Exploring phage mechanisms that enhance activity

by

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Abstract

In recent years, there has been a renewed interest in using bacteriophages as an alternate agent for the treatment of multi-drug resistant bacteria. One such highly drug-resistant group of bacteria is the *Burkholderia cepacia* complex (Bcc), which causes chronic infections in cystic fibrosis (CF) patients. Bcc consist of twenty-two Gram-negative species that are especially problematic because of their person-to-person transmissibility and an ability to generate a rapidly fatal acute infection. Presently, phage therapy is being developed as an alternative treatment for Bcc infections. However, for phage therapy to be most effective, strategies to enhance efficacy of treatment need to be explored. One of the methods we examined was the synergistic effect of certain antibiotics on Bcc phages KS12 and KS14. Phage-Antibiotic Synergy (PAS) was observed for both of the phages as noted by increases in phage plaque sizes and phage titers. A significant increase in the survival of Bcc-infected *Galleria mellonella* larvae was observed when treated with meropenem or minocycline in combination with phage, as compared to phage or antibiotics alone. Another strategy examined was to test the effects of phage-encoded MazG on phage production. MazG is a pyrophosphohydrolase which can hydrolyse GTP and ATP. MazG encoded by Bcc phages KL1 and AH2 was tested on phage production in overnight cultures of *B. cenocepacia* C6433 and K56-2. Significantly higher numbers of phages were observed upon infection of stationary phase Bcc cells transformed with cloned KL1 or AH2 *mazG.* This indicates that MazG can provide an advantage to phages infecting slow-growing Bcc bacteria, which likely exist in stationary phase

during chronic infections. These findings suggest that different strategies can be employed to increase the activity of phages and thus can be used to enhance the efficacy of phage treatment.

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Table of Contents

List of Figures

List of Tables

List of Abbreviations

ANOVA	analysis of variance
Ara4N	4-amino-4-deoxyarabinose
BCC	*Burkholderia cepacia* complex
BRED	bacteriophage recombineering of electroporated DNA
BZK	benzyldimethylalkylammonium chloride
℃	degrees Celsius
CAZ	ceftazidime
CF	cystic fibrosis
CFTR	cystic fibrosis transmembrane conductance regulator
CFU	colony forming units
CGD	chronic granulomatous disease
CIP	ciprofloxacin
CK13	cytokeratin-13
DLA	double-layer agar
EPS	Exopolysaccharides
ET-12	Electrophoretic Type 12
g or mg or μg	gram or milligram or microgram
IAA	indole-3-acetic acid
KO	D-glycero-α-D-talo-oct-2-ulopyranosylonic acid
LB	Luria-Bertani
LD50	50% lethal dose

LEV	levofloxacin
LPS	lipopolysaccharide
MBEC	minimum biofilm eradication concentration
MDR	multidrug-resistant
MEM	meropenem
MIC	Minimum inhibitory concentration
MIN	minocycline
MOI	multiplicity of infection
PAS	Phage-Antibiotic Synergy
PBS	phosphate-buffered saline
PFU	plaque forming units
PHDC	Philadelphia-District of Columbia
RND	Resistance-Nodulation-Division
ROS	reactive oxygen species
SAR	systemic acquired resistance
SCI	Single colony inserts
SD	standard deviation
TET	tetracycline
TNF	tumor-necrosis factor
XDR	extremely drug-resistant

Chapter 1 : Introduction

Burkholderia cepacia complex

Burkholderia cepacia was discovered as the causative agent of onion rot in 1950 by William H. Burkholder. He noted that the rotted onion bulbs had a smelly, yellow appearance that was different from the already known watery, glassy appearance caused by *Pseudomonas alliicola*. He described the bacteria as a strictly aerobic, Gram-negative, uni- to triflagellate rod with `rounded ends and named it *Pseudomonas cepacia* (*B. cepacia*). He tested the *B. cepacia* on various media and found it able to grow using different nutrients as sources of carbon and nitrogen (Burkholder, 1950). It is now known that *B. cepacia* is obligately aerobic and can survive on a wide range of nutrients. It can use a large number of organic compounds as its sole carbon and energy sources for growth, including a large variety of carbohydrates, monocarboxylic and dicarboxylic acids, monoalcohols and polyalcohols, aromatic compounds, amino acids, and amines (Stanier et al., 1966; Lessie and Gaffney, 1986; Vermis et al., 2003). This allows *B. cepacia* to adapt to a wide range of environments.

In nature, members of the *B. cepacia* complex (Bcc) are ubiquitous and have been isolated from soil, plants and water (Fiore et al., 2001; Miller et al., 2002; Vermis et al., 2003). The *Burkholderia* genus was proposed by Yabuuchi et al. in 1992 and contained the seven species *B. cepacia*, *B. mallei*, *B. pseudomallei*, *B. caryophylli*, *B. gladioli*, *B. pickettii* and *B. solanacearum.* These species were moved

from rRNA group II of the *Pseudomonas* genus based on 16sRNA sequences, DNA–DNA homology values, cellular lipid and fatty acid composition, and phenotypic characteristics (Yabuuchi et al., 1992). Additional *Pseudomonas* species were reclassified as *Burkholderia* species by Gillis et al. using DNA-rRNA hybridization studies with *B. cepacia* rRNA probes (Gillis et al., 1995). Following this, phenotypically similar but genotypically different species were divided into five distinct genomovars (Vandamme et al., 1997). Following this, four more genomovars were added and based on tests that allowed discrimination, all have been formally named as distinct species. Over the past several years, more species, isolated from either environmental or human clinical sources, have been found to belong to Bcc bringing the current number to twenty (Spilker et al., 2015).

Environmental importance

Bcc are found ubiquitously in the environment. They have been isolated from the soil, water and plants, especially in the rhizosphere of crops (Mahenthiralingam et al., 2005). Though *B. cepacia* was initially isolated from infection in onions, there are several beneficial uses attributed to them. Members of the Bcc can colonize plant roots resulting in stimulation of root growth which helps plants absorb nutrients (Chiarini et al., 1998). In these plants, Bcc exist as symbionts protecting the plants from fungal infections and helping growth by mobilizing nutrients. Some endophytic Bcc species promote growth by fixing nitrogen (Reis et al., 2000). Other ways Bcc could help plant growth is through production of the plant hormone / auxin indole-3-acetic acid (IAA) and the prevention of growth of deleterious bacteria. It is

hypothesized that the Bcc induce systemic resistance in plants similar to pathogen-induced systemic acquired resistance (SAR) (van Loon et al., 1998). The rhizobacteria-mediated induced resistance in plants has been attributed to bacterial determinants such as lipopolysaccharides, siderophores, and salicylic acid. Members of the Bcc can also suppress soil-borne disease occurrence in plants through their antagonistic effect on soil-borne pathogens (Thomashow et al., 1990; Haas and Défago, 2005). Some Bcc species can produce antimicrobial compounds such as pyrrolnitrin, which was demonstrated to inhibit the growth of mould on fruits such as apples and pears (Janisiewicz, 1988). The Bcc can also prevent damping-off disease of seedlings caused by *Pythium* species and *Rhizoctonia solani*, which affects a wide variety of crop plants (Hebbar et al. , 1992). Kang *et al*. (1998) discovered a non-ribosomal synthesized lipopeptide AFC-BC11 which inhibits the growth of numerous fungi. These compounds have been proposed as supplements or alternatives to chemical fungicides (Parke and Gurian-Sherman, 2001). In addition to their beneficial association with plants, the Bcc has also been shown to degrade ground water contaminants (Sangodkar et al., 1988; Yee, et al., 1998). This is due to their ability to use >200 compounds as a carbon source. These beneficial attributes have led to the commercial use of several Bcc strains as biological control agents in the United States (Parke and Gurian-Sherman, 2001). However, after a risk assessment in 1999 of Bcc bacteria as model opportunistic pathogens with biopesticidal uses, the United States Environmental Protection Agency placed a moratorium on the new registrations of products containing these bacteria (Mahenthiralingam et al., 2008).

Some *Burkholderia* species have been identified as plant pathogens. A few well-known species are *B. caryophylli*, which has been reported to cause onion rot (Ballard et al., 1970), *B. plantarii,* which causes blight of rice seedlings (Azegami et al., 1987), and *B. glumae,* which is the main causal bacterial agent of rot of rice grains and seedlings (Goto and Ohata, 1956; Nandakumar et al., 2009).

Human significance

The Bcc do not generally infect healthy individuals but have been associated with respiratory infections in those who are immuno-compromised or suffer from cystic fibrosis (CF) or chronic granulomatous disease (CGD) (Isles et al. 1984; O 'Neil et al. 1986). CF is an autosomal recessive disorder that is most common in Caucasian population affecting 1 in 3000 individuals (O'Sullivan and Freedman, 2009). CF is caused by a mutation in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, which mainly functions as a chloride channel but also maintains other regulatory roles such as the inhibition of sodium transport. A defect in the CFTR causes abnormalities in the transport of electrolytes across epithelial cells within the airways, pancreas, sweat ducts, and the intestinal tract (Robinson and Bye, 2002). This results in the accumulation of thick mucus, which inhibits mucociliary clearance in the lungs of CF individuals thus providing favorable conditions for colonization by bacteria (Robinson and Bye, 2002). In healthy individuals, transient bacteria are cleared by normal mucociliary activity (Govan and Deretic, 1996).

CGD occurs in 1 in 250,000 individuals in North America and results from a mutation in the gene encoding nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, which is involved in the generation of reactive oxygen species (ROS) within phagocyte lysosomes (Johnston and Newman, 1977). Without ROS, phagocytes cannot kill endocytosed microorganisms, which results in increased recruitment of phagocytes and a prolonged, damaging inflammatory response.

Staphylococcus aureus and *Haemophilus influenza* are two of the most common pathogens found in young CF patients. These are replaced by *Pseudomonas aeruginosa* infections causing 80% of infections in adult CF patients (Lyczak, et al., 2002). In the past 30 years, Bcc have emerged as an important opportunistic pathogen in cystic fibrosis (CF) patients. *B. cepacia* was isolated from the respiratory tract in an increasing percentage of CF patients (Isles et al., 1984). This prevalence was confirmed by another study soon after (Tablan et al., 1985). These reports described the virulent nature of *B. cepacia* infections, which in severe cases leads to a rapid deterioration of pulmonary function and a significantly reduced life expectancy for CF patients. This has been termed "the cepacia syndrome" and its disease progression is in direct contrast to chronic infections with other CF pathogens such as *P. aeruginosa*. Some of the symptoms associated with cepacia syndrome are high fever, severe progressive respiratory failure, a decline in leukocytes and erythrocytes, and in some cases, bacteremia resulting in rapid death. As compared to *P. aeruginosa*, the Bcc infects only a small proportion of CF patients, however, the Bcc have been shown to have a significant impact on survival (Liou et al., 2001). In addition to the virulent nature of these infections, it was also discovered that that Bcc bacteria are

capable of transmission through social contact, as confirmed by molecular epidemiology. One group of *Burkholderia cenocepacia* strains are known as Electrophoretic Type 12 (ET12), and these were found to be responsible for an epidemic among CF patients in Eastern Canada and the United Kingdom between 1986 and 1992. Social contact between patients was found to be the cause of the spread (Govan et al. 1993). Another well-known group of *B. cenocepacia* strains that has been associated with CF patients in the eastern United States is the Philadelphia-District of Columbia strain, abbreviated as PHDC (Coenye and LiPuma, 2003). These strains have also been found in CF patients in three European countries: France, Italy and the UK, suggesting that they are widely distributed (Coenye et al., 2004). The spread of infection through social contact is considered an alarming problem for the CF community, and infected individuals are asked to follow stringent infection control practices to prevent patient-to-patient transmission (Saiman and Siegel, 2004).

Although social contact is one of the frequent modes of spreading of Bcc in CF patients, it is not the only one. Bcc can also be acquired from contaminated surfaces such as nebulizers, nasal sprays, ultrasound gels, hospital water, and disinfectants, since the Bcc can survive on various surfaces for several hours (Drabick et al., 1996; Vonberg and Gastmeier, 2007). Recently, the Bcc have been observed to have an intrinsic resistance to benzyldimethylalkylammonium chloride (BZK), which is a compound found in most commercial disinfectants and industrial sanitizers (Ahn et al., 2016). This resistance makes bacteria of the Bcc one of the most difficult microorganisms to eradicate. Although there have been no reports to

definitively prove acquisition from the environment, some Bcc strains, such as PHDC, have been isolated from soil, which may suggest this route of transmission is a possibility (LiPuma et al., 2002).

