Broadening the host range of lytic phage against Methicillin-resistant *Staphylococcus aureus*

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Declaration

I, Eimear Ní Mhaoldomhnaigh/Moloney, do hereby declare that this thesis is my original work, has been written by me in its entirety and has never been published and/or submitted for reward to any other institution before.

Signature

Date

Acknowledgements

Firstly, I would like to express my sincere gratitude to my advisor Prof. Paul Ross for this opportunity and the support and guidance he extended to me during the duration of this thesis. Secondly, I would like to acknowledge the help and guidance of Prof. Colin Hill and all those in lab 3.43.

List of Abbreviations

Abi	Abortive infection
AIDS	Acquired immune deficiency syndrome
ATP	Adenosine triphosphate
BHI	Brain Heart Infusion
BIM	Bacteriophage insensitive mutants
Cas	CRISPR associated system
CDC	Centre for Disease Control and Prevention
CFU	Colony forming unit
CRE	Carbapenem-resistant Enterobacteriaceae
CLSI	Clinical and Laboratory Standards Institute
CRISPR	Clustered regularly interspaced short palindromic repeats
DNA	Deoxyribonucleic acid
DSDNA	Double stranded DNA
dTMP	Deoxythymidine monophosphate
EOP	Efficacy of plaquing
GRAS	Generally recognised as safe
GTP	Guanosine-5'-triphosphate
HGT	Horizontal gene transfer
HIV	Human immunodeficiency virus
HNH	Homing endonuclease
HPA	Health Protection Agency
IgM	Immunoglobulin M
IgG	Immunoglobulin G
LPS	Lipopolysaccharide
LPSS	Lipopolysaccharide Smooth

LPSR	Lipopolysaccharide Rough
MDR	Multidrug Resistant
MOI	Multiplicity of infection
MRSA	Methicillin-resistant Staphylococcus aureus
OD	Optical Density
Omp	Outer-membrane protein
PEG	Polyethylene glycol
RBP	Receptor-binding protein
RM	Restriction Modification
RNA	Ribonucleic acid
RNase	Ribonuclease
SDS	Sodium lauryl sulphate
SIE	Superinfection exclusion systems
TSB	Tryptic Soy Broth
VRDB	Virulence factor database
VRE	Vancomycin-resistant Enterococcus faecium
WHO	World Health Organisation
WTA	Wall teichoic acids

Aim of Thesis

The specific aims of this thesis were to characterise two lytic phage, phage K and Phage B1, to increase their host range and thus enable them to infect and kill a wider range of pathogenic staphylococci.

Abstract

Introduction

In recent years the rise in antibiotic resistance in pathogenic bacteria has meant that significant research has gone into finding alternative treatments. The higher mortality and morbidity associated with multidrug resistant bacteria suggest that solutions are urgently needed. The World Health Organisation (WHO) recently published a list of 12 bacteria which are considered the greatest threat in this regard (a list which includes drug resistant *Staphylococcus aureus*). Unless a viable alternative to antibiotics is found global human health will suffer and we may see a return to mortality and morbidity rates similar to those seen in the pre-antibiotic era. Despite the development of vaccines, and improved hygiene and living standards, bacterial infections remain a very real treat. In particular the developing world is most at risk of unchecked antibiotic resistance disease due to insufficient access to health care, overcrowding and decreased water sanitation.

Objectives

Bacteriophage targeting of specific pathogens may provide an alternative to antibiotic therapy in certain clinical settings. However specific issues may limit their use. The high specificity of phage for their host makes them potentially impractical for therapeutic use, without first identifying the causative agent/strain and then having access to a phage that infects it. The current study therefore investigated the factors that affect phage specificity for *S. aureus* isolates which vary at the strain level. The study also investigated whether bacteriophage populations can evolve to overcome bacterial defence mechanisms and whether it is possible to use this ability to increase or shift host ranges. We hypothesize that p cocktails of phage targeting different hosts may be the solution to overcoming narrow host ranges of individual phage. In numerous previous studies phage cocktails have been shown to

have success in treating infections (O'Flynn *et al.,* 2004; Gu *et al.,* 2012; Chan & Abedon, 2013; Örmälä & Jalasvuori, 2013; Niu *et al.,* 2014; Chadha *et al.,* 2016;).

Methods

In this study the host range of the phage was characterized by plaque assay, the anti-biotic resistance profile of the bacterial sample set examined by agar disc diffusion method and a method was used whereby MRSA phage were co-cultured with susceptible and un-susceptible strains of MRSA of clinical importance. Following the generation of mutants in Phage B1 and phage K with favourable adaptations to host range, genetic sequencing was carried out and hypothesis proposed as to what changes lead to the altered host range.

Results

It was found that after prolonged exposure phage evolved to be able to infect formerly non-susceptible strains. We analysed the host range of a novel staphylococcal phage, Phage B1 in comparison with reference phage commonly used in the literature. The study established key parameters such as the exposure time required and the ratio of target strains: permissive strains that provided for optimal phage adaptation. Some of the mutants had the ability to infect previously insensitive strains and others showed increased efficacy. In practical therapeutic terms, this would mean that it is possible to rapidly select adapted phage isolates to target previously non-permissive pathogenic bacterial strains. Sequencing of the original phage, phage B1 as well as the mutated/adapted phage derivatives, phage B1 0.0066 and phage B1 3488 revealed insights into genomic variation of phage during adaptation to previously non-permissive hosts. Acquisition of specific genes (including genes encoding alternative phage tail proteins) could explain altered host infection potential in the mutated/adapted phage, although further work is necessary to associate altered genes with functional properties of the phage.

Conclusion

The current study established the host range of a novel staphylococcal phage, phage B1 which has potential to be used in phage therapy. Whilst some *S. aureus* isolates were resistant to targeting by phage B1 we established conditions under which we could rapidly select for phage variants to target such strains. Genomic analysis of both phage B1 and phage variants (phage B1 0.0066 and phage B1 3488) provided molecular insights into the process

of phage adaptation. Overall, the results suggest the potential for phage adaptation and rapid isolation of new phage variants in the clinical setting.

1. Introduction

1.1 Introduction to phage therapy

Bacteriophage were first described in 1915 and 1917 by Frederick Twort and Félix d'Hérelle, respectively. Despite being discovered before antibiotics their use as therapeutics has not been common outside of certain parts of Eastern Europe and Russia and have rarely been used in Western countries (Shasha *et al*., 2004; Lin *et al.* 2017). Phage therapy is a term used to describe the use of bacteriophage to treat bacterial infections, essentially "infecting the infectious entity". Phage therapy became popular in certain countries even before their modes of action were even remotely understood (Chan, *et al.,* 2013).

Bacteriophage are viruses defined by their tail morphology which infect bacteria. They are abundant in the biosphere and are estimated to outnumber bacteria tenfold (Brussow & Hendrix, 2002). The order *Caudovirales* accounts for 96% of all known bacteriophage, the remaining 4% either belong to the order *Ligamenvirales* or another not yet assigned order (Ackermann, 2009). The *Caudovirales* are divided into three families according to tail morphology: *Myoviridae* (long contractile tail), *Siphoviridae* (long non-contractile tail) and *Podoviridae* (short non-contractile tail) (Niu *et al*., 2014).

Phage therapy is being re-examined in the light of the increasing emergence of antibiotic resistant bacteria such as Methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococcus* (VRE), and multidrug-resistant *Mycobacterium tuberculosis* (MDR-TB), means that new therapies, for example, the use of nanoparticles (Rudramurthy *et al.,* 2016), for the control of bacterial infections are urgently needed to avoid returning to the "pre antibiotics" era and associated mortality (Sulakvelidze *et al.,* 2001, Pincus *et al.,* 2015; Lin *et al.,* 2017). The use of and overuse of antibiotics may also have a detrimental effect on the microbiome (Nilsson 2014; Pincus *et al.,* 2015; Langdon *et al.,* 2016). The World Health Organisation (WHO) recently ranked 12 antibiotic resistant bacteria according to their threat to human health globally, with the aim of focusing research and

development on these as a priority. They are classified in order of priority, critical, high, and medium, with MRSA being considered high risk (Kahlmeter & Singh, 2017). According to a review by The Infectious Diseases Society of America MRSA is now responsible for more deaths than HIV/AIDS and tuberculosis combined (Boucher, *et al.,* 2009).

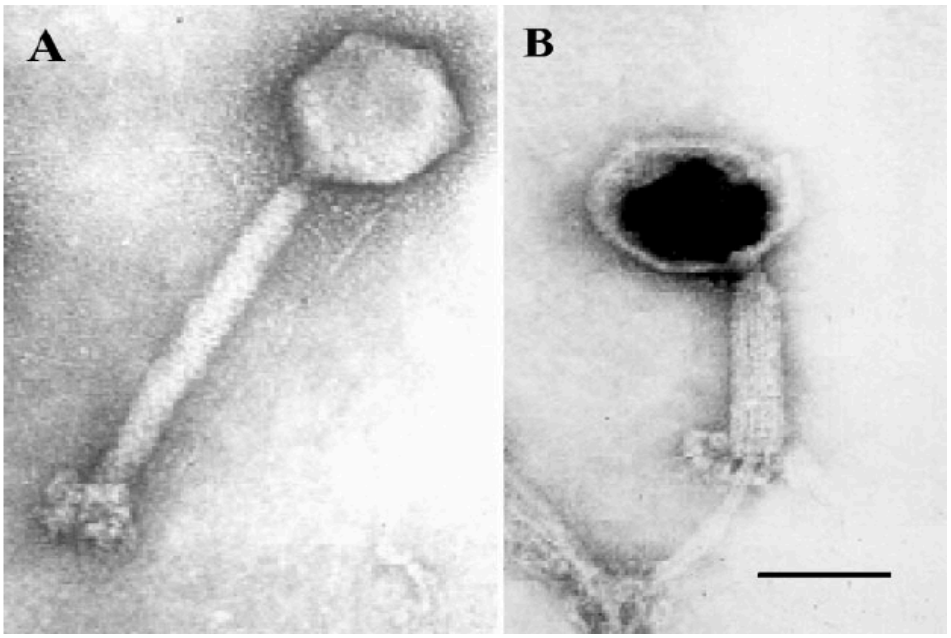


Figure 1.1 Electron micrograph images of phage K. (A) Phage K with a contractile tail. (B) Phage K with tail contracted and empty phage head. Scale bar, 100 nm (O'Flaherty *et al*., 2004).

Phage therapy is a viable alternative to antibiotics although its mode of action differs considerably from other antimicrobials. Thus, bacteriophage are once again gaining attention for their potential as alternative therapeutic agents against infectious disease (Smith & Huggins, 1982, 1983; Merril *et al.*, 1996; OFlynn, 2004; O'Flaherty *et al.,* 2005, 2009; Mattey & Spencer, 2008; Ly-Chatain, 2014; Lin *et al.,* 2017).

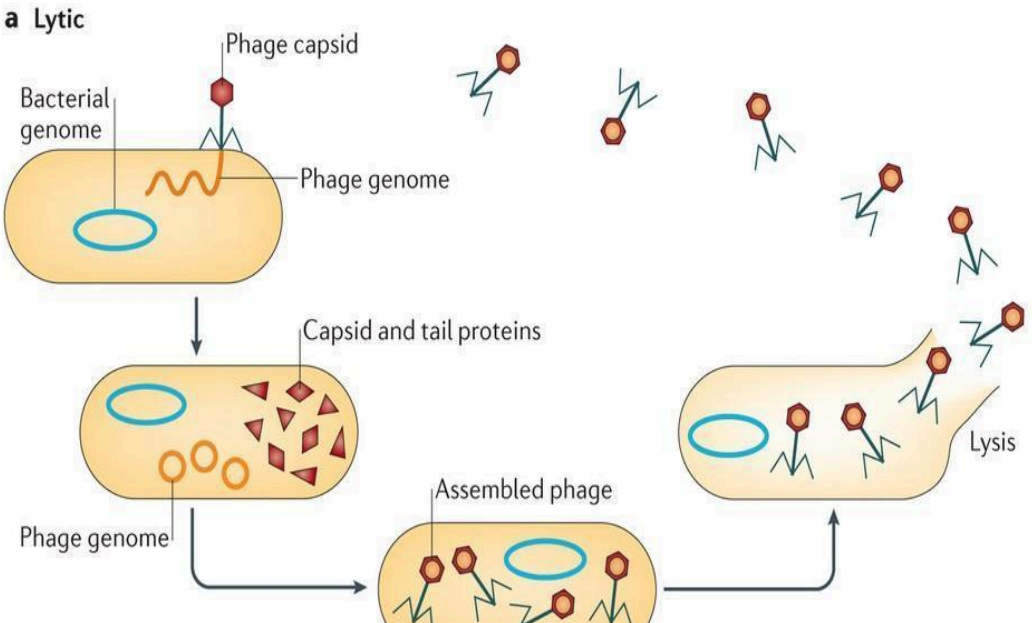


Figure 1.2 Phage lytic life cycle (Feiner, *et al.* 2015), showing attachment, penetration, synthesis, assembly and the release of new phage.

Phage can have lytic or lysogenic life cycles, virulent phage such as Phage B1 and K only use the lytic cycle to replicate, see fig. 1.2, (Salmond *et al.,* 2015). Lytic phage infect the bacterial host, disrupt bacterial metabolism, replicate within the cell and quickly kill (often within an hour) via lysis thus releasing more phage to infect and replicate (Sulakvelidze *et al.,* 2001; Nilsson, 2016). This method of replication means that not only will the bacterial population be reduced by phage infection, but phage also can increase in number within the body after administration (Weld *et al.,* 2004). It is assumed that the phage population will increase in accordance with the level of infection, for example if the infection is severe and the bacterial load is high, the phage will increase in number to combat the infection. The rise in phage numbers results in a reduction of bacterial numbers which in turn reduces phage numbers as there are less hosts available for phage replication, thus once the infection is cured, they will pass from the body or decompose (Bull & Gill, 2014; Nilsson, 2016). This of course contrasts with antibiotics which may pass through the body and persist in nature (Nilsson, 2016). This is an important characteristic of lytic phage that makes them suitable for use in medical therapy (Clokie *et al.,* 2011). Few studies have been carried out to examine phage-bacterial infection dynamics (Levin & Bull, 2004; Weld *et al.,* 2004; Abedon, 2011).

Lysogenic phage, however, are not suitable candidates for phage therapy as the phage genetic material can become incorporated into the host bacterium's genome to form a prophage. The prophage can then be copied during bacterial reproduction and passed into the daughter cell (Salmond & Fineran, 2015). This ability to integrate into the host's genome is one which is not desirable when considering the use of phage as therapeutics as a potential effect could be shedding of bacteria from the body which now contain prophage. This could disrupt wild bacterial populations and/or possibly contribute to the development of bacteriophage resistance in a similar way to the presence of antibiotics in drinking water has accelerated the spread of antibiotic resistance (Sanganyado& Gwenzi, 2019).

In virology, superinfection refers to the process in which a bacterial cell that has already been infected by a virus becomes co-infected with another strain or virus. Superinfection immunity is a process unique to bacteriophage and one which is best understood in lambda phage. A bacterial strain is said to have superinfection immunity when

infection by a lysogenic phage prevents it from being infected by another closely related species (Moineau & Lévesque, 2005). The prophage either inhibits attachment of secondary phage or allows them to attach to and inject their DNA into the bacterial cell but then repress the viral DNA meaning the phage are unable to reproduce lytically (Blasdel & Abedon, 2013). Although superinfection immunity does confer some selective advantage to both the prophage and the host, it is quite limited as it only protects the bacteria from infection by strains closely related to that of the initial infection (Brüssow & Kutter, 2005; Moineau & Lévesque, 2005).

Several studies have been carried out examining the interactions between phage K against *Staphylococcus aureus,* both *in vivo* and in vitro (O'Flaherty *et al*., 2004, 2005; Gill *et al.,* 2006). The origins of phage K are not known, but it was likely originally isolated by André Gratia in the early 1920s (Burnet & Lush, 1935; Rountree, 1949). Phage K is a lytic phage of the *Myoviridae* family, genus *Kayvirus.* Studies have increasingly shown that lytic phage are successful at killing antibiotic resistant bacteria, such as phage ENB6 against vancomycin-resistant *Enterococcus faecium* (VRE) and Phage B1 and phage SATA-8505 against methicillin-resistant *Staphylococcus aureus* (Biswas, 2002; O'Flaherty *et al.,* 2005; Sulakvelidze, 2005; Eyer, 2007; Sabouri Ghannad & Mohammadi, 2012; Pincus *et al.,* 2015). For almost all bacterial species there is at least one phage that can exclusively target, kill and infect it (Sabouri Ghannad & Mohammadi, 2012).

Phage therapy is not without its issues and many studies have documented its advantages and disadvantages (Cairns & Payne, 2008; Mattey & Spencer, 2008; O'Flaherty *et al.,* 2009; Chan et al., 2013; Örmälä & Jalasvuori, 2013; Bull & Gill, 2014; Ly-Chatain, 2014; Chadha *et al.,* 2016; Nilsson, 2016). Phage generally exhibit a narrow spectrum of inhibition, only infecting a subset of strains which make up a species, but some can infect entire genera. However, there are very few broad-spectrum phage to rival antibiotics, consequently phage treatment should not cause collateral damage to the surrounding microbiota, a factor which is now considered a major drawback for broad spectrum antibiotics (Chan *et al.,* 2013; Bull & Gill, 2014; Nilsson 2014). Damage to the normal bacterial flora in the patient can have a detrimental effect on host immunity, may lead to secondary infections from opportunistic infections and can affect the body's ability to process food (Nilsson 2014; Langdon *et al.,* 2016). It is very important that phage as an antibacterial

agent applied to living tissue shows selective toxicity and does not cause damage to the human host (Chan *et al.,* 2013).

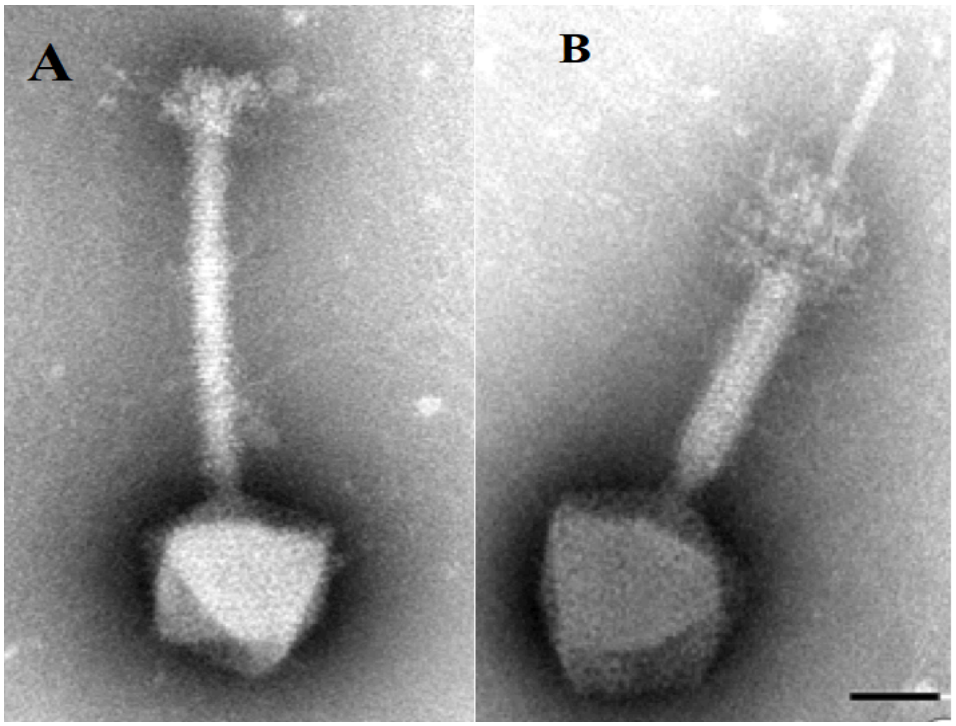


Figure 1.3. Electron micrograph images of Phage B1 (A) Phage B1 with a contractile tail. (B) Phage B1 with tail contracted and empty phage head. Scale bar, 100 nm. Courtesy of Aidan Coffey, Cork Institute of Technology.

