was measured by serial dilution and plating. The cultures were then diluted such that each culture was approximately 10^8 CFU/mL. Four replicate populations were established for each of the six treatments. 100 μL of the purified phage lysate corresponding to each treatment was added to the cell culture and for the control M9 solution was added.

2.3.5 Measuring resistance

The bacteria must be tested to observe if there measurable change in resistance caused by the transfer of the plasmid. Thirty minutes after the addition of the phage lysate or M9, 100 μL was plated on LB miller agar containing 40 mg/L of Neomycin (0.5 $\times$ MIC of the plasmid bearing strain and 10 $\times$ MIC of the WT). The plates were then incubated for 24h at 37°C and the number of CFU's were counted.

2.3.6 Statistical methods

Colony counts were analysed using analysis of variance with poisson error structures. Post-Hoc Dunnett's tests performed using the no phage treatment as the control.

2.3.7 Temperate phage PCR analysis

In order to see if the phage picked up the plasmid, the DNA encapsulated by the phage was tested using PCR analysis. 1 mL of phage lysate was treated with 10 μL of DNase I and incubated at room temperature for 15 minutes. The DNase was heat-inactivated at 75°C for 5 minutes. This was to destroy any viable DNA

outside those protected by the phage capsids. The presence or absence of pNUK73 was verified using PCR analysis. The reaction was conducted using GoTaq Hot Start Green Master Mix (Promega, USA). The primer sequences used were specific for pNUK73 (pNUK73-forward: 5'-CGCTAAGGATGTTTACAC -3', pNUK73-reverse: 5'- CTCAACCGTTCTAGGATT - 3') The amplification conditions were: initial denaturation for 95°C, followed by 30 cycles of denaturation for 30s at 95°C, annealing at 60°C for 30s and extension at 72°C for 30s. For the final extension, the reaction was kept at 72°C for 5 min. The purified plasmid was used as a positive control. Water was used as a negative control.

2.4 Materials and methods: Lytic Phage

2.4.1 Ancestral Phage Purification

In order to test the transduction of the plasmid by lytic phage, the lytic phage must be grown. Six phage isolates were obtained, three from *podoviridae* (LUZ19, PEV2, LUZ7) and three *myoviridae* (LMA2, 14/1, ΦKZ). The phages were chosen as to give a good range of different orders and types while still having a large enough capsid to hold the plasmid. LUZ7 and PEV2 were chosen because they come from the N4 like group of phages, which do not require the host RNAP for transcription of its early genes. PEV2 was specifically chosen because it is capable of infecting PA01 anaerobically and aerobically, which is essential for clinical applications where infections in thick mucus are anaerobic[155]. LMA2 and 14/1 were chosen because they come from the PB1 like group of phages, where the tail exhibits no transverse striations but presents a criss-cross pattern, which is rare in phages[156]. LUZ19 has broad infectivity on both environmental and clinical isolates of *P.aeruginosa*[157]. ΦKZ was chosen because of its similarity to ΦPA3, which is proved as an effective transducer of bacterial genes in previous studies[115]. The biological details of the phages used can be seen in Table 2.2.

The samples were stored at 4°C as purified supernatants in liquid LB Miller. Each phage isolate was amplified from one randomly chosen plaque and introduced into a single overnight PA01 culture. After 24 h, 1 mL was added with 100 μL of

chloroform and vortexed. These samples were then centrifuged at 13300 rpm for 2 minutes. Then the cell-free supernatant was syringed through a 0.22 μm filter to remove any remaining bacterial matter. The samples were plated on LB miller agar to verify that there was no remaining bacterial matter. The samples were stored at 4°C as purified supernatants in liquid LB.

Table 2.2: Lytic phages used in transduction assay

Phage Name	Order	Type	Receptor
LMA2	Myoviridae	PB1 like ~74 kbp genome	LPS
14/1			
ΦKZ		ΦKZ like ~280 kbp genome	Not Known
LUZ19	Podoviridae	KMV like ~43 kbp genome	Type IV pili
PEV2		N4 like ~66 kbp genome	Possibly both LPS/type IV pili
LUZ7			

2.4.2 Establishing replicate populations of plasmid bearing bacteria

Populations of plasmid bearing bacteria which will be used to be infected by the phage must be grown. Single colonies of *P.aeruginosa* PA01/pNUK73 isolated from frozen stocks by plating on to an LB Miller agar plate and by incubating at 37°C for 24 h. Replicate populations consisting of single colonies were then were cultured in 14 mL Falcon tubes containing 3 mL LB Miller broth incubated at 37°C and 225 rpm continuous shaking for 24 h.

2.4.3 Infection of Plasmid bearing bacteria and phage purification

The bacteria containing the plasmid must be infected by the phage to observe whether the phage can pick up and transfer the plasmid. The cultures of PA01/pNUK73 were infected with 50 μL of phage. The bacteria and phage were incubated at 37°C and 225 rpm continuous shaking for 24 h. After 24 h, 1 mL was added with 100 μL of chloroform and vortexed. These samples were then centrifuged

at 13300 rpm for 2 minutes. Then the supernatant was syringed through a 0.22 μm filter to remove any remaining bacterial matter. The phages were plated on LB agar to verify that there were no remaining viable bacteria. The samples were stored at 4°C as purified supernatants in liquid LB. Phages density was then quantified by plating serial dilutions on soft agar. The phage lysates were then diluted such that the density was 10^7 PFU/mL

2.4.4 Soft Agar Overlay method

To measure the density of phage, the soft agar overlay method can be used. Soft agar plates are made with two different concentrations of LB miller agar on top of each other. The bottom layer is a hard (15 g/L agar) layer is overlain with a soft (7.5 g/L agar) layer. 30 mL of soft agar is mixed with 1 mL of an overnight culture of the ancestral bacterial strain. Phage lysate can then be plated on the top of the soft agar using dilutions at which single plaques are visible from which the density can be determined,

2.4.5 Establishing replicate populations of plasmidless bacteria

Populations of plasmidless bacteria which will be used to be infected by the phage must be grown. Single colonies of *P.aeruginosa* PA01 were isolated from frozen stocks by plating on to a Luria Bertani (LB) Miller agar plate (15g/L agar) and by incubating at 37°C for 24 h. Bacteria were cultured in 14 mL Falcon tubes containing 3 mL LB Miller broth incubated at 37°C and 225 rpm continuous shaking for 24 h. Plasmidless bacteria density was quantified by plating serial dilutions on LB miller agar. The bacterial cultures were then diluted such that the density was 10^8 CFU/mL.

2.4.6 Infection of plasmidless bacteria by plasmid-bearing phage and measurement of resistance

The plasmidless bacteria and the plasmid bearing phage are mixed to see whether there is any transfer of the plasmid from the phage to the bacteria. 500 μL of

bacteria was mixed with 500 μL of phage lysate (resulting in an M.O.I. of 0.1) and aspirated using a pipette. The M.O.I was kept at 0.1 to reduce the probability of one bacterium being infected by multiple phages, and therefore having a bacterium that may receive the plasmid being lysed by another phage. Using an M.O.I of 0.1 has been shown to increase transduction frequency by 3 times compared to a M.O.I of 10 in previous studies[115]. The phage and bacteria were placed on in a static incubator at 37°C. At 6 minute intervals, 100 μL of each of the samples were taken from the cultures and centrifuged at 13300 rpm for 1 min. The cell-free supernatant was discarded after which the culture resuspended in 900 μL of PBS in order to remove any unadsorbed phage. The intervals were chosen at 6 minutes in order to reduce the probability of subsequent infection cycles to take hold and lyse bacteria that may have received the plasmid. 100 μL were taken from each sample were taken and plated on LB miller agar plate containing 40 mg/L of neomycin (0.5 $\times$ MIC of the plasmid bearing strain and 10 $\times$ MIC of the WT). The plates were then incubated for 24h at 37°C and the number of CFU's were counted.

2.4.7 Statistical methods

Colony counts were analysed using analysis of variance with poisson error structures. Post-Hoc Dunnett's tests performed using the no phage treatment as the control.

2.5 Results

2.5.1 Temperate phages can pick up the plasmid

This experiment attempts to test the hypothesis that temperate phages can move the plasmid horizontally. Prophage exists in the chromosome and can enter the lytic cycle when stressed by antibiotics. The temperate phages in PA01 containing the plasmid pNUK73 were induced using various concentrations of antibiotics. The phages were then isolated from the bacteria. To test whether the phage capsids contained the plasmid, the DNA contained within them capsid needed to be isolated. DNase was added to the phage lysate degrading any naked DNA that is not protected by the phage capsid. The phage DNA was then extracted, and PCR was used with

appropriate primers to test for the presence of the plasmid. Figure 2.2 shows the resulting gel electrophoresis. PCR analysis confirmed the presence of the plasmid in all the samples at the different positive concentrations of antibiotic concentrations. It is not possible to conclude anything the phage density at each concentration. This is because to measure the phage density would require the soft agar overlay method, where plaques are counted. However, because the temperate phage cannot infect PA01 (due to superinfection exclusion), it is not possible to use this method. It is also not possible to see which of the prophage in the genome are picking up the plasmid, because once the DNA held in the capsid is extracted, it is not possible to differentiate between the different prophage.

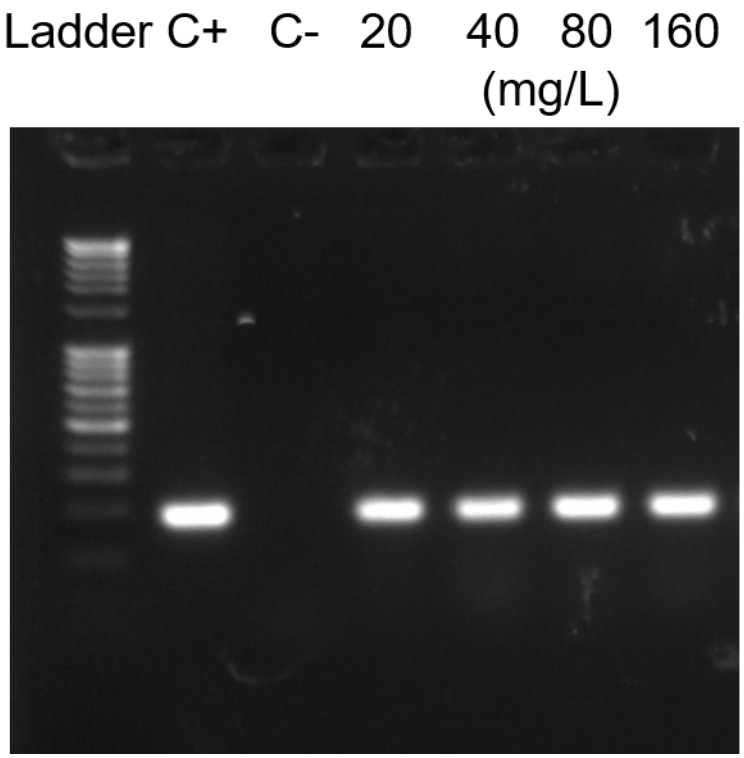


Figure 2.2: Image of the PCR to verify the uptake of the plasmid by temperate phage. The positive control is purified plasmid indicated by C+. The negative control is water indicated by C-. The temperate phage induced at different concentrations of neomycin is shown.

2.5.2 Temperate phage cannot move the plasmid between PA01

The plasmid containing phage lysate was then added to bacteria without the plasmid. These bacteria are chromosomally the same as bacteria with the plasmid and therefore contain the same three prophages. After the plasmidless bacteria were infected with the temperate phage, the bacteria and phage were plated on media containing neomycin. The neomycin is selective for the plasmid for 24 hours and then the resistant colonies are counted. The results are shown in Figure 2.3. The results show that temperate phage induced at various antibiotic is not able to produce a statistically significant ($p > 0.05$) amount of resistant colonies as compared to a phageless control, meaning they are not able to transduce the plasmid under those conditions. This was confirmed with a Dunnett's test.

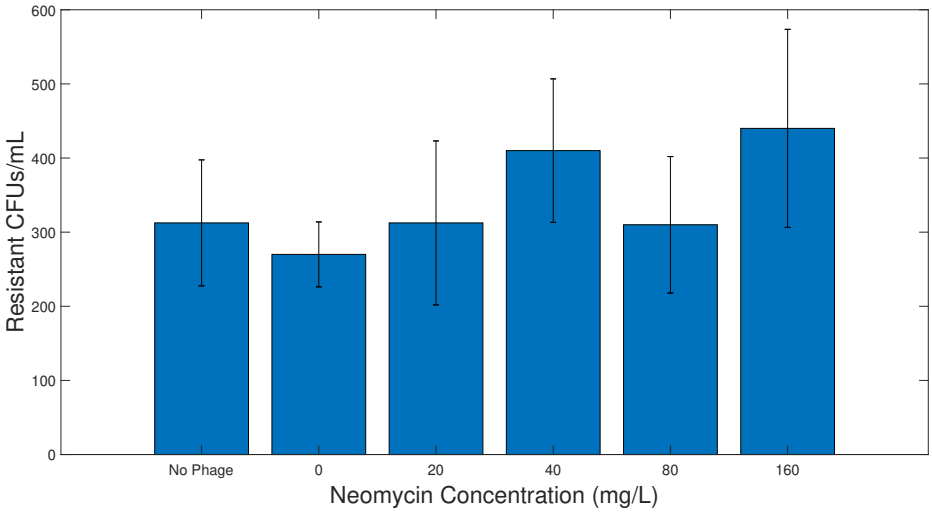


Figure 2.3: Number of colonies grown after 24 hours on Neomycin plates after being cultured with temperate phage. Error bars indicate 95 % confidence intervals. There were four replicates per treatment.

2.5.3 Lytic phages can to spread the plasmid

Lytic phages were trialled for their transduction capability. They were allowed to infect and lyse PA01 containing the plasmid pNUK73, and then these phages were isolated and then infected PA01 without the plasmid. In order to minimise

the possibility of multiple infections of one bacteria, the M.O.I. was kept at 0.1. Furthermore, to avoid bacteria being lysed by subsequent infection cycles, the time period of infection was kept to short intervals. Post-infection, the bacteria were plated on media containing neomycin. The number of colonies after incubation for 24 hours were counted. This gives a comparison of how effectively the plasmid has spread throughout the population when having a phageless control. The results are shown in Figure 2.4. Figure 2.4 shows that LUZ19, LUZ7 and 14/1 had significantly more ($p < 0.05$) resistant colonies upon plating. This was confirmed with a Dunnett's test with LUZ19, LUZ7 and 14/1 being different to the control.

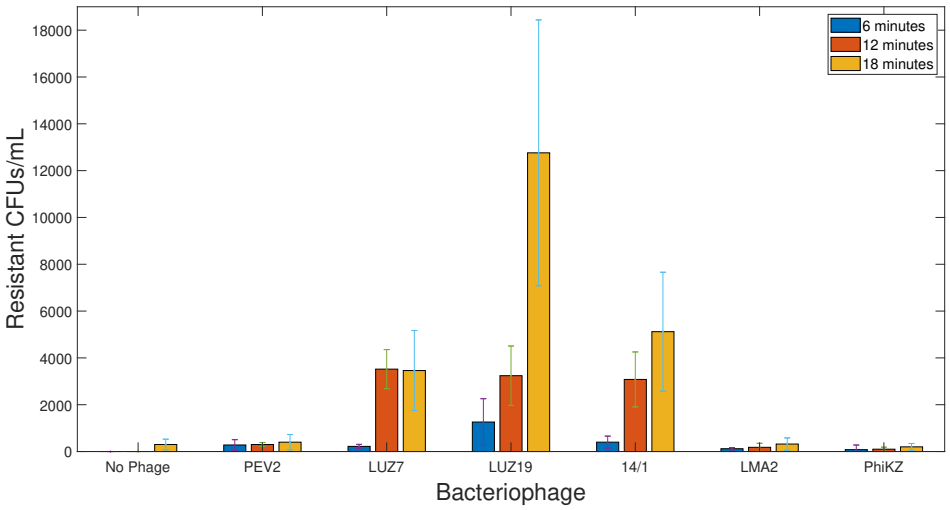


Figure 2.4: Number of colonies grown after at different time intervals on Neomycin plates after being cultured with different species of lytic phage. Error bars indicate 95 % confidence intervals. There were three replicates per treatment.

2.6 Discussion

The results show that there is no increase in resistance between a phageless control and phage induced at varying concentrations of neomycin, meaning that there is no significant transfer of the plasmid by temperate phage. One possible reason that may happen is the the frequency of plasmid encapsulation by phage is very low, and this may result in a low level of horizontal gene transfer. It is not possible to

disprove this theory because the number of phages produced cannot be measured, and the amount of plasmid picked by phage is not known.

Temperate phages do indeed pick up some amount of the plasmid but these phages cannot infect other bacteria and one theory that may explain this is superinfection exclusion. Superinfection exclusion is the prevention of infection of bacterial cells by phages which are already latent in the genome as prophages[158]. Various mechanisms exist for preventing superinfection that change depending on the species of phage[159][160][161]. Furthermore, one study[72] showed using different species of *P.aeruginosa* that many phages have evolved mechanisms that alter the bacteria they have infected. They make the bacteria resistant to infection by phage a that is different to it. That said, superinfection exclusion has been documented to not be perfect[162] and it is therefore theoretically possible for a temperate phage to move the plasmid between bacteria. In *Staphylococcus aureus* there are examples of superinfection exclusion failing and allowing antibiotic resistance genes to be transferred[51][52]. However, considering them both as two independent events that both need to occur (the probability that the phage will mistakenly pick up the plasmid and the probability that the immunity mechanisms that protect the bacteria from prophage fail), the total probability becomes very small. Another factor is that there is a possibility that the temperate phage may enter the lytic cycle when it enters the new host and then also start killing them off. Therefore, there may be temperate phage mediated transfer of the plasmid but detecting this transfer becomes very difficult.

It is important to state that these experiments do not prove that temperate phages do not perform transduction, merely that they cannot pass genetic material to identical clone-mates which contain the exact same prophage. One possible way to move the plasmid using temperate phage is to use a different recipient strain. Therefore the bacteria may not be immune to the phage. One possible option is using PA14, which has different prophages than PA01 and they have been shown to infect each other[72]. However, using different bacteria may not present a complete solution to this, either. It is not possible to deduce which phage

are moving the bacteria because multiple prophages will be excised. Once the phage DNA is extracted, it will not be possible to differentiate the one(s) that picked up the plasmid from the others that did not. Therefore, the phage which picks up the plasmid may be one that is resistant to superinfection by both strains. One other strategy that could be employed is to use strains lacking the prophage (using gene knockouts) and then looking at whether the plasmid is picked up. This would allow the clarification of which temperate phage is packaging up the plasmid, and a suitable recipient strain (that lacks that particular temperate phage) can be used to measure the transduction frequency.

The experiments have shown here that lytic phage can move the plasmid around, and the approximate rate at which this happens can be calculated by assuming that there will be an amount of spontaneous resistant mutants independent of phage infection. So, then it can be assumed that the number of resistant due to phage mediated transfer of the plasmid will be the resistant colonies per phage subtracted by the control. The starting concentration of bacteriophage is 10^7. Then the rate of transduction will be equal to:

$$\text{Transduction efficiency } = \frac{\text{CFU's at 18 minutes }_{Phage} - \text{ CFU's at 18 minutes }_{Control}}{10^7}$$

Table 2.3: Transduction Efficiencies for different phage

Phage	Transduction efficiency (c.f.u./phage)
LUZ7	1.44×10^{-5}
LUZ19	6.09×10^{-5}
14/1	2.26×10^{-5}

These rates are considerably higher than previous studies, which have used generalised transducing phages that have shown bacterial chromosomal DNA in *P.aeruginosa* is transferred at approx. between 10^{-7} and 10^{-9} c.f.u./phage[163][164][115]. There are possible reasons why the transduction efficiency may have been overestimated. There will be some spatial heterogeneity in the way that the antibiotic can select for true transductants. This is when clusters of (non-resistant) colonies are

able to grow because the antibiotic is titrated out due to a high number of bacteria, creating false positives. This will overestimate the rate of true transductants. Additionally, the transduction rate may be overestimated because the experimental design was done in such a way as to maximise the rate of transduction. Transduction is a rare event and in order to observe it, different aspects of the experiment were chosen to give phage mediated transfer an advantage. In order to avoid lysis of a cell that has received the plasmid, three things were controlled. The multiplicity of infection was kept at 0.1, meaning that there were ten plasmidless bacteria to one phage. This was to minimise the probability that any one bacteria would be infected by two phage, because if one of those phage carried the plasmid and infected the bacteria then that would be masked by the bacteria being lysed by the other phage. This method of using low M.O.I. may be one reason why certain studies have lower efficiencies, [164] used a M.O.I ranging from 5 to 32.3. [163] used a M.O.I ranging from 20 and 200, which may explain their maximal transduction rate of approx. 10^{-9} c.f.u./phage. Second, the intervals done at plating were such that a lytic phage would not have time to complete the infection cycle and then lyse the cell and have its progeny go on to infect and lyse a bacteria that had received the plasmid. [115] uses an interval of 30 minutes (but this is matched to the latent period of the phage that is used). Thirdly, before plating the bacteria, the culture was washed to remove any free phage such that when it was plated any bacteria could not be then infected and lysed. None of the other studies measuring transduction efficiency use this step. However, one difficult factor to control when using such a short period between infection and plating is the delay between when the bacteria acquire the plasmid and when they become phenotypically resistant to the antibiotic. There are examples of phenotypic delay being of the order of 3-4 generations in bacteria[165], meaning that these transduction rates may be underestimated. Lastly, another reason that may cause the horizontal gene transfer rate to be higher than previously suggested is that the plasmid being transferred has a copy number of approximately 11. In contrast, previous estimates of transduction rates are with chromosomal markers. This is a plausible explanation because this is an order of magnitude difference

Table 2.4: Latent Periods and Burst Sizes of lytic phages that were used for the transduction assay for which there is data available

Phage	Latent Period (min)	Burst Size (phage/cell)
LUZ19	80-100	17
PEV2[155]	25 - 30	100-150
ΦKZ[166]	30	100
LMA2[167]	35	65

in physical genetic material. This adjustment to the measured transduction rates would bring it more in line with other measured rates.

Experiments which are shown in Appendix B.2 describe experiments done to measure the latent period and burst size of LUZ19. The results of this posed the idea that phage life history may play a part in determining the transductive ability of phages. To explore this, the life history data for LUZ19 (the best performing phage in the transduction assay) was compared to the latent period and burst size of other phages in the trial for which there is published data.

Table 2.4 shows the comparison between phages and there is a clear discrepancy between three phages that did not perform well and LUZ19 which did. This could potentially be because a more infectious phage (with a lower latent period and higher burst size) with the same affinity for the plasmid will a transfer the plasmid at a similar rate than a phage with a lower burst size and higher latent period, but the effective rate of horizontal gene transfer is lower. This is because with a more infectious phage, the bacteria that receive the plasmid are more likely to be lysed themselves by the more infectious phage. Moreover, the more infectious phage will further reduce the number of donor and receptor bacteria. This will further reduce the potential for horizontal gene transfer. It is difficult to make any concrete conclusions due to the lack of data, but this idea is explored further using modelling techniques in Chapter 4.