Distribution in CF

Taxonomically, the Bcc comprises at least twenty phenotypically similar but genotypically different species (Spilker et al., 2015). Most of the Bcc can colonize CF patients but *B. multivorans* (formerly genomovar II) and *B. cenocepacia* (formerly genomovar III) account for 70% of the infections (LiPuma et al., 2001). In Canada and some European countries, *B. cenocepacia* has been recovered in higher proportions from CF patients. However, over recent years there has been a shift in prevalence to *B. multivorans*, with an increased number of infection cases over *B. cenocepacia* (Reik et al., 2005). It has been implied that this is because of the stringent measures placed in effect to control patient-to-patient spread of the epidemic *B. cenocepacia* strains. Although *B. multivorans* account for a large number of infections, it is uncommon to identify strains from this species being shared by multiple CF patients. Transient co-infection with different strains or species of the Bcc sometimes occurs in some patients, but chronic infection by a Bcc strain almost always occurs with a single strain (Yang et al., 2006).

Virulence and Pathogenesis

Several virulence factors have been described for the Bcc. After entering the respiratory tract, Bcc bacteria need to bind to host mucosal or epithelial surfaces. In

CF patients, thick mucous provides an ideal environment for bacteria to adhere to and cause infection which is made easier by defective mucous clearing and increased inflammatory response (Boucher, 2007). Though a number of virulence factors have been identified for the Bcc, their exact role in bacterial pathogenesis is not clear.

Extracellular factors

Extracellular lipase, metalloproteases and serine proteases are thought to be involved directly in the interaction with epithelial cells. Phosphoplipase C is an enzyme that cleaves phosphatidylcholine, a major lung surfactant, to yield phosphorylcholine and diacylglycerol, and has been associated with cytopathology of lung tissue. It has been reported that *B. cepacia* lipase inhibits the phagocytosis of rat alveolar macrophages (Straus et al., 1992). Lipase production is broadly distributed in the members of the Bcc and is implied to play a role in invasion of the host epithelial cells. Inhibition of lipase in *B. cenocepacia* resulted in reduced invasion of host cells. (Mullen et al., 2007). Metalloproteases and serine proteases play a role in proteolysis of the extracellular matrix and are produced by many members of the Bcc. Some of the Bcc species, namely *B. cepacia*, *B. cenocepacia*, *B. stabilis*, *B. ambifaria*, and *B. pyrrocinia* produce metalloproteases such as zinc metalloprotease encoded by *zmpA* and *zmpB*, which have been shown to contribute to virulence in chronic infections (Corbett et al., 2003; Kooi et al., 2006). A periplasmic serine protease encoded by *htrA* has recently been identified as a virulence factor in *B. cenocepacia* strain K56-2. It was shown to be important for infection in a rat agar bead model of chronic infection (Flannagan et al., 2007).

Extracellular structures

Bacterial surface structures like the lipopolysaccharide (LPS), flagella and pili are also important in the interaction with the CF host. LPS is a major component of cell surface in gram-negative bacteria and consists of O antigen polysaccharide, core oligosaccharide, and lipid A. The LPS of the Bcc is highly inflammatory and can cause cell damage. *B. cepacia* LPS affects levels of pro-inflammatory cytokines, tumor-necrosis factor (TNF) and interleukins. Both interleukins IL-6 and IL-8 were significantly up-regulated as were levels of TNF (Hutchison et al., 2000; Bamford et al., 2007). *B. cenocepacia* LPS can activate immune cells through Toll-like receptor 4-mediated signaling (Bamford et al., 2007). Bcc LPS has been found to be five times more toxic than the LPS of *P. aeruginosa* which is another major CF pathogen (Shaw et al., 1995). The structure of Bcc LPS differs from the LPS of other gram-negative bacteria. It contains less phosphate with the unusual sugar D-glycero-α-D-talo-oct-2-ulopyranosylonic acid (KO) present in the inner core oligosaccharide, and 4-amino-4-deoxyarabinose (Ara4N) residues bound to phosphates of the lipid A. These modifications lower the anionic charge of the Bcc cell surface inhibiting the binding and subsequent effects of cationic antimicrobial peptides (Vinion-Dubiel and Goldberg 2003). It has been shown that amino-arabinose enzymes which modify the LPS lipid A are essential for viability in *B. cenocepacia,* implying that Ara4N modification might be important for maintaining outer membrane stability (Ortega et al., 2007).

Flagella, pili and a 22kD adhesin help the Bcc bacteria with motility and adherence to host cells, and are essential for invasion of epithelial cells of the

respiratory tract. Flagella have also been shown to be involved in bacterial pathogenesis aside from their main role in motility. Flagella consist of three parts: a basal body, the hook, and the filament. A *fliG* mutant strain of *B. cenocepacia* strain J2315 showed reduced invasion of host lung cells (Tomich et al., 2002). J2315 belongs to the epidemic ET12 Bcc group. The mutation in *fliG,* which encodes the basal body of the flagella, caused the bacteria to become non-motile. Disruption of flagella in *B. cenocepacia* K56-2 resulted in a non-motile strain that was found to be avirulent in a murine agar bead model of infection. The major flagellin subunit *fliCII* in K56-2 was mutated and tested for virulence by examining cytokine production and inflammation response in mice. All the mice that were infected with the *fliCII* mutant K56-2 survived whereas mice infected with wildtype K56-2 had only a 60% survival rate three days post-infection (Urban et al., 2004).

There have been five types of pili identified in Bcc strains but only cable pili have been associated with *B. cenocepacia* epidemic strains. Cable pili are found peritrichously, can tether aggregates of cells, and have been observed to increase adherence to epithelial cells (Sajjan et al., 2000). These, along with adhesin AdhA, have been shown to bind cytokeratin-13 (CK13), an intermediate filament protein as a host cell receptor. An increase in CK13 has been observed in CF respiratory tract epithelial cells (Sajjan et al., 2000). This might provide an advantage to *B. cenocepacia* strains that can utilize CK13 as a receptor to establish an infection. In the epidemic *B. cenocepacia* ET12 strains, cable pili, and the *B. cenocepacia* pathogenicity island (discussed later) occur in a unique combination making it more virulent than other Bcc members (Mahenthiralingam et al., 2005).

Exopolysaccharide

Exopolysaccharides (EPSs) are branched, repeating polysaccharide subunits that are secreted by bacteria to help in their adaption to different stressful environments and the establishment of symbiotic and pathogenic relationships with the hosts. EPS has also been suggested to be a major component of bacterial biofilms. It has been proposed to play a role in imparting protection against the host immune system and antimicrobial agents. Desai *et al.* (1998) tested the susceptibility of biofilm- and planktonic- grown Bcc bacteria to different antibiotics. They observed a 15-fold increase in resistance against ciprofloxacin and ceftazidime for biofilm-grown Bcc cells over their planktonic counterparts during exponential phase growth (Desai et al., 1998). These findings demonstrate that Bcc EPS is capable of forming a protective barrier that provides resistance to various antibiotics.

Members of the genus *Burkholderia* produce seven different kinds of EPS. Bcc species produce four types of EPS: PS-I, cepacian (PS-II), levan and an unnamed polymer (Chiarini et al., 2004). Some strains produce a single exopolysaccharide while others might produce more than one. *B. cenocepacia* strain C9343, for example, produces PS-I, cepacian (PS-II), and α-1,6-glucan (Ferreira et al., 2011). Most of the Bcc strains, both clinical and environmental, have been found to produce cepacian. About 80% of the Bcc clinical isolates from Portuguese CF patients were found to produce variable amounts of cepacian (Richau et al., 2000). Cepacian is made up of a branched acetylated heptasaccharide repeat-unit with D-glucose, D-rhamnose, D-mannose, D-galactose, and D-glucuronic acid in the ratio of 1:1:1:3:1 (Cescutti et al., 2000). A virulent mutant of *B. cenocepacia* C1394, which produced

increased amounts of cepacian, was observed to have increased persistence in a mouse infection model (Chung et al., 2003). These observations were confirmed by another study that showed that the EPS produced by a *B. cenocepacia* clinical isolate interfered with phagocytosis of bacteria by human neutrophils and facilitated bacterial persistence in a mouse model of infection (Conway et al., 2004). In addition, cepacian was also found to inhibit the function of human neutrophils in vitro and also inhibited chemotaxis and the production of oxygen reactive species, both essential components of innate neutrophil-mediated host defenses (Bylund et al., 2006).

Genomic islands

Bcc bacteria have large genomes ranging from 6 to 9 Mb, made up of multiple large replicons. The genome sequence of *B. cenocepacia* J2315 has recently been completed. It consists of three large chromosomal replicons and a 92 kb plasmid. It encodes more than a dozen genomic islands (Holden et al., 2009). One genomic island, the *Burkholderia cepacia* epidemic strain marker (BCESM) has been shown to play a role in virulence. BCESM appears to act as a negative transcriptional regulator and is encoded by many strains isolated from individual CF treatment centres (Baldwin et al., 2004). The BCESM genes encode an amidase (AmiI), an outer membrane porin (OpcI), and the cciRI quorum-sensing system (Baldwin et al., 2004). Recently, a different genomic island has been identified in *B. cenocepacia* K56-2 which contains a putative regulatory protein called Pbr (Ramos et al., 2010). A K56-2 *pbr* mutant exhibited a number of phenotypes including impaired survival to either oxidative or osmotic stress, or aromatic amino acid or prolonged nutrient starvation

periods. In addition, the *pbr* mutant exhibited decreased virulence in the nematode *Caenorhabditis elegans* infection model. The *pbr* mutant also shows an inability to synthesize phenazines, which are antibiotics that are thought to interfere with electron transport and impair respiration (Ramos et al., 2010).

Antibiotic resistance

Bcc strains demonstrate a high level of innate resistance to antibiotics, which severely limits therapeutic options and therefore may be considered an important virulence factor. Bcc are capable of using penicillin G as a sole carbon source (Beckman and Lessie, 1979). This fact highlights the enormity of antibiotic resistance commanded by Bcc bacteria. Bcc are intrinsically resistant to aminoglycoside antibiotics and have multiple mechanisms for resistance to other classes of antibiotics (LiPuma 1998). Resistance can be attributed to primary resistance mechanisms, such as decreased permeability of the bacterial cell membrane structure, enzymatic modification, alteration of drug targets, and the activation of efflux pumps. Enzymatic modification of the drugs and alteration of drug targets results in resistance to specific antibiotics or a class of antibiotics whereas decreased permeability provides broad-spectrum protection against different antibiotics.

Enzymatic modification and altered drug targets have been observed for β-lactam and aminoglycoside antibiotics. A majority of Bcc strains have inducible chromosomally encoded β-lactamases (Chiesa et al., 1986). In addition, altered penicillin-binding proteins have been reported as another mechanism of β-lactam resistance. Most Bcc strains possess a resistant dihydrofolate reductase enzyme,

which is the targeted enzyme for the antibiotic trimethoprim (Burns et al., 1989). The outer membrane has decreased permeability relative to some other bacteria and the unique structure of the LPS results in resistance to multiple classes of antibiotics, including β-lactams and cationic peptides (LiPuma 1998; Burns et al., 1989). As mentioned earlier, the modifications in the LPS lower the anionic charge of the Bcc cell surface, which inhibits the binding and subsequent effects of cationic antimicrobial peptides (Vinion-Dubiel and Goldberg, 2003).

Bcc efflux pumps have been associated with resistance to chloramphenicol, quinolone antibiotics, and trimethoprim (Burns et al., 1996; Zhang, et al., 2001). The genome of *B. cenocepacia* J2315 contains coding sequences for all five major families of efflux systems (Holden et al., 2009). One of the major efflux systems used by bacteria is the Resistance-Nodulation-Division family (RND) efflux pump (Poole et al., 2004; Bazzini et al., 2011). RND efflux pumps are tripartite complexes formed by an outer membrane channel protein, a periplasmic membrane fusion protein and an inner membrane transporter protein. Bazzini et al. identified 16 putative RND pumps in J2315 genome, and the functionality of some of these efflux pumps has been studied. It has been shown that the transfer of the *ceo* operon (RND-10) to an antibiotic susceptible strain conferred resistance to chloramphenicol, trimethoprim, and ciprofloxacin in *B. cenocepacia* isolates tested (Nair et al., 2004). Additionally, Buroni et al. (2009) demonstrated that deleting two other pumps RND-3 and RND-4 in J2315 resulted in an increased sensitivity to aztreonam, chloramphenicol, gentamicin and various fluoroquinolones. Recently, Tseng et al. confirmed high-level of expression of efflux pumps in Bcc isolates and found that mutations in the RND-3

efflux pump regulator gene was one of the major causes of efflux pump activity, resulting in the resistance to antibiotics in clinical Bcc isolates (Tseng et al., 2014). These results suggest that RND pumps contribute significantly to the resistance exhibited by the Bcc against different classes of antibiotics.