The risk of emerging phage resistant bacteria is a concern and one which much be thoroughly investigated to avoid repeating the mistakes of the past (Merril *et al.,* 1996; Sulakvelidze *et al.,* 2001; Inal, 2003; Shasha *et al.,* 2004; Sulakvelidze, 2005; Eyer, 2007; O'Flaherty, 2007; Cairns & Payne, 2008; Mattey & Spencer, 2008; Labrie *et al.,* 2010; Moradpour & Ghasemian, 2011; Gu *et al.,* 2012; Sabouri Ghannad & Mohammadi, 2012; Chan *et al.,* 2013; Örmälä & Jalasvuori, 2013; Fair & Tor, 2014; Ly-Chatain, 2014; Nilsson, 2014; Chadha *et al.,* 2016; Langdon *et al.,* 2016). Under laboratory conditions bacterial resistance to phage arises quickly. However, it is important to emphasise that resistance has not been seen in applied phage therapy, unlike bacterial resistance to antibiotics which is a major problem in the treatment of infectious diseases (Örmälä & Jalasvuori, 2013; Fair & Tor, 2014). Phage resistant bacteria which do emerge are often found to be less virulent (Gu *et al.,* 2012, Oechslin, 2018). A greatly decreased risk of phage resistance development is

noted when bacteriophage are administered in a phage cocktail. A phage cocktail consists of various types of phage which infect the same species/strain, due to the presence of different phage types the likelihood of a resistant bacterial cell is much less probable (Gu *et al.,* 2012; Chan & Abedon, 2013; Örmälä & Jalasvuori, 2013).

The lower risk of phage resistance is not the only reason why phage cocktails are preferable to singular phage administrations. As already mentioned, phage are very specific and tend to have narrow host range, so by using a combination of phage a synergistic treatment can be developed which has a broader spectrum of activity. The use of phage cocktails means that the exact strain of pathogen does not need to be known to begin initial treatment as it targets a variety of strains within the species or even closely related species (Chan & Abedon, 2012). In terms of practical application, this means that firstly, a huge repository of individual phage to target different strains should not be needed and secondly, as already stated treatment can begin before the exact strain has be identified, this is of particular importance when dealing with diseases which have high mortality or morbidity rates (Chan & Abedon, 2012), such as carbapenem-resistant Enterobacteriaceae (CRE) which has a rate of mortality in infected patients of 50% according to the Centre for Disease Control and Prevention (CDC). Phage cocktails are not without their drawbacks either, every phage needs to be characterised in terms of host range, effectiveness and toxicity, there is a lot of work to be done before our knowledge of phage is sufficient for them to be used in a commercial and wide scale manner (Nilsson, 2014). Some, *in-vitro* studies have suggested that competition between the different phage strains in a phage cocktail may hinder their effectiveness (Niu *et al.,* 2014).

Antibiotics have been used extensively across the globe since their discovery. Although the use of phage cocktails decreases the risk of phage resistance, if phage were to be used to the same extent is it just a matter of time before we see significant levels of resistance? (Örmälä & Jalasvuori, 2013). Would the increased use of phage, which are known to drive bacterial evolution, cause the pathogens they are used to treat to develop resistance over time? Understanding phage defence systems in pathogenic bacteria mechanisms is critical to the success of phage therapy since it may lead to rational approaches to counteract it (Barrangou, 2007; Hyman & Abedon, 2010; Labrie *et al.,* 2010; Örmälä & Jalasvuori, 2013). Bacteria have a plethora of phage resistance mechanisms, including adsorption inhibition, restriction modification, CRISPR-cas and abortive infection (Samson *et al.,* 2013).

However, phage can evolve against these mechanisms resulting in an escalation of bacteria-phage coevolution, referred sometimes as an "arms race" (Weitz *et al.,* 2005). The dynamic relationship between bacteriophage (treatment) and bacteria (infection/host) must be understood and respected for history not to be repeated with the same mistakes made as with antibiotics (Barrangou, 2007; Eyer, 2007; Hyman & Abedon, 2010; Labrie *et al.,* 2010; Hall *et al.,* 2012; Örmälä & Jalasvuori, 2013). It is unlikely widespread bacteriophage resistance would be seen as even if bacterial diseases develop resistance to bacteriophage commonly used in hospital settings given the abundance of environmental bacteriophage the isolation of new bacteriophage effective against most bacterial targets should be possible indefinitely (Örmälä & Jalasvuori, 2013).

In this study, Phage K, a polyvalent virulent phage (Burnet & Lush, 1935) and a novel phage named Phage B1 have been characterised and developed for use against MRSA and other staphylococci. Phage B1 was obtained from The George Eliava Institute of Bacteriophage, Microbiology and Virology otherwise known as the Tbilisi Institute. When considering phage for therapeutics there are various factors which must be considered. Phage factors such as, host range, phage stability, likelihood of phage resistance development, rate of phage adsorption, phage survival *in vivo* and fecundity rate are all considered important, even if not fully understood (Bull & Gill, 2014). In this review we focus on the process of phage adsorption, which essentially dictates the host range of a phage and examine the various factors affecting it (Goldberg, 1994; Jończyk *et al.,* 2011; Longdon *et al.,* 2014). It is also very important to investigate side-effects or adverse reactions. As already stated, phage therapy has been common in certain countries for many years and orally administered phage treatments are generally considered to be safe, i.e. GRAS. The possibility of phage translocation across the intestinal epithelium and into the patients' blood is something which requires future study to understand fully the immunological response elicited by the administration of phage preparation (Górski *et al.,* 2006; Pincus *et al.,* 2015; Lin *et al.,* 2017).

1.2 Phage adsorption

Phage adsorption describes the initial attachment of the phage to the host and is the first step in the infection process. Despite being the first, however, it is the least understood step in the viral life cycle (Jakutyte˙ *et al.*, 2011), with most knowledge gleaned from research on *E. coli* phage (Randall-Hazelbauer & Schwartz, 1973; Montag *et al.*, 1989; Gibbs *et al.*,

2004; Edgar *et al*., 2008). Firstly, the phage recognises a particular host-specific cell component and adsorb to the permissive host (Baptista *et al.*, 2008; Rakhuba *et al*., 2010). Recognition of host cell receptors is primarily carried out by phage tail fibres which are also known as attachment proteins (Rakhuba *et al*., 2010; Katsura, 1983; Goldberg *et al.,* 1994; Hu *et al.,* 2015). Once the phage has recognised its specific host receptor, it will then inject the phage DNA into the cell, in some cases bacteria employ various adsorption-blocking mechanisms to protect itself, these include but are not limited to the blocking of receptors, production of competitive inhibitors and the production of extracellular matrix (Hyman & Abedon, 2010; Labrie *et al.,* 2010). The phage will have to overcome these if present to inject into the host cell.

Phage cannot infect and thus replicate and spread unless it is able to recognise receptors on the bacterial host, attach to them and be adsorbed. Receptors on the host are recognised by attachment proteins located on the end plate of the phage tail (Katsura, 1983; Goldberg, 1994). The specificity of the phage-bacteria interaction is a direct result of the specificity of phage adsorption, which is a direct result of the structure of receptors on the cell surface (Rakhuba *et al,* 2010).

Phage adsorption is composed of 2 stages or phases, the first is reversible and the second is irreversible (Jakutyte ̇*et al.,* 2011). These stages are specific and distinct to individual phage and can vary significantly, especially between members of different taxonomic groups (Rakhuba ̇*et al.,* 2010). In most cases, the phage tail is responsible for attachment, binding and injection of genetic material into the bacterial cell (Katsura, 1983; Goldberg, 1994; Jakutyte ̇*et al.,* 2011). Phage employ one of two strategies when adsorbing to the host: either the phage reversibly binds to a receptor before irreversibly binding to it or the phage reversibly binds to a receptor and then irreversibly binds to another receptor or cell component. Both strategies require different phage adsorption machinery varying from a single receptor-binding protein (RBP) to complex baseplates with several RBPs (Jakutyte ̇*et al.,* 2011). It is not just the nature and structure of the receptors which is important but also their quantity, location, and density (Tetart *et al.,* 1996; Edgar *et al*., 2008; Hu *et al.*, 2015).

In recent years, the rise of antibiotic resistance has meant that the use of phage, as therapeutic agents to control bacterial infections has become a hot topic (OFlynn, 2004; O'Flaherty *et al.,* 2005, 2009; Mattey & Spencer, 2008; Ly-Chatain, 2014). In particular, lytic phage are of interest as they cannot integrate their genetic material into the bacterial

chromosome as such they always lyse and kill the host (Guenther, 2009). Their specificity is both a negative and positive feature. Being so specific to their host means the bacteriophage will only infect their target bacteria and not affect other bacterial cells in the vicinity (Guenther, 2009). Their narrow host range can be a disadvantage as in medical application where the exact phage for the specific bacteria causing the infection is needed for the infection to be treated. Early studies had success in treating *Escherichia coli* infections in animal models (Smith and Huggins 1982, 1983; Merril *et al.* 1996), which prompted testing of phage against other bacterial species, eg mouse trials (Soothill, 1992) using bacteriophages against *Acinetobacter baumanii, Pseudomonas aeruginosa* and *Staphylococcus aureus,* these unfortunately provided mixed results.

The success of phage therapy depends on many factors, which include the bacteria being treated, the phage being used to treat it and the method of administration among others. Phage are natural enemies of bacteria and are very specific to their host, (Guenther, 2009), so factors which affect their adsorption are of utmost importance. Phage will only be successful in treating an infection if they are able to attach to and thus be adsorbed by the bacteria causing the infection. Since the density of the host affects adsorption rate, this is also an important factor because it determines the dose of phage that must be administered to treat the infection (Ly-Chatain, 2014, 2008). It has been shown in several studies that multiple doses are more successful than single doses (Biswas *et al*., 2002; Ly-Chatain, 2014,), but this can vary depending on when the first dose is administered (Huff *et al.,* 2003; Pincus *et al.,* 2015). Administering a higher dose of phage does not necessarily equate to more effective treatment, it may have a negative effect as any negative side effects would be exacerbated by the larger phage load (Pincus *et al.,* 2015).

1.3 Rate of adsorption

The rate by which phage find and attach to a host receptor is assumed to follow mass-action kinetics (Shao, 2008). This, and in turn the rate of adsorption is related to host density, where higher density is assumed to equal higher adsorption rates. Phage do not possess the necessary structures for independent movement thus rely on random collisions between the phage and the bacterial cell (Rakhuba˙*et al.,* 2010). Thus, it can be reasoned that higher bacterial and phage numbers enable more collisions and higher rates of adsorption. Other factors also affect the rate of adsorption and can be both physical and chemical e.g.,

pH, temperature, presence of certain ions and chemical and host factors. Phage strains with a higher adsorption rate have a shorter optimal lysis time and vice versa (Shao, 2008).

The use of bacteriophages against pathogenic bacteria has been studied using two different approaches, namely, passive, and active (Cairns & Payne, 2008; Abedon & Abedon, 2010). In the case of the passive approach, the bacteriophages are added to the system at a level sufficient to ensure that all target bacteria are infected and lysed in a short period of time. On the contrary, active biocontrol relies on the addition of a small amount of phage. Bacterial elimination, in this case, supposes the replication of phage over several generations at a rate faster than bacterial replication (Cairns & Payne, 2008). The capacity of new replicated phage to access target bacteria could be weakened by the biochemical and physico-chemical characteristics of the environment such as viscosity. Passive treatment is more time efficient than the active one since it does not require cycle(s) of phage multiplication through the host.

1.4 Factors affecting adsorption.

1.4.1 Gram positives versus Gram negatives

There are several differences between the structure of Gram positive and Gram-negative bacteria. Gram negative bacteria have a double layered cell membrane, a thin peptidoglycan layer, an outer cell membrane and are less rigid. Porins and lipopolysaccharides (LPS) are also present but teichoic acid is not and their cell wall is less susceptible to degradation by lysozyme. Proteins present in the cell membrane and lipopolysaccharides may act as bacteriophage receptors; indeed, many phage require both types of molecules for efficient adsorption (Lindberg, 1973). In contrast, Gram positive bacteria have a thick peptidoglycan layer, are more rigid, lack porins and lipopolysaccharides (in most cases). They possess no outer cell membrane; their cell wall is more susceptible to degradation by lysozyme and teichoic acid is present in the cell wall. The difference in the structure of the cell wall between Gram negative and Gram-positive bacteria means that phage must employ different mechanisms in order to gain entry to the host (Jakutyte *et al.,* 2011).

Using fluorescently labelled bacteriophage, it has been shown in both Gram negative and Gram-positive bacteria that phage generally prefer to bind at the poles of the bacterium.

Lambda phage binds to the pole of *E. coli* because it is here that ManY, a protein required for lambda entry is located. In addition, another protein LamB is required for irreversible binding and is located throughout the membrane. LamB rapidly but temporally relocates to the poles and binds to Lambda phage (Gibbs, 2004; Edgar, 2008). Although polar binding has also been shown to be the preferred option in Gram positives (Jakutyte *et al.,* 2011), the rigid structure of the cell wall means that movement of proteins/receptors as in Gram negative is not possible. This process was studied in Phage SPP1 and its host *Bacillus subtilis.* SPPI reversibly binds to cell wall teichoic acids (WTA) allowing the phage to scan the surface of the bacterium for its receptor YueB to which it irreversibly binds (Baptista *et al.,* 2008).

1.4.2 Host Receptors.

1.4.2.1 Protein: There are 5 classes of phage protein receptors located in the outer membrane layer of Gram-negative bacteria: porins, substrate receptors (no known example of phage using these), structural proteins (e.g. transmembrane proteins), transport proteins and enzymes (proteases can act as phage receptors). Porins are only found in Gram negative bacteria and in *E. coli* for example the OmcC protein found in porins acts as a receptor for certain phage while T4 for example uses it along with LPS (Rakhuba *et al.,* 2010). As stated above the well-known phage, phage lambda adsorbs to the transport protein, LamB (Randall-Hazelbauer & Schwartz, 1973). A phage protein called gpJ recognises LamB and is thus responsible for phage specificity (Nikaido H, 2003).

1.4.2.2. Lipopolysaccharide (LPS): Proteins are not the only receptors found in the outer membrane of Gram-negative bacteria, LPS also serve as a phage receptor, either independently or in tandem with proteins such as OmpC (Yu & Mizushima, 1982). Lipopolysaccharides, also known as endotoxins, are large molecules, they are a complex polymer consisting of a lipid (A), an inner and outer core and a polysaccharide composed of O-antigen. Endotoxins elicit strong immune responses in animals and play a role in bacterial pathogenicity. There are 2 types of LPS, smooth (S) and rough (R). Some bacteriophage are able to adsorb to both and thus have larger host ranges, followed by phage that absorb to R-type. Phage that absorb to S-type are very specific due to the variation in O-antigen structure in different bacteria. Many phage which use O-antigens as their receptors are seen to have their adsorption trigger specific enzymatic activity within the LPS; e.g. Bacteriophage Sf6 which infects *Salmonella flexneri* (Lindberg *et al.,* 1978). R-type does not possess the

O-antigen, so this factor does not affect absorption. Many T-phage's such as phage T4 bind to their *Escherichia coli* host via components of the R-type LPS (Rakhuba *et al.,* 2010).

1.4.2.3 Teichoic acid: In Gram positive bacteria it is the teichoic acids, but not lipoteichoic acid, that comprise the majority of known surface antigens (Rakhuba *et al.,* 2010; Xia *et al.,* 2011). Teichoic acids are anionic glycopolymers which play an important role in determining the physiology of Gram-positive bacteria and regulation of cell division. They also play an important role in bacterial pathogenesis and their resistance to antibiotics (Brown *et al.,* 2013). Many strains of *Staphylococcus aureus* phage including phage K are absorbed via teichoic acid (Xia *et al.,* 2011). Different molecules within the teichoic acid function as receptors for different phage strains, the anionic backbone of wall teichoic acid functions as the receptor for phage K (Xia *et al.,* 2011). It wasn't until a recent study by Xia *et al.,* 2011, that the molecular interactions between *Staphylococcus* phage and the host receptors to which they bind were understood, it was through the use of *Staphylococcus aureus* teichoic acid mutants which had wall teichoic acid (WTA) knocked out, that the importance of WTA in phage binding was proven. It is interesting that although they can act as receptors for bacteriophage adsorption some teichoic acids along with other molecules, such as peptidoglycan can deactivate phage (Rakhuba *et al.,* 2010).

1.4.2.4 Other: Phage can also adsorb to other molecules on the bacterial host including pili, flagella, and various types of polysaccharides (capsular and slime). In the case of flagella, the phage either move down the flagella and are adsorbed through the baseplate or initially attach via the head leaving the distal tail free for adsorption. So called flagellatropic phage usually have a broad host range but a noted exception to this is phage iEPS5 which can only infect a very small number of *Salmonella enterica* serovar Typhimurium (Choi *et al.,* 2013).

Many bacteria have external protection in the form of capsule or slime layers which can inhibit the movement and attachment of bacteriophage, by blocking the cell wall. However, components of these layers, such as Vi-antigen, also serve as the adsorption site for phage (Rakhuba *et al.,* 2010). Receptors located in the capsule layer tend to require enzymatic activity to assist in phage adsorption. As with the flagella, the capsule only acts as a receptor for initial attachment; components within the cell wall are essential for the next phase. Some DNA and RNA phage can bind to pili, although they only bind to sex pili, which

are able to absorb hundreds of phage virions. The best studied pili binding phage are *E. coli* phage such as M12 (Rakhuba *et al.,* 2010).

1.4.3 Phage Factors

In order for a phage to successfully adsorb onto and be injected into a bacterial cell it must undergo significant structural remodelling, with the most obvious changes often seen in the tail (Hu *et al.,* 2015). Studies on lambda phage, particularly those by Hendrix and Duda 1992, showed the importance of side tail fibres on adsorption rate. The loss of these fibres caused a reduction in the rate of adsorption of lambda phage onto the cell surface of its *E. coli* host. It has been shown in Phage T4 that duplication or mutation of the gene coding for tail fibres expands its host range, and not just to closely related species (Tétart *et al.,* 1996). Cryo-electron microscopic imaging has shown that T4 undergoes structural remodelling during the initiation of infection of the host cell. Unexpectedly, most long tail fibres bend back upon themselves and do not make contact with the host cell before the short tail fibres bind. The sheath then contracts and drives the tail tube into the cell through the peptidoglycan layer and into the periplasm. This contraction is triggered by structural changes within the T4 baseplate. The host cell responds to this attachment in one of 3 tail–membrane conformations.

In the first and least common, the tail tube is level with the cell wall. The second involves the tail tube being inside the periplasm and does not reach the inner membrane. In the third conformation, the tail tube is inside the periplasm and has fused to the inner membrane which curved towards it. Thus, T4 phage has the ability to cause significant remodelling of the inner membrane of the host cell (Hu *et al.,* 2015). The ability of T4 to initiate structural changes within itself and its host play a crucial role in phage adsorption, without it would not be able to attach and replicate, thus it is an important factor in phage adsorption.