The most straightforward why phage can fail to transfer the plasmid is that the plasmid does not fit into their capsid. However, this can't be the case here because all the possible phages tested have genomes that are far larger than the size of the plasmid. Therefore, the rate of horizontal gene transfer is likely to depend

on the affinity between the phage and the plasmid, more specifically between the phage and the *pac* site on the DNA of the plasmid. One possible analysis to do would be to look for homology between pNUK73 and the sequences of the phages that performed well or poorly in the transduction assay. One possibility is that there is a greater degree of homology between pNUK73 and LUZ19 that helps pNUK73 to hijack it in order to be transferred. This homology may be greater than that of pNUK73 and the other phages.

2.7 Conclusion

Phages are the most numerous biological entity on the planet, and these results provide evidence that both temperate and lytic phage could play a role in the dissemination of antibiotic resistance genes.

Temperate phage in the genome of bacteria can pick up the resistance plasmid, however they are unable to move the plasmid to identical clonemates that contain the same prophage. This is possibly due to superinfection exclusion and leaves open the possibility that they are able to infect other strains of bacteria. This could be tested using other recipient strains such as PA14 that have other prophages in its genome. Another way to further explore how the temperate phage pick up the plasmid is to use gene knockouts of each prophage. The remaining temperate phage would be induced in bacteria containing the plasmid. This would allow the examination of which temperate phage are able to pick up the plasmid and the measurement of the rates at which they pick them up.

Lytic phage on the other hand have varying abilities to move the plasmid, and the efficiencies at which this happens varies with different species of phage. The efficiencies that are measured here are larger than what has been reported previously. This is potentially because the conditions under which the experiment was carried out, which was stacked in favour of increasing the rate of transduction. However, it should be stated that there are issues with measuring the rate of horizontal gene transfer. Unlike mutation rates, the rate of horizontal gene transfer is shaped by many ecological factors. These ecological factors, to a certain extent, have been set

and controlled here, but they are variable. The results here show that horizontal gene transfer rates vary considerably temporally, and between phage. Therefore, it becomes challenging to gain an accurate method to measure the rates of transfer.

There are indications that phage life history may play a role in determining the effectiveness of transduction. A cursory comparison of the latent period and burst size of different phage shows that having a low burst size and high latent period may increase transduction rates. This is because more infective phage may kill off transductants and impose a higher selective pressure on the population. However, this idea requires more investigation and a survey of life histories of phage that are not yet published is needed to see if this trend holds.

3

Coevolution of Bacteria and Phage: its role in Transduction

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3.1 Abstract

Bacteriophages are the most numerous biological entity on the planet; and there are an estimated 10^{16} phage mediated gene transfer events per second, globally. However, it is not clear whether transduction could maintain a costly non-mobilizable plasmid (which requires episodes of horizontal gene transfer to persist) in the long term, when bacteria may develop resistance to phage. Here, I examine how pNUK73, a small plasmid that confers resistance to neomycin could be moved between strains *Pseudomonas aeruginosa*; one that has costless chromosomal resistance to rifampicin and one that is the wild type but containing the plasmid. I used serial transfers to test the persistence of the plasmid with and without phage over four days. After four days I found using DNA sequencing that the phage has managed to move the plasmid into the other strain. I also find evidence of the evolution of resistance to phage by the bacteria. However, I find that there is a stable rate of transfer of the plasmid over time, suggesting that the evolution of resistance to the phage may not be hindering transduction.

3.2 Introduction

Coevolution is the reciprocal adaptation and counter-adaptation of species that interact[168]. Whenever bacteria and phage encounter each other ecologically, there may likely be some form of coevolution. Bacteria can readily evolve resistance to infection and lysis by phage using mutations and other defence mechanisms[91]. Phage in turn may develop mechanisms to overcome this resistance. There are potentially endless cycles of evolution and counter-evolution[91]. This behaviour has been shown many times in different bacteria-phage systems[100][169][170], and not just in model systems either. Coevolution has been reported to occur in the gut where the presence of their respective antagonistic species has caused an expansion in bacteriophage host range and bacterial genetic variability[171].

Bacteria and bacteriophage coevolution is a considerable driver in shaping the diversity of both organisms and this happens on a within population level as well[172]. However, how these dynamic infectivities and resistances alter how transduction occurs has not been explored. Coevolution should affect transduction, and any examination of transduction must also explore how the coevolutionary dynamics between bacteria and phage affect it.

Coevolution can alter the susceptibility of the bacteria to the phage and the infectivity of the phage to the bacteria. These dynamic resistances and susceptibilities may vary temporally. Transduction, by definition, can only occur between bacteria that are sensitive to the phage. The donor and the recipient cells both need to be susceptible. However, bacteria may evolve resistance to the phage [100], meaning that the rate of transduction should also decrease. This is a dual effect because phage density should also decrease when resistance increases, as the vector mediating transduction is less. Assays have measured transduction rates over short time periods, but have not looked at how these may change over the longer term[27][163][164]. All things being equal, it is predicted that the rate of horizontal gene transfer by phage should decrease over time. To test this hypothesis, this experiment was set up to observe transduction over a more extended time period.

However, there is another factor adding a further layer of complexity is that if a bacteria is sensitive to transduction, it is also sensitive to being infected and lysed by the phage. In the experiment in the previous chapter, the M.O.I. was set to 0.1 such that if a bacteria was infected by a plasmid bearing phage, it was unlikely that it would be infected by another phage that was purely lytic. Additionally, because the timescale of the experiment was short, it reduced the chance of the infection progressing such that there are new phages as a result of newly burst bacteria. Over a much longer timescale, these conditions may not hold true. The infection and lysis of the bacteria may likely play a large role in determining the transduction dynamics. Moreover, because the infection and lysis dynamics may be many orders of magnitude higher than those of transduction, it should have a large effect on every part of the system. Transduction events may be masked by

the recipient cell being killed, and the overall magnitude of transduction occurring could be hidden. One study[111] has explored the lytic effects that phages have on the horizontal transfer of a conjugative plasmid. It was found that the presence of the phage increased the loss of the plasmid, by reducing the possibility of horizontal gene transfer by killing off the donors and receptors. However, it is not known how bacteriophage will affect the persistence of a non-mobilizable plasmid, where transduction is the only way in which the plasmid may move horizontally. It is hypothesised that transduction will allow the plasmid to be maintained longer than if the phage was not present, but the selection pressure caused by lysis may reduce the total amount of resistant bacteria. This is because horizontal gene transfer by phage will help the plasmid to persist, but the lytic effects of phage will also kill off transductants. It is important to note that these lytic effects are part of the ecology of the system, and that is different from the evolution of resistance or susceptibility. Nevertheless, they may also play a large part in the temporal dynamics.

This chapter describes an experiment that aims test the effectiveness of the lytic phage LUZ19 as a way to transfer the plasmid pNUK73, which confers resistance to neomycin, between strains of *Pseudomonas aeruginosa* PA01. One of the strains (PA01:rpoBH531C) contains a costless and uniquely identifiable chromosomal mutation that provides resistance to rifampicin. The other strain is wild-type PA01, but containing the plasmid pNUK73. The two strains were added in equal proportion along with the phage and then passaged for 4 days in a variety of conditions with a serial transfer at the end of each day. At the end of each day the cultures were plated on media containing both antibiotics to measure the amount of doubly resistant colonies grown, as well as the overall bacterial and phage titre. This work showed evidence of transduction as well as suggesting that the evolution of resistance to the phage by bacteria does indeed reduce the rate of transduction occurring.

3.3 Materials and methods

3.3.1 Isolating bacterial colonies and culture growth conditions

Replicate populations of the two bacterial strains must be grown in order to observe the movement of the plasmid by phage. Single colonies of *P.aeruginosa* PA01/pNUK73 and PA01:rpoBH531C[150] were isolated from frozen stocks by plating on to a Luria Bertani (LB) Miller agar plate (15g/L agar) and by incubating at 37°C for 24 h. PA01:rpoBH531C[150] was chosen because the rpoB marker contains two different nucleotide substitutions in the same codon, meaning it is uniquely identifiable. Bacteria were cultured in 14 mL Falcon tubes containing 3 mL LB Miller broth incubated at 37°C and 225 rpm continuous shaking for 24 h. The bacteria density was measured using serial dilution and diluted to 10^9 CFU/mL.

3.3.2 LUZ19 bacteriophage stocks

Replicate populations of the phage must be grown to observe their transductive ability. LUZ19 was previously investigated for its transductive ability of pNUK73 on *P.aeruginosa*, and therefore was chosen to be used in this experiment. Each LUZ19 isolate replicate was amplified from one randomly chosen plaque and introduced into an overnight PA01 culture. After 24 h, 1 mL was was added with 100 μL of chloroform and vortexed. These samples were then centrifuged at 13300 rpm for 2 minutes. Then the supernatant was syringed through a 0.22 μm filter to remove any remaining bacterial cells. The samples were stored at 4°C as purified supernatants in liquid LB. The phage density was measured using serial dilution and diluted to 10^9 PFU/mL.

3.3.3 Establishing replicate populations

The replicate populations of each bacteria and phage must be added together in order to observe any changes in resistance and movement of the plasmid. Each replicate consisted of a 2.5 mL LB Miller broth microcosm inoculated with 10 μL of each culture from different replicates. For the phage treatments, 5 μL of phage stock

was added. For the antibiotic treatments, 0.5 × MIC of PA01 WT of both antibiotics was added. Six replicate populations were established for each of the four treatments. The microcosms were then incubated at 37°C and 225 rpm continuous shaking.

3.3.4 Treatments

- **Control:** PA01/pNUK73 and PA01:rpoBH531C only.
- **Antibiotics:** Two strains of bacteria with 0.5 × MIC of the WT of both antibiotics (2 mg/L Neomycin and 8 mg/L Rifampicin).
- **Phage only:** Two strains of the bacteria with phage.
- **Antibiotic and Phage:** Two strains of bacteria with phage and 0.5 × MIC of the WT of both antibiotics (2 mg/L Neomycin and 8 mg/L Rifampicin)

3.3.5 Serial Passage

In order to see any changes in resistance over time, the bacteria and phage must be grown in fresh media every day. The microcosms were passaged by incubating them at 37°C and 225 rpm continuous shaking for 24 h under the treatments described above, vortexing and then transferring 50 μL into fresh 2.5 mL media (with and without the antibiotics depending on the treatment).

3.3.6 Measuring bacterial titre

The bacterial density was measured by plating dilutions on agar. 1 mL aliquot of the overnight culture was taken and centrifuged it at 13300 rpm for 5 minutes. The supernatant was discarded and the pellet was resuspended in 1 mL of PBS. This process was done twice more to ensure that any ensure any phages would be removed from the sample. The sample was then plated at serial dilutions of 4 μL per droplet on LB miller agar. The plates were then incubated overnight at 37°C and the number of CFU's were counted at the lowest dilution still containing at least one CFU.

3.3.7 Measuring phage titre

The phage density was measured by plating dilutions of phage isolate on soft agar. The overnight culture was vortexed, and a 1 mL aliquot was taken. This was added to 100 μL chloroform and these samples were then centrifuged at 13300 rpm for 2 minutes. The supernatant was then passed through 0.22 μ m filter. This sample was plated at serial dilutions of 4 μL per droplet on 30 mL LB miller soft agar (7.5 g/L) containing 1 mL of WT PA01. The plates were then incubated overnight at 37°C, and the number of distinct plaques was counted at the lowest dilution containing at least one plaque.

3.3.8 Measuring double resistant population

If the plasmid has been transferred, the bacteria will be resistant to both antibiotics. The double resistant population was measured by taking 200 μL aliquots of the overnight culture at then centrifuging it at 13300 rpm for 5 minutes. The supernatant was discarded, and the pellet was resuspended in 800 μL of PBS. This process was done twice more to ensure that any ensure any phages would be removed from the sample. Then 50 μL was plated on LB miller agar plates that contained 0.75 $\times$ MIC of the resistant strains of both antibiotics (60 mg/L Neomycin and 48 mg/L Rifampicin). The plates were then incubated overnight at 37°C and the number of CFU's were counted.

3.3.9 Sequencing of double resistant populations

To verify the movement of the plasmid, on Day 4 colonies from the phage treatment were taken and sequenced. From each of the 6 replicates, 5 colonies were taken at random. I used PCR to test for transductants in the populations that were exposed to phage. DNA was extracted from 5 colonies from each of the 6 replicate cultures using DNeasy Blood and Tissue Kit (QIAGEN, USA).

PCR analysis was conducted using GoTaq Hot Start Green Master Mix (Promega, USA). The primer sequences used were specific for pNUK73 (pNUK73-forward:

5'-CGCTAAGGATGTTTACAC -3', pNUK73-reverse: 5'- CTCAACCGTTCTAG-GATT - 3') The amplification conditions were: initial denaturation for 95°C, followed by 30 cycles of denaturation for 30s at 95°C, annealing at 60°C for 30s and extension at 72°C for 30s. For the final extension, the reaction was kept at 72°C for 5 min. The purified plasmid was used as a positive control. Water was used as a negative control. To test for the rpoB:H531C SNP, bacterial DNA was amplified with primers (rpoB-forward: 5'-GTTCTTCAGCGCCAGCG -3', rpoB-reverse: 5'-GCGATGACGTGGTCGGC - 3') that target the rpoB gene. The amplification conditions were: initial denaturation for 95°C, followed by 35 cycles of denaturation for 30s at 95°C, annealing at 50°C for 30s and extension at 72°C for 30s. For the final extension, the reaction was kept at 72°C for 10 min.

PCR products were sequenced using ddNTP terminators by (SourceBioscience, UK) and the resulting sequences were aligned to the rpoB sequence in Geneious (Biomatters, NZ).

3.3.10 Statistical Methods

To statistically demonstrate the differences in the treatments; bacterial density, phage density and number of double resistant cells were measured as response variables. Number of double resistant cells were converted to a density and combined with overall bacterial density to provide a proportion of double resistant bacteria. General Linear Models were used to analyse the variation of bacterial density, phage density, and proportion of double resistant bacteria. Poisson error structure was used the analyse the bacterial and phage densities. Binomial error structures were used to analyse the proportion of double resistance. Models were fitted with treatment and replicate populations (nested within treatments) as factors. Furthermore, time (in terms of Day) was fitted as a continuous variable with linear terms. In order to test for differences between treatments among each time-point, an analysis of variance test was used. Full statistical analysis tables can be viewed in Appendix A.1.

3.4 Results

3.4.1 Variation of different treatments across the time-course of the experiment

Bacterial Cell Density

To measure the total bacterial density, samples from the cultures were plated on agar plates. The bacterial density will vary across the treatments and days because of antibiotic action, phage predation and coevolution. In order to understand the relative levels of the resistances within each of the populations, it is vital to understand the size of each of the populations. By plating using serial dilutions, the density of the populations in each of the treatments was measured. Figure 3.1 shows the time course of bacterial titre for the different treatments.

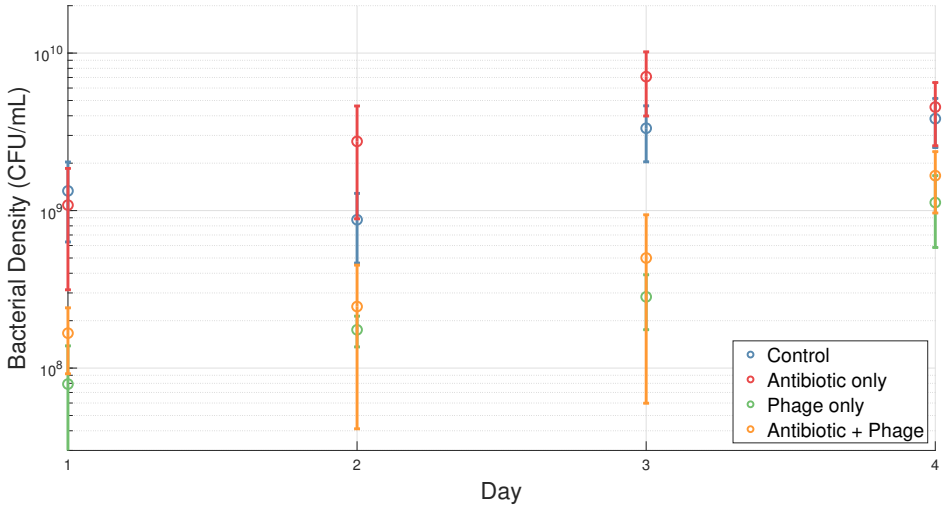


Figure 3.1: The total bacterial density on the different days of the experiment. Error bars indicate standard error.

The bacterial cell density varied significantly ($p < 0.001$) between all four treatments on all four days (Day 1: $df = 20$, $p < 2 \times 10^{-16}$, Intercept = 21.01; Day 2: $df = 20$, $p < 2 \times 10^{-16}$, Intercept: 20.59; Day 3: $df = 20$, $p < 2 \times 10^{-16}$, Intercept = 21.93; Day 4: $df = 20$, $p < 2 \times 10^{-16}$, Intercept = 22.07).

As expected, the bacterial titres were lower in the presence of phage, but the extent to which the phage suppress the bacteria reduces over time. Furthermore,

as time goes on, the difference in bacterial density between phage and control treatments becomes less. This loss of suppression of bacterial density suggests that there has been an evolution of resistance to the phage.

It is also clear that the antibiotic concentration in the antibiotic treatment has minimal effect on bacterial growth. The antibiotic treatment does not suppress the bacterial densities compared to the control and therefore is not hindering growth. This shows that the antibiotic concentrations used in this treatment were essentially non-toxic. However, it does appear that the antibiotic has an effect on phage predation, because the bacterial density in the antibiotic and phage treatment is higher than the phage only treatment. This means that the bacteria may be suppressed to a lesser degree by the phage when antibiotic is present.

Bacteriophage density

To measure the phage density, phages were isolated from the cultures in the phage treatment. These were plated on soft agar containing PA01 using serial dilutions. Bacteriophage density will vary through time as a result of reproduction, decay and the coevolutionary dynamics. The phage density will also determine the potential for horizontal gene transfer as when there is no phage present there can be no phage mediated transfer of the plasmid. Figure 3.2 shows the time course of phage density in both of the phage treatments.

The phage density varied significantly ($p < 0.001$) between both treatments on all four days (Day 1: $df = 10$, $p < 2 \times 10^{-16}$, Intercept = 24.84; Day 2: $df = 10$, $p < 2 \times 10^{-16}$, Intercept: 19.46; Day 3: $df = 10$, $p =< 2 \times 10^{-16}$, Intercept = 20.81; Day 4: $df = 10$, $p < 2 \times 10^{-16}$, Intercept = 7.76).

The phage densities in the phage only treatment displayed very high variability throughout the length of the experiment, changing by over three orders of magnitude. The phage started off at a very high density compared to the following three days. The second day resulted in a crash in density and displayed the lowest density throughout the experiment. The phage manages the rise consecutively

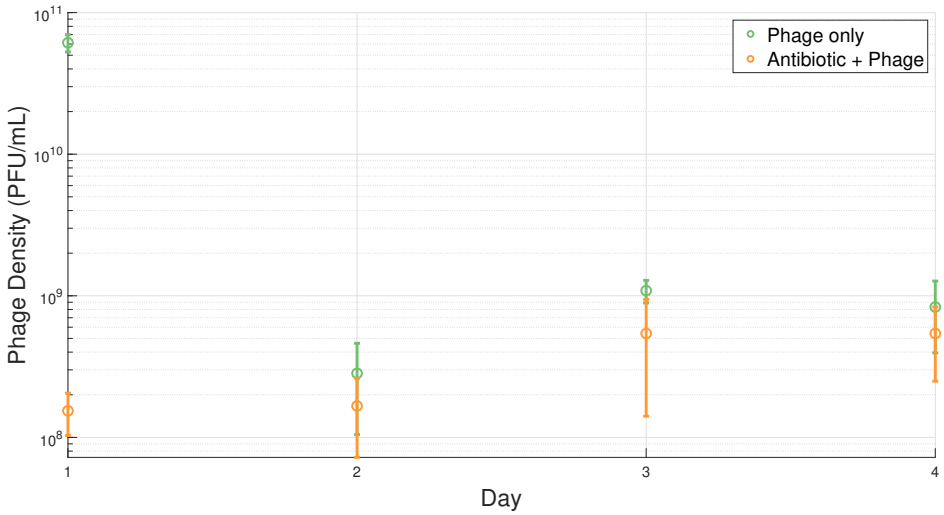


Figure 3.2: The total phage density on the different days of the experiment. Error bars indicate standard error.

for the following two days indicating that the phage has regained some of its ability to reproduce.

The results confirm that phage infection is inhibited by the presence of antibiotics, where the phage titre for the treatment containing antibiotic is about three orders of magnitude lower than the treatment with phage alone on Day 1. Even on the following three days, the phage density is lower on the antibiotic and phage treatment. Initially it was hypothesised that this was because the concentration of bacteria is lower in the treatment with the antibiotic, meaning there was less opportunity for the phage to reproduce. However, upon examining the bacterial densities (and the titration effects of the antibiotic with the densities involved), this is not the case and the bacteria in the treatment with both antibiotic and phage is at a higher density.

This means that the antibiotic is hindering phage reproduction, and this is why the bacterial densities are higher in the antibiotic and phage treatment, because there is less suppression by phage due to their lower densities. The lower phage densities in the antibiotic treatment could be due to the mechanism of action of both of the antibiotics which is to negatively affect the protein synthesis, which the phage needs to reproduce. Neomycin negatively affects translation of tRNA by the ribosome and rifampicin affects transcription by binding to RNA polymerase. Both

of these are required for phage replication, so when they are negatively affected then the phage infection process does not yield as many phage as without the antibiotics. It has been noted in a number of previous studies that phage replication can be inhibited by antibiotics[173]. Additionally, it is possible that on the first day, phage growth was initially limited by antibiotics but on the subsequent days it was limited more by the emergence of resistance to the phage; however, this is only speculative and impossible to prove without time-shift assays. There are other possible phage by antibiotic interactions that are going on that could be affecting the results. Phage infection is known to suppress the SOS response in uninfected cells[174]. The suppression of the SOS response renders the bacteria far more susceptible to antibiotic treatment[175], which in turn could reduce the efficacy of the phage lytic cycle, reducing their density.

The proportion of Double Resistant Bacteria

To measure the abundance of transductants, samples of the cultures were plated out on agar plates containing both neomycin and rifampicin. From this measurement, the density of double resistant bacteria could be calculated. Then, using the total bacterial density, the proportion of double resistant bacteria was determined. The proportion gives a measure that is easily comparable across treatments: what is the frequency of double resistance in the population. While the colony count on agar gives an absolute measure of the number of resistant cells in the population, this will be affected by the size of the total population and therefore, proportion of double resistance is a more accurate measure. Double resistance as a phenotype could emerge from transductants: bacteria that already have the chromosomal resistance that have received the plasmid, but they could also arise spontaneously through mutation. Figure 3.3 shows the number of double resistant cells divided by the total cell population or the proportion of double resistant cells of the total population.

