Burkholderia cenocepacia requires complete lipopolysaccharide to resist bacterial killing agents

by

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Abstract

Antibiotic-resistant infections are a growing concern, if this issue is not addressed by the year 2050 these infections will cause ~10 million deaths per year. Traditional antibiotics targeting essential life processes have demonstrated a rapid rate of resistance development. Modern antibiotics should therefore focus on targeting non-essential processes to prevent or at least slow the development of resistance. For this reason, many researchers have placed emphasis on identifying drugs which target virulence processes of antibiotic-resistant bacteria. One process of interest is innate immune resistance as it is the first line of defense against invading pathogens. This study used human serum to simulate innate immune defense including antimicrobial peptides and the complement system. Bacteria have evolved a variety of serum-resistance factors which can be used as novel antibiotic targets. *Burkholderia cenocecpacia* is an extremely multi-drug resistant (MDR) opportunistic pathogen causing severe and lethal infections in patients with cystic fibrosis (CF). This bacterial species is also capable of producing highly fatal bloodstream infections. Many virulence factors have been characterized for *B. cenocepacia* yet research on complement-resistance is limited. The purpose of this thesis was to identify serum-resistance factors of *B. cenocepacia* and potential antibacterials with mechanisms of action involving these factors. Following a mutant library screen, a variety of serum-resistance factors were identified but genes relating to lipopolysaccharide (LPS) biogenesis were recovered with the highest frequency. The inner core portion of the LPS had previously been characterized as playing a role in complement-resistance of *B. cenocepacia*. However, this study confirmed that the entirety of the LPS is required for survival in the presence of serum *in vitro* and *in vivo.* It was also found that complete LPS is necessary for *B. cenocepacia* to resist the antibiotic colistin. Bacteriophages, or phages, are viruses of bacteria which have long been known to be capable of

killing MDR bacteria. There has been a renewed interest in phages as a potential solution to the antibiotic resistance crisis. LPS is a common receptor for phages and following screening of a library of phages four were found to bind the LPS to initiate bacterial infection. Isolation and characterization of phage-resistant mutants revealed that they had truncated LPS and were sensitive to colistin. Combinations of colistin and LPS-binding phages were found to exhibit synergistic killing effects against *B. cenocepacia* likely through a phage steering mechanism. Altogether, this study associated several genes and their respective known/putative functions with *B. cenocepacia's* ability to resist bacterial killing agents in serum. Additionally, a novel treatment combination targeting a serum-resistance factor was identified which has the potential to prevent *B. cenocepacia* from causing sepsis.

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List of Symbols and Abbreviations

Symbols

σ: Standard Deviation

:: Interruption of the genetic locus by insertion

Abbreviations

(A_{575}) Absorbance at 575 nm

(Ara4N) 4-amino-4-deoxyl-L-arabinose

(BLAST) Basic Local Alignment Search Tool

(Bcc) *Burkholderia cepacia* Complex

(C4BP) C4-binding Protein

(CDC) Center for Disease Control

(CF) Cystic Fibrosis

(CFTR) Cystic Fibrosis Transmembrane Conductance Regulator

(CFU) Colony Forming Units

(DNA) Deoxyribonucleic Acid

(HGT) Horizontal Gene Transfer

(HHK) Hybrid Histidine Kinase

(LB) Luria Brittani

(LD_{50}) Lethal Dose for 50% of population

(LPS) Lipopolysaccharide

(MAC) Membrane Attack Complex

(MASP) MBL-associated Serine Protease

(MBL) Mannose Binding Lectin

(MCF) Microcentrifuge

(MDR) Multi-drug Resistant

(MIC50) Minimum Inhibitory Concentration where bacterial growth is reduced by 50%

(Mla) Maintaining lipid asymmetry

(MOI) Multiplicity of Infection

(MTT) 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide

(NCBI) National Center for Biotechnology Information

(NHS) Normal Human Serum

(OD_{600}) Optical Density at 600 nm

(PAS) Phage-antibiotic Synergy

(PCR) Polymerase Chain Reaction

(PFU) Plaque Forming Unit

(SEM) Standard Error of the Mean

(SM) Suspension Media

(Tet) Tetracycline

(Tn) Transposon

(Tp) Trimethoprim

(WT) Wildtype

(2CS) Two-component System

Chapter 1 General Introduction

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The Antibiotic Resistance Crisis

Antibiotic resistance is one of the largest threats the human population will face soon. It has also been labelled one of the biggest clinical, public health and scientific challenges that must be overcome [1,2]. Infectious disease is the second leading cause of death in the world with around 17 million people dying each year [3,4]. In the United States alone the most recent report from the Center for Disease Control and Prevention (CDC) indicated that per year three million people are infected with antibiotic-resistant bacteria and of those 35,000 will die [5]. This is an increase from the report preceding listing two million infections per year and that at least 23,000 individuals had died from antibiotic-resistant infections [6]. Antibiotic resistance has been and remains to be a growing concern for which no significant solutions have been implemented.

The antibiotic resistance era began over 20 years ago and few new drugs with novel mechanisms of action have been developed and brought to market [7]. This is the result of many factors however the most notable is that pharmaceutical companies have halted or significantly reduced research and development efforts to identify new antimicrobials [2]. This is likely due to the predicted failure rate of antimicrobial development being 95% [2]. One reason for this is that up until 1996 80% of clinically used drugs were either natural or natural-inspired compounds making it extremely difficult to isolate not only novel natural products but, ones that also exhibit microbial killing activity with little to no deleterious effects on human cells [3]. Some companies and academic research groups have screened robust synthetic chemical libraries returning meager promising antibiotic compounds [3,8]. Additionally, the increased cost and time to bring a new drug to market has likely contributed to pharmaceutical companies decreased interest in developing novel antimicrobials [3]. The estimated cost to bring a drug to market in 2017 was $1.2 billion USD taking anywhere between 14-22 years [3]. This is a huge monetary and time

investment companies must make into a drug that will be used short term for which resistance will likely develop quickly after clinical application. Therefore, many companies have shifted their focus to development or improvement of drugs used to treat chronic diseases and illnesses with higher investment returns. This leaves the responsibility of identifying novel antibiotics largely up to academic researchers with fewer available resources.

Unfortunately, bacteria have a leg up in the arms race to develop novel antibiotics as resistance is ancient pre-dating human use of antibiotics to treat infections [9]. Resistance genes have been identified in bacterial genomes isolated from permafrost and enclosed caves which were untouched by humans [10,11]. Interestingly, these genes also share high sequence homology with modern-day bacterial resistance genes suggesting there has been little to no evolution of these ancient genes [9]. However, with the development of novel synthetic antibiotics not found in nature bacteria have proven they are capable of developing resistance shortly after the antibiotics first use [12]. Resistance is also capable of spreading to neighboring bacteria through horizontal gene transfer (HGT), which is largely responsible for the widespread of antibiotic resistance seen today [7,9,13–19]. As a result, many commensal and pathogenic bacteria now possess the molecular machinery to resist multiple chemical structure classes of antibiotics [7,18].

Molecular Mechanisms of Antibiotic Resistance

In order to address the issue of antibiotic resistance, one must first understand the molecular mechanisms behind this phenomenon. Although antibiotic-resistant bacterial infections may be difficult to treat with current therapeutics, often requiring the long-term application of several complementary frontline antibiotics, the real issue is developing antibiotics for which resistance does not yet exist. There are multiple instances where pan-resistant Gram-negative bacteria have

been clinically isolated making them a growing concern [3]. The United States CDC has declared MDR Gram-negative pathogens as the most substantial threat to humanity during the antibiotic-resistance era [5]. There has been a significant increase in Gram-negative bacterial infections worldwide in the past several decades, and the majority of hospital-acquired or nosocomial infections are now caused by Gram-negative bacteria [20,21].

There are multiple mechanisms Gram-negative bacteria can use to resist different classes of antibiotics which fall into two main categories: intrinsic and extrinsic. Extrinsic mechanisms of antibiotic resistance were acquired by the bacterium at some point during its evolution either through spontaneous mutation or HGT [9,22–24]. Intrinsic resistance occurs when the bacteria can resist the antibiotic without acquiring genetic modifications. Extrinsic mechanisms of resistance include: antibiotic target modification, overproduction of the antibiotic target, modification, inactivation, or degradation of the antibiotic via enzymes and lastly acquiring new metabolic pathways that were previously inhibited by the antibiotic [13]. Extrinsic resistance mechanisms are generally more specific providing resistance to a handful of antibiotics. On the contrary intrinsic resistance typically provides resistance to multiple chemical classes of antibiotics.

Intrinsic antibiotic resistance is more commonly associated with Gram-negative bacteria due to their impermeable cell envelope [7,9,16,18]. Experts hypothesize that the Gram-negative cell envelope evolved in response to the selective pressure of antibiotics [25]. Together, the inner phospholipid bilayer membrane, the thin layer of cross-linked peptidoglycan, and the asymmetrical outer membrane significantly decreases membrane permeability [7,18]. The tight packing of lipid A molecules in the LPS layer of the outer membrane is largely responsible for the impenetrability of these cells [16–19,26]. Many Gram-negative bacteria also modify the chemical structure of the LPS providing increased resistance to certain antibiotics, mainly antimicrobial

peptides[27]. Additionally, Gram-negative bacteria utilize a variety of efflux pumps spanning either the entire, or portions of the cell envelope to remove various substrates from the cytosol and periplasm. Efflux pumps typically exhibit substrate promiscuity and can efflux a diverse assortment of compounds including antibiotics [7,16–18,22,28,29]. Furthermore, efflux pumps are highly redundant where some Gram-negative bacterial genomes encode as many as 16 different systems which overlap in substrate specificity [30,31]. Efflux pumps also play a role in transporting quorum sensing autoinducers which can help coordinate the bacterial population to better respond to antibiotic challenge [28,29,32]. In combination the relatively impermeable cell envelope structure, plus the variety of efflux pumps extruding a wide range of chemical compounds, account for most of the intrinsic resistance in Gram-negative bacteria.

Multiple mutagenesis studies have been conducted on a variety of Gram-negative bacterial species to identify their complete intrinsic resistome. These studies identified genes canonically involved in antibiotic resistance however, they also revealed a variety of hypothetical proteins with no predicted function or conserved domains previously known to be involved in resistance [33–37]. Additionally, some studies identified many intergenic regions required for intrinsic resistance [35–37]. Lastly, these studies associated many of these genes with resistance to multiple different chemical classes of antibiotics suggesting general mechanisms of resistance [35,37]. This indicates that there are many unknown and/or uncharacterized intrinsic resistance factors which contributes to the complexity of designing effective novel antibiotics against Gram-negative bacteria.

Another mechanism of both intrinsic and extrinsic antibiotic resistance is biofilm formation. Biofilms are complex microbial communities surrounded by an exopolysaccharide-based matrix which are irreversibly attached to either an abiotic or living surface [38–40]. Bacteria

in biofilms are more resistant, or tolerant, to antibiotics than cells growing in a planktonic state [4,38–41]. Biofilms are composed of heterogenous subpopulations of bacteria with different phenotypes and genotypes [40]. Heterogeneity of the subpopulations stems from the numerous distinct microenvironments that exist in a biofilm which have differing levels of nutrients, oxygen and pH all influencing gene expression and metabolic activity [38–40]. These microenvironments can affect gene expression by increasing expression of efflux pumps, antibiotic degradation enzymes and stress-related genes contributing to increased resistance [4,38–41]. Also, subpopulations growing in a nutrient-limited microenvironment will exhibit different metabolic activity which can decrease the effectiveness of certain antibiotics [38–40]. Further, antibiotics do not penetrate or diffuse through biofilms efficiently decreasing interaction with their antibiotic target [38,39]. Lastly, biofilms also act as a reservoir from which resistance can be acquired due to increased HGT and mutation of antibiotic targets [37–40]. Altogether the complex bacterial structure and diversity of biofilms culminate resulting in increased antibiotic resistance.

Gram-negative bacteria inhabiting environmental niches demonstrate an extraordinary ability to resist antibiotics. Some environmental bacteria are also opportunistic pathogens capable of infecting immunocompromised individuals. Opportunistic pathogens are of great concern as they typically possess many intrinsic and extrinsic antibiotic-resistance factors [42–44]. These bacteria are generally ubiquitous in the environment and can survive disparate conditions [45]. As a consequence environmental Gram-negative bacteria, especially those which inhabit soil, possess a large abundance of diverse antibiotic resistance mechanisms [9,15,46]. Further pathogenic bacteria have an increased likelihood of acquiring novel resistance factors due to the selective pressure imposed by human use of antibiotics [9,15,46]. Therefore, opportunistic pathogens which are adapted to both environmental and clinical habitats have been heavily selected to resist many antibiotics.

Burkholderia cenocepacia

B. cenocepacia is part of the *Burkholderia cepacia* complex (Bcc) which is a group of at least 20 phenotypically similar species which are ubiquitous in the environment and can cause lethal infections in immunocompromised individuals [47,48]. Opportunistic pathogens of this group typically infect the lungs of patients with CF or chronic granulomatous disease [49,50]. CF is a systemic disease caused by various mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene [51–54]. However, abnormalities in the lung are the major cause of morbidity and mortality due to excessive inflammation and highly viscous secretions from airway epithelial cells [54,55]. Additionally, mutations in the CFTR gene negatively affect innate and adaptive immune defenses rendering these patients immunocompromised [56]. The combination of these symptoms makes these patients highly susceptible to lung infections and *B. cenocepacia* is one of the main causative agents of such infections [44,57].

B. cenocepacia is also able to inhabit a diverse array of environments. These bacteria have been isolated from water, soil and various plant species [58–60]. This species is also known to colonize medical devices and solutions, including disinfectants [50]. These are common sources of *B. cenocepacia* outbreaks however infections may also be acquired from environmental niches [58]. Further, *B. cenocepacia* is highly contagious and can be transmitted from other colonized individuals with or without CF causing implementation of various strict infection control protocols for CF patients [61]. The capacity of this species to survive and move between distinct habitats is a testament to its versatility and ability to respond to various selective pressures.

B. cenocepacia encodes a relatively large, functionally diverse genome of ~7Mb composed of two to three chromosomes and sometimes endogenous plasmids, depending on the

strain [45]. There are multiple genomic islands and insertion sequences throughout all three chromosomes thought to play a role in this species high genomic plasticity [45]. Further, investigation into these genomic islands revealed a pathogenicity island on chromosome two with genes responsible for a variety of virulence phenotypes [45,62]. It was also discovered that the entire third chromosome in *B. cenocepacia* of the ET-12 outbreak lineage was dispensable as a virulence plasmid [63]. Loss of this chromosome resulted in virulence attenuation in animal infection models ranging from *Galleria mellonella* to rats [63]. Of particular importance *B. cenocepacia* dedicates ~7% of its large genome to the production of antibiotics therefore it must also encode the necessary resistance factors [64].

B. cenocepacia infections are incredibly difficult, and sometimes even impossible, to treat mainly due to MDR provided by a multitude of mechanisms (Figure 1.1). This bacterium uses numerous antibiotic modifying enzymes to resist certain chemical classes of antibiotics. First, *B. cenocepacia* has two distinct periplasmic β-lactamases which degrade these antibiotics [44]. Additionally, there is evidence to suggest the presence of aminoglycoside modifying enzymes encoded in the genome [65,66]. *B. cenocepacia* has also acquired mutations in DNA gyrase and dihydrofolate reductase genes to resist fluroquinolones and trimethoprim, respectively [42]. These specific mechanisms provide resistance to at least four antibiotic classes.

