Potential of *Klebsiella pneumoniae* Phage Depolymerases as Antimicrobial Agents.

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Potential of *Klebsiella pneumoniae* Phage Depolymerases as Antimicrobial Agents.

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Abstract

Klebsiella pneumoniae is a pathogenic Gram-negative bacterium which is prevalent in hospitals and infects immune-compromised individuals. It primarily causes pneumonia but also infects the urinary system and the liver. Due to overuse and misuse of antibiotics, multidrug resistant strains have arisen that threaten the effectiveness of last-line antibiotics. One of the most prevalent being sequence type (ST)258 strains which mainly impacts Europe and North America.

Bacteriophage (phage) are viruses that infect bacteria and are currently under scrutiny as antimicrobial agents to increase the efficacy of antibiotics against resistant pathogens or take over entirely against those that no longer can be treated by antibiotics. They produce depolymerase enzymes to hydrolyse the capsular polysaccharide of the bacterium allowing the phage to contact the bacterial cell surface.

In this study seven phage which infect ST258 have been isolated and eight putative depolymerase genes identified. The aim of this study is the expression of these proteins and the characterizing and comparison of their efficacy at treating four *K. pneumoniae* isolates. One 'high' and one 'low' biofilm producer from each group, of the serotypes KL106 and KL107 within the multi locus sequence type (MSLT) group ST258 and comparison of the depolymerases to the whole virion.

The production and isolation of these depolymerases was not achieved in this study, but the efficacy of the whole virion was tested in both planktonic and biofilm assays. KP5 was the only

phage to show the ability to reduce biofilm growth in its host after 24h and to infect all hosts in the planktonic assay. Future work should include the production and characterization of the depolymerase enzymes. Then the most appropriate could be used in a cocktail or in conjunction with antibiotics and tested *in vitro* and *in vivo.*

Acknowledgements

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1.0 Introduction

1.1 Antibiotic Resistance (AR)

The discovery of penicillin by Alexander Fleming in 1928 revolutionised humanity's ability to treat disease and infection, drastically reducing the risk of death from surgery allowing non-essential operations to become commonplace. Penicillin and antibiotics discovered soon afterwards provided effective treatments for infectious diseases such as typhoid, pneumonia and tuberculosis, making a large contribution to increases in life expectancy from the 1950s onwards. It was also discovered that the use of antibiotics allowed livestock to gain weight faster and increased their survivability (Limmathurotsakul et al., 2019). This led to massive overuse for both clinical therapeutic, livestock therapeutic and prophylactic purposes. In the UK in 2017 36 percent of antibiotics used were on livestock (Limmathurotsakul et al., 2019).

Even at the dawn of the antibiotic era the danger of antibiotic resistance (AR) was acknowledged by Fleming, in his Nobel Prize acceptance speech he warned of the creation of resistant bacteria by the reckless use of antibiotics (Levy, 2014). This warning was not heeded as most assumed the frequency of mutations generating resistant bacteria would be negligible (Davies and Wright, 1997) but AR has emerged to become a major global threat to human health, with over two million cases of AR reported yearly in the US alone (CDC, 2018). Some strains have become resistant to practically all antibiotics spawning 'superbugs' such as Methicillin-resistant *Staphylococcus aureus* (MRSA) and ESBL (extended-spectrum β-lactamases)-producing Enterobacteriaceae (such as *Klebsiella pneumoniae*). AR is one of the largest threats to humanity in the 21st century due to its multifaceted impact on health, food security and development (WHO, 2019).

There are 7 main methods by which bacteria have developed resistance to antibiotics (van Hoek et al., 2011). The first is through permeability changes in the bacterial cell wall which restrict antimicrobial access to target sites. The second is through active efflux of the antibiotic from the cell. The third and fourth are through enzymatic modification or degradation of the antimicrobial agent. The fifth is the acquisition of alternative metabolic pathways to those the drug inhibits. The sixth is through modification of the antibiotic target, and the final is the overproduction of the target enzyme.

1.1.1 The Spread of Antibiotic Resistance

There are two methods of gaining AR, either by mutation or Horizontal Gene Transfer (HGT) (Martínez and Baquero, 2014).

Mutation is the simpler of the two as all it required is two things; a random change in the DNA of the bacteria which confers upon it one of the methods of resistance listed above, and the selection pressure (presence of the antibiotic) to make such a trait advantageous (Martinez and Baquero, 2000).

HGT is more complex and has more mechanisms by which it can be accomplished; transduction (which can be generalized or specialised), transformation and conjugation.

Transduction is the first mechanism; this is when bacterial DNA rather than bacteriophage (phage) DNA is packaged into a phage head and injected into a different bacterium. There are two types of transduction, generalized and specialised, the former being when a random segment of DNA is packaged and the latter when DNA next to the phage insertion site is

packaged (van Hoek et al., 2011). There have been examples of full genetic elements being mobilised by transduction (Del Grosso et al., 2011).

The next is transformation, this is the uptake of short pieces of naked DNA by naturally competent bacteria (Andam et al., 2011). Some bacteria are only competent at certain stages of their life cycle, and others require specific sequences to allow uptake. An example is β-lactam resistance in *Streptococcus pneumoniae* caused by alterations to PBPs caused by recombination with resistant Streptococci of other species (van Hoek et al., 2011).

The last is conjugation, this involves the transfer of DNA via the sexual pilus and requires cell to cell contact. It is the passing of conjugative plasmids via a DNA rolling circle replication system linked to a type IV secretion system (Durão et al., 2018). It can also be carried out by integrative conjugate elements which reside in the host chromosome but retain the ability to excise and transfer (Wozniak and Waldor, 2010). This means that they do not have to have compatible replication machinery with the host so they have a larger host range which is an advantage over the plasmids (van Hoek et al., 2011).

The origin of resistance genes could be from non-pathogenic flora (Sommer et al., 2009) or environmental microbiota (Nolan et al., 2016) also exposed to the selection pressure of the presence of the antibiotic. This means that if antibiotics are to be used then their impact on the whole microbial community needs to be appreciated, not just on the target microbe (Martínez and Baquero, 2014).

1.2 *Klebsiella pneumoniae*

The genus *Klebsiella* is a part of the Enterobacteriaceae family. They comprise of Gram-negative, non-motile, encapsulated, rod-shaped bacterium. They typically colonise mucosal surfaces in mammals but can also survive in environmental reservoirs such as soil and water (Gupta et al., 2003).

Klebsiella pneumoniae is the most significant member of the genus in terms of human impact. It is a part of the average human microbiome but can cause clinical issues if aspirated or in nosocomial infections in immune weakened individuals, resulting in high mortality (Tumbarello et al., 2012). The most common condition caused is pneumonia, but also it frequently infects the urinary tract, liver abscesses, surgical wound sites and the bloodstream (Paczosa and Mecsas, 2016). Transmission can occur through human to human contact or contact with contaminated surfaces of instruments.

Multilocus sequencing (MLST) profiles seven different housekeeping genes in *K. pneumoniae* to characterise isolates into sequence types (ST) (Diancourt et al., 2005). There are many ST's within *K. pneumoniae* but in Europe the most prevalent is the sequence type 258 (ST258) clone. This is of particular interest and is prevalent worldwide in terms of clinical relevance (Chen et al., 2014). This ST is notable for its global prevalence, due to human-mediated spread, and potential for antibiotic resistance. There is evidence of widespread resistance to antimicrobials such as colistin from the polymyxin class, which is used almost solely against *K. pneumoniae* since the 1990s (Woodford et al., 2011). Isolates have been found producing

extended-spectrum beta-lactamases (ESBL) and carbapenem resistance, through carbapenemases (KPC), which both cause antibiotic resistance.

1.2.1 Clinical Importance

The mortality rates for *K. pneumoniae* can be extremely high, reportedly up to over 70%, in many instances (Tumbarello et al., 2012). One of the largest problems associated with *K. pneumoniae* is acquisition by immune-compromised individuals through nosocomial infection or community-acquired. The addition of antibiotic resistance also makes this pathogen much harder to treat, a study showed that the mortality within 21 days for non-resistant *K. pneumoniae* infection was 37%, but this increased to 51% for those isolates caused by ESBL-producing *K. pneumoniae* (Tumbarello et al., 2012). With the current rise of antibiotic-resistant strains, this pathogen is now also problematic for healthy non-immunocompromised individuals broadening its scope (Paczosa and Mecsas, 2016).

1.2.2 Antibiotic resistance in *K. pneumoniae*

The emergence of antibiotic resistance in *K. pneumoniae* infections contributes massively to its clinical importance, especially carbapenem-resistant and ESBL-producing *K. pneumoniae*

ESBLs are enzymes that are able to hydrolyse most antibiotics containing the beta-lactam ring, this includes penicillins and cephalosporins (Ghafourian et al., 2014). This means that bacteria producing ESBLs are resistant to many front-line antibiotics, which can increase their associated mortality drastically (Tumbarello et al., 2006), this is where last-resort antibiotics

such as carbapenems are used. It is when *K. pneumoniae* strains are both resistant to carbapenems and ESBL- producing that they become a major concern.

Carbapenems are a class of antibiotics reserved for use against multidrug-resistant bacteria, and are commonly referred to as the 'last line of defence'. The main method of *K. pneumoniae* carbapenem resistance is through *K. pneumoniae* carbapenemase (KPC) production, which is a transmissible plasmid-encoded enzyme (Arnold et al., 2011). Carbapenem-resistant *K. pneumoniae* (CRKP) is associated with high mortality rates and limited antimicrobial treatment options. There are improvements needed in effective preventative measures and early identification (Spagnolo et al., 2014).

1.2.3 Virulence Factors

Virulence factors are variable across different strains and infection sites for *K. pneumoniae* due to its many methods of pathogenicity and the genetic diversity ensuing from its worldwide coverage. They also differ between immuno-healthy and compromised hosts but the four best characterised and well-understood factors are the capsule, lipopolysaccharide (LPS), fimbriae, siderophores (Paczosa and Mecsas, 2016).

The capsule is a polysaccharide layer that is outside the cell envelope and coats the cell, promoting survival through a number of mechanisms. It is hydrophilic which aids in preventing desiccation of the cell, and helps block phagocytosis by macrophages. It also hinders complement-mediated lysis (Paczosa and Mecsas, 2016). It physically excludes viruses and hydrophobic toxic materials and can also prevent lipopolysaccharide (LPS) recognition by the host immune system. Capsules are also very important in biofilm formation, for example

it has been shown to be a major virulence factor in the colonization of the urinary tract (Struve and Krogfelt, 2003). The capsule has been shown to be particularly crucial to *K. pneumoniae*'s evasion of the host immune system, as opposed to the active suppression of phagocytes by other bacteria pathogens, by making it harder for the bacteria to be bound and ingested by phagocytes (Paczosa and Mecsas, 2016). The roles of the capsule are shown below (Figure 1).

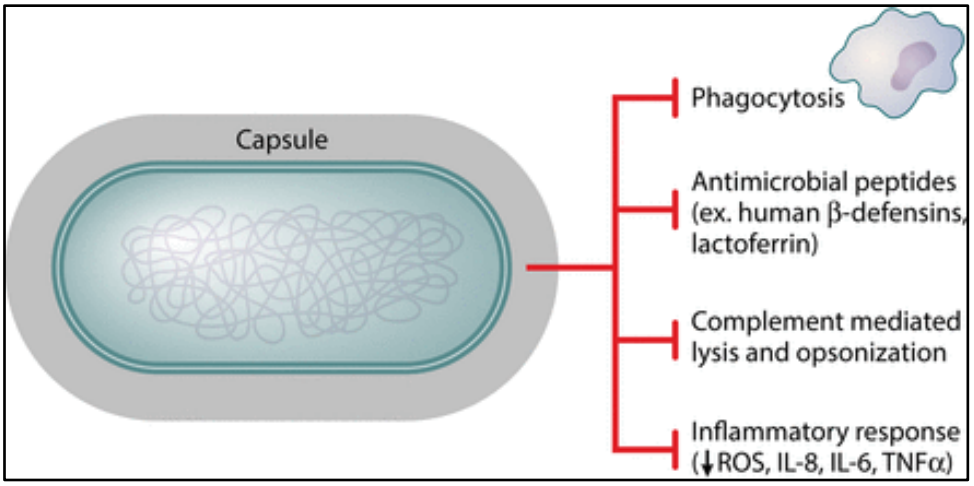


Figure 1. The role of the capsule in K. pneumoniae host immune evasion and virulence. From (Paczosa and Mecsas, 2016)

It has been suggested that the capsule is a crucial virulence factor contributing to the pathogenesis of *K. pneumoniae* (Lawlor et al., 2005) and in mice, acapsular strains have been shown to have significantly reduced virulence and a significantly higher chance of being phagocytosed (Lawlor et al., 2006).

As shown in Figure 1 the capsule also helps reduce the inflammatory response by the host, this reduces the intensity of the immune response (Evrard et al., 2010). Studies have shown that pro-inflammatory interleukins, such as IL-10 and IL-6 are expressed in drastically lower

levels when exposed to the wild type pathogen compared to acapsular (Xu et al., 2014) (Yoshida et al., 2001).

LPS is a component of the cell membrane, and though its structure is variable its typical composition is comprised of three subunits: lipid A, an oligosaccharide core and O-antigen. The lipid A is a component of the cells outer leaflet and aids pathogen survival in the hostile host by camouflaging the pathogen from host immune detection, promoting antimicrobial peptide resistance and by altering outer membrane properties (Llobet et al., 2015), the oligosaccharide provides a link to the O-antigen, of which serotypes O1, O2 and O3 account for 80% of infections (Follador et al., 2016). The lipid A component helps protect the bacteria against host defences but is also a strong immune activator so hinders the evasion of the immune system. The O-antigen protects against complement-mediated lysis (Bengoechea and Pessoa, 2019).

There are two main types of fimbriae that are classed as virulence factors for *K. pneumoniae*, type 1 and type 3, which are crucial for cell adhesion and colonisation.

Type 1 fimbriae are filamentous and composed of multiple FimA subunits that are membrane-bound and topped with a FimH subunit on the tip. These fimbriae help with bladder colonization and cell invasion, they are also found to aid in the colonization of abiotic surfaces. The FimH subunit gives the adhesive properties to these fimbriae but it also increases the likelihood of the cell being recognised and cleared by the host immune system (Paczosa and Mecsas, 2016).

Type 3 fimbriae are also membrane-bound, like type 1, but their structure differs as they are helical and composed of multiple MrkA subunits with a MrkD subunit on the tip. The MrkD subunit has been shown to aid in biofilm formation, specifically through binding to the host extracellular matrix, which is prevalent in damaged tissue, such as surgical sites and medical inserts. The MrkA subunits bind abiotic surfaces, such as surgical instruments. These fimbriae can increase the presence of reactive oxygen species (ROS), produced by the host as an immune defence (Paczosa and Mecsas, 2016). Type 3 fimbriae have been shown to be far more important in terms of biofilm formation in contrast to type 1 (Schroll et al., 2010).

The bacterial cell can also regulate its production and expression of these fimbriae so that they are only on the cell surface when they can help with invasion, an example is in the bladder. Primarily type 1 is expressed but when colonizing the lungs or intestinal tract type 3 is solely expressed. This helps increase the chance of immune evasion and cell survival (Struve et al., 2009).