The proportion of double resistant bacteria varied significantly ($p < 0.001$) between all four treatments on all four days (Day 1: $df = 20$, $p < 2 \times 10^{-16}$, Intercept

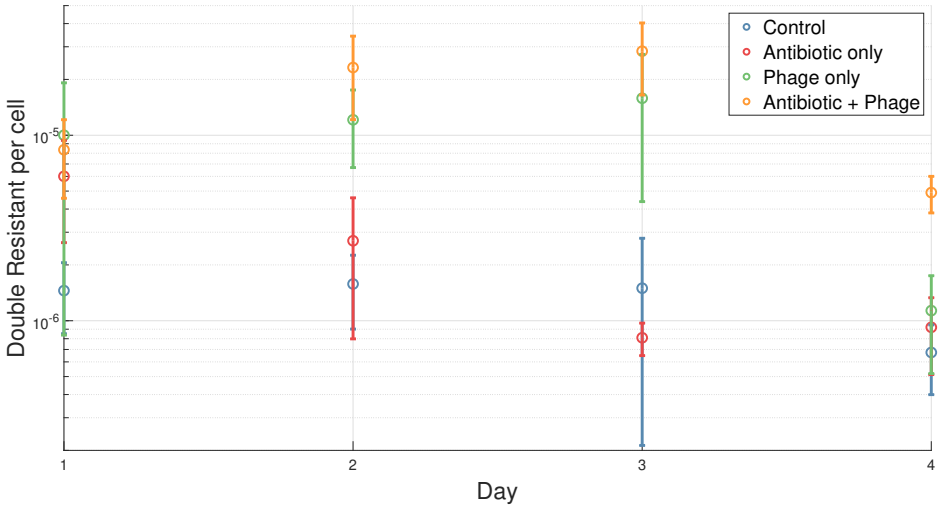


Figure 3.3: The proportion of double resistant bacteria on the different days of the experiment. Error bars indicate standard error.

$= -13.55$; Day 2: $df = 20$, $p < 2 \times 10^{-16}$, Intercept: -13.51; Day 3: $df = 20$, $p < 2 \times 10^{-16}$, Intercept $= -14.23$; Day 4: $df = 20$, $p < 2 \times 10^{-16}$, Intercept $= -14.30$).

The control does not change very much over the four days indicating that it is a not a large change in the frequency of spontaneous mutants that are double resistant. The antibiotic treatment has a far higher proportion of double resistant bacteria on the first day but that drops to closer to the control on the following three days. For the first three days, the proportion of bacteria that are resistant to both antibiotics are about an order of magnitude larger in the phage treatment as opposed to the control. On the fourth day, there is not a large degree of difference between the phage and the control. Therefore, the difference in proportion of double resistant bacteria between the phage and control treatments is stable for the first three days before falling on the fourth. This could indicate that transduction in the phage treatment is reducing past the fourth day.

The antibiotic and phage treatment has a consistently high proportion of double resistant bacteria throughout all four days. On the first three days, this is within half an order of magnitude in similarity to the phage only treatment. However, on the fourth day, it stays similarly high while the phage only treatment reduces by

Replicate Number	1	2	3	4	5	6
Number of True Transductants (out of a total of 5)	0	2	2	0	1	2

Table 3.1: The number of true transductants (containing both the chromosomal mutation and the plasmid) contained within each of the individual replicate cultures in the phage treatment, as verified by PCR and sequencing analysis.

an order of magnitude. This could be because the antibiotic selection is helping to maintain the plasmid, preventing its loss (as opposed to the phage only treatment).

3.4.2 Sequencing of double resistant bacteria

To test the hypothesis that double resistant bacteria in the phage treated populations were true transductants, I tested for the presence of pNUK73 plasmid and the rpoB:H351C SNP in 5 colonies from each of the 6 replicate populations. These colonies had their DNA extracted before being analysed through PCR and sequencing techniques. I found that out of a total of 30 colonies that were chosen, 7 were true transductants. These were from each replicate as shown in Table 3.1

3.4.3 Evidence of Transduction

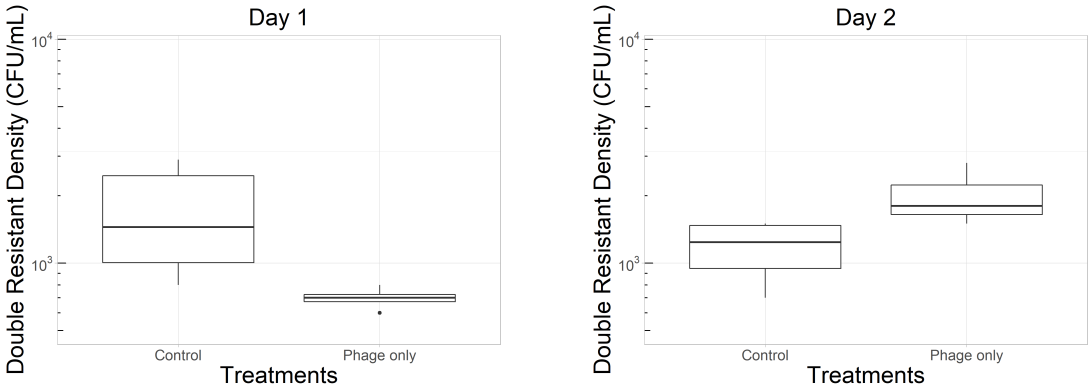


Figure 3.4: The density of double resistant bacteria on Day 1 and Day 2 of the experiment. Each day is further sub-divided into the control and phage treatments that were used during the experiment. Box plots indicate minimum, maximum, median, upper and lower quartiles.

Throughout the experiment, dual resistance to neomycin and rifampicin could arise through either mutation or transduction. To test whether transduction was

occurring, on Day 4, putative transductant colonies were sequenced. The sequencing of the double resistant bacteria shows that bacteria with the costless chromosomal mutation conferring resistance to rifampicin has obtained the plasmid pNUK73 which confers resistance to neomycin. This means that transduction has occurred. Notably, the rpoB marker that was used is uniquely identifiable because contains two different nucleotide substitutions in the same codon, meaning that the presence of this marker is indicative of transduction, and not spontaneous mutation. It is possible that transduction happened the other way around, where the chromosomal mutation was transduced to bacteria that contained plasmid. However, transduction of the plasmid is more likely because of the high copy number of the plasmid and lack of needing to recombine into the chromosome.

As seen in Figure 3.3, in the first three days of the experiment, the proportion of double resistant bacteria is higher in the phage treatment than the control by approximately an order of magnitude. It may be misleading as to why the phage treatment has a higher proportion than the control. The density of double resistant bacteria for the first two days is shown in Figure 3.4. On the first day the density of double resistant cells in the phage treatment is less than the control. However, because the overall bacterial density is proportionally lower due to the lytic effects of the phage, the proportion of resistant bacteria is greater. This does change over the next two days as the density of double resistant bacteria in the phage treatment becomes more in line with the control even while the overall bacterial density is still about an order of magnitude lower, resulting in a higher proportion of double resistance. The overall amount of transduction might be underestimated across all days but especially on the first (because phage predation is highest on this day). Transductants themselves may be lysed (because they are sensitive to transduction occurring).

3.4.4 Trends pointing to Coevolution

There is evidence that coevolution is occurring. The densities of bacteria and phage will vary as a result of the evolution of bacterial resistance and the reciprocal

evolution to overcome that resistance by phage. The bacterial density in the treatment with phage can be observed in Figure 3.1. The bacterial density on Day 1 starts at an order of magnitude lower than the control treatment. This reflects the substantial effect that phage predation has on the population numbers. However, over the following days, the bacteria develop resistance and the density monotonically increases. By Day 4, it is just over an order of magnitude more than on Day 1.

Furthermore, the variation of phage density with time reflects these dynamics. The phage density starts off very high on the first day relative to the next three days, where is drops approximately two orders of magnitude. However, this is not the complete picture. The densities do not give an accurate indication of the current infectivity of the phage relative to the resistance of the bacteria. The phage is sampled at the end of each day and then this seeds the next day's culture. It is then possible to see how the phage grows through the day, and this gives a measure of the infectivity of the phage on that day. The phage density at the start of the day is calculated by multiplying the density of the previous day by the dilution factor. The ratio of phage density is then:

$$Ratio = \frac{Density_{end}}{Density_{start}}$$

Ratios above 1 indicate positive phage growth and an infectious phage because the density is higher at the end of the day than at the start. Higher ratios indicate more growth. Conversely, ratios between 0 and 1 indicate that the phage has not grown because the phage density is lower at the end of the day than at the start. The graph showing the change in phage growth with time is shown in Figure 3.5.

On Day 1 there is a lot of phage growth, and this is the highest of all days because no bacterial resistance has yet emerged. On the second day the growth rate is less than one, showing that the phage density at the start of the day is more than at the end. This means that phage growth is not enough to overcome the phage decay, and consequently that bacterial resistance is very high relative to the day before. On the third and fourth days ratio is more than 1, showing there has been phage growth. This means that the phage can multiply and become infectious again.

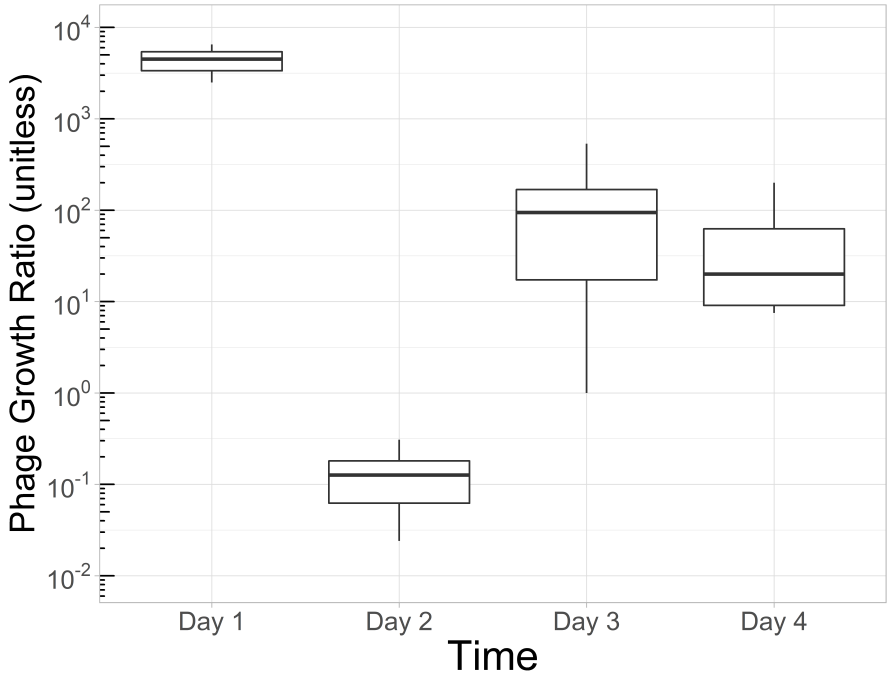


Figure 3.5: Ratio of phage growth between the start of the day and the end of the day. Box plots indicate minimum, maximum, median, upper and lower quartiles.

Therefore, taking the densities and growth of the two antagonistic species over the four days, it supports the conclusion that coevolutionary changes are going on but that the bacteria are seeming to win the evolutionary arms race.

However, there is another way to interpret the data and that is it just reflecting the ecological dynamics occurring between bacteria and phage. The phage and bacteria could simply be predator and prey, and the Lotka-Volterra[176] dynamics that describe those interactions could explain the trends here. In bacteria and phage interactions, the primary dynamic will be ecological, and the secondary interaction will be the evolutionary one that feeds back into changing the ecology. There are some indications such as phage growth ratio with time shown in Figure 3.5 which does display some damped oscillatory behaviour, like in predator-prey dynamics. Similarly, the bacterial density in the phage treatment could also be interpreted as a damped oscillation. Nevertheless, the sampling of the different densities was done once a day, and this is too coarse to see any possible oscillatory dynamics associated with predator-prey definitively.

3.4.5 Coevolution and its impact on transduction

For the first three days, the proportion of double resistant bacteria in the phage treatment stayed reasonably stable. This is not what was expected, as that the proportion of bacteria that are double resistant in the phage treatment were expected to have gone down with time as resistance to the phage went up. As there is more resistance to the phage, there will be less sensitive donors and recipients. This will reduce the magnitude of transduction. Figure 3.6 shows the variation of double resistant density with time and it mirrors the monotonic increase in bacterial density over the first three days. This shows that the emergence of bacterial resistance has not had a large effect on transduction. It is not immediately clear why the emergence of phage resistance does not result in reduction in transduction because mechanistically, it should. One possible reason is that the emergence of resistance is not at a high enough proportion to affect transduction. Transduction is by its very nature a rare event, and perhaps the population of bacteria are not resistant to a large enough degree that transduction is measurably affected. Another possible reason is that perhaps the sampling of bacteria and phage are too coarse and infrequent to fully measure the array of coevolutionary changes that are going on. Coevolutionary changes have been found to occur within a day[101], and perhaps there is phage sensitivity occurring that has not been detected. This phage sensitivity could allow transduction to take place.

However, on the fourth day there is a dramatic drop in the proportion of the double resistant cells in the phage treatment which is approximately an order of magnitude (which is equivalent to a 90% drop). This is not mirrored by a drop in bacterial density found in the first three days. There are two possible reasons for this (or indeed a combination of both). One reason is that in the absence of antibiotics, there is no selection for the plasmid and it is therefore lost from the population due to the fitness cost it imposes. Previous studies have shown that 70% of pNUK73 bearing PA01 have lost the plasmid by the fourth day (in the absence of positive selection)[135] (the chromosomal resistance to rifampicin is stable in the absence of positive selection[150]). This is corroborated by Figure

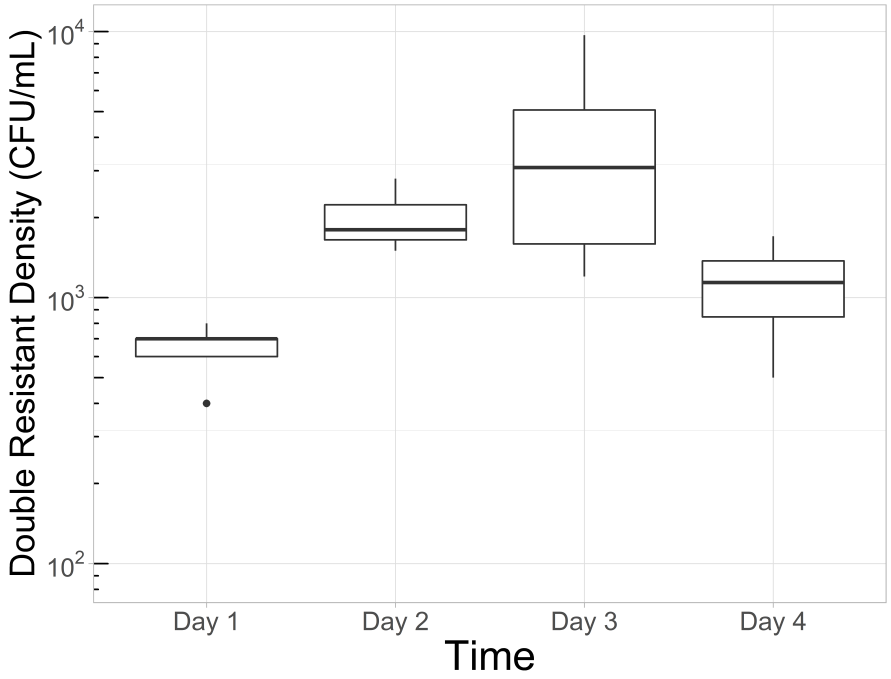


Figure 3.6: Double Resistant Density in Phage Treatment. Box plots indicate minimum, maximum, median, upper and lower quartiles.

3.3, which shows the variation of the proportion of double resistant bacteria with time in the control treatment. This shows that in the absence of any selective pressure that the proportion of double resistant bacteria goes down. By Day 4, the proportion of double resistant bacteria has dropped by approximately 50% compared to the first day. This could be because the plasmid is steadily lost in the bacteria because of the fitness cost that it imposes. There is more evidence that supports the plasmid being lost on the fourth day because treatment with antibiotics still contains a much higher proportion of double resistant bacteria compared to the control, approximately one order of magnitude (or 90% more).

While plasmid loss can explain some of the drops, another reason is that resistance to the phage has emerged and is stopping transduction from occurring. Transduction can only transfer the plasmid when there are both sensitive donor and recipient cells, and if resistance to the phage has emerged then the rate of transduction will go down. The emergence of phage resistance has a dual effect because phage resistance causes a reduction in phage density and this means there are fewer vectors by which to transfer the plasmid. The results have shown evidence

of the development of resistance to phage by bacteria. However, while bacterial density increases monotonically over the four days, and there is no evidence this is dramatically increased on the fourth day. Furthermore, there is a large drop in phage density from the first day to the subsequent three, but the fourth day is not dramatically different to the second and third.

3.5 Discussion

3.5.1 The factors that drive an abundance of transductants

In a conjugation assay when you mix donors and recipients and you plate it out, what results is the conjugation frequency. This is not necessarily measuring the rate of conjugation. Mostly, what this is measuring is the growth of the transconjugants. The same is valid here. So, for example, if a higher antibiotic dose had been used in the antibiotic treatment, it would be expected that more double resistant colonies would be formed because there is more benefit to be double resistant. For the dose of antibiotic used in the antibiotic treatment, there looks like there was no real benefit to being double resistant because the frequency of double resistance did not increase with time. Furthermore, the presence of low dose antibiotics does not appear to have suppressed the density of bacteria, meaning that there was no real selective pressure.

Therefore, the differences that have been measured between the control and the phage only treatments (in other words, mutation in the control versus transduction and mutation in the phage treatment) are capturing the underlying differences between them. It is measuring transduction and not selection for transductants.

Although that may not be the whole picture. It has been shown in another study involving a conjugative mercury resistance plasmid that positive selection actually inhibits horizontal gene transfer[177]. This experiment was done in soil bacteria, with a conjugative mercury resistance plasmid. However, in the presence of positive selection, the bacteria are suppressed meaning that the donor and recipient bacteria are suppressed. The relative absence of donor and recipient bacteria mean that the effective rate of horizontal gene transfer is reduced. The results from the experiment suggest that plasmids are more likely to be mobilised

when they are parasitic rather that when they are beneficial. This is analogous to the treatment with both antibiotic and phage, where there is positive selection (although the antibiotic only treatment shows that there is no measurable selective pressure) and horizontal gene transfer. Although the treatment appears to show the greatest proportion of double resistant bacteria on the last three days, this may not in fact be because the largest amount of horizontal gene transfer is occurring. Further complicating and compounding the effect of reduced transfer is that the presence of antibiotic appears to inhibit phage replication, which in turn reduces the potential for gene transfer to occur.

3.5.2 Coevolution between PA01 and LUZ19

It is important to stress that coevolution between phage and bacteria has not been definitively proven in this experiment, only that the trends shown by the data could be associated coevolutionary patterns associated with bacteria and phage in literature. To definitively associate the trends arising from the data, the presence or absence of evolution would need to be determined. Detecting coevolution requires the use of time shifts experiments to detect the different phenotypic motifs during the evolution of host resistance or phage infectivity[91]. Therefore, one way to verify the emergence of host resistance is to have taken bacteria on Day 4 of the experiment and tested the infectivity to the ancestral phage, and to see that the resistance went up compared to Day 1.

While it is not yet possible to say with certainty whether coevolution has occurred, there is excellent precedent in the literature for bacterial resistance increasing with time [178]. In addition, the data in experiments point towards that being the case. Experiments with bacteria and phage have shown the evolution of resistance to phage by the fifth day, and this has been shown to occur on a genomic level. Moreover, experiments specifically with PA01 and LUZ19 have shown real evidence of coevolutionary behaviour. Time-shift assays were used to show that there are dynamic changes in resistance and infectivity between bacteria and phage, even over a single day. Minima and maxima that vary with time were observed during

these experiments and these that are associated with local temporal adaptation[101]. Figure 3.5 does show that the experimental system presents oscillatory dynamics, which can be interpreted as coevolutionary changes. However, it must be stressed that these are not definitive without time-shift assays.

3.5.3 Maintenance of the plasmid in the presence of phage

One study that is worth comparing these results to is one that examined the persistence of a conjugative plasmid that conferred mercury resistance in the presence of phage. It was found that bacteriophages limit the conditions by which conjugative plasmids can exist by increasing their loss under weak selection conditions[111]. This is primarily because phage predation is reducing the amount of donor and receptor bacteria and thereby reducing the overall horizontal gene transfer. This is similar to the idea that is explored in this experiment, except rather than a conjugative plasmid, a non-mobilizable plasmid is used here. In this experiment, phage presents an avenue for horizontal gene transfer as well as suppressing bacterial populations through predation. The results show that there is a higher proportion of transductants in the phage treatment as opposed to the control. This shows that phage can transfer and help maintain a plasmid that has no other means of transferring itself horizontally. The predatory effect of the phage will reduce the overall density of double resistant bacteria (as evidenced by the first day of the phage treatment), but the plasmid will persist longer than if it had no avenue to move around bacteria. This effect is stronger in the presence of positive selection in the form of low dose antibiotics, which selects for the plasmid. This helps maintain the plasmid, compared to treatments without antibiotic. This increases the probability that the plasmid is transferred to bacteria which have the chromosomal mutations producing dual resistant transductants. This explains why the proportion of transductants is higher in the presence of both phage and antibiotics as opposed to purely phage.

3.6 Conclusion

This chapter describes an experiment that was designed to test the effectiveness of the phage LUZ19 in maintaining an antibiotic resistance plasmid over a period of four days. Two strains of bacteria were used to inoculate the culture on the first day: PA01/pNUK73 that confers resistance to neomycin and PA01:rpoBH531C that confers resistance to rifampicin. The bacterial and phage densities, as well as the frequency of double resistant bacteria, were measured.

Sequencing of putative transductants at the end of the experiment showed that there were bacteria that had the chromosomal resistance to rifampicin and the plasmid. This shows that transduction of the plasmid has indeed occurred.

There are clear trends that arise from the data. The steady increase with time of bacterial densities with the treatment containing phage, shows that the bacteria are developing resistance to the phage. The ratio of phage densities between the start and the end of the day shows that there is a reciprocal evolution of the phage to infect the bacteria. However, it should be stated that it is not possible from the present data to disentangle the evolution of resistance and infectivity from the typical predator-prey interactions that will occur between bacteria and phage. It is plausible that all the data could be explained by the ecological dynamics, but past studies have shown that coevolution does occur.

The likely evolution of resistance of bacteria to the phage has implications for transduction. Transduction can only occur between bacteria that are sensitive to phage infection, and the evolution of resistance to the phage reduces the amount of transduction that can occur. The experimental data does not support this. The difference between the proportion of double resistant bacteria in the control treatment and the phage treatment stays stable with time, except for the fourth day. However, there is no clear evidence that this drop is due to the evolution of resistance by the bacteria. Furthermore, this could also be caused by plasmid loss in the absence of positive selection, and the fall in the proportion of double resistant bacteria in the control treatment as well as the stability of the plasmid in the antibiotic and phage treatment provides some evidence of this. Nevertheless, as

transduction can, by definition, only occur between phage sensitive bacteria and this has not been proven by these experiments. This relationship between phage resistance and transduction merits further investigation.

In summary, this work shows that transduction does indeed move an antibiotic resistance plasmid between two strains of bacteria over more extended periods of time. However, there are few indications that coevolution plays a large part in driving the dynamics of transduction and that bacterial resistance to phage hinders the movement of the plasmid.