The most prominent resistance factor used by *B. cenocepacia* is an impenetrable cell envelope. This is largely derived from LPS which is modified with 4-amino-4-deoxyl-L-arabinose (Ara4N) coating the bacterial surface in a positive charge decreasing electrostatic attraction of positively charged antimicrobial peptides [67–70]. Interestingly, the gene cluster responsible for adding Ara4N to the LPS has been characterized as essential for LPS export to the outer membrane and cell viability [68,70]. Additionally, the maintaining lipid asymmetry (Mla)

pathway has been identified as essential to intrinsic resistance to a variety of antibiotics for *B. cenocepacia* [71]. The Mla pathway is known to maintain outer membrane lipid asymmetry in Gram-negative bacteria and some genes are dispensable for growth under lab conditions with no antibiotic present [71,72]. Disruptions to this pathway render *B. cenocepacia* sensitive to many antibiotics including those effective against Gram-positive bacteria [71]. Lastly, *B. cenocepacia* encodes 16 different resistance nodulation division (RND) class efflux pump open reading frames which many are known to play a significant role in resisting at least five different classes of antibiotics [30,31,42,44]. Efflux pumps have also been characterized as being involved in miscellaneous virulence phenotypes [28,29,32,73].

As previously alluded to *B. cenocepacia* encodes and utilizes a variety of virulence factors to cause infection (Figure 1.1). Most importantly *B. cenocepacia* has two distinct and functional quorum sensing systems which control expression of almost all virulence factors and is necessary for the bacterium to form good biofilms and establish infections in the CF lung environment [74–77]. Additionally, *B. cenocepacia* mounts an abnormally large inflammatory response which is primarily associated with the lipid A and O-antigen portions of the LPS and the many secreted zinc metalloproteases [67,78]. *B. cenocepacia* is also able to inhabit host epithelial cells using both cable pili and flagella to attach and invade cells, respectively [67]. *B. cenocepacia* uses Type III, IV and VI secretion systems in addition to zinc metalloproteases to survive within host cells [67,78–82]. Metalloproteases are also used to degrade multiple host defense proteins including host produced antimicrobial peptides [67,83–85]. Expression of these virulence factors and MDR allows *B. cenocepacia* to cause life-threatening infections in patients with CF.

Cepacia Syndrome

In addition to its canonical infections *B. cenocepacia* can cause a unique and fatal infection called Cepacia Syndrome [45,86]. The first report of cepacia syndrome was in 1984 where clinicians reported symptoms of high fever, significant impairment of lung function and a high fatality rate [87]. It is now better characterized as the combination of necrotizing pneumonia and sepsis leading to rapid decline of health and causing death in ~75% of cases [45,86]. Cepacia syndrome is a growing concern where in 2015 it was reported that ~20% of Bcc infections in patients with CF progress to cepacia syndrome [88]. Additionally, there have now been multiple documented cases of cepacia syndrome in individuals without CF and one case has been reported in an immunocompetent patient [86,89,90]. Some case studies have reported successful treatment of the infection using a combination of steroids and aggressive antibiotic treatment courses with extremely negative side effects [91–93].

Limited research has been conducted on what phenotypic differences exist between non-cepacia syndrome vs cepacia syndrome causing isolates of *B. cenocepacia*. One transcriptomic study compared isolates retrieved from the bloodstream and sputum of a patient experiencing cepacia syndrome [88]. The authors identified gene expression differences in canonical virulence factors such as quorum sensing, exopolysaccharide production, Type III secretion and decreased flagellar gene expression [88]. Another study compared the first *B. cenocepacia* isolate to a later one causing cepacia syndrome identifying upregulation of efflux pumps, likely due to aggressive antibiotic treatment, iron uptake and adhesion genes [94]. One thing however, which has been largely overlooked in *B. cenocepacia* virulence research is investigation into how these bacteria are able to evade innate immune defenses highly concentrated in the bloodstream, such as complement, to cause cepacia syndrome infections. Of particular interest is how this species is

adept at escaping the complement system, which is said to be present in negligible amounts in the CF lung [95].

The Complement System

The complement system, also termed the complement cascade, is a component of the innate, humoral immune system that plays a variety of roles in maintaining homeostasis [96]. The complement system is the first line of bodily defense against pathogenic invaders, including viruses, bacteria, fungi, and parasites including protozoa, helminths, and ectoparasites. The complement system achieves this by bridging the gap between innate and adaptive immunity [97]. The complement system comprises 60 different effectors, mostly proteins, that are directed towards tagging and destroying pathogens [98]. However, the complement system by itself is only capable of directly killing certain classes of pathogens, including Gram-negative bacteria. Due to their outer membrane and thin layer of peptidoglycan, Gram-negative bacteria are the major class of pathogens predominantly susceptible to lysis by the process of complement-mediated killing [99].

Complement can recognize a pathogen using one of three pathways, the alternative, classical and/or lectin pathways, that each lead to a common terminal cascade. Figure 1.2 outlines how these first three pathways identify and tag a pathogen for killing by the terminal pathway, which in turn make up the complement cascade. The alternative, or surveillance, pathway, is constitutively active and does not identify any specific bacterial surface structure, but instead, active C3b will bind to –OH or –NH2 groups on the amino acids of bacterial surface proteins [96]. The classical pathway uses C1q to initiate the cascade by binding to either LPS, bacterial outer membrane proteins or an antibody bound to a bacterium [96,100]. C1q will then

recruit serine proteases C1s and C1r to the bacterial surface which will in turn recruit C3 & a C3 convertase [99]. Lastly, the lectin pathway uses mannose binding lectin (MBL) to recognize specific molecular patterns on the surfaces of pathogens leading to the formation of the MBL-associated serine protease (MASP) complexes [97,99]. The MASP complex will then recruit C3 & a C3 convertase as in the classical pathway [99].

Once a pathogen has been recognized by one of the three pathways, the complement cascade converges at the cleavage of C3 protein into its active constituents, C3a and C3b, using a C3 convertase [96]. The alternative pathway uses the C3Bb convertase generated from either the Factor B or Factor D serine proteases [96]. The classical and lectin pathways utilize the C4b2a C3 convertase generated from the cleavage products of C4 and C2, recruited by each of the pathways respective serine proteases [96,99]. Once the active C3b molecule is generated and deposited onto the bacterial surface, the terminal pathway will be initiated.

Once a Gram-negative bacterium has been opsonized by multiple C3b molecules, the C3 convertases exhibit decreased specificity and begin to cleave C5 into C5a and C5b [96,99]. C5b then serves as a molecular scaffold for the construction of the membrane attack complex (MAC) [101]. C5b is an unstable molecule until bound by C6 creating the stable, soluble C5b-6 complex [101]. Next, C7 is added rendering the complex lipophilic, which is required for its insertion into the amphipathic outer membrane [101]. Then, C8 is added to form the C5b-8 complex, which is inserted into the outer membrane [101]. Lastly, multiple copies of the C9 protein are added to form a ~10 nm pore in the outer membrane resulting in cell lysis [100,101].

Although insertion of the MAC results in cell death, the molecular mechanism by which this occurs has yet to be experimentally confirmed. The most accepted theories suggest that insertion of MAC into the outer membrane causes a decrease in membrane potential and a large

influx of water which results in lysis [100]. Many experts argue that insertion of the MAC into the outer membrane alone would not be sufficient to cause cell lysis and suggest that the MAC actually spans across the outer membrane, peptidoglycan layer and the inner membrane [100]. Others argue that the initial insertion of the MAC into the outer membrane allows for further MACs to assemble in the inner membrane [100]. Additionally, some suggest that insertion of the MAC into the outer membrane is detected by the bacterial cell and causes initiation of an apoptosis-like response leading to cell destruction [102].

Although the main objective of the complement system is to defend against pathogenic invaders, the response may also indirectly result in host cell damage. This damage is caused by the generation of anaphlyatoxins C3a and C5a from cleavage of C3 and C5, respectively. When present in high enough concentrations, these molecules initiate an inflammatory response that leads to the generation of free radicals that damage cells non-specifically [99,103]. Bloodstream infections are particularly dangerous, as the complement system mounts a large response towards the bacterial invader thus generating large amounts of C3a & C5a [103]. When present in the blood these inflammatory molecules will disseminate rapidly throughout the body and can cause systemic organ failure, as seen in cepacia syndrome [103]. However, production of C3a has also been demonstrated to have additional antibacterial effects [100,104]. First, it was observed that C3a was structurally homologous to other known antimicrobial peptides, and subsequent experiments concluded that C3a was able to kill both Gram-positive and Gram-negative bacteria [104]. It is likely that generation of C3a in small to moderate amounts would be beneficial in helping the host protect itself from bacterial pathogens.

Because complement can cause life-threatening reactions, the system must be carefully monitored and regulated to keep the cascade directed towards the proper cells. Since complement

is a completely humoral system, it requires numerous regulatory proteins to differentiate healthy host cells from invading pathogens [99]. There are three general classes of complement-regulator molecules: fluid phase, host surface attached, and integral membrane complement clearance receptors which are mostly proteins [105]. Surface attached and integral membrane regulators protect against attack from all three complement pathways and are for the most part unable to be recruited by bacteria to protect themselves against complement [105]. In contrast, individual fluid phase regulators are specific and provide protection against one or two of the complement pathways and are easily recruited to the outer membrane of Gram-negative bacterial pathogens to disguise themselves as host cells [105].

Bacterial Complement-Resistance Mechanisms

Since most invading bacteria will meet the complement system, they have evolved multiple mechanisms to avoid complement-mediated killing which are summarized in Figure 1.3. All bacteria must be able to evade the alternative or surveillance pathway due to its constitutive activity. However, most bacteria will also be under attack from either or both the classical and/or lectin pathways following two to three days of infection [106]. There are also examples of pathogens who do not mount any defenses towards the lectin and/or classical pathways as there may not be any response amounted to them in this fashion [107,108]. There are three commonly used ways with which Gram-negative bacteria resist the complement cascade interfering at many different stages of the cascade between tagging of the bacterium and insertion of the MAC. The most common mechanism to evade complement killing is binding a host produced fluid-phase regulator. The most commonly acquired fluid-phase regulators are Factor H or Factor H-Like proteins as they are the most abundant regulators of complement [107,109–114]. Next, many bacteria also express complement-degrading proteins typically in the form

of metalloproteases or serine proteases which have been shown to cleave complement components into inactive forms [115–118]. Another mechanism mostly used by Gram-negative bacteria is physical hinderance imposed by the LPS which prevents the formation and/or insertion of complement components, specifically MAC in the outer membrane [119–122]. The inner core of the LPS so far is the only confirmed complement-resistance factor of *B. cenocepacia* [123,124].

Phage Therapy & Current Strategies

Bacteriophages, or phages, are viruses of bacteria for which there is a renewed interest in their therapeutic application against MDR bacteria. Phages undergoing the lytic life cycle will bind to specific bacterial cell surface structures, inject their DNA to be transcribed and translated into phage proteins which will then be assembled into complete phage particles [125–127]. In contrast, lysogenic phages can integrate their DNA into the bacterial host genome or circularize it to be replicated alongside the bacterial DNA [127]. Lysogenic phages will enter the lytic life cycle upon sensing bacterial stress, typically DNA damage [128]. Phages are being heavily investigated as potential novel therapeutics due to their ability to lyse bacteria through a different mechanism than traditional antibiotics. However, there are multiple scientific and regulatory facets which need to be considered before they can be applied for therapy.

There are several benefits to phage therapy which make it more appealing than traditional antibiotic treatment. The first is that phages can kill MDR bacteria using a different mechanism than traditional antibiotics. Most phages are highly specific and are only capable of infecting one or two bacterial species [125–127]. Therefore, phages delivered against pathogenic bacteria will have little to no effect on beneficial or commensal bacteria in the microbiome which has been shown to be important for overall health [129,130]. Although high specificity of phages is beneficial for

maintaining a “healthy” microbiome it can also be disadvantageous as it requires isolation and characterization of new phages for each bacterial species and even for specific strains. Further, there have been multiple case studies where phage therapy was applied to treat pan-resistant infections which were successful in clearing the bacteria [131–134]. Whilst there are many advantages to phage therapy there are also a myriad of diverse disadvantages and complications to overcome prior to their widespread use.

Phages must be carefully characterized prior to their use in therapy as they are well-known to act as reservoirs for virulence and antibiotic resistance genes [135–138]. Bacteria which are lysogenized by phages carrying such genes can become more virulent or resistant making these phages incompatible for therapy. These genes, in addition to others in the host genome, can be transferred via transduction through phages to new bacteria contributing to increased virulence and antibiotic resistance [135,139]. Specifically, there are two documented instances of a phage carrying genes related to complement-resistance [140,141]. For these reasons phage must be well characterized prior to being used in a clinical setting.

Recently, there has been an explosion of research characterizing interactions between phages and eukaryotic cells. It was previously thought that due to the high specificity of phages they could not have a significant effect on eukaryotic cells and processes [142]. Many beneficial and deleterious interactions between eukaryotic cells and phages have recently been confirmed [143]. Phages are now known to induce an immune response and phage-specific antibodies have been identified following administration of phage-based products [144]. These antibodies are in part responsible for clearance of phages from the body in addition to other immune system components [143,144]. There is mixed evidence on the half-life of phages within the human body where some cite it as being a few hours while other have shown stability for days [143,145]. It

appears the dosage and route of administration play the most significant roles in phage stability within the human body [143]. Phages administered through inhalation into the lung demonstrate relatively long term stability with activity up to 72h post-administration [145]. Multiple studies have shown inhalation of phages is an effective treatment to clear *B. cenocepacia* in mouse models indicating that phage stability is not an issue [146,147]. These studies also demonstrated that the phages used do not cause any detectable negative side effects on the animals short-term health [146,147]. Nevertheless, the potential of phages to interact with and potentially cause deleterious effects on eukaryotic cells should be considered for phage therapy.

The cardinal disadvantage of single phage therapy is that resistance develops to phages just as quickly as it develops toward traditional antibiotics [148]. Bacteria typically develop resistance to phage through receptor alteration although other mechanisms exist as well [125]. This can be mitigated by using multi-phage cocktails targeting different receptors [125,133,149–152]. This requires isolation and characterization of multiple phages active against a single species or strain. These cocktails require the same level of investigation as the phages within them in addition to optimization the dosage of each phage. Phage cocktails can also help mitigate the high specificity of phage therapy by increasing the host range of a single product. However, phage cocktails can have unpredictable pharmacokinetics derived from their ability to replicate at the site infection [153]. Although phage cocktails can increase efficacy of phage therapy it can be inconsistent and difficult to fully characterize.

A newer strategy to circumvent phage resistance is phage steering. This approach can be used when a phage utilizes a receptor involved in virulence and/or antibiotic resistance, this strategy may also be called anti-virulence [154,155]. The pioneering paper for phage steering described a phage which uses an efflux pump as a receptor which when phage-resistant mutants

arose this efflux pump was either lost or no longer functional rendering the bacteria sensitive to multiple antibiotics which it was previously resistant to [156]. Combining phages and antibiotics to increase killing efficiency so that it's greater than the additive killing effect of either treatment alone can also be called phage-antibiotic synergy (PAS) [149,150,157]. PAS can result from a wide range of mechanisms which should be characterized prior to their use in therapy. Both treatment approaches impose a decreased selection pressure on the bacteria as it is not targeting essential functions like traditional antibiotics decreasing the likelihood of resistance developing and/or prevent resistance from spreading [158–160].