Siderophores are used by the cell to acquire iron from its host. This is difficult as the mammalian innate immune system limits the amount of iron freely available to pathogens restricting their potential for growth (Zauberman et al., 2017). Humans transport iron through transport proteins such as transferrin, or when a pathogen is detected lactoferrin which has a higher affinity for iron further reducing its availability to the pathogen. Two-thirds of the available iron in a human body is also haem-bound in proteins such as haemoglobin (Miethke and Marahiel, 2007). This has led to the evolution of siderophores, which are secreted molecules that have an extremely high affinity for iron to take it off transport proteins, such as transferrin and lactoferrin. *K. pneumoniae* expressed four main siderophores (Paczosa and

Mecsas, 2016): Enterobactin which is the primary siderophore, Salmochelin which is a c-glycosylated form of Enterobactin which cannot be inhibited by the same host immune protein. Yersiniabactin which scavenges iron from the environment and Aerobactin which has been shown to be crucial for hypervirulent *K. pneumoniae* (Russo et al., 2015). Once bound to iron these molecules are re-absorbed by the pathogen via the use of transporter molecules.

K. pneumoniae employs many other mechanisms to aid in its virulence, such as porins, iron transport systems and efflux pumps (Paczosa and Mecsas, 2016).

1.2.4 Biofilm Features

One of the key reasons *K. pneumoniae* is so virulent as a pathogen is its ability to form a biofilm. A biofilm is a community of bacterial cells, which can contain many different species of bacteria, that have adhered to a surface and interact with each other. The method of biofilm formation is shown below in Figure 2.

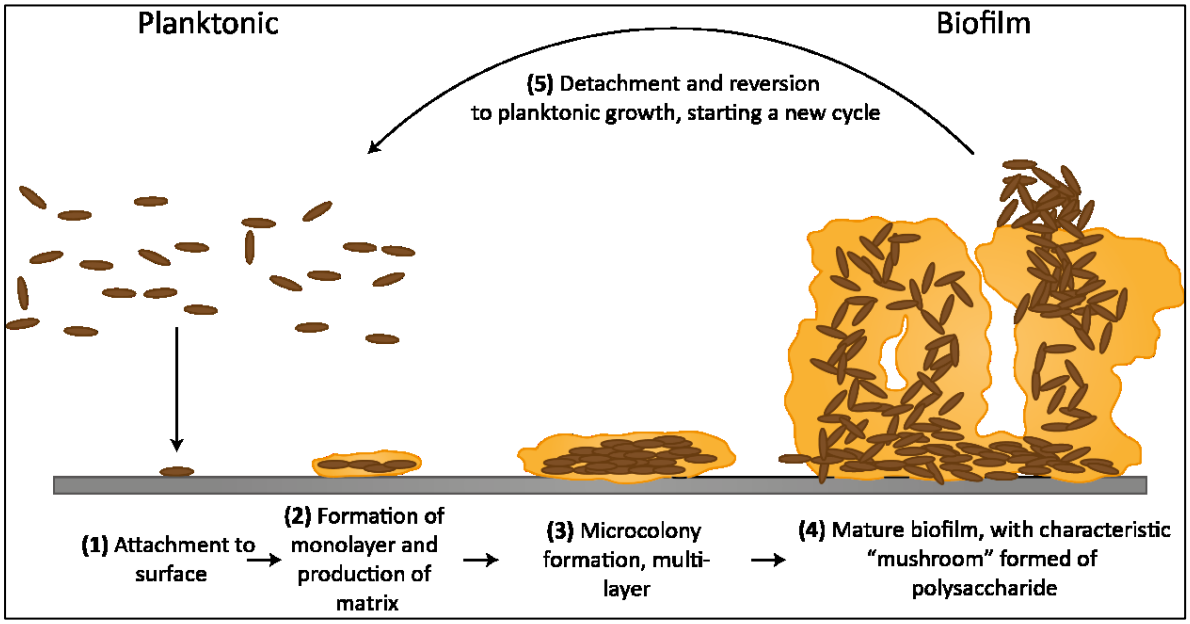


Figure 2. The method of biofilm formation. From (Vasudevan, 2014)

Biofilms can contain bacteria of many different species and are held together by a hydrated matrix of extracellular polysaccharides and proteins. Their formation is associated with chronic infections and can increase levels of antibiotic resistance up to 1000 fold, compared to planktonic bacteria (Stewart and Costerton, 2001). The formation of a biofilm, such as in stage 4 of Figure 2, also provides shielding and defence from the host immune systems. Antibiotics can manage the symptoms of a pathogenic biofilm, by killing the planktonic bacteria, as shown in stage 5 of Figure 2, that are shed but simultaneously failing to eradicate the embedded cells which in the most extreme cases leads to surgical removal of the infected surfaces.

The mechanism for the massively increased tolerance to antibiotics by bacteria when in a biofilm has not been conclusively proven but there are three main theories (Singh et al., 2017). The first being that the antibiotics fail to penetrate all the way into a mature biofilm, as shown in stage 4 of Figure 2, but as the matrix is hydrated there is no real barrier for diffusion, though the antibiotic could be deactivated upon entry. The second being that microenvironments form within the biofilm, of differing pH, oxygen levels or osmotic conditions as a few examples. This could lead to the inactivation of the antibiotic or reduced susceptibility of the bacteria to the antibiotic due to phenotypic changes (Schroll et al., 2010). The final explanation is linked to this as it is a change in the bacterial phenotype, this could be due to lack of nutrients or differing microenvironments as mentioned above (Singh et al., 2017). If the cell enters a spore-like, non-growing state then their uptake of any molecules is greatly reduced which leads to reduced sensitivity to antibiotics and increased resistance (Lewis and Manuse, 2019).

The most likely of these explanations is the last, that the bacteria enter their stationary phase. This makes sense as cells can be dormant due to others, who may have greater access to nutrients or other resources, to propagate their genes and in reverse, the active cells can depend on the dormant ones to survive if they are killed due to their increased susceptibility to antibiotics. This is supported by the knowledge that most *K. pneumoniae* biofilms are formed from clonal growth from a single attached cell, rather than recruitment of other planktonic cells, which leads to extreme genetic similarity within the biofilm (Schroll et al., 2010). Experimental evidence supports this theory in *K. pneumoniae* as it has been shown that ciprofloxacin can penetrate a *K. pneumoniae* biofilm in its entirety but fails to kill the dormant cells. Then when these cells become active and are dispersed from a biofilm they quickly regain their susceptibility, showing it is not genetic resistance to the antibiotic but circumstantial (Hall and Mah, 2017).

1.2.5 Sequence Type 258 (ST258)

This study focuses on *K. pneumoniae* isolates of MLST, sequence type (ST) 258. This group is prevalent worldwide, especially in Europe and northern America, and is considered a major driver of the global spread of KPC-production (Chen et al., 2014).

ST258 is one of the most pathogenic lineages of *K. pneumoniae* for example, it is the cause of over 50% of antibiotic-resistant *K. pneumoniae* infections in Italy (Fasciana et al., 2019). *K. pneumoniae* in general, has been shown to be especially adept at rapidly acquiring DNA and changing its genotype and phenotype to increase its durability and pathogenicity. Combining this with the ST258s antibiotic resistance shows why this pathogen is so prevalent globally

(Pitout et al., 2015). For example members of the ST258 group have been shown to be totally resistant to neutrophils, in mouse models, which negates a large part of the immune response to infection (Xiong et al., 2015).

ST258s carbapenem resistance is thought to stem from a plasmid-encoded transmissible gene called *bla*$_{KPC}$, first isolated in the eastern states of America. It has spread both clonally and through horizontal gene transmission to the point now where it is frequently found world-wide and in a range of pathogens (Cuzon et al., 2010). The plasmid encoding *bla*$_{KPC}$ is thought to have contributed significantly to the success and rapid dissemination of ST258s through the 2000s (Pitout et al., 2015).

ST258 has been shown to be a multi-clonal group as there is considerable variation within isolates. It evolved from a recombination event between ST442 and ST11. Roughly 80% of its genome is from ST11 strains and the other 20% from ST442 strains (Chen et al., 2014). The ST258 group can be further split into two 'clades' dependent on which *cps* gene they express. These are then further classified into individual serotypes. It appears that clade I evolved from clade II as a result of a *cps* region swap with an ST42 strain (Chen et al., 2014), while the *cps* region expressed by clade II is similar to that of ST442 (Paczosa and Mecsas, 2016). The *cps* gene encodes for the capsular polysaccharides, which as mentioned above are a key part of this pathogen's virulence and host immune evasion.

1.3 Bacteriophage

Bacteriophage are viruses that infect bacteria and archaea. They are the most common biological entities on the planet and are universal to practically all species of bacteria. They replicate via hijacking of the bacterial cell 'machinery' for use in copying their genetic information and producing copies of the original virion. The ability of phage to slow the growth, increase susceptibility to antibiotics or outright kill bacteria means they have been under renewed investigation for use against antibiotic-resistant bacteria, such as *K. pneumoniae*, since the rise of resistance in the late 20th century. Previously phage had been considered as antimicrobial agents from the early 20th century up until the discovery of Penicillin and subsequent antibiotics whereby western scientists lost interest, though Russians and eastern Europeans continued researching the field. It is only with the growing threat of antibiotic resistance that phage therapy is once again being considered in the western world.

1.3.1 Phage Therapy

The emergence of antibiotic-resistance in bacteria is one of the largest threats facing humanity in modern times, which is why other avenues for the treatment of pathogenic bacteria are being explored. One such alternative is the use of bacteriophages to help with detection, identification and treatment of antibiotic resistant bacteria.

The detection of an infection using phage is not currently in widespread clinical use but due to its ability to detect far faster than traditional culture methods it could improve the speed, and therefore effectiveness, of treatment. There are a few examples of pathogens where

phage diagnostics are currently in use, one of which is *Mycobacterium Tuberculosis*, which causes tuberculosis (TB). Current detection methods can take up to 8 weeks and as TB is prevalent mainly in developing countries there often is not the expertise or funding to allow it.

Due to phage's host specificity they are also useful in the identification of pathogens, they can be used to identify antibiotic resistance quickly and easily, allowing appropriate treatment to begin sooner and therefore leading to greater success. Traditionally the process takes around 3 weeks and is extremely labour intensive, the FASTPlaqueTB ^{tm} assay mentioned above can complete this in around 10 days which mean faster identification and appropriate treatment of the pathogen, leading to increased survivability (Schofield et al., 2012). The only downside is the lower accuracy of this compared to the traditional method, which could be why it is not employed against more pathogens. This technology has not yet been successful enough for real-world use against *K. pneumoniae* but it has great potential for future applications, as shown by the successful use of a depolymerase as a capsular type identifier (Lin et al., 2014).

The treatment of antibiotic resistant pathogens is the main aim for most research done into phage therapy. This is due to its potential to help negate antibiotic resistance and provide another line of defence against multidrug resistant bacteria, such as *K. pneumoniae.*

Whole phage application involves the use of the entire virion to treat an infection. There are currently no treatments that have been certified for use in humans but many promising studies have been done in animal models that show the effect whole phage can have on

infection. One such case study focussing on *K. pneumoniae* showed a 100% efficacy at treating pneumonia in mice, but the high success rate is due to the phage being administered at an unrealistic timescale after *K. pneumoniae* infection so the clinical relevance of this is somewhat mitigated (Chhibber et al., 2019).

Another option is the use of phage-produced proteins for treatment of infection. This would help prevent the development of phage resistance, and once their efficacy is proven they could be manufactured synthetically making them far easier to translate to large scale industrial use (Salalha et al., 2017). One such protein are endolysins, which the phage produces to cleave the bacterial cell wall and could be applied to the same effect medically, this is particularly well studied against Gram-negative bacteria (Klyachko et al., 2010) (Shannon et al., 2020). Another option would be the isolation and use of depolymerases, such as attempted in this study, as this could increase antibiotic impact on cells in biofilm form and help reduce innate immune evasion by planktonic cells via stripping away of the cell capsule to expose the cell to immune defences such as macrophages.

A particularly interesting area is the use of phage therapy to treat pathogenic biofilms. Phage are extremely adept at infecting bacteria in biofilm, this is due to the close proximity of their hosts and as phage are non-motile this allows a much larger increase in random meeting events when compared to planktonic cultures. The phage can be used on its own, which is not very effective due to rapid mutation of a resistant phenotype, in combination with antibiotics or in a phage cocktail.

The phage may aid antibiotics in treating biofilm infections by providing a method of penetration and disruption, which may lead to dormant bacterial cells becoming active and in a growing state so therefore once again receptive to the antibiotic. A depolymerase producing phage was shown to significantly increase the microbial efficacy of ciprofloxacin on a *K. pneumoniae* biofilm which the antibiotic alone had no effect upon (Verma et al., 2010). Another study showed that using a phage in combination with amoxicillin was more effective than the antibiotic alone, it also raises the point that by using combination therapy it is less likely a resistant mutant will form compared to when using either treatment alone (Bedi et al., 2009).

A phage cocktail is a preparation containing three or more phage, all of which can infect the target bacterial strain and is far more effective than monophage treatment due to the drastically decreased chance of a resistant mutation occurring able to combat all phage present. A phage cocktail has been shown to protect mice from a lethal *K. pneumoniae* infection more effectively than monophage treatment, and also be able to be administered later, which is more useful in real-world settings due to delays in detection and identification (Gu et al., 2012).

A potential phage development pathway is shown below (Figure 3). Phage preparations have been shown to only have a limited time window of use before they become ineffective, this means they would require frequent ‘re-training’ or replacement. If legislation from the European Medicines Agency (EMA) and Food and Drug Administration (FDA) is changed to be more similar to that for developing new influenza vaccines, which also requires rapid adaptation, then this would be a feasible method of large-scale phage production (Pirnay et

al., 2011). A recent study shows that *K. pneumoniae* is one of the easier pathogens for on-demand isolation of infecting phage making it a prime candidate for this model (Mattila et al., 2015). It has also been suggested that a generic phage cocktail could be modified to give it specificity as opposed to individual creation for every case, this would drastically reduce the required work and make it far more feasible in the large-scale (Chan et al., 2013).

This would also require a large shift in research priorities by big pharmaceutical companies, of which only a few have currently shown interest. Personalised development of phage cocktails would be the most effective method of utilising phage but the funding to set up such facilities and expertise on a large scale would be significant. Another significant factor that would discourage companies investment in this is that it is extremely hard to copyright, through intellectual property protection and trademarks, this is due to the principle of phage therapy being common knowledge and as the full phage virion is just an organism it is not patentable. Though preparations containing phage-derived products, such as depolymerases or lysins could be patented and therefore are much more attractive to private companies looking to license and profit from their research (Pirnay et al., 2011).