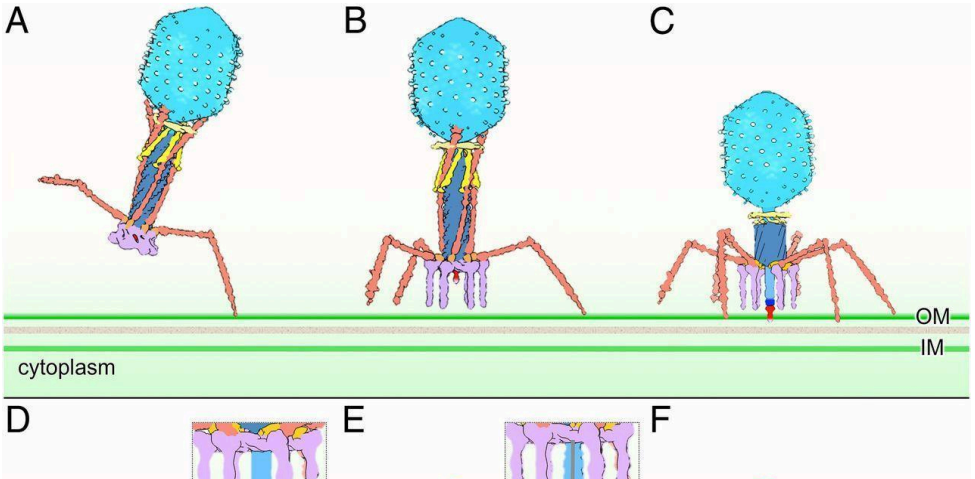


Figure 1.4: Schematic of the structural remodelling T4 phage undergoes

during initiation of infection based on study by Hu *et al.,* 2015 which used Cryo-electron tomography (cryo-ET) to provide information on 3D structures.

1.4.4 Environmental Factors

There are various environmental or external factors which affect phage survival including temperature, pH, humidity, salinity, and other ions (Jończyk *et al.,* 2011). The caudovirales have been shown to be the most stable under adverse conditions (Ackermann *et al.,* 2004). The larger the capsid head the more resilient the phage but there seems to be few other correlations between structure and stability. Phage which are structurally very similar may be dissimilar with regards their ability to withstand suboptimal conditions. Even closely related phage can have very different external requirements (Jończyk *et al.,* 2011). Given that phage are composed of protein it is not implausible that they would be denatured by high temperatures, but they have been found to be for the most part very thermostable. Unlike bacteria which tend to have a narrow range of temperatures in which they can survive, certain phage have been found to remain intact at temperatures up to 90°C and as low as 0°C (Buzrul *et al.,* 2007). It has been shown that phage reproduce in higher numbers at their specific desired temperature and at less-than-optimal temperature less genetic material penetrates the bacterial cell. Despite being quite thermostable, the temperature at which phage are stored long term has a notable effect on their activity (Jończyk *et al.,* 2011).

Various phage have different tolerances to salt concentrations but what most phage do have in common is that a rapid change in osmotic pressure, due to an increase or decrease in salt concentration is likely to cause osmotic shock (Lark & Adams, 1953). A study found *Staphylococcus* phage can tolerate high salt concentrations in the free state but cannot function as well at higher concentrations, often losing the ability to infect certain strains thus narrowing host range (West *et al.,*1963). Salt concentration in the growth media influences the switching on and off of genes in bacteriophage lambda (Li *et al.,* 2015).

It has long been understood that the presence of certain ions is essential for some bacteriophage to propagate and form plaques. These cations, also known as co-factors, can increase the size of plaques even in phage which do not require them. The two most used are

calcium and magnesium ions. The calcium ion requirements of a phage can vary, even when infecting closely related bacterial species. Mycobacteriophage L5 requires a higher concentration of Ca++ to be able to infect slow growing *Mycobacterium tuberculosis* compared to *Mycobacterium smegmatis* (Fullner and Hatfull, 1997). Phage Anti-R cannot bind to its host *Salmonella typhi* unless Mg++ and certain other divalent metal ions are present. Phage require cofactors for a variety of different reasons, they help with stability in the free state, attachment to host and gaining entry into the host (Tucker, 1961).

Phage K was found to be inactive in raw bovine milk (O'Flaherty *et al.,* 2005). In this case, the authors suggested that it can be assumed that there are immune factors present that affect phage adsorption in raw milk. Another study showed that bacterial numbers varied when incubated with phage in various media. Only 22% of bacteria survived when grown in TSB in the presence of Phage K, while 21-100 % survived in raw milk and 94% in bovine serum. The large range of bacterial inhibition in raw milk is due to the raw milk coming from different sources. The composition of the milk will vary from cow to cow. The higher bacterial survival rates show that there is something present in the raw milk and bovine serum which inhibits phage attachment (Gill *et al.,* 2006). It was found that it was the whey in the milk that was inhibiting phage growth. How whey inhibits phage was studied by measuring bacterial growth after the whey had been subjected to different chemical and physical treatments, different exposure times and separation of the whey to determine which subcomponents of whey were responsible for inhibition of phage. Proteins in the whey bind to the surface of *S. aureus* and inhibit phage attachment (Gill *et al.,* 2006).

The acidity of the environment is another important factor to take into account when considering phage stability. Various studies have shown that phage generally prefer pH close to neutral (Sharp *et al.,* 1946; Kerby *et al.,* 1949; Feng *et al.,* 2003; Jończyk *et al.,* 2011). There have been phage found surviving in more acidic environments, Lu *et al.,* 2003, showed that phage could survive at less than pH 3.5 even for long periods of time. Many studies have been carried out examining how phage survive in urine, that found there is a strong association between temperature and pH which phage can withstand (Höglund *et al*., 2002; Vinnerås *et al.,* 2008).

The nature of the environment in which phage and host are located also affects the rate of adsorption. Studies have shown that bacteriophage diffusion was delayed or inhibited in solid foods (Guenther *et al.,* 2009). There are issues with the use of the gelling agent, agar,

in phage assays. Purified agar, known as agarose, which has had most of the agaropectin removed which is thought to inhibit virus replication, is the prefered gelling agent especially when trying to grow newly discovered phage. It was found during this study that although agarose was not used, the use of lower-than-normal concentration of agar in the overlay layer yielded larger plaques, this is in accordance with previous studies (Serwer *et al.,* 2007). Although phage can move freely in blood and soft tissues, penetration of solid tissues is challenging (Sulakvelidze, 2005; Ly-Chatain, 2014). A possible solution is the use of non-pathogenic bacteria as carriers to carry bacteriophage into the infected cell (Inal, 2003).

1.4.5 Mammalian immune response to phage

In vivo bacteriophage may trigger an immune response but phage are capable of adapting and overcoming host response (Hyman, 2010), thus it can be considered a dynamic relationship. It is important to understand not just how bacterial hosts respond to phage, but also how the human patient responds, as this is crucial to use of phage therapy. In mouse trials looking at phage therapy against vancomycin-resistant *Enterococcus faecium* (VRE), it was noted that titers of IgG and IgM against the phage which was injected monthly for 5 months increased after the third injection. IgM levels increased 5-fold compared to normal while IgG increased nearly 4000-fold. This huge increase was not accompanied by other host immune responses though such as temperature increase, no adverse effects such as anaphylactic response were seen either (Biswas *et al.,* 2002).

1.5 Bacteriophage resistance mechanisms

There are five types of bacterial mechanisms against phage attack: adsorption inhibition, restriction modification, CRISPR-cas, abortive infection and recently, a novel phage resistance termed BREX was identified on the genome of *Bacillus cereus* (Goldfarb, *et al.,* 2015). There are 2 main methods of adsorption inhibition, as already mentioned phage bind to the host cell via receptors but bacteria are able to block these receptors by changing their structure or three-dimensional conformation. An example of this method of defence can be seen in *S. aureus* which releases a cell-wall anchored virulence factor, immunoglobulin G-binding protein A. When lower levels of protein A are released phage adsorption increases, thus it can be concluded that protein A masks the phage receptor in some way (Labrie *et al*., 2010). In some *E. coli* strains a lipoprotein, outer-membrane protein TraT modifies the outer-membrane protein A (OmpA), a known receptor for many *E. coli* phage. It is also seen

in *E. coli* how other molecules normally present in the bacterial environment can inhibit phage binding. FhuA, an iron transporter which serves as a phage receptor is also used as a receptor for antimicrobial molecule microcin J25 which can outcompete the phage and bind to FhuA (Labrie *et al.*, 2010). Another method of adsorption resistance stops the phage from being adsorbed by the release of structured extracellular polymers which can form a physical barrier between the phage and their receptors. Some phage are able to overcome this by releasing enzymes (hydrolases and lyases) which recognise and degrade the polymers (Labrie *et al.,* 2010).

Restriction modification comes into play post phage adsorption and after injection of phage DNA into the bacterial cell. A host-encoded restriction enzyme cleaves invading phage DNA at specific recognition sites while the host DNA is protected by methylation (modification) of certain residues. However, phage have evolved several anti-restriction strategies to overcome restriction–modification (RM) (Samson *et al.,* 2013). One such method is the absence of endonuclease recognition sites in the phage genomes. The viral double stranded DNA reduces the effectiveness of the RM protection system. Phage K is able to survive RM as it does not possess *Sau3*A, BamHI, PvuI and DpnI site points, due to accumulation of point mutations (O'Flaherty, 2004).

Clustered regularly interspaced short palindromic repeats (CRISPR-cas) are segments of prokaryotic DNA containing short, repetitive base sequences (21–48 bp) followed by segments of non-repetitive spacers (26–72 bp) of similar length. Cas (CRISPR-associated system) genes are located in clusters of 4-20 next to the CRISPR sequence. CRISPR and the CRISPR-associated (cas) genes have been shown to influence phage multiplication as shown by Barrangou *et al.*, 2007. The CRISPR/Cas system functions as a prokaryotic immune system providing resistance to invading genetic elements such as phage genomes and plasmids. The extra mechanism by which CRISPR/Cas confers phage resistance is not fully understood, it is now known that CRISPR-Cas mechanism cleaves invading foreign DNA and is guided by spacer sequences complementary to sequences in the foreign DNA (protospacers). Laboratory testing of a phage-sensitive strain of *S. thermophilus* showed that when challenged with a virulent phage, bacteriophage insensitive mutants (BIM) will emerge (Mills *et al.,* 2010). Examination of these BIMs shows that the newly resistant bacterium has acquired at least one new repeat-spacer unit at the 5` end of the repeat-spacer region of a

CRISPR locus. This new spacer is identical to a sequence located on the phage genome; different BIMs can acquire distinct spacers from the same phage genome.

Another method of defence is superinfection exclusion (Sie) which protects the host cell using membrane associated proteins which prevent the phage DNA from entering it. Many of these Sie systems have been identified, more often in Gram negatives than Gram positives but only a small number have been characterised (Labrie *et al.,* 2010).

Abortive infection resistance results in the death of both bacteria and phage, this is the least desirable method from the point of view of the bacterium as it results in cell death (Hyman *et al.,* 2010). Abortive infection (Abi) systems use a variety of heterologous proteins which usually target and interrupt a specific stage of phage multiplication such as translation. Although Abi systems have been known about and studied for years they are not fully understood. The best characterised Abi system is the Rex system found in phage λ-lysogenic *E. coli* strains (Molineux, 1991). The Rex system is a two-component system which reduces ATP which is needed by both the host cell and phage for multiplication (Snyder, 1995).

1.6 Bacteriophage insensitive mutants

Bacteriophage insensitive mutants (BIMS) are phage resistant derivatives which most commonly arise due to chromosomal mutations of surface molecules or antigens. Phage attachment to the receptor exerts selective pressure on the bacterium, causing it to alter or down regulate expression of said receptor thus resulting in the inability of the phage to attach to the bacterial host (Labrie *et al*., 2010). These mutations are not without a cost, evolving to be phage resistant may result in the loss of or reduction of efficiency of another trait, this is known as genetic trade off (Goldhill, 2014). For example, BIMs of the multidrug resistant MDR *Pseudomonas aeruginosa* are antibiotic sensitive (Chan *et al.,* 2017). Thus, the combined use of phage and antibiotics could enable treatment of antibiotic resistant bacteria. Firstly, phage could infect and lyse bacterial cells thereby reducing the bacterial population and when the remaining bacteria mutate and develop resistance to phage, they may then lose antibiotic resistance and be killed (Chan *et al.,* 2016). Restoration of antibiotic sensitivity is unfortunately not a common side effect of the generation of BIMs, but more studies should be carried out to see if this phenomenon can be replicated using other strains of bacteria.

1.7 Phage Host switching

Bacteriophage do not generally cross taxonomic boundaries (Guenther, 2010) but some phage do have the ability to switch hosts, this is called host shift. Host shift is often responsible for new viral pathogen emergence. Viruses have been seen to shift across mammalian hosts such as HIV jumping from chimps to humans. Host shift can be used in the development of phage therapy to expand/shift phage host range or create a generalist bacteriophage which can thrive on a wide range of species of bacteria (Nilsson, 2014). Host shifts are common in the development of pathogens, but how and why can viruses jump between some species and not others? (Longdon, 2014). It is most likely that the new host will be closely related to the original. Past host shifts can be identified by examining the topologies of host and parasite phylogenies and if they differ this is known as phylogenetic incongruence. Differences between host and parasite phylogenies are common, over 90% of studies looking at such found evidence of host shifts, although host shift is common, it can come at a cost to overall fitness. Examples of viruses and hosts whose phylogenetic pathways match completely are rare. The susceptibility of new hosts varies, but hosts which are closely related to the original are more likely to be susceptible than those which are not. Close relatives are more likely to be similar to each other and thus the virus must make fewer changes to be adapted to the novel host. This phylogenetic distance effect has been shown in many studies- the greater the genetic distance the less likely the virus is able to infect the host. There are some exceptions to this rule of course, species that are not closely related may be susceptible, in these cases it is likely that the other members of the clade are also susceptible, this is known as phylogenetic clade effect (Longdon, 2014).

There are many factors that affect a phage's ability to shift host, being able to attach to a new host is just the first step, what about after adsorption, are the correct mechanisms in place to allow the phage to reproduce within its new host? The mutations needed in order for the phage to switch host, may affect the phage's fitness, more specific mutations are needed to increase how efficiently the phage infects its new host. Not all non-specific mutations reduce phage fitness, studies looking at bacteriophage/bacteria coevolution saw increase in phage fitness in many instances (Bull, 1997). The first mutation which results in the jump to a new species is common to all host shift but after this the mutations are specific, in that the phage must adapt to suppress its new host's immune system or evade it. The phages ability to bind to a host can be determined by examining mutations that occur in molecules involved in

cell binding. Alterations in amino acids sequences in tail-fibre proteins and capsid proteins which are considered high-binding proteins are often seen in phage that can switch host (Longdon, 2014). This trend is seen in both spontaneous mutations and experimental mutations.

The probability of phage being able to adapt to a new host is dependent on several factors. The less the number of mutations required to adapt means it is more likely for a successful adaptation to occur, as does a high population, high mutation rate, (not all mutations are advantageous, many are deleterious or even lethal, but the higher the mutation rate the more likely a beneficial mutation will occur), high number of suitable mutation sites and a short generation time. If a combination of mutations is required or the trade off in fitness is too much it is unlikely that this shift in host will be successful or sustainable. A common trade-off is not being able to infect the original host, mutations which do not decrease fitness are rare. Mutational target size, (the number of sites which can be mutated to adapt to a new host) is something which is best studied in phage, where convergent evolution is often seen. Convergent or parallel evolution refers to mutations which occurred independently in different species but at the same site in the genome. Host shift is often the result of the same mutations or combination of mutations, these mutations become fixed.

Studies have shown that generalist phage are more likely to emerge from long term coevolution with their hosts than through once off spontaneous adaptation to a single host (Hall, 2011). Some bacteriophages (first seen in *Bordetella* strains) have been discovered to have unique genetic elements known as diversity generating retroelements which promote genetic variability. These phage have the ability to switch host by using a template-dependent, reverse-transcriptase-mediated process which substitutes nucleotides in a variable region of phage gene *mtd* which codes for the gene responsible for host recognition (Liu *et al*., 2002; Doulatov *et al*., 2004).

The objectives set out at the start of this study, were to characterize two phage, Phage K and Phage B1. They were be characterized by their host range, through testing against a sample of *S. aureus* and other staphylococcal strains, whose antibiotic resistance profile had been established. Once their host range was established, they were then subjected to co-culturing methods, growing them in broth with permissive and non-permissive strains to drive mutants favourable to expansion or shifting of host range. Genetic analysis was conducted to examine, where in the genome these mutants occurred, what genes were lost or

gained that were associated with the ability of the phage to infect host's that it previously could not.

2. Methods and Materials

2.1 Bacterial strains and growth media.

Staphylococcal strains are listed in Table 2.1. Strains with the prefix DPC are held in the Dairy Products Research Centre culture collection. Human MRSA strains were obtained from the Antibiotic Resistance Monitoring and Reference Laboratory, Health Protection Agency (HPA), Colindale, UK and the National Reference MRSA Collection, National MRSA Reference Laboratory, St. James's Hospital, Dublin 8. Strains were grown at 37°C in brain heart infusion (BHI) broth (Oxoid, Basingstoke, Hampshire, United Kingdom), shaking at 150 rotations per minute. Solid media contained 1.5% (wt/vol) bacteriological agar (Oxoid) and semi solid contained 0.4%. All strains were stocked in tryptic soya broth (TSB) containing 50% glycerol and stored at −80°C. BHI plates contained 20 ml of 1.5% BHI agar. All phage propagated and stocked in BHI and stored at 4°C. 25ml Mueller –Hinton agar plates used for antibiotic sensitivity testing.

Table 2.1 *Staphylococcus* strain details

Host	**Strain**	**Strain Details**	**EOP of Phage B1**
S. aureus	ST290	Human[a]	N/A
S. aureus	ST291	Human[a]	N/A
S. aureus	ST295	Human[a]	N/A
S. aureus	ST299	Human[a]	N/A
S. aureus	ST355	Human[a]	N/A

S. aureus	ST528	Human[a]	N/A
S. aureus	ST530	Human[a]	N/A
S. aureus	ST535	Human[a]	N/A
S. aureus	ST544	Human[a]	N/A
S. aureus	ST550	Human[a]	N/A
S. aureus	25949	Human[a]	N/A
S. aureus	35197	Human[a]	N/A
S. aureus	Newman	MSSA	N/A
S. aureus	RF122	MSSA	N/A
S. aureus	DPC5247	Bovine[c]	N/A
S. aureus	DPC5971	Bovine[c]	N/A
S. aureus	DPC5243	Bovine[c]	N/A
S. aureus	DPC3971	Bovine[c]	N/A
S. aureus	DPC5245	Bovine[c]	N/A
S. aureus	DPC5246	Bovine[c]	N/A
S. aureus	0220(II) ST5[d]	Human[b]	1.3×10^9
S. aureus	0.1345(II) ST5 [d]	Human[b]	NC
S. aureus	3596(IIV) ST8 [d]	Human[b]	5.4×10^9
S. aureus	3045(IIV) ST8 [d]	Human[b]	1.2×10^9
S. aureus	3488(IVV) ST8 [d]	Human[b]	NC
S. aureus	3144(IIV) ST8 [d]	Human[b]	4.2×10^8
S. aureus	E1038(IV) ST8 [d]	Human[b]	NC

S. aureus	E1185(IV) ST12 [d]	Human[b]	NC
S. aureus	E1174(IV) ST22 [d]	Human[b]	2.2 x 10^9
S. aureus	0242(IV) ST30 [d]	Human[b]	1.5 x 10^9
S. aureus	3594 (II) ST36 [d]	Human[b]	5.8 x 10^9
S. aureus	E1139(IV) ST45 [d]	Human[b]	3.4 x 10^6
S. aureus	0.1239(III) ST239 [d]	Human[b]	6.7 x 10^8
S. aureus	0.0066(IIIV) ST239 [d]	Human[b]	NC
S. aureus	0104(III) ST239 [d]	Human[b]	8.4 x 10^8
S. aureus	0073(III) ST239 [d]	Human[b]	4.1 x 10^9
S. aureus	M03/0073(III) ST239 [d]	Human[b]	NC
S. aureus	0308(1A) ST247 [d]	Human[b]	2.4 x 10^9
S. aureus	3581(1A) ST247 [d]	Human[b]	3.4 x 10^9
S. aureus	0.1206(IV) ST250 [d]	Human[b]	3.7 x 10^9
S. aureus	E1202(II) ST596 [d]	Human[b]	NC
S. epidermidis	DPC 6010	unknown[c]	N/A
S. saprophyticus	DPC 6011	unknown[c]	N/A
S. chromogens	DPC 6012	unknown[c]	N/A
S. Capitis	DPC 6013	unknown[c]	N/A
S. hominis	DPC 6014	unknown[c]	N/A
S. haemolyticus	DPC 6015	unknown[c]	N/A
S. caprae	DPC 6016	unknown[c]	N/A
S. hyicus	DPC 6017	unknown[c]	N/A

[a] Antibiotic Resistance Monitoring & Reference Lab, Health Protection Agency, Colindale, UK. Samples held in UCC culture collection.
[b] National Reference MRSA Collection, National MRSA Reference Laboratory, St. James's Hospital, Dublin 8.
[c] Dairy Products Research Centre (DPC), Fermoy, County Cork, Ireland
[d] MRSA strain names abbreviated to numbers before the brackets in rest of paper e.g. 0220(II) ST5[d] referred to as 0220.