Current treatment for CF infections

Over the past four decades, the survival of patients with CF has improved dramatically with median survival now approximately 40 years or age. The therapy for CF involves traditional methods such as nutritional support and mechanical mucus clearance along with aggressive antibiotic treatments aimed to suppress or eradicate bacterial colonization. In addition, new ways to improve muco-ciliary clearance and reduce inflammation are being used (George et al., 2009). CF patients colonized with Bcc bacteria have a much poorer outcome following lung transplantation than their non-infected counterparts, which is the only therapeutic option for patients with end-stage pulmonary disease (Aris et al. 2001; LiPuma 2001).

CF infections with Bcc increase patients' morbidity and mortality rates. Bcc have a very high innate resistance to antibiotics and can develop further resistance over the course of treatment. This limits the choice of antibiotics that can be used for therapy. Antimicrobial agents to which Bcc bacteria are often susceptible include semisynthetic penicillins (such as ticarcillin), carbapenems (such as meropenem), cephalosporins (particularly ceftazidime), quinolones (such as ciprofloxacin) and trimethoprim/sulfamethoxazole (Speert, 2002). CF patients receive multiple courses of oral, intravenous, and aerosolized antibiotics (Gibson et al., 2003). Combination

therapy is proposed as a way to circumvent the problem of antibiotic resistance evolution. Although combination of two antimicrobial agents is generally recommended for treatment of a CF pulmonary exacerbation (Gibson et al., 2003), there are relatively few published reports describing treatment of Bcc bacteria in CF patients.

Blumer *et al*. studied the safety and efficacy of antibiotics meropenem and tobramycin in 19 patients with Bcc bacteria. Even though a reduction in bacterial density was observed, there was no reported improvement in lung function (Blumer et al., 2005). Aaron et al. tested 10-15 different antibiotics against 119 multi-drug resistant Bcc isolates using a method called multiple combination bactericidal test (MCBT). The combinations resulted in either synergistic, additive or antagonistic effects. Their findings showed that all of the Bcc isolates were inhibited by a combination of three drugs whereas 8% were resistant to two-drug combinations and 50% were resistant to single drug treatment (Aaron et al., 2000). The double antibiotic combinations of meropenem–minocycline, meropenem–amikacin and meropenem–cefazidime were found to have the most bactericidal effects whereas triple-antibiotic combination of tobramycin, meropenem, and an additional antibiotic were most effective (Aaron et al., 2000).

Another study by Zhou et al. tested 18 antimicrobials and carried out checkerboard synergy tests for 23 combinations against *B. cepacia* isolates. Minocycline, meropenem, and ceftazidime were noted to be the most effective (Zhou et al., 2007). At present, current treatment protocols include tobramycin, trimethoprim, ceftazidime, meropenem and minocycline, although Bcc resistance to

these antibiotics is not uncommon (Peeters et al., 2009). This illustrates that the management of multidrug-resistant Bcc bacteria remains as a challenge in the CF population and that there is a need for newer treatment options.

Phage Therapy

One of the proposed alternative strategies to combat multi-drug resistant bacteria is to use bacteriophages for therapy. Bacteriophages (or phages) are viruses that specifically infect bacteria and can lyse these cells to cause death. Phages are specific for the host and are found abundantly in the environment.

History

Bacteriophages were co-discovered by Frederick W. Twort in London (Twort, 1915) and Felix d'Herelle in Paris (d'Herelle, 1917) in the early 1900s. Twort noticed zones of clearance with micrococcus bacteria while growing viruses under laboratory conditions, which he described as transmissible glassy transformation (Twort, 1915). The article published by d'Herelle reported treatment of bacterial dysentery using an oral phage preparation (d'Herelle, 1917). Following this discovery, d'Herelle performed a number of trials and field experiments and determined the effectiveness of bacteriophages as antimicrobial agents. Some of his early experiments included using phage to treat avian typhosis in chickens, shigella dysentery in rabbits and bacillary dysentery in humans. He found phages to be many times more potent against bacteria than any agent known at the time (d'Hérelle, 1921). Reports of the success of d'Herelle motivated the British medical officer Lieutenant Colonel

Morison to use phage for the treatment of cholera epidemics in India from 1930 to 1935. In 1932, Morison reported fewer cholera deaths in the phage-treated Naogaon region compared to the 474 deaths recorded in the Habiganj region which refused treatment (Summers, 2001).

Reports of these successes led several commercial laboratories and companies in the United States, France and Germany to produce phage preparations (Straub and Applebaum, 1933; Merril et al., 2003; Kutateladze and Adamia, 2010). In the 1940s, the Eli Lilly Company (Indianapolis, Ind.) produced seven phage products for human use, including preparations targeted against staphylococci, streptococci, Escherichia coli, and other bacterial pathogens. These preparations were made using sterile broth cultures of the targeted bacteria that had been lysed by phages, or the same preparations in a water-soluble jelly base. They were used to treat various infections, including abscesses, suppurating wounds, vaginitis, acute and chronic infections of the upper respiratory tract, and mastoid infections (Sulakvelidze et al., 2001). Although there were reports of successful phage treatment, there were some failures too. These inconsistencies in phage treatment success were due to a lack of understanding of phage biology, such as phage specificity, which resulted in the use of a phage for the wrong bacterial strain or species. Other problems included improper phage preparations resulting in phage inactivation or an failure to produce high volumes of purified phage (Sulakvelidze et al., 2001). Around the 1940s, broad-spectrum antibiotics were developed, which made bacterial infections more readily treatable. This provided an alternative to phage therapy that was not as expensive and easily available. After the discovery of antibiotics, the mixed success of phage

therapy at the time led to its abandonment in the U.S and Western Europe (Merril et al., 2003). Despite the widespread use of broad-spectrum antibiotics, research into the therapeutic use of bacteriophages has continued in Eastern Europe, particularly in Tbilisi, Georgia at the Eliava Institute. This institute was founded in 1923 by Giorgi Eliava and Felix d'Herelle (Sulakvelidze et al., 2001).

Mechanism of phage infection and therapy

Bacteriophages infect bacteria, replicate, and lyse the host cells to release the phage progeny. Typically, a bacteriophage consists of a head (or capsid) containing the genetic material, and a contractile or non-contractile tail. Bacteriophages can be classified as virulent (lytic) or temperate (lysogenic) depending upon their lifestyle. Lytic phages infect the host bacteria and lyse the bacterial host, whereas temperate phages infect the bacterial cell and integrate their genetic material into the host genome, which gets replicated passively along with the bacterial genome. These phages remain dormant as a prophage until adverse conditions trigger the phage to reenter the lytic lifestyle (Kropinski, 2006).

Lytic phages are considered to be the best candidates for phage therapy (Skurnik and Strauch, 2006). The first step in phage infection is binding to a receptor on the host bacteria. In gram-negative bacteria such as the Bcc, the LPS or outer membrane proteins can serve as phage receptors (Lindberg, 1973). This is followed by penetration of the phage genome into the bacterial cell. This process varies with different phage groups. Phages can be classified as belonging to *Myoviridae*, *Siphoviridae*, or *Podoviridae* family depending on the tail lengths. Phages belonging

to these three families have medium-length contractile, long non-contractile, or short non-contractile tails, respectively (Ackermann, 2003). Once receptor recognition happens, the phages attach to the bacterial surface using the tail fibers and base plate. In general, *Myoviridae* phages insert genetic material across the bacterial cell membranes with a syringe-like movement of the tail. After the base plate is attached irreversibly, the phage uses energy from ATP to drive the contraction of the tail and insertion of its genetic material (Rossmann et al., 2004; Sharma et al., 2017). *Podoviridae* and *Siphoviridae* phages do not possess a contractile tail and insert their genetic material using capsid pressure, after enzymatically degrading a portion of the bacterial cell membrane (Rakhuba et al., 2010). Once in the cytoplasm, the phages' early gene products are expressed, which disrupt normal bacterial processes and begin replication of the phage genome. The phages' late gene products are responsible for phage assembly and maturation, and host cells lysis (Skurnik and Strauch, 2006).

Temperate phages are less preferred for phage therapy as they can remain as dormant prophages for long period of time and do not kill the bacterial cells immediately, as required for efficient treatment (Skurnik and Strauch, 2006). Since temperate phages can produce proteins that inhibit infection by similar phages, the infected bacterial cell exhibits a phenotype termed "superinfection immunity". As well, incorporation of phage genetic material into the host bacterial genome can cause other phenotypic changes in the infected bacteria, termed "lysogenic conversion", which may result in the bacteria becoming more virulent (Sharma et al., 2017). The inserted genomes of temperate phages are also now capable of transducing nearby

bacterial genes to a new host cell, including those capable of increasing virulence or antibiotic resistance (Lynch et al., 2010a). Despite these drawbacks, temperate phages have been shown to be effective in experimental phage therapy (Seed and Dennis, 2009; Lynch et al., 2010a). Phage treatment of a clinical infection consists of three basic steps: identification of the causal bacteria, choice of phage or phages for therapy, and appropriate administration of the phage (Semler et al., 2011).

Advantages of phage therapy over traditional antibiotics

Despite the potential limitations associated with phage therapy, there are several advantages to using phages for treatment of clinical infections. Phages interact with the bacteria in a completely different way from antibiotics. Phages are more specific than broad-spectrum antibiotics and usually infect a single species or strain of bacteria. This permits the treatment of an infection without adversely affecting the normal microflora of the patient (Semler et al., 2011). There have been no reports of side-effects caused by phages as compared to antibiotics, which have many including allergies and intestinal disorders (Rhoads et al., 2009). Another advantage to using phages is that phages follow different pharmacokinetics than drugs. Phages can replicate after the original dose and increase exponentially, as long as there are host cells present and available, until the infection is cleared (Semler et al., 2011). This is in direct contrast with drugs, where the drug titres must be maintained by repeated doses. As with drugs, it is possible that bacteria can develop resistance to phages. However, unlike antibiotics, phages can co-evolve with the host bacteria to adapt to mutational changes in the host cells (Stern and Sorek, 2011).

Another way to circumvent the occurrence of resistance would be to use a phage cocktail made up of multiple phages that target different receptors, or phages plus antibiotics. Several studies have shown that phage cocktails are efficacious in preventing the development of resistance (Chan et al., 2013).

Renewed interest in phage therapy

Even though phage therapy was abandoned in the West after the introduction of broad-spectrum antibiotics, the treatment of infections using phages has continued in Eastern Europe, especially in Poland and the Republic of Georgia (Carlton, 1999). In the mid-1980s, a Polish group led by Prof. S. Slopek and his colleagues Dr. M. Mulczyk and Dr. B. Weber-Dabrowska at the Hirszfeld Institute of Immunology and Experimental Therapy, published a series of reports (Slopek et al., 1987). These papers reported phage treatment of suppurative or pus-causing bacterial infections, which included empyemas, peritonitis, and osteomyelitis in humans. Most of these cases involved chronic infections, were resistant to all available antibiotics, and had used phage therapy as a last resort. The infections were caused by the bacterial pathogens *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Escherichia coli*. These papers reported the success of phage therapy for approximately 90% of the cases, where a cessation of the suppuration was observed (Slopek et al., 1987). Recently, a review of the institute's performance recounted phage treatment of 1307 patients with suppurative bacterial infections caused by multidrug-resistant bacteria of different species. Phage therapy was reported to be highly effective with full recovery noted in nearly 86% of the cases (Weber-

Dąbrowska et al., 2000). Unfortunately, there were no placebo control groups conducted for these treatments, which calls into question the rigour of the experimental design.

Besides Poland, phage therapy continues to be practiced at the Eliava Institute in the Republic of Georgia. Here, phage therapy is a part of the standard medical practice used for both prophylactic and treatment purposes. Over several decades, there has been reports of successful phage treatment for infections of the lungs, eyes, skin and wounds, the gastrointestinal and urinary tracts, and various chronic bacterial diseases (Sulakvelidze and Morris, 2001). However, most of these reports are anecdotal and lack statistical analysis. The majority of phage treatments carried out at Eliava involve phage cocktails (Kutter et al., 2010). Two of the cocktails available commercially from Eliava are 1) Pyophage, which targets bacteria of suppurative infections, including *S. aureus*, *E. coli*, *P. aeruginosa*, two *Proteus* species, and several species of *Streptococcus*; and 2) Intestiphage, which targets approximately 23 different enteric bacteria, as well as gut-derived strains of *S. aureus* and *P. aeruginosa*. Intestiphage is used to treat traveler's diarrhea and other gastrointestinal upsets by both native Georgians as well as visitors (Kutter et al., 2010). Even though the use of phages for therapy in Poland and Georgia have lacked proper controls, which would be required to prove efficacy as in a clinical trial, the encouraging results still suggest that phage therapy holds great promise.

The rise of antimicrobial resistance and lack of development of new antibiotics in the recent times (Butler et al., 2017) has resulted in a renewed interest in phage therapy against multi-drug resistant bacteria in the West. Technological

advancement has led to an increase in basic knowledge and understanding of phages (Keen, 2015), which has fostered significant development of the field of phage therapy. There have recently been several reports about the successful treatment of infections in animals and humans using phages (Roach and Debarbieux, 2017; UC San Diego Health, 2017). However, before using phages for widespread treatment of human infections, safety and efficacy needs to be determined, and this is usually achieved through the successful completion of animal and clinical trials.