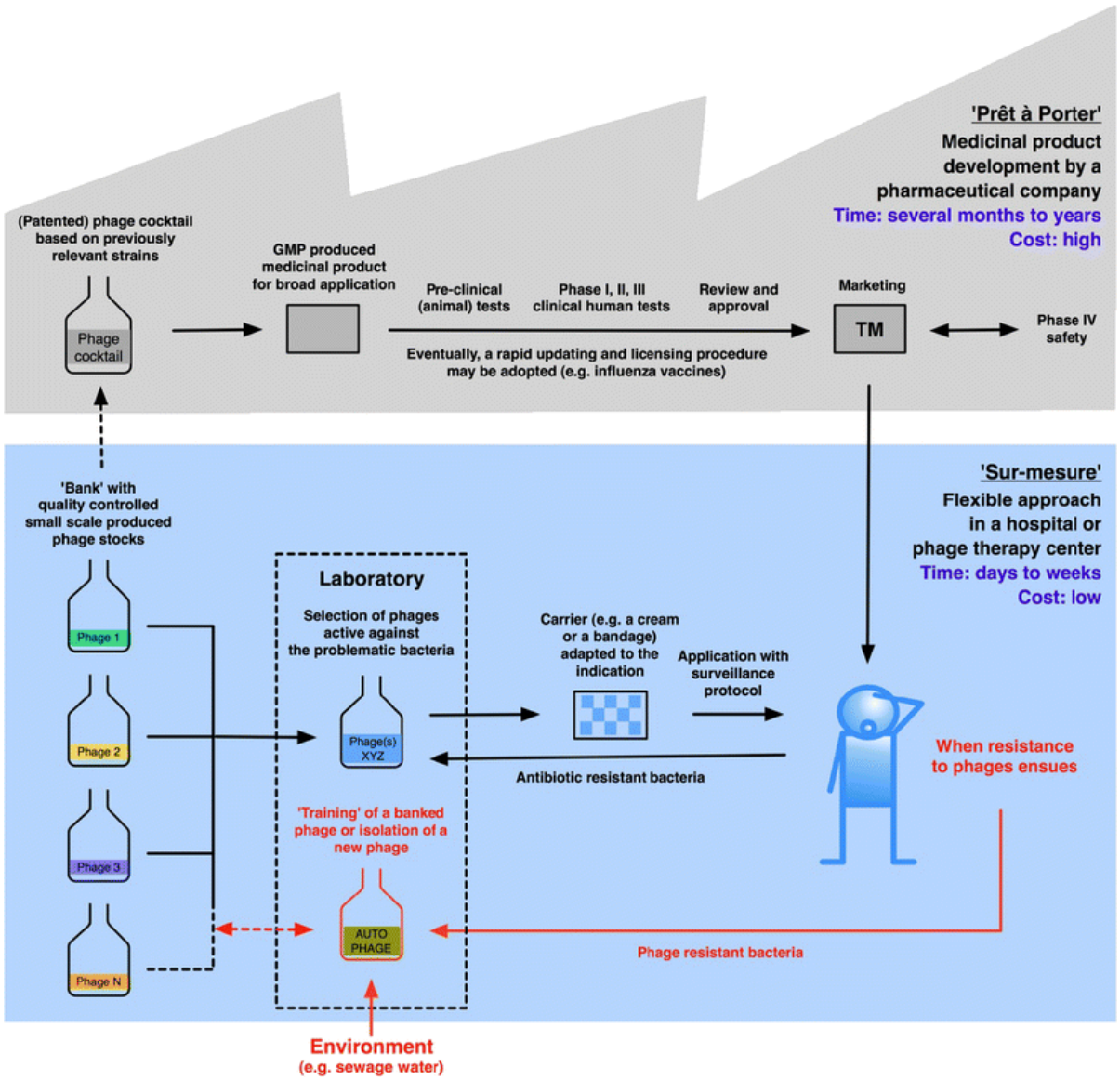


Figure 3. Showing a potential development pathway for the complementary use of generalised phage therapy and specialised in the treatment of antibiotic resistant pathogens such as K. pneumoniae. From (Pirnay et al., 2011).

1.3.2 Phage Life Cycles

Phage can have two types of life cycle, lytic or lysogenic. Which is employed is influenced by factors such as phage to host ratio and governed by the use of a small-molecule signalling system between phage (Erez et al., 2017).

The lytic cycle is the 'classic' cycle, where the phage hijacks the host cell and uses its resources to proliferate, subsequently leading to the lysis of the cell and the release of thousands of copies of the phage. The stages of the lytic cycle are shown below in Figure 4.

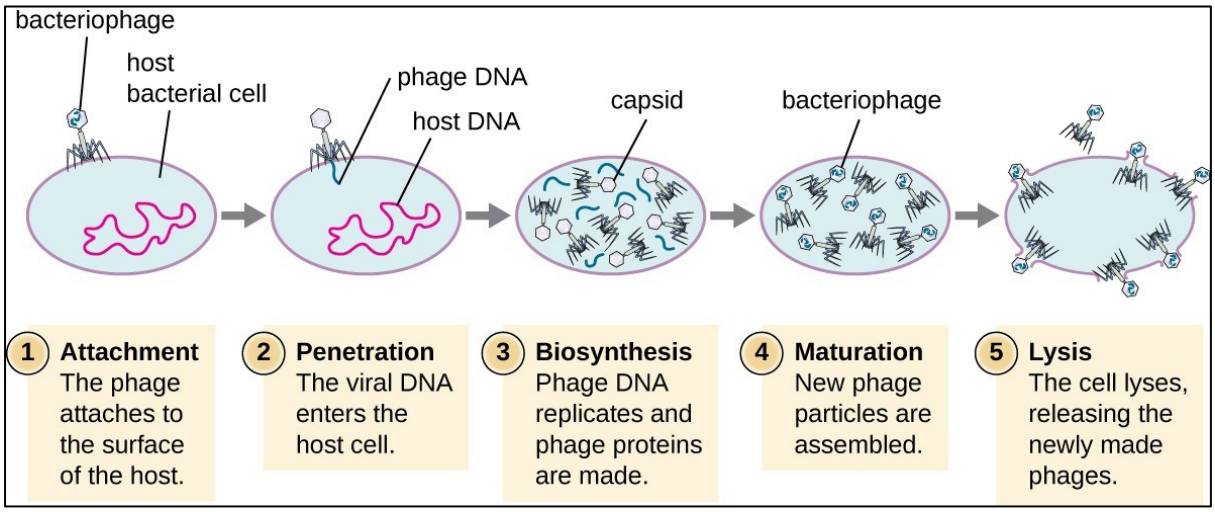


Figure 4. The lytic life cycle of a bacteriophage when infecting a host bacterium. From (Microbiology, 2005).

The other type of life cycle phage can employ is lysogenic. In this the phage follows the first two steps as lytic (Figure 4) but then it recombines its DNA with the bacterial chromosome, forming a prophage. This is not expressed by the bacterial host when it reproduces but lies dormant in all daughter cells produced until activated by external factors, such as DNA-damaging UV radiation or chemicals, at which point it completed steps 3, 4, and 5 of the lytic

cycle (Figure 4) (Abedon et al., 2011). Only some phage's have the ability to use the lysogenic cycle, those used in this study cannot use the lysogenic cycle and only the lytic.

Phage follow a specific growth pattern over time which can be expressed as a one-step growth curve (Abedon et al., 2011). It starts with the latent, or eclipse, period, in which the virion is penetrating the host capsule, attaching to cell surface and injecting its genetic material into the host. Then the replication process has to occur and no new virions are released until the end of this period where the host cell lyses and thousands of new virions are released. This marks the start of the rise period in which the number of free virions increases exponentially until there is a shortage of hosts available as more and more are lysed and not replaced, this leads to the numbers of virions stabilising and no new virions being released due to lack of hosts. The burst size refers to the number of new virions released per bacterial host infected and lysed (Abedon et al., 2011).

1.3.3 Phage Depolymerases

As mentioned in section 1.2.3, *K. pneumoniae* produces a thick capsule, or polysaccharide layer, as a virulence factor to help immune evasion and also exclude phage from the cell membrane so as to not let them bind and infect the cell. Phage have evolved depolymerases that allow then to degrade these polysaccharides which removes the physical barrier to their entry of the cell.

There are two main classes of bacteriophage encoded depolymerases, which are divided by their mode of action, they are hydrolases and lyases. Both cleave the polysaccharide into soluble oligosaccharides which allows the virion access to the cell surface. The hydrolases do

this by using a water molecule to cleave the O-glycosidic bonds of the polysaccharide, while the lyases cleave the same bond without use of a water molecule (Latka et al., 2017).

These depolymerases can either be released by the virion or can be virion-associated (Latka et al., 2017), the *K. pneumoniae* phage depolymerases used in this study were tail fibre proteins, so are virion-associated. Tail fibres and spikes are used for bacterial cell recognition, attachment, and tail-insertion which is why these depolymerases exhibit high specificity to bacterial capsular types, which limits the viruses host-range, and also could allow potential future use as a tool for characterizing a *K. pneumoniae* infections capsular type to allow greater specificity of treatment (Pan et al., 2019). There is also evidence of these depolymerases being used to facilitate penetration of biofilms, by cleavage of the extracellular polysaccharides that the matrices are formed of (Fernandes and São-José, 2018). This makes them an interesting target for potential use in conjunction with antibiotics, to increase their efficacy at treating biofilm infections, which are notoriously resistant to antibiotics as stated in section 1.2.4. A depolymerase producing phage was shown to significantly improve treatment of a biofilm when paired with ciprofloxacin in *K. pneumoniae,* compared to a non-depolymerase producing variant (Verma et al., 2010).

The target depolymerases for this study range between lengths of 345 amino acids to 2634 amino acids. They have presumed capsular type specificity for either *Klebsiella pneumoniae* bacteriophage serotypes KL 106 and KL 107 or both.

1.4 Conclusion

To conclude, the spread of antibiotic resistance (AR) to pathogenic bacteria is one of the largest threats facing mankind. It has come about through reckless use of antibiotics, the two main problems being livestock treatment and unnecessary use or misuse. AR can occur through random mutation or horizontal gene transfer from another bacterium.

K. pneumoniae is a human pathogen that has gained widespread AR, it causes many ailments including pneumonia and surgical site infection and traditionally is prevalent in only immunocompromised communities. This is changing, due to its worldwide prevalence and AR it is starting to become of clinical importance for even healthy individuals. The major issues being that its ESBL-production gives it resistance to most front-line antibiotics and its widespread KPC-production gives it carbapenem resistance, which are the antibiotics of last-resort. One of the most widespread lineages is ST258, which is particularly prevalent in Europe and North America. ST258 is considered the cause of the worldwide KPC-production in *K. pneumoniae* and is one of the most difficult strains to treat clinically.

When bacteria form a biofilm, they can become up to 1000 times more resistant to antibiotics. *K. pneumoniae* biofilms are practically untreatable with antibiotics and often require surgical removal from the patient.

Due to the rise in AR, other treatments such as phage therapy are coming under scrutiny. Bacteriophage (phage) are viruses that infect bacteria and can lyse and kill them. Their high specificity, impact on biofilms and ability to infect AR resistant bacteria mean they are a prime

candidate as a last resort option for otherwise untreatable pathogens. Phage therapy could consist of using the full virion in conjunction with antibiotics, to increase their efficacy against pathogens in biofilm, or just treatment using a phage 'cocktail' of three or more phage, which reduces the likelihood of resistance developing. Phage-produced proteins, such as lysins or depolymerases, could also be isolated used to treat *K. pneumoniae* biofilms as they degrade the extracellular matrix and polysaccharide layer, allowing easier access for the phage or antibiotic. These proteins are also more economically viable as they are easier to synthetically manufacture and patent so are more attractive to private companies.

1.5 Objectives

The overall aim of this study is the isolation, cloning and expression of 8 putative depolymerases from 7 *Klebsiella pneumoniae* bacteriophage and testing their efficacy compared to the whole virion against serotype KL 106 and KL 107, of the multidrug resistant MLST258 sequence type (ST258) of *Klebsiella pneumoniae,* both planktonically and in biofilm.

1.6 Ethics

This study does not involve the use of animals, humans or either of their tissues and there are no other ethical considerations.

EthOS number: 6054

2.0 Methods

2.1 Chemicals and Reagents

All chemicals and media were sources from Sigma Aldrich (Gillingham, UK) and Oxoid (Basingstoke, UK). All media and solutions were sterilized by autoclaving at 121^{0}C and 15 psi for 20 minutes. Media was allowed to cool to 60^{0}C before the addition of antibiotics where appropriate.

2.2 Bacterial Strains

All *Klebsiella pneumoniae* strains used were obtained from Professor Enright. These were KH 41, 42, 44, 45, 48, 55, 85 and 86 of KL 106 and KH 17, 67, 72, 79, 82, 87, 92 and 96 of KL 107. They were grown in Tryptone Soya Broth (TSB) or on Tryptone Soya Agar (TSA) plates. Overnight cultures were obtained from -80^{0}C freezer stock and grown at 37^{0}C in aerobic conditions shaking at 260rpm for 16 hours. Cultures were inoculated from a single colony. When required selective LB agar plates containing 100µg/ml ampicillin were also used. For long term storage of stock cultures, overnight cultures were mixed in a 1:1 ratio with glycerol and frozen at -80^{0}C. Streak plates were kept refrigerated for no longer than a month.

2.3 Preparing Phage Lysates

The Klebsiella phage host (KH) strains were taken out of the -80^{0}C freezer and streaked upon a TSA plate and incubated overnight at 37^{0}C. A colony was then picked and added to 5ml of TSB and incubated overnight in an orbital incubator at 260rpm and 37^{0}C. The Klebsiella phage (KP) lysates of KP 5, 10, 21, 29, 40, 47 and 62 were removed from the fridge and a serial

dilution in $^{-2}$ increments was made down to 10^{-10} using PBS. 100µl of KH+ 100µl KP dilution +3ml soft agar was poured onto TSA plates and incubated overnight at 37^0C. An appropriate dilution with a phage plaque lawn was chosen and 5ml of SM buffer, for phage storage, was added and the plate was stored overnight in the fridge. The SM buffer was removed and spun in a centrifuge at 4200 rpm for 20 minutes. The supernatant was removed and filtered through a 0.45µm syringe filter.

The titre of new phage lysates was then checked by re-plating out as described above and counting Plaque Forming Units (PFU) from an appropriate dilution.

2.4 Polymerase Chain Reaction (PCR)

2.4.1 Primer Design

The primers for the target genes were created using the NCBI primer blast webtool and manufacturer by Thermo Fisher. They were designed within the guidelines stated in the pEXP5-NT/TOPO® and pEXP5- CT/TOPO® TA Expression Kits for later use in these kits (Invitrogen, 2006). This meant that the first 3 nucleotides of the forward primer must ensure that the PCR product encodes the ATG initiation codon. The forward primer also had to place the initiation ATG codon of the desired protein 6-10 base pairs from the RBS sequence. The reverse primer also had to be designed to include coding for a stop codon. It was also important when synthesizing PCR primers, not to add 5´ phosphates to the primers as this will prevent the synthesized PCR product from ligating into the pEXP5-NT/TOPO® or pEXP5-CT/TOPO® vector. Table 1 shows the primers used in this study. The use of both a C-terminal

and N-terminal primer set allows a greater chance of success in transforming the target DNA into the cloning plasmid and then allows greater options for later downstream purification using tags. The preferred end was not known for the recombinant proteins in this study and as they are quite sensitive both were used. Using both also allows us to test which gives better yield without disrupting protein function and which is easiest to purify. Tagging can be extremely useful since it simplifies the purification protocol and improves the yield and solubility of your protein of interest.

Table 1. The 5’ to 3’ sequence of the primers used for PCR of the putative depolymerase genes. F is the forward primer for both N and C terminus. N_R is the N-terminus reverse and C_R is the C terminus reverse.