2.2 Phage Sources and Propagation

Phage K was obtained from the American Type Culture Collection (ATCC 19685-B1). Phage's B1 and JA1 were isolated from a commercial phage cocktail purchased from the George Eliava Institute of Bacteriophage, Microbiology and Virology, Tbilisi, Georgia. Phage B1 and K were routinely propagated on *S. aureus* DPC 5246 in BHI broth. The protocols used were in accordance with those described previously (O'Flaherty *et al*., 2004). Briefly Phage B1 and K were propagated by inoculating 20 ml BHI containing 10 mM $CaCl_2$ with 400µl of DPC5246 and incubating aerobically at 37°C. When an optical density of 0.4 OD_{600nm} was reached 400µl of respective phage was added. Samples were incubated aerobically at 37°C for 4 hours, at which point clearing should be seen. Samples were then centrifuged at full speed for 15 minutes and filter sterilized by 0.45µm-pore-size filter.

Propagation on solid medium was also carried out using the following method. 50 ml of BHI was inoculated with 100 µl of DPC 5246 and incubated at 37°C with shaking until desired optical density, 0.4 OD_{600nm}, is reached. 200 µl of this culture, 10 mM of $CaCl_2$ and 200 µl of phage was then added to 3 ml molten soft agar BHI and poured onto BHI plates. The plates were left to dry for 10 minutes before being inverted and incubated at 37°C overnight. At this point the phage were either collected in one of two ways depending on the purpose of propagation. In some cases, the soft agar layer on plates that showed semiconfluent lysis was carefully scraped off using a sterile hockey stick and transferred to a 15ml cone bottomed flask for centrifugation before being filtered through a 0.45-µm-pore-size filter. In other cases, distinct plaques were picked using a heated metal inoculating loop and resuspended in 1 ml $NaCl_2$ solution, left stand for at least 30 minutes and then vortexed.

2.3 Phage Plaque Assay

Phage plaque assays and phage sensitivity tests were performed as described previously (Kropinski *et al.,* 2009). Briefly, 100µl of the appropriate overnight culture, and 100 µl of the appropriate phage dilution were added to 3 ml of BHI overlay (0.4% agar). The contents were mixed and poured onto BHI plates, allowed to harden, then inverted and incubated at 37°C for 18 h. Following incubation plaque numbers counted and plaque forming units (PFU) calculated. Plaque assays were carried out in triplicate. (PFU/ml) = (no. of plaques/ (dilution x volume).

2.4 Efficiency of plating

This was done under the same conditions as the plaque assay, each phage was tested three times for each of the five different dilutions. The five phage lysates were 10^{-3} – 10^{-7} times dilutions from the relevant phage stock. The EOP assay replicates for a particular phage were done in parallel on all bacterial strains tested. The plates were incubated overnight at 37°C and the number of PFU counted for each, an average of the three was taken and used to calculate efficiency of plating (EOP). The EOP was calculated (average PFU on target bacteria / average PFU on host bacteria).

2.5 Spot Assay

200 µl of appropriate overnight culture was added to 4 ml of semi-solid agar and poured onto BHI plates. Plates were dried for 30 minutes after which 10 µl of appropriate phage was spotted onto the surface of the plates. The plates were left to dry before being inverted and incubated overnight at 37°C. Following incubation plates were inspected for lysis and zone of clearing measured.

2.6 Antibiotic susceptibility testing (Agar disk diffusion method)

All strains were tested for methicillin resistance using the Kirby-Bauer method. Media was prepared according to the National Committee for Clinical Laboratory Standards. Each strain to be tested was streaked onto BHI plates and incubated overnight at 37°C. 4 or 5 well isolated colonies were picked with an inoculating loop and transferred to a 1.5ml tube of

saline solution and vortexed. 1 ml of suspended saline solution transferred to cuvette and optical density read. The solution must have an OD_{635nm} of between 0.08 – 0.1. More saline solution was added if turbidity found to be too high or more bacterial colonies if found to be too low. Using a sterile cotton swab a sample of each strain was streaked onto Mueller-Hinton plates, with rotation, ensuring full coverage. One 1 µg Oxacillin disk (Oxoid) was placed in the centre of each plate with a sterile forceps and pressed into the agar. The plates were inverted and incubated at 30°C for 24 hours. After incubation the diameter of zones of inhibition were measured in millimetres and recorded (Andrews, 2001).

2.7 Isolation of Phage Mutants

Propagation of Phage B1 on staphylococci which exhibited reduced phage sensitivity was achieved initially by incubating 200 µl of phage with 10 ml of BHI broth containing 1ml from an overnight culture of host strain, DPC 5246 and 1ml of a resistant strain. Samples were incubated aerobically at 37°C overnight. Samples were then centrifuged, the supernatant was filter sterilized and phage lysate used for the next propagation. Plaque assays were carried out after each round of co-culturing, and this was carried out for 20 propagations. Modified phage were named according to the propagating strain, e.g., phage co-cultured with MRSA 3488(IVV) 5 was called Phage B1 5246/3488; in this paper they are referred to by the shortened version, Phage B1 3488 and Phage B1 0.0066.

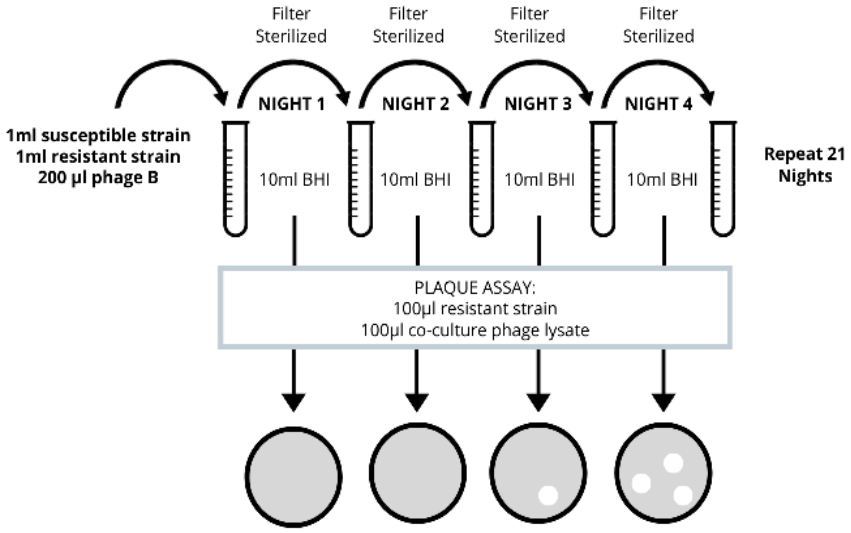


Figure 2.1 Diagram showing co-culturing method. A known volume of a susceptible strain and a resistant strain were incubated overnight with phage. After each night the samples were checked for any change in activity by plaque assay. The samples were filter sterilized and the supernatant used for the next night of co-culturing.

The second round of liquid co-culturing experimented with different ratios of permissive to nonpermissive hosts. The 50/50 co-culture was carried out using 200 µl of phage with 10 ml of BHI broth containing 500 µl from an overnight culture of host strain, DPC 5246 and 500 µl of a resistant strain. The 20/80 co-culture was carried out using 200 µl of phage with 10 ml of BHI broth containing 200 µl from an overnight culture of host strain, DPC 5246 and 800 µl of a resistant strain. Phage K was also tested as were non-*S. aureus* staphylococcal strains (See Figure 2.1).

2.8 Isolation of Bacteriophage insensitive mutants (BIM's)

BIMs were isolated according to protocol described previously (Chan *et al.*, 2016). Briefly the 5 MRSA strains of interest, 0220, 3594, 0242, E1174 and 0308 were grown to exponential phase in 25 ml of BHI and infected with phage at MOI of ~ 0.1 and incubated for 12 hours at 37^{o}C shaking. After 12 hours of incubation 100 µl of culture was plated on BHI agar and incubated for 12 hours. Individual colony forming units (CFU's) were collected and tested for phage resistance by spot assay. Each bacterial isolate was spread on BHI to create a lawn onto which phage were then spotted. Clearing indicates bacterial sensitivity while no clearing is indicative of bacterial resistance, i.e. a bacteriophage insensitive mutant.

2.9 Phage Propagation and Preparation of Phage DNA

Isolation and propagation of phage and preparation of phage genomic DNA was carried out using modified versions of previously described protocols (Sambrook & Russell, 2001). Individual plaques were picked using a pipette tip suspended in 1 ml of SM buffer and replated. This was repeated twice to purify the plaques. Phage lysate with a high titre was then used for extraction according to protocol, using DNA extraction kit from Qiagen with additional steps. Briefly, 10 ml of phage lysate was treated with 10% w/v PEG-8000 and salt (5M NaCl) to precipitate the DNA. 20 µl 10% SDS and 2 µl Proteinase K were added to digest contaminating proteins. Phenol:chloroform:isoamyl (25:24:1) was used to purify the DNA, twice.

2.10 Phage Genome Sequencing

Approximately 10 µg of phage DNA was sent for genetic sequencing in Moorepark Food Research Centre, Teagasc, Fermoy, using Illumina MiSeq. The assembled phage genomes had coverage as follows, phage 0.0066 had a 20x coverage and phage 3488 had a 50x coverage. Phage B1 which was previously sequenced by another party whose work has now been published (Ajuebor, *et al.*, 2018). and used for comparative purposes had 3200x coverage.

2.11 Phage ORF Prediction and Analysis

Initial genome annotation was completed using myRAST (Aziz, 2008). Predicted proteins were scanned for homologs using BLASTP and PSI-BLAST (Altschul *et al.*, 1997). Protein analysis and gene functional annotation was carried out using methods and applications developed by The National Centre for Biotechnology Information (NCBI) and The UniProt Consortium (2017). The appropriate rasta, blast and predicted gene files were then fed into Artemis Comparison Tool (ACT) for visual genome comparisons (Rutherford *et al.*, 2000). The comparative genome map was constructed using the Mauve2.3.1 proGram.

3. Results

3.1 Antibiotic Sensitivity

Antibiotic sensitivity of each *Staphylococcus* strain (Table 2.1) was ascertained using the Kirby-Bauer method, briefly each strain was plated on Mueller-Hinton agar, as a bacterial lawn and a 1 µg Oxacillin disk placed in the centre. Oxacillin is analogous to methicillin, as MRSA is resistant to all β-lactams due to the presence of *mecA*, a gene that produces a penicillin-binding protein (PBP2a) with low affinity for β-lactam antibiotics (Becker & Christof, 2011). The level of antibiotic sensitivity determined by measuring the zones of clearing surrounding an oxacillin antibiotic disc, in millimetres, after incubation, are presented in Fig.3.1. If the zone measured less than or equal to 10 mm the strain is said to be resistant, a zone of clearing measuring between 10 and 13 mm indicates intermediate resistance while a zone of 13 mm or more means the strain is susceptible to antibiotic. All clinical strains were confirmed to be MRSA, 11 of the 20 UCC culture collection strains were found to be resistant, of the 8 non-*S. aureus* strains from the Moorepark culture collection all were found to be susceptible except *S. epidermidis* DPC 6010 which was found to be intermediately resistant. The results of antibiotic testing are presented in Table 3.1.

Figure 3.1: Disc diffusion method showing antibiotic susceptibility of *Staphylococcus aureus* strains, 0.1206(IV) ST250 and DPC 5245 (or 317(T)) to 1 µg Oxacillin disk (Oxoid). The former, a human clinical isolate shows complete resistance to the antibiotic with no zone observed. The later, a bovine isolate is considered susceptible having an average diameter of 24.33 mm.

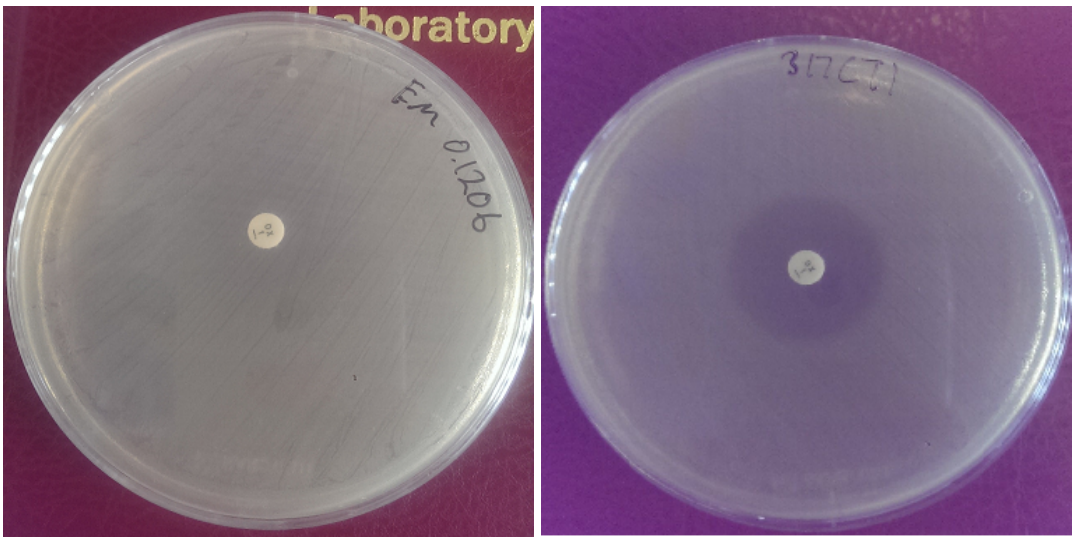


Table 3.1 Oxacillin resistance testing against *Staphylococcus* Bank

UCC culture collection			**MRSA Reference Laboratory**		
ST290	*S. aureus*	0^r	0220(II) ST5	*S. aureus*	0^r
ST291	*S. aureus*	0^r	0.1345(II) ST5	*S. aureus*	0^r
ST295	*S. aureus*	0^r	3596(IIV) ST8	*S. aureus*	0^r
ST299	*S. aureus*	0^r	3045(IIV) ST8	*S. aureus*	0^r
ST355	*S. aureus*	0^r	3488(IVV) ST8	*S. aureus*	0^r
ST528	*S. aureus*	16.66^s	3144(IIV) ST8	*S. aureus*	0^r
ST530	*S. aureus*	0^r	E1038(IV) ST8	*S. aureus*	0^r
ST535	*S. aureus*	0^r	E1185(IV) ST12	*S. aureus*	0^r
ST544	*S. aureus*	0^r	E1174(IV) ST22	*S. aureus*	0^r
ST550	*S. aureus*	0^r	0242(IV) ST30	*S. aureus*	0^r
25949	*S. aureus*	0^r	3592 (II) ST36	*S. aureus*	0^r
35197	*S. aureus*	30.66^s	E1139(IV) ST45	*S. aureus*	0^r
Newman	*S. aureus*	26.66^s	0.1239(III) ST239	*S. aureus*	0^r
RF122	*S. aureus*	0^r	0.0066(IIIV) ST239	*S. aureus*	0^r
DPC5247	*S. aureus*	22.33^s	0104(III) ST239	*S. aureus*	0^r
DPC6971	*S. aureus*	23.66^s	0073(III) ST239	*S. aureus*	0^r
DPC5243	*S. aureus*	23.33^s	M03/0073(III) ST239	*S. aureus*	0^r

DPC3971	*S. aureus*	24.66[s]	0308(1A) ST247	*S. aureus*	0[r]
DPC5245	*S. aureus*	24.33[s]	3581(1A) ST247	*S. aureus*	0[r]
DPC5246	*S. aureus*	23.33[s]	0.1206(IV) ST250	*S. aureus*	0[r]
			E1202(II) ST596	*S. aureus*	0[r]
Non-S. aureus strains from Moorepark culture collection					
DPC6010	*S.epidermidis*	12.66[r]	DPC6014	*S. hominis*	24[s]
DPC6011	*S. saprophyticus*	24.33[s]	DPC6015	*S. haemolyticus*	30.66[s]
DPC6012	*S. chromogens*	25.66[s]	DPC6016	*S. caprae*	25[s]
DCP6013	*S. Capitis*	31.33[s]	DPC6017	*S. hyicus*	33[s]

[a] Average of 3 mm
[r] Resistant. Zone diameter of ≤14 mm
[s] Susceptible. Zone diameter of ≥ 15 mm

3.2 Phage Sensitivity/Characterization

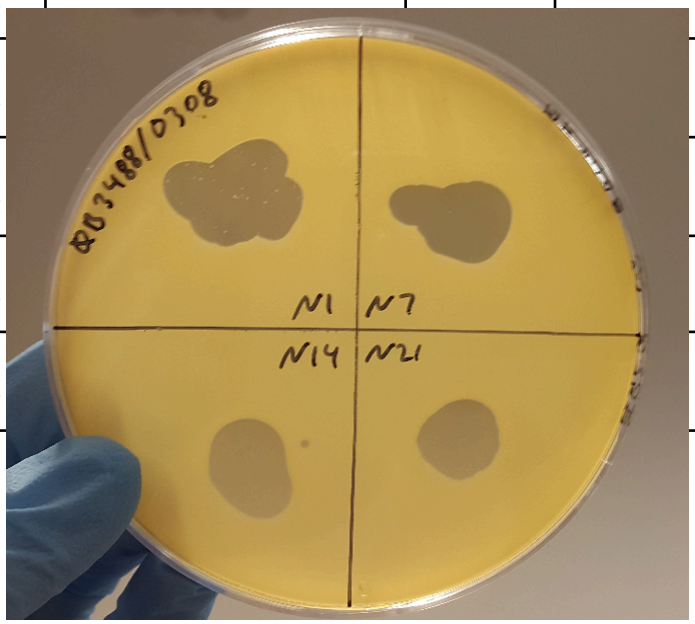
Phage B1 (George Eliava Institute) and phage K (ATCC), were tested against the full bank of *S. aureus* strains and other staphylococcal strains by spot assay and plaque assay. These assays were to determine which bacterial strains were permissive hosts (i.e. the phage could replicate in the strain) and which strains were nonpermissive hosts (i.e. the phage could not replicate in the strain). Assays were performed in triplicate. In plaque assays the plaques of Phage B1 and Phage K, against the different strains, varied between 0.5-12 mm in size and varied in opacity, being either uniformly clear or hazy. The results of these plaque assays on *S. aureus* can be seen in Table 3.2 and individual plaque morphology for the susceptible strains show in Figure 3.3.