One of the most well-known studies on phage therapy was carried out by William Smith and colleagues in Great Britain in the early 1980s. They reported the successful treatment of *E. coli* infection in mice using phages. They found that a single dose of *E. coli* specific phage was more effective than multiple doses of antibiotics tetracycline, ampicillin, chloramphenicol, or trimethoprim plus sulphafurazole (Smith and Huggins, 1982). This was followed by another study by the same group where farm animals were treated with a mixture of phages for *E. coli* induced diarrhea. In this study, it was found that the phage treatment reduced the bacterial numbers in the alimentary canals of calves, lambs and piglets, and all the animals survived the infection (Smith and Huggins, 1983).

Another group led by James Soothill investigated the use of phages against antibiotic resistant infections caused by *P. aeruginosa,* especially those associated with burn patients. They found that phage-treated skin grafts in a guinea pig model were protected from the *P. aeruginosa* infections (Soothill, 1994). Besides *Pseudomonas*, another organism that can cause antibiotic resistant infection in burn patients is *Acinetobacter baumanni.* Soothill and colleagues also tested phage

treatment against these in generalized infections of mice as a potential therapy in human infections. They observed protection against several fold LD_{50} numbers of the bacteria, and confirmed the *in vivo* multiplication of the phages (Soothill, 1992).

Clinical trials

In recent years, there have been clinical studies conducted to study the safety and efficacy of phage therapy. In a study performed by Bruttin and Brussow (2005) at the Nestlé Research Center in Switzerland, *E. coli* phage T4 was studied for safety in humans. Fifteen healthy adult volunteers received T4 phage in their drinking water. The water contained either a lower phage dose (10^3 PFU/ml), a higher phage dose (10^5 PFU/ml), or a placebo. The volunteers were tested for recovery of fecal phage. Phage was only detected in those who ingested the phage orally and the recovery was found to be dose-dependent. Interestingly, oral phage application did not cause a decrease in total fecal *E. coli* counts showing that phage did not affect the natural flora. Also, no adverse effects related to phage application were reported. The volunteers were tested for their serum transaminase levels as an indicator of liver toxicity and these levels all remained in the normal range. Also, there were no antibodies against T4 phage detected in the participants' serum demonstrating that T4 phage did not elicit an immune response. This was one of the first studies in modern scientific literature that proved the safety of phage therapy.

In 2009, three reports of other clinical trials against antibiotic resistant infections were published. Rhoads *et al*. (2009) carried out a phase I safety trial to examine the safety of a bacteriophage-based preparation for difficult-to-treat wounds

such as venous leg ulcers in 39 patients. A phage cocktail WPP-201, consisting of eight lytic phages that targeted *S. aureus*, *P. aeruginosa* and *E. coli* was used in the study. Either the phage cocktail or a saline control was applied to ulcers for 12 weeks and the trial was continued for a total of 24 weeks. There were no adverse events attributed to the phage cocktail.

Another smaller phase I study was carried out by Merabishvili *et al.* (2009) in which nine patients at the Burn Wound Centre of the Queen Astrid Military Hospital in Belgium were locally treated with a phage cocktail BFC-1. The cocktail consisted of three lytic phages: a *Myovirus*, a *Podovirus* against *P. aeruginosa*, and a *Myovirus* directed against *S. aureus.* The cocktail was applied in a single spray application over a large burned section and a distant part of the burn wound was treated as control. None of the patients reported any safety issues concerning the phage application.

Wright *et al.* (2009) performed another phase I/II trial in patients suffering from chronic otitis caused by *P. aeruginosa*. The patients were treated with cocktail Biophage-PA, which consisted of six phages effective against *P. aeruginosa*. A single dose of Biophage-PA or a placebo was applied directly in the ears of the 24 volunteers. The bacterial colony counts decreased significantly in the phage treated group. Patients in this group also reported lower intensity of symptoms such as discomfort, itching, wetness, and unpleasant odor. Also, the phage counts increased after the initial application and persisted until the infection was cleared. There were no reported adverse reactions to the phage application.

The Nestlé Research Center has been involved with treating diarrhea in Bangladesh for the past 20 years. In a recent trial, a cocktail of nine T4-like phages at

oral doses of 3×10^7 and 3×10^9 PFU/ml was given to healthy adults from Bangladesh (Sarker et al., 2012). More phages were detected in feces samples of the individuals who received higher titres in the oral dose. It was noted that only one percent of the phage survived unprotected in the gastric passage. There were no adverse events reported either by clinical examination or by using tests for liver, kidney, and hematology function.

Another study was performed for safety analysis of a commercially available Russian phage cocktail of ten phages (McCallin et al., 2013). The cocktail is produced by a company called Microgen and is used for treating infections caused by *E. coli*/*Proteus* bacteria. The cocktail was found to consist of seven phages belonging to *Podoviridae*, four *Myoviridae* and two *Siphoviridae* as confirmed by metagenome analysis. The cocktail was administered orally at a dose of 10^9 PFU to adult volunteers in Bangladesh. There were no significant differences observed in the natural microflora between the phage treated and the placebo treated group, and no adverse effects were reported.

Following up on the above studies, the same group conducted a phase II trial where 120 children were treated with a previously studied T4-like phage cocktail or the Microgen cocktail for *E. coli* induced diarrhea (Sarker et al., 2016). Both phage treated groups demonstrated significant fecal phage titre increases compared to placebo recipients. However, no treatment effect of either oral phage cocktail was observed. One reason why phage therapy was not effective in this trial was hypothesized to be the low level of *E. coli* infection, which resulted in low phage amplification after the initial phage dose. Another reason for the observed phage

therapy failure could have been due to co-infection with another bacteria insensitive to the phages.

A major phage therapy trial currently in progress is “Phagoburn” in Europe. Phagoburn is the first large-scale European Union-funded randomized phase I/II single-blind trial using phage therapy to treat burn wounds infected with *P. aeruginosa*. It is currently being conducted in 11 hospitals in France, Switzerland, and Belgium with results expected to be published this year (Roach and Debarbieux, 2017).

Other recent promising cases

A recent compassionate case trial was carried out for treatment of diabetic foot ulcers in nine patients (Fish et al., 2016). The patients had poorly perfused toe ulcers containing *S. aureus* infection of the bone and soft tissue. The infection failed to be cleared by the recommended antibiotic treatment, and so phage therapy was conducted. A commercially available preparation of staphylococcal phage Sb-1 was used. Phage solution was administered by topical application to the ulcers once a week. It was noted that all infections responded well to the phage treatment with the ulcers healed in an average of seven weeks. This study showed the promise of the use of staph phages to treat diabetic foot diseases, which will be further investigated in a future clinical trial.

Staph phage Sb-1 has also been employed in another case where it was used to treat a 7-year old CF patient (Kvachadze et al., 2011). The patient was suffering from chronic colonization of the lungs with *S. aureus* and *P. aeruginosa* and had been

undergoing antibiotic treatment for years. The phage treatment was administered once every 4-6 weeks using a nebulizer for a total of nine times. The phages used were the commercially available Pyophage and Sb-1 effective against *P. aeruginosa* and *S. aureus* respectively. When Pyophage was given alone in the first dose, a significant decrease in *P. aeruginosa* titres was observed but no decrease in titre for *S. aureus* was observed. When both of the phages were combined, a significant reduction in the numbers of both of the bacteria was observed. There were no adverse reported for the application of phages and the patient's general condition improved post treatment. Another important result from this case study was the reduction by half in the amount of antibiotics used for the patient's treatment. This study shows a potential for using phage therapy in future treatment of CF patient infections.

Bcc Phage therapy

Although human trials have not yet been conducted for phage therapy of *B. cepacia* infections, there have been several studies in animal models showing promise. One of the models used for Bcc is an immuno-compromised mouse infection model where bacteria and phages are administered to the mouse lungs, and subsequently bacteria and phage titres are measured to determine the efficacy of the treatment (Carmody et al., 2010; Semler et al., 2014). In the study conducted by Carmody *et al*., mice were infected with *B. cenocepacia* through a tracheotomy and phage BcepIL02 was administered 24 h post-infection either by intranasal inhalation or intraperitoneal injection. Mice lungs were assayed for bacteria and phage titres 48 h post-infection. A significant drop in Bcc titres was observed for the treated mice,

with a two-fold reduction in those that received the intraperitoneal phage treatment compared to a one-fold reduction in bacterial titres for the mice that received the intranasal phage treatment (Carmody et al., 2010). In contrast, Semler *et al.* demonstrated that aerosol delivery of the phages showed significantly better results for phage therapy of *B. cenocepacia.* The mice were treated with one of five *B. cenocepacia* phages and bacterial and phage titres were monitored 2 days post infection. A significant four-fold decrease in bacterial titres was observed for the mice that received phages through aerosol versus controls or the mice that received phages intraperitoneally (Semler et al., 2014).

Galleria mellonella (Greater waxworm) larvae model is another animal model (Seed and Dennis, 2008) that can be used for testing phage therapy against the Bcc. In the *G. mellonella* larvae infection model, the phages and bacteria are injected into the larvae and the efficacy of phages is determined by survival of infected larvae 48 h post-infection. The model was initially assessed for *B. cenocepacia* strains K56-2 and C6433 administered at lethal concentrations (Seed and Dennis, 2009). An increased larval survival was observed with increased phage multiplicity of infection (MOI) for KS12 against K56-2. This model has been successfully used in the lab for testing the efficacy of different phages active against Bcc (Lynch et al. 2010; Lynch et al. 2013; Kamal and Dennis 2015) .

Summary

Members of the Bcc are highly antibiotic resistant, can cause chronic lung infections in patients with CF, and therefore are an excellent candidate for phage

therapy. There is a need to determine the efficacy of phage treatment in Bcc in the animal models before treatment in humans. In our lab, several Bcc phages have been isolated and characterized, and some have shown promise in both *G. mellonella* and mouse models of infection.

In order to be able to use phages for therapy, high titres of phages are required to obtain the desired phage multiplicity of infection (MOI), as total phage numbers have been shown to have an impact on the effectiveness of the phage treatment (Seed and Dennis, 2009). In the following chapters, different strategies to increase the efficacy of phage therapy in Bcc will be discussed. The use of phages in combination with antibiotics to increase the production of phages and improve treatment efficacy will be described in Chapter 2. The role of the phage encoded MazG protein in enhancing phage production in stationary phase bacterial cells will be explored in Chapter 3.

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Chapter 2 : *Burkholderia cepacia* complex Phage-Antibiotic Synergy (PAS): Antibiotics stimulate lytic phage activity

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Introduction

The *Burkholderia cepacia* complex (Bcc) is a group of closely related Gram-negative bacterial opportunistic pathogens that can cause chronic lung infection in cystic fibrosis (CF) and immuno-compromised patients (Isles et al., 1984; LiPuma, 1998; Mahenthiralingam et al., 2005). As mentioned in Chapter 1, Bcc rarely infect healthy individuals but can be problematic in CF patients. At present, there are 20 species that have been identified as members of the Bcc (Coenye et al., 2001; Gross et al., 2006; Vanlaere et al., 2009; Peeters et al., 2013; Spilker et al., 2015). Although several Bcc species have been shown to infect CF patients, *B. cenocepacia* and *B. multivorans* have been found to be the most prevalent among them and account for approximately 85%- 97% of all Bcc infections (Reik et al., 2005; Drevinek et al., 2010). These two species have not been found to be more abundant in the natural environment (Ramette et al., 2005). In CF patients, Bcc infection occurs at a lower prevalence than *S. aureu*s and *P. aeruginosa* infections but the possibility of a "cepacia syndrome" associated with Bcc drastically increases the associated morbidity and mortality. "Cepacia syndrome" is characterized by a rapid pulmonary deterioration leading to death by an invasive infection and has been observed in up to 20% of CF patients particularly those infected with *B. cenocepacia* (Isles et al., 1984; LiPuma, 1998; Lipuma, 2010; Drevinek et al., 2010). Another reason Bcc bacteria are problematic in infections is due to their ability to spread between people (LiPuma et al., 1990; Govan et al., 1993; Pegues et al., 1994). They can be transmitted between patients via direct contact (LiPuma et al., 1990) as well as through a contaminated environment

(Drabick et al., 1996). Members of the Bcc have high levels of innate resistance to both antibiotics (Lewin et al., 1993) and biocides (Rose et al., 2009). Previous reports have demonstrated high antibiotic resistance ranging from 50% to 100% of all antibiotics tested (Aaron et al., 2000). This exquisite resistance is thought to be due to both intrinsic and acquired resistance mechanisms. Bcc bacteria also have the ability to form biofilms, which makes it harder to treat these infections since biofilms provide physical protection to embedded cells (Conway et al., 2002). Clinical treatment of Bcc-infected patients typically involves trimethoprim-sulfamethoxazole as the main therapeutic option (Avgeri et al., 2009). Since resistance occurs widely, combination therapy with three or more antibiotics is used as an alternative strategy (Aaron et al., 2000; Mahenthiralingam et al., 2002; Zhou et al., 2007), even though synergistic activity is rarely observed (Zhou et al., 2007) and multidrug resistant isolates are not uncommon (Aaron et al., 2000). Only 23-38% of clinical Bcc isolates are significantly inhibited by ceftazidime, meropenem and minocycline, the antibiotics commonly used to treat Bcc infections (Zhou et al., 2007).