Primer	Sequence (5'->3')
KP5_00046_F	GTG GAT TCT TTT TCC TGG TGT AC
KP5_00046_N_R	GCT TTG TGC TAT CCG GCT G
KP5_00046_C_R	GTA TCC GGC TGA AGA GAA TCG TTC
KP5_00047_F	ATG ACA ACT AAC GTT TCA AAA GAG TTT GC
KP5_00047_N_R	GGA GTT AAC ATA TTA CCG TCC AC
KP5_00047_C_R	GTA CCG TCC ACT TTT CAA GGT TGC
PnkP-KP10_00062_F	CCA ATG AAT AAA AAA TGT ATC TGC ATT TTC G
PnkP-KP10_00062_N_R	CAT TTT TCA TTT GTC TAC TCC GTG AG
PnkP-KP10_00062_C_R	GCA TTT GTC TAC TCC GTG AG
KP21_00004_F	ATG TCG TTG GTG GAT TTA GTT ACA GC
KP21_00004_N_R	GTG CGC CCT ACA CTG TCA CAG
KP21_00004_C_R	GTA CAC TGT CAC AGG GTT AGC
KP29_00078_F	ATG GCA CTA TAC AGA CAA GGC
KP29_00078_N_R	CCA ATC GGC CCC TTT GTT AC
KP29_00078_C_R	GTA CAT CTC TAA CTT TTC TTC AAT GGC
KP40_00001_F	ATG TCT TTA GTT AAT GTG ATA TAT CCC GCG AAT G
KP40_00001_N_R	GCT GAG TTA GCC CAG TAG TAA CG
KP40_00001_C_R	GTA CTG GGC TAA CTC AGC AAT GTA CTG
KP47_00023_F	ATG GCA ACA ATT AGC GAC CAA CTC G
KP47_00023_N_R	CAA CCG GCC CCT TTT TTT ATT CCC CTT TCA G
KP47_00023_C_R	GTA TTC CCC TTT CAG CTC CTT CAA TTC
KP62_00128_F	ATG AAA AGT CAG TTT AAT CAA CCC CAA GG
KP62_00128_N_R	CAC ATT CAT AGT ATA CAT GAG TTA CTC CAG
KP62_00128_C_R	GAG GTA CTC CAG GAT TAA AGA TAA TGT TAA AC

2.4.2 PCR Conditions

For each PCR amplification reaction, a master mix was prepared to consist of: 10 µl of 5x My Taq buffer, 1µl of Taq Polymerase (50 unit working stock), 0.1µl of High Fidelity Taq Polymerase (25 unit working stock) and 36.9 µl of Molecular grade water per sample. This protocol was adapted from the pEXP5-NT/TOPO® and pEXP5- CT/TOPO® TA Expression Kits manual (Invitrogen, 2006) after initial failure using their suggested protocol and adaptation with the help of Dr James Redfern.

To each PCR tube 48µl of the master mix was pipetted, then 1µl of the appropriate DNA template (between 10-100µg/ml concentration) and 1µl of forward and reverse primers added (from 10µM working stock). This brings the total volume of the reaction to 50µl. The tubes were then centrifuged briefly and put into the thermocycler. The PCR conditions were 1 initial denaturing cycle for 2 minutes at 94°C, then twenty five cycles of 1 minute at 94°C (denaturing), 1 minute at 55°C (annealing), and 1 minute at 72°C (extension), followed finally by one 7 minute cycle at 72°C for final extension.

2.5 Agarose Gel Electrophoresis

To prepare gels, 1g of agarose was added to 100ml TAE buffer (1%w/v) and microwaved until clear. 5µl of Midori Green DNA stain (NIPPON genetics) was then added to the gel when cooled slightly and it was poured into the mould and the combs inserted. Once set, the combs were removed to form the wells and the gel was added to the tank and covered in 1x TAE buffer (Tris base, acetic acid and EDTA). To prepare the PCR products they were mixed in a 1:1 ratio with 5X loading dye (Bioline) and 20µl of each was loaded into each well. 5µl of 1Kb Hyperladder (Bioline) was added to a well. The gel was run at 100 volts for 60 minutes then removed and pictured on a Bio-Rad GelDoc system.

2.6 TOPO Cloning Reaction

2.6.1 Setting Up

Using the pEXP5-NT/TOPO® and pEXP5- CT/TOPO® TA Expression Kits as per the manual (Invitrogen, 2006) the cloning reaction was prepared as such for transforming into One Shot® TOP10 chemically competent *E.coli* for each PCR product: 4µl of fresh PCR product, 1µl of salt solution and 1µl of TOPO vector. The cloning reaction was then mixed gently and incubated at room temperature for 5 minutes then placed on ice. The plasmids used for both N- and C-terminus expression are shown in Figure 5.

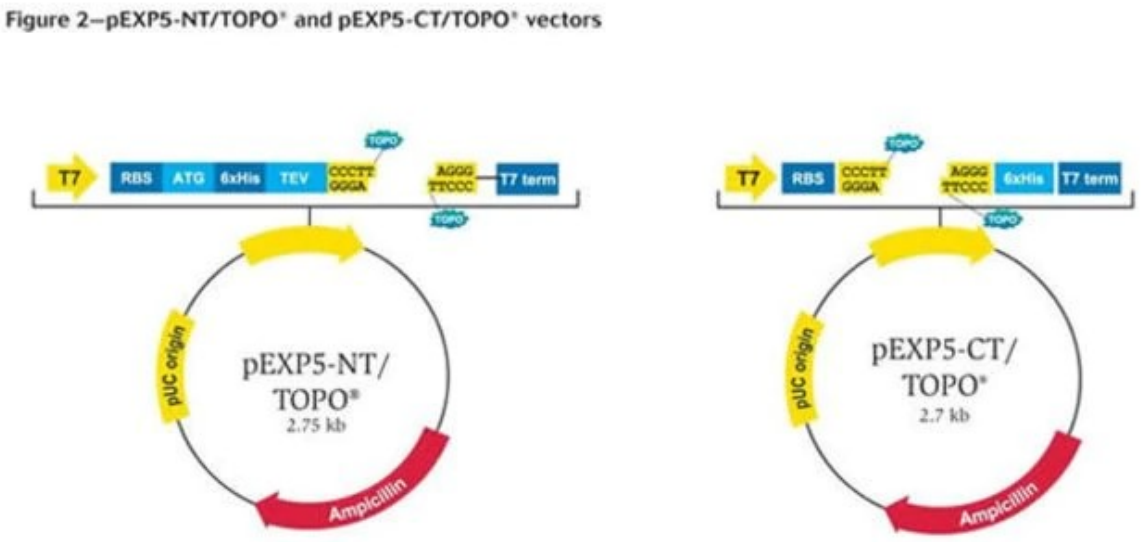


Figure 5. Plasmid maps for both the N- and C-terminal. From (Invitrogen, 2006).

2.6.2 Transformation

2µl of the TOPO cloning reaction was then added to the cloning reaction which was mixed gently and incubated at room temperature for 5 minutes then placed on ice for 10 minutes. Then the cells were heat shocked for 30 seconds in a 42^0C water bath without shaking, after which they were immediately transferred to ice. 250µl of room temperature S.O.C medium was then added and the tube shook horizontally at 200 rpm and 37^0C for an hour. 50µl of each transformant was then spread on a prewarmed LB plate containing 100 µg/ml ampicillin and incubated overnight.

2.6.3 Analysing Transformants

Five colonies from each transformant were picked and cultured overnight in LB broth containing 100µg/ml ampicillin at 37^0C in an orbital incubator. A patch plate was also prepared to allow the creation of glycerol stock of the bacterium, these were incubated for 24 hours at 37^0C. A reaction mix containing 300 - 500 ng plasmid DNA with 0.4 µM forward or reverse primer and sterile water in separate 0.5 ml microcentrifuge tubes were sent to the

DNA Sequencing Facility provided by University of Manchester. This was to check the quality of the plasmid construct and that no insertions, deletions or substitutions had occurred.

2.7 Purifying Plasmid DNA

The cultures containing the transformants were then used in a QIAprep® Spin Miniprep Kit as according to the manual for isolation of our target plasmid and purification of its DNA (QIAGEN, 2010). The success of this purification was determined using PCR of the purified DNA. A master mix was prepared, as explained in section 2.4.2, per sample and 1µl of the purified DNA from the QIAprep® Spin Miniprep Kit was added with 0.5µl of the appropriate T7 primer from the pEXP5-NT/TOPO® and pEXP5- CT/TOPO® TA Expression Kits and 0.5µl of the appropriate reverse primer. The PCR conditions were also the same as laid out in 2.4.2 and the product of this was analysed using agarose gel electrophoresis, set up in the same way as laid out in section 2.5.

2.8 Protein Expression

This was achieved using an Expressway™ Maxi Cell-Free E. coli Expression System, the following protocol is adapted from the manual (Invitrogen, 2011).

The *E. coli slyD*- Extract, E. coli Reaction Buffer (-A.A.), and 2X Feed Buffer were thawed on ice. Then a master mix was created of 20µl of *E. coli slyD*- extract, 20 µl of 2.5X IVPS *E. coli* reaction buffer (-A.A.), and 1.25µl of 50 mM Amino Acids (-Met).

42.5 µl of the master mix was added to a 1.5ml Eppendorf tube and 1µg of DNA template was added. Dependant on the concentration of the DNA template, verified by the use of a Thermo Scientific™ NanoDrop™ One^{C} Spectrophotometer, the final volume for each sample was made up to 50µl using DNase/RNase-free Distilled Water.

The tubes were then incubated in a shaking incubator set to 300rpm and either 25/28/30 or 37^{o}C. After 30 minutes 50µl of the feed buffer composed of 20µl of 2X IVPS Feed Buffer, 1.25 µl of 50 mM Amino Acids (-Met), and 23.75 µl of DNase/RNase-free Distilled Water was added. The tubes were then returned to the incubator for a further 3 hours 30 minutes. After which they can be stored by freezing at -20^{o}C till needed. The expected sizes of the overexpressed proteins are shown below in Table 2.

Table 2. The expected size of the overexpressed proteins.

Protein and terminus used	Size (kDa)
KP 5-46 C-terminus	13.4
KP 5-47 C-terminus	28.5
KP 5-47 N-terminus	29.9
KP 10 C-terminus	19.5
KP 10 N-terminus	20.9
KP 40 C-terminus	59.4
KP 62 C-terminus	73.9

2.9 Protein Analysis

Protein samples were analysed using SDS-PAGE following acetone precipitation, 5µl of the protein reaction product was added to 20 µl of 100% acetone and centrifuged for 5 minutes at 12,000rpm. The supernatant was then removed and the pellet resuspended in 20µl of 1x SDS-PAGE sample buffer. This was then heated for 10 minutes at 80^0C.

10% Mini-PROTEAN® TGX™ SDS-PAGE (Bio-Rad) gels were prepared and 5µl of the ColorBurst™ 8-220 kDa protein ladder loaded along with 10µl of each sample. The gels were run at 120 V for around 70 minutes using a Tris-glycine running buffer. They were then removed and washed with water. Then they stained using an all in one Coomassie blue stain (Thermo Fisher) and put on a Gyro-Rocker for 4 hours to stain. Once complete they were once again washed with water and photographed.

2.11 Planktonic Assay

An overnight culture of the KH strain was grown at 37^0C in a shaking incubator. A Sarstedt 96 well plate was used, all the edge wells being filled with 200µl of sterile water. 198µl of TSB was added to the wells, 2µl of the overnight culture was then added to them, giving a 1:100 dilution. The plate was then put into a FLUOstar Omega where it was incubated at 37^0C while shaking for 1.5 hours. A growth curve was set up by measuring the OD at 600nm every 6 minutes. After 1.5 hours, 2µl of phage was added to the appropriate wells and the OD was measured for a further 17.5 hours while still at 37^0C and shaking.

Plaque forming units (PFU/mL) could not be calculated for all KPs in this study due to inconsistency in phage growth on host strains so using a standardised amount of 2µl in the planktonic assay was decided upon.

2.12 Biofilm Assay

2.12.1 48h to Determine High/Low Biofilm Producers

KH strains were removed from the -80^{0}C freezer and streaked upon a TSA plate then incubated overnight at 37^{0}C. A single colony of each KH strain was inoculated in 5ml of TSB and incubated overnight in an orbital incubator at 260rpm and 37^{0}C. The overnight cultures were then diluted 1:10 in fresh TSB and their OD read and standardized to 0.6 at 600nm on a spectrophotometer (using TSB as a blank). 2µl of standardized culture was then added to 198µl of TSB in a Nunclon 96 well plate. Blank wells were filled with 200µl of TSB. The plate was then sealed with parafilm and wrapped in moist paper towels before placing it in a sealable plastic container and being incubated at 37^{0}C for 24 hours. The plate was then removed and 50µl of TSB was added to all wells before a further 24 hours of incubation at 37^{0}C. The cultures were then discarded by inversion into a waste container and the plates dried on paper towels. All test and control wells were rinsed twice with 250µl PBS, emptying and drying between each as described previously. All wells were then stained with 260µl of 0.1% crystal violet stain and allowed to stand for 10 minutes. The plate was then submerged in 3 respective water baths and then inverted and dried upon paper towels. 270µl of 30% acetic acid was added to each well and allowed to stand for 10 minutes. The solution in each well was pipetted to solubilize and 200µl transferred into a new 96 well plate. The optical

density of the solubilised biofilm was then read in a FLUOstar Omega plate reader at 550nm and the results blank corrected.

2.12.2 72h to Determine Effect of Phage on Biofilm

This method was identical to the one listed in section 2.12.1 but at the 48-hour step, where the biofilms were stained and quantified, instead 2µl of the respective phage were added to the appropriate wells, these were then incubated for a further 24 hours at 37^0C after which the staining steps were followed as detailed above.

Plaque forming units (PFU/mL) could not be calculated for all KPs in this study due to inconsistency in phage growth on host strains so using a standardised amount of 2µl in the biofilm assay was decided upon.

3.0 Results

3.1 Phenotypic Analysis of KL 106 and KL 107 via Biofilm Assay

An initial biofilm assay was carried out to determine if there was a trend within the MLST's for 'high producers' or 'low producers' of biofilm. This is an arbitrary judgement based on statistical testing done on the results, namely a 2-way ANOVA with Tukey's multiple comparisons (Figures 6 and 7).

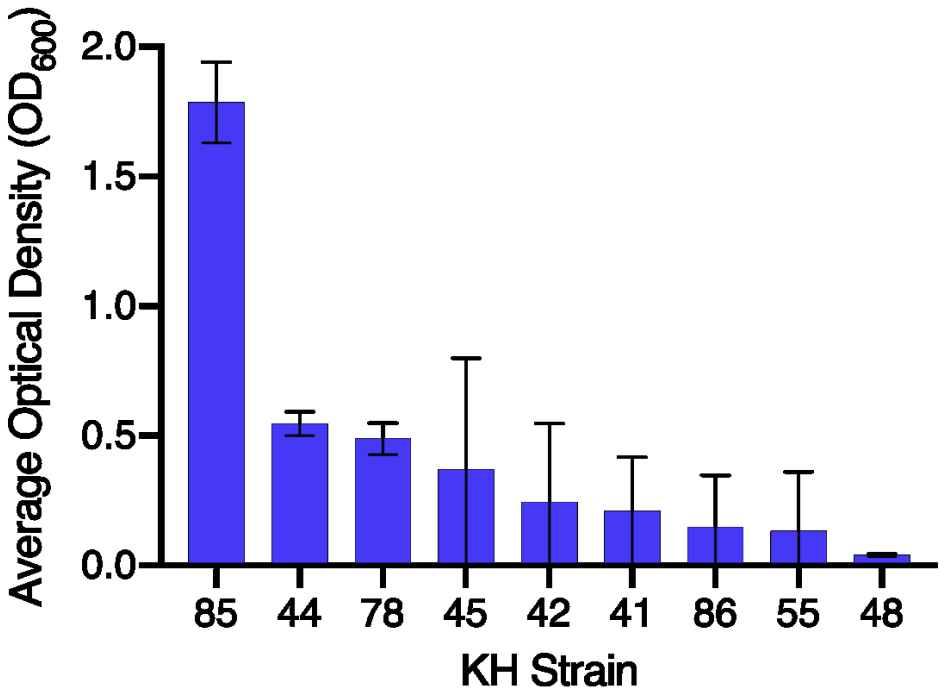


Figure 6. The biofilm growth of the KL 106 group, measured through absorbance at 600nm (OD). The error bars are Standard Deviation. All repeated in quadruplicate.