Quantitative results are shown where '-' means no clearing, 'X' means small, weak or hazy plaque and 'XX' indicates a clear plaque or good size, approximately 10 mm or above. see Fig. 3.2. One of the Phage B1 and Phage K mutants when plates produced plaques with a halo appearance, this may indicate the production of lysin. EOP results are presented in Table 2.1.

Table 3.2 Sensitivity of MRSA to Phage B1 and Phage K (5246)

Host	**Strain**	**Phage B1**	**Phage K**
S. aureus	5246	XX	XX
S. aureus	0220	XX	XX
S. aureus	0.1345	-	-
S. aureus	3596	X	X
S. aureus	3045	X	X
S. aureus	3488	-	-
S. aureus	3144	X	X
S. aureus	E1038	X	X
S. aureus	E1185	-	-
S. aureus	E1174	XX	-
S. aureus	0242	XX	XX
S. aureus	3594	X	X
S. aureus	E1139	-	-
S. aureus	0.1239	X	-

Host	Strain	Phage B1	Phage K
S. aureus	0.0066	X	X
S. aureus	0104	X	X
S. aureus			
S. aureus			
S. aureus			
S. aureus			



3.3 Phage B1 host switching.

Figure 3.2 Spot test of phage B1 mutant after being co-cultured with 3488 after 1, 4, 14 and 21 rounds tested against strain 0308.

Shifting of the host range was achieved by repeated rounds of co-culturing; incubating the phage in a relatively large volume of BHI broth, with a host (H, susceptible) strain, DPC5246 and a resistant (R) strain, in varying ratios (H:R > 50:50, 20:80). Plaque assays were carried out after each round of co-culturing as per protocol in section 2.3. Spot assays of first-round Phage B1 mutants were carried out at different time points, day 1,7, 14 and 21, as per protocol in section 2.5. The results are presented in Figure 3.2. They showed no increase in phage plaquing ability with future exposure after initial plaques were first seen. Round two Phage B1 mutants, those with higher ratio of a resistant strain, were found to overcome resistance quicker and affected a larger number of strains. Spot assays of all phage B1 mutants on the MRSA bank were performed, the results of which are presented in Table 3.3, a comparison of the host ranges of phage B1, K and selected mutants can be seen in Table 3.5. Individual plaque morphology for the susceptible strains is shown in Figure 3.3.

Additionally, one of the Phage B1 and Phage K mutants when plated produced plaques with a halo appearance, this may indicate the production of lysin. EOP results are presented in Table 2.1.

Table 3.3 Sensitivity of MRSA to Phage B1 Mutants

		Phage mutant name and ratio of host strain to resistant strain				
Host	**Strain**	**ØB 3488 20:80**	**ØB 3488 50:50**	**ØB 0.0066 20:80**	**ØB 0.0066 50:50**	**ØB 0.1345**
S. aureus	5246	XX	XX	XX	XX	X
S. aureus	0220	XX	XX	X	XX	XX
S. aureus	0.1345	-	X	-	-	-
S. aureus	3596	XX	XX	-	-	-
S. aureus	3045	X	XX	X	X	X
S. aureus	3488	X	X	-	-	-
S. aureus	3144	X	XX	X	X	X
S. aureus	E1038	-	X	-	-	-
S. aureus	E1185	-	-	-	-	-
S. aureus	E1174	XX	XX	XX	XX	XX
S. aureus	0242	XX	XX	X	XX	X
S. aureus	3594	X	X	X	X	X
S. aureus	E1139	-	XX	-	-	-
S. aureus	0.1239	X	X	X	X	-
S. aureus	0.0066	-	-	-	-	X
S. aureus	0104	-	-	-	-	-

S. aureus	0073	XX	XX	XX	XX	XX
S. aureus	0308	-	-	-	-	-
S. aureus	3581	X	X	-	X	-
S. aureus	0.1206	XX	XX	XX	XX	X

> - no plaque, X weak plaque, XX clear plaque

3.4 Phage K host switching

Shifting of the host range was achieved by repeated rounds of co-culturing; incubating the phage in a relatively large volume of BHI broth, with a host strain and a resistant strain, in varying ratios (as described for phage B1) Plaque assays were carried out after each round of co-culturing as per protocol in section 2.3. Spot assays of first-round Phage K mutants were carried out at different time points, day 1, 7, 14 and 21, as per protocol in section 2.5. As with Phage B mutants no notable increase in phage plaquing ability was seen after initial plaques appeared. Round two Phage K mutants, those with higher ratio of a resistant strain, were found to overcome resistance quicker and affected a larger number of strains. Spot assays of all phage K mutants on the MRSA bank of strains were carried out, the results of which are presented in Table 3.4, a comparison of host ranges selected mutants, phage B1 and K can be seen in Table 3.5. Individual plaque morphology for the susceptible strains is shown in Figure 3.3.

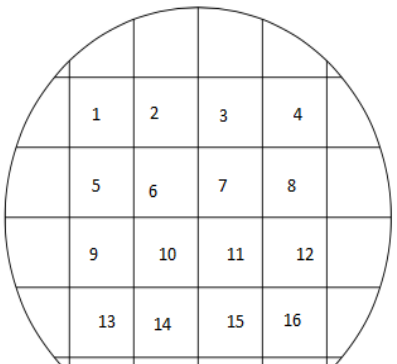
Table 3.4 Sensitivity of MRSA to Phage K Mutants

	Phage mutant name (combination of original name and the bacterial co-cultured strain) and the culturing ratio								
Strain	**Ø K S. hom**	**Ø K S. epi**	**ØK 3488 20:80**	**ØK 3488 0:50**	**ØK 0.0066 20:80**	**ØK 0.0066 50:50**	**ØK 0.1206**	**ØK E1139**	**ØK 0.1345**
5246	XX	XX	XX	XX	XX	XX	XX	XX	XX
0220	XX	XX	XX	XX	XX	XX	XX	XX	X
0.1345	XX	X	XX	XX	-	XX	XX	-	X

3596	X	X	XX	XX	-	-	XX	X	X
3045	XX	X	X	XX	X	XX	XX	X	XX
3488	-	-	XX	X	X	X	XX	-	X
3144	XX	XX	XX	XX	X	XX	XX	X	XX
E1038	X	X	XX	XX	X	X	X	-	X
E1185	-	-	-	-	-	-	-	-	-
E1174	XX	XX	XX	XX	XX	XX	XX	XX	XX
0242	XX	X	XX	XX	XX	X	XX	XX	XX
3594	X	X	X	X	X	X	X	X	X
E1139	X	-	XX	XX	-	XX	XX	-	X
0.1239	X	X	X	X	X	X	X	X	X
0.0066	XX	XX	XX	XX	XX	-	X	-	-
0104	X	X	X	X	X	-	X	-	-
0073	XX	XX	XX	XX	XX	XX	XX	XX	XX
0308	XX	XX	XX	XX	XX	XX	XX	X	X
3581	XX	X	XX	XX	XX	XX	XX	X	-
0.1206	XX	XX	XX	XX	XX	XX	XX	XX	X

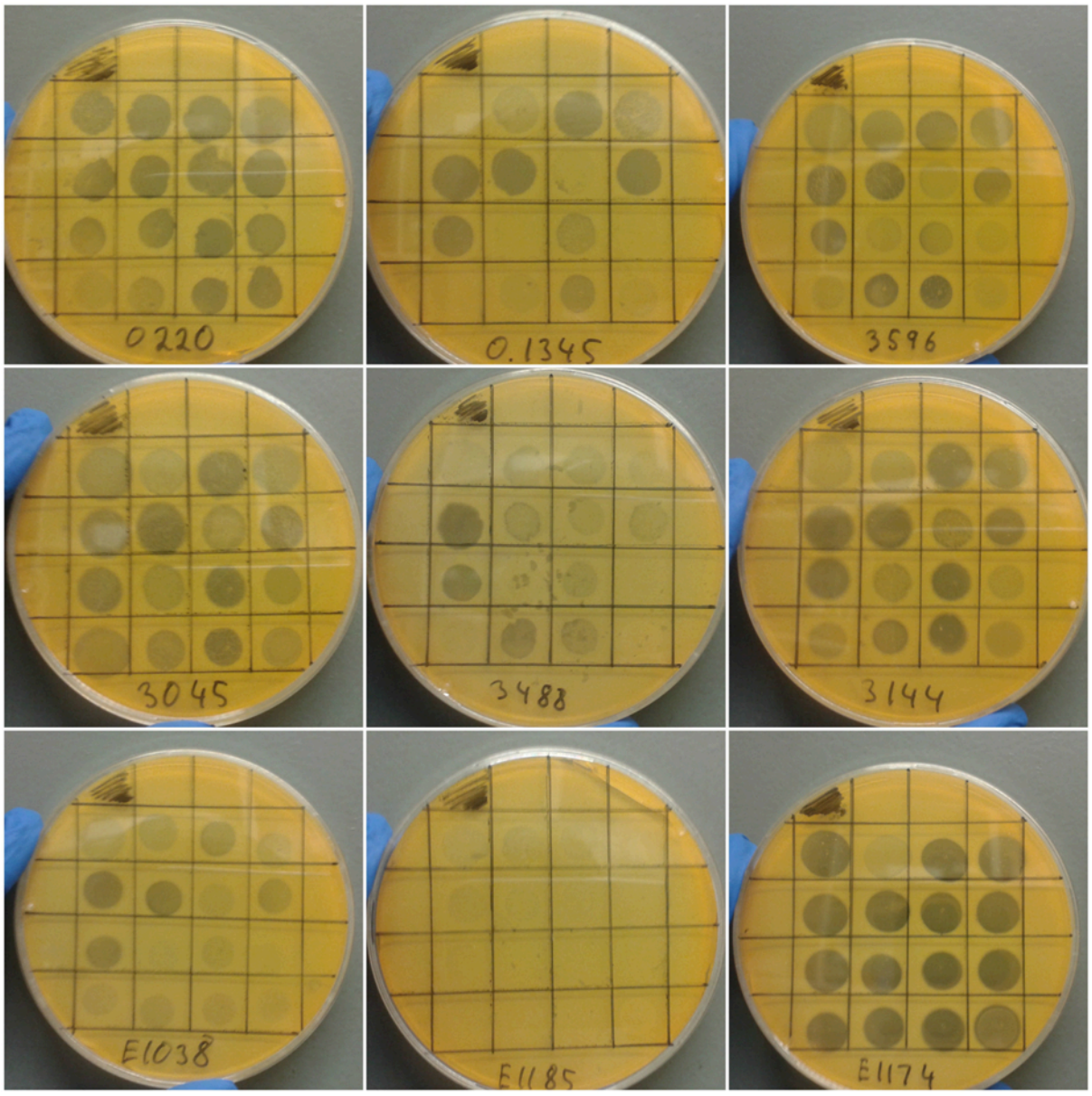
> - no plaque, X weak plaque, XX clear plaque

Figure 3.3. Photos of phage B1, K and phage mutants spot assays after 21 nights. Filtrates of the original phage and their mutants were spotted onto different strains (see Table 2.1 for strain details). The results of these are listed in Table 3.2, 3.3 and 3.4. The strains were plated as follows:



1. ØB 5246
2. ØK 5246
3. ØK 5246/*Staphylococcus hominis*
4. ØK 5246/*Staphylococcus epidermidis*
5. ØK 5246/0.1206(IV) ST250
6. ØK 5246/E1139(IV) ST45

7. ØK 5246/0.1239(III) ST239
8. ØK 5246/3488(IVV) ST8 20:80
9. ØK 5246/3488(IVV) ST8
10. ØK 5246/0.0066(IIIV) ST239 20:80
11. ØK 5246/0.0066(IIIV) ST239
12. ØB 5246/0.0066(IIIV) ST239 20:80
13. ØB 5246/0.0066(IIIV) ST239
14. ØB 5246/3488(IVV) ST8 20:80
15. ØB 5246/3488(IVV) ST8
16. ØB 5246/0.1345(II) ST5



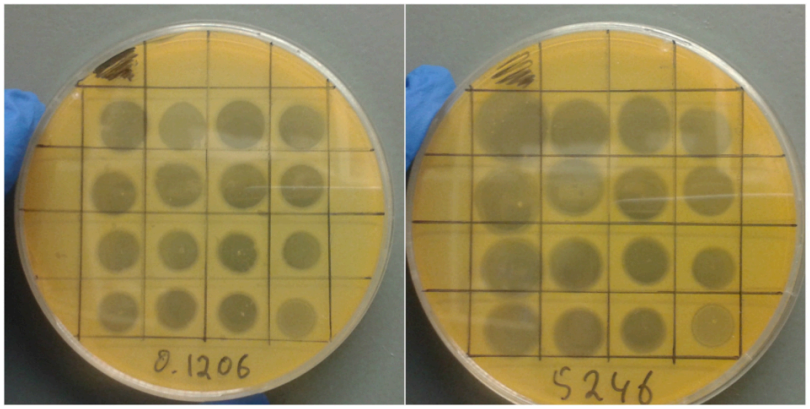

D.1206
5246

Table 3.5 Comparison of host range of Phage B1, phage K and selected mutants

		Phage name			
Host	**Strain**	**Phage B1**	**ØB 3488 50:50**	**Phage K**	**ØK 3488 50:50**
S. aureus	5246	XX	XX	XX	XX
S. aureus	0220(II) ST5[d]	XX	XX	XX	XX
S. aureus	0.1345(II) ST5 [d]	-	X	-	XX
S. aureus	3596(IIV) ST8 [d]	X	XX	X	XX
S. aureus	3045(IIV) ST8 [d]	X	XX	X	XX
S. aureus	3488(IVV) ST8 [d]	-	X	-	X
S. aureus	3144(IIV) ST8 [d]	X	XX	X	XX
S. aureus	E1038(IV) ST8 [d]	X	X	X	XX
S. aureus	E1185(IV) ST12 [d]	-	-	-	-
S. aureus	E1174(IV) ST22 [d]	XX	XX	-	XX
S. aureus	0242(IV) ST30 [d]	XX	XX	XX	XX
S. aureus	3594 (II) ST36 [d]	X	X	X	X
S. aureus	E1139(IV) ST45 [d]	-	XX	-	XX
S. aureus	0.1239(III) ST239 [d]	X	X	-	X
S. aureus	0.0066(IIIV) ST239 [d]	X	-	X	XX
S. aureus	0104(III) ST239 [d]	X	-	X	X
S. aureus	0073(III) ST239 [d]	XX	XX	-	XX
S. aureus	0308(1A) ST247 [d]	XX	-	XX	XX
S. aureus	3581(1A) ST247 [d]	XX	X	XX	XX
S. aureus	0.1206(IV) ST250	XX	XX	X	XX

> - no plaque, X weak plaque, XX clear plaque

Phage B1 3488 and phage B1 0.0066 were selected for further analysis because they exhibited the most encouraging results. After co-culturing both were able to infect strains that were previously permissible and appeared to have greater virulence against strains that they could previously infect, as witnessed by larger zones of clearing.

The genomes of the parent phage B1 and the mutant phages B1 3488 and B1 0.0066 were sequenced and compared to determine if their genomes differed.

3.5 Genome of Phage B1

Phage B1 was isolated from a Fersisi commercial phage mixture. The genome of phage B1 is 140,808 bp in size. Genome analysis of Phage B1 revealed it to be very similar to the genome of a phage called team 1 and phage G1. Phage team 1 is a hospital isolate from the Republic of Georgia (El Haddad, *et. al.*, 2014). Phage G1 was first isolated from a mixture of *S. aureus* bacteriophages (Staphylococcal bacteriophage, lot no. 361098, BioPharm, Tbilisi, Republic of Georgia) (Khan, *et. al.,* 2005)

Phage B1 and phage team 1 are compared in Figure 3.4. They have 99-100% shared identity and the red colour in the diagram indicates that they have the same orientation. Approximately 45% of the ORFs encoded in the genome have unknown functions.

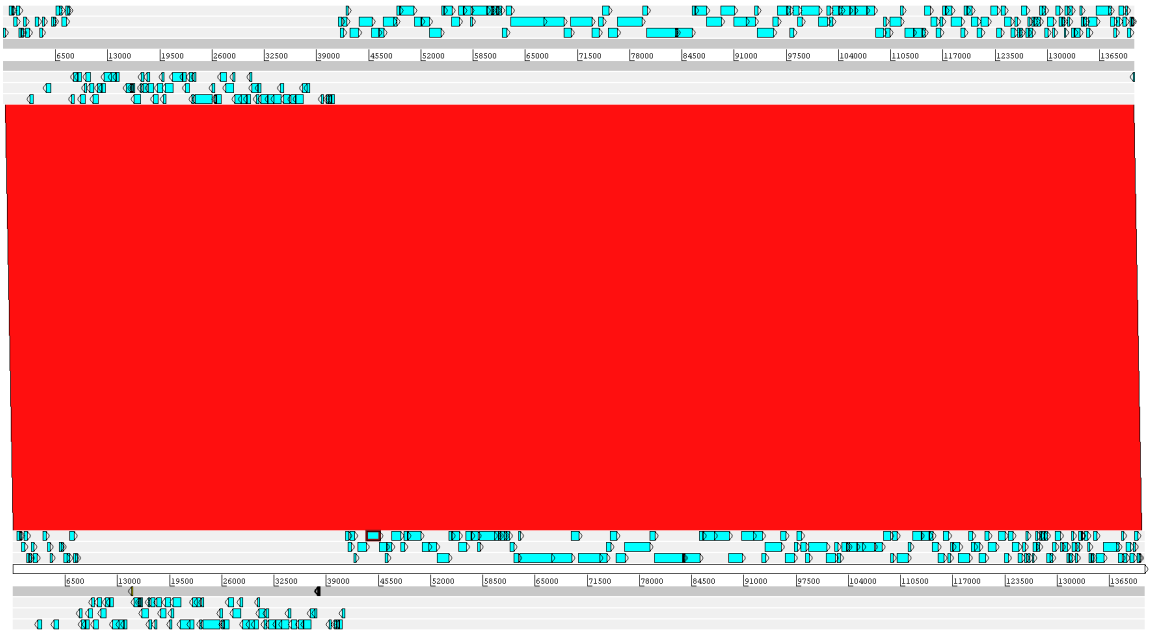


Figure 3.4 Open reading frame map comparing the genome of Phage B1(top) to the genome of phage team 1 (bottom). The direction of the teal arrows represents forward and reverse open reading frames. The red colour indicates that genes are arranged in the same orientation.