Thesis Objectives

Objective 1: Identify genes related to serum-resistance in *B. cenocepacia* by constructing and screening a mutant library.

Objective 2: Investigate genes recovered from the library screen for potential exploitation as therapeutic targets for novel antibacterials, focusing on phage therapy, against *B. cenocepacia* using the anti-virulence strategy.

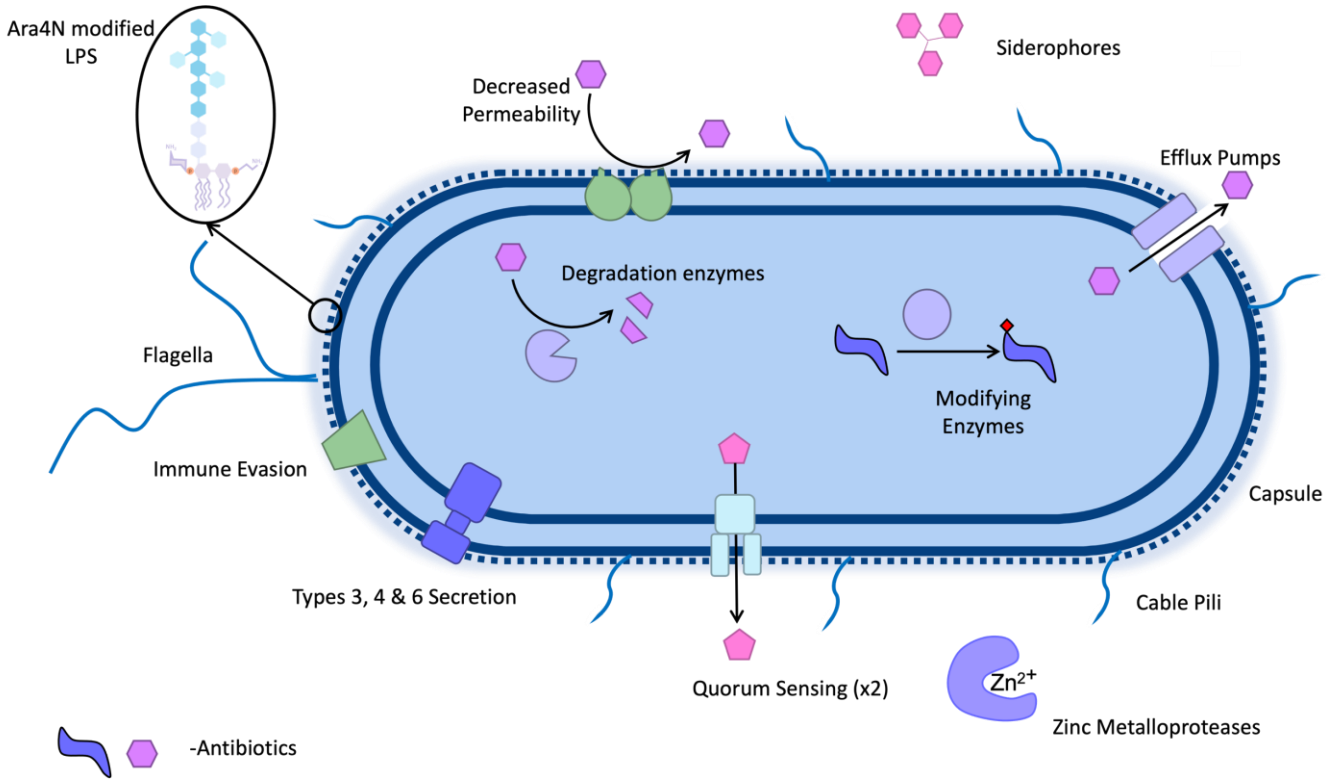

Figure 1.1 Antibiotic resistance and virulence factors of *B. cenocepacia*.

Antibiotic resistance factors include efflux pumps, antibiotic modifying or degradation enzymes, decreased permeability provided by Ara4N modified LPS and other outer membrane proteins such as porins. Virulence factors which help *B. cenocepacia* cause disease comprise multiple secretion systems (Types III, IV and VI), redundant quorum sensing systems, zinc metalloproteases, motility, and adhesion structures such as flagella and cable pili, protective capsule, iron acquiring siderophores, LPS and lastly uncharacterized immune evasion structures.

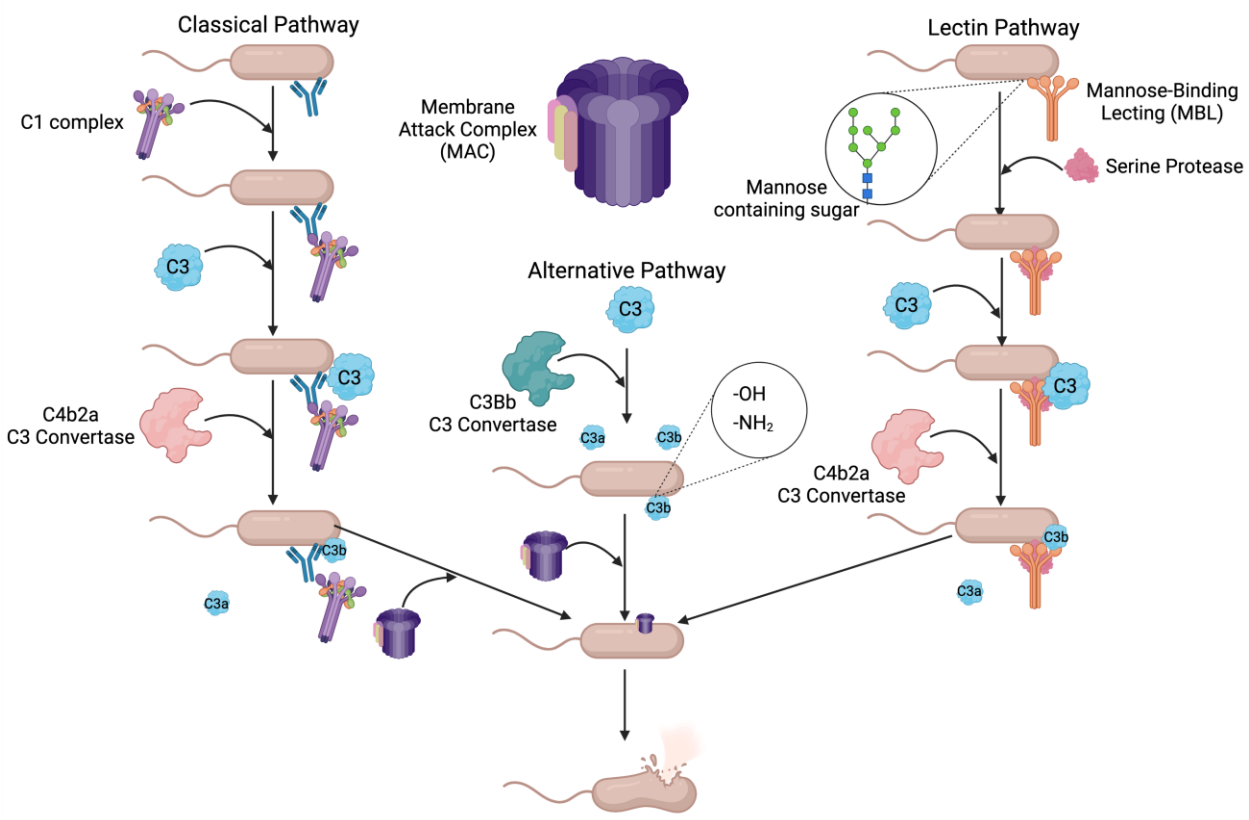

Figure 1.2 Overview of complement cascade in relation to cell lysis of Gram-negative bacteria.

Complement uses three different pathways to recognize bacterial pathogens: classical, alternative and lectin. The classical pathway uses antibodies to recognize specific antigens on a bacterium. The alternative pathway is constitutively active and deposits C3b molecules on bacterial surfaces with -OH or NH_2 groups exposed. Lastly, the lectin pathway will recognize specific sugar structures on the outer surface of the bacteria. Once the bacterium has been identified and tagged by the initial components of the respective complement pathway, they all converge at the deposition of C3b on the outer membrane. Once C3b has opsonized a pathogen the terminal pathway will begin MAC assembly leading to the insertion of a pore in the outer membrane which results in cell lysis.

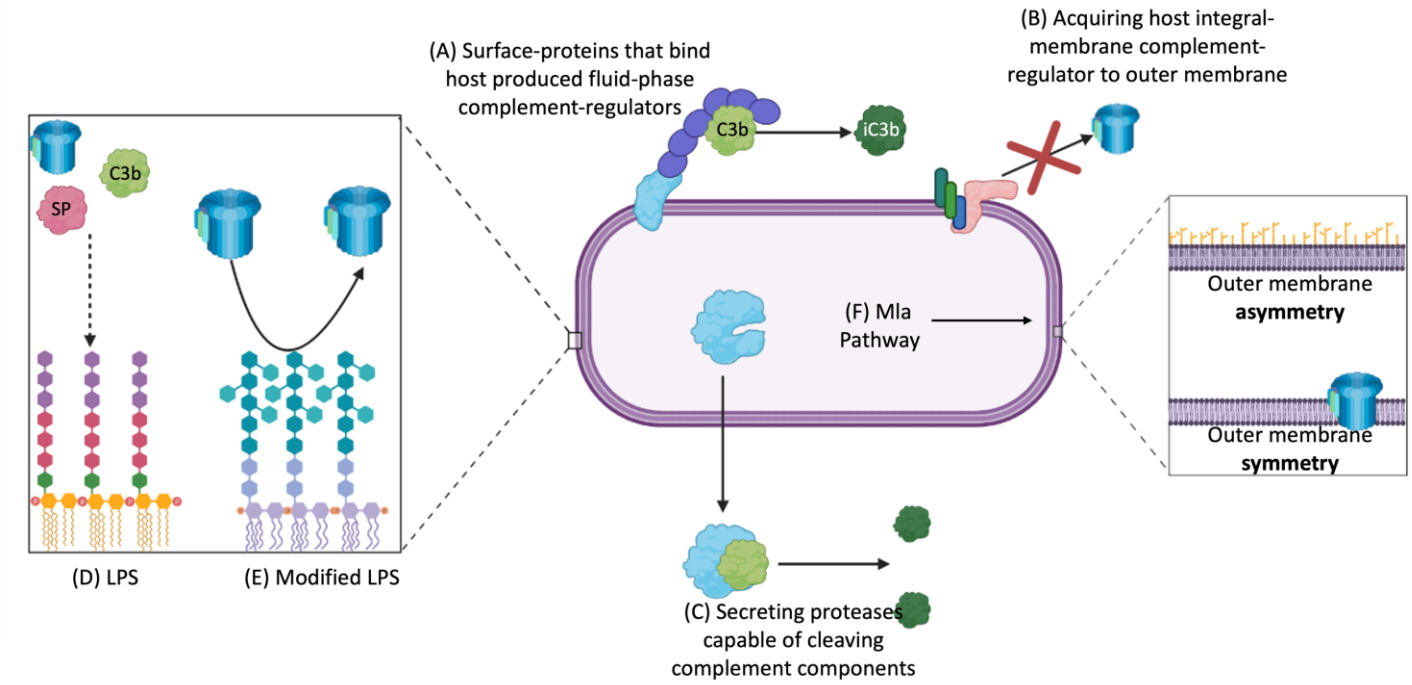

Figure 1.3 Bacterial mechanisms used to evade complement-mediated killing.

A. Production of surface-exposed proteins that can bind host-produced fluid-phase complement-regulators which typically inactivate specific complement components. B. Acquiring host integral-membrane complement-regulator to prevent MAC insertion into outer membrane. C. Secretion of proteases that can cleave complement components into inactive forms. D. LPS causes steric hinderance making it more difficult for complement components to bind or be inserted to/into outer membrane, some insertion still occurs with traditional LPS, however. E. LPS modified with additional sugars increases steric hinderance and prevents MAC insertion. F. Mla pathway is responsible for maintaining lipid asymmetry in the outer membrane, i.e., LPS can cause steric hinderance preventing MAC insertion, when Mla pathway is non-functional due to mutation or blockage in the pathway the outer membrane is composed of phospholipids only and MAC can be inserted.

Chapter 2 LPS-binding phages exhibit synergy with the antibiotic colistin via a phage steering mechanism

Methods:

Strains and Growth Conditions

Bacterial strains for this study are listed in Table 2.1. *B. cenocepacia* K56-2 and variants were routinely cultured in ½ strength Luria-Brittani (LB; 10g/L tryptone, 5g/L yeast extract, 5g/L NaCl) solid or liquid medium, at 37ºC with aeration at 225 RPM for 18h unless otherwise stated. Media was supplemented with 100-150 μg/mL of tetracycline (Tc) or trimethoprim (Tp) for plasmid maintenance or selection. All statistical analyses for this paper were conducted using GraphPad Prism 9 (Graph-Pad Software Inc., San Diego, CA, USA).

Mutant library construction and screening

A transposon (Tn) mutant library was constructed in *B. cenocepacia* K56-2 by electroporation of the pTnMod-OTp' plasposon vector [161]. Recovered transformants were plated on LB+150 μg/mL Trimethoprim (Tp) and incubated at 37ºC for 2 days. Single colonies were handpicked and inoculated into 200 μL of LB +150 μg/mL Tp in 96-well microplates. Plates were then incubated 16-18 hours at 37ºC with shaking at 225 RPM. Next, a microplate replicator was swirled in grown overnight cultures and used to inoculate 200 μL of LB, sterilized and swirled in overnights again to inoculate 200 μL of LB + 30% pooled Normal Human Serum (NHS) purchased from BioIVT with 1x 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) dye (Biolog, CAT No. 74221). Plates were incubated at 37ºC for 24h then absorbance was read at 600 nm for LB plates and 575 nm for LB+30% NHS plates in a PerkinElmer VICTOR X3 plate reader. Total growth was calculated by subtracting the pre-incubation reads from the final growth reads and data was normalized using methods previously described [162]. Mutants demonstrating growth lower than 2.5 standard deviations away from the

mean in 30% NHS and above 2.5 standard deviations in LB were chosen for further analysis. Screen hits were confirmed by repeating the same screening procedure experiment, but growth was measured using colony forming unit (CFU) counts, identifying mutants which were unable to survive in the presence of NHS.

In order to identify the mutated genes in the serum-sensitive mutants of interest, we used protocol similar to one which was previously described [161]. Briefly, genomic DNA was isolated from a single colony using a phenol-chloroform extraction, if DNA purity was low, an ethanol precipitation was performed prior to downstream steps. Next, 1 μg of DNA was digested using the PstI Fast Digest enzyme (ThermoFisher Scientific, CAT No. FD0614) for 1h at 37ºC. The restriction enzyme was removed using the QIAgen polymerase chain reaction (PCR) Purification kit. Digested DNA was ligated using T4 ligase (New England Biolabs (NEB), CAT No. M0202S) for 8hrs at 16ºC followed by heat inactivation of the enzyme at 65ºC for 10 minutes. Ligated DNA was transformed into chemically competent *Escherichia coli* DH5α cells. Recovered transformants were plated on LB+100 μg/mL Tp and incubated at 37ºC overnight. Resulting colonies were inoculated into 5mL of LB+100 μg/mL Tp and grown overnight as described above. Cultures were then subject to QIAgen Miniprep plasmid extraction kit and the resulting plasmid was sent for Sanger Sequencing at Molecular Biology Services Unit at the University of Alberta using JD47 (5'-TTTATCCTGTGGCTGC-3') and JD28 (5'-GGGGAAACGCCTGGTATC3') primers. Resulting sequence was inputted into NCBI BLASTn searching in the *B. cenocepacia* K56-2 genome to identify the mutated gene. If a gene function was not listed in the K56-2 genome, the homologous gene was found in *B. cenocepacia* J2315 genome to identify a putative function.

