Engineering ϕX174 to create a biocontained phage therapy model.

A thesis submitted in partial fulfillment of the degree of Master of Research.

Pamela Rea Tsoumbris

Department of Molecular Sciences

Faculty of Science and Engineering

Macquarie University

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Statement of Originality

This thesis entitled "Engineering ϕX174 to create a biocontained phage therapy model" is representative of the research study conducted between July 2020 and May 2021 for the completion of Master of Research degree in the Molecular Science department at Macquarie University, New South Wales, Australia. The work presented in this thesis is certified to be original by the author, unless otherwise referenced in the literature and/or acknowledged of personal advice and suggestions.

This thesis is formatted according to Master of Research guidelines prescribed by the Faculty of Science & Engineering and Department of Molecular Sciences and has not been submitted for qualification or assessment to any other institution.

Sincerely,

Pamela Rea Tsoumbris

SID: 45892539

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Table of Contents

Statement of Originality ii

Acknowledgments iii

Abstract vi

Chapter 1: Introduction 1

1.1 Bacteriophage 1

1.1.1 Bacteriophage as Natural Predators of Bacteria 3

1.1.2 ΦX174 and interactions with its E. coli hosts. 4

1.2 Antibiotic and Multidrug Resistant Bacteria 7

1.2.1 Antibiotics and the microbiome 8

1.3 Phage Therapy 9

1.3.1 Utilising Natural Predation for Phage Therapy 11

1.4 Engineering Phage 12

1.4.1 Biocontainment 14

1.5 Orthogonal Translation Engineering 15

1.6 Project Aims 16

Chapter 2: Methods and Materials 17

2.1 Bacterial strain handling, glycerol stocks and creating competent cells. 18

2.1.1 Bacterial Strains 18

2.1.2 Creating C70 and MP strains 18

2.2 Creating amber initiated ϕX174 Variants 19

2.2.1 Plaque Assays (Double Agar Overlays) 19

2.2.2 Engineering of Mutant Bacteriophage 19

2.2.3 Propagation and purification of F amber initiator mutant (F.am phage) 20

2.2.4 ϕX174 lysis curve 21

2.3 Determining Antibiotic Minimum Inhibitory Concentration 21

2.4 Determining Phage Minimum Inhibitory Concentration 21

2.4.1 Identification of phage resistant bacterial revertants 22

2.4.2 Identification of wild-type revertant phage 22

2.5 Determining Phage Stability 22

2.6 Phage Attachment Assay 23

Chapter 3: Results 23

3.1 Design and construction of a non-replicating biocontained bacteriophage. 24

3.2 Building i-tRNA$_{CUA}$ plasmids 26

3.3 Comparison of the effects of antibiotics and non-replicating phage F.am on *E. coli* 30

3.4 Phage Revertants 33

3.5 Host Resistance 34

3.6 Phage Stability 34

3.7 Phage Attachment Assay 35

3.8 RNA structures 36

Chapter 4: Discussion 37

4.1 Potential effects of using amber initiation tRNA. 38

4.2 The strength and efficiency of F.am lysis. 40

4.3 Potential for Host Resistance 43

4.4 Potential for revertant phage 44

4.5 Does the F.am phage efficiency decrease due to stability or attachment? 45

4.6 What can this be used for? 45

4.7 Conclusion 46

References 46

Abstract

Antibiotic resistant bacteria are an increasing concern amongst hospitals worldwide, with many patients presenting infections that are nonresponsive to current treatments. To resolve this problem, many scientists are turning to phage therapy for treatment. Unfortunately, phage therapy harbours drawbacks including fear of gene transfer between different microbes within human microbiomes, and the potential for off-target interactions interrupting other microbial niches within the body. Engineered phage may reduce some of these drawbacks, creating phage with broader host range or the ability to degrade biofilms, but to realise these benefits outside the lab, engineered phage need to show they cannot spread widely in the environment. In this thesis I created a biocontained phage therapy model to attempt to address these concerns. In employing the non-canonical amber start codon (TAG) at the beginning of ϕX174 capsid genes, I engineered a phage that could only replicate when provided with the mutant initiator $tRNA_{CUA}$. As a result, I was able to provide a method of biocontainment which maintained phage stability, maintained its ability to adsorb to host cells, and prevented it from replicating, although host-killing efficiency was reduced from wild-type phage. This study provides a platform technology for the effective containment of bacteriophages and takes the first step towards enabling phage therapy to become an accessible treatment option.

Chapter 1: Introduction

1.1 Bacteriophage

Bacteriophages (phages) are viruses restricted to the exclusive infection of bacteria. One of the most diverse biological entities, they can be found in most environments and hence influence evolution and global ecology (Clokie et al., 2011; Sutton and Hill, 2019; Santiago-Rodriguez et al. 2015; Cobian et al., 2016). Whilst there are many theories surrounding their origins (Kuprovic et al., 2019), phages tend to contain few genes, and therefore protein products, which are conserved amongst the clades. This makes them difficult to categorise as metagenomic analyses are required for the differentiation of many types of phages, and numerous protein products have a hypothetical function (Van de Bossche et al., 2014). Additionally, genes are often transferred between different types of phages which gives the illusion of evolutionary similarity, meaning that whilst it is possible to group them via common proteins or genes, it is still difficult to definitively differentiate them (Rohwer and Edwards, 2002). Furthermore, morphological and phenotypic differences can assist in differentiating phages from each other as many can be compared structurally (Dion et al., 2020). However, individually these methods are not sufficient to be a sole determinant of phylogeny due to convergent evolution and the effects of horizontal gene transfer, resulting in the need to use multiple methods (Jurczak-Kurek et al., 2016). As a result of this wide diversity, some phages will have a high specificity for a small number of hosts (e.g. ϕX174 phage) whilst others will have a significantly broader host range (e.g. P-27 phage) each with their own associated benefits and costs (Dion et al., 2019; Ross et al., 2016).

Phages can be placed into one of three morphological categories dependent on their capsid structure. These include (i) an icosahedral capsid with a tail, (ii) an icosahedral head without a tail or (iii) a filamentous capsid. Genomically, phage can vary greatly in many aspects. Some contain very small genomes (<6 kb) whilst others are extremely large (>200 kb). Additionally, they may be comprised of single-stranded (ss) DNA, double stranded (ds) DNA, ssRNA or dsRNA. Regardless of this large variation and diversity amongst the types of phages, all follow the common process of infecting a host cell, replicating the viral genome through the hijacking of host mechanisms, packaging the genome in a protein structure known as a capsid, followed by lysis of the host cell to release progeny. Not all phages are strictly lytic though, with many able to undergo the lysogenic cycle (Figure 1). The lysogenic cycle enables the phage to integrate the viral genome into that of

the host, where it lies dormant until environmental conditions are favourable for phage release (Howard-Verona et al., 2017). For the purpose of this thesis, only lytic phages will be discussed.

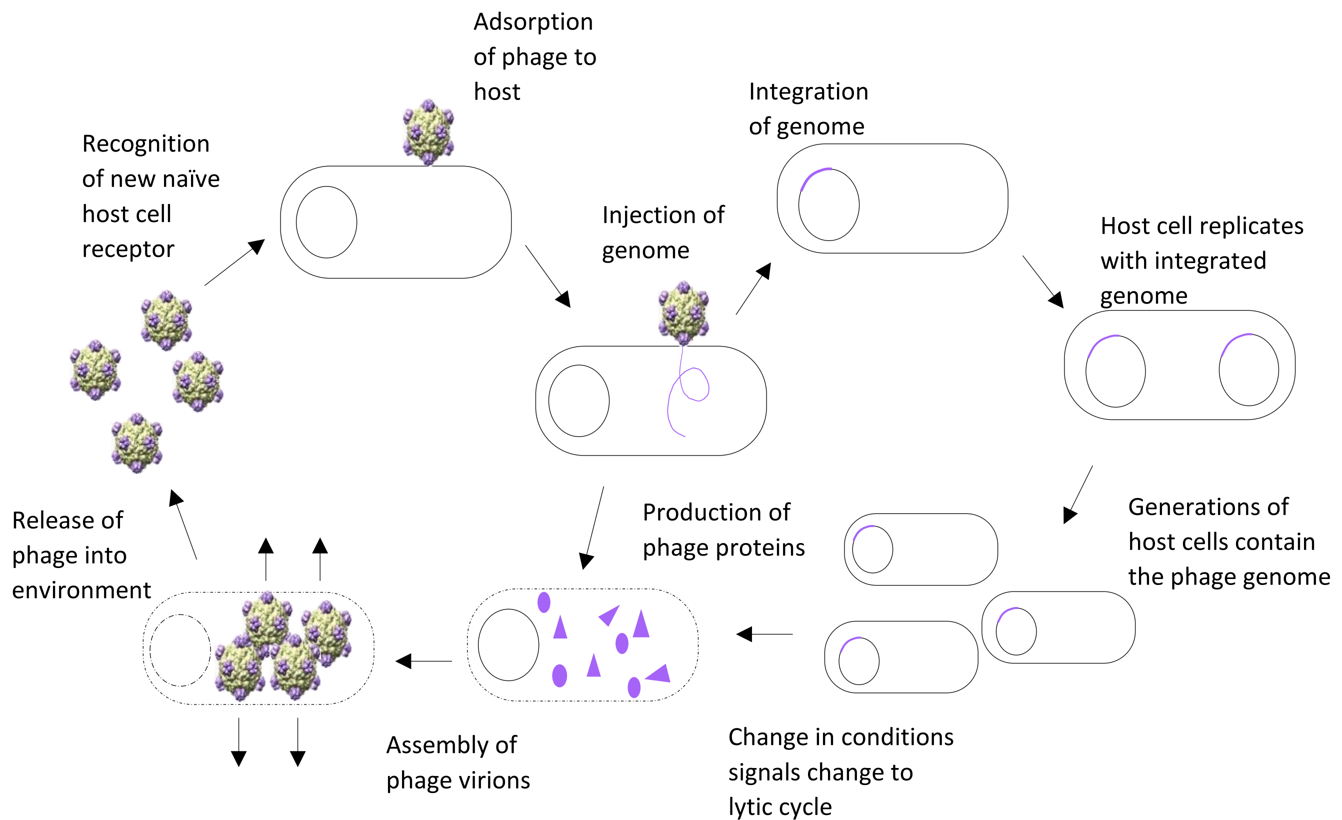

Figure 1: The lytic and lysogenic cycles of phage. After adsorption to the host cell and injection of the genome, the phage may integrate their genome with that of the host (right) or induce an immediate production of virion proteins for the assembly and subsequent release of phage via lysis (left).

The lifecycle of a phage begins with adsorption. To infect a host, phage capsid proteins must recognise the correct receptors on the host to enable adsorption to the cell surface (Bertozzie Silva et al., 2016). Once the genome is injected into the bacterial cells, the phage can either undergo a lytic or lysogenic cycle (Figure 1). The lytic cycle is as follows. Once the host cell is recognised, the phage will inject their genome into the cytoplasm and hijack the bacterial replication machinery. Whilst some phages are large enough to encode certain replication, transcription and translation factors (Sokolova et al., 2020), many instead rely on bacterial resources to replicate their genome. As the viral genes are transcribed and translated, capsid structures begin to form inside the cell. Once the viral genome is packaged into the capsid structure, lysis is induced to allow the phage virions access to new hosts in the wider environment.

As viral genes are amplified and their protein products expressed by the bacterial machinery, virions are assembled, and the viral genome packaged. Once cell lysis is induced, progeny are released into the environment to infect naïve cells and the environmental conditions support the continued propagation of phage within hosts.

1.1.1 Bacteriophage as Natural Predators of Bacteria

Bacteria and phage naturally co-exist in nearly all environments. The dynamic interactions between them entail a continual evolution and adaptability between the bacteria's ability to resist infection and the phage's ability to infect (Shkoporov and Hill, 2019). Inhabiting the same space, phage and bacteria have been shown to survive in a dependent and coevolutionary manner (Koskella and Brockhurst, 2014) which is largely influenced by the composition of species within the community, the density of these populations, and is defined by the antagonistic and mutualistic interactions of the ecological community (Silveira et al., 2020; Hall et al., 2020). The complicated relationship between microbes and phages relies on the constant evolution of both species, with phage being known to influence the diversity and quantity of bacterial strains in niches (Rodriquez-Valera et al., 2009; Koskella and Brockhurst, 2014). Receptor binding proteins found on the external surface of phage allow recognition of bacteria and target a range of receptors such as lipopolysaccharides (LPS), membrane proteins, flagella or pili (Bertozzie Silva et al., 2016). Consequently, these targeted regions are also frequently observed as high mutational areas (Betts et al., 2018) which suggests a parallel evolution of host receptors allowing for the evasion of phage infection. As previously discussed, successful recognition of the host allows the phage to adsorb to the cell and inject their genome for replication and translation by host machinery. Following a lytic infection, the release of newly assembled virions occurs via cell lysis. It is through this lytic process that phages modulate the composition of the bacterial community within an environment.

Conversely, constant predation upon host bacteria selects for mutations which ensure the continued survival of the host. There are many mutations that take place in bacteria which affect phage infection and bacterial biochemical processes. Internal resistance mechanisms employ mutations in the replication, transcription and translation processes whilst external resistance mechanisms involve changes in the receptor proteins. One such example is the mutation of receptors that prevent phage adsorbing to the bacterial host such as those seen in *Escherichia coli*. In the presence of λ phage, *E. coli* induce loop deletions in the LamB receptor to elude recognition

by the phage tail proteins (Berkane et al., 2006). Expectedly, this mutation also causes selection for phage with mutated LamB recognition proteins such that alternative receptors such as OmpF are used instead (Meyer et al., 2012).

Internal mechanisms often confer a cellular defence to prevent successful phage infection. The specificity of these mechanisms is mediated by the diversity of microbes in the surrounding environment. In low diversity environments, bacteria undergo mutations that confer resistance to specific phages, whereas in high diversity environments, mutations acquired are generalised to a range of different phages (Friman and Buckling, 2012). Cellular defence mechanisms by the host vary from small amino acid substitutions (Betts et al., 2016), to drastic cellular changes such as degradation of phage genome or infected cell suicide (Dy et al., 2014). Many of these internal mechanisms have been extensively reviewed elsewhere (Dy et al., 2014; Fernandez et al., 2018; Labrie et al., 2010). It should also be noted that the intense coevolution that drives bacteria-phage interactions is the defining factor of mutational rates. In highly diverse mixed populations, phages are exposed to an increased degree of competition which escalates the rates of coevolution through differing degrees of general selection pressures (Brockhurst et al., 2003).