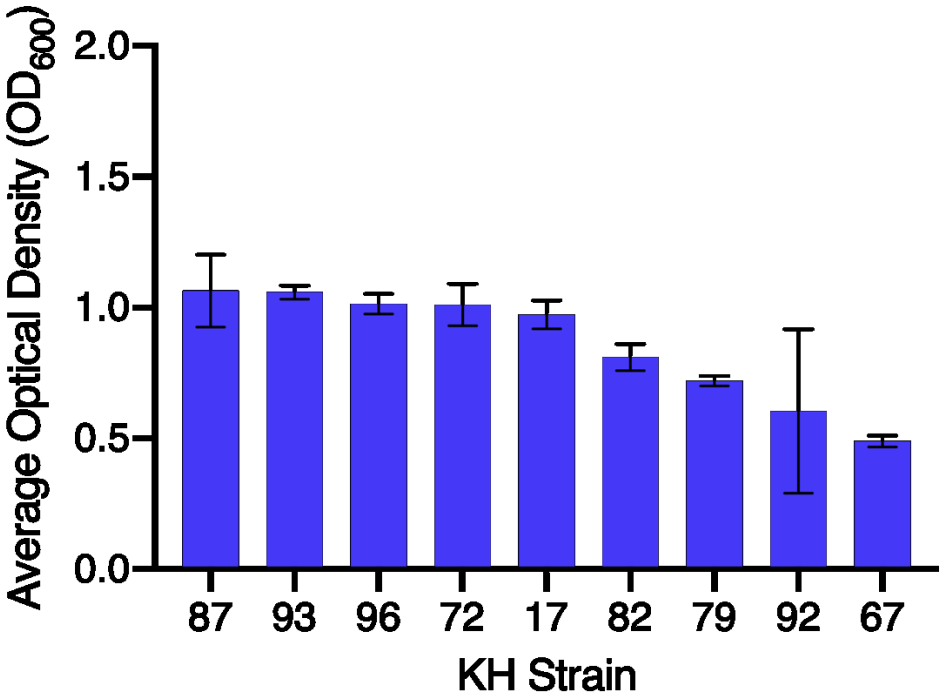
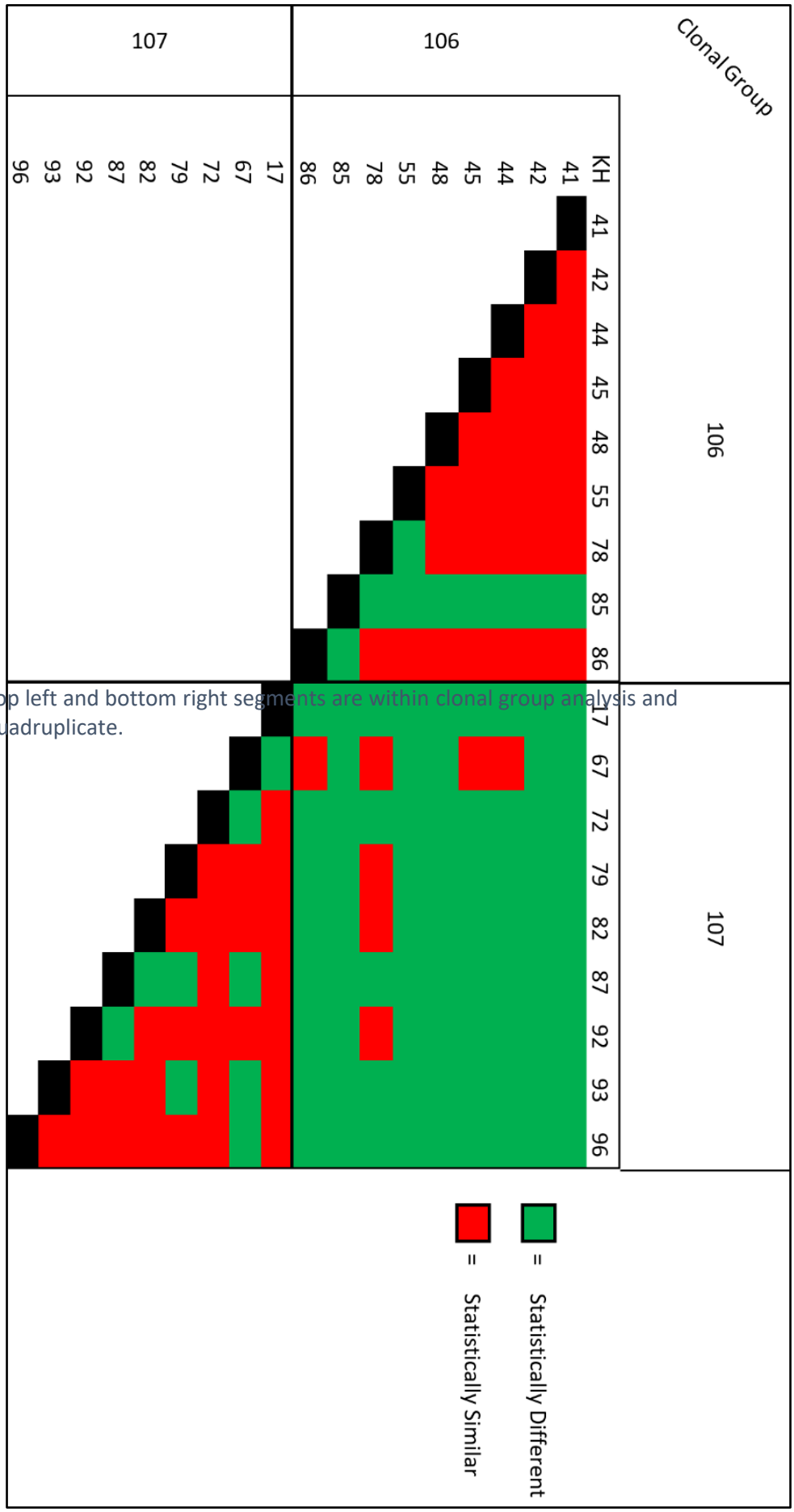


Figure 7. The biofilm growth of the KL 107 group, measured through absorbance at 600nm (OD). The error bars are Standard Deviation. All repeated in quadruplicate.

As there was no clear grouping, a 2-way ANOVA with Tukey's multiple comparisons was carried out (Figure 8). This figure then confirms that KH 85 is statistically different from the rest of the serotype KL 106 group, which is clear from Figure 7. It is also statistically significantly different than all of the serotype KL 107 group, showing it is significantly the highest biofilm producer out of the 18 strains tested. Strain KH 85 has a distinct phenotypic difference compared to the rest of its clonal group. In the rest of the serotype KL 106 strains there is no significant difference between any, with the exception of KH 55 and 78. This means for the purpose of this study these will all be classed as low biofilm producers, with KH 55 being the lowest and KH 85 the highest in this phenotypic bracket. KH 48 is shown to have no significant growth at all, that is why it was excluded from the rest of this study as the effect of phage would be difficult to determine on such low initial growth.

The KL 107 group is less easy to divide into distinct groups than the KL 106, there is no clear statistical divide between low and high biofilm-producing phenotypes. There is a significant difference between multiple strains but no clear way to divide these into the arbitrary low or high biofilm-producing groups. This means that for the continuance of this study KH 87, the strain with the highest mean, and KH 67, with the lowest mean, in Figure 7, will be used as representative high and low biofilm producers.

It has to be noted that these results are only comparable within this study and not across studies, this is due to the arbitrary nature of the 'low' or 'high' biofilm-producing label and that such a subjective phenotypic measure cannot be extrapolated upon.



Tukey's multiple comparison. The top left and bottom right segments are within clonal group analysis and measurements were repeated in quadruplicate.

3.2 Comparison of Phage Activity on Selected KL 106/107 KH Strains in Biofilm

The 72-hour biofilm assay was carried out on KH 55 and 85 of the KL 106's and KH 67 and 87 of the KL 107's as the nominated 'high' and 'low' biofilm producers of each group. All KH's were tested against KP 5, 10, 21, 40 and 62 to see if they reduce the growth of biofilm, a control was also set up to show the growth of biofilm after 72 hours without phage, for comparison (Figure 9).

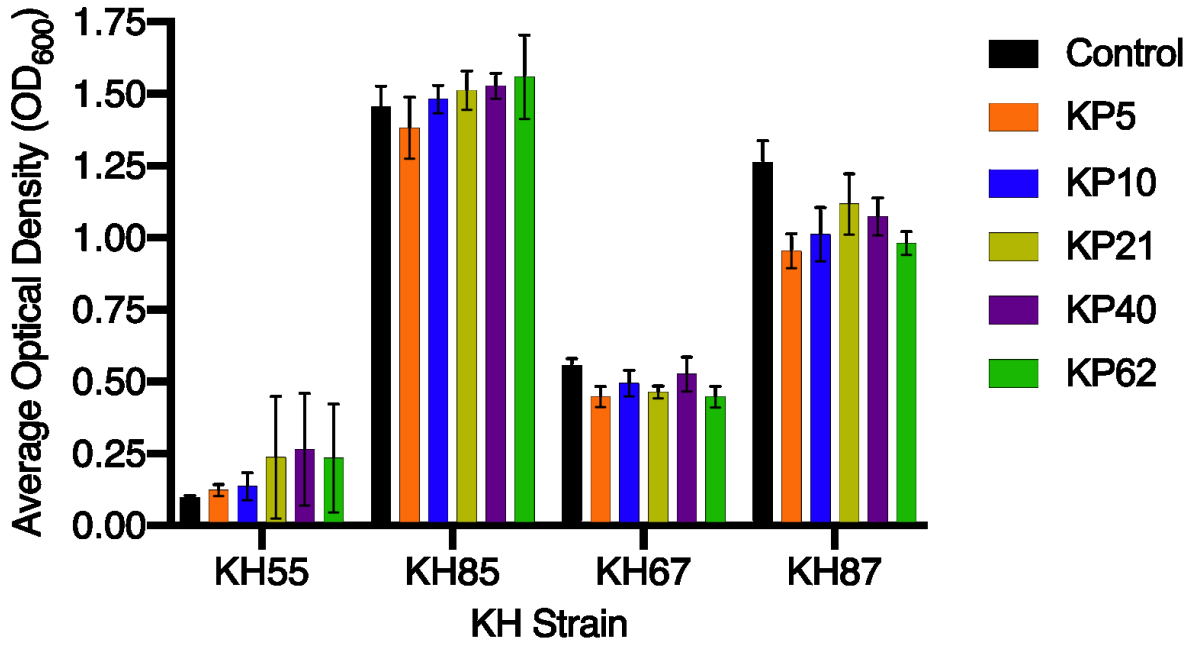


Figure 9. The effect of phage on biofilm formation after 72 hours.

The error bars shown are standard deviation and all results were done in quadruplicate. A 2-way ANOVA with Dunnett's multiple comparisons was carried out. It showed there was no significant difference between the control and phage groups for any of KH 55, 85, and 67, this means that the phage had no effect on overall biofilm growth after 72 hours. KH 87 had its

biofilm formation significantly reduced by all phage except KP 21, which means that KP5, 10, 40 and 62 all reduced this strains ability to form a biofilm ($p<0.05$).

3.3 Effect of Phage on Planktonic Growth

The 96-well plate planktonic growth assay was carried out upon KH 55 and 85 of the KL 106 group and KH 67 and 87 of the KL 107 group as the nominated 'high' and 'low' biofilm producers of each group. This was to judge the phage's effect on planktonic cell growth. For all the following figures the error bars are shown for every 5th timepoint, which is every 30 minutes, and all results were done in quadruplicate. A 2-way ANOVA with Dunnett's multiple comparisons was carried out on each time point for the control versus the separate phage treatments to show any difference in growth throughout the test. Any significance reported was to the $p<0.05$ level.

Figure 10 shows that the phage had an effect on the planktonic growth of KH 55. The most distinct was that of KP 5, in Graph A, which statistically significantly reduced the growth of KH 55 from 2.5h, which is an hour after phage addition, throughout the entire test. KH 10, Graph B, also significantly reduced the bacterial growth from 2h after phage addition to the end of the test. The only phage to have no statistical effect on the growth of KH 55 was KP 21, through a dip at the end of the exponential phage was seen in Graph C, its statistical importance could not be proven. Both KP 40 and KP 62, Graphs D and E respectively, slowed the growth during the bacterial exponential phase of growth, roughly between 1 to 4h after phage addition. The resistant mutant that arises against KP 40 appears to be able to grow to a higher cell count in Graph D, but there was no statistical difference between that and the control.

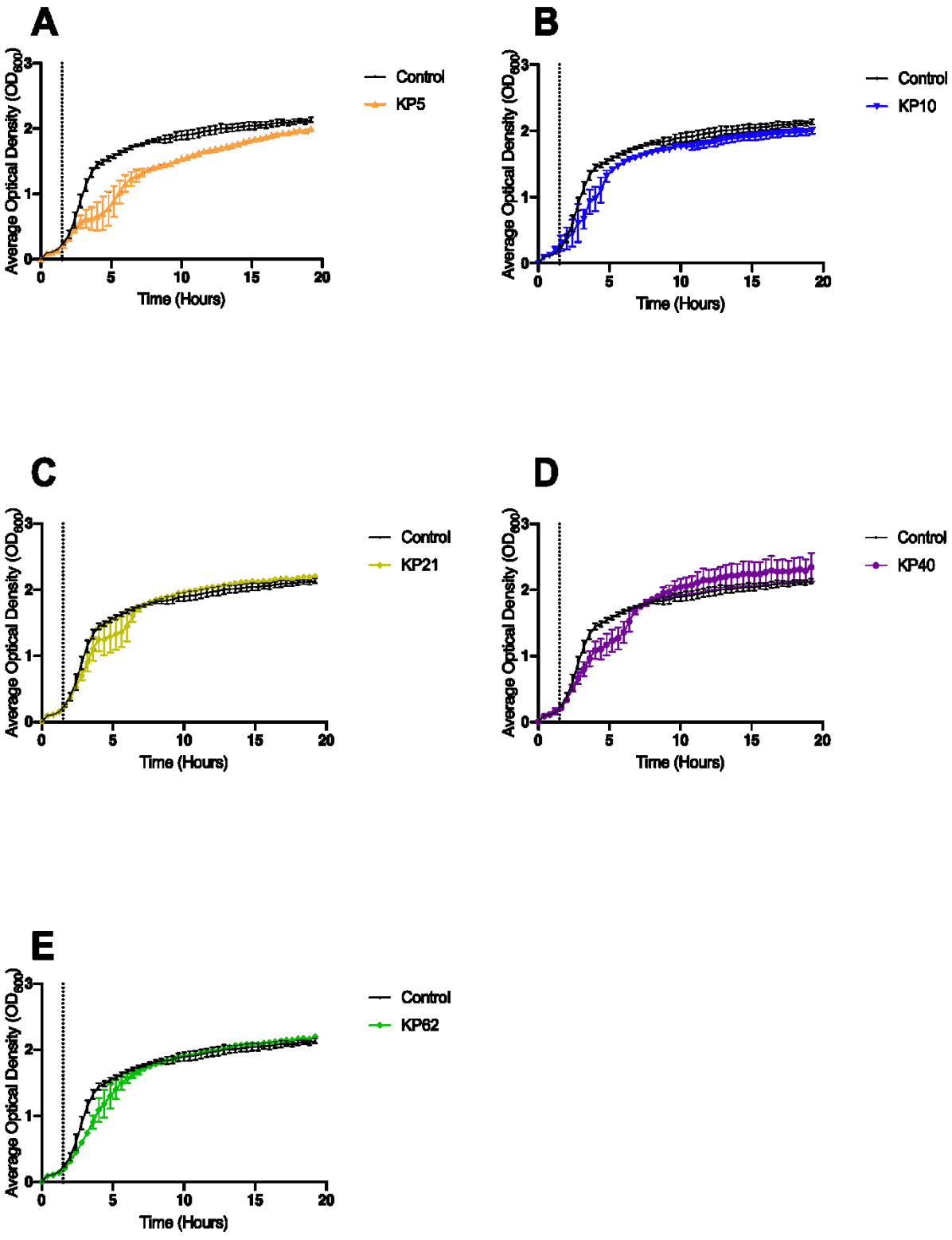


Figure 10. The effect of the phage on the growth of KH 55, the 'low biofilm producer' of KL 106. Graphs A, B, C, D and E showing the effect of KP 5, 10, 21, 40 and 62 respectively on host growth.