Phage propagation

Phages used in this study are listed in Table 2.2. Phages were propagated at 37ºC either using double agar overlays or in liquid as previously described [163]. For titering, phage stocks were serially diluted in suspension media (SM; 50mM Tris-HCl [pH 7.5], 100mM NaCl, 10mM $MgSC_4$) and spotted onto soft agar overlays of K56-2. Alternately, 100 µL of diluted phage was mixed with 100 µL of K56-2 overnight culture, incubated for 7 minutes on the bench, combined with 3 mL of ½ LB soft agar and poured onto ½ LB agar plates. For both titering methods, plates were incubated at 37ºC overnight and enumerated the next morning.

Bacterial growth curves

WT K56-2 and LPS mutant overnight cultures were standardized to an OD_{600nm} of 0.5 and then used to inoculated fresh ½ LB media (1:100) to a final volume of 200 µL in a 96-well microplate. Surrounding wells were filled with mQH_2O to prevent edge effects. The plate was incubated in an EPOCH BioTek microplate reader at 37ºC for 48hrs with shaking at 225 RPM. OD_{600nm} measurements were taken every 30 minutes. Plates were observed for biofilm growth at the end of the experiment which were taken into consideration if the data resulted in large error bars.

Serum survival

Serum survival assays were conducted as previously described [163]. Briefly, overnight cultures of WT K56-2 and LPS mutants were used to inoculate a subculture (1:100), which was grown to mid-log phase and then standardized to an OD_{600nm} of 0.1. NHS was prepared in various concentrations ranging from 0-100% diluted in ½ LB. In 96-well plates, 198 µL of ½ LB + NHS was inoculated with 2 µL of subculture. Plates were incubated for 2h at 37ºC, then a 20

μL aliquot was taken from plates, serially diluted, and spotted for CFU counts. CFU plates were incubated overnight at 37°C and enumerated the next day.

Galleria mellonella LD_{50}

G. mellonella trials were conducted as previously described with some modifications [164–166]. WT K56-2 and LPS mutants were cultured overnight and serially diluted in ½ LB to achieve final CFU's of 10^1-10^8 CFU/mL. Larvae were raised in-house at 30°C in the dark with artificial food (264g wheat germ, 132g brewer's yeast, 210g beeswax, 132g glycerol, 132g honey, 66g water). Larvae weighing ~280mg were injected using a 250 μL Hamilton syringe with a repeating dispenser with 5 μL of bacteria or 1x phosphate buffered saline as a control. Larvae were incubated at 37°C for 24h and scored for growth. Worms which exhibited movement upon touch were scored as alive and those that did not were scored as dead. The LD_{50} for each strain was recorded as the injected CFU resulting in survival of 50% of the worms in each trial. Each LD_{50} was determined in biological triplicate.

Minimum inhibitory concentration (MIC_{50}) of antibiotics

MIC50s were performed using methods previously described by Weber et al. (2020) with some modifications. Overnight cultures of WT K56-2 and LPS mutants were used to inoculate subcultures 1:100, which were standardized to an OD_{600nm} of 0.1. Antibiotics tested included: colistin sulfate (MP Biomedicals, CAT no. 194157) in mQH_2O, ciprofloxacin (Sigma-Aldrich, CAT no. 17850-5G-F) in 0.1M HCl, tetracycline (Sigma-Aldrich, CAT no. T7660-5G in 70% ethanol, chloramphenicol (Sigma-Aldrich, CAT no. C0378-25G) in 100% ethanol, erythromycin (MP Biomedicals, CAT no. 190197) in 100% ethanol, rifampicin (Sigma-Aldrich, CAT no. 236-312-0) in methanol and sulfamethoxazole (Sigma-Aldrich, CAT no. S-7507) in dimethyl

sulfoxide. Antibiotic stock solutions were prepared at 25.6 mg/mL, then further diluted in ½ LB. Twenty microliters of the diluted antibiotic stock was added to a 96-well plate containing 180 µl of standardized bacterial culture, producing final antibiotic concentrations of 0.125-256 µg/mL. Plates were incubated overnight at 37ºC with shaking at 225 RPM and then read using PerkinElmer VICTORx3 plate reader at OD_{600nm}. Growth was normalized to the no antibiotic controls.

Identification of LPS-binding phages

Phage receptors were characterized using a previously described protocol [167]. Phage adsorption assays were performed using *B. cenocepacia* K56-2 cells treated with either periodate or proteinase K. Periodate treatment was performed by centrifuging 1 mL of overnight culture at 6,000xg for 3 minutes, removing the supernatant and resuspending in 1 mL 100 mM periodate in 50 mM sodium acetate [pH 5.2] or 50 mM sodium acetate [pH 5.2] and incubated on the bench away from light for 2h. A 20 mg/mL stock of proteinase K (Applied Biosystems) was prepared, and 10 µL of this stock was added to 1 mL of overnight culture and incubated at 37ºC for 3h. Following treatment, cells were spun down at 6,000xg for 3 mins, washed 3x in ½ LB, and standardized using OD_{600}. 500 µL of washed cells were transferred to fresh Microcentrifuge (MCF) tubes and 100 µL of $1x10^6$ PFU/mL phage stocks were added and incubated on the bench for 30 mins. Samples were centrifuged at 21,130xg for 3 minutes and titered to determine the PFU/mL of unadsorbed phage. As a control, 500 µL of ½ LB was incubated with 100 µL of a $1x10^6$ PFU/mL phage stock, spun down, and titered as described above. Data was normalized to the ½ LB control, and the assay was completed in biological and technical triplicate.

To determine the specific portion of the LPS bound by each identified LPS-binding phage, phage stocks were spotted onto K56-2 LPS mutants with various LPS truncations (Table 1). Briefly, $10^7 – 10^8$ PFU/mL phage stocks were spotted onto soft agar overlays of WT K56-2 or LPS mutants harbouring empty vector or complementation plasmids. Plates were incubated at 37ºC overnight and observed for clearing. Spotting was done in biological triplicate.

Phage resistant mutant/lysogen isolation and analysis

Phage-resistant K56-2 mutants were isolated using previously published protocols with some modifications [168,169]. Overnight cultures of K56-2 were diluted 1:10 in ½ LB to a final volume of 100 µL, mixed with 100 µL of 10^7-10^8 PFU/mL phage stock, and incubated for 20 minutes on the bench. After incubation, 3mL of ½ LB soft agar was added and poured onto ½ LB agar plates. Plates were incubated at 37ºC for 2 days and monitored for complete clearing of the bacterial lawn after one day, and the emergence single colonies after two days. Plates were then flooded with 3 mL of ½ LB, swirled, and pelleted in a MCF tube by centrifuging 1mL aliquots. Pellets were resuspended in ½ LB, serially diluted, and plated on ½ LB for single colonies. Overnight cultures of resulting colonies and WT K56-2 were overlayed with soft agar, and 5 µL of 10^8 PFU/mL phage stock was spotted onto the overlays. Plates were incubated overnight at 37ºC and observed for clearing. Bacterial variants showing no clearing were screened using PCR with phage specific primers listed in Table 2.3 to detect lysogens. Variants lacking the presence of a phage-genome band were considered resistant mutants, while strains showing phage-genome bands were considered lysogens. If a lysogen was detected, the colony was passaged 3x on ½ LB to isolate stable lysogens. No stable lysogens for KS9 and KS5 were identified.

Phage-resistant mutants' LPS was extracted using a hot-phenol extraction protocol detailed in McKay et al. (2003). LPS extractions were diluted 1:1 with Laemmli loading buffer (BioRad, CAT no. 161-0737) and run alongside a protein ladder on a polyacrylamide gel comprised of a 5% stacking gel and a 16% separating gel in 1x sodium dodecyl sulfate buffer. The gel was silver stained as per [171] and imaged on a white background using an iPhone 11 camera.

Phage, Antibiotic and Phage-antibiotic Kill Curves

WT K56-2 were cultured overnight followed by subculturing 1:100 in ½ LB media for 3h and standardized to an OD_{600nm} of 0.1. Standardized cultures were diluted in ½ LB for a final concentration of $1x10^6$ CFU/mL. Phages were diluted in ½ LB to titers of $1x10^7$-$1x10^9$ PFU/mL. 100 µL of diluted bacteria was added to a microplate followed by a 100 µL of phage at various concentrations resulting in multiplicities of infection (MOI) of approximately 10, 100 and 1,000. If a dual phage treatment was used, 50 µL of the correct phage dilution was added for a total MOI of both phages combined of 10, 100 and 1,000. If antibiotic was added to the solution, 99 µL of bacteria and phage were added with 2 µL of antibiotic, resulting in colistin concentrations of 128-256 µg/mL. As a control, 100 µL of bacteria was mixed with 100 µL of sterile ½ LB. The microplate was then incubated in an EPOCH BioTek plate reader for 48h at 37ºC with shaking at 225 RPM with OD_{600nm} measurements taken every 30 minutes. Each treatment was done in biological triplicate.

Growth curve data was analyzed to calculate growth reduction % of treated conditions in a similar fashion to [172,173] with some modifications. Endpoint OD_{600nm} (t=48h) values were used to calculate growth reduction % using the following formula: $\frac{(Untreated-Treated)}{Untreated}\ x\ 100\%$. To

calculate if a dual treatment exhibits synergy, the growth reduction percentages were compared to the expected additive effect of the treatments which was calculated as follows: $\frac{Growth\ reduction\ \%\ A + Growth\ reduction\ \%\ B}{Number\ of\ Treatments}$. Actual growth reduction percentages were then compared to the expected additive effect using an unpaired parametric T-test. Any dual treatment which demonstrated a significantly higher growth reduction than the expected additive effect was deemed to be synergistic.

Results and Discussion:

Complete LPS is required for full virulence *in vitro* and *in vivo*

Screening of over 7,000 *B. cenocepacia* Tn mutants identified 171 strains of interest which were sensitive to humoral immune factors in NHS, demonstrating OD_{600nm} >0.54 in LB media and A_{575nm}<0.26 in LB+30% NHS (Figure 2.1A). Most Tn mutants grew to a normalized OD_{600nm} of ~1.0 in LB, while more variation existed between mutants when grown in LB+30% NHS as demonstrated by the histograms in Figure 2.1A. Following verification that mutants of interest were completely dying in LB+30% NHS, the mutated genes were recovered. Screen hits are summarized in Table 2.4. Genes relating to LPS biogenesis were recovered at the highest frequency (Figure 2.1B). LPS has already been indicated in protection of *B. cenocepacia* against complement using a *wbiI* mutant lacking O-antigen [174]. To further investigate the role of other portions of LPS in complement-resistance, a variety of strains with various levels of LPS truncation were tested for their ability to survive in various concentrations of NHS (Table 2.1) [124,175]. Figure 2.2A confirmed previous work and screen results whereby any truncation to the LPS resulted in complete sensitivity to serum even at low concentrations of 10%. This indicates that LPS plays an essential role in survival of *B. cenocepacia* K56-2 in the presence of serum.

To further confirm that LPS is necessary for full virulence, we calculated LD_{50} of each LPS mutant in the *G. mellonella* wax worm model. *G. mellonella* have a sophisticated immune system, including pathogen detection and destruction using humoral and cell based immune responses [176,177]. Mutants with truncated LPS required a minimum of ~200x more cells than WT to kill 50% of the injected population, suggesting that complete LPS is required for infection *in vivo* (Figure 2.2B). It has been previously discovered that inner core LPS mutants are unable to cause infection in a rat-agar bead model of infection, however, no previous evidence suggests complete LPS is necessary for *B. cenocepacia* to cause infection *in vivo* [175]. The decreased virulence *in vivo* of LPS mutants is likely due to decreased steric hinderance, allowing complement factors to bind to and insert themselves into the outer membrane, leading to complement-mediated lysis. Additionally, LPS mutants lacking O-antigen may result in increased phagocytosis of the bacterial cells as O-antigen is known to play a role in preventing this process [178].

Due to the role of LPS in membrane stability, overall cell wall biogenesis and the consequences of toxic and/or mislocalized LPS intermediates, we wanted to investigate the effects of these LPS-related mutations on overall growth [179,180]. Aside from XOA8 (p-value=0.0689), mutants with truncated inner and outer core or lacking O-antigen resulted in a statistically significant growth defect (Figure 2.2C), confirming that complete LPS is required for WT growth. This growth defect is likely due to incomplete LPS biogenesis, which may cause accumulation or increased time of intermediates in the inner membrane or periplasm, activating stress responses and effecting membrane potential [180]. As previously stated, perturbations to the LPS affect a variety of outer membrane structures and overall outer membrane integrity, which can have an effect on overall growth [180]. In contrast, RSF19 (*wbxE*:: pRF201), which has a

truncated O-antigen that cannot be polymerized [124,174], demonstrated growth equivalent to WT (p-value 0.9084, Figure 2.2C). Due to the small effect of this truncation on overall LPS structure, it is unlikely that this mutation effects LPS trafficking or overall outer membrane structure and integrity enough to cause a growth defect. Altogether, complete O-antigen is not required for WT growth, however, lack of O-antigen and any further truncation to the LPS results in growth deficiencies.

LPS mutants are sensitive to the antibiotic colistin

Next, we wanted to see if *B. cenocepacia* with truncated LPS were more sensitive to different chemical classes of antibiotics in comparison to WT. Screening of eight different classes of antibiotics identified that all LPS mutants demonstrated increased sensitivity to colistin (Figure 2.2D, Figure 2.3). Colistin is a polymyxin class antibiotic which was canonically known to bind the lipid A portion of the LPS, creating small pores in the outer membrane, increasing osmotic pressure, and ultimately leading to cell lysis [182]. A recent study by Fu et al. (2022) revealed that polymyxin class antibiotics cause lipid scrambling in the outer membrane resulting in cell death [183]. Binding of a polymyxin antibiotic to the LPS relieves tight packing of LPS, then negatively charged phospholipids from the inner leaflet flip to the outer leaflet. This leads to phase separation of the LPS and phospholipid molecules increasing permeability and allowing polymyxins to move to the inner leaflet of the outer membrane. From there the polymyxin may be translocated to the inner membrane having a similar effect. Polymyxin class antibiotics have been largely disfavored in clinical settings due to their negative side effects, which are harsh but reversible [182,184]. However, due to a lack of development of novel antibiotics, colistin is now being used as a last resort to treat MDR infections [182,185–187]. Additionally, new polymyxin analogues are being created which demonstrate lower toxicity while maintaining bacterial killing activity *in vitro* [188,189].