Table 3.6 Genome of Phage B1 with ORF description

Gene/Protein Name	Function (when known)	% Identity	Hit Length	E-value
ORF128			112	5.00E-70
ORF162	transfer protein	100	206	7.00E-148
ORF039	protein coding	100	278	0
ORF202	protein coding	100	71	9.00E-45
tail sheath protein	tail sheath protein	100	587	0
ORF105	membrane protein	100	142	4.00E-99
ORF293		100	46	2.00E-22
ORF093	dTMP biosynthetic activity	100	152	5.00E-107
ORF215		100	64	9.00E-34
ORF141	portal protein	100	103	3.00E-65
ORF095	ATP binding, DNA binding	100	152	2.00E-104

Gene/Protein Name	Function (when known)	% Identity	Hit Length	E-value
ORF145	structural protein	100	102	4.00E-68
ORF074	protein coding	100	178	4.00E-125
ORF001	tail tape measure protein	100	1352	0
ORF005	amidase; secretory antigen	100	808	0
ORF033	DNA replication, oxidation/reduction	100	295	0
ORF004	glycerophosphoryl diester phosphodiesterase	100	848	0
ORF043	putative tail tube associated base plate protein		263	0
ORF078		100	174	1.00E-120
ORF052	LysM domain	100	234	2.00E-170
ORF027	protein coding	100	348	0
conserved hypothetical protein		100	1019	0
ORF159	anti-sigma factor	100	94	3.00E-62
ORF079	major capsid protein	100	173	1.00E-121
ORF002	Virulence-associated protein	100	1152	0
ORF262	putative site-specific DNA ndonuclease	100	52	3.00E-26
capsid and scaffold protein	capsid and scaffold protein	100	640	0
ORF117		100	124	4.00E-83
putative structural protein	putative structural protein	99.78	458	0
ORF012	Type III restriction enzyme; DEAD/DEAH box helicase	100	582	0
ORF013	Putative conjugative transposon protein	100	537	0
ORF015	AAA ATPase	100	480	0
ORF028	exonuclease	100	345	0
ORF221	endonuclease activity	100	63	2.00E-36
ORF110		100	125	9.00E-85

Gene/Protein Name	Function (when known)	% Identity	Hit Length	E-value
ORF009	exonuclease	100	639	0
ORF067	DNA binding	100	198	2.00E-141
ORF026	DNA primase	100	355	0
ORF127	putative superinfection exclusion	100	112	5.00E-71
conserved hypothetical protein	penicillin-binding	100	150	1.00E-99
ORF064	protein coding	100	202	5.00E-147
ribonucleotide reductase stimulatory	Ribonucleotide reductase; NrdI	100	143	2.00E-96
ORF006	Ribonucleotide reductase large subunit	100	704	0
putative ribonucleotide reductase minor subunit	Ribonucleotide reductase minor subunit	100	349	0
ORF085	protein coding	100	162	3.00E-113
ORF130	protein coding	100	109	7.00E-72
thioredoxin-like	thioredoxin-like protein	100	106	2.00E-68
ORF066	protein coding	99.49	198	3.00E-138
ORF147	Integration host factor	100	101	2.00E-65
ORF035	DNA polymerase	100	290	0
hypothetical protein		99.38	170	7.00E-110
PolA	DNA polymerase I	100	1072	0
putative HNH endonuclease	HNH endonuclease	100	269	0
ORF037	DNA polymerase	100	286	0
ORF181	HNH endonuclease	10	80	3.00E-49
hypothetical protein		100	52	5.00E-26
ORF089	membrane protein	100	160	4.00E-114
ORF020	phage major tail protein	100	423	0
recombinase a	RecA bacterial DNA recombination protein	100	74	2.00E-43
endonuclease	endonuclease activity	100	322	0

Gene/Protein Name	Function (when known)	% Identity	Hit Length	E-value
recombinase a	RecA bacterial DNA recombination protein	100	315	0
ORF121	protein coding	100	117	2.00E-78
ORF056	RNA polymerase sigma	100	220	3.00E-155
ORF059	putative structural protein	100	210	7.00E-149
putative major tail protein	major tail protein	100	173	3.00E-115
ORF189	major tail protein	100	75	3.00E-44
ORF297		100	43	2.00E-20
ORF174	protein coding	100	86	4.00E-54
ORF046	DNA binding	100	251	3.00E-179
ORF022	metallophosphoesterase	100	416	0
ORF118	protein coding	100	122	1.00E-79
ORF143		100	103	2.00E-67
ORF075	protein coding	100	178	2.00E-126
ORF045	protein coding	100	255	0
ORF099	protein coding	100	148	1.00E-102
ORF036		100	287	0
ORF047	envelope protein	100	243	4.00E-172
ORF135	tail tube terminator protein	99.07	107	9.00E-70
ORF094	protein coding	100	152	2.00E-104
ORF100	DNA binding, nuclease binding	100	147	8.00E-98
ORF053	protein coding	100	234	2.00E-167
ORF108		100	132	2.00E-89
ORF182		100	80	1.00E-47
ORF252	putative HNH homing endonuclease	100	54	8.00E-29
ORF240		100	58	6.00E-32
ORF076	late transcription factor	100	177	3.00E-122
hypothetical protein		100	82	3.00E-44

Gene/Protein Name	Function (when known)	% Identity	Hit Length	E-value
ORF241	ATP binding, DNA binding	100	58	2.00E-30
hypothetical protein phageK gp017		100	78	4.00E-46
ORF152	DNA binding/intergration	100	98	1.00E-62
MbpK		100	63	2.00E-32
ORF119		100	122	4.00E-81
ORF124	putative ribonucleotide reductase minor subunit	100	115	7.00E-76
putative membrane protein MbpI	putative membrane protein	100	92	2.00E-55
ORF140		100	101	3.00E-66
ORF122		100	116	2.00E-75
ORF065	protein coding	100	200	7.00E-144
ORF237	lysozyme activity	100	59	8.00E-34
ORF107		100	133	7.00E-86
ORF092		100	156	2.00E-103
ORF173	glutaredoxin	100	86	2.00E-52
ORF157	Iteron-binding	100	95	2.00E-58
ORF362	protein coding	100	38	3.00E-14
ORF170	DNA endonuclease	100	87	3.00E-52
ORF236		100	59	1.00E-28
ORF171		100	87	6.00E-55
ORF137	protein coding	100	105	3.00E-67
ORF055	protein coding	100	226	3.00E-160
ORF263	protein coding	100	52	1.00E-22
ORF211		100	66	2.00E-40
ORF150	protein coding	100	99	2.00E-63
ORF166	putative transmembrane protein	100	89	2.00E-19
ORF155		100	96	2.00E-58
ORF144	terminase large subunit	100	102	9.00E-63

Gene/Protein Name	Function (when known)	% Identity	Hit Length	E-value
ORF031	phosphoribosyl transferase domain protein			
putative nicotinate phosphoribosyltransferase	Nicotinate phosphoribosyltransferase			
ORF178	membrane protein			
ORF113	translation regulation			
ORF194	putative fiber assembly protein			
ORF142	endonuclease			
ORF082	protein coding			
ORF131	hydrolase activity			
hypothetical protein				
gpORF179				
ORF139	protein coding			
ORF225	protein coding			
ORF445	protein coding			
hypothetical protein SA5_0153/152				
ORF088	protein coding			
ORF109				
ORF103	protein coding			
ORF224				
conserved hypothetical protein				
ORF104		100	143	7.00E-97
ORF073		100	180	2.00E-124
TreB		100	62	2.00E-29
ORF086	GTP cyclohydrolase	100	162	4.00E-116
ORF111		99.18	132	1.00E-80
ORF051	Serine/threonine-specific protein phosphatase	100	235	2.00E-169

Gene/Protein Name	Function (when known)	% Identity	Hit Length	E-value
ORF070		99.46	184	9.00E-128
ORF138		100	105	2.00E-66
ORF071		100	182	9.00E-123
ORF201		100	72	1.00E-42
ORF218		100	64	4.00E-37
ORF050		100	245	2.00E-173
ORF437		100	34	1.00E-13
ORF156		100	96	6.00E-60
gpORF020		100	82	7.00E-50
ORF114		100	129	3.00E-88
ORF245	Immunodominant antigen A	100	57	1.00E-33
ORF090		100	160	1.00E-108
ORF072		100	180	3.00E-122
ORF077		100	177	6.00E-123
ORF256		100	54	1.00E-28
ORF163		100	91	2.00E-52
ORF038		100	281	0
ORF024	Nitric oxide reductase	100	372	0
ORF158		100	95	7.00E-60
ORF134		100	108	3.00E-72
ORF106		100	138	2.00E-94
ORF149	DNA-binding	100	100	2.00E-63
ORF228		100	62	4.00E-34
ORF259		100	53	1.00E-28
ORF007		100	682	0
ORF172		100	87	2.00E-54
hypothetical protein		100	57	3.00E-31
ORF068		100	192	4.00E-129

Gene/Protein Name	Function (when known)	% Identity	Hit Length	E-value
ORF061		100	208	1.00E-149
ORF154		100	97	1.00E-62
ORF032	DNA ligase/mRNA capping enzyme	100	298	0
hypothetical protein		100	74	2.00E-39
ORF049	PhoH-like protein	100	246	0
ORF063		100	204	2.00E-143
ORF096	RNase H	100	141	1.00E-93
ORF222		100	63	1.00E-36
ORF057		100	213	9.00E-143
ORF187	Helix-turn-helix motif	100	76	4.00E-46
ORF190		100	75	3.00E-44
ORF054	Transglycosylase-like; Immunodominant antigen A	100	230	2.00E-166
ORF175		100	85	1.00E-53
ORF058		100	211	7.00E-151
ORF044	Prohibitin; Band 7 protein	100	263	0
ORF146		100	102	3.00E-64
ORF060		100	209	2.00E-153
ORF084	Amidase	99.4	166	7.00E-114
ORF042	HNH endonuclease;	100	267	0
ORF083	Amidase	100	167	3.00E-115
ORF233		100	61	4.00E-33
ORF200		100	72	1.00E-44
ORF207		100	69	8.00E-42
ORF183		100	79	5.00E-49
ORF209		100	110	1.00E-68
hypothetical protein		100	108	5.00E-70
ORF168		100	128	2.00E-53

Gene/Protein Name	Function (when known)	% Identity	Hit Length	E-value
ORF161		100	92	5.00E-60
ORF133		100	136	8.00E-91
terminase large subunit	large terminase	100	65	9.00E-40
hypothetical protein		100	323	0
Ter	terminase	100	605	0
hypothetical protein		100	273	0
ORF091		100	159	7.00E-109
ORF125		100	115	8.00E-76
hypothetical protein		100	376	0
ORF120		100	116	4.00E-73
hypothetical protein		100	103	7.00E-67
ORF014		100	563	0
ORF048		100	257	0
ORF029	Topoisomerase; Autolysin; Internalin	100	318	0
ORF016	major capsid protein	100	463	0
ORF151		100	98	4.00E-59
ORF030		100	302	0
ORF034		100	292	0

3.6 Genome of Phage B1 3488

Phage B1 3488 is the novel phage generated by co-culturing Phage B1 with S. aureus strain 3144 (IIV) ST8[d]. In Figure 3.5 we can see that although Phage B1 3488 has decreased in size the areas which have remained are highly conserved and show 99-100% identity with phage team 1. The areas in red have retained the same orientation while those in blue have reversed. Areas in the genome with no similarity are shown on the image as sections where there is no block connecting the two genomes. To the right of the image there are no lines or blocks connecting Phage B1 3488 with phage team 1, this presents the bulk of

new genes that Phage B1 3488 acquired, which are not present in either Phage B1 or Phage B1 0.0066.

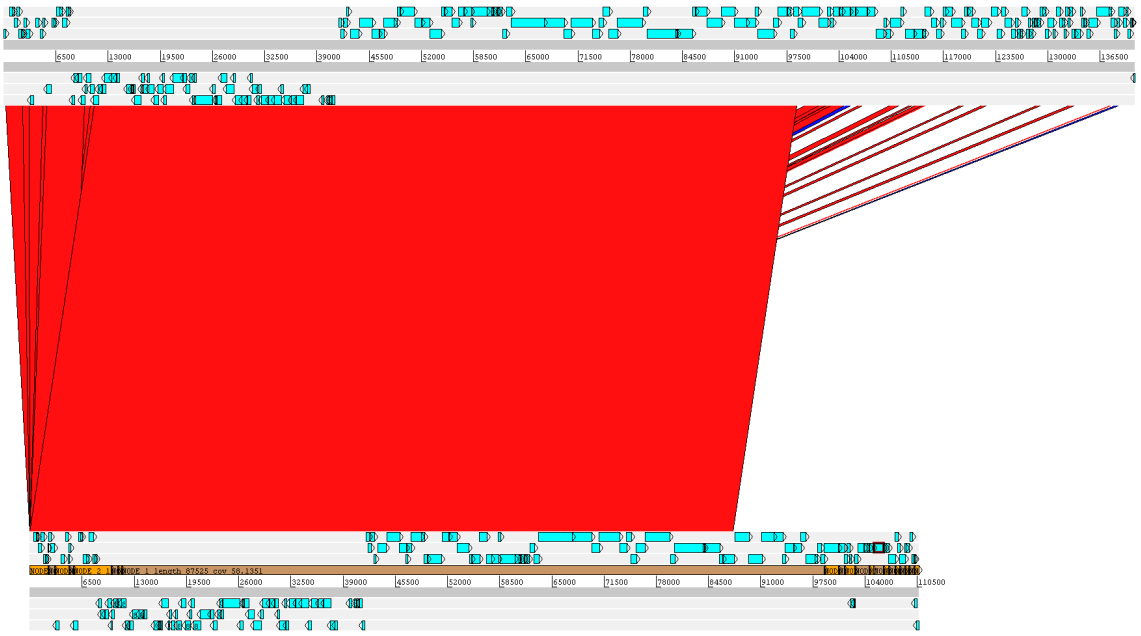


Figure 3.5 Opening reading frame map comparing phage team 1 (top) with Phage B1 3488 (bottom). The direction of the teal arrows represents forward and reverse open reading frames. The red colour indicates that genes are arranged in the same orientation, the dark blue colour represents a reversal in orientation of the genes.

Table 3.7 Genome of Phage 3488 with ORF description

Gene/Protein Name	Function (when known)	% Identity	Hit Length	E-value
recombinase a	DNA repair	100	315	0
putative ribonucleotide reductase minor subunit	putative ribonucleotide reductase minor subunit	100	349	6.00E-173

Gene/Protein Name	Function (when known)	% Identity	Hit Length	E-value
hypothetical protein		100	144	5.00E-08
ORF224		100	63	3.00E-35
conserved hypothetical protein		100	161	2.00E-104
ORF171	protein coding	100	87	6.00E-55
ORF137	protein coding	100	105	4.00E-47
ORF121		100	117	1.00E-51
endonuclease	endonuclease activity	99.05	322	1.00E-68
ORF036	large terminase	100	287	2.00E-133
ORF178	membrane protein	92.16	81	6.00E-23
hypothetical protein		74.6	103	2.00E-26
ORF194	putative fiber assembly protein	100	73	1.00E-43
DNA polymerase	DNA polymerase	100	416	1.00E-59
ORF118	Protein coding	100	122	4.00E-51
ORF221	endonuclease activity	100	63	4.00E-36
DNA polymerase I	DNA polymerase I	100	298	4.00E-124
ORF073	protein coding	100	180	2.00E-116
ORF050	DNA coding	100	245	2.00E-173
ORF012	Type III restriction enzyme; DEAD/DEAH box helicase	100	582	0
ORF013		100	537	0
ORF015	AAA ATPase	100	480	0
ORF028	Exonuclease	100	345	0
ORF110		100	125	9.00E-85
hypothetical protein		100	639	0

Gene/Protein Name	Function (when known)	% Identity	Hit Length	E-value
ORF009	Exonuclease	100	639	0
ORF067	DNA binding	100	198	2.00E-141
ORF026	DNA primase	100	355	0
hypothetical protein		100	112	9.00E-56
ORF437		100	34	1.00E-13
conserved hypothetical protein		100	150	1.00E-99
ORF064	protein coding	100	202	4.00E-100
gpORF020		100	82	7.00E-50
ORF114	transferase activity	100	129	3.00E-88
ORF245	Immunodominant antigen A	100	57	1.00E-33
ORF090	phosphorylated membrane protein	100	160	4.00E-109
ORF072	transcription termination factor	100	180	3.00E-122
ORF077		100	177	6.00E-123
ORF256		100	54	1.00E-28
ORF163	probable conjugal transfer protein	100	91	2.00E-52
ORF086	GTP cyclohydrolase	100	162	4.00E-116
ORF038	protein coding	100	281	0
ORF024	Nitric oxide reductase	100	372	0
ORF134	protein coding	100	108	3.00E-72
ORF106		100	138	2.00E-94
ORF149	DNA-binding	100	100	2.00E-63
ORF228		100	62	4.00E-34
ORF259	protein coding	100	53	1.00E-28
ORF007	N-acetylmuramoyl-L-alanine amidase activity	100	682	0

Gene/Protein Name	Function (when known)	% Identity	Hit Length	E-value
ORF172	protein coding	100	87	2.00E-54
hypothetical protein		100	57	3.00E-31
ORF111	protein coding	100	132	6.00E-88
ORF068	protein coding	100	192	4.00E-129
ORF061	protein coding	100	208	1.00E-149
ORF032	DNA ligase/mRNA capping enzyme	100	298	0
hypothetical protein s		100	74	2.00E-39
ORF049	PhoH-like protein	100	246	0
ORF063		100	204	2.00E-143
ORF096	RNase H	100	141	1.00E-93
ORF222		100	63	1.00E-36
ORF057	protein coding	100	213	9.00E-143
ORF187	helix-turn-helix motif	100	76	4.00E-46
ORF051	serine/threonine-specific protein phosphatase	100	235	5.00E-170
ORF190		100	75	3.00E-44
ORF054	transglycosylase-like;immunodominant antigen A	100	230	2.00E-166
ORF058	protein coding	100	211	7.00E-151
ORF044	prohibitin; Band 7 protein	100	263	0
ORF146		100	102	3.00E-64
ORF060	amidase	100	209	2.00E-153
ORF084	HNH endonuclease; Intron encoded nuclease domain	99.4	166	7.00E-114
ORF042	DNA binding	100	267	0
ORF083	holin	100	167	3.00E-115
ORF233	DNA binding	100	61	4.00E-33

Gene/Protein Name	Function (when known)	% Identity	Hit Length	E-value
ORF070	RNA polymerase subunit	99.46	184	9.00E-128
ORF200	DNA binding/intergration	100	72	1.00E-44
ORF207	putative Gp5 baseplate hub subunit and tail lysozyme	100	69	8.00E-42
ORF209		100	110	1.00E-68
hypothetical protein		100	108	5.00E-70
ORF169		100	88	2.00E-49
ORF168	protein coding	100	128	2.00E-53
ORF161	nucleotide binding	100	92	7.00E-60
ORF133	protein coding	100	136	8.00E-91
terminase large subunit	terminase large subunit	100	65	9.00E-40
hypothetical protein		100	323	0
ORF138		100	105	2.00E-66
Ter	DNA replication terminus site-binding protein	100	605	0
hypothetical protein		100	273	0
hypothetical protein		100	57	4.00E-29
ORF091	transcription regulation	100	159	7.00E-109
hypothetical protein		100	376	0
ORF120		99.14	116	1.00E-72
phypothetical protein		100	126	1.00E-84
ORF014	phage portal protein	100	563	0
ORF048	major capsid	100	257	0

Gene/Protein Name	Function (when known)	% Identity	Hit Length	E-value
ORF029	topoisomerase; autolysin; internalin	100	318	0
ORF071	protein coding	100	182	5.00E-123
ORF016	major capsid protein	100	463	0
ORF151		100	98	4.00E-59
ORF030		100	302	0
ORF034	protein coding	100	292	0
ORF062	protein coding	100	206	7.00E-148
ORF039	protein coding	100	278	0
ORF202	methyltransferase	100	71	9.00E-45
tail sheath protein	major tail sheath protein	100	587	0
ORF105	membrane protein	100	142	4.00E-99
ORF293		100	46	2.00E-22
ORF201	carboxy-terminal domain	100	72	1.00E-42
ORF093	dTMP biosynthetic process	100	152	5.00E-107
ORF215		100	64	9.00E-34
ORF141	portal protein	100	103	3.00E-65
ORF095	DNA helicase	100	152	2.00E-104
ORF074	protein coding	100	178	4.00E-125
ORF001	tail tape measure protein	100	1352	0
ORF005	amidase; secretory antigen	100	808	0
ORF033	DNA replication, oxidation/reduction	100	295	0
ORF004	glycerophosphoryl diester phosphodiesterase	100	848	0
ORF043	putative tail tube associated base plate protein	100	263	0
ORF218	protein coding	100	64	4.00E-37

Gene/Protein Name	Function (when known)	% Identity	Hit Length	E-value
ORF078	protein coding	100	174	1.00E-120
ORF052	LysM domain	100	234	2.00E-170
ORF027		100	348	0
hypothetical protein		99.9	1019	0
ORF079	major capsid protein	100	173	1.00E-121
ORF002	virulence-associated protein	100	1152	0
ORF262	putative site-specific DNA endonuclease	100	52	3.00E-26
capsid and scaffold protein	capsid and scaffold protein	100	640	0
ORF117		100	124	4.00E-83
putative structural protein	putative structural protein	99.78	458	0
ORF119		100	122	3.00E-24
ORF124	putative ribonucleotide reductase minor subunit	100	115	6.00E-76
ORF104		100	143	7.00E-97
ORF085	HNH endonuclease	100	162	3.00E-113
ORF088	protein coding	100	161	1.00E-109
ORF109	protein coding	100	135	1.00E-92
ORF103		100	143	2.00E-93
hypothetical protein		100	52	5.00E-26
ORF297		100	43	2.00E-20
ORF135	tail tube terminator protein	99.07	107	9.00E-70
hypothetical protein CPT phageK gp017		100	78	4.00E-46
ORF092		100	156	2.00E-103

Gene/Protein Name	Function (when known)	% Identity	Hit Length	E-value
ORF166	protein coding	100	89	2.00E-19
hypothetical protein		100	62	8.00E-32
hypothetical protein SA5_0153/152		100	240	9.00E-49
ORF150	enhancin-1	100	99	2.00E-63
TreB	Terminase B	100	62	2.00E-29
ORF156	putative thymidylate kinase	100	96	6.00E-60
ORF158	protein coding	100	95	7.00E-60
ORF154		100	97	1.00E-62
ORF175		100	85	1.00E-53
thioredoxin-like protein		100	106	2.00E-68
ORF066	protein coding	99.49	198	3.00E-138
ORF147	Integration host factor; Histone-like bacterial DNA-binding protein	100	101	2.00E-65
hypothetical protein pSco10_57		98.53	290	1.00E-38
ORF128	ribonucleotide reductase, small subunit	100	112	5.00E-70
ORF145	structural protein	100	102	4.00E-68
ORF159	anti-sigma factor B	100	94	3.00E-62
hypothetical protein GH15_004		100	63	5.00E-04
ORF006	ribonucleotide reductase large subunit	100	704	0
ORF025	ribonucleotide reductase minor subunit	100	265	1.00E-54
ORF130		100	109	7.00E-72

Gene/Protein Name	Function (when known)	% Identity	Hit Length	E-value
hypothetical protein pSco10_60		100	106	2.00E-04
hypothetical protein		98.57	290	1.00E-91
PolA	polymerase A	100	1072	9.00E-08
ORF020	RNA binding	100	423	0
hypothetical protein		100	65	1.00E-05
ORF183		100	79	5.00E-49
ORF125	l-shaped tail fiber	100	115	8.00E-76

3.7 Genome of Phage B1 0.0066

Figure 3.6 Open reading frame map comparing the genome Phage B1 (top) to the genome Phage B1 0.0066 (bottom). The direction of the teal arrows represents forward and reverse open reading frames. The blue colour indicates that genes are arranged in the opposite direction.