Figure 11 shows the effect of the phage on the planktonic growth of KH 85. In Graph A, KP 5 showed no difference at the start of the exponential phase and only 8h after phage addition does it cause a significant difference from the control, through to the end of the test, resulting in KH 85 reduced ability to grow planktonically. Though Graph B showing KP 10 looks like there is no slowing of growth, they were statistically different around 2.5 to 4h after phage addition, which is at the end of the exponential growth phase. Graphs C and D showed KP 21 and KP 40 respectively having no significant impact on the growth of KH 85. In Graph E, KP 62 does not affect the growth curve of KH 85 until the 15^{th} hour after phage addition, where the OD is higher than that of the control until the end of the test.

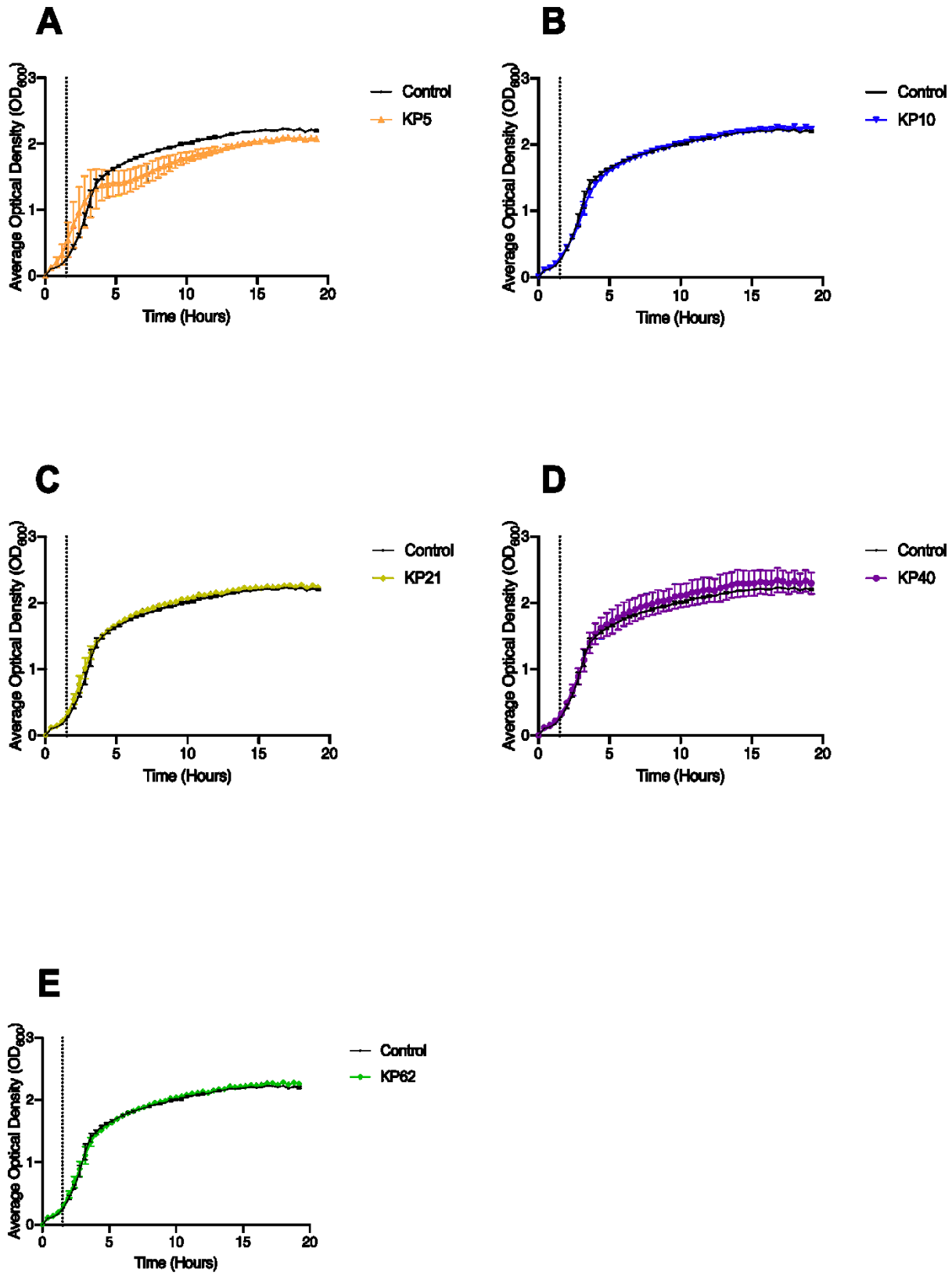


Figure 11. The effect of the phage on the growth of KH 85, the 'high biofilm producer' of KL 106. Graphs A, B, C, D and E showing the effect of KP 5, 10, 21, 40 and 62 respectively on host growth.

KH 67 was the least affected host strain out of the four tested (Figure 12). Graph A shows KP 5 significantly reduced bacterial growth from 4h post-phage addition through to the end of the test. Graphs B, C and D respectively show KP 10, 21 and 40 having no significant difference throughout the test, compared to the control. Graph E shows, KP 62 having a period of reduced growth, between 2 and 3.5 hours after phage addition, during the exponential phase. The KH 67 mutant that arose from this went on to show significantly increased growth from the 16th-hour post-phage to the end of the test.

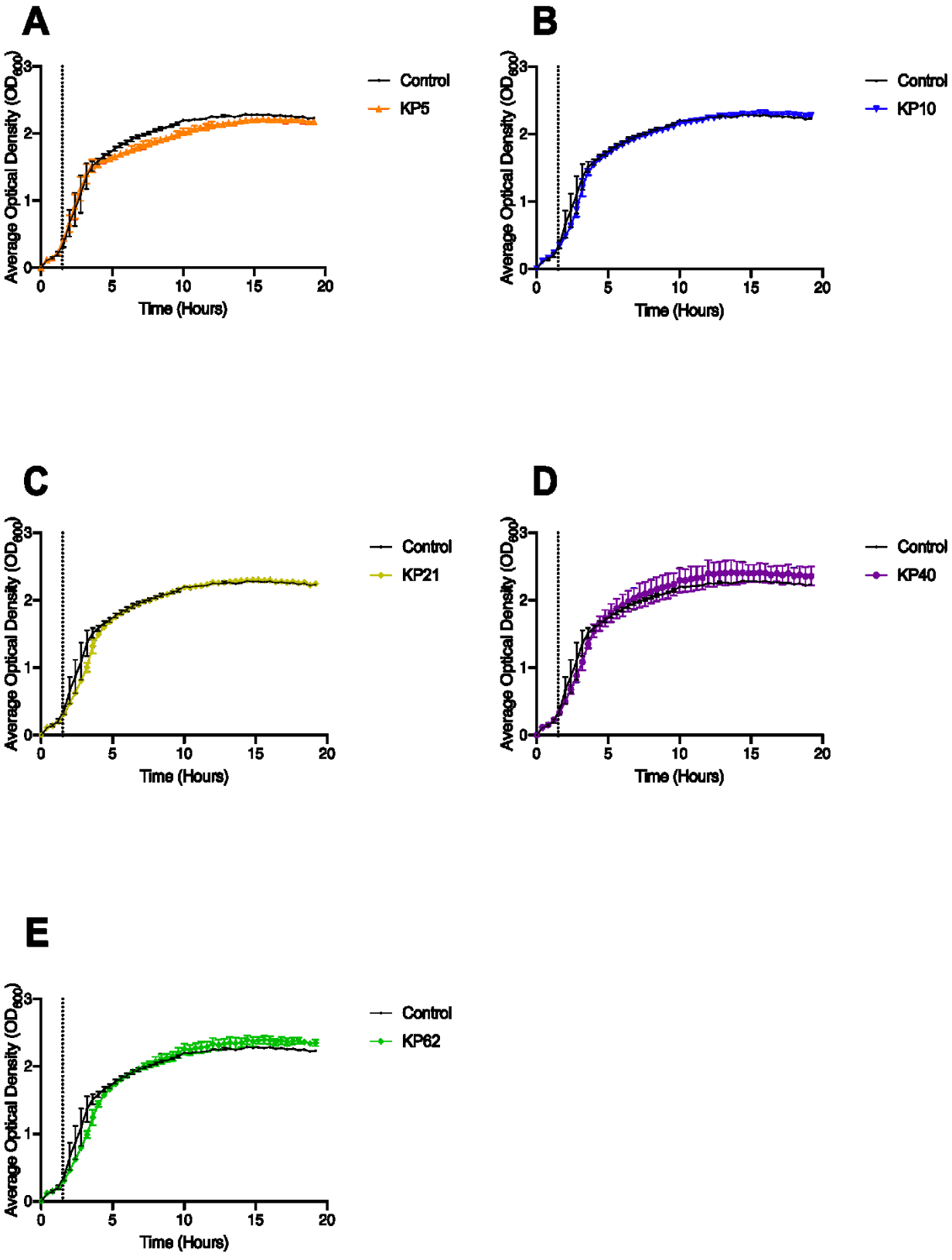


Figure 12. The effect of the phage on the growth of KH 67, the 'low biofilm producer' of KL 107. Graphs A, B, C, D and E showing the effect of KP 5, 10, 21, 40 and 62 respectively on host growth.

Figure 13 shows that as with the previous host strains tested KP 5 had the largest effect on the planktonic growth. In Graph A it significantly reduced growth from 1.5h post-phage addition throughout the rest of the test. Between 1.5h and 4h after the addition of the phage the growth of KH 87 was stopped entirely and the OD remained constant. Graph B shows KP 10 reduced the growth of KH 87 for half an hour, two hours after phage addition. The KH 87 mutant that arose from this then showed an increased capacity to grow planktonically and had a significantly higher OD than the control from 6h to 10h after the addition of phage and then also from 14.5h post-phage to the end of the test. In Graphs C and D, KP 21 and KP 40 respectively both significantly slowed the growth of KH 87 from between 1.5 to 4.5h post-phage. Finally Graph E shows KP 62 reduced the growth between 1.5 and 2.5h post-phage, the KH 87 mutant that arose from this was a better planktonic grower than the control and showed significantly higher OD from 10h post-phage to the end of the test.

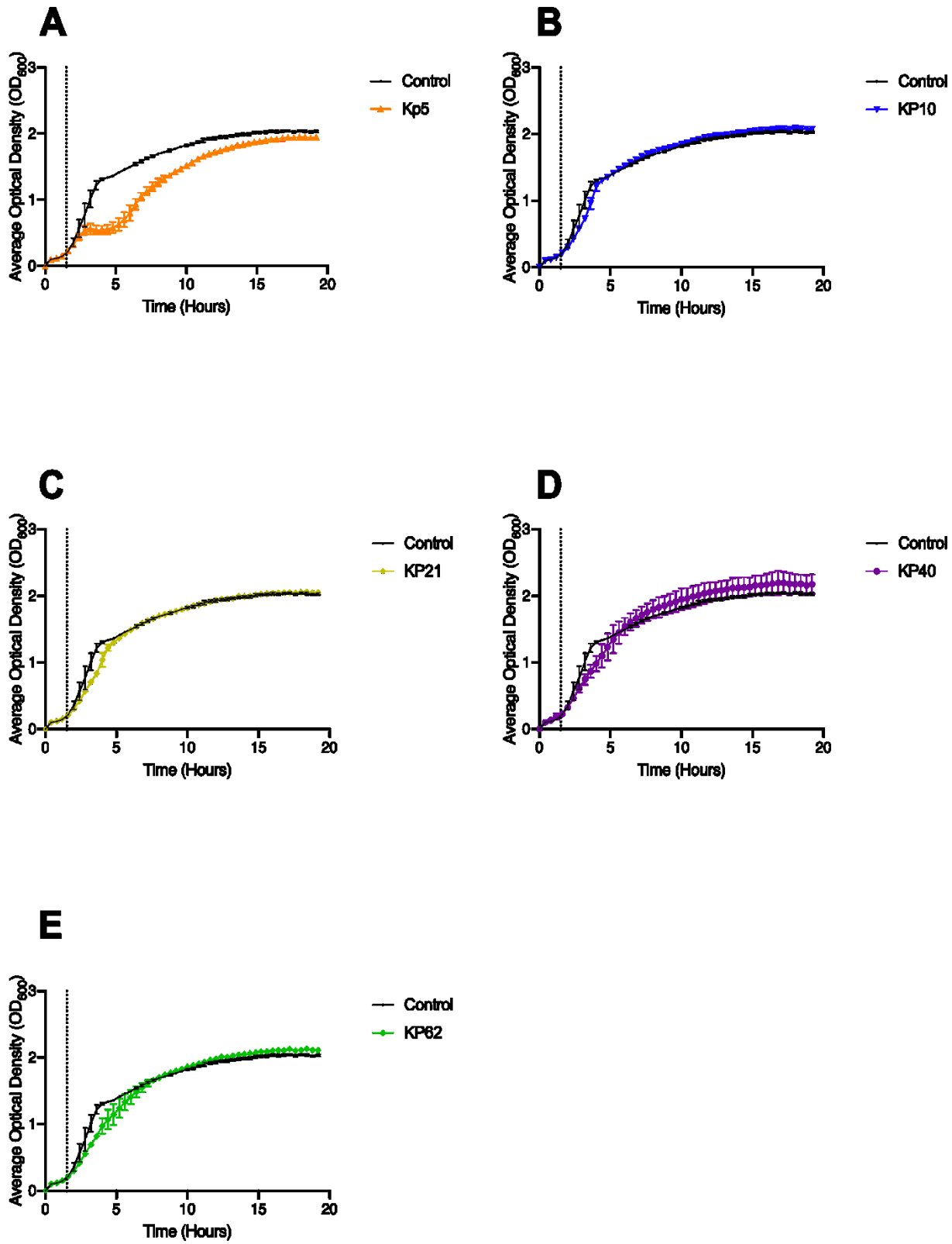


Figure 13. The effect of the phage on the growth of KH 87, the 'high biofilm producer' of KL 107. Graphs A, B, C, D and E showing the effect of KP 5, 10, 21, 40 and 62 respectively on host growth.

3.4 PCR of Target Genes for Transformation into *E.coli*

A Polymerase Chain Reaction was carried out to determine if the putative depolymerase genes had been successfully transformed into the plasmid. Amplification of the product indicative of depolymerase was successful for all N and C terminus primers except KP40 N-terminus and KP62 N-terminus, in lanes 8 and 10 (Figure 14). For KP 29 and KP 47 neither C- or N- terminus primers produced product so were excluded from the rest of this study. Both N- and C- terminals were used as recombinant proteins are quite sensitive and the preferred end was unknown for all our primers. Using both gave the best chance as at a higher yield without disrupting protein functions and for ease of purification. Table 3 shows the expected size of the amplicons.

Table 3. Expected size of amplicon (base pairs).