Polymyxin class antibiotics cannot be used against *B. cenocepacia* due to the extreme resistance provided by modified LPS, metalloproteases and efflux pumps [69]. More specifically, it has been hypothesized that the Ara4N modification of lipid A bestows Gram-negatives with polymyxin resistance because it is positively charged, therefore repelling cationic AMPs and hindering colistin-lipid A interactions [69,182]. However, due to the essentiality of this modification in *B. cenocepacia* LPS export and biogenesis, it has been speculated that the modification is not playing a role in polymyxin resistance [69]. Previous studies have identified a role for the core oligosaccharide in *B. cenocepacia* polymyxin resistance but, to our knowledge, no studies have shown that any truncation to the LPS results in colistin sensitization [69,175]. The increased sensitivity to the antibiotic likely results from increased permeability of the outer membrane, allowing colistin easier access to its lipid A target. Unexpectedly, the mutant with the most severely truncated LPS (CCB1) did not demonstrate the lowest MIC50 (Figure 2.2D). Instead strains XOA15 & XOA17 with longer LPS had the lowest colistin MICs at 4 μg/mL, while CCB1's MIC50 was 128 μg/mL (Figure 2D). This may be explained by the low maximum OD_{600nm} of the XOA17 mutant (OD_{600nm}=0.42 for XOA17) in comparison to the average of 0.56 for other mutants with growth defects (Figure 2.2C). Additionally, these XOA15 and XOA17 strains may have decreased cell envelope integrity in comparison to other mutants which may affect outer membrane proteins playing a role in resistance. Regardless of the mechanism, all the tested LPS mutants were sensitive to colistin, whereas WT *B. cenocepacia* was not.

LPS binding phages can be used to steer the bacterial population towards mutants with truncated LPS

The Dennis lab possesses a library of previously characterized phages, many of which have been hypothesized or partially determined to use LPS as a receptor [190–192]. To definitively identify

the receptor, two assays were used. First, adsorption assays were performed on proteinase K and periodate treated *B. cenocepacia* K56-2, degrading cell surface proteins or sugars respectively. Four phages, KS5, KS9, JG068 and KS4-M, were shown to use a carbohydrate-based structure as a receptor (Figure 2.4A, B, C, D). Second, spotting these phages on the various LPS mutants showed that KS9, JG068 and KS4-M utilize the outer portion of the O-antigen and that KS5 utilizes the inner core of the LPS as a receptor (Figure 2.4E, Table 2.5). This finding is in contrast to previous work that predicted JG068 used the inner core of LPS as a receptor, but did not have sufficient evidence to confirm this prediction [191]. Updated host range screening of 150 *Burkholderia* spp. indicated that KS5 infects 90 strains, KS9 infects 11 strains, KS4-M infects 16 strains and JG068 infects 50 strains (unpublished data). Therefore, KS5 and JG068 are the most promising therapeutic candidates due to their broad host range.

Next, phage-resistant mutants were isolated to determine if they possess truncated LPS. As hypothesized, results showed that phage-resistant mutants do not possess complete LPS (Figure 2.5A). Resistant mutants were not able to be isolated from KS4-M as all survivors following infection were lysogens. For this reason, KS4-M was not pursued further in this study. Two of three KS5-resistant mutants had truncated LPS core without O-antigen present, and the other had truncated core and a lesser amount of O-antigen compared to WT when the gel was overstained as it is in Figure 2.5A. All three JG068-resistant mutants exhibited truncated core and lacked O-antigen (Figure 2.5A). Lastly, KS9-resistant mutants had a complete core but lacked O-antigen (Figure 2.5A). These LPS truncations were expected, as receptor alteration is the most common mechanism of phage-resistance used by bacteria [193]. Nonetheless, these resistant isolates were not sequenced, so it is uncertain which LPS biogenesis genes are mutated and what other mutations are present which could be contributing to this phenotype.

Subsequently, phage-resistant mutants were challenged with colistin to determine if they were sensitive to the antibiotic and to confirm that there were no other mutations conferring colistin resistance. JG068-resistant mutants had colistin MIC50s of 64 μg/mL and KS5-resistant mutants had MIC50s of 128 μg/mL (Figure 2.5B & C). These results are consistent with each strain's LPS profile (Figure 2.5A) and with the MIC50's determined for the LPS mutants (Figure 2.2B), indicating that the truncated LPS is likely the reason for the colistin sensitivity. In contrast, two out of three KS9-resistant mutants lacking O-antigen exhibited decreased growth in the presence of 256 μg/mL colistin but did not show a MIC50 (Figure 2.5D). This result conflicts with our previous finding that the XOA7 LPS mutant lacking O-antigen had an MIC of 16 μg/mL (Figure 2.2B). Furthermore, none of the phage-resistant mutants with truncated LPS exhibited MIC50s like constructed mutants with the same LPS modifications (Figure 2.2B & D). Other non-LPS resistance mechanisms against polymyxin class antibiotics have been previously discovered for *B. cenocepacia* and may be playing a role for these mutants. A previous study identified genes involved in polymyxin B resistance when incomplete LPS was expressed and found that there are likely a wide variety of other factors that may contribute to resistance [194]. Specifically, they identified degradation enzymes, isoprenoid biosynthesis and genes under the control of the *rpoE* and *BCAL2831* regulons as possible contributing factors [194]. Additionally, mutations in the *suhB* gene, which plays a role in protein secretion, were found to increase sensitivity to polymyxin B [195]. Further, capsular polysaccharide and efflux pumps have been shown to contribute to polymyxin resistance in other species [196–199]. Thus, there are many factors contributing to polymyxin resistance, and it remains unclear why the phage resistant LPS mutants do not exhibit MIC50s comparable to those of the constructed LPS mutants.

As a follow-up to these analyses, we wanted to know if the KS9 and KS5 lysogens had altered LPS profiles and to determine if they had increased resistance to colistin. However, after many attempts to isolate stable lysogens, none were identified. Considering the transience of these lysogens, it is likely not necessary to characterize them further.

LPS-binding phages can exhibit phage-phage synergy

Due to the ease with which bacteria can develop resistance to a single antibacterial treatment (Figure 2.6), interest in combination treatments against MDR bacteria has grown. One such combination is the application of phage cocktails, which utilize two or more distinct phages to target bacteria. This approach increases the number of strains that the treatment can kill and decreases likelihood of phage-resistance evolving [49,200]. These treatments are most effective when acting on different molecular targets [150,157,201]. Two different treatments combining a phage using the inner core (KS5) as a receptor and a phage using the O-antigen (KS9 or JG068) were tested. Notably, KS9 and KS5 can undergo the lysogenic life cycle, while JG068 is a lytic phage and these lifestyles affect growth reduction % [190,191]. Synergy was defined as when the measured growth reduction % was greater than the growth reduction % of the additive effect of both treatments combined. Phages KS5 and KS9 did not synergize with one another, whereas KS5 & JG068 showed synergism (Figure 2.7). Because both combinations did not exhibit synergy, it was predicted that the synergy seen between KS5 and JG068 is more likely a result of combining a lysogenic and a lytic phage which use different portions of the LPS as a receptor rather than the use of different portions of the LPS as a receptor alone [202].

LPS-binding phages and colistin can exhibit synergy

Next, we tested whether LPS-binding phages could synergize with colistin provided at either the MIC or half MIC of the antibiotic of their respective phage-resistant mutants. KS5+128 μg/mL colistin exhibited the highest growth reduction % of 92.6 at an MOI of 100 and demonstrated the strongest synergistic effect (Figure 2.8A & E). With JG068, we used a colistin concentration of 128 μg/mL as 64 μg/mL had little to no effect on overall killing effect of the treatment. JG068+128 μg/mL colistin produced a growth reduction % of 47.98 and demonstrated synergy (Figure 2.8B & E). The highly variable resistant outgrowth of this treatment suggests JG068 could be using a primary and secondary receptor to infect, creating a variety of different resistant mutants. Additionally, because JG068 utilizes the O-antigen portion of the LPS this could be generating a variety of mutants with varying LPS structures. JG068+KS5+128 μg/mL colistin produced a growth reduction % of 85.7 and showed synergy with the antibiotic (Figure 2.8C & E). The growth reduction % of the two-phage + antibiotic treatment group was not significantly different when compared to KS5+128 μg/mL (p-value=0.4038) but was significant when compared to JG068+128 μg/mL (Figure 2.8E). Additionally, the growth curve for the two-phage + antibiotic treatment resembled that of KS5 + colistin and differed from that of JG068 + colistin. These analyses suggest that KS5 largely drives the killing effect of the two phages + antibiotic treatment. No treatments exhibited large differences between different MOIs, suggesting that this effect does not depend on phage dosage. Based on our previously discussed findings, we predict that the mechanism driving this synergy is phage steering, whereby the phage controls the WT population and creates colistin-sensitive mutants. Many previous works have demonstrated similar effects using phage to generate more antibiotic-sensitive bacteria [156,203–205]. To our knowledge, this is the first instance of phage steering in *B. cenocepacia*.

Conversely, KS9+256 μg/mL colistin had a much lower growth reduction % of 28.9 at an MOI of 100 and did not exhibit a synergistic interaction (Figure 2.8D & E). We calculated growth reduction %s for KS9 + 256 μg/mL colistin at MOIs of 10 and 1000 to determine whether the treatment was phage dose-dependent, but no significance was found between additive and actual growth reduction %. This result is consistent with the KS9-resistant mutants as none of them demonstrated a MIC50 (Figure 2.4D). Since KS9 is unable to form stable lysogens, it is unlikely that lysogeny is favored in the presence of colistin.

Alternatively, results seen for KS9 could be due to unknown mutations. We previously discussed the role of the alternative sigma factor RpoE, which is essential for *B. cenocepacia* growth stressful environments, in colistin resistance. [194,206]. It is not known which specific LPS-related gene is mutated in the phage-resistant strains or if any other mutations are present which could be affecting colistin sensitivity and/or resistance. Furthermore, previous work has shown that phage U136B requires both TolC and LPS for infection and generates mutations in both structures [203]. In the study, LPS mutants were outcompeted by the *tolC* mutants due to decreased fitness associated with LPS perturbations [203]. In this study, a similar phenomenon could be happening if KS9 uses a secondary receptor to inject its DNA. Future investigation into the long-term evolution of these phage-resistant mutants is required to determine why the KS9+256 μg/mL colistin treatment is less effective and lacks synergism.

Figures & Tables:

Table 2.1 Bacterial strains used in this study

Strain name	Characteristics of Interest	Source/Reference
K56-2 WT	None	CF-e patient, Canada / (Lewenza et al., 1999)
K56-2 XOA7 (*waaL*::pGPΩTp)	Lacks O-antigen of LPS, Tp^R	[174]
K56-2 XOA15 (*wabR*::pGPΩTp)	Truncated outer core of LPS, Tp^R	
K56-2 XOA17(*wabS*::pGPΩTp)	Truncated outer core of LPS, Tp^R	
K56-2 XOA8 (*wabO*::pGPΩTp)	Lacks outer core, truncated inner core of LPS, Tp^R	
K56-2 CCB1 (*waaC*::pGPΩTp)	Lacks outer core & inner core of LPS, Tp^R	
K56-2 RSF19 (*wbxE*:: pRF201)	Truncated O-antigen of LPS, Tp^R	[175]
K56-2 WT + pSCRhaB2	Tc^R	[163]
K56-2 XOA7 + pSCRhaB2	Truncated outer core of LPS, Tp^R & Tc^R	
K56-2 XOA15 + pSCRhaB2	Truncated outer core of LPS, Tp^R & Tc^R	
K56-2 XOA17+ pSCRhaB2	Truncated outer core of LPS, Tp^R & Tc^R	
K56-2 XOA8 + pSCRhaB2	Lacks outer core, truncated inner core of LPS, Tp^R & Tc^R	
K56-2 CCB1 + pSCRhaB2	Lacks outer core & inner core of LPS, Tp^R & Tc^R	
K56-2 RSF19 + pSCRhaB2	Lacks O-antigen of LPS, Tp^R & Tc^R	
K56-2 XOA7 + pSCRhaB2-*waaL*	Tp^R & Tc^R	
K56-2 XOA17 + pSCRhaB2-*wabS*	Tp^R & Tc^R	
K56-2 XOA15 + pSCRhaB2-*wabR*	Tp^R & Tc^R	
K56-2 XOA8 + pSCRhaB2-*wabO*	Tp^R & Tc^R	
K56-2 CCB1 + pSCRhaB2-*waaC*	Tp^R & Tc^R	
K56-2 RSF19+ pSCRhaB2-*wbxE*	Tp^R & Tc^R	

Table 2.2 Bacteriophages used in this study

Name	Morphology/Life cycle	Reference
KS4-M	*Myoviridiae*/Lysogenic	[165]
KS5	*Myoviridiae*/Lysogenic	[192]
KS9	*Syphoviridiae*/Lysogenic	
JG068	*Podoviridiae*/Lytic	[191]

Table 2.3 Primers used in this study to detect presence of phage genomic DNA in surviving K56-2 following phage infection.

Name	Sequence (5'-3')	Annealing location in genome (bp)
KS1/KS5 F	GCCCGCATGCTGAAACTGTAC	35695-35715
KS1/KS5 R	GCCGTCCAATCAGCGTTACAAG	36011-36032
KS4/KS4-M F	TGTTCAGAGATGCGTTCGAC	8335-8354
KS4/KS4-M R	ATGGCGCTTGACAGGTAATC	9194-9213
JG068 F	GCACAACTGGAGTAACGATCA	1570-1590
JG068 R	CTCGCTGAAGAAGTGATCCTC	2521-2541
KS9 F	GTTTTACGACGGAGGGAATGCG	26529-26550
KS9 R	TTGTCGAAGTACACCATGCCGG	27474-27495

Table 2.4 List of genes recovered from serum screen.