Phage B1 0.0066 is the novel phage generated by co-culturing Phage B1 with host strain,DPC 5246 and a resistant strain 0.0066(IIIV) ST239 [d] as per methods and materials section 2.3. In Figure 3.6 we can see that Phage B1 0.0066 has marginally increased in size, by gaining 44 new genes, its genome is mostly the same as that of the genome of Phage B1 but that it has reversed as indicated by the blue colour. The gene's it acquired exhibited no homology with Phage B1 or Phage B1 3488.

Table 3.8 Genome of Phage 0.0066 with ORF description

Gene/Protein Name	Function	% Identity	Hit Length	E-value
hypothetical protein CPT_phageK_gp 010		100	102	9.00E-08
ORF150		100	99	2.00E-63
ORF002	virulence-associated	100	1152	0
ORF079	major capsid protein	100	173	1.00E-121
conserved hypothetical protein		100	1019	0
ORF027	protein coding	100	348	0
ORF052	LysM domain	100	234	2.00E-170
ORF078		100	174	1.00E-120
ORF043	putative tail tube associated base plate protein	100	263	0
ORF004	glycerophosphoryl diester phosphodiesterase	100	848	0
ORF033	DNA replication	100	295	0
ORF005	amidase; secretory antigen	100	808	0
ORF445		100	33	7.00E-13
ORF001	tape measure protein	100	1352	0
ORF074	protein coding	100	178	4.00E-125
ORF095	RNA interference	100	152	2.00E-104
ORF141	portal protein	100	103	3.00E-65
ORF215		100	64	9.00E-34
ORF093		100	152	5.00E-107
ORF293		100	46	2.00E-22
ORF105	membrane protein	100	142	4.00E-99
tail sheath protein	tail sheath structure/function	100	587	0

Gene/Protein Name	Function	% Identity	Hit Length	E-value
ORF202	protein coding	100	71	9.00E-45
ORF225	protein coding	100	55	2.00E-29
ORF039	protein coding	100	278	0
ORF062	protein coding	100	206	7.00E-148
ORF034	protein coding	100	292	0
ORF030	protein coding	100	302	0
ORF151		100	98	4.00E-59
ORF016	major capsid	100	463	0
ORF029	topoisomerase; autolysin; internalin	100	318	0
ORF048	major capsid	100	257	0
ORF014	phage portal protein	100	563	0
hypothetical protein		100	103	7.00E-67
ORF139	protein coding	100	104	8.00E-66
ORF120	protein coding	100	116	4.00E-73
hypothetical protein		100	376	0
ORF091	DNA templated transcription regulation	100	159	7.00E-109
hypothetical protein		100	273	0
Ter	DNA replication	100	605	0
hypothetical protein		100	323	0
terminase large subunit	viral genome encapsidation	100	65	9.00E-40
ORF133	protein coding	100	136	8.00E-91
ORF161	nucleotide binding	100	92	5.00E-60
ORF168	protein coding	100	128	2.00E-53
gpORF179		100	69	4.00E-36

Gene/Protein Name	Function	% Identity	Hit Length	E-value
hypothetical protein		100	108	5.00E-70
ORF209	membrane protein	100	110	1.00E-68
ORF207	baseplate hub subunit and tail lysozyme	100	69	8.00E-42
ORF200	DNA binding	100	72	1.00E-44
ORF233	DNA binding, transcription regulation	100	61	4.00E-33
ORF083	holin	100	167	3.00E-115
ORF042	amidase	100	267	0
ORF084	HNH endonuclease; intron encoded nuclease domain	99.4	166	7.00E-114
ORF060	amidase	100	209	2.00E-153
ORF146		100	102	3.00E-64
ORF131	hydrolase activity	100	109	1.00E-72
ORF044	prohibitin; band 7 protein	100	263	0
ORF058	protein coding	100	211	7.00E-151
ORF054	transglycosylase-like; immunodominant antigen A	100	230	2.00E-166
ORF190	virion core protein	100	75	3.00E-44
ORF187	helix-turn-helix motif	100	76	4.00E-46
ORF057	protein coding	100	213	9.00E-143
ORF222	lyase activity	100	63	1.00E-36
ORF096	RNase H	100	141	1.00E-93
ORF063	protein coding	100	204	2.00E-143
ORF049	PhoH-like protein	100	246	0
ORF082	protein coding	100	169	2.00E-117
hypothetical protein		100	74	2.00E-39
ORF032	DNA ligase/mRNA capping enzyme	100	298	0
ORF061	protein coding	100	208	1.00E-149

Gene/Protein Name	Function	% Identity	Hit Length	E-value
ORF068	protein coding	100	192	4.00E-129
hypothetical protein		100	57	3.00E-31
ORF172	DNA-binding	100	87	2.00E-54
ORF007	n-acetylmuramoyl-l-alanine amidase activity	100	682	0
ORF259	protein coding	100	53	1.00E-28
ORF228	protein coding	100	62	4.00E-34
ORF149	DNA-binding	100	100	2.00E-63
ORF142	endonuclease	100	103	4.00E-65
ORF106		100	138	2.00E-94
ORF134	protein coding	100	108	3.00E-72
ORF024	nitric oxide reductase	100	372	0
ORF038	protein coding	100	281	0
ORF163		100	91	2.00E-52
ORF256		100	54	1.00E-28
ORF077		100	177	6.00E-123
ORF072	transcription termination factor	100	180	3.00E-122
ORF090	membrane protein	100	160	1.00E-108
ORF245	immunodominant antigen A	100	57	1.00E-33
ORF194	putative fiber assembly protein	100	73	1.00E-43
ORF114	transferase activity	100	129	3.00E-88
gpORF020		100	82	7.00E-50
ORF437		100	34	1.00E-13
ORF050	DNA binding	100	245	2.00E-173
ORF218	protein coding	100	64	4.00E-37
ORF201	carboxy-terminal	100	72	1.00E-42
ORF071	protein coding	100	182	9.00E-123
ORF138		100	105	2.00E-66

Gene/Protein Name	Function	% Identity	Hit Length	E-value
ORF070	polymerase subunit	99.46	184	9.00E-128
ORF051	serine/threonine-specific protein phosphatase	100	235	2.00E-169
ORF113	translation regulation protein	100	130	9.00E-86
ORF111		99.18	132	1.00E-80
ORF086	GTP cyclohydrolase	100	162	4.00E-116
ORF073	protein coding	100	180	2.00E-124
ORF104	plasmid protein	100	143	7.00E-97
conserved hypothetical protein		100	161	1.00E-108
ORF224		100	63	3.00E-35
ORF103		100	143	2.00E-94
ORF109	protein coding	100	135	1.00E-92
ORF088	protein coding	100	161	1.00E-109
hypothetical protein SA5_0153/152		100	240	9.00E-49
ORF128		100	112	5.00E-70
ORF178	membrane protein	100	81	9.00E-51
hypothetical protein		100	62	8.00E-32
ORF125	L-shaped long tail fibers	100	115	8.00E-76
ORF236		100	59	1.00E-28
ORF170	endonuclease activity	100	87	3.00E-52
ORF362	protein coding	100	38	3.00E-14
ORF157	iteron-binding	100	95	2.00E-58
ORF173	glutaredoxin	100	86	2.00E-52
ORF107		100	133	7.00E-86
ORF237	lysozyme activity, cell wall process	100	59	8.00E-34

Gene/Protein Name	Function	% Identity	Hit Length	E-value
ORF065	terminase large subunit	100	200	7.00E-144
ORF122		100	116	2.00E-75
ORF140	envelope protein	100	101	3.00E-66
ORF183	protein coding	100	79	5.00E-49
putative membrane protein MbpI	membrane protein	100	92	2.00E-55
ORF124	putative ribonucleotide reductase minor subunit	100	115	7.00E-76
ORF119		100	122	4.00E-81
hypothetical protein		100	82	4.00E-34
ORF076	late transcription factor	100	177	3.00E-122
ORF240		100	58	6.00E-32
ORF252	putative HNH homing endonuclease	100	54	8.00E-29
ORF182		100	80	1.00E-47
ORF108		100	132	2.00E-89
ORF053	protein coding	100	234	2.00E-167
ORF175		100	85	1.00E-53
ORF100	DNA/nucleotide binding, exonuclease activity	100	147	8.00E-98
ORF094	protein coding	100	152	2.00E-104
ORF047	envelope protein	100	243	4.00E-172
ORF036		100	287	0
ORF099	phosphate starvation-inducible protein	100	148	1.00E-102
ORF045	protein coding	100	255	0
ORF075		100	178	2.00E-126
ORF143		100	103	2.00E-67
ORF118		100	122	1.00E-79
ORF022	metallophosphoesterase	100	416	0

Gene/Protein Name	Function	% Identity	Hit Length	E-value
ORF154		100	97	1.00E-62
ORF046	DNA binding	100	251	3.00E-179
ORF174		100	86	4.00E-54
ORF189	major tail protein	100	75	3.00E-44
putative major tail protein	tail structure and function	100	173	3.00E-115
ORF059	putative structural protein	100	210	7.00E-149
ORF056	RNA polymerase sigma	100	220	3.00E-155
ORF121	protein coding	100	117	2.00E-78
recombinase a	DNA repair	100	315	0
endonuclease	restriction enzyme	100	322	0
recombinase a	DNA repair	100	74	2.00E-43
ORF158	protein coding	100	95	7.00E-60
ORF020	phage major tail protein	100	423	0
ORF089	membrane protein	100	160	4.00E-114
ORF181	DNA binding	100	80	3.00E-49
ORF037	DNA-directed DNA polymerase	100	286	0
putative HNH endonuclease	putative HNH endonuclease	100	269	0
PolA	DNA polymerase I	100	1072	0
hypothetical protein		99.38	170	7.00E-110
ORF035	DNA polymerase	100	290	0
ORF147	Integration host factor; Histone-like bacterial DNA-binding protein	100	101	2.00E-65
ORF066	protein coding	99.49	198	3.00E-138
ORF156	dTMP catalyze	100	96	6.00E-60
thioredoxin-like protein	thioredoxin-like protein	100	106	2.00E-68
ORF130	protein coding	100	109	7.00E-72

Gene/Protein Name	Function	% Identity	Hit Length	E-value
putative ribonucleotide reductase minor subunit	putative ribonucleotide reductase minor subunit	100	349	0
ORF006	ribonucleotide reductase large subunit	100	704	0
ribonucleotide reductase stimulatory protein	ribonucleotide reductase	100	143	2.00E-96
ORF064	protein coding	100	202	5.00E-147
hypothetical protein		100	150	1.00E-99
ORF127	putative superinfection exclusion protein	100	112	5.00E-71
ORF026	DNA primase	100	355	0
ORF067	DNA binding	100	198	2.00E-141
TreB	Tre-specific PTS enzyme II	100	62	2.00E-29
ORF009	exonuclease	100	639	0
ORF110		100	125	9.00E-85
ORF028	exonuclease	100	345	0
ORF015	AAA ATPase	100	480	0
ORF013	translation regulation	100	537	0
ORF012	Type III restriction enzyme	100	582	0
putative structural protein	putative structural	99.78	458	0
ORF117		100	124	4.00E-83
capsid and scaffold protein	capsid and scaffold protein	100	640	0
ORF262	putative site-specific DNA endonuclease	100	52	3.00E-26
hypothetical protein		100	77	2.00E-45
protein Rep	replication initiation	98.11	64	9.00E-29

Gene/Protein Name	Function	% Identity	Hit Length	E-value
membrane protein	membrane protein	100	57	1.00E-29

3.8 Comparative Analysis

Comparative computational genomic analysis for the genomes of Phage B1, Phage B1 0.0066 and Phage B1 3488 were carried out and the results of this analysis are visually represented in fig 3.7.

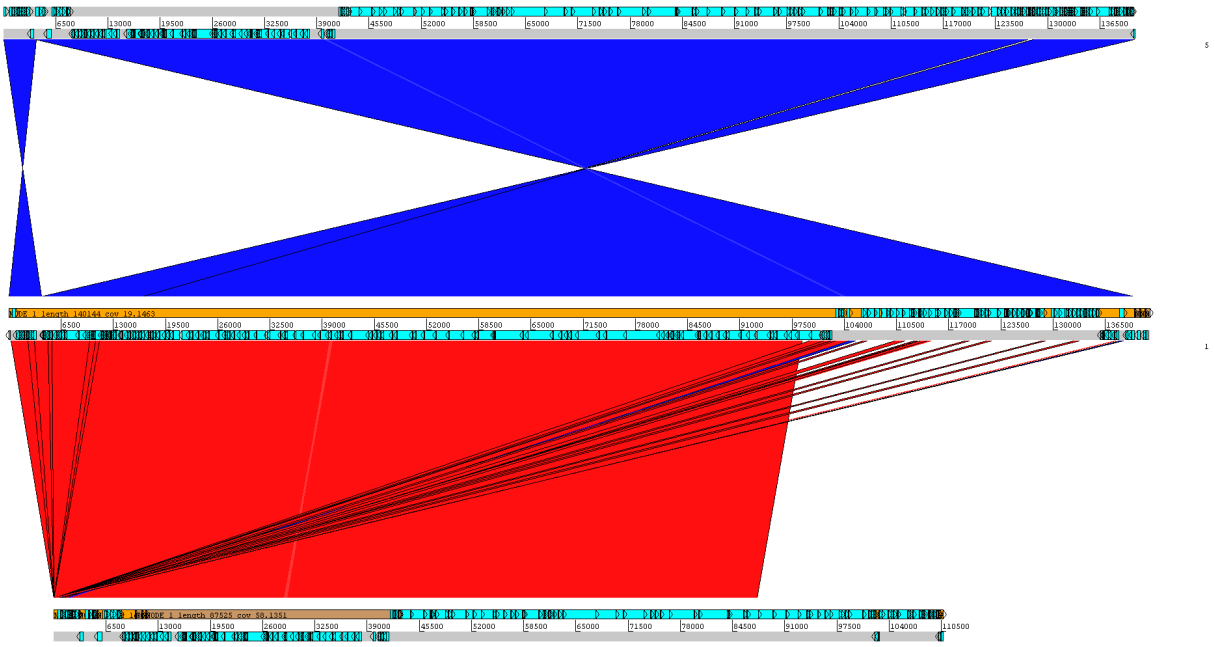


Figure 3.7. Open reading frame map showing comparison between Phage B1 (top), phage 0.0066 (middle) and phage 3488 (bottom). The direction of the teal arrows represents forward and reverse open reading frames. The red colour indicates that genes are arranged in the same orientation, the blue colour represents the reversal of orientation.

The genome for Phage B1 0.0066 appears to have acquired 44 new genes that were not present in Phage B1 and increased in size while Phage B1 3488 appears to have acquired

9 new genes that were not present in Phage B1 and decreased in size. None of the new genes acquired by Phage B1 0.0066 and Phage B1 3488 are the same, they both acquired different genes, see Table 3.9.

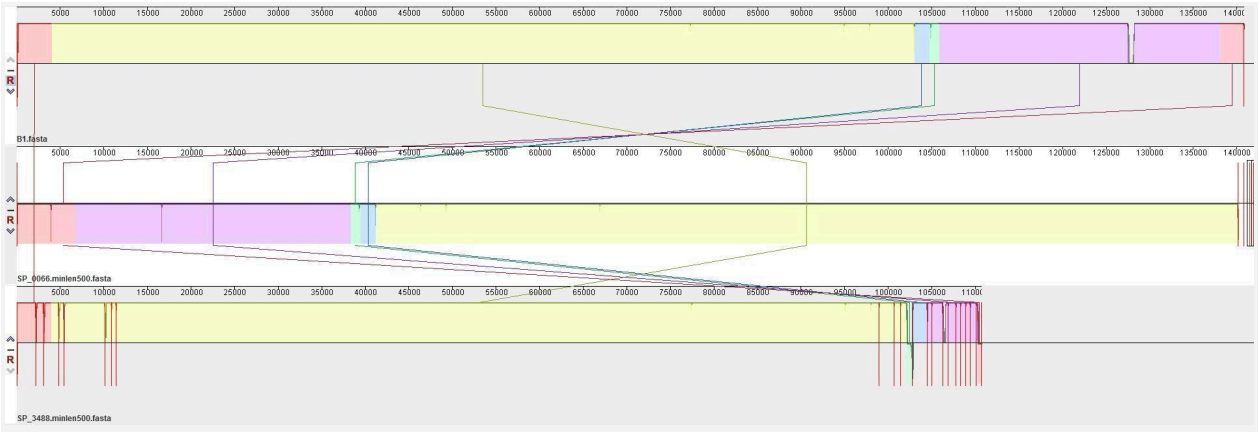


Figure 3.8 Comparison of genomes of Phage B1, Phage B1 0.0066 and Phage B1 3488 respectively. The coloured regions represent homologous regions and lines connect them between genomes. The height of the bar in each region represents homology. The red lines represent breaks in the assembly.