Amplicon	Size (base pairs)
KP 5-46	345
KP 5-47	753
KP 10	492
KP 21	1974
KP 29	2634
KP 40	1677
KP 47	2028
KP 62	492

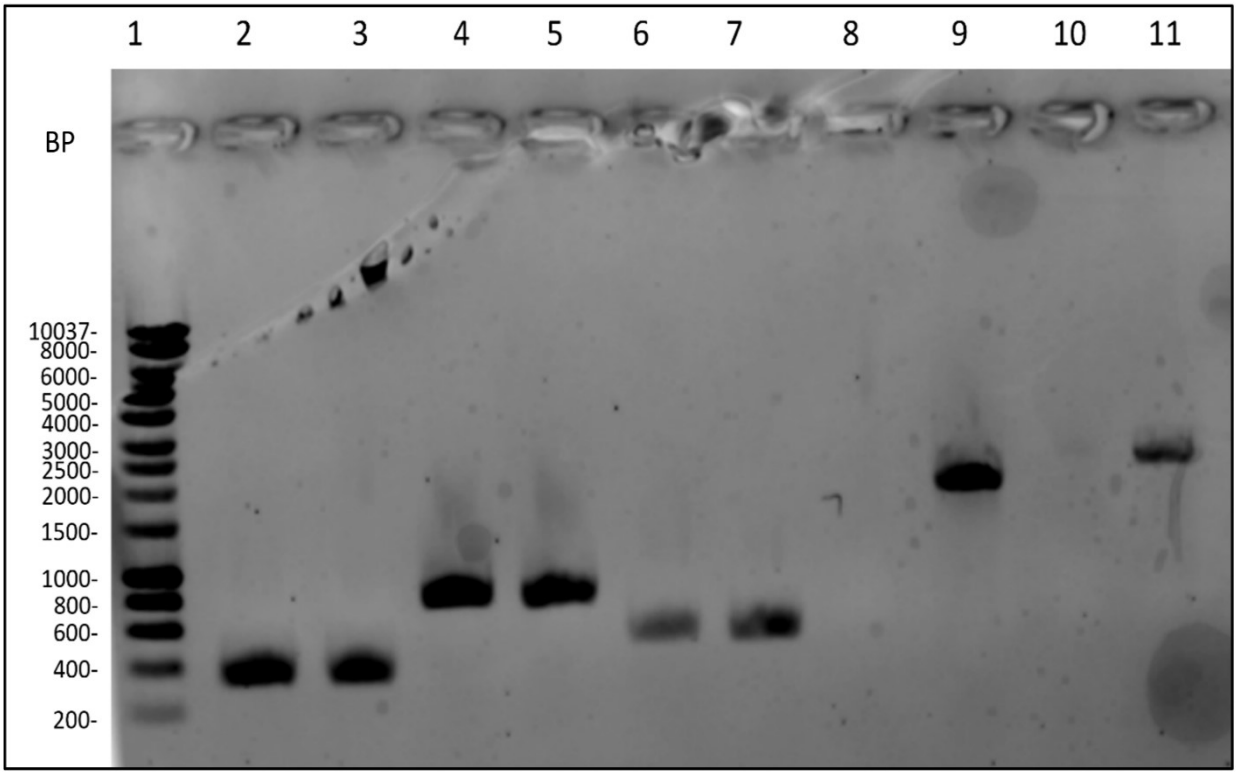


Figure 14. Confirmation of the presence of the PCR products using the primers designed to be used for insertion into a vector and transformation. Examined using 1% agarose gel electrophoresis ran at 100v for 60 minutes. Lane 1 contains the 1 kB Hyperladder marker. Lanes 2-11 were the N-terminus then C-terminus for KP5-46, 5-47, 10, 40 and 62 sequentially. Product was produced for all bar KP40 N-terminus and KP62 N-terminus. The product size is shown in number of base pairs (BP).

3.5 Quality Control of the Transformants

After transformation, the isolated plasmid DNA was checked for the target genes via PCR. Figures 15 and 16 show that the putative depolymerase genes were successfully transformed into plasmids for all Klebsiella phages. There was only C terminus success for KP 5-46, 40 and 62 but success in both terminuses for KP 5-47 and 10.

A maximum of two colonies for each terminus of each gene were sequenced via Sanger sequencing (DNA Sequencing Facility, University of Manchester) to check the orientation of the inserted PCR product and that there had been no insertion, deletion or substitutions

which might adversely affect the produced protein. Seven colonies passed this quality control stage and were used moving forward (Table 4).

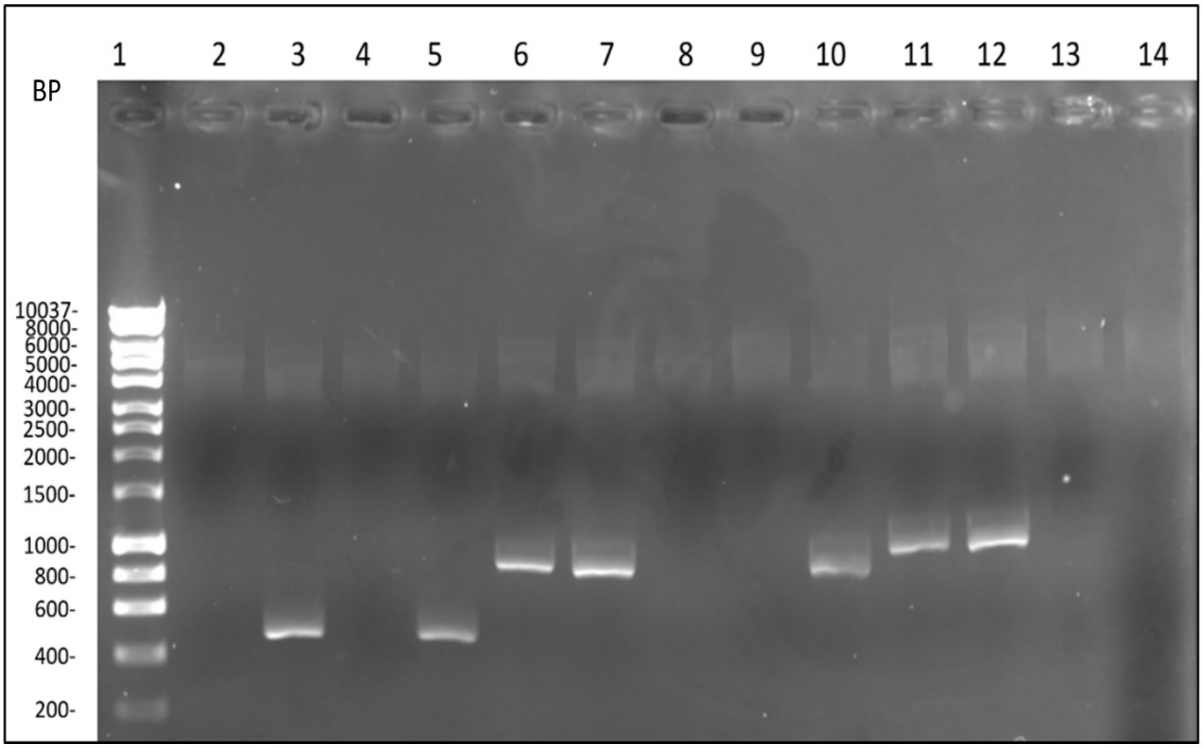


Figure 15. The product of a PCR amplification reaction with the plasmids purified by the QIAprep® Spin Miniprep Kit to show the presence of the target genes in the plasmid (Gel 1). The output of the QIAprep® Spin Miniprep Kit was examined using 1% agarose gel electrophoresis ran at 100v for 60 minutes. Lane 1 was the 1 kB Hyperladder marker. Lanes 2-5 were KP 5-46 C terminus colonies, of which colony 2 (C2) and C4 contained the plasmid. Lanes 6-10 were the KP 5-47 C terminus colonies, of which C1, C2 and C5 contained the plasmid. Lanes 11-14 were the KP 5-47 N terminus colonies, of which C1 and C2 contained the plasmid. The product size is shown in number of base pairs (BP).

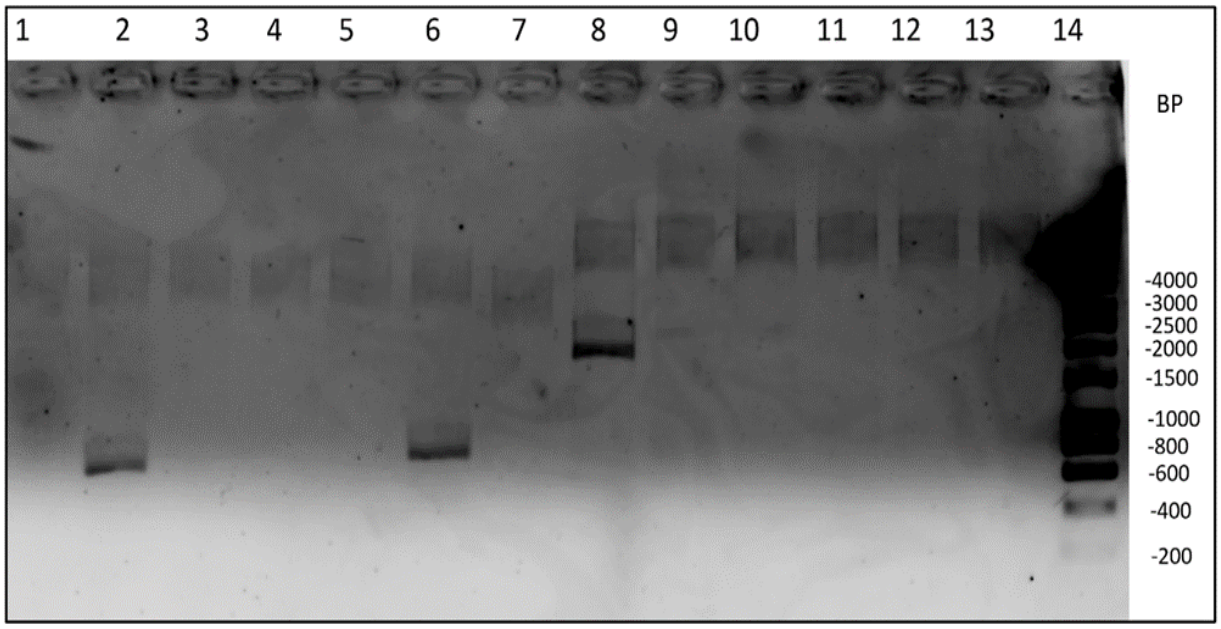


Figure 16. The product of a PCR amplification reaction with the plasmids purified by the QIAprep® Spin Miniprep Kit to show the presence of the target genes in the plasmid (Gel 2). The output of the QIAprep® Spin Miniprep Kit was examined using 1% agarose gel electrophoresis ran at 100v for 60 minutes. Lane 1 was the KP5-47 N terminus colony 5 (C5), which contained the plasmid. Lane 2 was KP10 C terminus C1, which contains the plasmid. Lanes 3-7 were KP10 N terminus colonies, of which only C4 contains the plasmid. Lane 8 was KP 40 C terminus C1, which contains the plasmid. Lane 9-13 was KP62 C terminus colonies, of which only C1 contained the plasmid, though banding was faint. Lane 14 was the 1kB Hyperladder marker. The product size is shown in number of base pairs (BP).

Table 4. The colonies selected to move forward after the screening of their genetic code to check for potential changes such as insertions/deletions/replacements.

Gene	C-terminus Colony	N-terminus Colony
KP5-46	C4	x
KP5-47	C5	N2
KP10	C1	N4
KP40	C1	x
KP62	C1	x

3.6 Protein Production

The protein reaction product generated using the use of the Expressway™ Maxi Cell-Free *E. coli* Expression System was assessed using SDS-PAGE. None of the SDS-PAGE gels showed the production of the desired proteins (Figure 17).

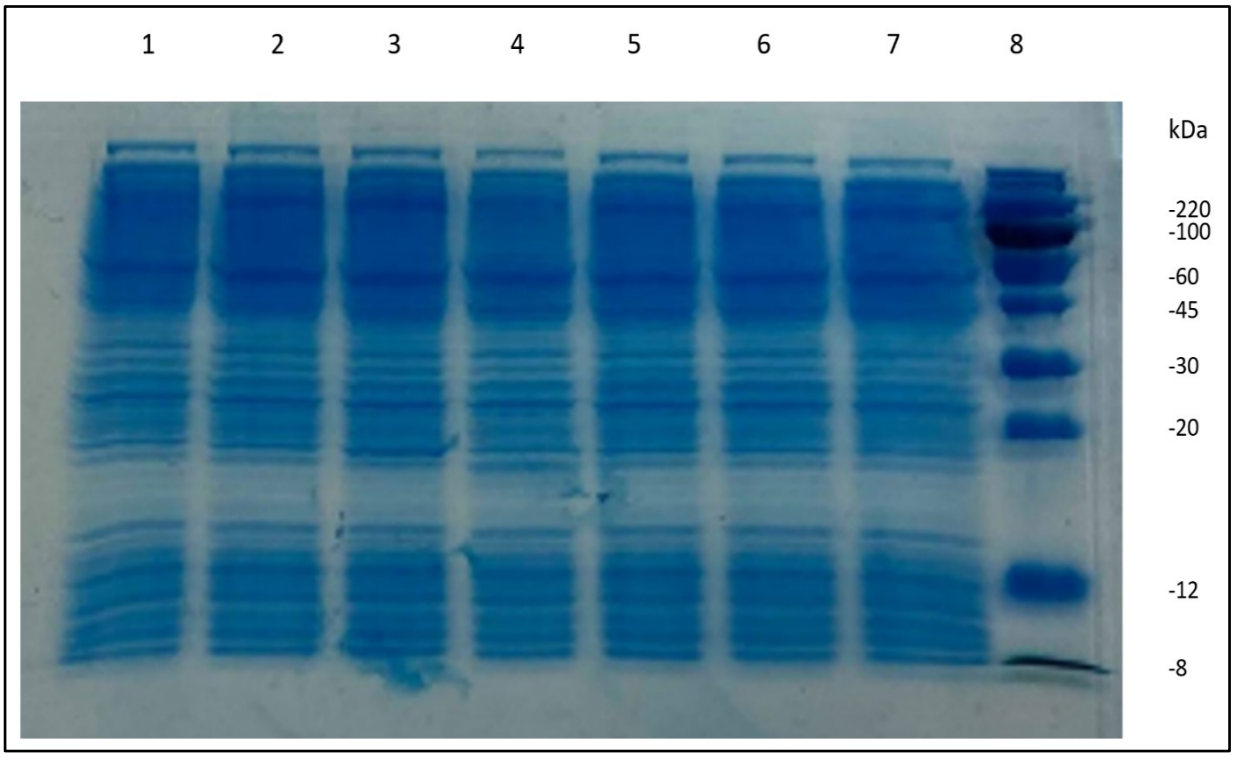


Figure 17. The SDS-PAGE gel showing the failure of the Expressway™ Maxi Cell-Free E. coli Expression System reaction to produce the target proteins. Examined using SDS-PAGE ran at 120v for 70 minutes. The lane contents from 1-7 were as follows: pEXP5-NT/CALML3 control vector, KP 5-47 C5, KP 5-47 N1, KP 10 C1, KP 10 N4, KP 40 C1 and KP62 C1. None were successful. Lane 8 contains the ColorBurstTM 8-220 kDa protein ladder. The product size is shown in kiloDaltons (kDa).

Figure 17 is a representative gel, as regardless of conditions used none were successful. Some conditions changed were: amino acid concentration variation from 1mM to 4mM, incubation from 30-37^{0}C and overall incubation time from 3-6 hours, as suggested by the Expressway™ Maxi Cell-Free E. coli Expression System reaction manual. The plasmids containing the

transformants were used in the Expressway™ Maxi Cell-Free E. coli Expression System reaction, done at 25^{o}C.