1.1.2 ΦX174 and interactions with its E. coli hosts.

ϕX174 is an icosahedral bacteriophage known to infect a small number of *E. coli* and *Salmonella* strains (Michel et al., 2010; Inagaki et al., 2000; Lindberg, 1977). Consisting of only 11 genes (Table 1), this positive (+) single-stranded 5.5 kb DNA genome contains many overlapping essential genes. There are only three non-overlapping genes (Figure 2A). These are genes F, G and H and all are involved in capsid formation. Most importantly, each of these gene products are capable of forming proteins which aggregate into small protein complexes which then combine to form a prophage (50S complex) and eventually a mature phage virion (Hayashi et al., 1988). Each viral particle consists of 5 subunits which totals to 60 F proteins, 12 spikes formed by five G proteins, 60 copies of protein J and 12 copies of protein H (Hayashi et al., 1988).

Genome replication involves the use of the bacterial host DNA polymerase associated with protein A to perform rolling circle replication. Once the phage genome is inserted, the ssDNA is converted into dsDNA via primosome activity. The newly synthesised (-) strand is then used as a template for RNA polymerase transcription (Logel and Jasckey, 2020; Wright et al., 2020). Replication of the ϕX174 genome begins at the origin of replication located within the A gene and involves the use of

the bacterial host DNA polymerase associated with protein A to perform rolling circle repliations (Table 1). The resulting (+) strand is bound to the H protein as a small subunit and packaged into the 50S protein complex (Hayashi et al., 1988). The virion matures from a procapsid to a viable virion when protein D is shed from the external surface. Greater detail of the lifecycle of ϕX174 and the molecular mechanisms involved in the propagation can be found in other literature (Fane et al., 2005; Cherwa and Fane, 2011; Hayashi et al., 1988).

Table 1. Genes of ϕX174 and their associated function (From Cherwa and Fane, 2011)

Gene	Function	Role in Phage
A	DNA replication at both stage II and stage III (origin of replication)	Replication
A*	Inhibition of DNA unwinding to prevent host cell replication	Replication
B	Internal scaffolding for procapsid formation	Scaffolding
C	Facilitates DNA replication from stage II to stage III	Replication
D	External scaffolding protein for procapsid formation	Scaffolding
E	Mediates host cell lysis	Lysis
F	Major protein forming the capsid	Capsid
G	Major spike protein responsible for interaction between the phage and host cell	Capsid
H	DNA pilot protein and H-tube formation for genome transportation	Capsid
J	Bind and package DNA	Replication
K	Considered non-essential, influential in burst size propagation	Capsid

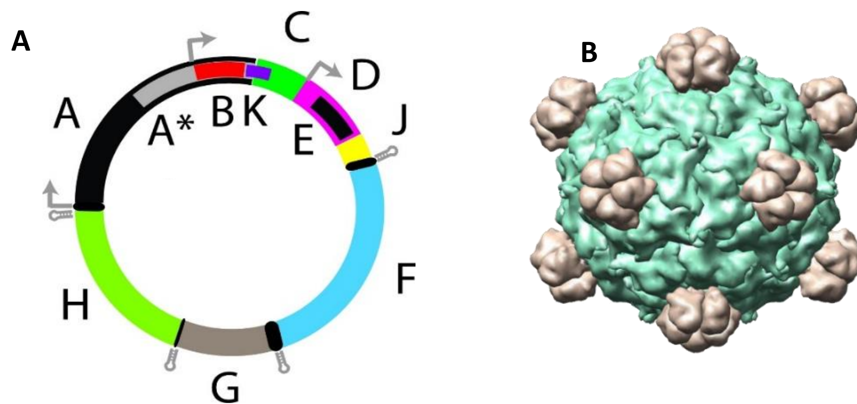

Figure 2. The genome and structure of ϕX174. The genome of ϕX174 is depicted on the left with the overlapping and non-overlapping genes evident in the simplified circular depiction. The structure of the virion is depicted on the right (PDB: 1RB8). ΦX174 is comprised of a total of 60 units of capsid protein F (green) and 60 units of capsid protein G (white) which comprise the external capsid structure.

Gene E is one of the overlapping genes which plays an essential role in the propagation of phage virions (Table 1). Responsible for the lysis of the host bacterial cell, gene E is found on the most abundant transcript (Blasi et al., 1990; Logel and Jaschke, 2020), however due to a poor ribosomal binding site, has the least amount of protein expression. This suggests translational regulation of the gene to ensure sufficient time is granted to the host to create phage virions. Once sufficient protein levels accumulate to a point capable of inducing cell lysis, the translocase present in the host cell is inhibited (Bernhardt et al., 2001) and the bacterial wall becomes increasingly susceptible to the osmotic pressure of the external environment, eventually leading to lysis.

Naturally found within the human gut, the main bacterial host of ϕX174 is *E. coli* C strain. In particular, the laboratory cultivated C122 is the most susceptible strain, with decreased virulence for ROAR340, ROAR375, M1402, C474, VDG388, and VDG401 strains recorded as well (Michel et al., 2010). Although these strains differ in their LPS composition, each is comprised of either an R1, R2, R3 or combined LPS core type which can be recognised by ϕX174 (Michel et al., 2010). Furthermore, these hosts all exhibit a rough O-antigen phenotype.

The G protein is responsible for the interaction of the phage with its host as only one of the 12 protein spikes will interact with the LPS and allow the phage to adsorb (Sun et al., 2017). This suggests that the most variable regions of the capsid are more integral to LPS recognition than the more conserved N-terminal region of the G protein (Kawaura et al., 2000). Once the phage has adsorbed to the external surface of the bacterial host, protein G dissociates leaving only the protein F structure to maintain contact (Sun et al., 2017). Subsequently, the integral H-tube (formed by the H protein) is engaged and deposited into the membrane of the host cell, allowing the phage access to the cytoplasm so that it may transfer its genome (Sun et al., 2014). Once the genome has been transferred to the bacterial cell, the H-tube dissociates and the empty capsid remains irreversibly attached via the F proteins (Sun et al., 2014). Upon lysis of the cell, all ϕX174 progeny are released into the local environment and the process repeats until susceptible hosts are exhausted.

1.2 Antibiotic and Multidrug Resistant Bacteria

Since the initial use of penicillin as an antibiotic in the 1940s, the treatment of microbial infections with drugs has drastically risen (Collignon, 2015). Historically, broad-spectrum antibiotics have been administered to treat a wide variety of diseases and proven highly effective in many early cases. The successful treatment of these diseases elicited the wide-spread use of antibiotics for all types of illnesses which unfortunately led to excessive and unregulated administration on a global scale. Applying this selective pressure upon bacteria has resulted in their evolution to ensure their continued survival. Many of these mechanisms involve the acquisition of antibiotic resistance genes. The rise of antibiotic resistance and in many cases multidrug resistance (MDR) continues to spread throughout various bacterial species which influence the environment, agriculture and human health. As pathogenic strains develop these resistances, they begin posing a serious threat to overall human health making the treatment of bacterial infections exceptionally difficult. Although the widespread use of antibiotics can be observed in human medicine, MDR has been further encouraged by the excessive administration in the agricultural industry, an increased positive selection for microbes containing at least one resistance, as well as a drastic decrease in the availability of new antibiotic treatments (Gordillo Altamirano and Barr, 2019).

The inability to treat common diseases due to the high degree of resistance has become a serious issue for both less developed and highly developed countries. Developing countries such as India

have shown an increase is pathogenic bacteria such as *E. coli* in urinary tract infections due to a lack of regulation in antibiotic administration. This is especially detrimental as these strains have become largely untreatable due to the development of resistances to last resort drugs such as ciprofloxacin and fluoroquinolones (Mandal et al., 2012). In more developed countries such as Australia, the United States and the United Kingdom, antibiotics are more stringently regulated yet resistances continue to spread throughout pathogenic bacterial strains. As of 2019, estimated deaths in the US resulting from infection by antibiotic resistant pathogens totalled over 35 000 (CDC, 2019). The most urgent threats arise from several bacteria containing carbapenem resistance, whilst those comprising the slightly less serious threats are resistant to a combination of vancomycin and other drugs, making most pathogens MDR. A study conducted by AURA revealed that Australia was amongst the top countries administering the highest amounts of antibiotics to patients, suggesting that specifically carbapenem and vancomycin resistant bacteria are especially significant threats (AURA, 2019). Likewise, the UK have reported a 12% increase in the presence of MDR *E. coli* causing bloodstream infections (ESPAUR, 2020), with the total death rate caused by antibiotic resistant microbes estimated to rise to approximately 10 million by 2050 (IACG, 2019). This suggests that although stringent regulations have been introduced to decrease the irresponsible use of antibiotics, MDR is a persistent problem plaguing multiple countries.

1.2.1 Antibiotics and the microbiome

The human microbiome is comprised of various strains and species of prokaryotes which contribute to the regular organ function of humans. Comprised of a large variety of strains (Qin et al., 2010), the microbiome is highly complex and mutualistic interactions between the different strains can directly impact an individual's overall health. This is especially observed in environments such as the gastrointestinal tract (Shkoporov and Hill, 2019; Dahlman et al., 2021). Administration of broad-spectrum antibiotics therefore threaten this delicate balance. Whilst antibiotics are administered to target pathogenic bacterial strains, the wide variety of commensal bacteria living in these environments are also generally susceptible to the selective pressures exerted by this treatment (Zhang and Chen, 2019). Off-target effects that occur as a result from antibiotic exposure have been linked to numerous other diseases related to changes in the composition of the microbiota (Le Chatelier et al., 2013) and include increased mortality (Weber et al., 2017), increased likelihood of developing long term obesity (Cho et al., 2012), thrombosis (Zhu et al., 2016), gastrointestinal disease (Frank et al., 2007) and poor nutrient utilisation (Turnbaugh

et al., 2016). Furthermore, altering the composition of the gut microbiota has also demonstrated detrimental lasting effects on immune response and homeostasis (Willing et al., 2011). Although there have been various methods suggested as alternative treatment options (Ghosh et al., 2019), none have proven to be as effective as broad-spectrum antibiotics thus far.

1.3 Phage Therapy

In the 1920s the notion of using phage as therapy against bacterial infections was proposed and the idea was set to revolutionise medicine in the Western world (Fruciano and Bourne, 2007). Whilst the idea of phage therapy never gained sufficient traction in Western medicine due to the popularity and easy administration of newly discovered antibiotics, Eastern European medicine sought to use phage to treat many different diseases (Rohwer and Segall, 2015). The decline in popularity in the West was due to a number of reasons, the main being the inability to replicate outcomes reliably and a lack of understanding regarding the mechanics of phage infection. Viral ecology, enabled by advances in sequencing and general molecular biology techniques, has progressed in the 21st century and the role of phage within the biosphere is widely accepted and better understood. These discoveries lend themselves to the possibility of changing the way the scientific community looks at phage and employing them as potential therapies to treat MDR bacterial infections.

There are many advantages to using bacteriophage in human medicine as compared to antibiotics alone (Table 2). These include a highly specific and targeted host range, a robust nature and tolerance of bioengineering, a self-limiting nature which prevents survival without a host and different lysis mechanisms to antibiotics. Additionally, phages have also been shown to be highly effective when used in conjunction with antibiotics (Chen et al., 2020; Manohar et al., 2018; Liu et al., 2020). Unfortunately, there are also numerous drawbacks (Table 2). These include a narrow or limited host range, wide range of inappropriate phages and a small range of appropriate ones, the development of phage resistance mechanisms, potential release of endotoxins following bacterial cell lysis and the inability definitively confine bacteriophage to the targeted pathogenic bacteria (Loc-Carillo and Abedon, 2011; Principi et al., 2019). Advantages and disadvantages are further summarised in Table 2. To successfully employ phage as a routine therapy, these obstacles need to first be addressed. Many studies have demonstrated that these limitations should not prevent the use of phage therapy. The efficacy of phage therapy has been demonstrated in *in vivo* models (Khalifa et al., 2018, Manohar et al., 2018; Betts et al., 2016; Yehl et al., 2019) as well as in model organisms and human infections (Regeimbal et al., 2016; Schooley et al., 2017; Chan et al.,

2018; Dedrick et al., 2019; Ferry et al., 2020). In scenarios where patients (human or model organisms) were severely infected, the application of phage therapy has seen the full recovery of individuals (Ferry et al., 2020, Schooley et al., 2017; Regeimbal et al., 2016). Furthermore, constructing a cocktail of phage with various types of phages has shown increased efficiency as opposed to single phage therapies (Yang et al., 2020; Khalifa et al., 2018; Manohar et al., 2018; Zalewska-Piatek and Piatek, 2020).

Within Australia specifically, the application of phage therapy is a rare situation that may be employed as clinical trials for compassionate cases (McCallin et al., 2019). Currently, there is only one reported phage therapy that has been approved for clinical trial on Australian patients (clinicaltrials.gov, NCT04323475). As the necessity for phage therapy increases, synthetic biology will need to be employed in order to abate fears and address disadvantages.

Table 2: Some of the advantages and disadvantages of using phage for therapy and how synthetic biology can be used to assist in its development. (From Nilsson, 2019; Bichet et al., 2020; Gordillo Altamirano and Barr, 2019; Nobrega et al., 2015).

Advantages	Disadvantages	Engineering Solutions
High specificity for hosts	Narrow host range	Changing tail fibres for a broader/different host range
Self-limiting (will not persist after extinction of host)	Self-limiting	Inclusion of biocontainment
Ability to isolate new phage if resistance is developed	Many phages are uncharacterised/partially characterised	Inclusion of differing phage structures/recognition sites
Co-evolution offers easy mechanism to mutate phage if host becomes resistant	Bacteria may develop mechanisms against infection	Directed evolution studies can assist in the discovery of new phages
Lysis of bacterial cells ensures definitive treatment	Not all phage are appropriate for use in therapy (e.g. temperate)	Engineering temperate phages so they are appropriate may assist in broadening the availability
Able to restore antibiotic sensitivity to pathogenic bacteria	Release of endotoxins upon lysis	Can be engineered to carry genes that encourage the restoration of antibiotic sensitivity
Highly effective against biofilms	Strict requirements which prevent extensive modification or engineering	
Use as an adjuvant	Variable pharmacokinetic and pharmacodynamic properties	

1.3.1 Utilising Natural Predation for Phage Therapy

Given the rapid increase in the presence of pathogenic MDR microbes within nosocomial environments, it is comparatively easier to find phage capable of targeting these strains than to develop new and effective antibiotics. This can be demonstrated by the isolation of the *Enterococcus faecalis* bacteriophage EFLK1 (Khalifa et al., 2018). Upon exposure to the initially isolated EFDG1 phage, the vancomycin resistant *E. faecalis* began exhibiting a resistance to phage treatment. As a result, EFLK1 was rapidly isolated from sewage effluents and used to successfully

treat both planktonic and biofilm aggregates. This process occurred in a matter of weeks, which shows the potential for rapidly discovering phage able to target problematic bacterial infections. Additionally, in devising cocktails which comprise of multiple types of naturally isolated phages, the potential for the bacteria to develop a resistance is drastically decreased as there is an abundance of different phage receptors being targeted (Gordillo Altamirano and Barr, 2020).