Mutant ID	K56-2 locus tag	J2315 locus tag	Product description (gene name)
1BO2	K562_13054	BCAL3126a	glycosyltransferase family 4 protein (*wbxE)*
1E07	K562_13171	BCAL3243	putative capsular polysaccharide biosynthesis (*wcbC*)
1E09	K562_40004	NA	hypothetical protein on pBCK56
2A03	K562_12287	BCAL1492	methyltransferase
2A08	K562_22804	BCAM2739	hopanoid biosynthesis associated radical SAM protein HpnH (*hpnH)*
2E06	K562_11327	BCAL2403	Putative LPS core biosynthesis protein
2E09	K562_12325	BCAL1455	fusaric acid resistance protein
2F05	K562_12080	BCAL1670	taurine catabolism dioxygenase (*tauD)*
2F10	K562_20237	BCAM0228	two-component response regulator protein

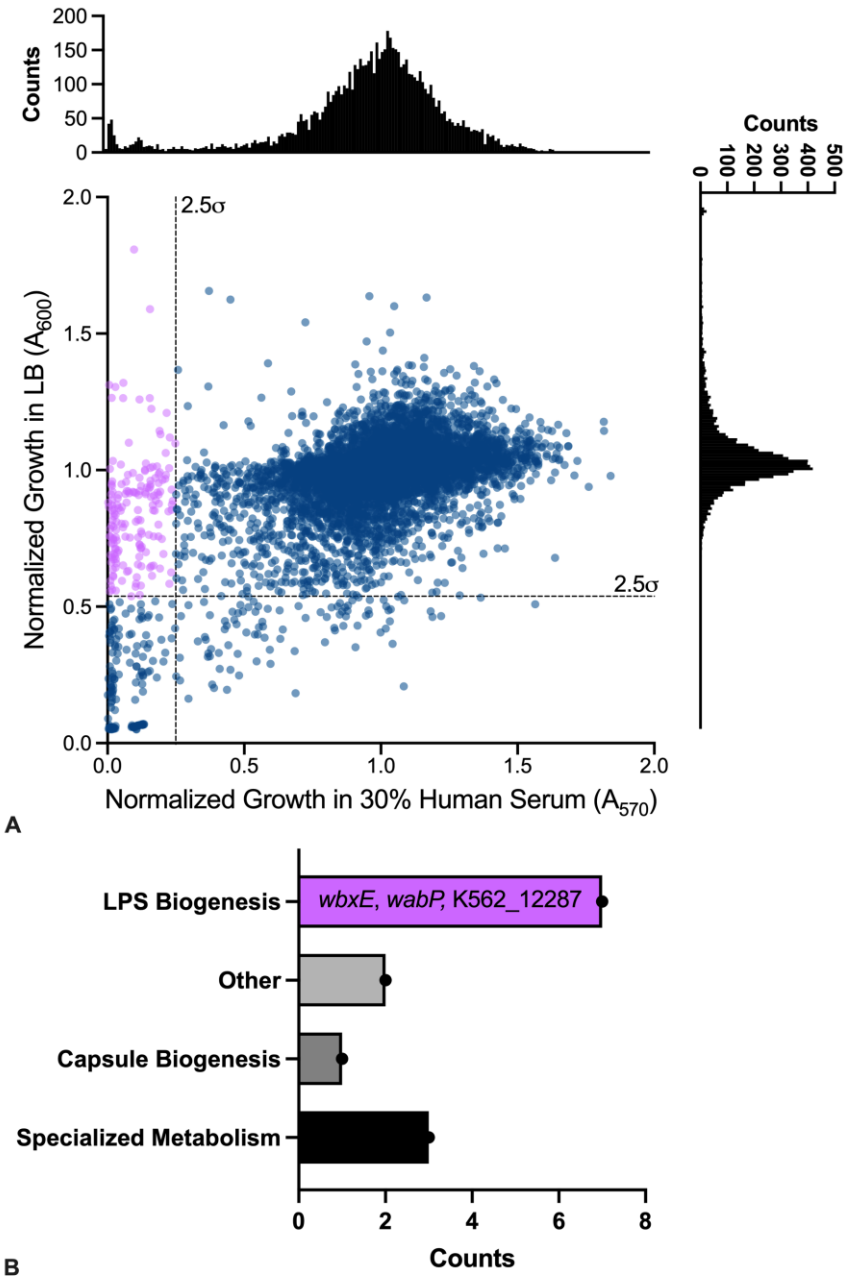

Figure 2.1 Tn mutant library screen in LB and LB+30% NHS media.

A Each dot represents one mutant's growth in LB and LB+30% NHS, purple dots represent hits which deviate 2.5 standard deviations away from the mean. Histograms represent counts with bin sizes of 0.01. **B** Genes of interest isolated from mutants which were completely killed in NHS.

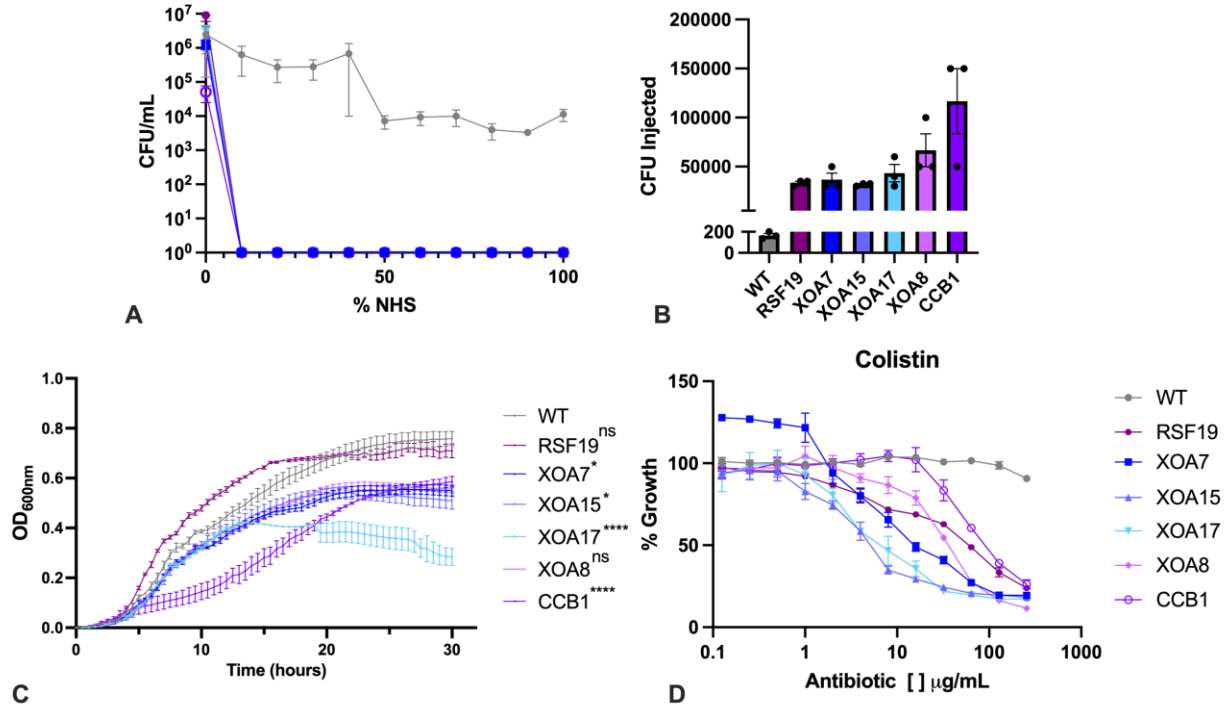

Figure 2.2 B. cenocepacia K56-2 LPS mutants exhibit decreased virulence phenotype in vitro and in vivo

A Confirmation of Tn mutant library screen using 2h serum survival test in various dilutions of NHS, all LPS mutants are incapable of surviving even 10% NHS. **B** LD_{50} of LPS mutants in G. mellonella model is significantly higher than WT for all mutants tested. Data collected from 3 biological replicates injected into 10 worms alongside 10 worms injected with 1xPBS. **C** Growth curve demonstrating 4/6 LPS mutants have a growth defect compared to WT, ordinary one-way ANOVA with multiple comparisons to WT. (*=p-value<0.05; ****=p-value<0.0001) **D** All LPS mutants tested have a colistin MIC between 8-128 µg/mL compared to WT MIC of >256 µg/mL. All experiments were completed in biological triplicate (n=3); SEM error bars plotted.

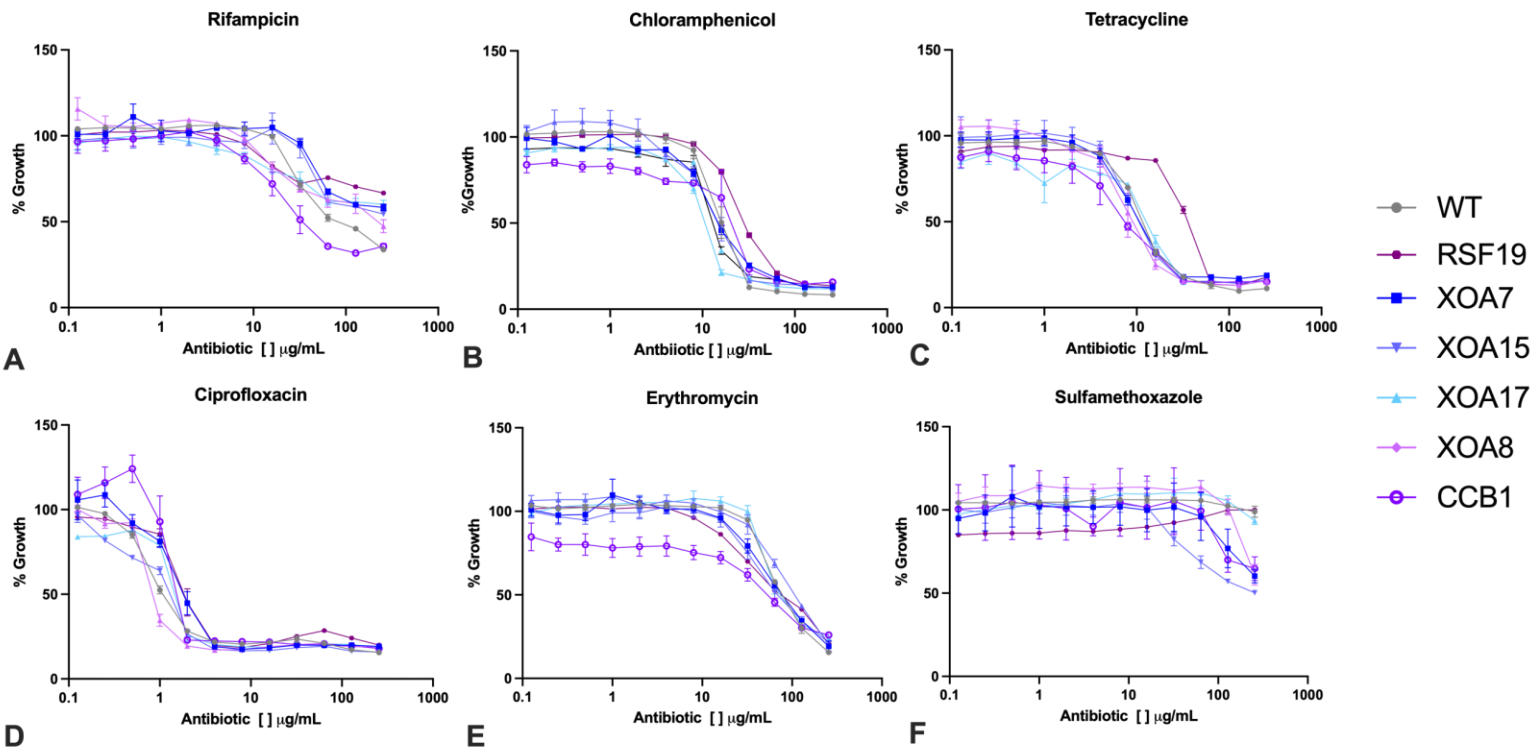

Figure 2.3 : Screening LPS mutants' sensitivity to various classes of antibiotics

MIC_{50}s of LPS mutants and WT *B. cenocepacia* K56-2 for **A** Rifampicin, **B** Chloramphenicol, **C** Tetracycline, **D** Ciprofloxacin, **E** Erythromycin & **F** Sulfamethoxazole. LPS mutants exhibit no significant differences in MIC compared to WT for antibiotics tested. All experiments done in biological triplicate.

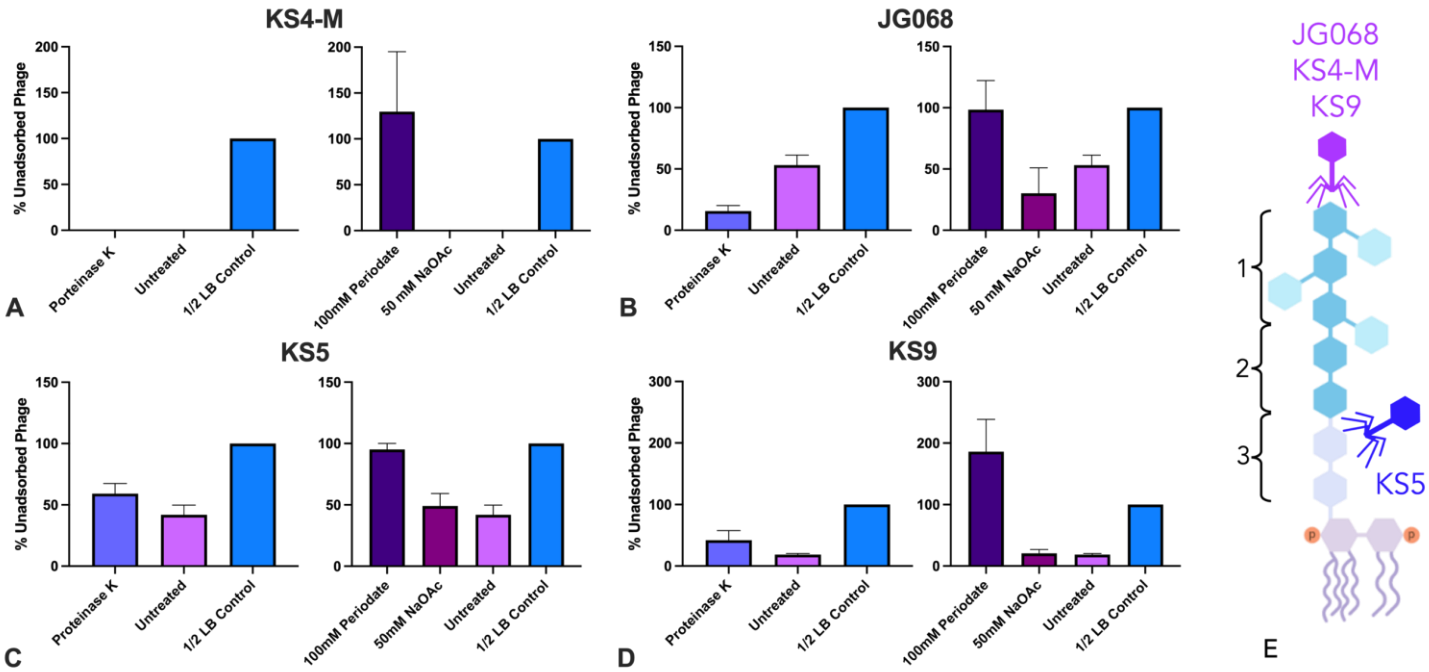

Figure 2.4 Phage-receptor characterization

Adsorption assays using proteinase K and periodate-treated cells determining that phages **A** KS4-M, **B** JG068, **C** KS5 & **D** KS9 adsorb to the LPS. Experiment was done in biological triplicate and titering was done in technical triplicate; SEM error bars plotted. **E** Representation of specific portion of LPS being bound by each phage as determined via spotting assays on LPS mutants (see Table S4). JG068, KS9 and KS4-M adsorb to the O-antigen (1) and KS5 adsorbs to the inner core (3) portion of the LPS. Spotting was done in biological triplicate.