Phage therapy

Because Bcc bacterial infections in CF patients are highly resistant to and cannot be cleared by antibiotics, one proposed alternative treatment strategy is that of phage therapy (Merril et al., 2003). Most phages are extremely specific, targeting only a subset of strains of a single bacterial species, but allowing treatment of a specific infecting strain without affecting a patient's normal microflora. Phages replicate exponentially in bacterial hosts resulting in increased phage titers over time

as they infect the host bacteria, replicating and lysing the host cells to release more phages. This dynamic effect enhances their therapeutic potential for treating bacterial infections. The phage cycle of replication continues until there are no longer bacterial host cells present. Phage therapy was commercially developed in the 1930s and has been continued particularly in the Republic of Georgia. Phage therapy was largely abandoned in Western countries following the discovery and widespread introduction of broad-spectrum chemical antibiotics (Alisky et al., 1998; Merril et al., 2003). With the recent emergence of multidrug-resistant (MDR) and extremely drug-resistant (XDR) bacteria, including those of the Bcc, there has been renewed interest in phage therapy. Since phages employ a completely different mechanistic strategy to chemical antibiotics, they can still be effective against antibiotic resistant bacteria. In a type of co-evolutionary arm's race, phages can rapidly adapt or evolve to counter improvements in bacterial resistance, which is in direct contrast to the static activity of chemical antibiotics (Alisky et al., 1998). With renewed interest in phage therapy, new clinical trials have been conducted that demonstrate phage therapy to be effective in the treatment of various bacterial infections. Recent experimental studies exploring the treatment of Bcc infections via phage therapy also appear promising (Seed and Dennis, 2009; Carmody et al., 2010; Lynch et al., 2010b, 2013; Semler et al., 2014), including our recent study showing Bcc phage efficacy in a mouse infection model where phages effectively clear *B. cenocepacia* infections as compared to controls (Semler et al., 2014).

Phage Antibiotic Synergy

Since phages and antibiotics work differently, they can be used in combination for dual treatment of multi-drug resistant bacteria. Previous reports have investigated how phages behave in the presence of chemical antibiotics. It has been reported that more phages are produced from bacteria in the presence of penicillin than in its absence (Krueger and Cohn 1948; Price, 1947b; Price, 1947c; Price, 1947a). These early studies showed an effect of penicillin on phages produced by *Staphylococcus aureus*. More recently, there have also been reports of the stimulation of phage development in *Escherichia coli* (Hadas et al., 1997) and *S. aureus* (Maiques et al., 2006) by β-lactam antibiotics. In 2007, Comeau *et al*. published a report on effect of sub-inhibitory concentrations of several antibiotics belonging to different classes for increased phage production. In this study, *E. coli* phage MFP and T4-like phages were observed to form larger plaques in the presence of sub-lethal concentrations of quinolones and β-lactam antibiotics tested (Comeau et al., 2007). This effect of the antibiotics on phages was termed Phage-Antibiotic Synergy (PAS). *E. coli* phage MFP was observed to display an increased plaque size and a seven-fold higher titer in the presence of a cephalosporin antibiotic, cefotaxime. A similar effect was also observed for T4-like phages (Comeau et al., 2007). Another recent study tested efficacy of PAS by combining sub-inhibitory concentrations of different antibiotics such as gentamicin, ceftriaxone, ciprofloxacin or polymyxin B with *P. aeruginosa*-specific phages (Knezevic et al., 2013). An increased killing was observed when ceftriaxone and a Siphoviridae phage σ-1 were applied together

(Knezevic et al., 2013). Ryan *et al.* also observed antimicrobial synergy between phage T4 and cefotaxime in the *in vitro* eradication of *E. coli* biofilms. Both increased plaque sizes and phage titers were noted with increasing sub-lethal concentrations of cefotaxime. A significant reduction in the minimum biofilm eradication concentration (MBEC) value of cefotaxime occurred in the presence of phage T4 (Ryan et al., 2012). The MBEC value was reduced from 256 μg/ml to 32 μg/ml in the presence of 10^7 PFU/mL of T4 phage. These reports suggest that under some circumstances the production of phages is stimulated by certain chemical antibiotics and that PAS can effectively enhance the killing of susceptible bacteria.

Objective

In this study, we proceeded to test a number of antibiotics to identify those that demonstrated an increase in plaque size, as expected for PAS. We demonstrate that some antibiotics appear to induce increased production of several Bcc phages. We also extend these findings to show PAS is operational against the Bcc in an *in vivo* infection model. *Galleria mellonella* larvae have previously been used for the study of pathogenic bacteria such as *P. aeruginosa* (Miyata et al., 2003), *Bacillus cereus* (Fedhila et al., 2006), *Francisella tularensis* (Aperis et al., 2007), and the Bcc (Seed and Dennis, 2008), and also to test phage therapy against clinically relevant Bcc strains (Seed and Dennis, 2009). We show PAS rescue of *B. cenocepacia* infected *G. mellonella* larvae using phage in combination with two different antibiotics.

Materials and Methods

Bacterial strains and phages

The Bcc bacterial strains used in this study were obtained from the *Burkholderia cepacia* complex experimental strain panel (Mahenthiralingam et al., 2000; Coenye et al., 2003). Bacterial cells were grown overnight aerobically in 10 ml half strength Luria Bertani (½ LB) broth at 30°C and 220 rpm in a gyratory shaker. The cells were diluted 1:100 in fresh medium and grown an additional 3.5 hours to an optical density value (OD_{600}) corresponding to exponential phase and a titer of approximately 2 X 10^8 CFUs. Optical density values were measured using a Victor X3 spectrophotometric plate reader (Perkin Elmer, Woodbridge, ON).

Phages KS12 and KS14 were previously isolated and characterized by members of the Dennis lab, and these results were previously published (Seed and Dennis, 2009; Lynch et al., 2010b; Semler et al., 2014). Phages KS12 and KS14 both contain double-stranded DNA nucleic acid, and are of the phage family Myoviridae (A1 morphotype). Phage KS14 (vB_BceM-KS14), isolated from *Dracaena* sp. (dragon tree) soil extract plated on *Burkholderia multivorans* C5393, is a *Peduovirinae* subfamily, genus "P2-like" virus (Lynch et al., 2010b) with a genome size of 32,317 base pairs encoding 44 proteins. KS14 can lysogenize host cells as a plasmid (Lynch et al., 2010b). Phage KS12 is a myovirus isolated from soil planted to *Dietes grandiflora* (wild iris) on *B. cenocepacia* K56-2, and its genomic content is currently unknown (Seed and Dennis, 2009). Based upon experimental data (not shown), KS12 has not been observed to lysogenize any of its known Bcc hosts.

Phage stocks were grown using an agar overlay method and the phage stock was filter sterilized using a 0.45 μm filter. Phage titers were determined using the double-layer agar technique (Kropinski et al., 2009). Briefly, 100 μl of phage was added to 100 μl of exponential phase bacterial cells and mixed in 3 ml top agar that was poured onto 20 ml agar plates. Plates were incubated overnight at 30°C, and plaques were identified and examined by visualization. For use in *Galleria mellonella* experiments, endotoxin was removed from the phage stock using Detoxi-Gel Endotoxin Removing Column (Thermo Scientific).

Media and antibiotics

Bacterial strains were grown in one-half-strength Luria-Bertani (1/2 LB) broth. For use in a double-layer agar (DLA) phage plaquing method (Kropinski et al., 2009), this same medium was supplemented with Select agar (Invitrogen, Burlington, ON) at final concentrations of 0.4% and 1.4% in the top and bottom layers, respectively. Ciprofloxacin, tetracycline, minocycline, levofloxacin and ceftazidime were purchased from Sigma-Aldrich Inc (St. Louis, MO), and meropenem was purchased from AstraZeneca Canada Inc. (Mississauga, ON). Minimum inhibitory concentrations (MICs) of antibiotics were determined using a 96-well micro-plate dilution protocol (Wiegand et al., 2008). Briefly, 5 μl of exponential phase cells were added to 100 μl of antibiotic prepared in Mueller-Hinton broth and grown overnight at 30°C and 220 rpm in a gyratory shaker. The MIC of an antibiotic towards a specific bacterial strain was determined to be the concentration of antibiotic at which the optical density (OD_{600}) was equal to a cell-free blank control.

Effects of different concentrations of antibiotics

For measurement of plaque sizes and phage particle number determinations in the presence of antibiotics, different concentrations of antibiotics were added only to the top agar in a total volume of 100 µl in a double layer agar assay. The antibiotic concentrations in the top agar ranged from 0.625 µg/ml to 10 µg/ml ciprofloxacin, 2.5 µg/ml to 40 µg/ml meropenem, 2.5 µg/ml to 40 µg/ml tetracycline for phage KS12. Similarly, the antibiotic concentrations in the top agar used for phage KS14 were 62.5 µg/ml to 1 mg/ml ciprofloxacin, 2.5 µg/ml to 40 µg/ml meropenem and 25 µg/ml to 100 µg/ml tetracycline. These correspond to 1/4X MIC to 4X MIC of antibiotics in liquid media for the bacterial hosts *B. cenocepacia* K56-2 and C6433. As a control, 100 µl of sterile water was added in place of the antibiotic suspension to account for agar dilution effects. No antibiotics were added in the bottom layer of the agar in the plates. This means the true concentration of antibiotics was lower than that added to the top agar as some diffusion occurs into the bottom agar layer. Phage plaques were backlit and viewed under the magnifying glass of a New Brunswick Scientific colony counter (model C110) and plaque size was measured using digital calipers manufactured by Tresna (Guilin, China).

For phage particle counts, 5 ml of ½ LB broth was added to each plate and the top agar layer was scraped off and added to 15 ml tubes. Phages were released into liquid media by gentle pipetting, solid debris was removed by low speed centrifugation, and the supernatant was filtered using EMD Millipore (Etobicoke, ON) 0.22 um syringe filters. Filtrates were serially diluted and used in double layer

agar assays. Differences in phage plaque sizes, phage titers, numbers of surviving bacterial cells, and numbers of surviving waxworm larvae were statistically analyzed by using one-way analysis of variance (ANOVA), as compared to controls. An ANOVA with Dunnett's posttest was used, with a P value of <0.05 being considered statistically significant. The analyses were performed using GraphPad Prism 6 (Graph-Pad Software Inc., San Diego, CA).

To ensure bacterial numbers were similar in the different concentrations of antibiotics tested, bacterial cells were initially grown to exponential phase. One ml of bacterial cells was added to different sub-inhibitory concentrations of antibiotics in 15 ml tubes and grown for an additional 6 hours. The antibiotic concentrations used were 1.25 μg/ml ciprofloxacin, 5 μg/ml meropenem and 5.5 μg/ml tetracycline. Cell counts were determined by taking samples at different time points and plating the samples after serial dilution. For analysis of the bacterial cells in the presence of different concentrations of antibiotics, 100 μl of cells grown overnight at 30°C were added to 3 ml of top agar containing sub-inhibitory concentrations of antibiotics and spread on 20 ml agar plates. The plates were incubated overnight at 30°C and cells were scraped and suspended in 4% paraformaldehyde in phosphate-buffered saline (PBS). Cells grown under different concentrations of antibiotics were stained with 2% phosphotungstic acid and visualized using transmission electron micrographs taken with an FEI / Philips (Hillsboro, OR) Morgagni Transmission Electron Microscope with a Gatan Digital Camera.

Combination treatment of *B. cenocepacia* infected *Galleria mellonella* larvae

G. mellonella larvae were purchased from Recorp Inc (Georgetown, ON). Larvae were stored in wood chips at 4°C. A 250 µl Hamilton syringe was used to inject 5 µl aliquots of *B. cenocepacia* K56-2 into *G. mellonella* larvae, as previously described (39). *B. cenocepacia* K56-2 suspended in 10 mM $MgSO_4$ plus 1.2 mg/ml ampicillin was injected into larvae via the last left proleg followed by injection of phage at an MOI of 100 or 0.31 µg meropenem or 1.5 µg minocycline resuspended in 5 µl sterile 10 mM MgSO4 in the next proleg through separate injections. As *G. mellonella* larvae contain approximately 50 µl of hemolymph, we estimate that the final concentration in the larvae was 6 µg/ml meropenem or 30 µg/ml minocycline alone, or in combination with phage KS12. Control larvae were injected with 5 µl of 10 mM MgSO4 plus 1.2mg/ml ampicillin. Larvae were placed in the dark in an incubator at 30°C. Ten larvae were injected for each treatment group and larvae were scored as dead or alive 48 and 72 h post-infection (p.i.). Larvae were assessed as expired if they did not respond to touch with movement.