The natural coevolution between species remains a constant event which continuously produces phage able to infect different strains of bacteria. In using naturally isolated phage to treat MDR infections, antibiotic sensitivity may once again be acquired (Chan et al., 2016; Gordillo Altamirano and Barr, 2019; Liu et al., 2020). Within phage therapy and medical treatments, phages can be administered as an adjuvant to antibiotics which takes advantage of this natural evolutionary interaction and increases the likelihood of successful targeting. Bacteria that have recently acquired a sensitivity to antibiotics can then be treated by a combined antibiotic/phage therapy regime.

Unfortunately, not all MDR bacteria will be susceptible to a wide range of bacteriophage. Quite frequently, the environment the phages are isolated from and where the infection was acquired will yield different results and an appropriate phage may not be isolated (Hyman, 2019). Furthermore, there are fears that using a naturally isolated phage will result in the widespread modification of commensal bacteria. For these reasons, synthetic biology has the potential to play an influential role in the improvement of phage therapy.

1.4 Engineering Phage

The potential exists to avoid some of the limitations of naturally isolated phage using synthetic biology to re-engineer phage with expanded host ranges, increased tolerance of environmental conditions (e.g. pH), biocontainment, increased specificity, low immunogenicity and decreased or lacking toxin production. Such methods include the employment of the CRISPR-Cas system (Cobb et al., 2019), swapping tail fibres between phages to broaden the host range (Ando et al., 2015), targeting different LPS core types (Yang et al., 2020), changing genetic codes such that synthetic amino acids are required for continued propagation (Hammerling et al., 2014), removal of lysogenic properties (Paul et al., 2011) and the expression of non-endogenous proteins and enzymes for a more targeted approach to therapy (Lu and Collins, 2007; Edgar et al., 2012). The engineerability of bacteriophage can be best demonstrated through a study where researchers

used the scaffold of *E. coli*-specific bacteriophages T7 and T3, and modified the C-terminal region of the tail fibre gene to increase the host range (Ando et al., 2015). This provided the *E. coli* phages with the tools to infect *Klebsiella* and *Yersinia* strains, respectively. Furthermore, infection by the modified T7 phage demonstrated the ability to lyse 99.95% of *Klebsiella* within an hour whilst T3 also demonstrated a similar efficiency for infecting *Yersinia pseudotuberculosis* as well as *E. coli*. This suggests that specific modifications of tail fibres can be performed that simultaneously shift and broaden the host range to include multiple bacterial species. Further research on T3 phage tail fibres have also been seen to alter host ranges through the construction of synthetic phagebodies (Yehl et al., 2019). These constructs aimed to increase the overall diversity of the phage population, whilst minimising structural changes made to the tail. Using a synthetic biology approach, researchers designed phagebodies by modelling and identification of host-range-determining-regions which were then subjected to minor mutations that maintained the structural integrity of the phage tail. These phage constructs maintained their ability to infect the wild type *E. coli* host by minimising the disruptions caused to the tails. Additionally, they also exhibited the ability to target hosts with mutated receptors (Yehl et al., 2019). These studies of changing phage tail fibres to increase host ranges are a powerful demonstration of the use of synthetic biology to tailor phage therapies. They suggest that the notion of a limited host range can be surpassed. Furthermore, these studies enable researchers to design and synthetically construct phage which may lose the ability to infect due to the coevolutionary manner of bacteria-phage interactions. In demonstrating the vast flexibility and engineerability that phages are capable of enduring, it can also be deduced that phage therapy can be created to be a highly effective treatment for many diseases.

Whilst there are numerous benefits which encourage the progression of research into phage therapy, there are also many drawbacks which need to be acknowledged when using engineered phages. Mainly, difficulty in approval for use through regulatory bodies and a wide hesitancy from the general public in using genetically modified organisms (Loc-Carillo and Abedon, 2011; Principi et al., 2019; Lin et al., 2021). Many of these disadvantages or worries could be abated by employing synthetic biology to engineer a biocontained phage.

1.4.1 Biocontainment

Biocontainment can be defined as engineering of containment methods such that potentially pathogenic or genetically modified organisms (GMOs) cannot interact with the environment upon release outside the lab (Kim and Lee, 2020). Whilst many GMOs are designed to specific display a function or modulate a process, they carry specific genes that may interfere with current environments if they escape controlled conditions. If these organisms escape into natural ecosystems there are two fears that may arise. The first concern relates to the out competition of wild type microbes from the GMO, whilst the second concern regards horizontal gene transfer and the potential for wild populations to acquire these altered genes/functions/enzymes. In both scenarios the repercussions could be devastating for natural ecosystems. For this reason, biocontainment has become a highly desirable goal in the field. In Australia, GMOs are approved on a case-by-case basis by the Office of the Gene Technology Regulator and the Therapeutic Goods Administration (Dedrick et al., 2019; Lin et al., 2021). Given microbial organisms have a rapid rate of mutation (Drake, 1991; Cuevas et al., 2009), the ability for mutational escape for any genetically modified microbe is a likely possibility.

The efficacy of biocontainment in synthetic organisms has been demonstrated in various studies concerning the recoding of genomes to incorporate non-standard amino acids (Gallagher et al., 2015; Rovner et al., 2015; Mandell et al., 2015, Fujino et al., 2020). In particular, engineering the regulatory machinery (riboregulators) in *E. coli* has proven extremely effective (Gallagher et al., 2015). Total dependence on synthetic or non-standard amino acids caused a metabolic cross-feeding and the transfer of genetic materials between different strains to become impossible in sustaining viable bacteria. In biocontaining *E. coli* in this manner, multiple synthetic molecules would be required by the bacteria creating a 3 and 4-layered safeguard which saw the escape frequency drop from 1.0×10^{-6} to $<1.3 \times 10^{-12}$ cells. This means that a mutation may occur within one of the riboregulators without compromising any biocontainment properties.

Within the context of phage therapy, the introduction of biocontainment aims to ensure that administration of the phage being used for treatment is done so in a controlled manner whereby the escape (or ability to infect other bacterial species and strains) of these constructs is minimised. In doing so, the phages are unable to transfer unique genes or properties to other phage, and they do not have time to adapt to target nonspecific hosts present in the natural population. Currently, there are only a few reports addressing different methods for biocontainment in phage, with many of them requiring the inclusion of non-canonical amino acids and an expanded genetic code

(Hammerling et al., 2014; Oller-Salvia and Chin, 2019) or the removal of lysogenic properties (Hagens et al., 2004). Biocontainment of phage is especially important as they have been known as vectors of horizontal gene transfer (Colomer-Lluch et al., 2011), which suggests that not enforcing limitations will potentially cause other strains to acquire engineered traits.

1.5 Orthogonal Translation Engineering

A potential solution to creating biocontained phage involves the utilisation of orthogonal cellular processes. Orthogonality can be defined by a network of synthetically engineered components which work parallel to a host system and does not interfere with native functions (Liu et al., 2018). Orthogonal systems can be designed to interact with machinery at the replicative, transcriptional, or translational levels (Rackham and Chin, 2005; Ravikumar et al., 2014; Chin, 2017, Dunkelmann et al., 2020). As a result, understanding in various biochemical pathways and the synthesis of new biological functions has been enabled.

A large part of creating orthogonal systems relies on the degeneracy of the genetic code. There are 64 possible codons that encode only 20 amino acids, which provides a large degree of redundancy that can be utilised in orthogonal systems. Of these 64 codons, only three of them uniquely code for termination in bacteria. Release Factor I (RFI) recognises UAG and UAA stop codons, whilst Release Factor II (RFII) recognises UGA and UAA stop codons. In the presence of a suppressor tRNA however, these stop codons allow for the continuation of translation through the suppressor tRNA decoding the stop codon as a sense codon (Varshney and RajBhandary, 1990). Each stop codon is referred to as the amber (UAG), ochre (UAA) and opal (UGA) stop codons, and they have been used in a wide range of experiments to determine the function of genes in both prokaryotes and eukaryotes (Denhardt and Silver, 1966; Smith et al., 1970; Laski et al., 1982; Declerck et al., 1990; Normanly et al., 1990). The implementation of these stop codons as nonsense codons within genes resulted in “conditional lethal” mutants, whereby survival of the engineered organisms was only viable under specific conditions (Stahl, 2012).

In bacteriophage, suppressor tRNAs have proven to be especially successful. Many studies have found that employment of the stop codons for incorporation of non-canonical amino acids (ncAAs) have allowed utilisation of the genetic code in such a way that bacterial hosts have been recoded to result in phae resistance (Lajoie et al., 2013; Hammerling et al., 2014; Ma and Isaacs, 2016).

Suppressor tRNAs can also be used to initiate translation when stop codons are put in the usual place of the AUG start codon (Varshney and RajBhandary, 1990). In particular, amber suppressor initiator tRNAs have proven to be the most useful with a strong initiation of translation from amber stop codons (Varshney and RajBhandary, 1990). When supplied with an amber initiator tRNA (Figure 3) containing a CUA anticodon (i-$tRNA_{CUA}$), resulting protein levels translated in *E.* coli have been significant, though not as efficient, to those produced by the native i-$tRNA_{CAU}$ (Vincent et al., 2019). Although the amber initiator tRNA has been shown to robustly initiate across many common *E. coli* strains (Vincent et al., 2019), thus far, suppressor tRNAs have never been used to direct protein synthesis within bacteriophage.

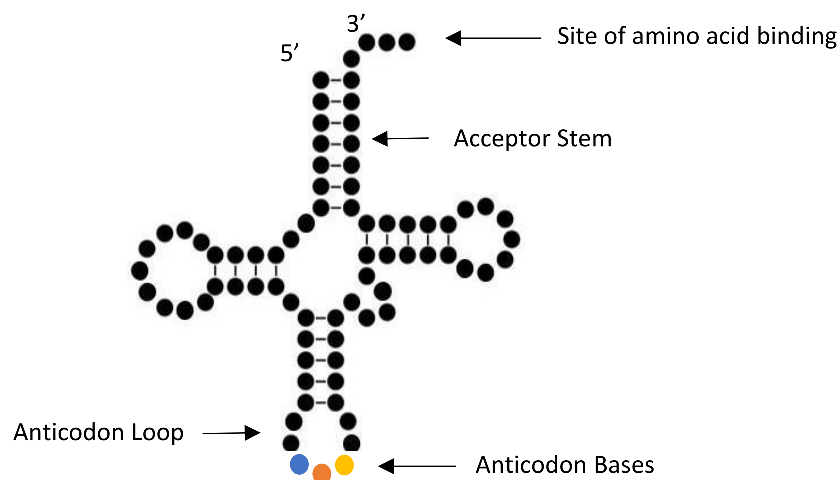

Figure 3. Cloverleaf secondary structure of a tRNA molecule. When charged with the complementary amino acid determined by the anticodon, the acceptor stem carries the amino acid and is responsible for its addition to the growing peptide chain. The amino acid is covalently bonded to the 3’ end of the structure. The anticodon loop contains the 3 complementary bases to that of the codon on the mRNA strand and allows for the recognition of the appropriate bases for amino acid addition. tRNAs will have a different sequence of anticodon bases and be charged with the appropriate amino acid based on the anticodon sequence.

1.6 Project Aims

Phage therapy is becoming an increasingly sought-after treatment for persistent MDR bacterial infections. As the field of synthetic biology advances, many problems that were previously proposed by phage therapy may potentially be resolved through engineered bacteriophage and the employment of orthogonal systems to create controlled treatment options. To demonstrate

the potential for creating biocontained phage using the amber suppressor initiator tRNA, I created three bacteriophage ϕX174 variants by changing the start codon (ATG) of the F, G and H genes to amber stop codons (TAG). I have hypothesised that each of these variants stop translation in the absence of i-$tRNA_{CUA}$ and hence model a biocontained phage therapy scenario within wild type bacterial hosts. I then show the amber initiator ϕX174 mutants can propagate in strains expressing the complementary amber initiator tRNA (i-$tRNA_{CUA)}$). Finally, I present the characterisation of the F amber initiator mutant ϕX174 (F.am) whereby I determined the minimum inhibitory concentration, attachment, and *in vitro* stability. As the modifications that would convert ϕX174 to F.am were minimal, I assumed that characterisation of the properties would reveal a similar, if not identical, pattern to that of the WT ϕX174.

Chapter 2: Methods

2.1 Bacterial strain handling, glycerol stocks and creating competent cells.

2.1.1 Bacterial Strains

This study employed the use of seven different E. coli strains: MG1655 (K-12), M1402, ROAR340, NCTC 122 (C122) from Public Health England (strain #NCTC122) and Nissle 1917 obtained from Mutaflor tablet (strain DSM 6601). Additionally, two strains of C122 were transformed with plasmids containing the $tRNA_{CUA}$ gene to express amber initiated ϕX174. These were C122(pULTRA::*metYp1p2-metY*$_{CUA}$) (hereby referred to as 'MP strain') and C122(pUC57::*proK-metY*$_{CUA}$) (hereby referred to as 'C70 strain'), The pUC57 plasmid was chosen as it is a high copy plasmid which enabled expression of the tRNA at a high concentration. The C70 strain was used for the propagation and purification of mutant phage. The pULTRA plasmid is a low to medium copy number plasmid and hence exerts less stress on the cells, enabling clearer plaque assays. The pULTRA::*metYp1p2-metY*$_{CUA}$ plasmid was gifted from a previous study (Vincent, 2017). All bacterial strains were tested for their susceptibility to wild type ϕX174 through a double agar overlay or via liquid culture.