4

Mathematical Modelling of Horizontal Gene Transfer and Transduction

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4.1 Abstract

Bacteriophages have complex and many layered interactions with bacteria such as predation, horizontal gene transfer and coevolution. However, it is difficult to systematically study these interactions as each of these processes may mask

the effects of the other ones. Mathematical modelling allows us to examine their dynamics in ways not possible experimentally. Here, I present three models that look at the phage mediated horizontal gene transfer of a costly antibiotic resistance plasmid at various levels of abstraction. The first model looks at horizontal gene transfer in general, and specifies the logical conditions under which an antibiotic resistance plasmid may persist. The second model looks at transduction in detail and presents the critical parameters of phage and bacterial biology that can allow for a plasmid to be maintained in a population of bacteria. The third model looks specifically at bacteriophage life history. It shows that a phage with a low burst size and long latent period will be more effective at transducing a plasmid through a population of bacteria than a phage with a high burst size and short latent period.

4.2 Introduction

Antibiotic resistance represents one of the greatest challenges to modern medicine. It is estimated that by 2050, there will be a death every three seconds as a result of a drug resistant infection. Furthermore, there are sizable economic implications from the emergence of large scale antibiotic resistance, as there will be an estimated yearly loss of over $100 trillion to global GDP[5]. In order to overcome antibiotic resistance, the processes that spread, promote or inhibit antibiotic resistance genes in the environment need to be better understood. It is still not completely clear how antibiotic resistance plasmids can persist in the long term, in a problem known as the 'plasmid paradox'. Resistance plasmids can impose a fitness cost on to the bacteria. Under conditions where there are no antibiotics and therefore no benefit provided by the plasmid, selection is in favour of the bacteria losing the plasmid. Conversely, if there are antibiotics present such that the genes on the plasmid provide benefit to the bacteria then those genes could also move to the chromosome leaving the plasmid redundant and still imposing a fitness cost. Essentially, plasmids initially provide a function that increases bacterial fitness but then when those genes move on to the chromosome, the plasmid becomes parasitic[179]. However, it has been shown empirically that efficient horizontal

gene transfer can maintain a plasmid in the long term[108]. Genetic sequencing has shown that 50% of plasmids are incapable of conjugation [20], so they must have to transfer horizontally in another way. Transduction is a form of horizontal gene transfer that is mediated by bacteriophages, but it is not clear what kind of conditions and parameters would promote the most efficient transfer, and allow the persistence of an antibiotic resistance plasmid. Furthermore, with few new antibiotics in the drug development pipeline, bacteriophages are increasingly thought of as a new way of combating drug resistant infections. This treatment is known as phage therapy and is where bacteriophages are used to kill the bacteria infecting the patient. There are even arguments that the efficacy of old antibiotics could be preserved by using phage therapy[175].

Bacteriophages can substantially alter the ecology of bacterial communities through predation (such as in phage therapy), horizontal gene transfer and coevolution. However, there are issues with studying these interactions as each of these processes may mask the effects of the others. Mathematical modelling can provide methods to study the dynamics in ways that are just not possible experimentally. Models are simplified descriptions of the phenomena that they are representing and can be used to show flaws in our understanding. This theoretical approach allows us to conceptualise what critical features in phage and bacterial ecology are required such that plasmids may persist.

Theoretical approaches have been used previously to explore how plasmids may persist in the long term. They have termed these the "existence conditions" of plasmids. One model published in 1977, used a model of population dynamics that looked at analysing how plasmids may persist through conjugative transfer[112]. They examined the parameter space in population growth, conjugation rate and segregation rate using biologically realistic values. The plasmid can be maintained only when the cell density and conjugational transfer rate are sufficiently large to overcome segregational loss and selection against the plasmid. This model was further expanded upon in another study[113], which subdivided the bacterial population into plasmid bearing and plasmid free types. They found that the

establishment of a population of plasmids may depend on them being introduced initially at a high enough number. However, countering that is a another study, published in 2000[110]. It cited empirical studies that found that conjugation does not occur at high enough rate to maintain plasmids. They modelled what happened to the plasmid using a population dynamics approach, and found that plasmids cannot persist indefinitely purely by carrying genes that are beneficial to the host. Instead, the beneficial genes will eventually move to the chromosome. They do go on to show that plasmids can be maintained if they can transfer local adaptation genes on to newly arrived bacteria that bear evolutionary advantages or, if they can transfer the genes intermittently between non-competing strains. Although, this model explicitly makes it impossible for the plasmid to be maintained through conjugation, even at maximum bacterial density. In fact, another study[114] took that model and expanded it and found parameter space required for maintenance is far less stringent than had been found previously. Furthermore, it found that parasitic plasmids may persist through conjugation.

It is important to state that none of these models explore the existence conditions for non-conjugative plasmids, and how other forms of horizontal gene transfer can maintain plasmids. Therefore, the relevance of these models to exploring transduction is minimal. One study that did look at the existence conditions of strictly non-mobilizable plasmids used a population dynamics approach and found that it is very difficult for these plasmids to persist in the long term[20]. This study suggested that the only way that costly plasmids can exist is for them to experience some form of horizontal gene transfer. Another study used modelling approaches to see the effect of phage predation on a conjugative plasmid[111]. They first used ordinary differential equations to show that the phage drive plasmid dynamics through the ecological effects that they have on bacteria, and they ultimately limit the existence conditions by killing off donors and receptors needed for horizontal gene transfer. However, in the presence of phage and no selective pressure they found bistability in the system (plasmid fixation and plasmid loss), meaning temporary maintenance of the plasmid followed by rapid loss. To explore this, they used an

individual based model which showed that conjugative plasmids could hitchhike with chromosomal mutations providing resistance to phage. These recurrent sweeps of resistance mutations drive the temporary maintenance of the plasmid followed by the its loss. This model deals with conjugative plasmids so does not explore the role that transduction may play in maintaining the plasmid. Additionally, this model describes the phage ecology using one parameter, and so loses resolution on the actual mechanistic basis of phage infection. Therefore, a model that looks deeper into the transduction of a non-mobilizable plasmid is warranted.

In this chapter, three different models are presented that use various and different mathematical techniques to reflect the underlying biology of horizontal gene transfer. All of the approaches model bacteriophage-bacteria interactions but use different methods to present the ecology at different levels of abstraction. This in turn allows them to provide a different insight. They have all been adapted to each specific situation that they are modelling, and therefore can be used as a collective to provide a more complete picture of what provides plasmid persistence. Additionally, these models can be used to elucidate the role that horizontal gene transfer and transduction play in bacterial ecology. The modelling here is used to provide both qualitative and quantitative conclusions which can be verified in an experimental setting.

The first model takes a more general view of the horizontal gene transfer of a antibiotic resistance plasmid in a population of bacteria. This model explores how and under what conditions can this resistance plasmid can persist. The second model looks more specifically at transduction and the role that different phage traits can have on bacterial ecology and the persistence of the plasmid. The third model takes an agent-based modelling approach to look at early phage infection and two phage traits in particular: burst size and latent period and the effect they may have on transduction.

4.3 Basic Model of Horizontal Gene Transfer

To explore the role that horizontal gene transfer has on the persistence of an antibiotic resistance plasmid in a population of bacteria, initially, a more general view of the ecological processes involved is taken. Presented here is a system of ordinary differential equations that models the horizontal gene transfer of a antibiotic resistance plasmid. The model is inspired by a previous model on social evolution[180], but it is itself a population dynamics model. A visual representation of the processes and interactions represented by the model is given in in Figure 4.1. The model is presented in its entirety in Equation Set 1. There is no specific method of horizontal gene transfer that is being modelled. Rather, the purpose of this model is to see the effect that horizontal gene transfer has on bacterial diversity and the persistence of an antibiotic resistance plasmid, when there is an antibiotic present or not.

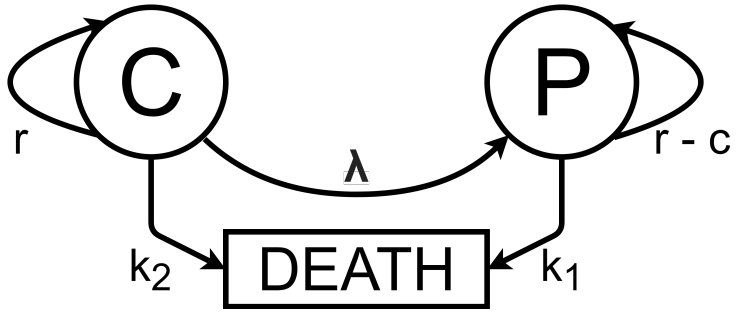


Figure 4.1: Illustration of the biological framework underpinning the basic model. This model examines how a resistance plasmid may persist using horizontal gene transfer.

4.3.1 Individual Dynamics underlying the Basic model

There are several different ecological processes that are being modelled here. These processes and the mathematical assumptions behind them are described in this section.

Bacterial Growth

P represents the population of plasmid bearing bacteria. C represents the population of plasmidless bacteria. In this model, the bacterial growth is exponential and not

nutrient or carrying capacity dependent. The growth rate is represented by r, and the cost of antibiotic resistance plasmid carriage is a reduction in the growth rate and this cost is represented by c. It is also assumed that there is no segregational loss of the plasmid during cell division. The overall dynamics of growth can be described by:

$$\frac{dP}{dt} = (r - c)P$$

$$\frac{dC}{dt} = rC$$

Antibiotic Action

I represents the concentration of antibiotic. k_1 and k_2 represent the rate of killing with and without the plasmid, respectively. The plasmid will confer protection against the antibiotic and parametrically that is presented through $k_2 > k_1$. It is assumed that antibiotic action has mass action kinetics with a rate k_i, and is proportional to the bacterial populations and antibiotic concentration. Additionally, it is assumed that there is no bacterial death than by antibiotics. The overall dynamics of antibiotic action can be described by:

$$\frac{dP}{dt} = k_1 IP$$

$$\frac{dC}{dt} = k_2 IC$$

$$\frac{dI}{dt} = -k_1 IP - k_2 IC$$

Horizontal Gene Transfer

λ represents the rate of horizontal transfer of the plasmid. It is assumed that horizontal transfer has mass action kinetics governed by the rate λ, and is proportional to the product of both plasmidless and plasmid bearing bacteria. The overall dynamics of horizontal gene transfer can be described by:

$$\frac{dP}{dt} = \lambda PC$$

$$\frac{dC}{dt} = -\lambda PC$$

4.3.2 Complete Basic Model of Horizontal Gene Transfer

The individual dynamics describing facets of the model are combined to give a complete model which is shown in Equation Set 1. The model uses a system of ordinary differential equations and there there are assumptions inherent to that. The first assumption is that the system is deterministic and there is no stochasticity. Furthermore, it is assumed that the system is well mixed and there is no spatial heterogeneity in the system.

$$\frac{dP}{dt} = (r - c)P - k_1 IP + \lambda PC$$

$$\frac{dC}{dt} = rC - k_2 IC - \lambda PC$$

$$\frac{dI}{dt} = -k_1 IP - k_2 IC$$

Equation Set 1: The Basic Model of Horizontal Gene Transfer

4.3.3 Analysis of the Model

The advantage of using a mathematical model to describe the temporal dynamics of a system is, that even in the face of changing parameters, the behaviour can still be analysed. It can be used to find the critical parameters in the system that permit certain and critical results. A vital question with biological relevance is under which conditions will an antibiotic resistance plasmid persist in the system, and therefore when will resistance persist in a population of bacteria? This is essentially

Table 4.1: List of parameters and variables in the basic model

P	Population of plasmid bearing cells
C	Population of plasmidless cells
I	Antibiotic concentration
r	Basal rate of growth
c	Metabolic cost of carriage
k_1	Killing rate by antibiotic with plasmid
k_2	Killing rate by antibiotic without plasmid
λ	Horizontal transfer rate of plasmid

the question that is of most relevance when studying antibiotic resistance ecology. Knowing the conditions that cause plasmid persistence can help mitigate the spread of resistance. Invasion by rare analysis allows us to find a single mathematical condition by which the plasmid bearing strain persists. Furthermore, the condition under which the plasmid persists.

Invasion by rare is a type of analysis that relies on linear algebra and has been used previously to analyse invasions into habitats by foreign species and in epidemiology [181]. It sets out the conditions by which a certain species will invade a foreign population given they started at a very rare level. By setting the antibiotic and plasmidless cells to an arbitrary amount I^* and C^*, I can linearize the equation for the plasmid bearing cells around zero and if this condition is greater than zero then the plasmid bearing species will invade from rare. The mathematical condition specifying how a plasmid bearing species can invade from rare is described by:

$$r - c - k_1 I^* + \lambda C^* > 0$$

The full mathematical derivation of the invasion from rare condition is described in Appendix B.1.

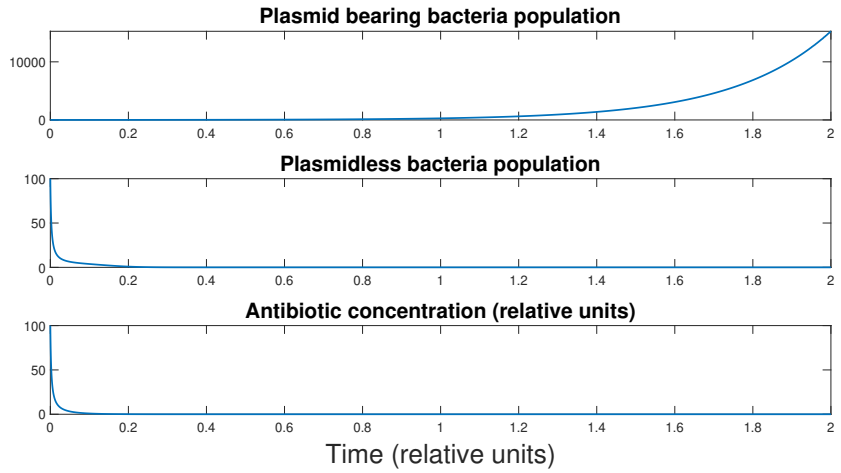
Invading from rare is equivalent to a species persisting and therefore specifies the boundary condition by which a species can persist in an environment. One of the most important questions concerning plasmids in the environment is how they manage to persist despite the fitness costs they impose on the cell, in what is

known as the 'plasmid paradox'. What the invasion from rare analysis provides is the logical conditions leading to persistence. This shows that the rate of horizontal gene transfer (λ) must be greater than the fitness cost (c) of the plasmid and the killing rate of the antibiotic (k_1) for it to be able to persist. This means that for antibiotic resistance plasmid persistence, it is not just it provides benefit against the antibiotic but that it must also experience sufficient horizontal transfer to overcome the fitness cost it imposes on the bacteria. Importantly, this shows that horizontal gene transfer is a critical mechanism by which plasmids persist in the environment. Crucially, this analysis shows that horizontal gene transfer is a possible solution to the 'plasmid paradox'.

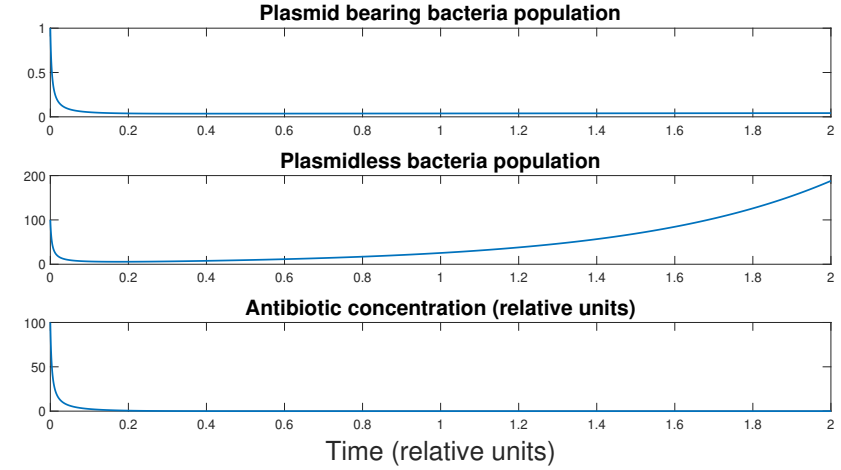
The conclusion of the invasion by rare analysis can also be confirmed by numerical analysis, where the temporal behaviour of the model can be solved by computation. Figure 4.2 shows that when the invasion condition is satisfied the amount of plasmid bearing bacteria rises exponentially. In contrast, the amount of plasmidless bacteria goes to zero. When the invasion condition is not satisfied, the opposite happens and the plasmid cannot persist in the bacterial population. It is essential to state that these are not parameters that are biologically realistic, they were just chosen to demonstrate the mechanisms involved in the process of plasmid persistence. Furthermore, because of the models underlying simplicity (and the lack of any feedback loops), the model will only have two outcomes, plasmid maintenance or the plasmid dying out. Therefore, the behaviour of the model will be dependent on the invasion by rare inequality, and then will conform to either of the outcomes.

4.4 Model of Transduction

While the basic model can provide neat analytical results, these results cannot accurately predict or model the temporal dynamics of phage mediated horizontal plasmid transfer due to the model's inherent simplicity. There are several different ecological processes that are involved in a system containing bacteria, bacteriophage, antibiotics, and antibiotic resistance plasmids. A visual overview of the processes



(a) Invasion condition satisfied: Plasmid persistence ($r = 5, c = 1, k_1 = 1, \lambda = 3$)



(b) Invasion condition not satisfied: Plasmid dies out($r = 2, c = 1, k_1 = 3, \lambda = 1$)

Figure 4.2: Numerical simulations when the invasion condition is satisfied and not. When the invasion condition is satisfied, the plasmid bearing species can invade from rare. When the invasion condition is not satisfied, the plasmid bearing species will die out.

being modelled can be seen in Figure 4.3. The model is first presented in its entirety in Equation Set 2.

4.4.1 Individual dynamics underlying the Transduction model

There are several different ecological processes that are being modelled here. These processes and the mathematical assumptions behind them are described in this section.

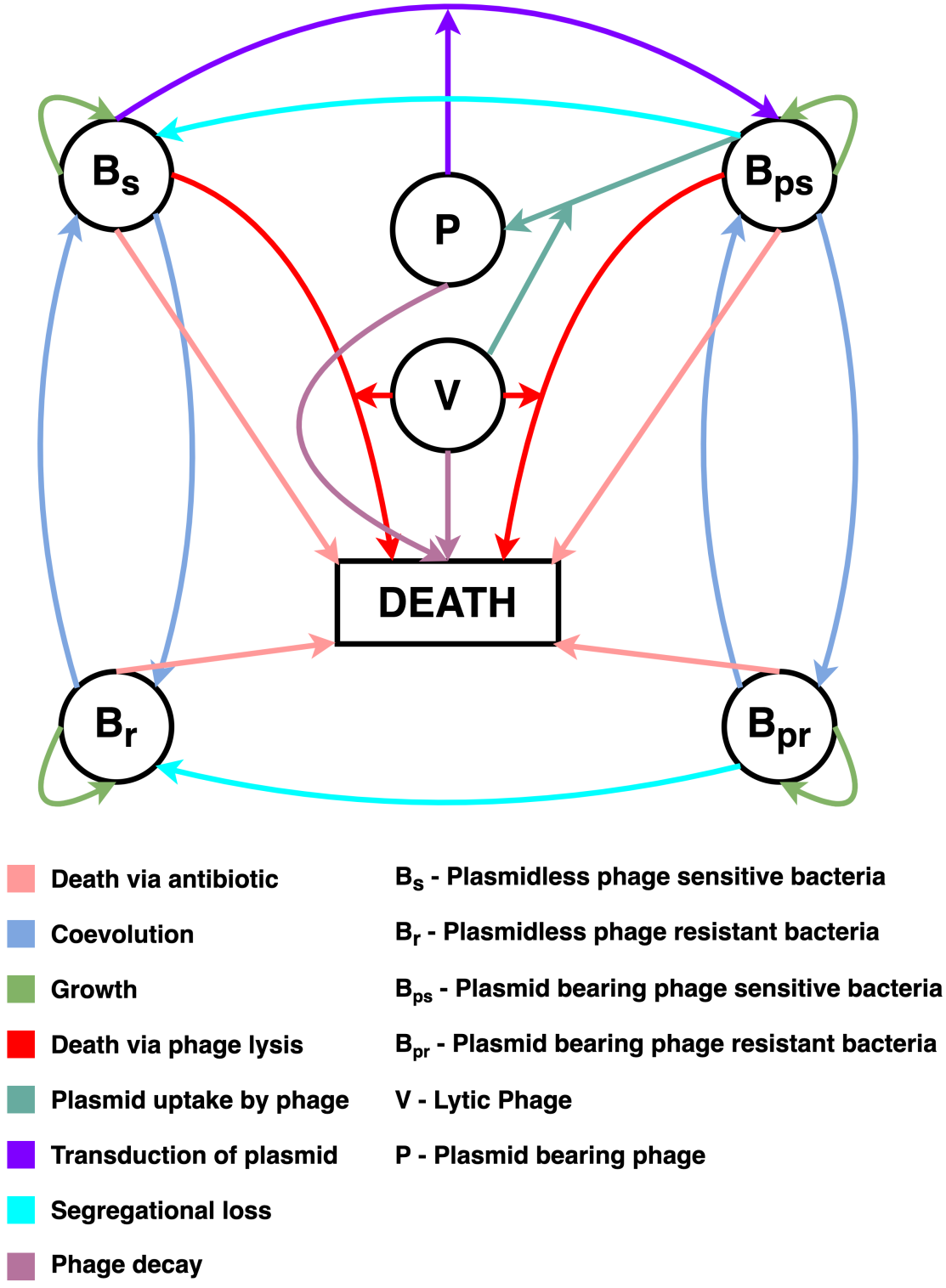


Figure 4.3: Illustration of the biological framework underpinning the transduction model

Lac-Monod Growth

The finite environmental resource concentration R is explicitly modelled, and consumption is modelled using Michaelis-Menten growth dynamics. The growth

rate is then the resource consumption rate multiplied by the resource conversion rate ρ. The different types of bacteria are represented by B_i, the specific affinity of bacteria for resource uptake is represented by $\frac{\mu}{K}$. The fitness cost of the plasmid is then presented through the slower rate of growth when carrying the plasmid such that $\rho_p\left(\frac{\mu_p R}{K_p+R}\right) < \rho\left(\frac{\mu R}{K+R}\right)$. Plasmid loss can occur through segregational loss during growth as the plasmid is assumed to lack any sort of partitioning or toxin-antitoxin system, which is an accurate representation of pNUK73[135]. The plasmid is assumed confer a single fitness loss. These dynamics can be described by:

$$\frac{dR}{dt} = \left(\frac{-\mu_i R}{K_i + R}\right) B_i$$

$$\frac{dB_i}{dt} = \rho_i \left(\frac{\mu_i R}{K_i + R}\right) B_i$$

Antibiotic action

The antibiotic concentration is represented by A. It is assumed that there is mass action kinetics between the antibiotic and bacteria. The presence or absence of the plasmid determines the killing rate κ. The protection that the plasmid confers is represented by a lower killing rate such that $\kappa > \kappa_p$. The plasmid is assumed to be single copy. It should be stated that in actuality the plasmid will not offer binary protection against the antibiotic. Instead, it will be function of the number of copies of the plasmid that the bacteria contains. However, the model will assume a binary response as an approximation of this, as described in a previous mathematical model of pNUK73[20].