Antibiotic resistant mutants

Plasmid pBBR1MCS-3 (Kovach et al., 1995) carrying a tetracycline resistant marker was electroporated into K56-2 cells and transformed resistant cells were selected on media containing tetracycline. In addition, wild-type K56-2 cells grown overnight with high concentrations of tetracycline in the media produced spontaneous mutants that were isolated. These resistant cells were grown overnight at 30°C and 220 rpm in ½ LB broth with tetracycline, and tested in PAS experiments. 100 µl of

phage KS12 were added to 100 µl exponential phase cells, mixed with 3 ml of top agar containing different concentrations of antibiotics and plated using DLA. Plaque sizes were measured as described above.

Results

Effects of different concentrations of antibiotics on Bcc PAS.

With the objective of developing phage therapy as an alternative treatment strategy for the Bcc, phages KS12 and KS14 were tested against *Burkholderia cenocepacia* strains K56-2 and C6433, respectively. The phages were initially tested for PAS in the presence of different concentrations of several different classes of antibiotics, including ampicillin, ceftazidime, ciprofloxacin, kanamycin, levofloxacin, meropenem, minocycline, piperacillin, and tetracycline. A PAS effect was observed with phages KS12 and KS14 for only six of these antibiotics: ciprofloxacin, meropenem, tetracycline, minocycline, levofloxacin and ceftazidime. As seen in Figure 2-1, an increase in plaque size was observed for the two phages that demonstrated PAS. Bacterial cells were grown to exponential phase and the Minimum Inhibitory Concentration (MIC) of the antibiotics was determined for liquid cultures. As observed in Table 2-1, strain C6433 MICs in liquid ranged from 0.125 µg/ml for minocycline to 250 µg/ml for ciprofloxacin and ceftazidime, and strain K56-2 MICs ranged from 0.25 µg/ml for minocycline to 500 µg/ml for ceftazidime.

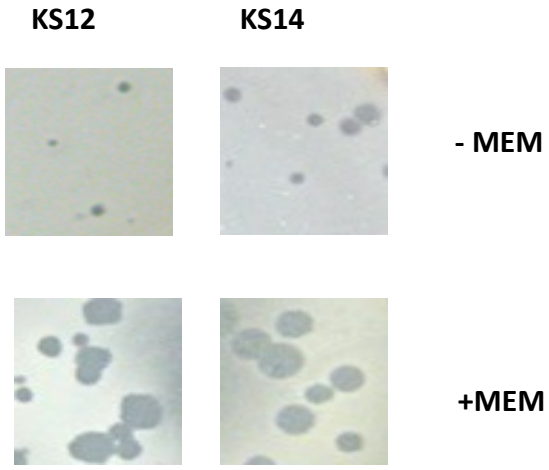

Figure 2-1: Plaque sizes of phages KS12 and KS14 in the presence or absence of meropenem (MEM) in the top agar of half-strength Luria plates. Phage KS12 was grown on *B. cenocepacia* C6433 at 30°C. All plaques were photographed at the same magnification.

Table 2-1: Antibiotic MICs for *B. cenocepacia* strains

B. cenocepacia strain	MIC (µg/ml)					
	CIP	MEM	TET	MIN	CAZ	LEV
C6433	250	10	100	0.125	250	125
K56-2	2.5	10	10	0.25	500	15

CIP, ciprofloxacin; MEM, meropenem; TET, tetracycline; MIN, minocycline; CAZ, ceftazidime; LEV, levofloxacin.

Bcc phages KS12 and KS14 plaque sizes and titers were determined on K56-2 and C6433 respectively. The phages showed an increase in plaque size in the

presence of different concentrations of antibiotics in the top agar as shown in Table 2-2, with a maximum increase in plaque size observed for meropenem, a β-lactam antibiotic. The increase in plaque size was concentration dependent up to a maximum size.

Table 2-2: Plaque diameter of phages KS12 and KS14 in the presence of different concentrations of antibiotics.

	Phage KS12 mean plaque diameter (mm) ± SD[a]					
DLA[b]	1.22 ± 0.12					
CIP (µg/ml)		0.625	1.25	2.5	5	10
		1.33 ± 0.07	1.45 ± 0.07	1.51 ± 0.11	1.82 ± 0.12	**** 2.23 ± 0.12
MEM (µg/ml)		2.5	5	10	20	40
		1.58 ± 0.08	1.76 ± 0.11	1.92 ± 0.26	2.19 ± 0.26	**** 2.37 ± 0.15
TET (µg/ml)		2.5	5	10	20	40
		1.41 ± 0.31	1.40 ± 0.32	1.42 ± 0.32	1.48 ± 0.08	**** 1.56 ± 0.11
	Phage KS14 mean titer (log PFU/ml) ± SD[a]					
DLA	2.81±0.23					
CIP (µg/ml)		62.5	125	250	500	1000
		3.20 ± 0.27	3.66 ± 0.30	3.67 ± 0.23	3.77 ± 0.27	**** 3.88 ± 0.26
MEM (µg/ml)		2.5	5	10	20	40

		3.52 ± 0.24	3.55 ± 0.27	3.73 ± 0.40	4.20 ± 0.39	**** 4.35 ± 0.50
TET (μg/ml)		25	50	100	200	400
		3.72 ± 0.28	4.05 ± 0.30	****4.19±0.22	ND	ND

[a] Plaque diameter values are the average ± standard deviation of 20 different plaques from three separate trials.

[b] DLA, double-layer agar technique.

CIP, ciprofloxacin; MEM, meropenem; TET, tetracycline; ND, not determined.

Statistical analysis performed using ANOVA with Dunnett's post test, **** $p<0.0001$.

For phage KS12, plaque diameter size increased from 1.22 mm without antibiotics to 2.37 mm when 40 μg/ml (4X MIC) of meropenem was added to the top agar, an increase of 97.1% (equal to a 94.3% increase in area). For phage KS12 plated on *B. cenocepacia* K56-2 in the presence of varying concentrations of antibiotics in the top agar (0.625 μg/ml to 10 μg/ml ciprofloxacin, 2.5 μg/ml to 40 μg/ml meropenem, or 2.5 μg/ml to 40 μg/ml tetracycline), there were statistically significant differences. For KS12 in the presence of 10 μg/ml ciprofloxacin in the top agar, a One-way ANOVA test with Dunnett's post test, was used to calculate the probability of a null hypothesis, that these plaque sizes are the same ($p<0.0001$). Similar values were calculated, for 40 μg/ml meropenem ($p<0.0001$), and for 40 μg/ml tetracycline in the top agar($p<0.0001$). Phage KS12 plaque size increases were

also observed for 0.25 μg/ml minocycline (1.37 ± 0.13 mm, $p<0.01$), 250 μg/ml ceftazidime (1.67 ± 0.12 mm), and 17 μg/ml levofloxacin (1.54 ± 0.20 mm) in the top agar.

Phage KS14 showed a plaque diameter increase in size from 2.81 mm in the absence of antibiotics to 4.35 mm in the presence of 40 μg/ml of meropenem in the top agar, an increase of 77.4% (equal to a 54.8% increase in area). For phage KS14 plated on *B. cenocepacia* C6433 in the presence of varying concentrations of antibiotics in the top agar (62.5 μg/ml to 1 mg/ml ciprofloxacin, 2.5 μg/ml to 40 μg/ml meropenem, or 25 μg/ml to 100 μg/ml tetracycline), significant differences (One-way ANOVA with Dunnett's post test) were observed for 1 mg/ml ciprofloxacin ($p<0.0001$), 40 μg/ml meropenem ($p<0.0001$) and 100 μg/ml tetracycline ($p<0.0001$). In addition, KS14 plaque size increases were also observed when 0.125 μg/ml minocycline (3.64 ± 0.32 mm, $p<0.0001$), 37.5 μg/ml ceftazidime (3.19 ± 0.22 mm), and 62.5 μg/ml levofloxacin (3.47 ± 0.17 mm) were added to the top agar.

Phage titers also showed an increase with increasing concentrations of antibiotics. As shown in Table 2-3, phage KS12 titers were significantly increased in the presence of 5 μg/ml ciprofloxacin ($p<0.0001$), 20 μg/ml meropenem ($p<0.0001$), and 20 μg/ml tetracycline ($p<0.0001$) in the top agar, as compared to controls not containing antibiotics. However, phage KS12 mean titers did not significantly increase in the presence of 250 μg/ml ceftazidime (6.00 ± 0.26 log PFU/ml) or 7.5 μg/ml levofloxacin (6.07 ± 0.22 log PFU/ml), and were significantly reduced in the presence of 0.25 μg/ml minocycline (4.80 ± 0.35 log PFU/ml, $p<0.01$) in the top agar.

In contrast, as also shown in Table 2-3, phage KS14 titers were significantly increased in 500 μg/ml ciprofloxacin ($p<0.05$), 20 μg/ml meropenem ($p<0.001$), and 100 μg/ml tetracycline ($p<0.05$), although little change in KS14 titer was found with 50 μg/ml ceftazidime (7.22 ± 0.51 log PFU/ml) or 62.5 μg/ml levofloxacin (7.44 ± 0.16 log PFU/ml) in the top agar as compared to controls. However, 0.062 μg/ml minocycline in the top agar again significantly reduced the titer of phage KS14 (6.11± 0.29 log PFU/ml, $p<0.05$) as it did for KS12, as compared to control conditions not containing antibiotics (7.31 ± 0.71 log PFU/ml).

Table 2-3: Titers of phages KS12 and KS14 in the presence of different concentrations of antibiotics in the top agar.

	Phage KS12 mean titer (log PFU/ml) ± SD[a]				
DLA[b]	5.99 ± 0.34				
CIP (μg/ml)		0.625	1.25	2.5	5
		6.02 ± 0.34	6.29 ± 0.41	6.32 ± 0.33	6.37 ± 0.27
MEM (μg/ml)		2.5	5	10	20
		6.55 ± 0.46	6.54 ± 0.25	6.84 ± 0.74	*6.85 ± 0.25
TET (μg/ml)		2.5	5	10	20
		6.05 ± 0.30	6.13 ± 0.25	6.13 ± 0.30	5.97 ± 0.05
	Phage KS14 mean titer (log PFU/ml) ± SD[a]				
DLA	7.31 ± 0.71				

CIP (μg/ml)		62.5	125	250	500
		8.55 ± 0.56	8.75 ± 0.43	8.84 ± 0.19	* 8.81 ± 0.30
MEM (μg/ml)		2.5	5	10	20
		9.01 ± 0.29	9.34 ± 0.28	8.97 ± 0.25	***9.61 ± 0.27
TET (μg/ml)		25	50	100	200
		8.97 ± 0.74	8.58 ± 0.23	* 8.71 ± 0.41	ND

[a] Plaque diameter values are the average ± standard deviation of 20 different plaques from three separate trials.

[b] DLA, double-layer agar technique.

CIP, ciprofloxacin; MEM, meropenem; TET, tetracycline; ND, not determined. Statistical analysis performed using ANOVA with Dunnett's post test, ***$p<0.001$ *$p<0.05$.

The changes to phage plaque sizes were independent of the antibiotic resistance levels of the bacterial cells hosting phage production. Phage plaque size increases were tested in the presence of different concentrations of antibiotics on antibiotic resistant bacterial cells. As compared to phage KS12 control conditions containing no antibiotics producing mean plaque diameters of 1.27 ± 0.09 mm on *B. cenocepacia* K56-2 harboring tetracycline resistant plasmid pBBR1MCS-3, tetracycline resistant K56-2 cells still showed increased KS12 plaque sizes in the presence of 10 μg/ml meropenem (2.21 ± 0.19 mm) or 40 μg/ml tetracycline (1.58 ±

0.09 mm) in the top agar. A *B. cenocepacia* K56-2 mutant expressing spontaneously acquired tetracycline resistance similarly showed increases in phage KS12 mean plaque sizes in the presence of top agar antibiotics: no antibiotic control, 1.20 ± 0.06 mm; 40 µg/ml tetracycline, 1.62 ± 0.10 mm; 10 µg/ml meropenem, 2.16 ± 0.24 mm.

Effects of sub-inhibitory concentration of antibiotics on host morphology and growth.

B. cenocepacia C6433 and K56-2 cells were visualized using transmission electron microscopy in order to observe changes in cell morphology when grown in the presence of sub-inhibitory concentrations of ciprofloxacin, meropenem and tetracycline. As shown in Figure 2-2, both C6433 and K56-2 cells appear filamentous in the presence of sub-inhibitory concentrations of ciprofloxacin, exist as chains of elongated cells in the presence of sub-inhibitory concentrations of meropenem, and as clusters of cells in the presence of tetracycline.