2.1.2 Creating C70 and MP strains

The *E. coli* C122 strain was made competent using the Zymo Research Mix and Go Transformation Kit (Zymo Research Cat. No. T3002). Using Lysogeny Broth Miller (LB) media, a 1:100 dilution from overnight culture was grown at 37°C until the OD_{600} read between 0.4-0.6. Manufacturer instructions were followed to create competent C122 cells which were then transformed with 20 ng of either pUC57::*proK-metY*$_{CUA}$ or pULTRA::*metYp1p2-metY*$_{CUA}$ plasmids. The cells were then heat shocked at 42°C for 30 seconds and cooled on ice. LB broth was added to a total of 900 µL and the cells left to incubate at 37°C for 1 hour shaking at 250 rpm. After incubation, 100 µL of the cells were plated onto LB agar plates containing either 50 µg/mL spectinomycin (for MP strain) or 50 µg/mL kanamycin (for C70 strain) and left to incubate at 37°C overnight. Subsequently, one colony was picked and grown overnight in LB broth at 37°C with shaking. Each strain was glycerol stocked for long-term storage by adding 900 µL of glycerol (60%) and 900 µL of overnight culture. Glycerol stocks were stored at -80°C.

2.2 Creating amber initiated ϕX174 Variants

The phage variants used in this study originated from the wild type (WT) ϕX174 designed to correspond to Jaschke et al. (Jaschke et al., 2019).

2.2.1 Plaque Assays (Double Agar Overlays)

To determine the titre of phage stock a double agar overlay was performed (Kropinski et al., 2009). LB media was supplemented with agar to form a 1.5% agar bottom layer and the molten top layer at 0.7% concentration. A volume of 3 mL top agar was supplemented with 2 mM $CaCl_2$ and 50 µg/mL spectinomycin and subsequently inoculated with 100 µL of fresh overnight MP strain culture and 10 µL of phage. The top agar mix was then poured over solidified 1.5% LB agar plates. The phage was serially diluted in SM Buffer (Bonilla et al., 2016) to determine titre. Plates were incubated at 37°C overnight. Plates with clear individual homogenous plaques were counted and plaque forming units per mL (PFU/mL) were calculated in accordance with conventional means. Diameter of the plaques were measured using the Plaque Size Tool (Trofimova and Jaschke, 2021).

2.2.2 Engineering of Mutant Bacteriophage

A Phusion High-Fidelity DNA Polymerase Chain Reaction (PCR) was used to amplify the wild type ϕX174 genome. Primers used were designed with 2 base pair mismatches to replace the ATG start codon of the ϕX174 F, G and H genes with a TAG sequence and contained a 5' phosphorylation (Table 3) (Integrated DNA Technologies, IDT). The PCR product was then run on a 1% agarose gel in 1X TAE buffer for 45 minutes and subsequently extracted using the Monarch DNA Gel Extraction Kit (New England Biolabs, NEB). From the gel extracted product 6 µL was added to 1X T4 ligase Buffer, 400 units T4 ligase and made to a total volume of 50 µL. The ligation mix was incubated at 16°C for 1 hour. The reaction was then inactivated at 55° C for 5 minutes, and 5µL of the assembly mix was transformed by heat-shock into 100 µL of *E. coli* MP host strain made competent using the Mix & Go Kit (Zymo Research Cat. No. T3002). An outgrowth step was performed where the transformation mix was resuspended with 900 µL of phage LB (Rokyta et al., 2009) and incubated for 120 mins at 37°C with constant shaking at 250 rpm. Aliquots of 500 µL of the outgrowth mix was added to 4 mL of phage LB top agar containing 100 µL log-phase MP strain cells and plated onto an LB agar plate. Plaque assays were left to incubate overnight at 37°C. Two individual plaques were picked from each successful transformant and subjected to Phusion PCR and Monarch PCR Clean Up (NEB) prior to sequencing via Macrogen.

Table 3: Primers used for site-directed mutagenesis of F, G and H amber mutants.

Name	Sequence
F.am Forward	AGGATAAATTtaGTCTAATATTCAAACTGGCGC
F.am Reverse	/5Phos/CAAGTAAGGGGCCGAAGC
G.am Forward	TAgtttcagacttttatttctcg
G.am Reverse	/5Phos/gattaaactcctaagcagaaaac
H.am Forward	GAGGTGATTTtaGTTTGGTGCTATTG
H.am Reverse	/5Phos/ACTTAAGTGGCTGGAGAC

2.2.3 Propagation and purification of F amber initiator mutant (F.am phage)

Propagation and purification of phage was adapted from the Phage-On-Tap protocol (Bonilla et al., 2016). The titre of the phage stock was determined through a plaque assay as previously described. Plaque assays were performed using a serial dilution of F.am phage and the starter phage lysate titre determined.

Using an overnight C70 strain culture, a 1:100 dilution was grown in 10 mL phage LB containing 50 µg/mL kanamycin at 37°C until mid-log phase was reached (approximately OD_{600} 0.5). A plaque was picked from one of the diluted plates and suspended in 1 mL of SM Buffer, and the 10 mL log-phase culture infected for an intermediate phage propagation. The phage suspension was incubated at 37°C until OD_{600} <0.5. Phage lysate was stored at 4°C until large scale phage propagation was performed.

From an overnight culture, 40 mL of host bacteria were used to spike 400 mL of phage LB. The culture was incubated at 37°C with agitation for 2 hours. The previously prepared phage lysate was subsequently added to the culture and incubated at 37°C with agitation. OD_{600} was taken every hour until the culture reached a reading of <0.5. A total of 40 mL of chloroform and 1 M NaCl was added to the supernatant and incubated on ice for 1 hour. Aliquots were then taken and centrifuged at 4,000 g for 5 minutes using a swinging bucket rotor. The supernatant was stored at 4°C until ultrafiltration.

To filter and concentrate the phage stock, 30 mL of the phage lysate was then aliquoted into 50 mL Amicon filters (Sigma-Aldrich, CAT #Z740208) and centrifuged at 4,000 g for 20 minutes. Subsequent aliquots of 15 mL were added to the Amicon filters and spun for 45-minute intervals until the total volume of lysate remaining was <10 mL. To store, 15 mL of SM buffer was added

and centrifuged at 4,000 g until the final phage lysate volume totals <10 mL. Glycerol was then added to a volume of 10% and the final phage titre determined through the plaque assays. The phage was then stored at 4°C until use.

2.2.4 ΦX174 lysis curve

From fresh overnight culture, 10 µL of MP strain culture was added to phage LB and grown to mid-log phase. An aliquot of the culture was taken and diluted to approximately OD_{600} 0.05. Purified F.am phage was serially diluted and used to infect diluted MP strain. Culture was incubated at 37°C shaking at 250 RPM for 15 hours and OD_{600} measured at 5 minute intervals. This process was then repeated with WT phage.

2.3 Determining Antibiotic Minimum Inhibitory Concentration

Antibiotic susceptibility testing to determine the minimum inhibitory concentration (MIC) were conducted on *E. coli* strains MG1655 (K-12), M1402, ROAR340, C122, C900 and Nissle 1917 (Wiegand et al., 2008). From fresh overnight cultures, the above strains were grown to an OD_{600} between 0.4-0.6 shaking at 37°C. Antibiotics concentrations were made to twice the expected MIC concentration based off known values (EUCAST, 2020) and diluted 2-fold to a final volume of 140 µL. Antibiotics tested were carbenicillin and chloramphenicol due to their comparative lysis activity to phages, and streptomycin due to the different inhibitory mechanism employed (Sigma-Aldrich Inc.). A 1:6000 dilution of the bacterial strains was made in LB broth and 10 µL aliquots added in triplicates to a 96-well plate. The plates were covered with a Breathe-Easy seal and left to incubate at 37°C overnight, shaking at 250 rpm. Isolate breakpoints were determined by measuring OD_{600} the following day. Overnight measurements >0.1 were taken to indicate growth of the bacterial strain and hence classified as non-susceptible or resistant. MICs were determined to be the lowest concentration of antibiotics where no growth (OD_{600} <0.1) was measured.

2.4 Determining Phage Minimum Inhibitory Concentration

Seven *E. coli* strains (C122, C900 (Bentley Fane, RY2731), MG1655 (K-12), Nissle 1917, M1402, ROAR 340) were chosen to determine the minimum inhibitory concentration of F.am phage, all of which had varying degrees of susceptibility. Susceptibility of bacteria to phage infection was

conducted through plaque assays using WT ϕX174. From fresh overnight cultures, 1:100 dilutions were made in phage LB and grown at 37°C shaking to OD_{600} of 0.5. The inoculum was then adjusted to contain approximately 100 cells per 190 µL and an overnight assay prepared. The bacterial culture was infected with 10 µL of phage at concentrations ranging 10^5 - 10^1 PFU/µL such that the multiplicity of infection (MOIs) of 10 000, 1000, 100, 10 and 1 were tested. The plate was then incubated at 37°C with double orbital shaking in a Biotek plate reader. Growth was monitored by measuring OD_{600} every 5 minutes for 15 hours. Measurements of OD_{600} <0.1 were concluded to be complete lysis (i.e. no bacterial growth was observed).

2.4.1 Identification of phage resistant bacterial revertants

Once MIC values were determined for infecting phage, each sample that recorded clearance from phage were tested for bacterial revertants. From the highest MOI in the MIC assay, 10 µL were streaked onto an LB plate and incubated overnight at 37°C. Any colonies formed were grown to a stationary phase in LB broth at 37°C, shaking at 250 rpm. A 1:100 dilution in fresh LB broth was made the following day and grown to early log phase. Cultures were subsequently infected with 1.0 x 10^4 PFU WT ϕX174 and left to incubate at 37°C shaking at 250 rpm for 4 hours. Cultures that showed no cloudiness were deemed susceptible to ϕX174 infection, whilst cultures that grew to cause a cloudy LB broth solution were recorded as resistant.

2.4.2 Identification of wild-type revertant phage

To identify whether there were any revertant phage, samples were taken from wells that showed clearance in the MIC experiment. The overnight MIC assay was shaken for 30 seconds at 250 rpm and a 10 µL sample used to infect stationary phase C122 and MP strain cultures in a plaque assay. Any plaque formation on C122 plates were indicative of mutant reversion, whilst plaque formation on the MP strain was indicative of free unbound phage present in the MIC sample.

2.5 Determining Phage Stability

To determine the stability of the F.am mutant phage over the course of the MIC experiment, an aliquot of phage was taken and mixed with phage LB to a concentration of approximately 10^5 PFU/µL. The phage was then subjected incubation at 37°C without shaking and 20 µL aliquots

taken at 2-, 4-, 6-, 8- and 24-hours post-incubation. A total of four 1:10 dilutions were made at each time point. Plaque assays were performed as previously described.

2.6 Phage Attachment Assay

The phage attachment assay was adapted from Wright et al. (Wright et al., 2020). A fresh overnight C122 culture was diluted in phage LB to a 1:100 concentration and grown to mid-log phase (OD_{600} 0.5-0.6). The cells were pelleted at 4,000 g for 8 minutes and washed with HFB Buffer (60 mM NH_4Cl, 90 mM NaCl, 100 mM KCl, 1 M $MgSO_4$, 1 mM $CaCl_2$ and 100 mM tris base, pH 7.4) (Fane and Hayashi, 1991). The pellet was then resuspended to 1:10 of the original culture volume with HFB Buffer and kept on ice. Phages were then added to the resuspended cells at an MOI of 1. Infected cells were incubated at 37°C with continuous agitation at 900 rpm for 5- and 10-minute intervals. After incubation, cells were immediately pelleted for 5 minutes at 4,000 g, 4°C. The top 100 µL of supernatant was removed and placed on ice, and double agar overlays performed as previously described.

Chapter 3: Results

3.1 Design and construction of a non-replicating biocontained bacteriophage.

To create a bacteriophage that is capable of replicating within the controlled conditions of a laboratory but not in the outside environment, I first chose to modify a well-studied phage. This was completed through the mutagenesis of standard canonical start codons (ATG) to amber stop codons (TAG) of three ϕX174 capsid genes. ϕX174 has been studied for over 50 years and enabled me to choose appropriate genes to mutate without disrupting phage propagation or its ability to lyse host cells. All overlapping genes were related to DNA replication or lysis, making them inappropriate for modification. Furthermore, replacing the ATG start codon in a gene which is overlapping may interrupt the translation of other genes and hence prevent a functional virion from being produced, which is why it was vital that the mutation occur within a non-overlapping gene. The three non-overlapping genes present were F, G and H and each are involved with capsid production making them amenable to modification. No promoters, terminators or origins of replication are found near the gene F, G or H start codon regions, making modifications to these sites likely benign (Figure 4). When in the presence of the suppressor tRNA, the codon will be aminoacylated and translation of the protein can occur. This in turn will create infectious phage virions (Figure 5). If no suppressor tRNA is present however, the amber initiated gene will not be translated, and as a result, no phage virions will assemble.

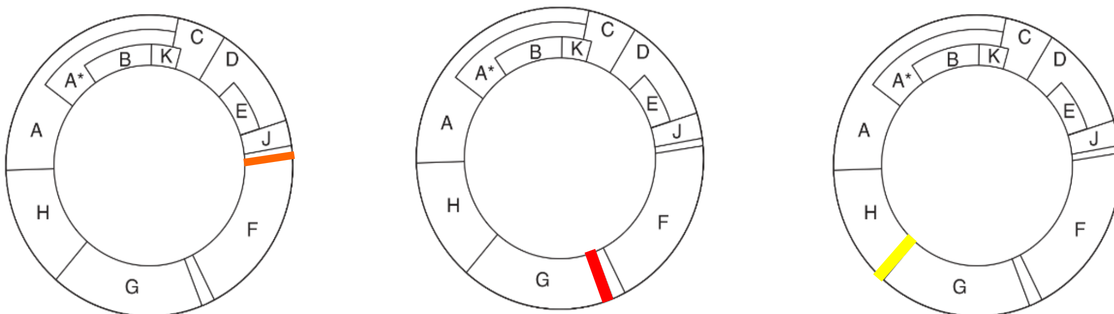

Figure 4. Individual mutant amber initiated mutants. From left to right, amber mutations present in the F, G and H genes.