The resistance to an antibiotic is via an enzymatic mechanism, where the cells produce enzymes that destroy the antibiotic molecule making it ineffective[182]. This resistance causes the rate of antibiotic degradation in the environment α to be higher with the plasmid ($\alpha_p > \alpha$). Antibiotic degradation without the plasmid is assumed to be due to the use of antibiotic when cells are killed. Additionally, it is assumed that the antibiotic has no impact on phage reproduction. As observed in

Chapter 3, this is not entirely accurate but it will be assumed here that there is no interaction because there is not enough data to describe the relationship between phage replication and antibiotics accurately. These dynamics can be described by:

$$\frac{dA}{dt} = -\alpha_i A B_i$$

$$\frac{dB_i}{dt} = -\kappa_i A B_i$$

Phage lysis, reproduction and transduction

The lysis of bacteria by phage is considered to be instantaneous (there is no explicitly modelled latent period), and there is a release of y viruses V by an single infection. It is also assumed that the phage reproductive cycle is not affected by antibiotic concentration. The kinetics of the phage infection is mass action, which is an accurate approximation of the dynamics because the reproductive cycle requires physical contact between the phage and the bacterial cell. The dynamics are mediated by a parameter p, which is the rate of lysis and is dependent on the strain of phage. It is also assumed that the rate of lysis does not change through time.

Transduction occurs when bacterial DNA is mistakenly packaged into the capsid of the phage, this phage then goes on to infect another bacteria, transferring the DNA. It is assumed in this model that there is no other DNA transduced than that due to the plasmid and that transfer of the plasmid can only be to plasmidless bacteria. The uptake of the plasmid can only occur from plasmid bearing bacteria. The number of phages that contain the plasmid that is represented by P and is controlled by the parameter z. This parameter z represents the probability that any single phage mistakenly packages up the plasmid DNA instead of its own. This parameter depends solely on the affinity between the phage and the plasmid DNA. The transfer of the DNA by a plasmid carrying phage to a plasmidless bacteria is controlled by the parameter γ. Again, this is controlled by mass action kinetics because there must be physical contact between the phage and plasmidless

bacteria to transfer the plasmid. The decay of the bacteriophage, independent of whether they carry the plasmid or not, is represented by the parameter d. These dynamics can be described by:

$$\frac{dP}{dt} = zpyVB_{ps} - \gamma PB_s$$

$$\frac{dV}{dt} = ypV(B_{ps} + B_s) - zpVB_{ps}$$

Coevolution

Coevolution is defined as the process of reciprocal adaptation and counter-adaptation between ecologically interacting species[89]. Antagonistic coevolution between bacteria and phage has been described by two canonical models: frequency dependent selection and arms race. Frequency dependent selection is driven by negative frequency dependence. Phage evolve to infect common bacterial genotypes, thereby selecting for rare resistance genotypes, which then become common. This creates sustained oscillations of phage and bacterial genotype frequencies. These dynamics do not lead to evolution of broad resistance and infectivity. In arms race, both bacteria and phage accumulate resistance and infectivity respectively through a series of selective sweeps. This leads to a broad range of bacterial resistance and phage infectivity.

There have been previous studies that have modelled the coevolutionary dynamics of bacteria and phage. One model[183], takes inspiration from the ecology of predator prey dynamics. They then designate a new state variable to each new strain of bacteria or phage that may arise from mutation. The bacteria differ between their resource uptake efficiency and resistance to phage. The phage alter their bacterial preference. Using a combination of parameters they also may adjust the dynamics between arms race and frequency dependent selection. However, this method of modelling the coevolutionary dynamics is unsuitable for looking at its impact on transduction because creating multiple strains of bacteria and phage

would increase the computational complexity, especially when the focus primarily lies on whether the bacteria contains the plasmid or not. Another model[184] uses stochastic modelling techniques to examine the coevolutionary dynamics between bacteria and phage. They use a single parameter to differentiate between the two modes of coevolution, such that are middling value can describe a combination of the two. There are two different populations of bacteria and phage with one variety of bacteria susceptible to both varieties of phage. The other variety of bacteria is only susceptible to one set of phage (which can infect both varieties of bacteria). This was therefore unsuitable because there was no bacteria that was totally resistant to phage predation at any one point, which was a critical point of the model in observing the effect of phage resistance on transduction. Therefore, a new novel approach to modelling transduction was required.

The coevolutionary dynamics between bacteria and phages are complex[172] and often do not on conform to one type of dynamics[102]. It has been suggested that the form of dynamics depend on the kind of receptor used by the phage when infecting the bacteria[101]. Furthermore, the type of dynamics that it undergoes may change throughout their time, as with *P.fluorescens* and $\Phi 2$, which changes from arms race to frequency dependent selection after 200-250 generations[102]. For the sake of simplicity it will be assumed that only one of these particular dynamics can be used to describe the coevolutionary behaviour within the complete model. It has been shown experimentally that LUZ19 which was used in the experimental work displayed peaks in bacterial resistance and phage sensitivity associated with frequency dependent selection [101], so these dynamics will be used.

In the dynamics of frequency dependent selection, the bacteria are resistant to the most recent phage and the phage is only infectious to the current bacteria. In modelling terms, this means that the bacteria and phage are memoryless. This allows the model to have two classes of bacteria with respect to phage infectivity, phage sensitive B_s and phage resistant B_r. The dynamics can then be mechanistically described as follows:

- When phage are more numerous than the sensitive bacteria, the sensitive bacteria become resistant to the phage. The resistant bacteria do not change. Sensitive bacteria evolve to overcome the infectivity of the phage.

- When resistant bacteria are more numerous than the phage, the resistant bacteria become sensitive to the phage. The sensitive bacteria do not change. This is the phage evolving to overcome to resistance of the bacteria.

These mechanisms are able to link the genetics of the bacteria with the ecology of phage. Hyperbolic tangent functions can be used to describe the changes of resistance and sensitivity, because they can act as gating functions as they vary between 0 and 1. The ratio between the phage and sensitive bacteria will control the evolution of bacteria to become resistant, the function will be $\approx c$ when phage $\gg$ sensitive bacteria. The present resistant bacteria will be left unchanged. The ratio between resistant bacteria to phage will control the evolution of the phage to make the bacteria sensitive, the function will be $\approx c$ when resistant bacteria $\gg$ phage. The present sensitive bacteria will be left unchanged. It is assumed that phage resistance is binary: bacteria are either fully sensitive or fully resistant. c represents the rate of coevolution and is used to alter the strength of these coevolutionary changes. The last point that should be emphasised is that phage resistance in this model does not bear a fitness cost. There is a precedent for this in real phage-bacteria interactions. Although evolving phage resistance by chromosomal mutations is associated with fitness costs, there are scenarios where acquiring phage resistance is likely to generate no additional cost. For example, if all bacteria in the population have a functional CRISPR-Cas system, and phage resistance results from the acquisition of a new spacer targeting the phage then it has been shown that there is no additional fitness cost[185]. Overall these dynamics can be described by:

$$\frac{dB_{(TOT)s}}{dt} = -cB_{(TOT)s}\tanh\left(\frac{V}{B_{(TOT)s}}\right)+cB_{(TOT)r}\tanh\left(\frac{B_{(TOT)r}}{V}\right)$$

$$\frac{dB_{(TOT)r}}{dt} = cB_{(TOT)s}\tanh\left(\frac{V}{B_{(TOT)s}}\right)-cB_{(TOT)r}\tanh\left(\frac{B_{(TOT)r}}{V}\right)$$

4.4.2 Complete Model of Transduction

The individual dynamics describing facets of the model are combined to give a complete model which is shown in Equation Set 2. The model uses a system of ordinary differential equations and there there are assumptions inherent to that. The first assumption is that the system is deterministic and there is no stochasticity. It is assumed that the system is well mixed and there is no spatial heterogeneity in the system. Additionally, it is assumed that there is no cell death other than by antibiotic or bacteriophage.

The model uses bacterial and plasmid parameters from a previous study[135]. For the phage related parameters, experiments were conducted using LUZ19, which is the phage that performed the best in the transduction assay (Chapter 2) and was used for the co-evolution experiment (Chapter 3). Two experiments were conducted that measured the decay rate of the phage and the burst size of the phage. The decay rate of the phage was done by sequentially measuring the phage titre at different time intervals. The burst size was measured by using a one step growth curve which allows the measurement of both the burst size and the latent period of the phage. The methods and results of these experiments are in Appendix B.2 and B.3.

$$\frac{dA}{dt} = -\alpha A(B_s + B_r) - \alpha_p A(B_{ps} + B_{pr})$$

$$\frac{dR}{dt} = \Big(\frac{-\mu R}{K+R}\Big)(B_s + B_r) + \Big(\frac{-\mu_p R}{K_p+R}\Big)(B_{ps} + B_{pr})$$

$$\frac{dP}{dt} = zpyVB_{ps} - \gamma PB_s - dP$$

$$\frac{dV}{dt} = ypV(B_{ps} + B_s) - dV - zpyVB_{ps}$$

$$\frac{dB_{pr}}{dt} = (1-\sigma)\rho_p\Big(\frac{\mu_p R}{K_p+R}\Big)B_{pr} - \kappa_p AB_{pr} + cB_{ps}\tanh\Big(\frac{V}{B_{ps}+B_s}\Big) - cB_{pr}\tanh\Big(\frac{B_{pr}+B_r}{V}\Big)$$

$$\frac{dB_r}{dt} = \sigma\rho_p\Big(\frac{\mu_p R}{K_p+R}\Big)B_{pr} + \rho\Big(\frac{\mu R}{K+R}\Big)B_r - \kappa AB_r + cB_s\tanh\Big(\frac{V}{B_{ps}+B_s}\Big) - cB_r\tanh\Big(\frac{B_{pr}+B_r}{V}\Big)$$

$$\frac{dB_{ps}}{dt} = (1-\sigma)\rho_p\Big(\frac{\mu_p R}{K_p+R}\Big)B_{ps} - \kappa_p AB_{ps} - pVB_{ps} + \gamma PB_s - cB_{ps}\tanh\Big(\frac{V}{B_{ps}+B_s}\Big) + cB_{pr}\tanh\Big(\frac{B_{pr}+B_r}{V}\Big)$$

$$\frac{dB_s}{dt} = \sigma\rho_p\Big(\frac{\mu_p R}{K_p + R}\Big)B_{ps} + \rho\Big(\frac{\mu R}{K + R}\Big)B_s - \kappa A B_s - pV B_{ps} - \gamma P B_s - cB_s \tanh\Big(\frac{V}{B_{ps} + B_s}\Big)$$

$$+ cB_r \tanh\Big(\frac{B_{pr} + B_r}{V}\Big)$$

Equation Set 2: The Model of Transduction

Table 4.2: List of parameters and variables of the Transduction model

A	Concentration of antibiotic	
R	Concentration of environmental resource	
P	Population of plasmid bearing phage	
V	Population of lytic phage	
B_{pr}	Population of plasmid bearing phage resistant bacteria	
B_r	Population of plasmidless phage resistant bacteria	
B_{ps}	Population of plasmid bearing phage sensitive bacteria	
B_s	Population of plasmidless phage sensitive bacteria	
α	Antibiotic degradation rate without plasmid	10^{-7}
α_p	Antibiotic degradation rate with plasmid	10^{-8}
μ_B	Maximum resource consumption without plasmid	0.0826×10^{-8}
μ_{Bp}	Maximum resource consumption with plasmid	1.529×10^{-9}
K	Half velocity constant without plasmid	1.0822
K_p	Half velocity constant with plasmid	2.3547
ρ	Resource conversion rate without plasmid	1.2138×10^{9}
ρ_p	Resource conversion rate with plasmid	0.9573×10^{9}
κ	Antibiotic killing rate without plasmid	0.1
κ_p	Antibiotic killing rate with plasmid	18×10^{-4}
σ	Segregational loss rate	0.00484
d	Decay rate of phage (See Appendix B.2)	0.44
y	Burst size upon cell lysis (See Appendix B.3)	17
p	Rate of lysis by phage	
z	Probability of plasmid uptake by phage in a given cell lysis	
γ	Rate of uptake of plasmid by bacteria from phage	
c	Magnitude of coevolution	

4.4.3 Numerical Methods

The numerical simulations were built to mirror an actual serial transfer evolution experiment, this was done for two reasons. One, this avoids computational power

issues when the frequencies of different species reach high magnitudes. Secondly, this reflects the experimental protocol in Chapter 3, where bacteria was passaged over several days with and without phage present. These methods mean that many analytical methods such as bifurcation diagrams or stability analysis cannot be used to describe aspects of the system, and therefore only analysis through numerics can be used to examine the system.

The system is numerically solved in discrete 24h intervals. At the start of which the resources and antibiotics are replenished to their full initial values and the different species are sampled at 1% of their final value at the end of the last interval. These samples are used to seed the new interval along with the full antibiotic and resource concentrations. The numerical solutions for each different interval are solved for a total of 15 intervals, to represent an experiment running for 15 days. The numerical simulations within each interval were performed with MATLAB's Ordinary Differential Equation Solver *ode*45, which is a non-stiff solver. Due to the specific simulation regime employed here and the rates of change are all of similar magnitudes, a non-stiff solver is adequate. The initial conditions (the initial inoculum) is set as the following: $[P, V, B_{pr}, B_r, B_{ps}, B_s] = [0, ic0/10, 0, 0, ic0/2, ic0/2]$, which results in a multiplicity of infection (MOI) of 0.1. $ic0$ was set at 2×10^7 to represent typical bacterial population inoculums in an experiment.

4.4.4 Analysis of Model

To see the effect of bacteriophage driven horizontal transfer of an antibiotic resistance plasmid, there needs to be a metric by which one can compare the effectiveness of such transfer or lack thereof. The metric used here is time to extinction (TTE). It is defined as the time at which the frequency of all plasmid bearing bacteria (B_{ps} and B_{pr}) falls to below 1% of the total bacteria. If the plasmid bearing species persists until the end of the experiment, then $TTE = 360$ hours (15 days $\times$ 24 hours)

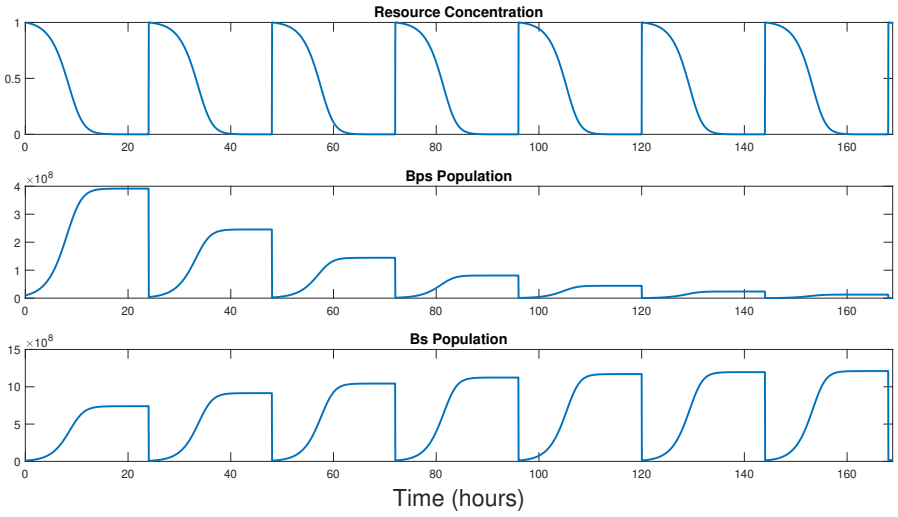


Figure 4.4: Simulation of the frequencies of the different bacterial species in the baseline case. All other species in the model are zero. Plasmid bearing bacteria are lost as a result of the fitness cost the plasmid carries

Baseline Case

To observe the effect that transduction and antibiotic selection has on the persistence of the plasmid, there needs to be a TTE that any selective pressure or gene transfer effect can be compared to. This can be considered the baseline case and will be referred back to throughout the results section. The baseline case is when the experiment runs without any antibiotics or lytic phage, and as a result there will be no cell death by phage or antibiotic action. As a result, there is no type of selection other than that which is operating on loss of the plasmid, and there will be no selection for the phage resistance. The frequencies of the different species varying with time are shown in Figure 4.4. This showcases the framework of the numerical methods, which is a serial transfer evolution experiment, with replenished resources at the start of every interval. In the absence of positive selection horizontal gene transfer, the fitness cost of the plasmid means that the rate of growth of the plasmidless bacteria exceeds the plasmid bearing variety. Therefore, over several transfers, the plasmid is lost. In the absence of any phage, there is also no selection for phage resistance, meaning the bacteria stay sensitive to the phage. The plateauing of the bacterial frequency at the end of an interval, is caused by the resource scarcity.

Figure 4.5 shows the proportion of bacteria that contain the plasmid through time. The percentage of plasmid bearing bacteria fall from 50% of the population to below 1% in just over eight days, equating to a $TTE = 168.8744$ hours.

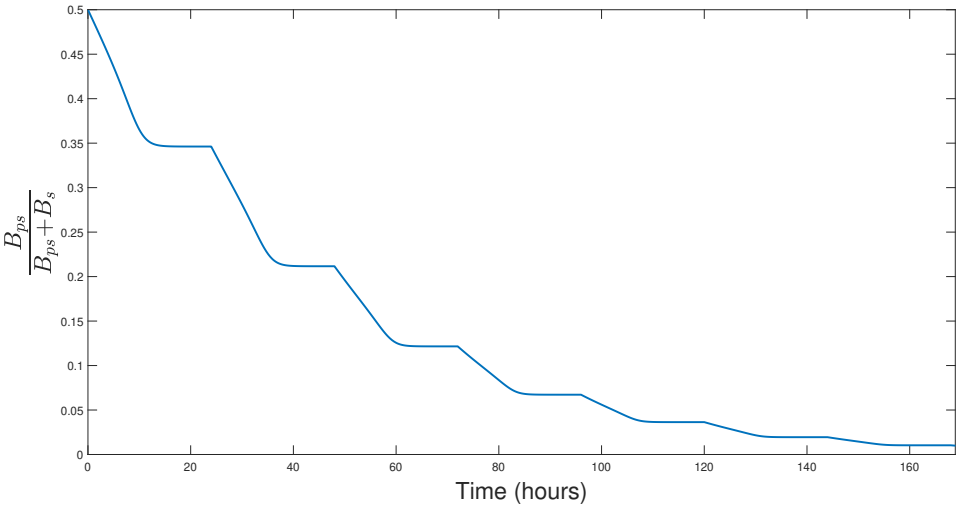


Figure 4.5: Simulation of the proportion of the total bacteria that contain the plasmid with time in the baseline case. Plasmid bearing bacteria fall to zero with time as a result of the fitness cost the plasmid carries.

Individual Parameter Sensitivity Analysis

Population biologists have long since stated that any changes to parameters driving selection pressure could influence population frequency[186]. A parameter sensitivity analysis allows us to examine the effect that changing different parameters have on the dynamics on the system[187]. By varying the parameters, I am testing changes in the biological characteristics and life history of the different members of the system. This allows us to see what kind of phage and bacteria will allow for the persistence of the plasmid.

Parameter Sensitivity: p

The parameter p determines the rate at which the phage infects the system. Mechanistically, this determines the rate of adsorption of the phage to the cell surface. Qualitatively, is gives an indication of "infectiousness" of the phage. When this rate is low, the phage will kill the sensitive bacteria slowly and this then

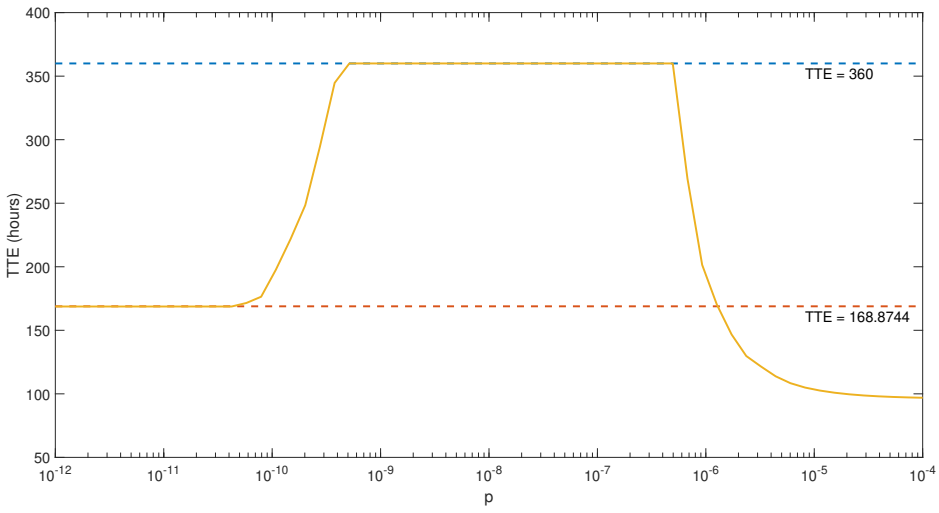


Figure 4.6: Parameter sensitivity analysis showing the change of TTE with p.

lowers the amount of plasmid bearing virions produced (which reduces the rate of transduction) as well. As seen in Figure 4.6, the TTE is virtually unchanged from the baseline case when the $p \leq 10^{-10}$. Essentially, because p is too small to cause any significant effect on the system, the infection spreads too slowly for the phage to multiply effectively and quickly die out. Coevolutionary changes in the bacteria do not occur because bacteria are not killed by the phage at a high enough rate and it is almost as if the phage were not even present in the system. Transduction becomes negligible and the plasmid is lost in the same way as the baseline case where the differences in fitness cause the plasmidless bacteria to thrive and the plasmid is lost over time. Then as p increases from 10^{-9} and until 10^{-6}, there appears to be a critical range in terms of plasmid persistence. This appears to be the range at which transduction are maximised. So much so that at $p = 10^{-8}$ the plasmid does not only manage to survive the experiment, but the transduction leads the plasmid bearing bacteria to dominate. This is shown in Figure 4.7. However, as the value of p increases further then the TTE decreases to well below the baseline case and causes the plasmid bearing bacteria fade out quickly. At $p > 10^{-5}$, the rate of infection is so high that it causes the death of bacteria very quickly. There is no chance for the resources to be used up and the growth of the bacteria stays consistently in exponential phase. This effect is compounded by the fact that as

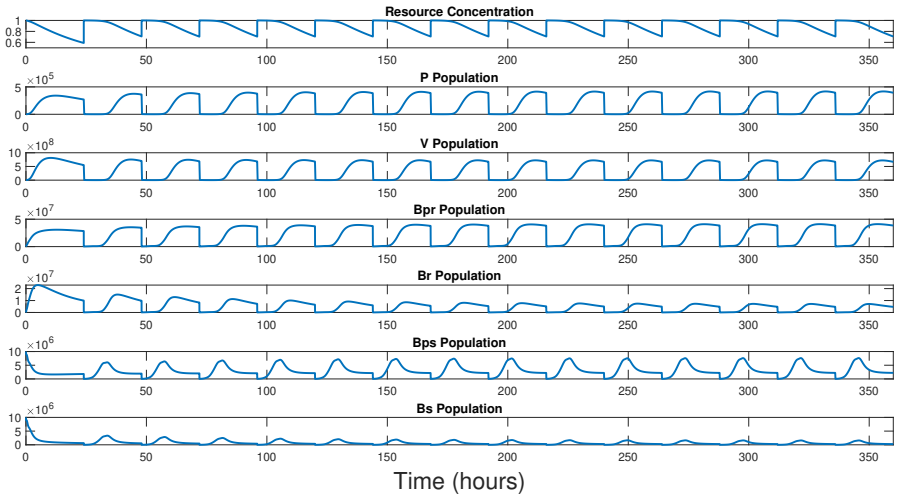


Figure 4.7: Simulation of the frequencies of the different bacterial species where $p = 10^{-8}$. All other species in the model are zero. The plasmid bearing bacteria dominates at these parameters.

soon as bacteria grow, they are likely to be killed off by the phage. This means that unlike where the bacteria reaches stationary phase at the end of an interval, the bacteria is constantly growing. Additionally, due to their fitness differences, the difference between the frequency of bacteria also grows exponentially. Hence, the frequency of the plasmid bearing bacteria quickly falls relative to the plasmidless bacteria. This results in a lower TTE. The TTE stays constant as p increases from this point. This is because there are diminishing returns to killing off the bacteria at this rate of infectiousness, and bacteria cannot be killed off any quicker.