Table 2.5 Spotting on B. cenocepacia K56-2 LPS mutants and complemented strains

Bacterial Strain + pSCRhaB2	KS4-M	KS 5	KS 9	JG068
WT K56-2 (complete LPS)	+	+	+	+
RSF19 (*wbxE*:: pRF201, truncated O-antigen)	-	+	-	-
XOA7 (*waaL*::pGPΩTp, lacks O-antigen)	-	+	-	-
XOA15 (*wabR*::pGPΩTp, truncated outer core)	-	+	-	-
XOA17 (*wabS*::pGPΩTp, truncated outer core)	-	+	-	-
XOA8 (*wabO*::pGPΩTp, lacks outer core, truncated inner core)	-	-	-	-
CCB1 (*waaC*::pGPΩTp, lacks outer core, truncated inner core)	-	-	-	-
+pSCRhaB2-complement				
RSF19	+	+	+	+
XOA7	+	+	+	+
XOA15	-	+	-	-
XOA17	+	+	+	+
XOA8	+	+	+	+
CCB1	+	+	+	+

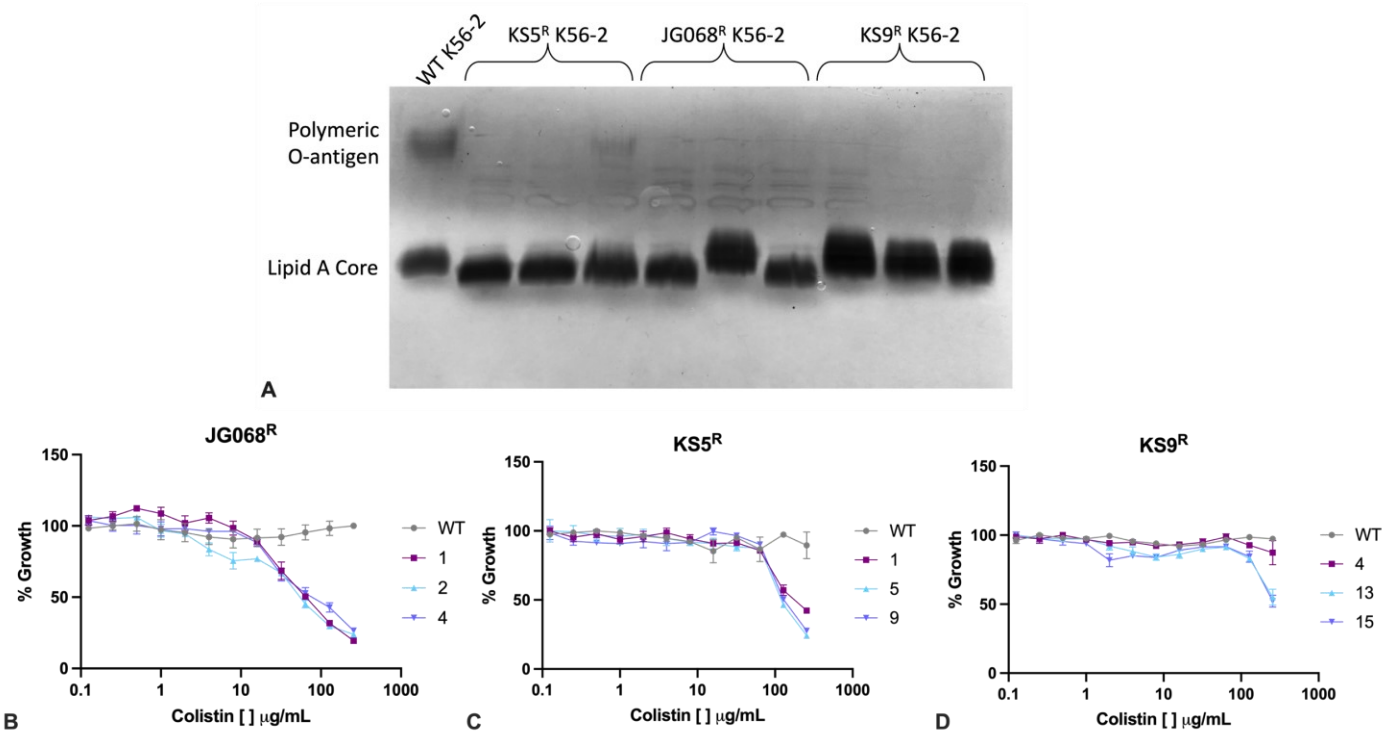

Figure 2.5 B. cenocepacia K56-2 phage-resistant mutant analysis

A LPS profiles of WT and phage^R mutants isolated from high MOI infections. LPS was extracted using a hot-phenol protocol, run on a PAGE gel (5% stacking; 16% resolving), and silver stained. All phage^R mutants demonstrate non-WT LPS profiles either lacking O-antigen and/or having a truncated lipid A core. **B, C & D** Colistin MIC50s of JG068, KS5 and KS9 resistant mutants respectively. JG068^R mutants MIC is 64 μg/mL, KS5^R mutants MIC is 128 μg/mL and KS9^R mutants do not demonstrate a true MIC with <50% growth, but 2/3 mutants demonstrate 55% growth at 256 μg/mL. All MICs done in biological triplicate; SEM error bars plotted.

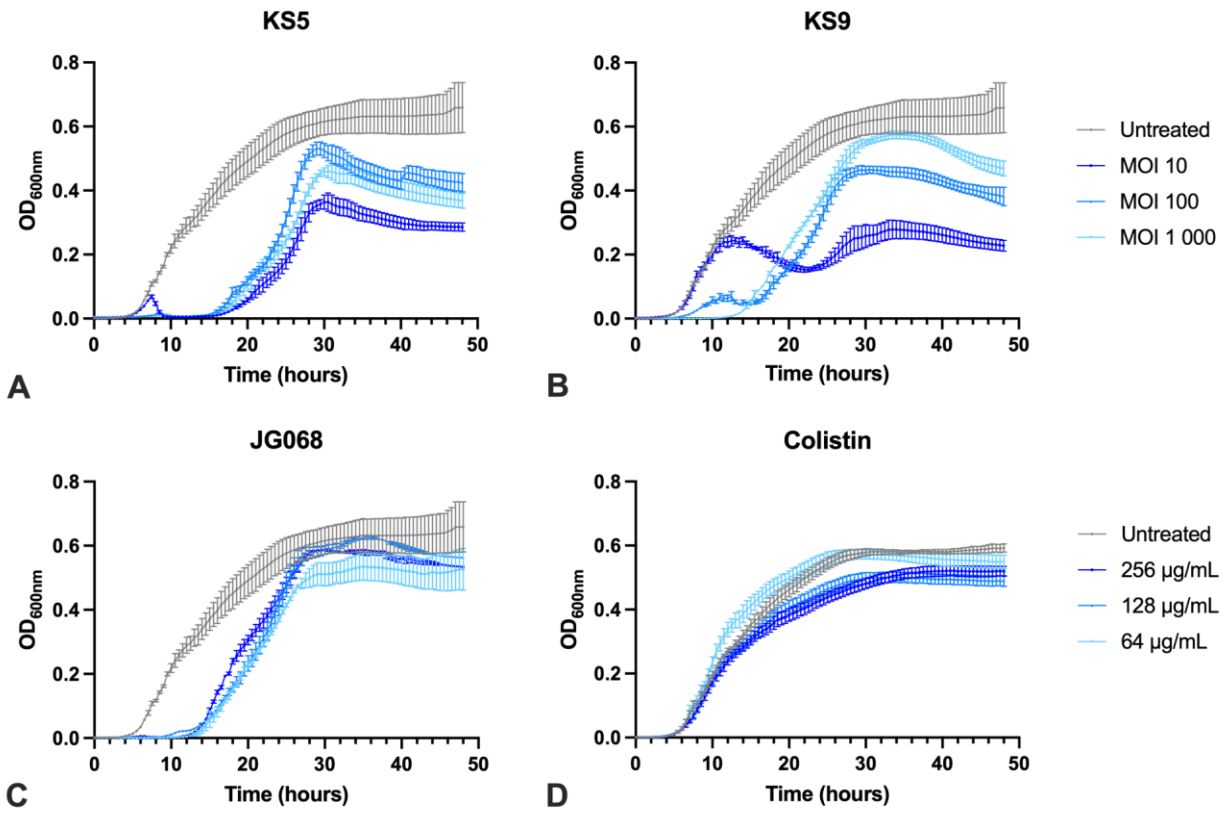

Figure 2.6 Single phage/antibiotic treatment growth curves

Log phase WT *B. cenocepacia* K56-2 was diluted and mixed with phages **A** KS5, **B** KS9, **C** JG068 or **D** Colistin at various MOIs or concentrations and growth was tracked over time using OD_{600nm}. Lysogenic phages KS5 and KS9 exhibit initial growth at MOIs of 10 and 100. All phage treatments exhibit resistance outgrowth starting between 14-16h except for KS9 at an MOI 10. Colistin does not demonstrate antibacterial effect on K56-2 at the concentrations tested.

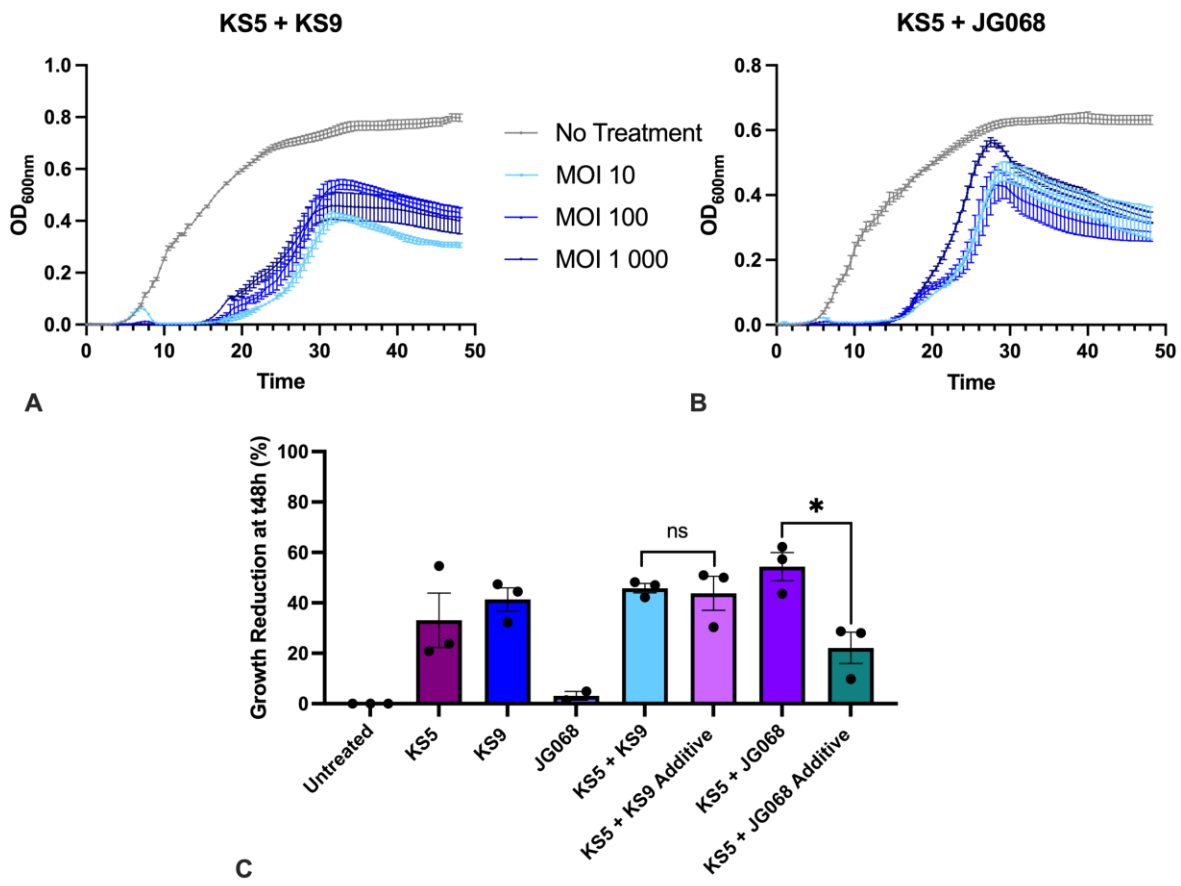

Figure 2.7 Phage-phage synergy between LPS-binding phages

A & B Growth curves of WT *B. cenocepacia* K56-2 treated with KS5 and KS9 or KS5 and JG68, respectively. Log phase subcultures of K56-2 were standardized via OD_{600nm} and mixed with phage combinations at various MOI. Note: MOI is total number of phages added to the treatment, i.e., MOI 10 = 5 JG068 & 5 KS9. **C** Growth reduction percentages for single (see Figure S2), actual dual phage and calculated additive dual phage treated K56-2 at an MOI of 100. KS5 & KS9 do not exhibit synergy, KS5 & JG068 do exhibit synergy. All experiments done in biological triplicate, SEM error bars plotted, and unpaired t-tests used to determine significance (*=p-value<0.05).

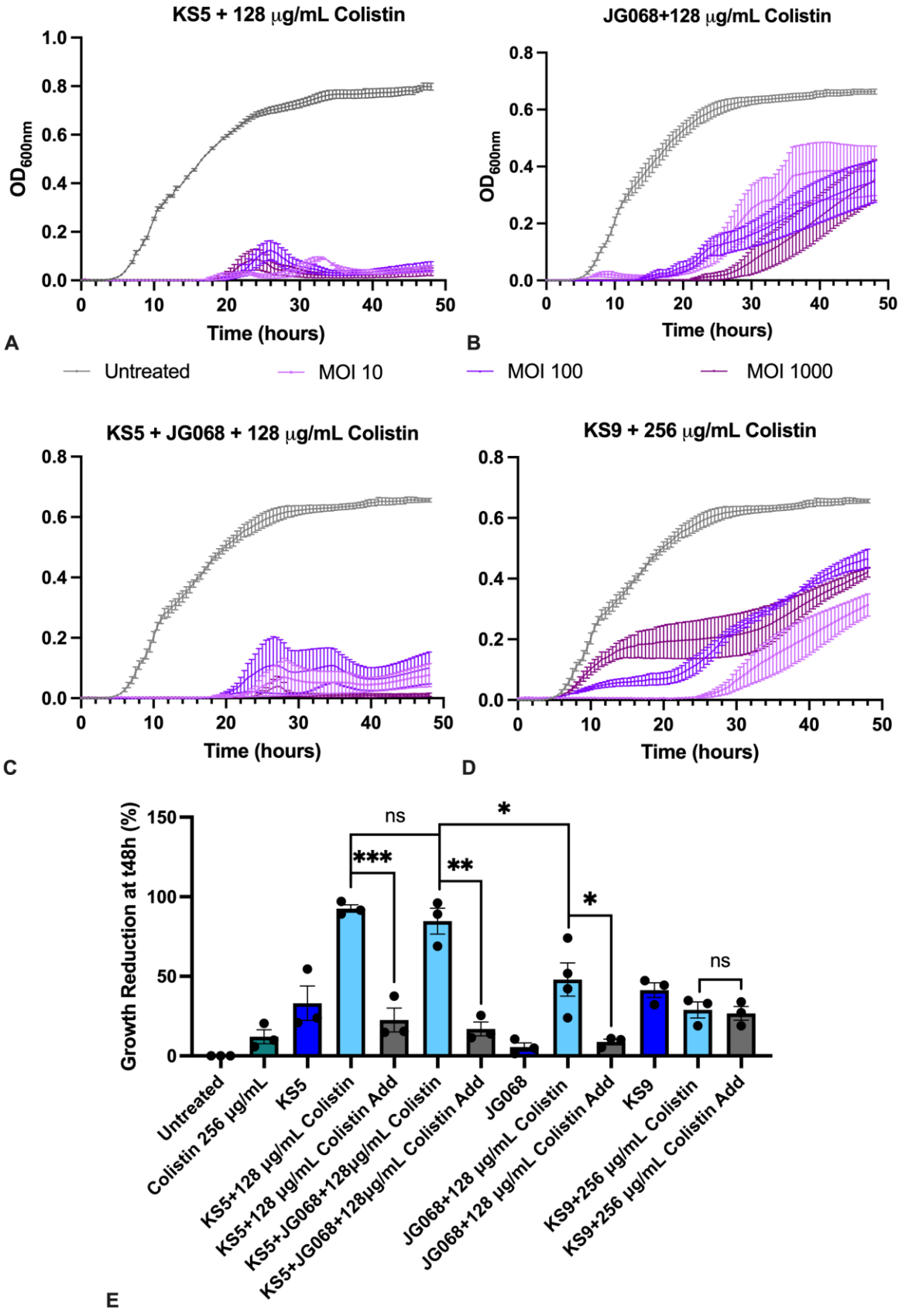
KS5 + 128 μg/mL Colistin
JG068+128 μg/mL Colistin
KS5 + JG068 + 128 μg/mL Colistin
KS9 + 256 μg/mL Colistin
OD600nm
Time (hours)
A
B
C
D
Untreated
MOI 10
MOI 100
MOI 1000
Growth Reduction at t48h (%)
ns
*
***
**
*
ns
Untreated
Colistin 256 μg/mL
KS5
KS5+128 μg/mL Colistin
KS5+128 μg/mL Colistin Add
KS5+JG068+128μg/mL Colistin
KS5+JG068+128μg/mL Colistin Add
JG068
JG068+128 μg/mL Colistin
JG068+128 μg/mL Colistin Add
KS9
KS9+256 μg/mL Colistin
KS9+256 μg/mL Colistin Add
E

Figure 2.8 LPS-binding phages and colistin synergy

A, B, C & **D** Growth curves of KS5+128 μg/mL, JG068+128 μg/mL, KS5+JG068+128 μg/mL and KS9+256 μg/mL respectively. Growth curves were done using the same protocol as in Figure 5. **E** Growth reduction %s at t48h at an MOI of 100. Unpaired t-tests were conducted using actual treatment growth reduction % and estimated additive growth reduction %. KS5+128 μg/mL (p-value=0.009), KS5+JG068+128 μg/mL (p-value=0.0018) and JG068+128 μg/mL (p-value=0.0254) exhibit synergy, KS9+256 μg/mL (p-value=0.7567) do not. KS5+JG068+128 μg/mL compared to KS5+128 μg/mL show no significant difference (p-value=0.4038) and KS5+JG068+128 μg/mL compared to JG068+128 μg/mL (p-value=0.0481). All experiments were done in biological triplicate except JG068+128 μg/mL (n=4). SEM error bars plotted.