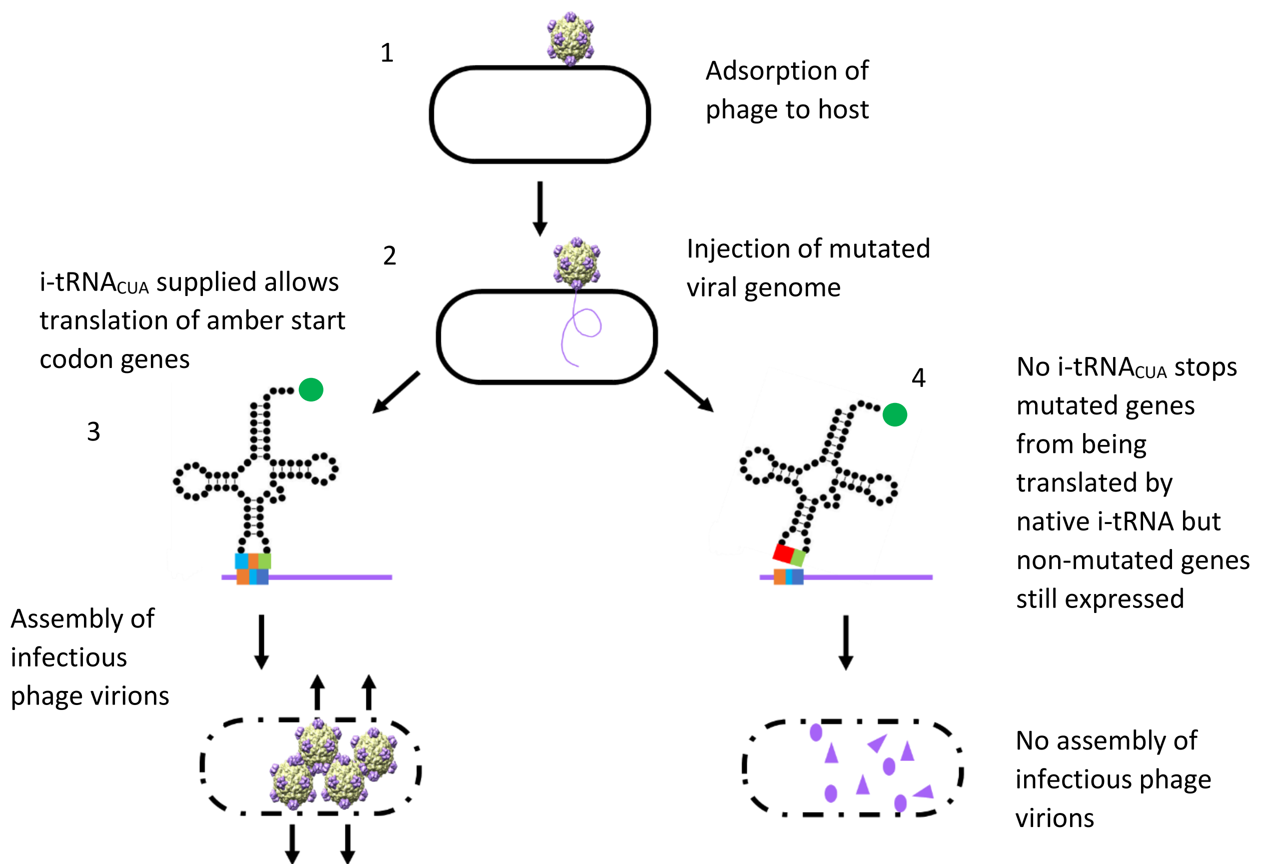

Figure 5. Biocontained phage model. (1) The phage adsorbs to host cells followed by (2) injection of the genome. (3) Translation of the mutant phage genome will only produce viable progeny able to infect subsequent cells if $tRNA_{CUA}$ is supplied by the bacterial cells (left). (4) When infecting host cells outside laboratory, genes containing an amber stop codon will not be expressed preventing the ϕX174 phage from assembling complete infectious virions (right).

Although the presence of one amber start codon would create a biocontained phage, there remains the potential for the phage to escape the containment conditions through mutation. This could involve a single base change from TAG to the third strongest start codon TTG (Hecht et al., 2017) which would allow the phage to propagate without the i-$tRNA_{CUA}$. Based on a phage therapy dose of 10^8 PFU with the assumption that burst size is 150 it can be calculated for single mutants that there are 150 390 revertant phage per 10^8 PFU used. Double mutants would result in 2.8 revertant phage and triple mutants would result in 0.00052 revertant phage per 10^8 PFU. To avoid frequent escape mutants, double and triple amber initiated mutants were planned. This would entail modification of the genome to contain different combinations of amber start codons for genes F, G and H (Figure 4). By including numerous amber start codons, spontaneous

mutations in the alternative codons could be avoided as multiple mutation events spread across different areas of the genome would need to occur for escape from biocontainment.

3.2 Building i-tRNA$_{CUA}$ plasmids

To propagate phage containing an amber start codon sequence, I first needed to create a host strain of *E. coli* capable of expressing the amber i-tRNA$_{CUA}$. The plasmid used to express i-tRNA$_{CUA}$ was obtained from a previous study (Vincent, 2017). I transformed competent *E. coli* C122 cells with pULTRA containing *metY$_{(CUA)}$* under the transcriptional control of the native *metYp1p2* promoter (Figure 6). This strain C122(pULTRA::*metYp1p2-metY(CUA)*) will be called MP.

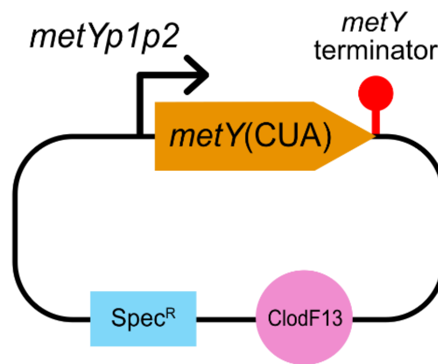

Figure 6. Amber initiator tRNA plasmid. The tRNA is encoded by the *metY(CUA)* gene, preceded by the native *E. coli* K-12 *metYp1p2* promoter and *metY* terminator. The plasmid encodes spectinomycin resistance (Spec^R) and contains a medium-copy ClodF13 origin.

To measure the effect of i-tRNA$_{CUA}$ expression on cellular growth, I ran growth curves overnight for the wild type C122 and MP strains. The MP strain grew identical to the C122 strain within error, with comparable timings for the log phase (Figure 7). These results confirm that expression of i-tRNA$_{CUA}$ does not interfere with growth and hence the MP strain is a suitable strain to use for infection model with amber start codon ϕX174 mutants.

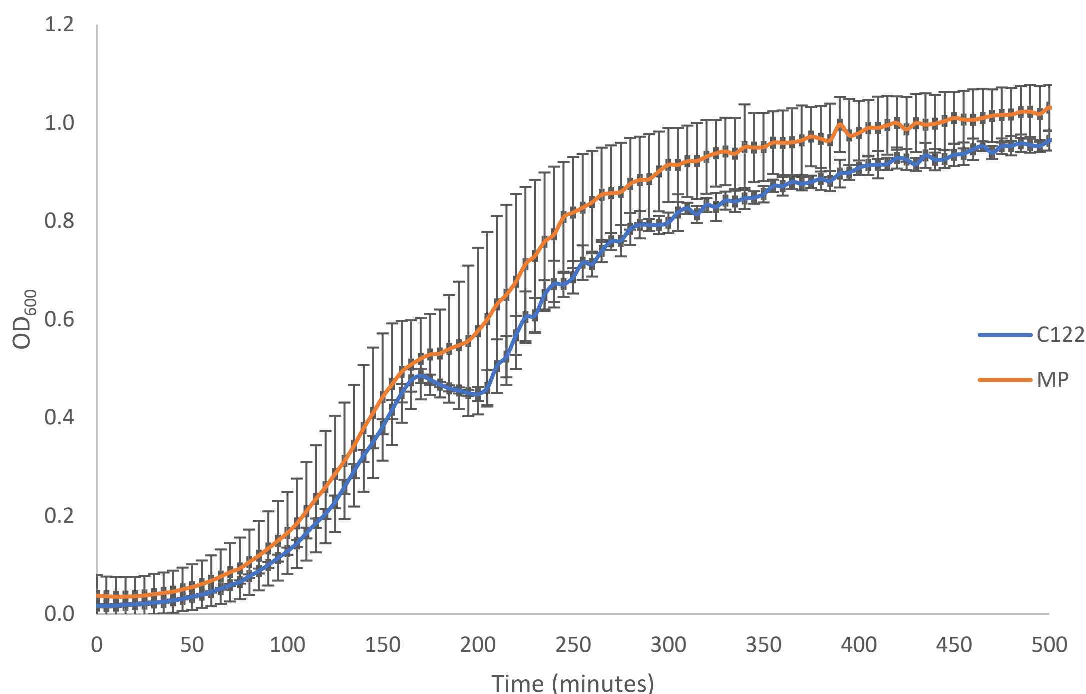

Figure 7. Comparison of C122 and MP growth strains shows no difference. A 10 µL aliquot of mid-log phase culture was diluted 1:100 into phage LB and incubated overnight at 37°C shaking at 200 RPM. Measurements of OD_{600} were taken at 5-minute intervals. It is expected that *metYp1p2* promoter is active throughout the growth cycle (n = 3).

To create an amber initiated mutant phage, I used site-directed mutagenesis to individually change the ATG start codons present at the beginning of the F, G, and H genes of WT ϕX174 to a TAG amber stop codon (Figure 8A). Following the ligation and subsequent purification of the mutant gene sequences with the WT ϕX174 genomes, I transformed the PCR products into competent MP strain and co-plated with a saturated culture of MP strain followed by a double agar overlay. Resulting plaques were used to infect C122 host lacking i-$tRNA_{CUA}$ to identify phage capable of producing plaques on MP strain but not on C122 (Figure 8B, C, D). Transformants that formed plaques on C122 were considered unsuccessful transformants (or wild type) as the mutant phage with amber stop codons in place of the usual start codons for F, G, and H genes would not be expected to create plaques in the absence of the amber initiator $tRNA_{CUA}$. Transformants passing this initial screen were sequence verified, amplified, and purified. I also attempted to construct F/G, G/H, F/H and F/G/H mutants from single mutants but was not able to recover any phage with these mutations after several attempts. Only single mutants were recovered as a result. This may indicate that the cost for phage to contain more than one amber initiated gene is

too high and as a result only single mutants can be created. I named the single mutant phages F.am, G.am and H.am (Figure 8).

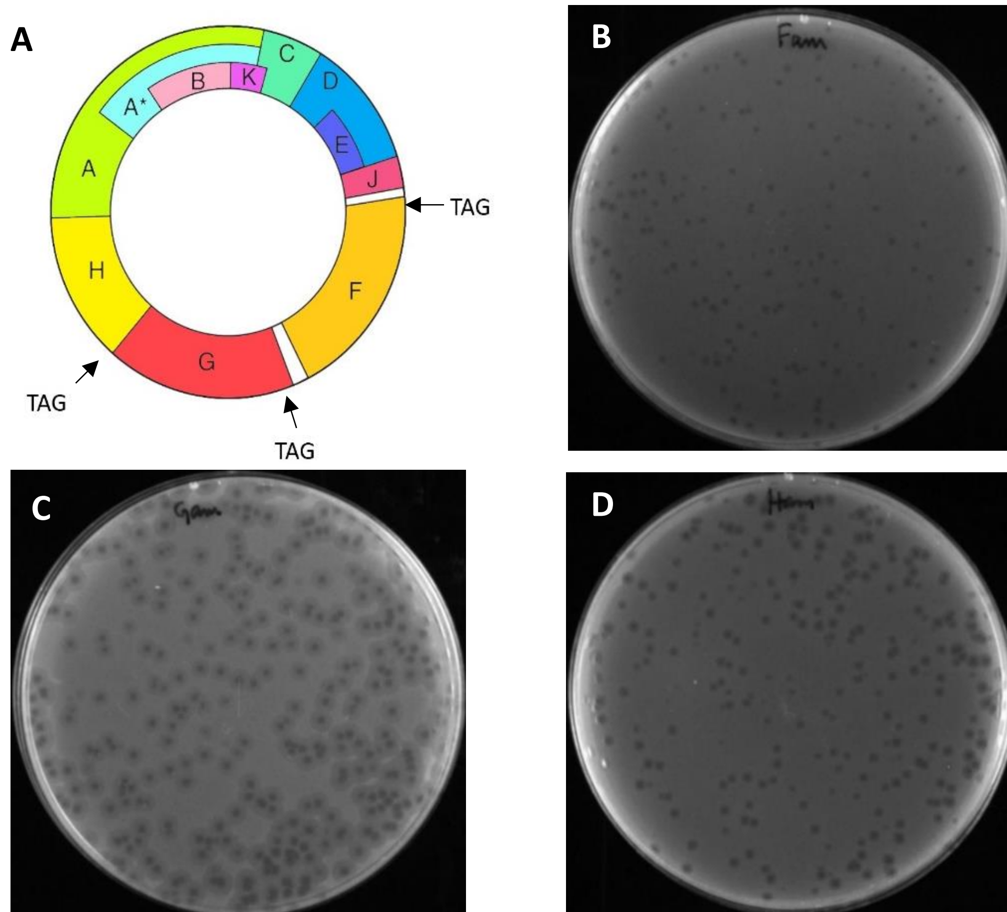

Figure 8. Mutant strains of F.am, G.am and H.am phages. Designed to contain an amber start codon at the beginning of the F, G or H genes (A). Mutant phage (B) F.am phage, (C) G.am phage and (D) H.am phage were only propagated when a double agar overlay was performed using a bacterial strain containing i-tRNA$_{CUA}$.

I next tested the growth characteristics of the mutant phages. Measuring the average diameter of plaques, F.am phage produced 1.45 mm, G.am phage produced 3.20 mm and H.am phage produced 1.70 mm.

I went forward further characterising the F.am phage as the F protein is the major capsid protein and lack of it would be expected to halt virion assembly at the earliest stage, giving the least chance for production of any particle containing nucleic acid to leave infected cells. I performed cell lysis curves across five multiplicity of infections (MOIs) and a no phage control on both the

wild type ϕX174 and F.am phages to measure replication efficiency. The phages were serially diluted and added to mid-log phase cultures of MP expressing i-$tRNA_{CUA}$ followed by measuring OD_{600} over 400 minutes (Figure 9). Wild type ϕX174 showed a rapid lysis of host cells, with the absorbance declining between 50 and 110 minutes (Figure 9A), similar to when infecting C122 lacking i-$tRNA_{CUA}$ (Logel and Jaschke, 2020). MOIs >0.01 proved to have the most efficiency of lysing with host cells reduced to a minimal absorbance between 100 and 130 minutes (Figure 9A). Regrowth of bacterial cultures were observed to enter log phase approximately 450 minutes after infection with WT ϕX174 (Figure 9A).

In contrast, F.am phage showed a slightly more delayed lysis time against the MP strain. Host cell growth began declining between 90 and 145 minutes, however MOI = 1 was the only concentration able to reduce absorbance back to baseline levels (Figure 9B). Furthermore, MOI = 0.0001 appeared to have no effect on bacterial cell growth with the rate and maximum absorbance comparable to that of uninfected MP (Figure 9B). Effective lysis was observed MOI >0.01 however saw the recurrent growth of bacterial cells at approximately 500 minutes (Figure 9B).