Parameter Sensitivity: z

The phage imposes a selective pressure by keeping populations low through lytic infection. Essentially, without the phage the two competing strains of bacteria would use up the resources and enter stationary phase before being sampled for the next interval. Whereas when phage is present, the bacterial populations are kept low meaning that the resources are never used up. This causes the bacteria to continuously grow in exponential phase where the fitness cost of the plasmid causes the plasmidless variety to grow much quicker causing the plasmid bearing variety to fall in proportion. The only way that the plasmid can be maintained

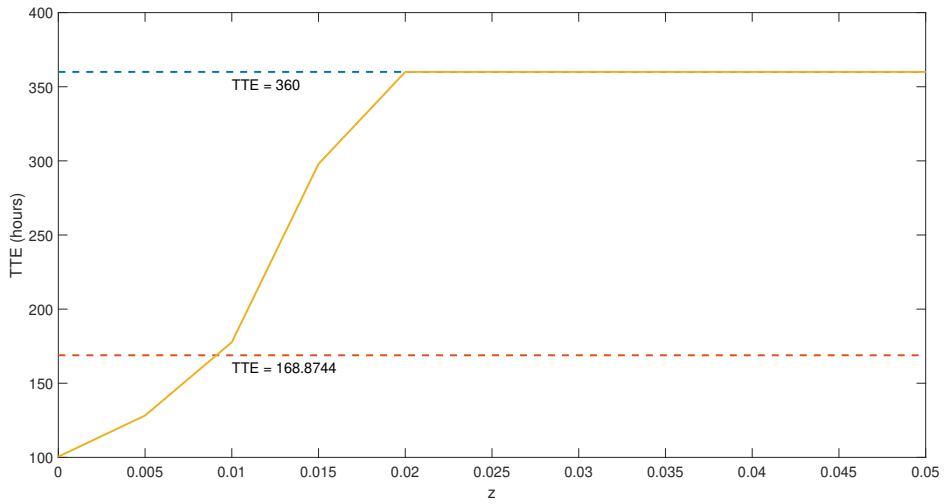


Figure 4.8: Parameter sensitivity analysis showing the change of TTE with z

at that point is through transduction. z determines the magnitude of possible transduction because z determines the probability that a plasmid is taken up by the phage. When the probability that the plasmid is taken up by the phage is too low ($z < 0.01$), the plasmid is quickly lost from the system. This is the region of parameter space where the TTE is lower than the baseline case. The temporal change of the different species when $z = 0.002$ is shown in Figure 4.9. While there is transduction present that maintains the plasmid, it is not picked up by the phage at a high enough rate. The lytic effects of the phage make it more difficult for the plasmid bearing species to multiply compared to when there is no phage.

However, when the probability that the phage picks up the plasmid increases then the effect of phage mediated transfer of the plasmid becomes important in driving the dynamics. The frequency of the plasmid bearing phage will increase with z, and this causes more plasmidless bacteria to become plasmid bearing bacteria. When z increases to 0.02 then it can cause the plasmid to survive the experiment ($TTE = 360$ hours). These dynamics are shown in Figure 4.10. When z goes up to 0.05, then the magnitude of transduction can drive the plasmid to fixation. These dynamics are shown in Figure 4.11. Even when the plasmid bearing bacteria reaches fixation at high levels of z, there are transient dynamics when the the plasmid bearing proportion drops slightly at the beginning of a new interval before

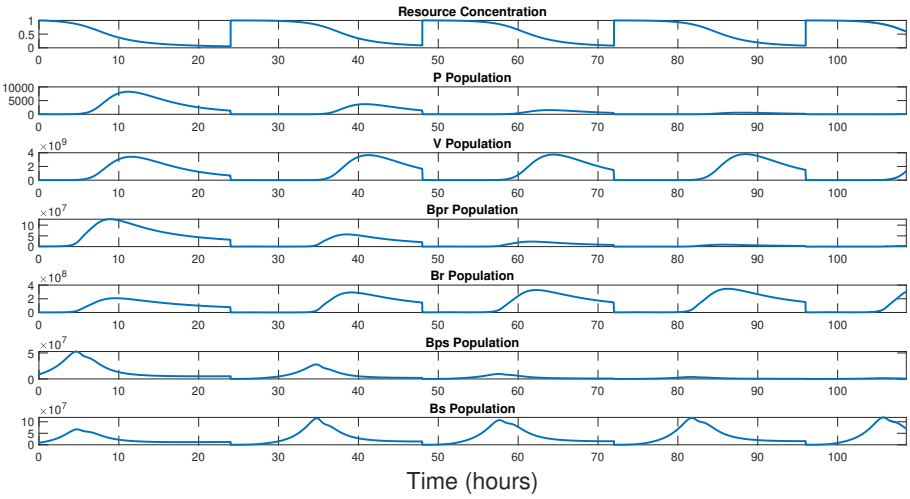


Figure 4.9: Simulation of the frequencies of the different bacterial species where $z = 0.002$. All other species in the model are zero. Transduction is too small to maintain the plasmid.

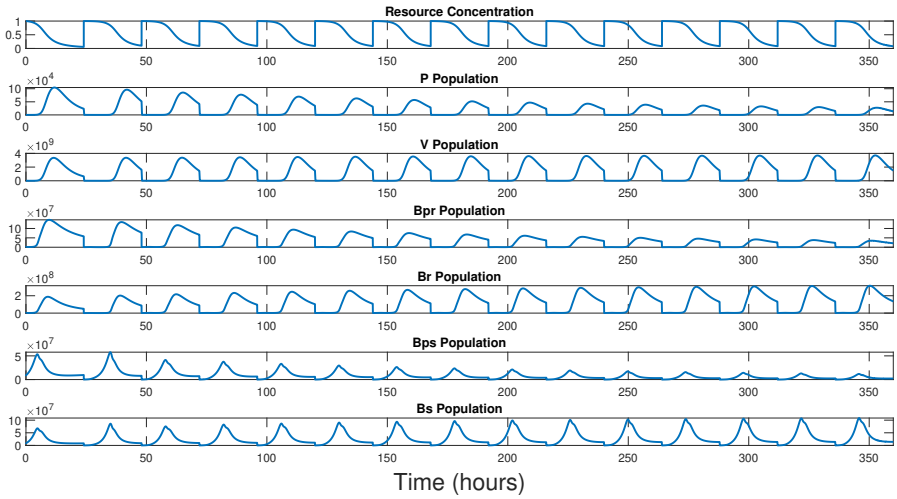


Figure 4.10: Simulation of the frequencies of the different bacterial species where $z = 0.02$. All other species in the model are zero. Transduction is large enough to allow the plasmid to survive the experiment

recovering to 100%. This occurs as at the start of a new interval the resources are replenished and the plasmidless bacteria are able outcompete the plasmid bearing ones, and due to the sampling, only a small portion plasmid bearing phage remain and amount of plasmid transfer is significantly reduced. Once the system has to grow within the interval, the plasmid bearing phage grows and the horizontal transfer of plasmid restores the plasmid bearing bacteria to fixation.

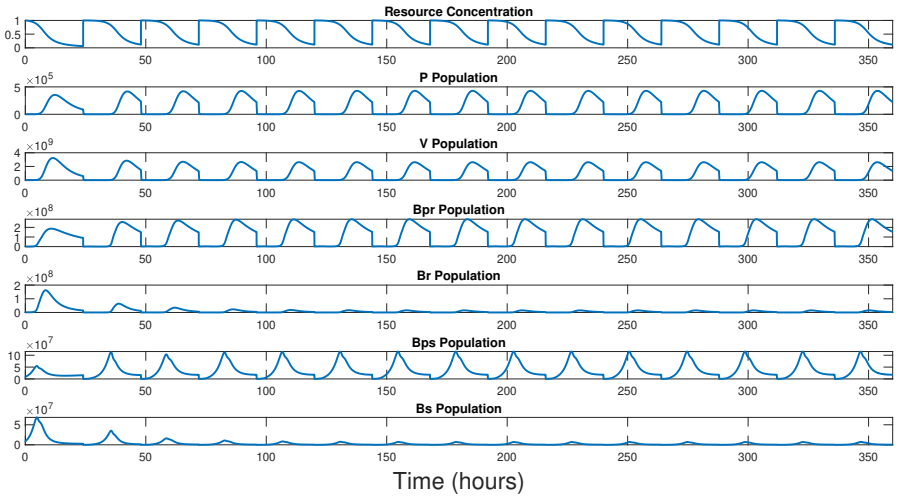


Figure 4.11: Simulation of the frequencies of the different bacterial species where $z = 0.05$. All other species in the model are zero. Transduction is so extensive that the plasmid bearing species are driven to fixation.

Parameter Sensitivity: c

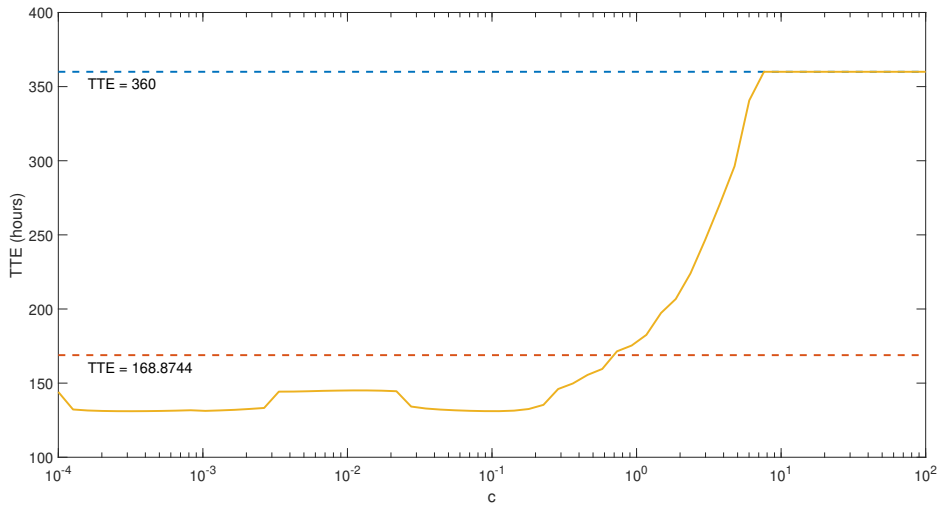


Figure 4.12: Parameter sensitivity analysis showing the change of TTE with c

c is the coevolution parameter and mediates the strength of coevolution, in effect how quickly the different bacteria switch between phage sensitivity or resistance. It has been observed in different systems that substantial evolution can happen over short time span[188].

The effect of the evolution of resistance is to provide a refuge for the plasmid,

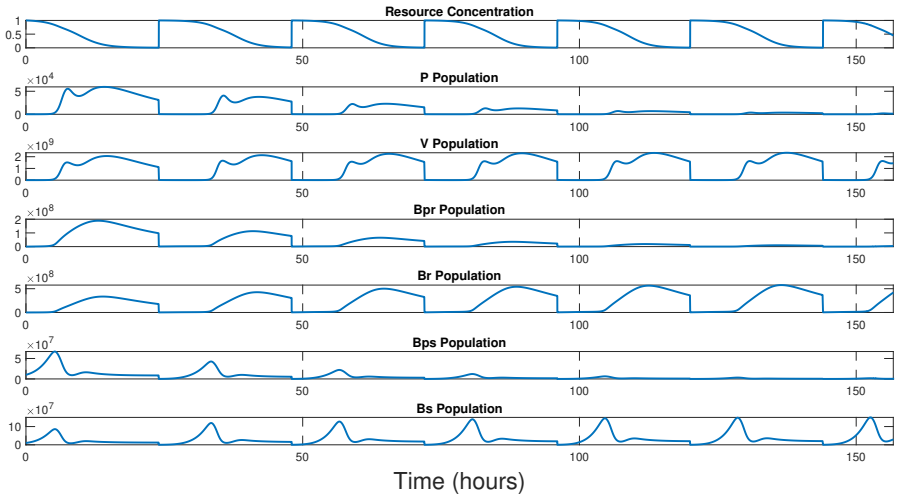


Figure 4.13: Simulation of the frequencies of the different bacterial species where $c = 0.5$. All other species in the model are zero. Coevolution is too small to maintain the plasmid.

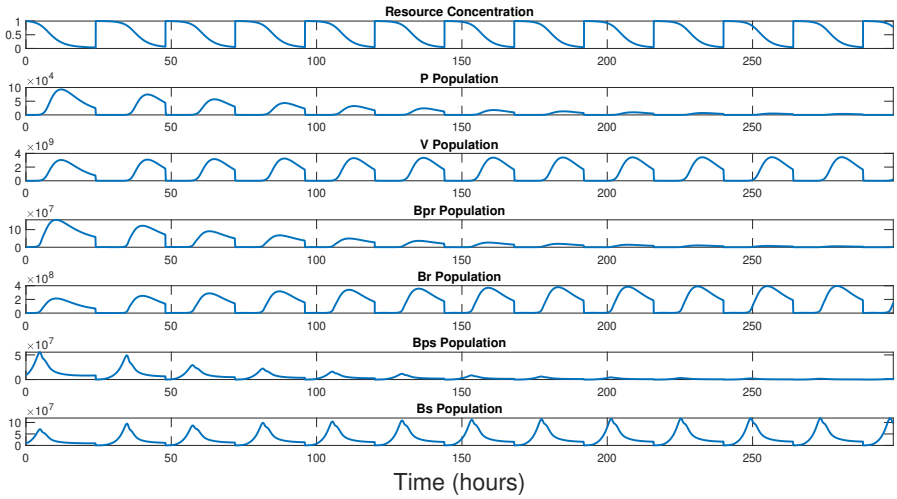


Figure 4.14: Simulation of the frequencies of the different bacterial species where $c = 5$. All other species in the model are zero. Coevolution is high enough to allow the plasmid to last longer than the baseline case.

where it can escape predation by phage. Refuges are traditionally thought of as any strategy that reduces the risk of predation for prey[189], and are common in ecology as ways to prevent prey extinction and ensure coexistence[190]. Refuges also occur in host parasite interactions, where there can be variation in the phenotypes of hosts and parasites. This can lead to the ability of parasitoids to parasitize hosts, and of hosts to escape parasitism[191]. This is presented in the model by

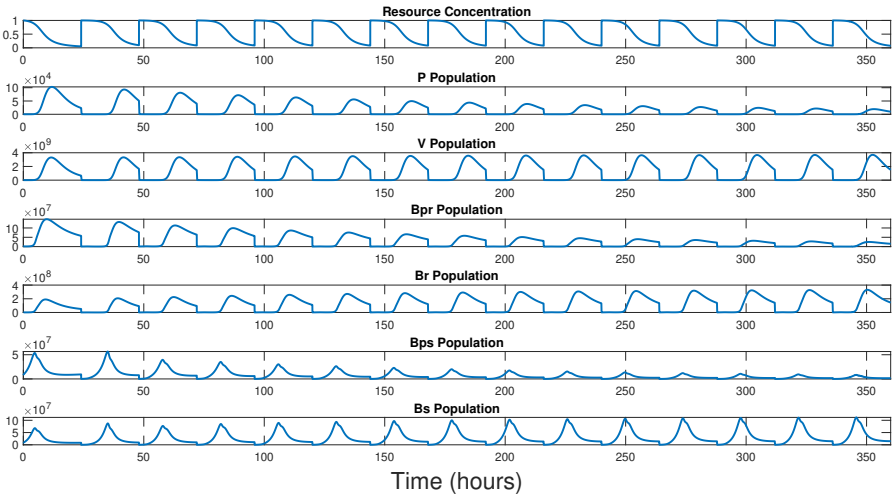


Figure 4.15: Simulation of the frequencies of the different bacterial species where $c = 50$. All other species in the model are zero. Coevolution is large enough to allow the plasmid to persist.

the presence of phage resistant bacteria B_r and B_{pr}, where the bacteria evades infection by phage through adaptations to the ensure that phages cannot infect them. This is known as an absolute refuge where the bacterial host is completely resistant to the parasitic phage, regardless of phage frequency[191]. The effect of bacterial resistance provides a refuge for those bacteria. But it also provides a refuge for the plasmid. When the plasmid is kept in the phage resistant bacteria, it is kept free of predation and therefore protected from loss. Then phage resistance becomes widespread, the plasmid is protected until the phage can evolve to infect the bacteria again, and the plasmid can spread through transduction. If coevolution was not a feature of the system (such as $c = 0$), the phage would continually kill the bacteria without resistance emerging and the fitness cost of the plasmid would mean it is quickly lost. The adaptation to phage resistance allows the bacteria to protect the plasmid against loss. The variation of TTE against the value of c can be seen in Figure 4.12. At low rates of coevolution, the TTE is less than the baseline case. This is because the phage is creating a selection pressure by reducing donors and receptors without transferring the plasmid at a high enough rate. Bacteria are not able to evade this selection pressure by becoming resistant because of the low magnitude of c. This is why the TTE is less than the baseline

case where there is no phage present. These dynamics are shown in Figure 4.13. As c increases (from 10^{-1} to 10^{1}), the TTE increases as it grows to more than the value for phageless system at approximately $c = 1$. These dynamics are shown in Figure 4.14. This increase in c results in only small increases in TTE until it levels out. This is because at approximately $c = 10$, the maximum amount of bacteria that can be made resistant by coevolution has been reached and the refuge effect can no longer increased. At this point, the limits of coevolution have been reached and the TTE can no longer increased. These dynamics are shown in Figure 4.15.

Parameter Sensitivity: γ

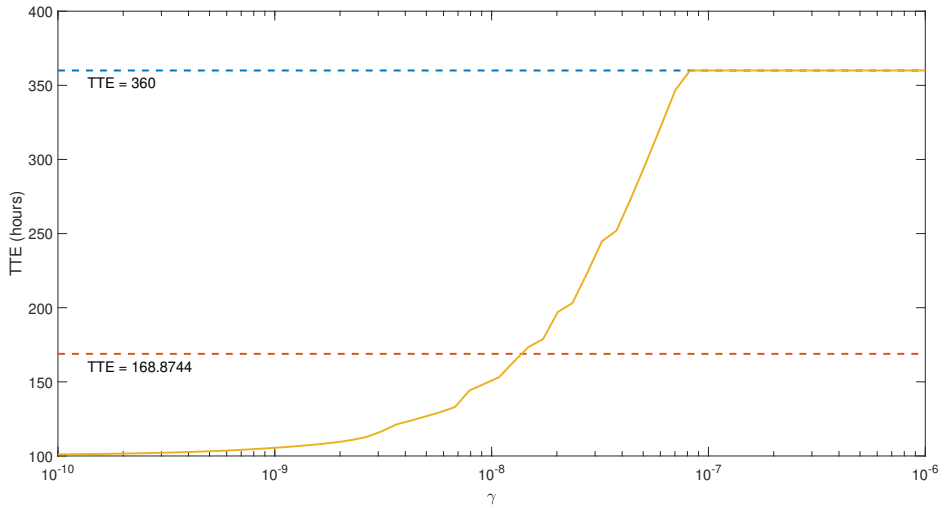


Figure 4.16: Parameter sensitivity analysis showing the change of TTE with γ

γ is the rate of transfer of the plasmid from plasmid bearing phage to phage sensitive plasmidless bacteria, turning them into phage sensitive plasmid bearing bacteria. More simply, this can be interpreted as the rate of horizontal gene transfer. It is the parameter that controls how quickly the plasmidless bacteria turns to plasmid bearing bacteria through transduction. It is the other parameter along with z and p, that controls the transduction process. Whereas z and p control the rate at which plasmid bearing phage are produced, γ controls the rate at which plasmid bearing phage are used up. The variation of this parameter and the resulting TTE is shown in Figure 4.16. As shown, there is large variability in the parameter range.

This happens mechanistically because when the value of γ is too low, the it there is a build up of plasmid bearing phage but that is not used to infect plasmidless bacteria, resulting in a low transfer of the plasmid and the plasmid bearing bacteria quickly being outcompeted by the plasmidless variety. However, as the magnitude of γ increases then the TTE increases as well until the TTE becomes more than the baseline phageless system. However, after this it reaches the point of diminishing returns, this is because at the current rate of z there is only a certain amount of plasmid bearing phage that are produced and after a certain point all of those phage are immediately used up to infect the plasmidless bacteria.

Parameter Sensitivity Heat map

For the previous analyses, the parameter sensitivity has been done in a manner to keep all other variable parameters at a single value and then varying the parameter of interest. However, the parameters are related and there are interactions between the parameters that can lead to different persistence effects. To observe the effects of different parameter combinations on plasmid persistence, a sweep over multiple dimensions in parameter space has been conducted. These parameter sweeps have been conducted to provide a heat map showing the different TTE at different combinations of parameters. The parameters that differentiate phage species (and to see the varieties of phage that are able to transduce the plasmid) are p and z.

As shown in Figure 4.17 there is parameter space where all manner of TTE exist. p and z are both phage related parameters and this shows the phage with the critical range of parameters that could allow plasmid persistence. Furthermore, dependent on the phage then there are different dynamics relating phage persistence. At low values of p, the TTE is no different than the baseline case, regardless of the value of z. This is because p is so low that it is like there is no phage in the system. So even if the plasmid is picked up by the phage at a high enough rate, it cannot contribute to plasmid persistence. At intermediate values of p ($10^{-10} < p < 10^{-6}$), there is a variation between TTE lower and higher than the baseline case. At low z, the lytic effects dominate and this results in a TTE lower than the baseline. However, as the

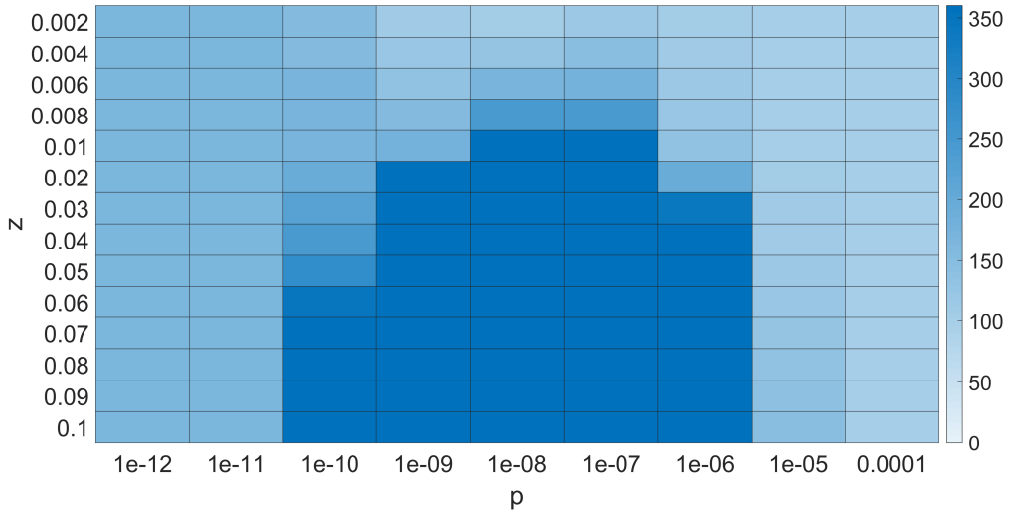


Figure 4.17: Heatmap of parameter sensitivity showing the variation of TTE with p and z. Colour bar ranges from 0 to 360 hours. There is only a certain region of parameter space that will allow plasmid persistence.

value of z increases the plasmid is picked up more and more and the transductive effects of the phage lead to TTE higher than the baseline. If the plasmid is picked up at a high enough rate ($z > 0.02$) then the plasmid can persist in the system. When p is high ($p > 10^{-5}$), this is a region of parameter space where the phage is so infectious that it kills all the bacteria and the plasmid with it. For all values of z, the transductive effects are ineffective to maintain the plasmid in the system.