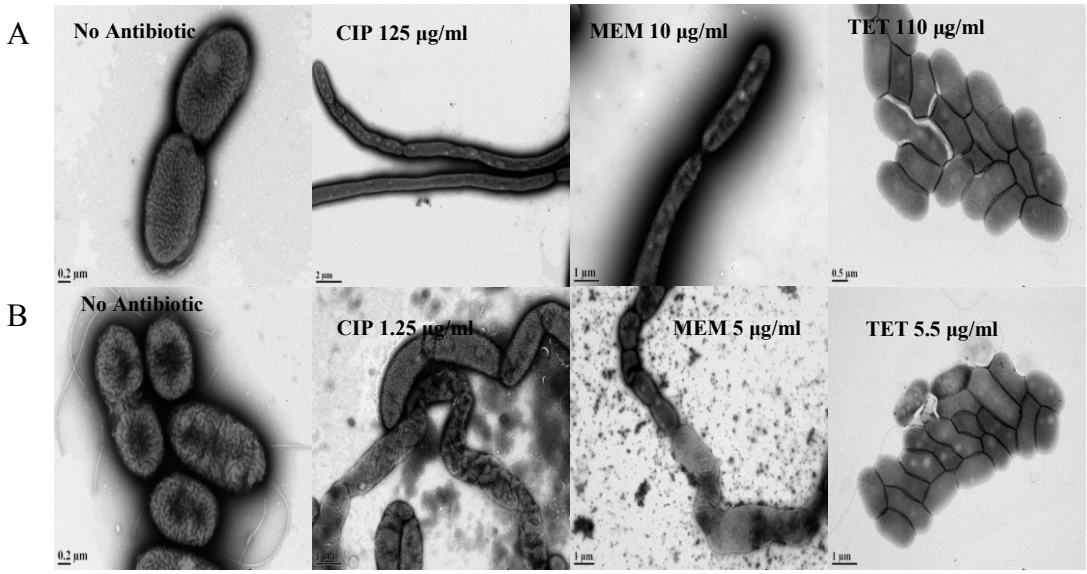

Figure 2-2: Transmission electron micrographs of *B. cenocepacia* strains C6433 (A) and K56-2 (B) in the presence of ciprofloxacin (CIP), meropenem (MEM) and

tetracycline (TET). Cells were stained with 2% phosphotungstic acid and imaged using an FEI / Philips (Hillsboro, OR) Morgagni Transmission Electron Microscope with Gatan Digital Camera.

Sub-inhibitory concentrations of antibiotics were added to exponential phase cells in vitro at 1/2 MIC: 1.25 μg/ml ciprofloxacin, 5 μg/ml meropenem or 5.5 μg/ml tetracycline and growth was monitored over time. Cells exposed to sub-inhibitory concentrations of antibiotics were determined to exhibit no growth rate defects as compared to controls containing no antibiotics (data not shown). There is an increase in cell concentration of both C6433 and K56-2 by 1.5 logs over 6 hours in presence of either 1/2 MIC of ciprofloxacin, meropenem or tetracycline. In contrast, K56-2 numbers in vitro decrease at 330 minutes with the addition of phage KS12, as shown in Figure 2-3, and decrease dramatically with the addition of KS12 in the presence of either 1/2 MIC of ciprofloxacin, meropenem or tetracycline.

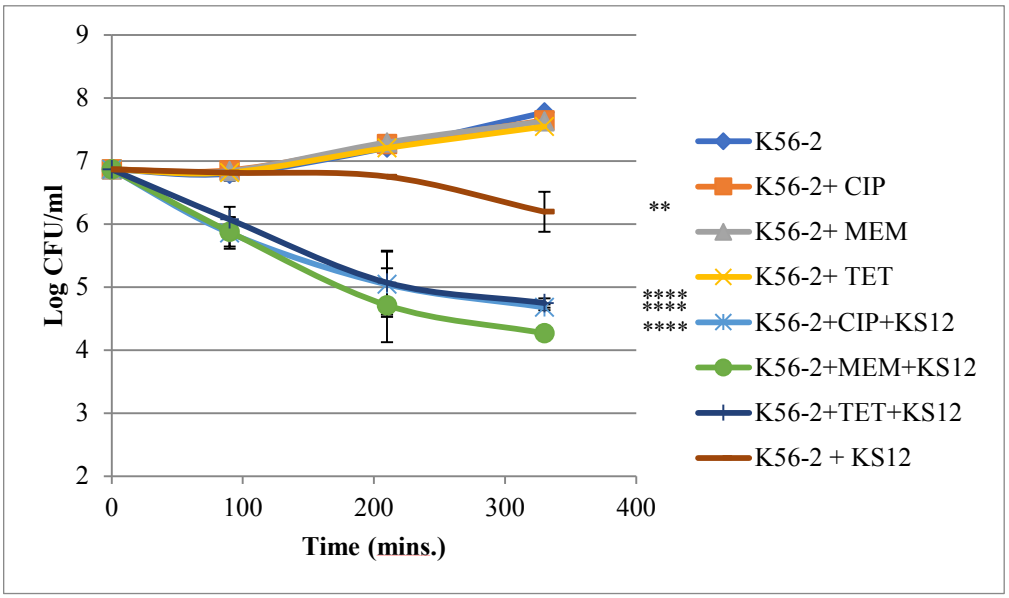

Figure 2-3: Killing effect of phage KS12 on *Burkholderia cenocepacia* K56-2 log phase cells in the presence of ciprofloxacin (CIP), meropenem (MEM), and tetracycline (TET). Cells were treated with 1.25 µg/ml CIP, 5 µg/ml MEM or 5.5 µg/ml TET. Phage KS12 was added at an MOI=1.

Values given are averages ± standard deviations for three replicates. Statistical analysis was performed using One-way ANOVA with Dunnett's post test. **$p < 0.01$, ****$p < 0.0001$, as compared with the K56-2 control.

Treatment of *B. cenocepacia* infected *Galleria mellonella* larvae with a combination of phage and antibiotics.

As shown in Figure 2-4, *G. mellonella* larvae exhibit increased survival when treated with a combination of phage and antibiotics as compared with no antibiotic or no phage controls. After 48 hours post infection, the mortality of larvae decreases from 80% when treated with meropenem alone, or 67% mortality when treated with

phage KS12 alone, to 22% upon treatment with phage KS12 and meropenem together. A similar decrease is observed with minocycline and phage KS12 in vivo: larval mortality at 48 hours is 76% with minocycline alone, but only 31% mortality when treatment with phage KS12 and minocycline is combined. These results are statistically significant, $p<0.0001$. After 72 hours, similar statistically significant results were observed, $p=0.004$. For meropenem, there was a decrease in mortality from 97% for KS12 alone to 57% for KS12 plus meropenem, and for minocycline, there was a decrease from 100% mortality with minocycline alone to 60% for KS12 plus minocycline. The rescue of *G. mellonella* with the addition of sub-inhibitory meropenem and KS12 is similar to that with minocycline and KS12. In contrast, the addition of both meropenem and minocycline showed only minor improvement (61% mortality at 48 hours) over either of the antibiotics alone (76% mortality for minocycline alone at 48 hours versus 80% mortality for meropenem alone).

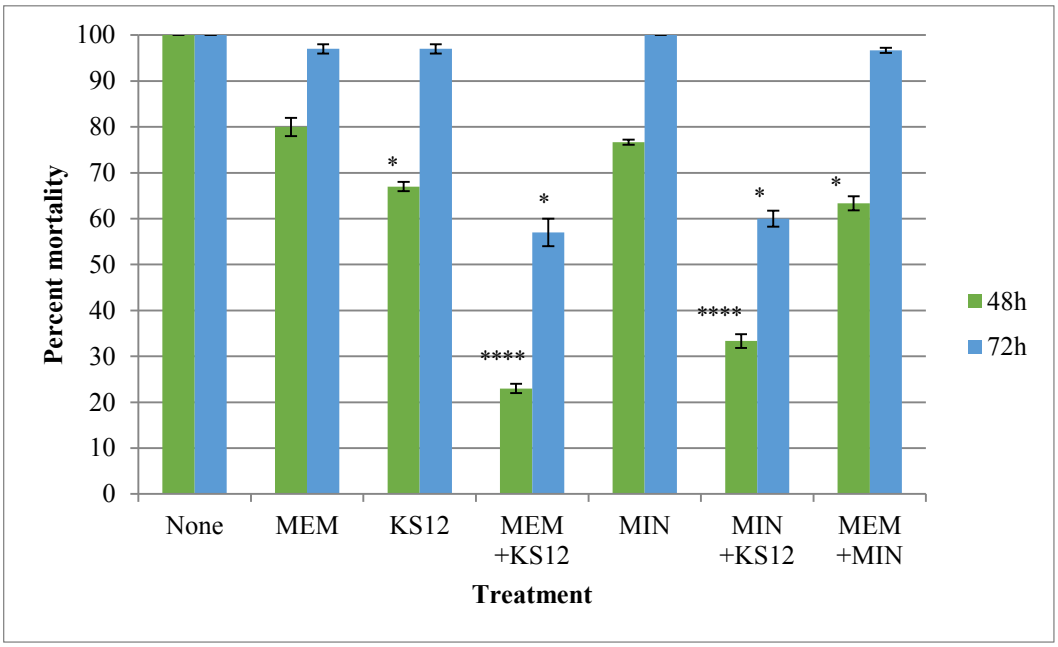

Figure 2-4: Mortality of *B. cenocepacia* K56-2-infected *G. mellonella* larvae treated with phage, antibiotic or a combination of both. Larvae were infected with *B. cenocepacia* K56-2 (9X LD_{50} = 9000 CFU), treated with 6.0 µg/ml meropenem (MEM) or 30 µg/ml minocycline (MIN) alone or in combination with phage KS12 (MOI=100).

Values given are averages ± standard deviations for three trials (n = 10 larvae per trial). Statistical analysis was performed using One-way ANOVA with Dunnett's post test. *$p < 0.05$, ****$p < 0.0001$, as compared to no treatment controls.

PAS in antibiotic resistant *B. cenocepacia.*

PAS in tetracycline resistant K56-2 cells was tested and as seen in Figure 2-5, there were no significant differences observed in KS12 plaque sizes when plated on tetracycline resistant K56-2. Both the pBBR-tet transformed K56-2 and spontaneously isolated tetracycline resistant mutants show similar increases in KS12 plaque sizes in the presence of tetracycline and meropenem as seen for the wild-type K56-2.

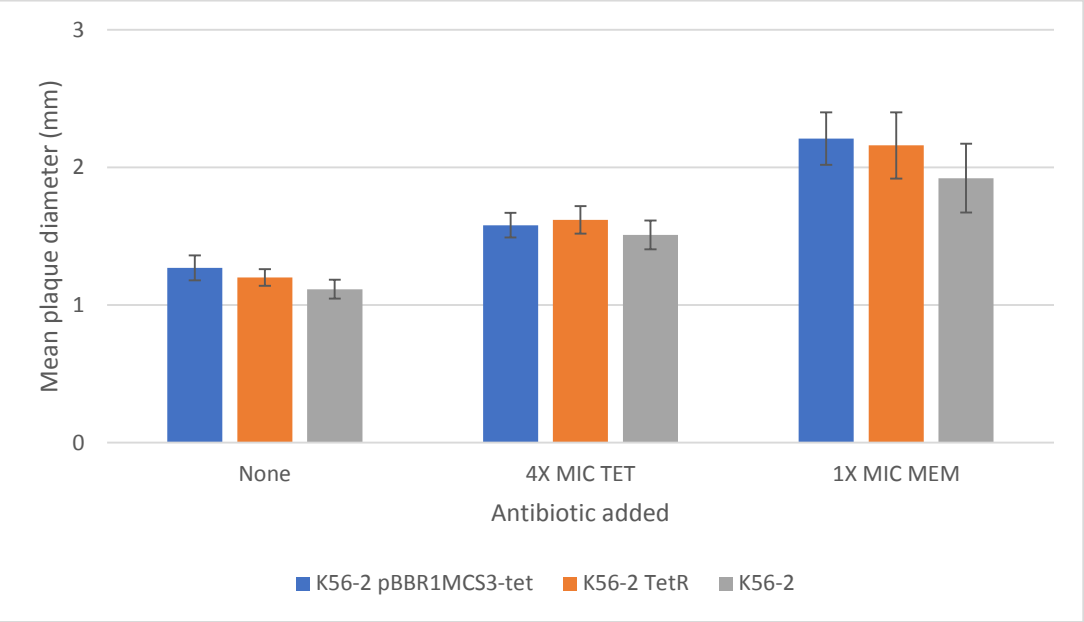

Figure 2-5: Plaque diameter of phage KS12 on tetracycline resistant *B. cenocepacia* K56-2 in the presence of 40 µg/ml tetracycline (TET) and 10 µg/ml meropenem (MEM) in the top agar.