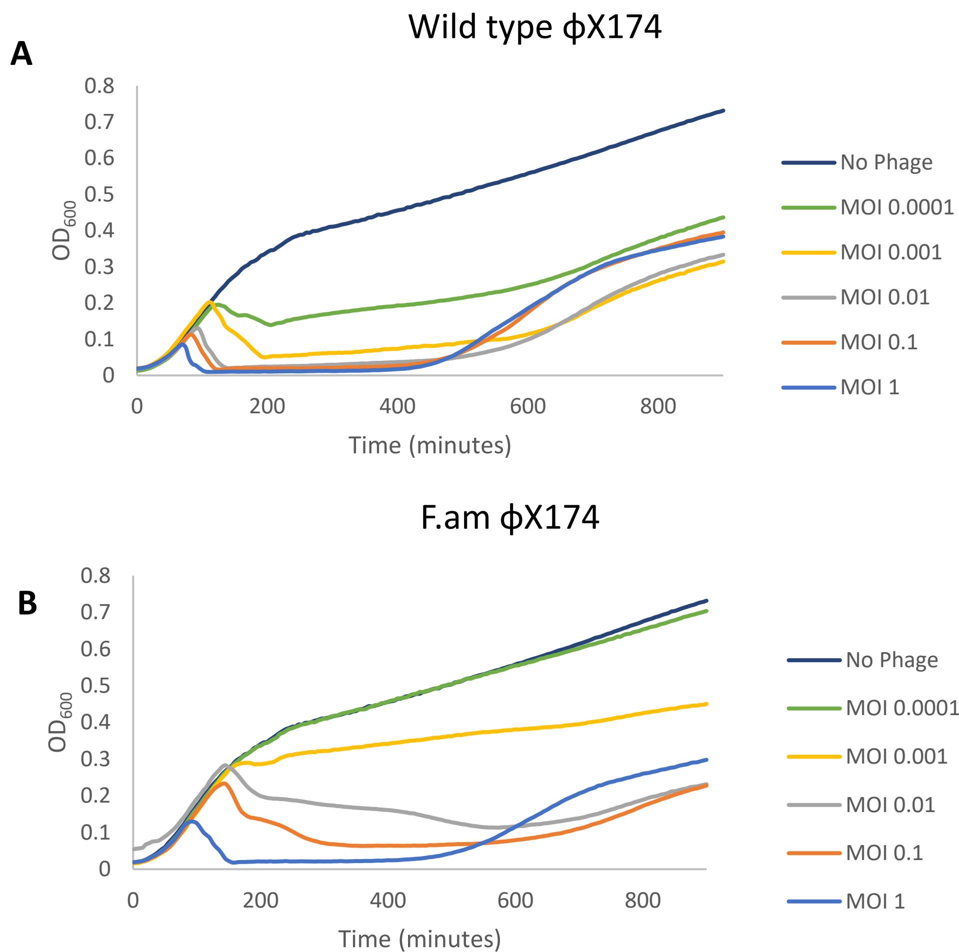

Figure 9. Lysis curve of WT ϕX174 and F.am phage shows the comparative lysis capabilities of the mutant phage. Phage was serially diluted in phage LB and added to a mid-log culture of the MP strain and monitored overnight. Cultures were incubated with shaking at 250 RPM, 37°C for 15 hours. Measurements of OD_{600} were taken at 5-minute intervals.

3.3 Comparison of the effects of antibiotics and non-replicating phage F.am on *E. coli*

As the aim of this work is to create a non-replicating phage suitable for deployment as a model therapeutic, the closest analogue currently in use would be antibiotics. To determine the relative sensitivity to antibiotics of the strains used in this study, I measured minimal inhibitory concentrations (MICs) for a range of antibiotics with either bacteriostatic or bactericidal properties (Table 4). Each of the strains tested were susceptible to expected published concentrations (EUCAST, 2020) apart from ROAR340 and Nissle 1917 (Table 4). Both strains exhibited an increased susceptibility to carbenicillin. Additionally, Nissle 1917 also showed a significantly increased susceptibility to chloramphenicol with the antibiotic becoming effective at the lower

concentration of 0.78 µg/mL (Table 4). The effectiveness of antibiotics on a molar concentration basis ranged from 2 to 33 nM.

Table 4. Minimum Inhibitory Concentration for strains tested against F.am phage.

	Antibiotic MIC (µg/mL)/(nM)		
Strain	CARB	CAM	STREP
C122	12.5/33	6.25/19	1.95/3
M1402	12.5/33	6.25/19	1.95/3
ROAR340	6.25/17	6.25/19	1.95/3
K-12	12.5/33	6.25/19	1.95/3
Nissle 1917	6.25/17	0.78/2	1.95/3

CARB = carbenicillin, CAM = chloramphenicol, STREP = streptomycin.

After determining the antibiotic concentrations that inhibited the *E. coli* strains used in this study, I next measured the MIC values of F.am phage against the same strains. F.am phage was used to infect host cells at varying MOIs under the same conditions as used in the antibiotic MIC experiment and was monitored overnight for trends in host growth. I found that growth of strain C122 was suppressed only at extremely high concentrations (MOI >700) of F.am phage (Table 5). To determine accurate MOI values for each replicate and strain, I plated 190 µL of the bacterial culture used to set up the MIC experiment on LB plates and counted the resulting colonies. M1402 and ROAR340 appeared to be less susceptible to F.am phage, requiring MOIs >7,000 to impede growth (Table 5). This result was tested by looking qualitatively at the efficiency of plating of ϕX174 on M1402 and ROAR340. When inoculated with WT ϕX174 on double agar overlays, M1402 and ROAR340 produced fewer plaques that were significantly smaller than those that formed on C122, suggesting ϕX174 is less virulent towards these strains. Measurements of M1402 efficiency of plating was 0.04 +/- 0.03 compared to C122, supporting this assessment.

To determine if the F.am phage was lysing host cells at these high MOIs due to action of the lysis protein E or were instead lysing cells "from without" due to external pressure from excessive phage adsorption (Abedon, 2011), I next tested MIC values against an *E. coli* C (C900) strain

containing a mutation in the *slyD* gene. The *slyD* mutation in C900 prevents lysis of the cell by ϕX174 due to increased E protein instability and degradation (Roof et al., 1994). The MIC measurements for C900 revealed similar results to that of C122. Growth was not observed between MOI 7,000-36,000, inhibited at MOI 700-3,600 and saw regular growth when MOI <360 (Table 5). These results imply that "lysis from without" is occurring whereby the host cell lyses due to abundance of phage binding to the external surface rather than the expression of the lysis protein.

Table 5. Growth of various strains tested against serially diluted F.am phage to determine MICs. Bacterial strains contained between 50-150 CFU/190 µL and were infected with 10-fold dilutions of mutant phage. Samples were incubated at 37°C overnight and OD_{600} measurements taken at 5-minute intervals and the end of 15 hours. Regular growth, inhibited growth and no growth were all determined in ranges of MOI infections. N/A = no clearance/ associated MIC value observed (n = 3).

	MOI			Molarity (fM)		
Strain	Regular Growth	Inhibited Growth	No Growth	Regular Growth	Inhibited Growth	No Growth
C122	<360	700 – 3,600	7,000 – 36,000	<0.014	0.83-0.84	>8
C900	<360	700 – 3,600	7,000 – 36,000	<0.019	0.83-0.84	>8
M1402	<3,600	7,000 – 36,000	N/A	<0.84	8.3-8.4	N/A
ROAR340	<3,600	7,000 – 36,000	N/A	<6	12-60	N/A
K-12	<36,000	N/A	N/A	<8.3	N/A	N/A
Nissle 1917	<36,000	N/A	N/A	<7.9	N/A	N/A

As the MIC values are only an endpoint measure, I next wanted to understand the dynamics of bacterial inhibition by F.am phage by monitoring growth over 15 hours after infection. Infecting various host cells at MOIs similar to the MIC experiment, bacteria-phage samples were incubated for 15 hours, with OD_{600} measurements taken every 5 minutes. C122 experienced a lag time of 5 hours for MOIs <30 which increased to 6.5 hours for MOIs ≥30 (Figure 10A). For the less susceptible M1402, MOIs <7,813 experienced lag times of 5 hours whilst MOI = 7,813 increased to 6.5 hours, similar to C122 (Figure 10B). Comparatively, the less susceptible ROAR340 exhibited a

4-5 hour lag time similar to that of the negative control Nissle 1917 strain (Figure 10C, 10D). These results suggest that lower MOIs do not inhibit growth phases over all susceptible strains.

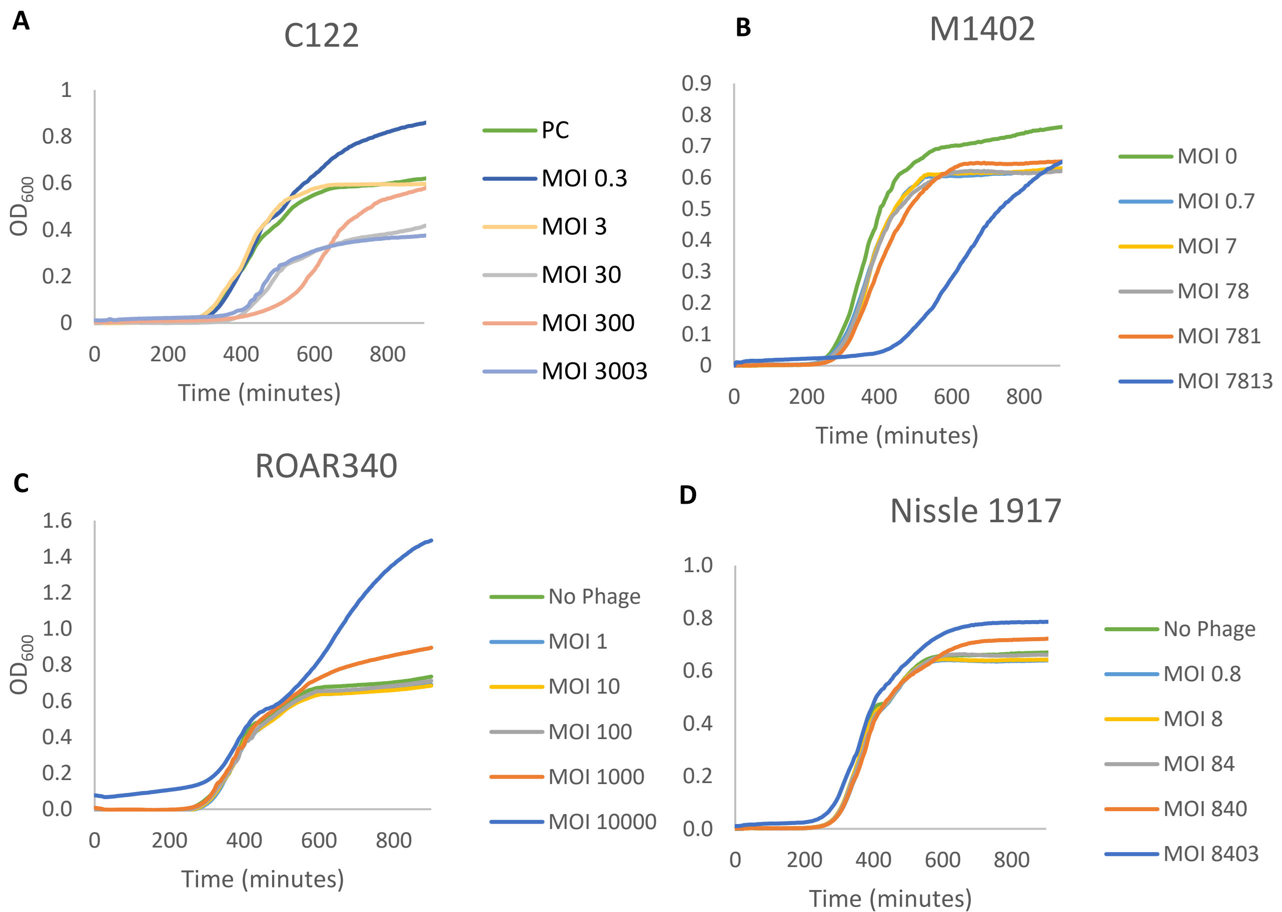

Figure 10. Representative growth curves for C122 and M1402 showed increased lag times for higher MOIs but showed no change for ROAR340 and Nissle 1917. Bacterial strains contained between 50-150 CFU/190 µL and were infected with 10-fold dilutions of mutant phage. Samples were incubated at 37°C overnight and OD_{600} measurements taken at 5-minute intervals.

3.4 Phage Revertants

A sample of the infected C122 was taken from the MIC experiment where no growth was evident (MOI = 3003) and used to infect fresh C122 and MP strains. Performing a double agar overlay, propagation of plaques on C122 would suggest the presence of revertant phage whilst plaques on the MP strain would suggest non-adsorbed viable F.am mutant phage were still present. Neither C122 nor MP plates produced plaques. These results imply that overnight incubation with 3.0 x

10^5 PFU does not produce any revertant phage under these conditions. Furthermore, it also suggests that all F.am phage within the assay were either irreversibly adsorbed to the host cells or had degraded.

3.5 Host Resistance

In order to identify the cause of growth suppression breakout after 6 hours of incubation in the phage MIC experiment, aliquots from the MIC experiment were taken from no growth wells and streaked out on LB agar. Fewer than 10 colonies grew over 4 replicates. When grown in liquid LB media, colonies tended to clump together, and agitation caused minimal dispersion throughout the media. Furthermore, growth was significantly slower than the F.am phage naïve C122. Aliquots of overnight culture were inoculated into fresh LB media and infected with WT ϕX174 for 4 hours. Bacterial growth was observed for both uninfected and infected samples suggesting that these slow growing C122 isolates had developed a resistance to ϕX174 infection.

3.6 Phage Stability

I next assessed the stability of the F.am phage over a 24-hour period in conditions mimicking those of the MIC experiment to see if virion viability loss over the long incubation could also contribute to the loss of growth suppression. I added F.am phage to phage LB and incubated at 37°C with samples taken at points between 2 - 24 hours. Double agar overlays were performed, and plaques enumerated. F.am phage maintained their stability between 2 – 6 hours but saw a 50% decrease between 6 – 8 hours (Figure 11). This decreased concentration was maintained until 24 hours post-incubation (Figure 11). These results suggest that the stability of the phage decreases between 6 - 8 hours at 37°C. This aligns with previous growth curves whereby bacterial growth of more susceptible strains began increasing 6 hours after initial infection with F.am phage (Figure 10).