Many of the genes acquired are hypothetical proteins thus their function cannot be determined. Phage B1 0.0066 acquired many genes which share a high level of homology with Staphylococcus bacterial genes and some which represent bacteriophage proteins in particular Staphylococcus phage proteins. Phage B1 3488 almost exclusively acquired genes which have high identity with phage proteins and in particular Staphylococcus phage proteins. These gens code for tail fibre proteins which play a role in primary attachment of viron receptors and ORK 085 which is a special spike-shaped protein complex. This protein complex forms an extension of the tail tube (the ‘cell-puncturing device’) that is used to pierce the other cell membrane of the bacterial host.

Both Phage B1 0.0066 acquired genes which exhibit high homology with genes from Staphylococcus phage which code for various tail proteins such as phage tail tape measure protein which controls the length of the tail, portal protein which plays a role in neck/tail attachment and genome ejection in the case of Phage B1 0.0066.

Table 3.9 Annotation/description of genes unique to Phage B1, Phage B1 0.0066 and Phage B1 3488

Phage	Gene/Protein Name	Function	% Identity
Phage B	No significant similarity found		N/A
	ORF 241	No function assigned	100
	ORF 152	No function assigned	100
	No significant similarity found		N/A
Phage B1 0.0066	transcriptional regulator	regulation of transcription	99
	replication protein	replication	100
	membrane protein	interacts with or is part of membrane	100
	uncharacterised protein	No function assigned	99
	hypothetical protein	No function assigned	100
	MFS transporter (Major facilitator superfamily)	membrane transport proteins that facilitate movement of small solutes across cell membranes in response to chemiosmotic gradients	100
	elongation factor GreAB	DNA binding, DNA templated transcription reg., translation elongation factor	99
	GntR family transcriptional regulator	dna binding, sequence specific DNA binding, transcription factor activity	100
	tyrosine--tRNA ligase/tyrosyl-t-RNA synthetase	ATP binding, nucleotide binding.tyrosine-tRNA ligase activity / catalyses tyrosyl-tRNA formation	100
	D-alanine-D-alanine ligase	d-alanine metabolism and peptidoglycan biosynthesis.	100
	XRE family transcriptional regulator	XRE family transcriptional regulator	100
	rha phage regulatory protein	interferes with infection of bacterial strains that lack integration host factor (IHF), which regulates the rha gene	99
	PhnP protein	Catalyzes the hydrolysis of the cyclic ribose-phosphate to alpha-D-ribose 1,5-bisphosphate	99

Phage	Gene/Protein Name	Function	% Identity
	FUSC family protein	fusaric acid resistance= membrane transporter proteins	100
	replication protein	replication	100
	ABC/ABC type AA transporter protein	amino acid transporter	95
	LsyR family transcriptional regulator	sequence specific DNA binding, transcription	100
	hypothetical protein	No function assigned	100
	hypothetical protein	No function assigned	98
	Homoserine kinase	catalyses ATP and L-homoserine, into ADP and O-phospho-L-homoserine.	98
	DNA-directed RNA polymerase subunit beta	DNA binding, ribonucleoside binding, transcription	100
	cell division protein Fic (filamentation induced by cAMP))	regulatory mechanism of cell division via folate metabolism	100
	phage tail tape measure protein	Controls the length of tail by stopping tail tube protein polymerization.	99
	TetR family transcriptional regulator	Tetracycline resistance genes	100
	pyroglutamyl-peptidase	catalyses Release of an N-terminal pyroglutamyl group from a polypeptide	100
	Phosphatase	enzyme that uses water to cleave a phosphoric acid monoester into a phosphate ion and an alcohol.	100
	dephospho-CoA kinase	catalyses ATP and dephospho-CoA, into ADP and CoA.	100
	ligand-gated channel	transmembrane ion channels	99
	N-acetylglucosamine transferase	transferase enzyme	99
	DNA topoisomerase	DNA topoisomerase	100
	LsyR family transcriptional regulator	sequence specific DNA binding, transcription	99
	UDP-N-acetylglucosamine diphosphorylase/glucosamine-	No function assigned	100

Phage	Gene/Protein Name	Function	% Identity
	1-phosphate N-acetyltransferase		
	Oligoendopeptidase F	Zinc binding	100
	No significant similarity found		
	No significant similarity found	major structural motif capable of binding DNA, involved in gene expression	
	helix turn helix	sequence specific DNA binding, transcription factor activity	100
	AraC family transcriptional regulator	metal ion binding	100
	heavy metal-associated domain protein	No function assigned	100
	hypothetical protein	No function assigned	100
	hypothetical protein	head assembly, genome packaging, neck/tail attachment, and genome ejection.	100
	portal protein	major structural motif capable of binding DNA, involved in gene expression	95
	No significant similarity found		
	No significant similarity found		
	No significant similarity found		
Phage B1 3488	ORF221	Protein Phosphatase	100
	ORF129	protein coding	100
	tail fibers protein	play a role in primary attachment of virion to host receptors	97
	DNA packaging protein	ATP/nucleotide binding, hydrolase activity, viral release from host cel	100
	No significant similarity found		
	ORF221	Protein Phosphatase	100
	ORF085	special spike-shaped protein complex, which forms an extension of the tail tube (the ‘‘cell-puncturing device’’), is used to pierce the outer cell membrane in the process of tail sheath contraction	100

Phage	Gene/Protein Name	Function	% Identity
	hypothetical protein	No function assigned	100
	No significant similarity found		

4. Discussion

This work examined the host range of two *S. aureus* phage (phage B1 and phage K) and demonstrated that the host range could be altered through co-culture with phage-resistant

S. aureus strains. Sequencing of resulting phage variants provided insights into the mechanisms underpinning the process of forced evolution of S. aureus phage. The ability to drive the evolution of phage to enable infection of previously/currently non-permissive hosts is crucial to the viability of phage therapy (Salazar *et al*. 2021). Bacteria, upon repeated exposure to phage, can and will develop resistance to the phage, appearing to create the same problem we have seen with antibiotics. Other studies have shown that though bacteria frequently develop resistance, this resistance can be repeatedly overcome, creating a new type of therapy that is adaptable and thus able to overcome the hurdles that antibiotics face (Salazar, *et al*, 2021). Not only can the resistance be overcome, but it also comes at a cost to the bacteria. To develop resistance to a phage, the bacteria may lose virulence potential become susceptible to a different phage or show increased sensitivity to antibiotics (Schooley, *et al*, 2017). Schooley, *et al*, also showed that custom applications can be developed on a patient-to-patient basis.

Unlike antibiotics which are broad spectrum and/or are often administered without knowledge of the exact strain causing infection, phage are very specific to their host so will only infect a very narrow spectrum of strains. While this specificity has benefits, in practice this is far from ideal as the causative agent of infection would first have to be identified before the appropriate phage could be selected to treat the infection. In this thesis we have provided an approach where new phages may be rapidly selected to treat previously resistant host strains by co-culturing a phage at different ratios with susceptible host and non-susceptible strain. We have proven that this technique can increase phage host range.

Since the interaction is so specific, for effective therapy, there would need to be a catalogue of phage available to treat whichever specific strain the patient presented with. If a previously unidentified strain was the causative agent of the disease, no phage might be available in the catalogue with the ability to treat the patient. Screening for compatible phage can be a lengthy and unsuccessful process so is certainly not an option when dealing with an actively progressing infection. Thus a more robust and practical alternative is needed. The ability to switch hosts as shown in this study means there is potential to create a comprehensive phage cocktail which could be able to treat most strains. When previously unidentified strains are isolated, it would be hoped that the same technique could be used to generate a suitable phage for their treatment. This is an area which requires more investigation to understand if the results achieved in this study are unique to the strains

examined, or if this is a phenomenon which could be replicated in not just other *Staphylococcus* phages, but phages specific to other species of bacteria. Indeed perhaps if we can force phage to switch host range and infect different strains of the same species, there may be potential to select phage with the potential to infect closely related species. The development of a more broad-spectrum phage treatment or a standard protocol for the manipulation of phage stocks to combat infections could be the answer to overcoming the issues which stand in the way of phage therapy.

4.1 Antibiotic resistance profile

The objective of our study was to target methicillin resistant *S. aureus* (MRSA) with novel phages which may have therapeutic potential. Methicillin resistance is genetically based and is encoded on mobile genetic elements (MGEs), namely the staphylococcal cassette chromosome mec (SCCmec). mec (SCCmec) is a genomic island of unknown origin containing the antibiotic resistance gene mecA (Lowy, 2003). Methicillin resistance is acquired by the acquisition and insertion of these mobile genetic elements into the chromosomes of strains that were previously susceptible.

It was important to initially confirm that the bacterial strains that were being used in this study exhibited resistance to the antibiotic. All strains were tested for methicillin resistance, according to CLSI guidelines, the results of which can be seen in Table 3.1. All of the strains from the National Reference MRSA Collection were confirmed to be methicillin resistant. 11 of the 20 strains from the UCC culture collection were found to be methicillin resistant. This was an unexpected result - as these stains have the antibiotic resistance monitoring and reference lab (HPA) named as their source and are listed as grade 2 pathogens according to the UCC culture collection database we would have expected all of the strains to exhibit resistance to methicillin.

All 8 of the staphylococcal strains from the Teagasc Moorepark culture collection (table 2.1) were found to be susceptible, with DPC 6010, a *S. epidermidis* strain being intermediately resistant. 75–90% of hospital isolates of *S. epidermidis* are resistant to methicillin which is even higher than the corresponding rate for *S. aureus* (40–60%) (Otto, 2009). However, the DPC 6010 strain used here was of animal origin and might not be expected to exhibit the same high rate of resistance as human-origin isolates.

4.2 Phage B1 and Phage K host switching.

The initial phage work examined the host range of Phage B1 and Phage K across our collection of Staphylococcus strains outlined above. Having identified that some strains are resistant to phage infection we demonstrated that the host ranges of Phage B1 and K can be altered and shifted to infect previously resistant hosts through co-culture. Relatively high volumes of media (10ml) phage (200 µl) and bacteria (1,000 µl) were used to increase the chances of achieving desired results; the higher number of bacterial and phage cells permitted higher levels of phage mutations. During each round of co-culturing the phage grew to large numbers on the permissive strain, DPC 5246. Exponential growth of phage depleted the DPC 5246 supply, once all the cells were lysed this applied selective pressure to the phage to evolve to be able to infect the non-permissive strain (Borges, 2021) If the phage did indeed switch hosts this could usually be seen before plating as clearing of the culture after overnight incubation. In the first round of co-culturing the permissive to non-permissive were inoculated in a 50:50 ratio. In subsequent rounds, different ratios were used to see if this affected the rate of host switching. It was shown that a higher ratio of non-permissive to permissive, 80:20 caused the phage to switch hosts more rapidly. This is most likely due to the large number of non-permissive bacterial cells being available for the phage to attempt to infect, the larger the number the greater the likelihood of observing the desired result. Similar studies have been performed in a range of bacteria (Borin *et al*, 2021, Borges, 2021) however the current work provides optimization of the conditions specific to these *S. aureus* phages which we feel show therapeutic potential.

An unexpected result of the generation of Phage B1 mutants was apparent lysin production by some of the phage mutants as indicated by the halo-like appearance of their plaques. Lysins, also known as endolysins, are hydrolytic enzymes produced by some bacteriophage at the end of the lytic cycle to cleave the host's cell wall (Vázquez *et al,* 2021). Phage K is known to be a lysin producer and is capable of producing LysK which is highly effective *in vitro* against *S. aureus* (Fenton *et al.,* 2010). There are advantages too the use of lysins in treating bacterial infection. In particular, bacterial resistance to lysin has not yet been reported (Oh *et al,* 2023).

Furthermore lysins, have potential for use in different environments including mammalian, food and notably biofilms, and could be used as a prophylactic treatment (Murray *et al*, 2021). The disadvantages of lysins as therapeutic agents include the inability

to target gram negative bacteria (that we know of), the inability to self-replicate/amplify and as proteins, they are susceptible to denaturation (O'Flaherty *et al.,* 2009). Despite these downsides, the putative production of lysin by phage B1 mutants is a promising result that deserves future investigation and could lead to the future exploitation of the novel lysin as a therapeutic agent.

4.3 Genomic Analysis

We performed phage genome sequencing of the original phage B1 and the two mutant derivaties phage B1 0.0066 and phage B1 3488. The sequence of phage B1 was found to be genetically similar to phage team 1 and phage G1. This is not surprising as they were all isolated from a mixture of *S. aureus* bacteriophages (*Bacteriophagum staphylococcum liquidum*) from the Eliva Institute. A recent study by Cui *et al*., 2017, which set out to assess the safety of *Staphylococcus* phage as therapeutic agents, showed that phage K, phage team 1 and phage G1 do indeed have a common ancestor, see Figure 4.1. Phage B1 was not included in this publication but it can be assumed that it shares the same ancestor given the high identity it exhibits with phage team 1 and phage G1. These three phages which are closely related to Phage K, have similar but not identical host ranges. Given their similarity it is likely that it may be possible to shift the host range of phage team1 and phage G1 using the protocol described here.

In terms of gene functionality, approximately 65% of phage B1 ORF's have functions assigned but many of these are too general to provide much insight into their role in phage virulence, e.g., ORF039's assigned function is simply 'protein coding'. Approximately 66% of the ORF's found in Phage B1 3488 have functional annotations while in the case of Phage B1 0.0066 the percentage of ORF's assigned a function is slightly higher at approximately 71%. This level of functional annotation is typical for phage genome as reported in other studies.

We were interested in how the phage genomes were altered by adapting phage B1 to new host strains. Phage B1 3488 gained 9 genes, despite decreasing in overall size while the size of Phage B1 0.0066 increased with a gain of 44 genes which are listed in Table 3.8. These acquired genes are unique to each mutant, there is no overlap between the genes gained by each phage; nor were they identified in the genome of Phage B1. Some of the genes showed no similarity to any known genes, others were hypothetical proteins, so it is not

possible in every case to hypothesise how the genomic difference influenced functional host adaption. However, some genes with interesting and potentially beneficial host-adaptive functions were identified. Phage B1 3488 acquired a gene encoding a tail fibre protein and it could be hypothesised that this could be responsible for the increased phage host range exhibited by this mutant, as phage receptor binding proteins have previously been identified as tail fibres. This mutant also acquired ORF 085 which codes for a special spike-shaped protein used for puncturing the outer membrane of bacterial cells during attachment and infection so this is also a possible candidate which may contribute to a broader host range (Browning, *et. al*, 2012). Phage B1 0.066 acquired a rha phage regulatory protein which is known to play a role in the infection of bacterial strains that lack integration host factor, (Henthorn & Friedman, 1995) which might possibly have contributed to its ability to infect new strains. It is possible that these new genes are derived from the host genomes of the bacterial strains. Indeed, Borin *et al.,* (2021) reported that a mutant phage exhibiting increased bacterial suppression compared with that of the parent phage may have been due to recombination with a gene in a defunct prophage in the bacterial genome. Sequencing the genomes of the resistant and susceptible strains and comparing the genomes with the sequenced phages should help to confirm this theory.

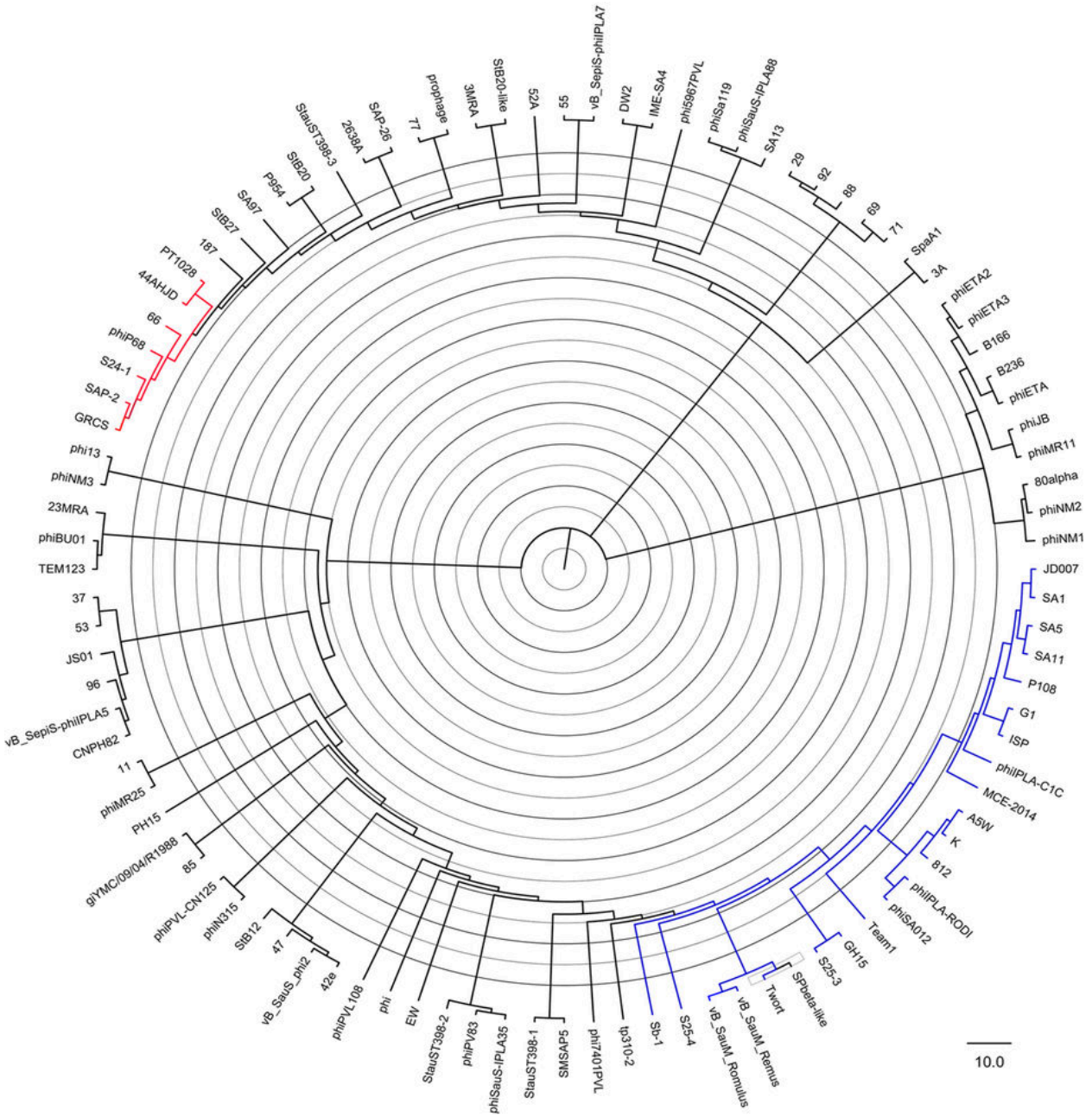


Figure 4.1 The phylogenetic tree of *Staphylococcus* phages of the Myoviridae family (Cui *et al.,* 2017)

4.4. Conclusion

The host ranges of 2 phages, B1 and K were examined in this study. It was found that phage B1, which was isolated from a commercially available phage cocktail purchased from the George Eliava Institute of Bacteriophage, had a larger host range than phage K. The host range of both phage was successfully shifted and expanded by co-culturing resistant strains with susceptible strains. Genomic analysis of phage B1, B1 0.0066 and B1 3488 showed that B1 0.0066 and B1 3488 acquired new genes during co-culturing including genes putatively encoding altered tail fibre proteins that could contribute to host range adaption. Further genetic work could be carried out to investigate which of these altered gene sets contribute to the expanded hot range. Overall, over all the work provides insights into host range adaption in *S. aureus* phages and suggests that rapid bespoke phage adaptation may be possible to tailor phage cocktails for use against specific MRSA strains in a clinical setting.

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