Effect of Low Dose Antibiotics

Examples of low dosing antibiotics affecting bacterial communities, whether intentional or not, have been observed in several different contexts[192]. Antibiotics are used for enhancing livestock growth in the farming industry[193] leading to runoff in the environment. A major effect of this is the expansion of the resistome[194]. The effects of antibiotics in the environment are more dramatic in aquatic environments because the presence of antibiotics can cause the aggregation of bacteria as a method to induce survival. This could possibly lead to the transfer of resistance genes through horizontal gene transfer, such as by bacteriophage.

The existence of the antibiotic resistance plasmid in this system means that in the presence of antibiotic, there will be selection for the plasmid. The plasmid can

reach fixation in such conditions. However, at smaller concentrations the dual effect of the transduction and low magnitude selection can help to maintain the plasmid.

There is an increasing body of research that suggests that that using phage therapy in tandem with antibiotics could be more effective at controlling an infection than either alone[36]. The idea stems from evolutionary theory which dictates that two different selective pressures are going to be more effective than either alone. The model allows us to to test the predictions of the persistence of the plasmid bearing species in presence of antibiotics and phage.

Under strong selection by antibiotics the plasmid will go to fixation. However, under no or weak selection by antibiotics the plasmid will be lost. The effect of low dosing antibiotics together with transduction on the persistence of the plasmid can be shown in change in in the parameter sensitivity heatmap shown in Figure 4.18. The heatmap shows that the conditions on the phage related parameters become less stringent to cause plasmid persistence as it is already under selection by the antibiotic.

At low rates of p ($p < 10^{-10}$) there is still no plasmid persistence, but TTE is slightly higher than the baseline case. This is because of the presence of the low dose antibiotic creates enough of a selection pressure that the plasmid bearing bacteria lasts longer through the experiment. At intermediate values of p ($10^{-10} < p < 10^{-6}$), the low dose antibiotics create enough of a selection pressure that the value of z required for the plasmid to survive the experiment is far lower. Essentially, the magnitude of transduction needed to maintain the plasmid becomes lower. At these values of p, there are dual synergistic forces helping to maintaining the plasmid, the antibiotic selection and transduction. At high values of p ($p > 10^{-6}$), where there is a 'deadly' phage that kills off the bacterial population and amplifies the fitness cost because the bacteria is constantly in exponential phase, the presence of low dose antibiotics becomes key. As the presence of low dose antibiotics nullifies the fitness advantage of the plasmidless bacteria and allows the plasmid bearing bacteria to outcompete it. The plasmid bearing bacteria survive the experiment at all values of z, meaning transduction is not even needed to maintain the plasmid.

This means that at high values of p, transduction does not play a large part but the lytic effects of the phage dominate and amplify the fitness differences between the bacteria. Where there are no antibiotics, the plasmidless strain easily wins and the plasmid bearing variety dies out quickly. In the presence of antibiotics, those fitness differences are switched and the plasmid bearing variety wins.

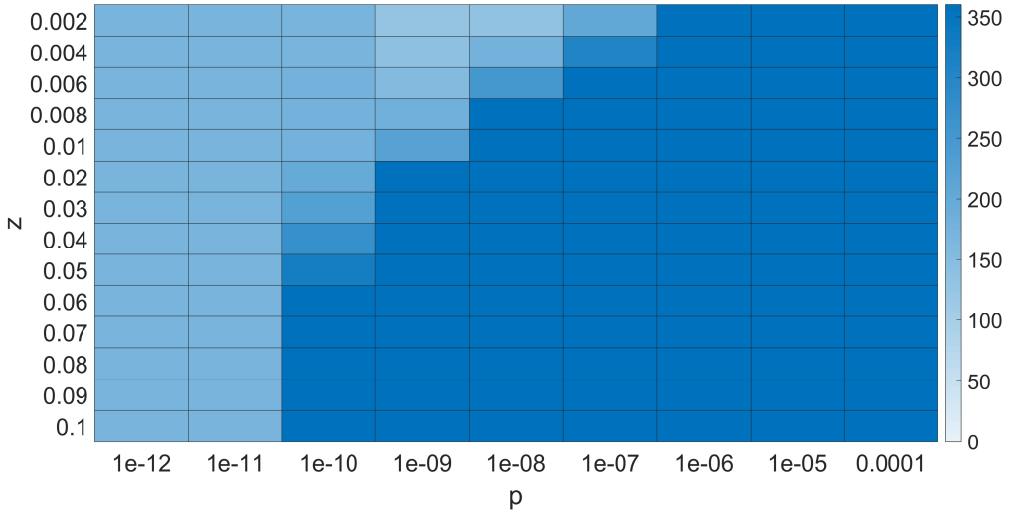


Figure 4.18: Heatmap of parameter sensitivity showing the variation of TTE with p and z when there is $\frac{1}{8}^{th}$ MIC of WT antibiotic. Colour bar ranges from 0 to 360 hours. The conditions on transduction are less severe than without the antibiotic.

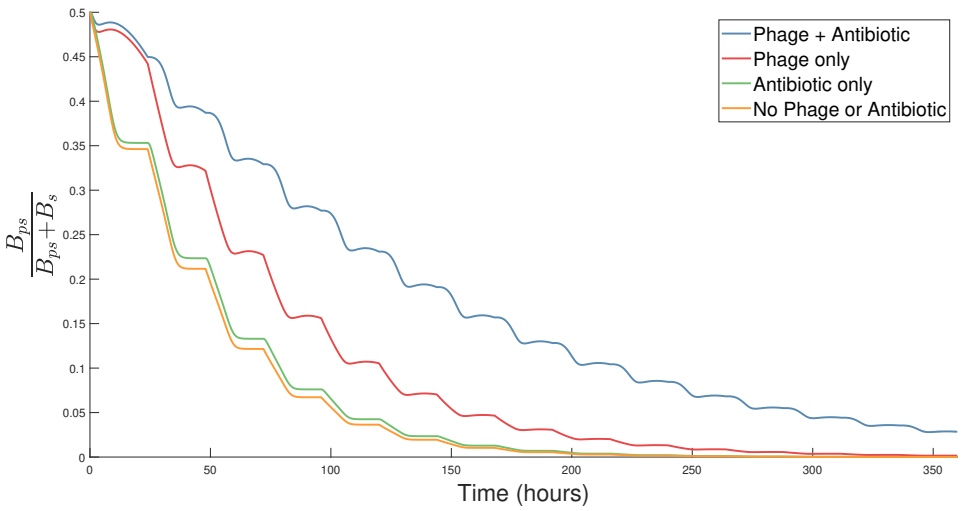


Figure 4.19: Proportion of Plasmid bearing bacteria with and without low dose antibiotics and phage. The antibiotic is equal to $\frac{1}{8}^{th}$ MIC of the WT. The phage parameters are $p = 10^{-8}$ and $z = 0.002$.

The synergistic effect of the horizontal gene transfer coupled with the positive selection by antibiotic can be further seen in Figure 4.19. This graph shows how the proportion of plasmid bearing bacteria changes with time throughout the length of the model experiment. The plasmid bearing species survives (due to the presence of phage and antibiotic) but the proportion is still falling with time. There are four different scenarios presented: one, where there is both low dose antibiotics and phage. Second, where there is the same phage but no antibiotic. Third, where there is low dose antibiotic and no phage. Fourth, where neither are present. The effect of the antibiotic and phage depends on the values of other species throughout the length on the 24 hour transfer period. Initially, the low dose antibiotic and phage work together to actually increase the proportion of the bacteria that contain the plasmid. However, the increasing numbers of plasmid bearing bacteria quickly cause the low dose antibiotic run out and the phage is left to maintain the plasmid. The difference in fitness between the strains allows the plasmidless bacteria to outcompete the plasmid bearing bacteria until the resources run out, which forces the proportion of plasmid bearing bacteria to fall. As soon as the resources run out, the proportion of plasmid bearing bacteria increases due to horizontal gene transfer. This cycle repeats when the resources and antibiotic are replenished. This cumulative effect between the phage and antibiotic in maintaining the plasmid is notable because the increase in TTE is more significant than the simple addition of the two separate increases in TTE from the two individually. This shows how the two work together to maintain the plasmid even when both are not strong enough to maintain it on their own individually.

4.5 Agent based model of initial phage infection

One of the short comings of the ordinary differential equation model of phage mediated horizontal gene transfer is that it models the steady state dynamics after the initial transient dynamics have died out. In doing that, it cannot actually model some of the the biological phenomena occurring, such as the latent period of phage infection. Furthermore, the ordinary differential equation approach assumes that

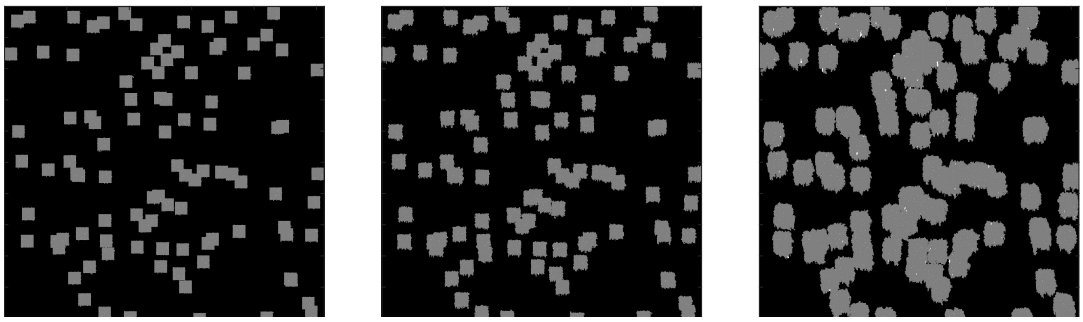


Figure 4.20: Temporal evolution of agent based model with a phage that has a low burst size and high latent period. The grey squares are plasmidless bacteria and the white are plasmid-bearing bacteria. The plasmid bearing bacteria are maintained.

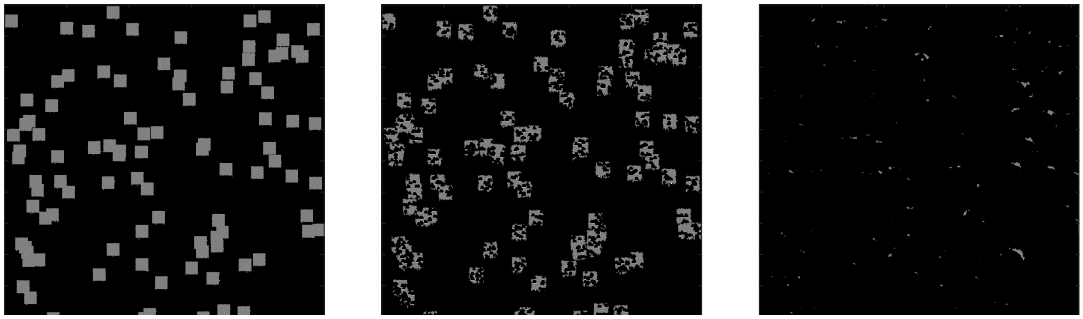


Figure 4.21: Temporal evolution of agent based model with a phage that has a high burst size and low latent period. The grey squares are plasmidless bacteria and the white squares are plasmid-bearing bacteria. The plasmid bearing bacteria are not maintained.

the system is well mixed and that there is no spatial variation. This, again, does not mirror reality. Horizontal gene transfer is an inherently spatially-dependent process and antibiotics can only act within their local spatial environment. To overcome these shortcomings, a rule-based cellular automata modelling approach is developed here, where spatial heterogeneity is explicitly defined. It is important to state that this model is not meant to supercede the model of transduction, but rather to supplement some of the limitations of it.

A cellular automata approach allows testing of the hypothesis that a less infective phage will be a better transducer. Phage infectivity is parameterized by two factors; burst size and latent period. A more infective phage will have a higher burst size and shorter latent period. It is hypothesised that a more infective phage with the same affinity for the plasmid will have a transfer the plasmid a similar amount than a phage with a lower burst size and higher latent period but the effective rate

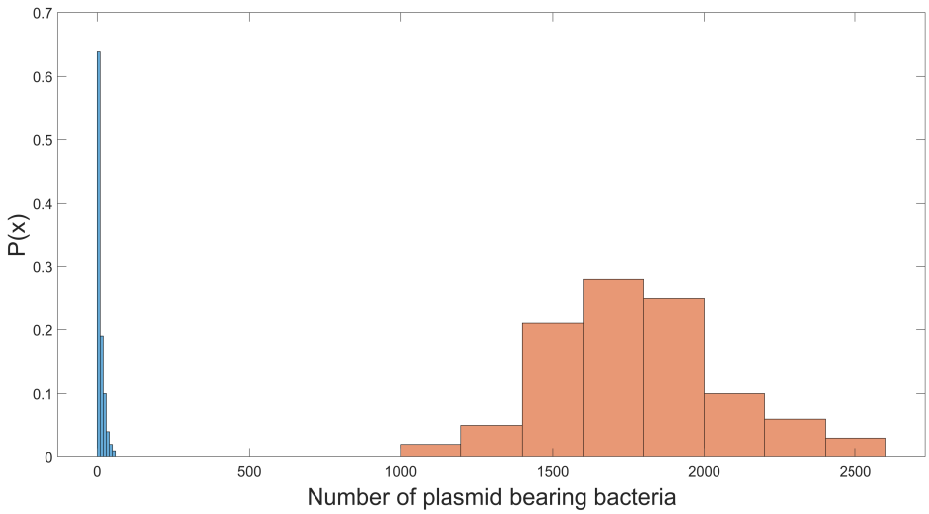


Figure 4.22: Probability histogram of numbers of resistant bacteria after 50 timesteps. The orange indicates a phage that has low burst size and high latent period. There is a relatively high number of resistant bacteria. The blue indicates a phage that has high burst size and low latent period. There is a relatively low number of resistant bacteria.

of horizontal gene transfer is lower. This is because with a more infective phage, the bacteria that receive the plasmid are more likely to be lysed themselves by the more infective phage. Moreover, the more infectious phage will kill of donors and receptors further reducing the potential for horizontal gene transfer

As this is a rule-based model, the rules under which this simulation will run must be defined. These rules will be chosen in such a way as to make them as close a mirror to the ones that actually govern bacterial ecology.

- Cell Growth and Death with associated fitness
- Horizontal Gene Transfer
- Lysis by phage
- Segregational Loss
- Selection via Antibiotics

The grid is a 1024×1024 square resulting in a maximum bacterial population of 1048576. The edges are treated as hard stops where there are no possible interactions beyond the edge. This was done as to mimic the edge conditions of a petri dish.

The temporal evolution of the system is iterated using the Gillespie algorithm[195]. The algorithm is used to simulate stochastic processes with computational efficiency in a way that is statistically correct. Using an initial spatial distribution of cells I can iterate through time with our set of predefined rules that operate stochastically see how bacterial cells grow and interact with each other as individuals within a population.

Stochasticity is an important feature of this model, because this added complexity can be a critical factor in biology. Bacterial ecology is not deterministic and even slight perturbations due to noise in the system can lead to vastly different long term outcomes. o model the inherent uncertainty of this system, each event is modelled as a poisson process with an associated rate of occurrence and the probability of that event is then $\frac{1}{rate}$. Poisson processes have mathematical properties such as stochastic independence mean that they are used frequently to model random events in biology [196].

What happens in practice is that the simulation is started with an initial distribution of cells and antibiotic. In the next time step a random number between 0 and 1 is generated and if that number is less than the probability of a certain ecological event happening, then that event (such as cell growth) will occur. The model will react dynamically to the changing landscape. For example, antibiotic usage will change depending on whether the cell that is on its space contains the plasmid or not. If the model is iterated through, a spatial description of what happens to the system with time is obtained.

However, the limitations of this model must also be acknowledged and emphasised. Because this model does not rely on actual biologically derived parameters, the extent to which it can produce biologically realistic results is limited. The parameters that are used here are arbitrary and are in fact only relevant, in magnitude, in relation to the other parameters used in the model. Therefore, this model cannot be used to produce granular data as such needed for a parameter sensitivity analysis. Rather, the objective of this model is to test a hypothesis about two phages that

have widely differing parameters and for that this model is appropriate because it is able to evaluate them accurately due to the wide gap between the parameters.

4.5.1 Analysis of Model

One of the insights generated by the one step growth curve experiment that was used to parameterize the ODE model is that the phage that performed best in the transduction assay is one with a relatively long latent period and a low burst size[197]. This raises the question of whether these properties are what helps this phage be effective at transducing the plasmid. The cellular automata model can be used to test the hypothesis that a lower burst size and latent period will result in a greater effective rate of transduction. The model is run similar to the transduction assay conducted experimentally in Chapter 1. There is a population of bacteria without the plasmid. Then a population of phage at a tenth of the magnitude of the bacteria (MOI = 0.1) is added. The population of phage will have some proportion of it containing the plasmid. The rule-based dynamics are then allowed to run and iterated through. Figures 4.20 and 4.21 shows how the model changes with time for two different phages, one that has high burst size and low latent period; and one that has a low burst size and high latent period. As the model is stochastic in nature, there will often be a distribution of possible end results when the simulation is run several times. The probability histogram of the two different phages is shown in Figure 4.22. These show that for there are significantly more resistant bacteria ($p < 0.001$) in the phage with a low burst size and high latent period. This is an interesting result because this underlines the importance of phage life histories in determining transduction. Naively, it would appear that the only feature of a phage that is important in determining transduction is the affinity that the phage has for the plasmid (in the transduction model, this is represented by z). However, this shows that ecology matters as well. When the phage has a low burst size and high latent period, this means that the phage infection will spread slowly and not killing off the bacteria. This is similar to having a p from the transduction model that is low.

4.6 Discussion

The basic model of horizontal gene transfer uses an invasion from rare analysis to look at how plasmids may persist in the environment. It finds an inequality that shows how horizontal gene transfer of any variety is crucial to the persistence of a costly plasmid. This is similar to the result of a previous population dynamics model that looked at conjugative plasmid existence conditions[112]. In that model, they find a similar inequality condition. Their inequality states that for a plasmid to persist, the cell density and conjugation rate must be sufficiently large to overcome segregational loss and the cost of the plasmid. The basic model of horizontal gene transfer is in agreement with this idea, except for that the model presented here takes into account the benefit of the plasmid conferred in the presence of antibiotics and describes horizontal gene transfer without referring to a specific form. This allows the model to incorporate how positive selection can help maintain the plasmid in tandem with horizontal gene transfer.

The transduction model looks at how the different parameters of phage and the strength of the coevolutionary dynamics may shape the persistence of the plasmid. They show that a combination of parameters is required for the plasmid to be maintained. Although, there is also a combination of parameters that allow the plasmid to be lost more quickly than if the phage was not present. In a previous study[111], the effect of phage on the persistence of a conjugative mercury resistance plasmid was observed. They found that the presence of phage reduced the existence conditions for the plasmid, whereby the phages drove quicker extinction of the plasmid in conditions without positive selection. However, the model presents a plasmid that is non-mobilizable. The distinction here is important because in the transduction model the plasmid may move horizontally using phage. Whereas in the model examining conjugative plasmids, phage only exist to suppress bacterial populations without moving the plasmids horizontally. Nevertheless, that being said, there are areas of parameter space in the transduction model where the same conclusions are reached as the model containing the conjugative plasmid. The transduction model shows that if you have a phage that is relatively very infectious

(large p) and not able to pick up the plasmid at a high rate (low z), then the plasmid will go to extinction more quickly than if the phage was not there at all, which is in agreement with the model with the conjugative plasmid. There are two mechanisms at work here. One, where the infectious phage will be imposing a selective pressure and will be reducing the amount of donors and receptors where the plasmid may be transmitted. Two, where the phage cannot pick up the plasmid at a high enough rate to be able to transmit the plasmid horizontally (which helps maintain it). However, if the phage is less infectious and is able to pick up the plasmid at a high enough rate, then the phage will be able to help the plasmid persist. Therefore, it would also be interesting to observe the impact that a less infectious phage may have on the persistence of a conjugative plasmid, because the model predicts that there should be a less of a limit on the existence conditions.

The transduction model showed how low dose antibiotics could maintain the plasmid in tandem with transduction. The same study that looked at the persistence of a conjugative mercury resistance plasmid in the presence of phage[111], tested how the plasmid prevalence changes over time with and without the presence of mercury. The treatment containing mercury with the phage was able to maintain the plasmid. In contrast, the treatment without mercury but still containing phage; the plasmid died out. This was because (in the presence of mercury) the mercury resistance plasmid bearing species was able to outcompete the plasmidless strain due to positive selection and evade the selective pressure of the phage. This is similar to the results shown in the modelling, that in the presence of positive selection of antibiotic, the fitness advantage conferred by the plasmid allows it to survive longer.

The agent based model further explores this idea of using phages with different infectiousness and their effect on the existence conditions for plasmids, but in a spatial environment. The infectiousness of a phage is defined here in terms of latent period and burst size, with a lower burst size and higher latent period being a lower infectious phage and vice versa. The model further supports the idea that because the less infectious phage is able to spread the plasmid more widely because it is not killing off donor and receptor cells and thereby reducing the capacity for

Table 4.3: Latent Periods and Burst Sizes of lytic phages that were used for the transduction assay for which there is data available

Phage	Latent Period (min)	Burst Size (phage/cell)
LUZ19	80-100	17
PEV2[155]	25 - 30	100-150
ΦKZ[166]	30	100
LMA2[167]	35	65

horizontal gene transfer to occur. Therefore, the agent based model is in agreement with the previous study looking at how phage reduce the existence conditions for plasmids[111], but also provides an insight in when that may not be the case and phages can allow plasmids to exist through horizontal gene transfer. If a phage has a high enough latent period and low enough burst size, it may change the results and actually help plasmids to persist. There is also some experimental evidence showing that this may the case, where previous experiments looked at how different phages transduced the plasmid pNUK73. Further details of this may be found in Chapter 2, but it is worth nothing that the phage LUZ19 performed the best. Experiments were performed in order to measure the latent period and burst size of LUZ19. The life history data for LUZ19 (the best performing phage in the transduction assay) was compared to the latent period and burst size of other phages in the trial for which there is published data.