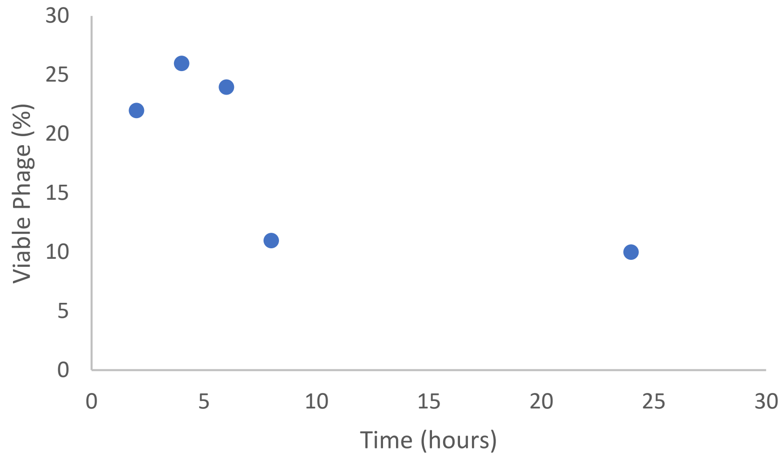

Figure 11. F.am phage shows a decrease in stability during incubation. An aliquot of F.am phage was added to phage LB broth and incubated at 37°C. A dilution series of plaque assays were performed at various points over 24 hours.

3.7 Phage Attachment Assay

Given the difficulty in identifying why the MIC experiments only cleared bacterial growth at extremely high phage concentrations and not at lower MOIs, I decided to interrogate the host binding capabilities of F.am phage. This would determine whether the TAG mutation interfered with capsid formation significantly enough to create virions with reduced attachment. I theorised that if the capsid structure were severely altered then the F.am phage would have a very low percentage of attached phage to C122 cells. Testing against C122 cells, I determined the percentage of attached phage for both WT and F.am phages at 5- and 10-minutes post infection. After incubation for the allocated times, unattached phages were subjected to plaque assays with C122 and MP strains respectively and enumerated.

Attachment after 5 minutes of incubation revealed wild type to reach nearly 100% binding capacity with only 1 plaque being produced amongst 4 replicates (0.0001% unattached). F.am phage showed 98% attachment (Figure 12). After 10 minutes of incubation no unattached phage were recovered for F.am phage, suggesting there was a 100% binding to C122 cells (Figure 12). This result implies that not only does the amber start codon not interfere with capsid formation and phage attachment to host cells, but also suggests that the capsid (which is mainly comprised of the F gene) remains comparable to that of WT ϕX174.

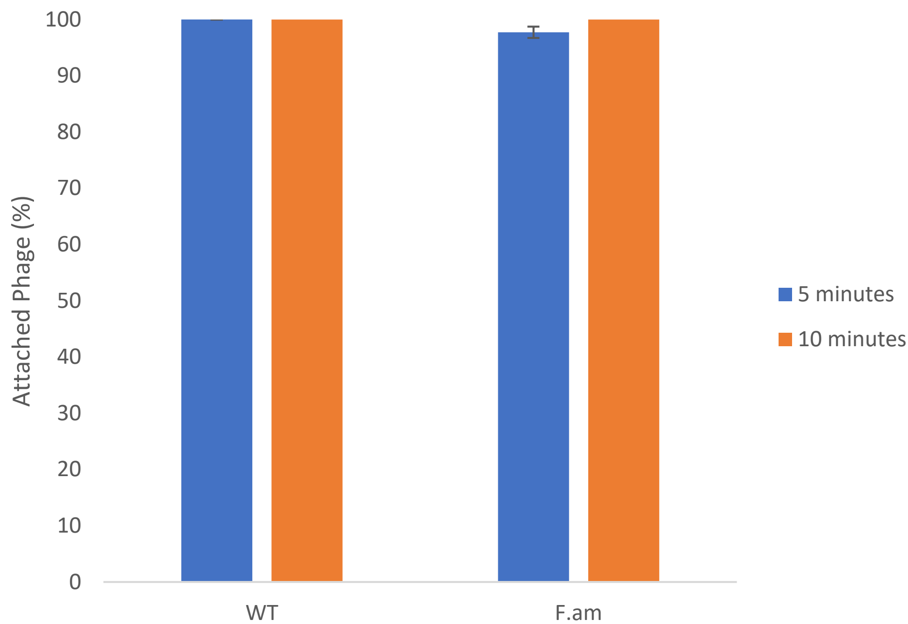

Figure 12. WT and F.am phage attachment to C122 cells. C122 cells were incubated at 37°C with either wild type or F.am phage for 5 minute or 10 minutes. The titre of unattached phages was then assessed via double agar overlay and plaque enumeration (n = 3, Std. Dev. WT = 0.005, F.am = 1).

3.8 RNA structures

Finally, to ensure that the mutant phages were not significantly altering the phage transcripts, they were analysed using NUPACK. The F.am phage showed a -1 kcal/mol difference when compared to wild type and a similar predicted base pairing, suggesting the inclusion of an amber initiator does not impact the stability of the RNA structures greatly (Figure 13). The G mutant however showed a smaller ΔG of -0.44 kcal/mol but significant changes in predicted base pairing as compared to wild type (Figure 13). These results imply that the RNA structure could be interfering with the translation of the amber initiated G gene. Finally, H.am phage showed no difference in base pairing or ΔG when compared to wild type (Figure 13).

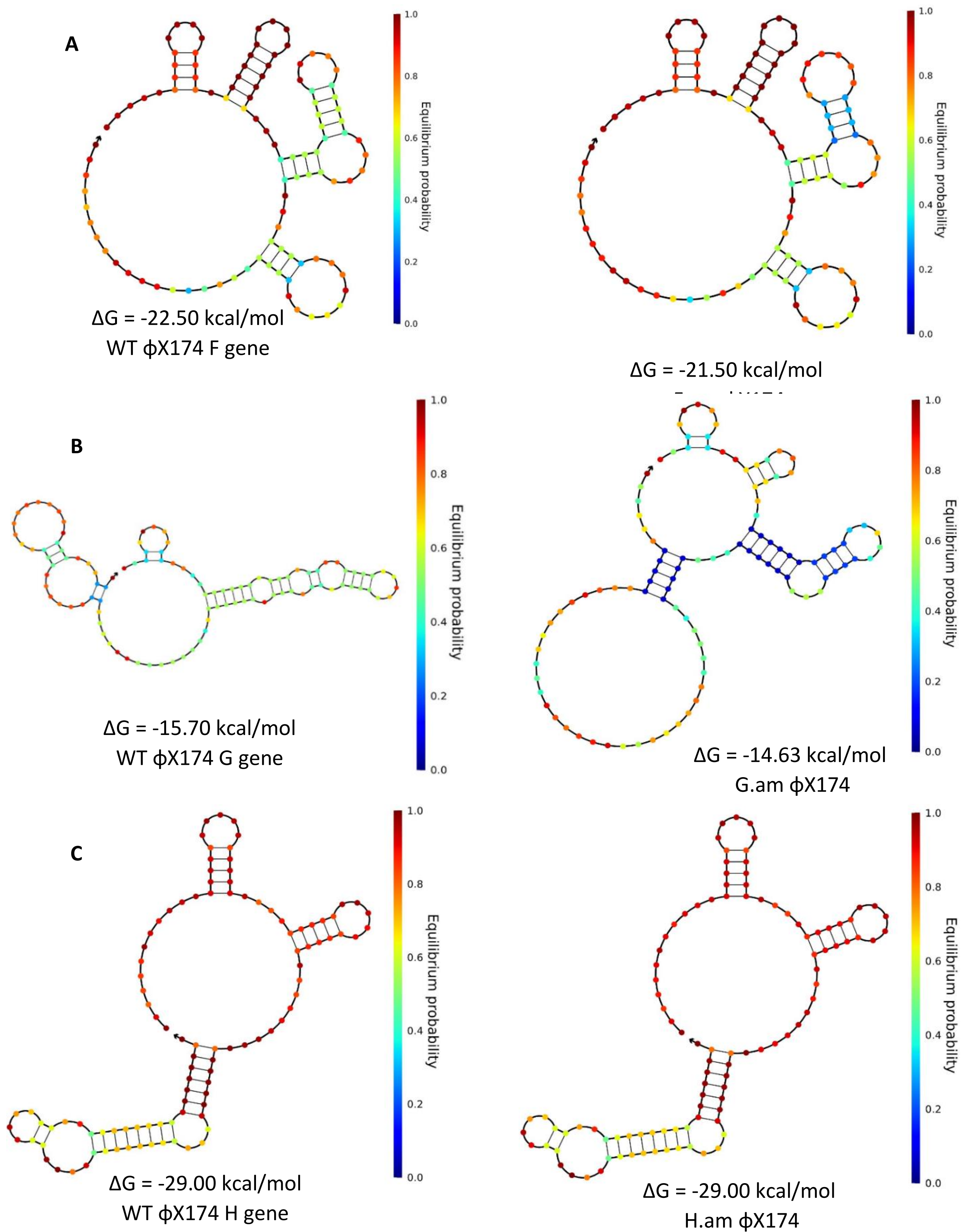

Figure 13. NUPACK RNA structures generated from an 83-nt window surrounding the start codons of genes F, G and H for WT and amber initiator mutants. (A) F.am and (C) H.am phages showed minimal structural changes whilst (B) G.am phage showed significant difference in structure.

Chapter 4: Discussion

Phage therapy is quickly becoming a much sought-after treatment for the growing number of antibiotic resistant infections developing worldwide. There is great potential for synthetic biology to enhance the speed and effectiveness of these therapies, but there is currently a fear around the accidental release of GMOs which prevents real world application – particularly in phage therapy.

In this thesis project I created a biocontained phage therapy model to address this problem. To do this I engineered the start codons of F, G and H capsid genes of phage ϕX174 from ATG to TAG, which resulted in phage that could only replicate in the laboratory host strain containing i-$tRNA_{CUA}$. I then characterised the F.am phage variant by determining the minimum inhibitory concentration (MIC) in comparison to antibiotics against a range of laboratory and environmental strains including M1402, ROAR340, K-12 and Nissle 1917. To further characterise F.am, I assessed stability of the phage and the attachment to *E. coli* host cells C122.

4.1 Potential effects of using amber initiation tRNA.

The use of the amber stop codon and associated amber suppressor tRNA has been an important tool to determine the function of genes, where it has been used in numerous phage experiments and assisted in developing an understanding of how phage work and which genes are responsible for given phenotypes (Denhardt et al., 1966; White and McGeoch, 1987; Cherwa et al., 2011). In 1990 the first amber stop codon suppressor was used as an initiator tRNA (Varshney and RajBhandary, 1990) and in turn created the potential for orthogonal systems in phage biology.

To the best of my knowledge, this work is the first demonstration of an amber stop codon being used at the beginning of phage genes to control replication through translation initiation of an essential gene. This has proven especially beneficial as a biocontainment method as ϕX174 has a constrained genome which is severely limited in the modifications it can endure. For this reason, employing the amber initiator system was a viable option. It would ensure that the phage could propagate properly as all biological constraints were maintained and supplementing the bacterial host with i-$tRNA_{CUA}$ would provide the proper scaffold for protein production of the mutated genes. This proved successful when changing the start codon of only one gene (Figure 4) however during this work I was not able to obtain double and triple mutants. A study conducted on ssRNA phage MS2 suggests that viruses have a selective pressure to minimise the presence of UAG

codons in their genome when UAG codons are absent from host cells (Ma and Issaacs, 2016; Ivanova et al., 2014). If this selective pressure is enacted upon in my mutant phage, then this observation could explain my failure to engineer double and triple ϕX174 capsid mutants. Alternatively, another explanation as to why only single mutants could be engineered would relate to competitive binding of the UAG start codon with Release Factor 1 (RF1) as opposed to the intended i-$tRNA_{CUA}$. As RF1 is naturally found to recognise UAG codons and signals translation termination through the addition and hydrolysis of the highly conserved GGQ motif (Zavialov et al., 2002), then competitive binding to the amber start codon on mutated ϕX174 transcripts is a possibility. This RF1 binding would interfere with regular translation of the phage components and hence alter the amount of protein produced. Consequently, incomplete proteins may then be degraded by host proteases preventing the formation of viable phage capsids.

It is unlikely however that the reason the phage is not proliferating as expected is due to an inadequate expression of amber start genes. Given that C122 has been shown to express amber initiated genes to a high degree of efficiency with protein expression resulting in comparable quantities to those expressed under the canonical AUG start codon (Vincent et al., 2019), the use of this orthogonal system should not be a limiting factor in protein production. Additionally, i-$tRNA_{CUA}$ expression does not inhibit cell growth in orthogonal systems (Vincent et al., 2019). Furthermore, all off-target initiation should be limited or nearly completely absent as has been shown in a previous study (Vincent et al., 2019). Off-target translation from an amber start codon requires Shine-Dalgarno sequences that typically range 3-15 nt and are present right before the start codon, allowing the ribosome to bind (Shine and Dalgarno, 1975). Given the small size of the F.am genome, it is unlikely that these sequences will be present prior to a UAG stop codon and hence it is unlikely that off-target translation would occur. Additionally, a previous study has shown that even in the presence of Shine-Dlagarno sequences, off-target translation of proteins from *E. coli* was not observed (Vincent et al., 2019). Thus, it can be suggested that the amber start codon may be influencing the copy number or translation capabilities of the phage transcripts, however off-target translation is not likely to occur.

Finally, there has been mixed evidence about whether a glutamine or a methionine is found at the N-terminal of an amber initiated protein (Vincent et al., 2019, Govindan et al., 2018; Varshney and RajBhandary, 1992). Whilst methionine is typically incorporated in canonical translation initiation, if a glutamine is incorporated instead this may have structural implications. Insertion of glutamine

could result in some steric hindrance or unfavourable amino acid interactions which interferes with the protein's integrity due to the differing polarity of the two amino acids. Additionally, inclusion of a glutamine amino acid at the N-terminal of any of these proteins could also affect the stability of the protein (Kunjapur et al., 2018). Current studies have shown that non-canonical ammino acids (ncAAs) incorporated at the N-terminus can be destabilising in nature, which in turn affects the efficiency of the orthogonal translation system employed (Kunjapur et al., 2018). This destabilisation results in rapid degradation of the protein. If this were the case for the F.am, G.am and H.am phages, then assembly of viable progeny would take longer or may not occur at all. If, however there were a methionine added to the N-terminal of these proteins then stability would be the same as wild type produced phage. It should also be noted that various amino acids have been located at the N-terminus of proteins expressed within bacteria and the resulting function appeared uninhibited (Tharp et al., 2020). This is not to say the incorporation of alternative or ncAAs do not hinder phage function, however it is an unlikely explanation if stability is maintained, and a glutamine is added instead.