In addition to the lack of band representing the target protein, the positive control, pEXP5-NT/CALML3 control vector in lane 1 of Figure 17, provided by the kit was also absent.

4.0 Discussion

The primary aims of this study were to isolate, express and test the efficacy of eight putative capsular polysaccharide depolymerase enzymes and seven phage against 18 *K. pneumoniae* host isolates of MLST sequence type (ST)258 capsular types KL 106 and KL 107. Recombinant enzyme expression was unsuccessful however testing of whole phage was completed in a number of planktonic and a biofilm assays.

4.1 Protein Expression

Due to time constraints, it was not possible to successfully express depolymerase proteins, though all steps prior to this were accomplished. There are numerous reasons which could explain why the expression of the depolymerases was not successful. The Expressway™ Maxi Cell-Free E. coli Expression System suggests that it could be due to DNA template or reagent contamination. It could also have been that the T7 enzyme used was inactive due to too many freeze/thaw cycles or contamination. It is also possible that DNA templates were not correctly configured but this cannot be the case in this study as the pEXP5-NT/TOPO® and pEXP5-CT/TOPO® TA Expression Kit is the suggested means of creating the appropriate template for use in the expression system (Invitrogen, 2011). More time is required to troubleshoot this system and so any future work into this study would have to be heavily focussed around this problem. The fact that the positive control provided along with the kit also did not work suggests it is one of the above listed problems rather than a methodological error in the steps leading up to this protein production.

If the putative depolymerases had been expressed, the planned work would have included characterizing the proteins size and general information such as the temperature and pH range in which it is effective, then the proteins host range would have been tested and compared to that of the full phage they were isolated from, a good example of a methodology for this is in (Majkowska-Skrobek et al., 2016). Then they would have been tested against whole bacterial cell and isolated capsular polysaccharides, a key virulence factor as stated in the introduction which can be isolated as per (Majkowska-Skrobek et al., 2016) and tested using a turbidity assay. The three key factors tested would be its effect on cell growth/ ability to kill, its effect on biofilm formation and its ability to strip away the cell's polysaccharides. These could have then been compared to whole phage particles to judge the usefulness of the isolated enzyme at treating *K. pneumoniae*.

The enzymes ability to decrease cell growth/kill could also have been tested against bacteria in the planktonic assay detailed above. This would have allowed a comparison between it and whole phage which have already been studied. Similarly, the ability to decrease biofilm formation would have been tested using the biofilm assay detailed above, this again would have allowed comparison between expressed recombinant depolymerase and whole phage. The ability to strip away bacterial capsular polysaccharides could have been confirmed using microscopy and further tested as detailed in (Majkowska-Skrobek et al., 2016) against purified CPS.

4.2 Planktonic Assay

This assay involved the testing of both a 'high' and 'low' biofilm producer from each KL 106 and KL 107 serotypes of ST258 against five whole phage particles. This dynamic assay was used as opposed to a static one such as one carried out on an agar plate. This is because it better mimics the infectious model within the human system where the interaction between phage and pathogen would be dynamic in such a medium as the blood for example.

The process of viral replication follows a one-step growth curve. There is an eclipse period, in which the virions are infecting the host and replicating within the cell, during this no viral particles are released. Next is the rise period, in which the number of infective virions is increased exponentially as the cells are lysed and the phage are released (Abedon et al., 2011).

The eclipse period for all phage except KP 10 was found to be around 1.5h post phage addition. This is the time when the rate of the OD increase is slowed as the cells are lysed and new virions released. KP 10 appears to have a slightly longer eclipse period of 2h. The length of the eclipse period has been shown to be affected by the availability of hosts, and as all KH strains were at roughly similar OD this could explain the similarities between the phages' eclipse periods (Abedon et al., 2001).

ST258 must be in the host-range for all phage though as they all slowed the growth during the exponential phase of at least one KH from each serotype KL 106 and KL 107, but it is possible that ST258 was not in the host range for the depolymerase, as they are more specific;

normally only infecting a single serotype each (Pan et al., 2019). As the phage were not initially isolated on ST258 strains the likelihood of their depolymerases being specific to this host is low. Testing this hypothesis would have been possible if the depolymerases were isolated, expressed and a separate host-range analysis carried out upon them as planned.

A reason for the resurgence of the bacterial population shown in all instance of phage infecting hosts (Figures 10, 11, 12 and 13) could be that biofilms were formed and reduced the access the phage to the bacterium in the ways discussed in the introduction. This is plausible as depolymerases are the main method of phage gaining access to bacteria in biofilm, and as previously discussed the specificity of these depolymerases could render the phage unable to infect bacteria in a biofilm allowing it to replenish the planktonic cells faster than the phage can lyse them, leading to the increase in OD seen in all test runs (Hall and Mah, 2017).

A common theme throughout is that KP 5 showed the largest effect on all host strains tested, no matter their biofilm-forming ability or serotype. It was the best phage at infecting all hosts tested and was the only one that significantly reduced all four hosts growth in the exponential growth phase and overall. This could be due to it containing two putative depolymerase enzymes, KP 5-46 and KP 5-47, both expressed as tail fibre proteins as opposed to the other phage only having one each. Previously *K. pneumoniae* phage have been shown to exhibit a diverse host range (Manohar et al., 2019) which may explain why this phage, which was isolated on a separate serotype, of a totally separate MLST group was able to infect these strains. The fact that these ST258 strains were not the original host may also explain why even though there was significant effect on all hosts growth by KP 5 there was no point at which

the bacterial cell count, represented by the OD, decreased. The largest effect was against isolate KH 87 in which growth halted totally for between 1.5h post phage addition to 3.5h. Even then the host recovered, and though the final OD was significantly lowered compared to the control, an exponential growth phase occurred and the host replicated (Figure 13-A). The lower final OD, compared to the controls, suggests that the mutants that arose for resistance to KP 5 have a fitness disadvantage. Another explanation as to why the resistant mutants that arose still grew to a high final OD is that in a rich broth, such as in this assay, the cost of resistance upon fitness has been shown to be far less than when in a nutrient-deprived environment (Sanchez, 2011). Though KP 5 did not eradicate the bacterial population it has been shown that the cost of phage-resistance can be associated with virulence reduction (Oechslin, 2018).

The phage least able to infect any ST258 strain, regardless of serotype, was KP 21. It only caused a significant slowing of the growth of KH 87 during the exponential phase and did not lower the end OD (Figure 13-C), which shows it had no overall significant effect on the growth of any the KH strains trialled. As previously mentioned, this may be due to the phage being isolated on a different host to the ST258 strains tested, which would mean this phage would not be a good candidate for use against the ST258 strains either for diagnosis or treatment.

The host that was most able to withstand phage infection was KH 67. Three out of five phage could not infect at all, and only KP 5 inhibited the overall growth of this isolate (Figure 12-A). Phage isolates KP 10 (Figure 12-B), 21 (Figure 12-C) and 40 (Figure 12-D) were unable to infect it at all and KP62 only slowed the growth during the exponential phase but then a significantly

higher final OD was recorded meaning the mutant that arose had a higher growth rate than the original strain (Figure 12-E).

KH 87 was infected by all phage at around 1.5h after phage addition (Figure 13). This means it was the least well equipped to deal with phage infection, as none of the phage were specific to any of the hosts so KH87 must have fewer capable defences against generalized phage infection. Interestingly two out of the five times KH 87 was infected the mutant that arose ended up with a higher final OD than the control, though this could also be explained as the host wasn't targeted at all and grew uninhibited. This could be due to within host KH 87 populations there being multiple genotypes encoding for the same original phenotype but upon phage addition this allows a genetically diverse response that means many different methods of resistance appear, which increases the chances of one of them having a higher fitness than the original strain phenotype (Williams, 2013).

KP 62 was interesting as it infected all hosts but three out of four hosts ended with a significantly higher OD compared to their control groups, this may mean that development of resistance to KP 62 leads to an increased fitness for the bacterial strains compared to the original sensitive strain. This means that specialising to resist this specific phage increases fitness, potentially by losing other fitness limiting mechanisms such as resistance to other phages'.

It does not appear that there is any clear-cut difference between the two isolates of each of the two serotypes - KL 106 or KL 107. Neither was clearly better or worse at defending against

phage infection. There was also no correlation between biofilm-forming ability and ability to defend against phage infection when in a planktonic culture.

A disadvantage of this assay was that the multiplicity of infection (MOI) of the phage to bacterial hosts was not found and so 2µl of phage was added. MOI is the ratio of phage to host and could not be worked out for this study as some phage were extremely sporadic in infecting the host they were initially isolated upon. Though MOI was not used, to standardise amount of phage added, the pfu/ml of the phage lysates used was similar which means that though it was not the exact same amount of phage added each time results are still comparable. Another method that could've been used to analyse these data sets is the Liquid Score Assay which measures phage ability to suppress host growth (Xie et al., 2018).

4.3 Biofilm Assay

This involved the testing of five phage against four *K. pneumoniae* strains, two from each KL 106 and KL 17 groups of ST258 in a biofilm assay. From each group was a designated 'high' and 'low' biofilm producer. This assay was reliable as both the control and treatment group for each host were grown on the same 96-well plate, so they were under identical conditions for the entirely of this run and the negative control on the plate allow allowed confidence that none were contaminated. To further ensure the accuracy of this assay, and to stop the cross-contamination of different phage the treatment wells were all spaced out to reduce the risk of aerosolised phage travelling from one well to another and contaminating a separate treatment group.

Only KH 87, the ‘high’ producer of the KL 107 group, had its ability to form a biofilm reduced by phage addition, all other hosts were statistically unaffected (Figure 9). All phage, except KP 21, significantly reduced KH 87’s ability to form a biofilm. The largest effect was from KP 5, which may be due to it containing two depolymerases which would help destroy more of the biofilm matrix giving access to more cells for the phage and allowing greater effect.

There was some correlation between the planktonic and biofilm assay, as mentioned above KH 87 was the worst at defending against phage infection in planktonic culture and was also the only host whose biofilm-forming ability was decreased by the phage. KP 5 was also the most effective phage in the planktonic assay, infecting all hosts and significantly reducing their ability to grow, and it also was the phage which reduced KH 87’s biofilm-forming ability the most drastically.

There could be multiple reasons why only KH 87 was affected in this assay, such as the other KH strains producing different extracellular polysaccharides, which the depolymerases were not specific for, or quick mutation by them to resist infection by the other phage. It could also be as the ‘high’ producer, it has forfeited some fitness limiting defences against infection to be able to grow more efficiently, which would explain why the genetically similar KL 107 strain KH 67 was not affected in this assay.

The limitation of this assay is that it takes place in a static environment, and as with the planktonic assay, a dynamic environment would better represent the human infectious model. Though the addition of 50µl of TSB at the 24-hour mark allowed for the environment the bacteria were growing in to constantly be high in available nutrients, to better mimic the

human body. Similarly working out the MOI would have allowed for comparison across to other similar studies, but as the MOI is unknown as just 2µl of lysate was added no comparisons can be drawn. It has been previously shown that sometimes use of a low MOI, such as 0.1 or even 0.0000001 can be more effective at halting bacterial growth when in conjunction with antibiotics (Tagliaferri et al., 2019). Another limitation was that the effect on biofilm was only judged 24 hours after addition, and as shown in the planktonic assay even when the KH strain is susceptible to the phage, a mutant quickly arises and the growth of the host is only slowed not totally inhibited. This means that it is possible the phage had an effect on the hosts' ability to form biofilm but it was not seen as the host had time to recover, so using different time points may be advisable in future work.

5.0 Conclusion

In conclusion, the aims of this study were to isolate, express and test the efficacy of putative depolymerase enzymes and full phage from seven *K. pneumoniae* phage against 18 hosts from the MLST258 capsular types KL 106 and KL 107.

The expression of the putative depolymerase enzymes was not possible due to time constraints; but the isolation and transformation steps were successful for five of these genes.

The efficacy of the phage at infecting *K. pneumoniae* in both planktonic and biofilm cultures was tested against one 'high' and one 'low' biofilm producer from each serotype, KL 106 and KL 107.

In the planktonic assay all phage infected and caused a slowing of growth in at least one host, but KP 5 infected all hosts and in KH 87 totally halted growth for a period. Though all phage could infect at least one host, their effect was not significant, if they effected at all then it would mainly be a slowing of the growth and a slight, but significant, drop in the final OD. None were able to completely kill the host or extremely hamper its growth. KP 21 was the least effective phage as it could only slow the growth of one host, KH 87, and had no significant effect on the overall growth of any host including KH 87. KP 62 was of interest as three out of the four strains it infected ended growing to a higher OD than the control, suggesting gaining resistance to this phage increases the fitness of the host. No correlation could be drawn between the 'high' and 'low' biofilm-forming isolates and their ability to defend against phage infection. Equally, there did not appear to be a difference between the

KL 106 and KL 107 groups, both seeming to be equally capable at defence against phage infection.

The biofilm assay showed that only KH 87's ability to form a biofilm was decreased 24h after phage addition and, all phage except KP 21 reduced its ability to grow significantly with KP 5 being the most drastic. This correlates with planktonic assay results, in which KP 5 was also the most successful. The lack of effect on the other hosts growth could be due to 24h being too long a time scale and the bacteria being able to be infected, mutate and regrow in the time before measurement, or it could be due to the phage being isolated on different hosts not from ST258, so their depolymerases, which are very specific, not being able to degrade the biofilm matrices and allow access to the bacterial cells within.

Overall, this study showed that KP 5 has the best potential as a therapeutic agent against *K. pneumoniae* of MLST 258, though phage isolated against these strains may be more effective than those tested.

5.1 Future Work

One of the aims of this study was the expression of the seven putative depolymerase genes from the six *K. pneumoniae* phage, this was not achieved due to time constraints so this is obviously the first piece of future work needing to be completed. As mentioned above these proteins would then have to be characterised and their host range compared to that of the whole phage they were isolated from. Then the depolymerases ability to reduce cell

growth/kill, its effect on biofilm formation and its ability to strip away the cell's polysaccharides would need to be tested and compared to the whole virion.

Another piece of work that would be key is to do the same study but with phage who were originally isolated on the ST258 strains, so we know that they are within their host range and that there are specific to this clinically prevalent strain of *K. pneumoniae.*

If the planktonic assay was to be repeated it would be useful to work out the MOI of the phage so that the results from it are comparable to other studies, and then test at differing MOI to see its effect. As mentioned above, it would also be interesting to do the same planktonic assay in a nutrient-limited environment to see if changing this factor would effect the growth of the resistant mutants that arise after phage addition, as the effect of resistance on host fitness is greater when in a nutrient-limited environment (Sanchez, 2011). Further to this, it would the also be of interest to test the resistant mutants that arose by redoing the assay with them, once with the same initial phage to see if they are totally resistant now and then again with the other phage to see if gaining resistance to one reduces their defences against the others. More work on KP 62 would be interesting to see if the reason behind why three out of four strains it infected ended growing to a higher final OD than their control could be discovered.

An improvement to the biofilm assay would be judging the effect of the phage against the biofilm at multiple time periods, not just 24h, for example after every two hours for 24h. This would be appropriate as the planktonic assay showed the eclipse time for these phages' to be roughly two hours and would allow greater insight into the effect on biofilm formation.

Another piece of future work would be the use of phage cocktails, which has been shown to reduce resistance to phage and that simultaneous exposure to multiple phage significantly weakens resistance to this treatment (Wright et al., 2019). Phage could also be used in combination with antibiotics, and not only their effect on increasing efficacy of treatment but also the level of antibiotic resistance gained could be tested.

Finally testing *in vivo* in animals, such as mice, would allow for the therapeutic applications of these phage to be judged properly in respect to use for humans in a clinical setting. This would only be done if the phage showed to be promising in the other work detailed above.

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