Table 4.3 shows the comparison between phages and there is an apparent discrepancy between three phages that did not perform well and LUZ19 which did. This is not at all complete picture, but perhaps stands as a starting point in investigating this relationship further. It is also worth stating that life history will not be the only deciding factor in determining transduction, as the phage's affinity for picking up the plasmid will also play a large role.

4.7 Conclusion

One of the most important routes for bacteria to evolve an antibiotic resistant phenotype is by acquiring resistance genes horizontally. However, it is unclear as to how these mobile elements such as plasmids can persist in the environment in

the long term. The modelling approaches presented in this chapter provide insights as to how this may happen. The basic model of horizontal gene transfer shows that general horizontal gene transfer is able to maintain the plasmid under certain conditions. If the rate of horizontal gene transfer is at a high enough rate, such that it is able to overcome the fitness cost of the plasmid and that the plasmid confers enough of a benefit in the presence of antibiotic, then the plasmid is maintained. The model of transduction produces many different insights. There is a critical range of infectiousness of phage where horizontal transfer is enough to maintain the plasmid. Below this range, the phage is not infectious enough to move the plasmid. Above this range, the phage is too infectious and all bacteria are kills too quickly. Coevolution is found to be a critical feature in maintaining the plasmid, because it allows the plasmid to be protected from predation. Low dose antibiotics are able to impose the selective advantage to the plasmid bearing species and this expands the parameter range by which transduction can maintain the plasmid. Lastly, the agent based model was used and insight from the experimental work to test the hypothesis that a phage that has a long latent period and a low burst size is better for transduction. This stochastic model showed that there was significantly more transfer of the plasmid with this kind of phage.

5 Conclusion

5.1 General Conclusions

The main objective of my thesis was to explore the role that bacteriophage could play in maintaining a small non-conjugative resistance plasmid through transduction; using both experimental and theoretical approaches. I use the plasmid pNUK73 (that confers resistance to the antibiotic neomycin) and the bacteria *Pseudomonas aeruginosa* PA01 as my model system. Bacteriophages represent the most abundant biological entity on the planet, so transduction could play a considerable role in the dissemination of resistance genes. Moreover, several studies of of phageomes around the world have shown the presence of antibiotic resistance genes. Therefore, bacteriophages could represent a sizable reservoir of resistance genes that could be moved to bacteria.

In Chapter 2, I start by examining how different types of phage could be used to transfer the plasmid between bacteria. I look first at temperate phage, which exist in the genome of the bacteria. I induce expression of the chromosomal prophage in plasmid bearing bacteria using different concentrations of antibiotic. This temperate phage was then isolated and used to infect bacteria without the plasmid. Then those bacteria were tested to see if there were any gains in resistance. The temperate phage were not able to increase the resistance, but they were found

to have packaged up the plasmid in their capsid. I then use lytic phages and use them to infect plasmid bearing bacteria. The phage were then isolated after the infection, and then used to infect plasmidless bacteria. Three phages were found to be very effective at increasing resistance in bacteria as compared to the control. These three phages were found to be transferring the plasmid at higher rates of transduction than other previously reported phages.

In Chapter 3, I look at whether the plasmid could be maintained over a longer period of time through transduction; and how coevolution may affect the plasmid dynamics. I use bacteria with a stable costless chromosomal resistance mutation to rifampicin in tandem with bacteria containing the plasmid. I use the lytic phage that performed the best in the transduction assay (LUZ19) to see whether it is able to transfer the plasmid to the other strain of bacteria, and to what extent it can maintain the plasmid over four days. I use sequencing techniques to show that the plasmid has transferred horizontally to the other strain of bacteria. Furthermore, I find evidence of coevolution occurring between the bacteria and phage, and especially the evolution of resistance to phage by the bacteria. However, there is little evidence that this loss of bacterial susceptibility lowers the rate of horizontal gene transfer occurring, with the proportion of double resistance stable with time except for the fourth day where it is lower.

In Chapter 4, I use modelling approaches to explore transduction and horizontal gene transfer in general. The first model looks at the conditions under which horizontal gene transfer can maintain a costly antibiotic resistance plasmid. I find that a plasmid can persist in conditions where the horizontal gene transfer and the benefit conferred by resistance to antibiotics is more than the cost imposed by the plasmid. The second model looks at specifically at transduction and examines how different aspects of phage and bacterial ecology can have on maintenance of the plasmid. I find that there are critical parameter ranges under which transduction is maximised and where a costly plasmid may be maintained. It also shows how phage and low dose antibiotics can act synergystically to maintain a plasmid. The third model uses an agent based modelling approach to test and validate

the prediction that a phage that has a long latent period and small burst size is better at maintaining the plasmid than a phage with a short latent period and large burst size. The model finds that the hypothesis is correct and the lower infectiousness of the phage reduces the selective pressure of the phage and allows the plasmid to be maintained through transduction.

5.1.1 Challenges of isolating and highlighting transduction events

The overwhelming challenge that arose from examining transduction experimentally is that transduction is in effect a lower level dynamic compared to infection and lysis. The sheer numbers involved show how large an issue this is. Transduction has been reported at around once in 10000 bacteria. However, bacteriophage can reduce bacterial populations by 1000 times. Moreover, if a bacteria is sensitive to receiving the plasmid, then it is also sensitive to being lysed. There is every chance that these a transduction event may be masked by that particular transductant being later infected and lysed. Therefore, the actual magnitude of transduction may be underestimated in the experiments that have been performed.

Further complicating this is the evolution of resistance of to phage, which can increase bacterial density over a 100 times over the course of an experiment. This posed difficulties in terms of experimental design that can capture the full range of interactions and biological phenomena occurring, especially when trying to capture resistance in bacterial densities that vary so much. There is a density dependence in the effect of antibiotics. If the density of bacteria is too high, then the antibiotic will be titrated out and bacteria that are not actual transductants will survive selection. Conversely, if the density of bacteria is too low, then the bacteria which are true transductants may be killed by the antibiotic. Therefore, the magnitude of transduction may be over or underestimated dependent on the bacterial density.

5.2 The implications of this work in understanding phage mediated horizontal gene transfer and the plasmid paradox

One of the original objectives of this work was to observe whether phage mediated transfer could help explain the plasmid paradox. It was hypothesised that the small plasmid pNUK73 could be maintained by transduction in the absence of positive selection and this could (at least, partly) explain the existence of small non-mobilizable plasmids.

The experiments in Chapter 2 show that the temperate phage do pick up the plasmid but are not able to spread it around within the same strain of bacteria, possibly due to super infection exclusion. However, the evidence shown here of the lack of ability of temperate phage to move around the plasmid in *P.aeruginosa* does contradict the vast array of studies showing temperate phage moving around genetic material in other bacteria like *Staphlycoccus aureus*. In *S.aureus*, there are very few conjugative plasmids. The other plasmids tend to move around using different forms of transduction using temperate phages[88] and superinfection exclusion is not a limiting factor. Furthermore, there are more mechanisms (termed lateral[52] and auto-transduction[51]) whereby temperate phage are able to move around genetic material, sometimes at several thousand times the rate of traditional transduction. So while transduction is an important mechanism for spreading genetic material in *S.aureus*, similar evidence has not been found in these experiments for *P.aeruginosa*. But it must be noted that these experiments only tested the movement of resistance between *P.aeruginosa* bacteria of the same strain (which contain the exact same chromosomal prophage), and it has not been tested whether using a different strain (with different chromosomal prophage) allows the temperate phage to spread the plasmid.

There is great precedent for lytic phage moving genetic material between bacteria in literature. Similarly, evidence for this is found in Chapter 2, where different phage can spread the plasmid at different transduction rates. However, the long term effects of the presence of phage on the maintenance of a non-mobilizable

plasmid had not been previously explored. The experiment in Chapter 3 showed that the phage is able to maintain the plasmid at a higher rate than a phageless control. A similar experiment using a conjugative mercury resistance plasmid has been has been previously performed[111]. It was found that that phage actually limit the the conditions by which conjugative plasmids can exist by increasing their loss under weak selection conditions, which is in opposition to the results shown in this work. The loss of the conjugative plasmid was primarily due phage predation which reduces the amount of donor and receptor bacteria and thereby reducing the overall magnitude of horizontal gene transfer. However, while this may be the case with a conjugative plasmid, the conditions will be different in a plasmid that is non-mobilizable and therefore have no other way to move other than the phage. The experiment in Chapter 3, shows that bacteriophage presents an avenue for horizontal gene transfer as well as suppressing bacterial populations through predation. The predatory effect of the phage will reduce the overall density of bacteria (including the density of transductants), but the plasmid will persist longer than if it had no avenue to transfer horizontally to other bacteria. Therefore, the phage can actually help maintain a plasmid that has no other means of transferring itself horizontally and can help explain part of the plasmid paradox in non-mobilizable plasmids.

It is also worth noting the importance that coevolution should play in these dynamics. Several studies have looked at measuring the transduction rate of lytic phage over a short time span[163][164][115], but none have measured how these may change over longer periods of time. The degree to which transduction can be a solution to the plasmid paradox is greatly attenuated by the evolution of resistance to the phage. This is why single rate of transduction measurements will, in general, overestimate the ability of phage to maintain a plasmid in the absence of positive selection over a longer period. Although, the experimental data did not support this, with the evolution of phage resistance not greatly altering the rate of transduction. Therefore, this relationship merits further investigation. Conversely, the modelling has shown that the evolution of phage to infect the bacteria (after the evolution of resistance by the bacteria to the phage) is necessary to keep the plasmid moving

horizontally in the longer term and therefore maintained. It is important to look at the horizontal gene transfer by phage not purely through the lens of transduction rates, but also examine the coevolutionary dynamics between the phage and bacteria.

5.3 The relevance of this work in the context of phage therapy and antibiotic treatment

Lytic bacteriophages are increasingly seen as a way of treating bacterial infections, in a treatment known as phage therapy. It used to be a niche treatment option, used predominantly in the former Soviet Union, but the increasing prevalence of antibiotic resistant infections means that it is now thought of as a more mainstream treatment option. The advantage in using bacteriophages is that there are virtually an infinite diversity of phages, so if one does not work in treating an infection, another can be found relatively easily. Furthermore, if bacteria become resistant to the phage, then phage have the ability to evolve *in situ* to overcome this resistance. There have been arguments for the use of phage as a complement to antibiotics[175], where there are used in tandem to treat bacterial infections. However, the work here may cast doubt as to the efficacy of such a treatment, or at the very least posit unintended consequences of such a treatment route. The experiments have shown that phage are able to maintain and spread antibiotic resistance genes, which could potentially reduce the effectiveness of the antibiotic part of the treatment. Moreover, the modelling has also shown how antibiotics and phage can act together to maintain a resistance plasmid. Especially, when either are not strong enough forces to maintain the plasmid on their own. Ultimately, phage have the potential to increase diversity in target pathogen populations, by moving around plasmids and by increasing recombination.

Cystic Fibrosis (CF) of the lung is an microbial ecosystem containing several different bacterial. In CF, *P.aeruginosa* (which is the predominant bacteria) is capable of sustaining chronic infections, which are highly resistant to multiple antibiotics[198]. A study looked at the diversity of 44 morphologically identical *P. aeruginosa* isolates taken from a single CF patient's sputum. The study found that

there were great levels of both phenotypic and genotypic diversity. The dominant driver of this diversity was found to be through recombination and not spontaneous mutation, showing the importance played by recombination in generating diversity in chronic CF infections[199]. The horizontal gene transfer effects of phages on CF infections will add to other effects that have been observed by other studies. One study found that (temperate) phages are able to drive adaptive evolution in cystic fibrosis. The study tested cystic fibrosis infections with and without a set of three temperate phages, and found that the bacteria in the treatment with phages showed more parallel evolution and faster selective sweeps than the bacteria without phages. This showed that phages allow quicker evolution of bacterial pathogens and contribute in their adaptation to the host environment and possible treatments[132].

When using antibiotics in complement to phage therapy, the experiments performed here have also brought another issue into prominence. The results have shown very clearly that phage growth is greatly inhibited by antibiotics. This could be due to the mechanism of action of both of the antibiotics used. Both of the antibiotics negatively affect bacterial protein synthesis, which the phages require for replication. The antagonistic effect of antibiotics on phage replication have been documented in other experimental studies[173]. If phage growth is reduced when antibiotics are present, then the efficacy of a treatment using both antibiotics and phages will be greatly reduced. Therefore, when choosing the specific antibiotic and phage preparation to be used in such a treatment, it is important to consider the antibiotic interference on phage replication. In order to maximise the efficacy of the bactericidal effect of the phage, an antibiotic must be chosen that minimises its antagonistic effect on phage replication.

5.4 Future Work

Within the scope of this thesis, transduction has only been explored on a superficial level and there are several avenues through which this work can be expanded on.

It would be quite interesting to look at the homology of the plasmid to the different phages that were tested in the transduction assay. Conventional wisdom

would say that there was quite a lot of sequence similarity between the plasmid and the *pac* sites in the phage genome, however it would be very interesting to verify this with bioinformatics analysis. Furthermore, it would be interesting to use qPCR to analyse the genetic composition of the DNA isolated from phage capsids after the phage has infected plasmid bearing bacteria. From this data, the parameter z from the transduction model would be able to be found. Moreover, the ratio of plasmid DNA to chromosomal DNA could be found, and it could be seen whether the plasmid is taken up by the phage at a higher rate.

The coevolution experiment could be expanded on further. It would be interesting to see what would happen over a period longer than four days. In the experiment, the plasmid frequency had dropped considerably by the fourth day in the absence of positive selection; and transduction had difficulty maintaining it. It would be fascinating to see if this trend continues five or ten days on. This would be useful to further investigate the relationship between the evolution of resistance to phage and its effect on transduction rates. Moreover, it would be useful to include time shifts to test the susceptibility and resistance of bacteria to phage over time. This would be helpful in determining whether and to what degree coevolution has occurred. This would also be able to disentangle the coevolutionary dynamics from the ecology.

The plasmid pNUK73 that was used in the experiment is clinically insignificant. Therefore, it would be interesting to repeat the experiments done here with a plasmid that has clinical relevance. Another step that could be taken is to use another model organism. Transduction is far more prevalent in *Staphylococcus aureus*, with it being far more widely reported and there being several mechanisms that it works under. Consequently, it would be interesting to see how a clinically relevant plasmid may be maintained in populations of *S.aureus* by phage mediated transfer only.

5.5 Concluding Remarks

My thesis has shown that transduction is able to move and maintain a costly antibiotic resistance plasmid between bacteria. Bacteriophages are the most numerous biological organism on the planet and could represent a substantial

reservoir of antibiotic resistance genes. As resistant infections become more common, knowledge of how resistance genes disseminate will be critical to understanding and overcoming widespread multi-drug resistance.

Appendices

A

Appendix to Chapter 3: Coevolution of Bacteria and Phage: its role in Transduction

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A.1 Statistical Tables

A.1.1 Bacterial Density

Source	**d.f.**	**Residual deviance**	***F***	**p**
Treatment	2	$1.3202e+11$	$3.8617e+10$	$< 2.2e-16$
Time	3	$1.6114e+11$	$1.6037e+10$	$< 2.2e-16$
Time x Treatment	7	$1.4894e+11$	$1.4894e+11$	$< 2.2e-16$
Replicate	17	$1.0714e+11$	6006500958	$< 2.2e-16$
Null	71	$2.0925e+11$		

In Pseudo-F tests, all terms were tested against the residual deviance. Analogous to mean sum of squares squares, mean deviances (deviance/d.f.) were used.

A.1.2 Phage Density

Source	d.f.	Residual deviance	F	p
Time	3	$1.8752e+11$	$3.0494e+11$	$< 2.2e-16$
Replicate	5	$9.3154e+11$	$3.416e+10$	$< 2.2e-16$
Null	23	$1.1023e+12$		

In Pseudo-F tests, all terms were tested against the residual deviance. Analogous to mean sum of squares squares, mean deviances (deviance/d.f.) were used.

A.1.3 Proportion of double resistant bacteria

Source	d.f.	Residual deviance	F	p
Treatment	2	200657	35693	$< 2.2e-16$
Time	3	229685	14120	$< 2.2e-16$
Time x Treatment	7	148376	17667	$< 2.2e-16$
Replicate	17	172422	5860.1	$< 2.2e-16$
Null	71	272043		

In Pseudo-F tests, all terms were tested against the residual deviance. Analogous to mean sum of squares squares, mean deviances (deviance/d.f.) were used.

B

Appendix to Chapter 4: Mathematical Modelling of Horizontal Gene Transfer and Transduction

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B.1 Mathematical derivation of invasion by rare condition of plasmid bearing bacteria in the Basic Model

Set the antibiotic and plasmidless cells to an arbitrary amounts I^* and C^* in the equation defining the temporal dynamics of the plasmid bearing bacteria.

$$\frac{dP}{dt} = (r - c)P - k_1 I^* P + \lambda P C^*$$

This equation is then linearised by taking the gradient around 0. This creates a linear representation of the function when the plasmid bearing species are very rare (i.e. close to zero).

$$\left.\frac{\partial \frac{dP}{dt}}{\partial P}\right|_{P=0} = (r - c) - k_1 I^* + \lambda C^*$$

If the gradient is positive, then the function will be increasing and the plasmid bearing species will invade from rare.

$$(r - c) - k_1 I^* + \lambda C^* > 0$$

B.2 Experimental methods to measure decay rate of phage

B.2.1 Materials and Methods

LUZ19 Purification

LUZ19 phage samples were amplified from one randomly chosen plaque and introduced into a single overnight PA01 culture. After 24h, the samples were were centrifuged at 13300 rpm for 5 minutes and the cell free supernatant was extracted. The samples were plated on M9 agar plates to verify that there was no remaining bacterial matter. Six replicate populations of phage were established and stored at 4°C.

Initial quantification of phage samples

Phage density of each of the samples were quantified using serial dilutions and plating those on a soft agar overlay. The phage were then diluted such that they all had density of the lowest density sample. This density was 2×10^{10} PFU/mL.

Measurement of phage density at sequential time points

For the duration of the experiment, the phage samples were incubated at 37°C and 225 rpm continuous shaking. The phage density was quantified by plating on soft agar at intervals of three hours for a total of 12 hours. The soft agar plates were incubated at 37°C.

B.2.2 Results

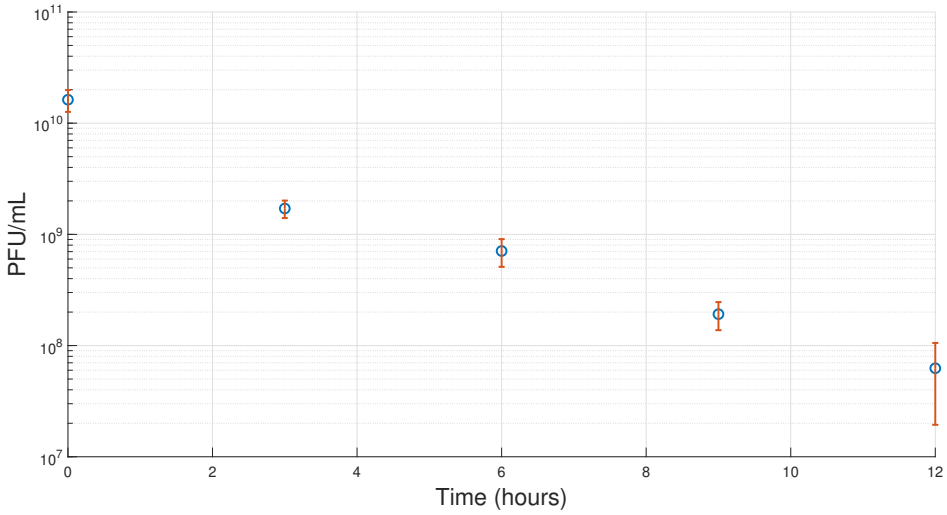


Figure B.1: Plot showing the temporal change of the density of LUZ19 with time during the decay experiment. Y axis is log scale. Error bars indicate 95% confidence intervals.

Figure B.1 shows the phage titre plotted against time. The decay process can be modelled as an exponential decay $P_t = P_0 e^{-dt}$. The natural log of both sides is taken and this results in $\ln \frac{N_0}{N_t} = -dt$. The decay rate is then the slope of the linear line of regression of the log plot of the data. This decay rate is estimated to be $d = 0.444h^{-1}$

B.3 Experimental methods to measure burst size of phage (One Step Growth Curve)

B.3.1 Materials and methods

Isolating bacterial colonies and culture growth cultures

Single colonies of *P.aeruginosa* PA01 were isolated from frozen stocks by plating on to a M9KB agar plate (15g/L agar) and by incubating at 37°C for 24 h. Six colonies were independently cultured in 14 mL Falcon tubes containing 3 mL M9 broth incubated at 37°C and 225 rpm continuous shaking or 24 h.

Phage adsorption

The overnight culture was diluted 100 fold and incubated until mid-exponential phase (~ 0.3 OD_{578nm}). 0.1 mL of phage lysate was added to 9.9 mL of exponential culture and incubated for 5 minutes to allow adsorption.

Isolating and quantifying adsorption control

0.1 mL of chloroform was added to 0.9 mL of phage+bacterial culture to kill off any bacteria and leave only unadsorbed phage. This was quantified on by plating using soft agar overlay.

Measurement at phage density at sequential time points

Phage titers were measured at time points by collecting samples at 15 minute intervals and plating them immediately by the overlay method.

B.3.2 Results

The results from the one step growth experiment can be observed in Figure B.2. The number of unadsorbed phage was determined by subtracting the number of phage in adsorption control by the phage titre before the latent period of the experiment (The average below minutes). The resulting value is then divided into the post-burst titre (The average above 150 minutes) to find the burst size. The burst size is estimated to be $b = 17$.

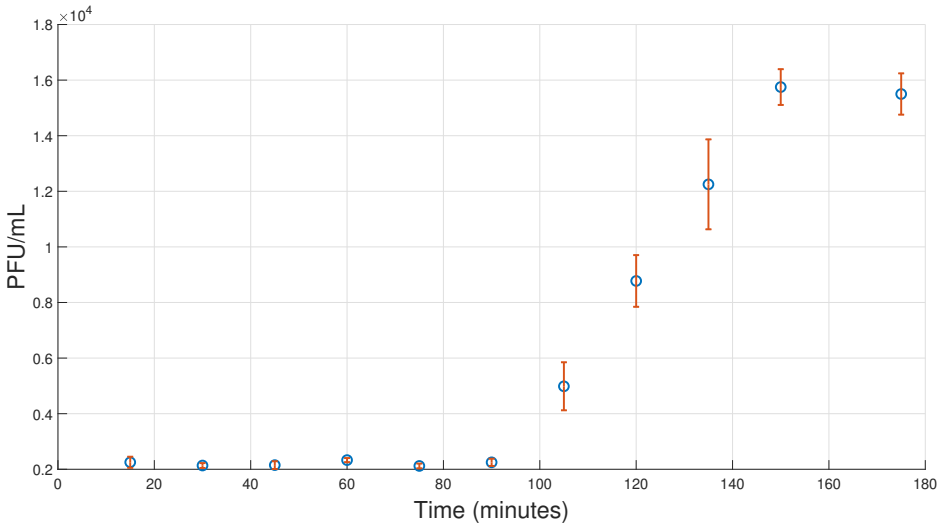


Figure B.2: Plot showing the temporal change of the density of LUZ19 during the one step growth experiment. Error bars indicate 95% confidence intervals.

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