4.2 The strength and efficiency of F.am lysis.

Typically, antibiotic breakpoints are assessed through conventional means of either a disk or a liquid broth in accordance with EUCAST guidelines (EUCAST, 2020). Those that contain antibiotic resistance can be categorised due to their lack of clearance above certain concentrations. Phage MICs however are more difficult to determine. Given their replicative nature within host cells, phage have a continuous cycle that exponentially produces more progeny capable of infecting naïve host cells. This biocontainment model aims to prevent the replicative abilities of the phage, thereby making it slightly easier to determine what a potential MIC may be. My MIC measurements of F.am phage showed decreased efficiency to wild type phage against C122 strain with high MIC values, which it would be expected to most easily adsorb to (Table 5) and infect. The results showed that growth could be inhibited for 5-6 hours (Figure 9), similar to the wild type ϕX174 which is able to replicate. This implies that the loss of growth inhibition is due to *E. coli* C122 host cells developing resistance. An additional factor that may increase the likelihood of growth inhibition loss is the degradation of the virions over the assay, which showed to be a 50% decrease between 6 - 8 hours post-incubation (Figure 10).

Monitoring the MICs overnight, it was revealed that the less susceptible strain M1402 and ROAR340 were only inhibited by the highest concentration of F.am phage (Table 5). ROAR340

occasionally showed no inhibition when infected with a high MOI (Figure 10). This is indicative of the lower susceptibility of these strains as high concentrations of WT ϕX174 were only able to produce smaller plaques when susceptibility of these strains were tested in a double agar overlay. Although ROAR340 and M1402 are known host strains of ϕX174 with susceptible core LPS types (Michel et al., 2010), it can be assumed that these core LPS sequences differ sufficiently resulting in decreased virulence of the phage. For this reason, it can be assumed that the host range of the phage used is very important as a biocontained therapy will prove ineffective if it does not adequately target the pathogen. Additionally, the K-12 and Nissle 1917 strains tested are not susceptible to infection by ϕX174 and depicted regular growth regardless of the MOI they were infected with (Table 5, Figure 10). This result implies that bacterial growth is inhibited as a result of specific receptor recognition, and as such supports the notion that phage resistance develops as a result of the mutation of receptors. Regarding Nissle 1917, growth of this strain is especially important contextually as it is frequently used as a probiotic due to its antagonistic nature with pathogenic bacteria (Sonnenborn and Schulze, 2009). This result implies that biocontained phage therapy has the potential to target specific bacterial strains without affecting commensal microbes.

Prior to this work I expected that an MOI of 1 or at most 10 would be sufficient to clear all viable bacteria in my MIC assays. This did not occur and instead, growth inhibition required MOIs of at least 70-fold higher. Using the lysis resistant C900 *slyD* strain I showed that the reason for this unexpectedly low efficiency is likely due to the lack of protein E expression or of the inability of F.am to inject its genome. This is because the growth inhibition of C900 was the same as that of C122 whereby lysis only occurred at the highest MOI (Table 5). Given that C900 cannot be lysed due to protein E expression (Roof et al., 1994), it can be assumed that the "lysis from without" mechanism is occurring. "Lysis from without" refers to the lysis of bacterial cells due to an extremely high degree of phage binding to the external membrane and hence exerting an immense extracellular force on the cell and is commonly observed at MOIs >100 (Abedon, 2011).

Injection of the phage genome into the host cell is essential in allowing ϕX174 to lyse a host cell. This DNA penetration occurs by passing through the tube formed by the H-protein (Sun et al., 2014) . It is therefore unlikely that a two base mutation at the beginning of the F gene would influence this process, however small mutations in the ϕX174 genome have previously been observed to alter the biophysical properties of virions (Hafenstein and Fane, 2002). Although I showed that F.am phage could attach to C122 cells with similar efficiency to wild type ϕX174

(Figure 12) I did not directly test the ability of F.am to inject its genome into host cells lacking the i-$tRNA_{CUA}$. Future experiments to confirm this would be required, but my experiments with infecting the C122 strain expressing i-$tRNA_{CUA}$ show that F.am is able to inject its DNA into the host cytoplasm, although perhaps at lowered efficiency to wild type ϕX174, as shown by delayed lysis curves (Figure 9).

Although it was not tested in the scope of this project, confirmation of gene E expression would be the next obvious experiment. To do this, I would begin by transforming C122 cells with ϕX174 DNA containing an amber mutation in the E gene (E.am) along with another condition where I transform F.am phage gDNA and positive control WT ϕX174 DNA. Comparison of these different conditions would show whether F.am expressed protein E. Incomplete or incorrect expression of protein E would result in the growth of C122 cells, as would be observed in E.am sample while no growth would suggest a completely functional protein. This would allow me to compare observations with C122 cells containing the F.am phage genome. As it is assumed that protein E is being produced by the F.am phage DNA, I would expect to observe lysis or limited growth from these samples. Additionally, regular growth would be expected from cells containing E.am DNA as the lytic properties of the phage have been disrupted. Overnight monitoring would allow for a definitive answer regarding whether the production of the lytic E protein in F.am phage is occurring.

Antibiotics are inherently labelled as either bactericidal or bacteriostatic in nature based on their modes of lysis (Wald-Dickler et al., 2018). In choosing three different antibiotics, both categories could be compared to the lysis ability of F.am phage. Chloramphenicol is a bacteriostatic antibiotic capable of binding to the 50S ribosome to prevent the translation of proteins. Carbenicillin is a representative of the penicillin group and is a bactericidal beta-lactam capable of inhibiting cell wall synthesis. Streptomycin was chosen as it is a bactericidal aminoglycoside that causes a translational frameshift ending in premature termination. It is commonly observed that the bacteriostatic antibiotics require a higher concentration to completely lyse cells (Wald-Dickler et al., 2018) which is reflected by chloramphenicol and streptomycin MIC values (Table 4). Carbenicillin however required the highest concentrations suggesting it is a weaker antibiotic. This has been observed in previous *E. coli* studies (Sulavik et al., 2001). MIC concentrations appeared in the nanomole region (Table 4). Comparatively, concentrations for F.am phage clearance appeared six orders of magnitude less with concentrations in the femtomole region (Table 5). This

result suggests that biocontained bacteriophage are for more efficient lysing agents than antibiotics and provides further proof to their use as an adjuvant treatment.

ΦX174 has a number of different promoters and terminators which vary in strength (Logel and Jaschke, 2020). Consequently, ϕX174 will produce a differing amount of transcripts which vary in length, thereby allowing a varying concentration of the subsequent proteins to be produced. Gene E is present on one of the most abundant transcripts in ϕX174 but does not produce excessive amounts of protein due to a poor ribosome binding site (Blasi et al., 1990; Maratea et al, 1985). As terminator read through is indeed an important factor in the production of various proteins (Hayashi et al., 1981), if RF1 is indeed outcompeting the i-tRNA$_{CUA}$ then there is a possibility that this is causing the decreased killing efficiency through a lack of adequate/stable protein E production. Furthermore, the J-F intergenic region provides some stability to transcripts (Hayashi and Hayashi, 1985). If there is a premature termination at the F site due to the presence of the non-canonical UAG start codon sequence, then this may also affect transcript stability. I could test this hypothesis in future with RNA-seq or qPCR measurements of transcript levels across wild type ϕX174 and F.am phage (Logel and Jaschke, 2020; Brown et al., 2010; Zhao et al., 2012)

4.3 Potential for Host Resistance

Colonies that grew from the highest cleared MOI samples (Table 5) showed a strong selection for slow growth and aggregation suggesting a change in the LPS structure. If a genetic analysis of these resistant colonies were to be performed, it is quite possible that mutations within the *rfaB*, *rfaC, rfaD, rfaE, gmhB* and *lpcA* genes would be observed (Michel et al., 2010). Specifically, *rfaC*, *rfaE* and *lpcA* are known to confer a deep rough phenotype due to an altered formation of the LPS which allows ϕX174 to infect them (Bauer and Welch, 1997; Austin et al., 1990). Mutations in these genes could explain the clumping nature of bacteria isolated from the higher MOI samples and the phage resistant behaviour observed. Additionally, mutations within the *mraY* genes are known to confer a resistance to ϕX174 infection (Chamakura and Young, 2019) and hence may also influence the resistance of the *E. coli*. The notion of phage resistant *E. coli* growing throughout lysis is also supported by the lysis curves performed using both WT and F.am phage (Figure 9). Regrowth was observed 6 hours after infection for all MOIs tested suggesting these prior-mentioned mutations are quite likely to occur in a short period of time from populations as small as 10^5 cells.

4.4 Potential for revertant phage

In this work I did not observe any revertant F.am phage despite infecting 100 cells with over 10^6 phage numerous times. The theoretical reversion frequency of F.am phage is based on a mutational rate of 0.81 mutated phage per infection. Given one base change is needed to convert TAG to TTG, one of the strongest and most common start codons of *E. coli* (Hecht et al., 2017), a total of 150, 390 revertants per 10^8 PFU used is to be expected. The fact that I did not observe any revertants points to the notion that the biocontainment model works on a small scale. Despite not seeing any revertants in this work it is expected that they would be inevitable given larger *E. coli* population sizes. As such, to avoid this, it has been suggested that multiple biocontainment safeguards be implemented in any GMO, with three to four layers giving the lowest possible escape probability (Gallagher et al., 2015). In my case I would create double and triple amber initiated mutants to achieve this. This is especially important given that phage and bacteria are constantly co-evolving and hence may undergo horizontal gene transfer events in phage which are not recoded (Ma and Isaacs, 2016) and whilst ϕX174 is not known to easily recombine with heterologous DNA, these gene transfers remain a potential concern.

To further confirm the rate of revertants and calculate the potential escapees, I would perform two experiments. The first would involve applying a very mild selective pressure by inoculating the F.am phage in the presence of i-$tRNA_{CUA}$ and allowing it to incubate for 6-8 hours. Every hour a double agar overlay would be performed using C122 and MP strain cultures. Any plaque formation on C122 would be indicative of start codon reversion. I expect that revertants would appear close to six hours after infection as the native i-$tRNA_{CAU}$ is still being expressed and can consequently recognise the canonical AUG start codon. The second option would be to infect a much larger population of *E. coli* C122 lacking the i-tRNA. This applies a stronger selective pressure as there is no i-$tRNA_{CUA}$ present to recognise the amber start codon and hence reversion to a canonical AUG or UUG start codon would be essential for phage survival. Performing a plaque assay on C122 culture every hour would reveal the rate of reversion and a revertant would be expected sooner than the previous method described. Furthermore, if double or triple mutants were to be produced then I suspect the reversion rate would decrease as has been suggested by other biocontainment models and inferred by my calculations (Gallagher et al., 2015; Agmon et al., 2017).

4.5 Does the F.am phage efficiency decrease due to stability or attachment?

To maximise usage outside a laboratory environment, biocontained phage must not lose core properties which in turn substantially decrease their efficacy. As engineering phage for therapeutic uses is still in its infancy, it is imperative to ensure that inclusion of amber start codons does not decrease the *in vitro* stability of the phage, or their ability to bind to the target bacteria. This is especially important as F.am phage is a non-replicating phage in wild-type *E. coli* and there have been studies which show that the killing efficiency of a phage is decreased when it is unable to replicate (Moradpour et al., 2009; Krom et al., 2015). Characterisation of F.am phage resulted in the relative stability of the mutant phage over a 2-6 hour period before a 50% decrease in viability was observed (Figure 11).

The RNA sequence of the F.am phage mutant was also processed through NUPACK and compared to the wild type F gene to ensure that the structure was not altered by the presence of the amber start codon. There was no formation of hairpin loops (Figure 13) and there was only -1 kcal/mol difference in structures which suggests only a slight increase in transcript stability.

Attachment assays to C122 are near comparable to those observed using wild-type ϕX174 (Figure 12), which suggests that the binding capabilities of F.am phage to host cells are only slightly decreased. Whilst the G protein predominantly recognises the LPS of the host cells (Sun et al., 2017), the F protein of ϕX174 assists in the irreversible binding of the phage (Sun et al., 2017). The F protein assists by creating loops by which the phage may interact, however the N-terminal region remains embedded inside the viral structure (Sun et al., 2017). If a significant decrease in binding ability were observed, it can be assumed that there was a change of structure in the N-terminal region of the phage which hinders its ability to attach. As the percentage of unattached F.am phage measured was only 2% (SD = 1%) as compared to the wild type 0.0001% (SD = 0.5%), it can be assumed that any structural changes that may have occurred due to the amber start codon did not decrease adsorption.

4.6 What can this be used for?

As previously discussed, biocontainment is very important for ensuring that escape of GMOs does not negatively impact native ecosystems. I have created a model that decreases the probability of escape whilst maintaining the least mutations possible, making it highly versatile. Application of this model could be used for numerous phage experiments, but most widely in scenarios where naturally isolated phages capable of infecting a pathogenic strain are lysogenic in nature. Studies

have shown that deactivating the lysogenic component of a temperate phage can cause it to maintain a lytic cycle (Paul et al., 2011). To ensure that integration of the genome does not occur the amber initiator model could be used in a similar scenario without applying too great of a selective pressure on the phage.

Additionally, in the event that only broad-spectrum or wide host range phage need to be used (engineered or naturally isolated), engineering a TAG amber start codon at the beginning of an essential gene will also ensure that phages are not active in environments such as the gut microbiome. This will decrease the possibility of pathogenic species outcompeting commensal bacteria and allow the virome to remain virtually unscathed.

4.7 Conclusion

In this study I designed a biocontainment model suitable for phage therapy which saw the employment of an orthogonal translational control. To my knowledge, this was the first time that the canonical ATG start codon was changed to an amber initiated TAG start codon at the beginning of capsid genes within phage. In changing the beginning of the capsid genes F, G and H, all other genes remained functional, allowing for the progression of the phage cycle. The phage cycle will halt in the absence of amber initiator tRNA which suppresses the TAG stop codon. I have shown that the amber initiator mutation creates a phage that is able to lyse cells at high concentrations whilst maintaining its stability and attachment properties, although lysing ability was decreased from that of wild type ϕX174 phage. This study provides a platform technology which can be further build on, allowing engineered phage therapy to thrive and as a result, become an accessible treatment for those suffering from antibiotic resistant infections.

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