Chapter 3 Conclusions & Future Directions

Conclusions

This study identified a range of serum-resistance genes in *B. cenocepacia,* in addition to a promising phage-antibiotic combination for use in therapy against MDR *B. cenocepacia.* Mutants that were killed by the presence of serum had mutations in a variety of genes, but genes related to LPS biogenesis were isolated with the highest frequency. Follow-up analyses using a panel of LPS mutants with varying LPS truncations concluded that the entirety of the LPS is required for *B. cenocepacia* to survive in serum. *G. mellonella* LD_{50} assays demonstrated that any truncation to the LPS results in significant attenuation of virulence. LPS mutants also exhibited an increased susceptibility to colistin, a polymyxin class antibiotic. Altogether, complete LPS is necessary for full virulence and antibiotic-resistance of *B. cenocepacia.*

To identify potential novel anti-virulence therapeutics, the Dennis lab's previously characterized phage library was screened identifying four phages which use the LPS as their receptor. Further investigation confirmed that three phages can generate phage-resistant mutants with truncated LPS. Phage KS4-M was unable to produce phage-resistant mutants and instead favored lysogeny. Mutants isolated from KS5, KS9 and JG068 phage infections all demonstrated LPS profiles with significant truncations when compared to WT. KS5 and JG068 phage-resistant mutants were more sensitive to the antibiotic colistin demonstrating decreased MIC_{50}. However, only one out of three KS9 phage-resistant mutants demonstrated growth reduction in the presence of colistin. Regardless, at least two of the identified LPS-biding phages were able to steer the bacterial population towards truncated LPS and increased sensitivity to colistin.

Phage-phage and phage-antibiotic synergy was demonstrated by some of the combinations tested. Phages KS5 and JG068 exhibited a synergistic interaction, however this cannot be solely attributed to binding different portions of the LPS. This relationship is likely due to combining

an obligately lytic phage and a lysogenic phage. Although KS5 can only form pseudolysogens, or non-stable lysogens, in the strain of interest it is probable that the presence of a lytic phage contributes to initial increased killing activity to control the population. Lastly, phages KS5 and JG068 demonstrate synergy with the antibiotic colistin, likely via a phage steering mechanism. This work provides *in vitro* evidence for a novel treatment combination against the MDR bacteria *B. cenocepacia,* which potentially has the capacity to prevent infections from spreading to the bloodstream where the concentration of complement components is much higher. This work provides hope for a treatment which prevents cepacia syndrome infections which are extremely difficult to treat and typically results in death.

Future Directions

LPS-binding phages and colistin project

The current work demonstrated short-term efficacy of LPS-binding phages and colistin to control the bacterial population and steer it towards decreased virulence and increased antibiotic susceptibility. A recent study was previously discussed which showed that long-term application of phages to bacteria will effect evolutionary outcomes [203]. Long-term experiments should be conducted challenging *B. cenocepacia* with either KS5 alone or KS5 and colistin tracking the evolution of the bacterial population. This is necessary as in a clinical setting it is common for patients to undergo long treatment courses to clear an infection [207]. Also, pre-treating the bacteria with various antibiotics prior to the application of KS5+colistin may alter evolutionary outcomes of phage steering and should be investigated as this is more representative of a clinical setting.

The CF lung is typically colonized by a diverse community of commensal and pathogenic bacteria. The evolution of various pathogenic bacterial species has been studied in numerous

fashions, demonstrating that *B. cenocepacia* and other pathogens undergo significant evolution during a chronic CF infection [208,209]. CF pathogens are also known to exhibit extreme within species diversification due to the various microenvironments that exist in the CF lung with different levels of nutrients, oxygen, and host defense systems [210]. Treatment trials should be conducted in both CF sputum media and CF mice, which more closely represent the complex lung environment as this will likely affect evolutionary outcomes of the targeted bacteria. Experiments in mice will also confirm the ability of the treatment combination to prevent infections from spreading to the bloodstream.

Intracellular pathogens remain one of the largest challenges of infection control to date and are typically the means with which septic infections commence [211]. *B. cenocepacia* is a facultative intracellular pathogen capable of surviving in both macrophages and lung epithelial cells [77,78]. More specifically, *B. cenocepacia* inhabits the vacuoles of macrophages preventing their maturation into lysosomes [206].The ability of *B. cenocepacia* to invade and survive within macrophages is critical for the bacterium's ability to colonize the bloodstream and cause cepacia syndrome [77].These infections are incredibly difficult to effectively treat due to the inability of traditional antibiotics to cross the eukaryotic cell membrane with high enough efficiency to achieve the necessary concentration of antibiotic for killing [212]. Additionally, drugs must be able to withstand the harsh conditions inside eukaryotic cells and/or cellular compartments to remain active [212]. Recent work on phage interactions with eukaryotic cells has revealed that they are able to cross the eukaryotic cell membrane via transcytosis at extremely low efficiencies of 0.1% [213]. Some phages have also been shown to maintain their bactericidal activity within eukaryotic cells; however, this is highly dependent on where the bacteria are located within the eukaryotic cell and individual phage properties [214,215]. Although phages can enter and remain active in

eukaryotic cells the frequency with which this happens is too low, therefore it is necessary to evaluate other means to deliver phages to their targeted bacteria.

Drug delivery systems are tools which can increase the efficiency with which drugs are delivered intracellularly [211]. Research has focused on macrophages as these are the most common eukaryotic cells pathogens invade and for their natural ability to internalize extracellular materials [211]. These systems can be highly specific, targeting particular eukaryotic cell types and cellular compartments, only releasing their contents upon sensing an inducing cue, ensuring the drug is delivered to the correct location [211]. These cues are commonly low pH, eukaryotic or bacterial enzymes or specific redox states of the surrounding area [211]. Various phage delivery mechanisms have been proposed and/or investigated including using attenuated bacteria, phage encapsulated in a liposome, phage loaded polymeric nanoparticles and lastly using engineered phage conjugated to various compounds recognized by eukaryotic cells for uptake [211,216–218]. For the purposes of the KS5+colistin treatment liposomes will likely be the best delivery method as colistin loaded liposomes have already been shown to be effective against numerous intracellular pathogens residing in macrophages [219,220]. Additionally, previous work on antibiotic loaded micelles revealed that this decreases the toxicity of colistin while maintaining bacterial killing activity allowing for larger doses to be used [221]. Future work should focus on investigating how phage-antibiotic treatments can be efficiently delivered to eukaryotic cells to treat dangerous intracellular pathogens.

Other screen hits

Many other genes were isolated from the initial serum screen, including a putative hopanoid biosynthesis gene. Hopanoids are similar in structure to sterols and exist in a variety of bacterial species contributing, to outer membrane fluidity and permeability as well as other physiological

roles [222]. Experts in the field have debated on whether hopanoids form lipid rafts in prokaryotic organisms like sterols do in eukaryotes [223]. Lipid rafts are domains within a membrane with relatively high stability where certain proteins localize to carry out specific functions [222]. One study confirmed the presence of lipid rafts created by hopanoids in bacteria, and a follow-up study connects these lipid rafts to specific physiological functions [224,225]. In the latter study, the authors demonstrated that the presence of hopanoids in the bacterial outer membrane increases stress tolerance and suggests that they might help modulate the functionality of membrane associated protein complexes, specifically efflux pumps [223,225]. Hopanoids play an important role in bacterial physiology and membrane integrity.

Hopanoids and hopanoid production have been previously evaluated in *B. cenocepacia* as *Burkholderia spp.* have one of the most well-conserved hopanoid biosynthesis pathways [222]. The gene identified in the serum screen was *hpnH,* which has been characterized as catalyzing the second step of the hopanoid biosynthetic pathway in *B. cenocepacia* [226]. Mutants unable to produce complete hopanoids are known to be sensitive to membrane stressors including low pH, detergents and antimicrobial peptides [226,227]. Interestingly, these mutants are also defective in swimming and swarming motility, suggesting that hopanoids may form lipid rafts necessary for the functionality of virulence factors [227]. Lastly, a metabolomic study revealed that *B. cenocepacia* demonstrated increased production of hopanoids when grown in synthetic CF sputum medium than in LB [228]. Altogether, hopanoids present a unique and novel serum-resistance factor which could play a role in a variety of virulence and antibiotic resistance phenotypes requiring future investigation.

An uncharacterized two-component system (2CS) was also identified in the serum-resistance screen. The gene *BCAM0228,* annotated as a 2CS response regulator, was recovered from a

mutant which was unable to survive in serum. Directly downstream of this gene is *BCAM0227,* which is annotated as a hybrid histidine kinase (HHK) response regulator. Canonical 2CSs are composed of a sensor histidine kinase, which typically exist as homodimers in the inner membrane [229]. These proteins sense specific environmental stimuli leading to autophosphorylation, this phosphoryl group is then transferred to the response regulator in the cytosol which will then alter gene expression [229]. However, the identified 2CS is a variation on the traditional 2CS and is a phosphorelay system. These systems are composed of HHKs, which have a C-terminal histidine kinase receiver domain similar to a response regulator [229]. Once the HHK has been autophosphorylated, the phosphoryl group will then be transferred to its receiver domain, then to a histidine phosphotransferase protein, which will finally deliver the phosphoryl to a response regulator [229]. Phosphorelay systems are more common in bacteria with larger genomes and/or inhabit a variety of environments, as they provide additional opportunities for regulation [229,230]. Many 2CSs have been characterized as controlling a plethora of phenotypes, including virulence and antibiotic resistance, sparking interest as potential antibiotic targets [229–234]. Of particular interest are 2CSs, which have been characterized as global virulence regulators controlling the expression of many genes related to virulence processes or factors [230,233,234]. For this reason, the *BCAM0227/8* encoded phosphorelay system should be further characterized for its role in serum resistance and overall virulence and antibiotic resistance.

Lastly, a putative fusaric acid resistance efflux pump system was identified in the screen. This gene is annotated as a putative fusaric acid resistance protein and is located between a putative fusaric acid resistance outer membrane efflux protein (K562_12325) and a membrane protein (K562_12328). K562_12327, otherwise annotated as aaeX has no conserved domains and is a relatively short gene of only 204 base pairs which is equal to the *Escherichia coli aaeX*

gene. In *E. coli* this gene is upstream of *aaeA* and *aaeB,* which are known to play a role in efflux of aromatic carboxylic acid compounds and biofilm formation [73,235]. From here K562_12325-12328 will be referred to as *fusA, fusB*, *fusC, fusD,* respectively, despite the operons putative function. NCBI protein domain databases were searched to gain more insight on the potential function of these genes. *fusA* has a conserved domain from a NodT family outer membrane factor lipoprotein, *fusB* has a FUSC family membrane transporter domain and *fusD* has a conserved domain from the EmrA superfamily which is common for MDR pumps. All together this strongly suggests that these genes encode some sort of tripartite efflux system, most likely an RND family pump. Initially it was hypothesized that this operon plays a role in serum-resistance by effluxing antimicrobial compounds found in human serum. This had been previously demonstrated in *Neisseria gonorrhoeae,* where an efflux pump actively removes AMP LL-37 [236]. However, after noticing a growth defect in this strain in LB media (data not shown), it is more likely that this efflux pump is functioning as a general metabolite waste efflux pump like the *aaeXAB* system in *E. coli* [235]. Further work should focus on identifying the substrate of this efflux pump and its potential role in biofilm formation.

Final Remarks

Identification and characterization of bacterial virulence factors is becoming increasingly more important in the antibiotic resistance era. Understanding the molecular machinery behind how bacteria cause infection can present novel therapeutic targets. Specifically, discovering factors necessary for bacteria to survive in the presence of complement, the body's first line of defense against pathogens, offers advantageous antibiotic targets [181]. Anti-virulence therapeutics are a novel way to decrease the likelihood of resistance developing to a treatment [154,155]. These medications could possibly prevent infections from progressing and spreading throughout the

body in the bloodstream. Although these therapies do not commonly kill the bacteria, when paired with traditional antibiotics they can be extremely effective.

Phage therapy alone has proven to be an effective alternative to traditional antibiotics, however is significantly more potent when administered in combination with antibiotics [149]. Therapeutic compounds consisting of phage and antibiotics targeting a virulence and/or antibiotic resistance factor demonstrate great killing capability [156]. The advent of phage steering allows for more treatment alternatives, which can attenuate and increase antibiotic sensitivity of previously pathogenic and MDR bacteria [156]. However, phages require intense characterization and for the purposes of anti-virulence/phage steering therapies their receptor must be a known virulence and/or resistance factor. This is limiting as many phage receptors are unknown or are not related to virulence/antibiotic resistance. Genetic engineering of phage is challenging, however can be accomplished and could help alleviate this limitation of phages being used as anti-virulence compounds. Also, traditionally, phages and most current antibiotics, are ineffective against intracellular pathogens. Advances in drug delivery systems have provided multiple options for delivering phages and antibiotics to the intracellular site of infection [211,216–218]. While traditional antibiotics remain the gold standard for treating bacterial infections, novel therapies with which resistance is unlikely to develop are necessary. Unconventional drugs such as phages, with novel mechanisms of action are essential to overcome antibiotic resistance and will become the future of infection control.

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