Plaque diameter values are the average ± standard deviation of 20 different plaques from three separate trials.

Discussion

Bcc strains are well known to possess impressive innate resistance to chemical antibiotics. This is attributed to several different mechanisms, some of which have been mentioned in Chapter 1. These include a number of biodegradation gene clusters on its large, multi-replicon ~8Mb genome. *B. cenocepacia* is inherently resistant to several different classes of antibiotics including aminoglycosides, polymyxins and most β-lactams (Drevinek et al., 2010), and can acquire increased resistance to many drugs following repeated exposure. Like most bacteria, Bcc bacteria possess aminoglycoside-inactivating enzymes that impart resistance to aminoglycosides. In addition, the Bcc have decreased outer membrane permeability as well as an amino-arabinose modified lipopolysaccharide layer that prevents entry of certain antibiotics and antimicrobial peptides (Mahenthiralingam et al., 2005; Cox and Wilkinson, 1991). Also, Bcc bacteria have multiple efflux pumps that remove the antibiotics from the cell thereby providing protection against various antibiotics (Rushton et al., 2013; Zhang et al., 2001). Although this high-level resistance reduces the available choice of antibiotics for therapeutic treatment, several antibiotics belonging to different classes have been used to suppress the Bcc in CF infections. These antibiotics were tested for efficacy individually as well as in combination in several studies. Antibiotics with synergistic activity were identified, the most effective combinations containing meropenem, which showed efficacy in 70% of 119 Bcc isolates tested (Zhou et al., 2007). However, 30% of Bcc strains tested retained

resistance to the most active triple combination synergistic antibiotic treatments, indicating that alternative therapies are desperately needed.

In this study, we have examined two strains of *B. cenocepacia*, strains C6433 and K56-2, since *B. cenocepacia* is one of the most prevalent Bcc species associated with CF infections (Reik et al., 2005). Sub-inhibitory concentrations of antibiotics have been shown to produce an increase in phage activity (Krueger and Cohn, 1948; Price, 1947b; Price, 1947c; Price, 1947a; Hadas et al., 1997; Maiques et al., 2006; Comeau et al., 2007; Knezevic et al., 2013; Ryan et al., 2012) and recently this was termed "phage-antibiotic synergy" or PAS (Comeau et al., 2007). In this study, we have tested ciproflocxacin (a fluoroquinolone antibiotic), meropenem (a carbapenem, which is a modified β-lactam antibiotic) and tetracycline (a protein translation inhibitor). Although these antibiotics all possess different mechanisms of action, all three antibiotics exhibit PAS with both phages KS12 and KS14, as detected by an increase in plaque size. Comeau *et al*. (2007) suggested that a change in the morphology of the bacterial cell, which occurs in the presence of sub-inhibitory concentrations of antibiotics, may be resposinble for PAS as these changes allow rapid phage maturation, and accelerated cell lysis. We performed transmission electron microscopy of Bcc cells in the presence of sub-MIC antibiotics to look for possible morphological changes. We found the cells of both Bcc strains C6433 and K56-2 to be elongated in the presence of ciprofloxacin and meropenem, and present in clusters in the presence of tetracycline. This is not surprising as cell filamentation has been previously associated with presence of sub-inhibitory concentrations of fluoroquinolones (Diver and Wise, 1986) and β-lactams (Spratt, 1975). Our data

confirms earlier findings that phages may have increased access to phage receptors on elongated or filamented cells, which leads to increased phage production and accelerated timing of lysis. However, we also note that PAS is observed in presence of sub-inhibitory concentrations of tetracycline, which causes cell clustering but not filamentation, indicating that filamentation is not a requirement for PAS. Instead, tetracycline PAS may suggest that cell clustering provides increased phage infection due to an ability of phages to travel laterally across adjoined cell surfaces, thereby enhancing contact with phage receptors on different cells. In each circumstance, the ability of phages to move along or between cells seems to be an attribute of PAS.

Interestingly, although PAS was exhibited by both the phages tested, there were some differences noted between the two phages. Whereas both phages KS12 and KS14 displayed increased mean plaque diameters in the presence of different antibiotics in the top agar (Table 2-2), only KS14 appeared to exhibit greatly increased phage titers when exposed to different antibiotics (Table 2-3). At this time, we have no explanation for this effect. A similar riddle exists for differences between antibiotics. Both phages exposed to minocycline produced increased mean plaque diameters in vitro, but minocycline also significantly reduced phage titers produced in each plaque. Curiously, in the *G. mellonella* infection model, minocycline was almost as effective at reducing larval mortality in combination with phage KS12 as meropenem at both 48 and 72 hours (although meropenem was used at sub-MIC 6 μg/ml, whereas minocycline had to be used at 30 μg/ml, or 120X MIC in order to rescue the larvae). This would suggest that despite the lower numbers of phages being produced through PAS by minocycline, there are still sufficient phage numbers to

produce a therapeutic PAS effect in vivo. Somewhat surprisingly, related drug tetracycline, with a similar chemical structure and mechanism of action as minocycline, produces “normal” PAS, increased plaque diameter along with increased phage titer, all at sub-MIC concentrations. This suggests that perhaps there is something unusual about the PAS effects observed with minocycline in vitro versus in vivo, and further investigation of this activity is required.

We had also tested other antibiotics namely gentamicin, streptomycin, ampicillin, piperacillin, kanamycin besides ciprofloxacin, meropenem, tetracycline, minocycline, ceftazidime and levofloxacin. Even though ampicillin and piperacillin belong to the same ß-lactams class of antibiotics like meropenem, pronounced PAS was observed for meropenem only. Similarly, a strong PAS effect was observed for ciprofloxacin but not levofloxacin though both antibiotics are fluoroquinolones and target DNA gyrase in the cells. Gentamicin, streptomycin and kanamycin are aminoglycosides and target the protein synthesis and did not produce a PAS effect whereas tetracycline which also affects protein synthesis did. As mentioned above, a difference in PAS was also observed for tetracycline analog minocycline which did not increase phage titers as observed for tetracycline. This shows that antibiotics belonging to the same class or with similar drug targets exhibit differences in PAS. A difference in PAS was also observed for the different phages tested KS5, KS10, KS12 and KS14. We have shown data for phages KS12 and KS14. Phage KS5 can infect both strains K56-2 and C6433 but did not exhibit PAS. Phage KS10 infects a different *B. cenocepacia* strain PC184. No increase in plaque size was observed for

KS10 in the presence of the antibiotics tested. Thus, PAS appears to occur in an antibiotic-dependent as well as phage-dependent manner.

Phage therapy against Bcc infections has produced promising results in both *G. mellonella* larvae (Seed and Dennis, 2009) and when aerosolized into lungs in a mouse infection model (Semler et al., 2014). In this study, we have combined phage therapy with doses of antibiotics to observe PAS in the treatment of a Bcc infection in *G. mellonella*. Our results show larval rescue from Bcc infection at even very low MOIs of KS12, with larvae mortality falling dramatically from 65% at 48 hours when treated with KS12 alone to 20% at 48 hours when treated with a combination of KS12 and meropenem. Moreover, both minocycline and meropenem with KS12 gave similar results in vivo, suggesting that PAS is operating similarly, even though the antibiotics' mechanism of action on the bacterial cells is different. This is similar to the results observed by Knezevic *et al* (2013) for the treatment of *P. aeruginosa* where cells treated with a combination of phage and antibiotic showed better clearance of infection than those treated with phage alone (Knezevic et al., 2013).

Interestingly, the antibiotic resistance status of the target cell does not change PAS. We observed no difference in PAS for cells that were made tetracycline resistant, either through mutation or transformation with a tetracycline resistant plasmid. This suggests that the cell's innate or acquired antibiotic resistance levels does not alter whatever effects sub-inhibitory levels of antibiotics are having on the phages or the cells to make them more sensitive to phages. It is encouraging to note therefore, that PAS could still be used for treatment of cells that have previously become resistant to antibiotic treatment. The Bcc causes chronic infections that

cannot be cleared from the lungs of CF patients using antibiotics. This indicates that all current antibiotic treatments in these patients are sub-inhibitory (at sub-MIC concentrations), and that PAS would appear to offer a realistic alternative to reduce bacterial numbers more than that which can be achieved through either antibiotic or phage treatment alone.

With the emergence of antibiotic resistance, phage therapy in combination with antibiotics may be useful as an alternative treatment. Another reason why members of Bcc have high innate antibiotic resistance is due to their ability to form biofilms (Conway et al., 2002). As some phages have been shown to readily penetrate bacterial biofilms (Hughes et al., 1998; Hu et al., 2012; Phee et al., 2013), it is anticipated that PAS will work as well against bacteria growing in biofilms as planktonic bacteria. In support of this hypothesis, PAS has been shown to work well against *E. coli* biofilms as evidenced by a decrease in minimum biofilm eradication concentration (MBEC) of the antibiotic cefotaxime when used in combination with phage T4 (Ryan et al., 2012). Thus, combining phage therapy with traditional antibiotics could help better manage antibiotic resistant bacterial infections, regardless of whether or not the cells are growing in bacterial biofilms.

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Chapter 3 : Phage encoded MazG affects phage infection and production in *Burkholderia cenocepacia*

Introduction

Members of *Burkholderia cepacia* complex (Bcc) are opportunistic pathogens associated with chronic lung infection in cystic fibrosis (CF) patients (Mahenthiralingam et al., 2005). *B. cenocepacia* has been identified as one of the most prevalent Bcc species in CF patient Bcc infections (Drevinek et al., 2010). Until now, all of the epidemic Bcc strains that have been identified are *B. cenocepacia* strains. These include the well know ET12 and PHDC (Coenye & LiPuma, 2003; Govan et al., 1993). Another reason that *B. cenocepacia* is one of the most problematic Bcc species is its association with "cepacia syndrome" (Mahenthiralingam et al., 2005). Since members of the Bcc have high levels of innate resistance to antibiotics (Lewin et al., 1993), current treatment involves combinations of different drugs, especially trimethoprim-sulfamethoxazole as the main therapeutic option (Avgeri et al., 2009). Combination therapy is considered to be a way to reduce the occurrence of resistance that occurs with repeated exposure to antibiotics (Aaron et al., 2000; Mahenthiralingam et al., 2002; Zhou et al., 2007), even though this does not prevent the development of multidrug resistant isolates (Aaron et al., 2000). Most Bcc strains are innately resistant to many antibiotics even when antibiotic combinations are used as treatment options (Aaron et al., 2000).

A proposed alternative treatment strategy for highly antibiotic resistant bacteria is the use of bacteriophages, which are viruses that infect specific bacteria (Alisky et al., 1998). There have been several studies that have demonstrated phage therapy to be a promising alternative for the treatment of Bcc infections including

reports from our lab (Seed and Dennis, 2009; Carmody et al., 2010; Lynch et al., 2010b, 2013; Semler et al., 2014), and including our recent study showing phage efficacy against *B. cenocepacia* in a mouse infection model (Semler et al., 2014). The ideal conditions required for optimal phage replication is the attachment and lysis of target cells growing rapidly as during exponential growth phase. However, in a chronic infection such as that associated with CF patients, the majority of the bacterial cells are likely present in stationary growth phase or in slow-growing biofilms.

(p)ppGpp and MazG in Bacteria

In many bacteria, during hostile or unfavorable growth conditions, amino acid starvation prompts the rapid accumulation of an unusual guanosine nucleotide, termed GDP 3'-diphosphate or GTP 3'-diphosphate, collectively referred to as (p)ppGpp. (p)ppGpp is now recognized as a global regulator of gene expression in bacteria (Braeken et al., 2006) and as an effector of the stringent response (Gaca et al., 2015). It is centrally involved in bacterial adaptation to a very wide range of environmental conditions. Accumulation of (p)ppGpp has been found to be associated with large-scale transcriptional alterations that result in a general repression of genes required for rapid growth, such as the genes encoding rRNA and amino acid biosynthesis as well as those involved in nutrient acquisition and survival under stressful conditions. Besides transcriptional control, (p)ppGpp has also been shown to directly inhibit the activity of several enzymes, including DNA primase, translation factors, and enzymes involved in GTP biosynthesis (Kanjee et al., 2012).

In the cell, (p)ppGpp is synthesized from GTP and ATP by the bacterial enzymes RelA/SpoT, which regulate its level (Cashel, 1975). RelA is responsible for (p)ppGpp synthesis in response to amino acid limitation (Wendrich et al., 2002) whereas SpoT is responsible for (p)ppGpp degradation in response to various stress conditions, including carbon, iron and fatty acid starvation (Cashel et al., 1996; Traxler et al., 2008). During growth conditions, SpoT is also involved in maintaining a basal level of (p)ppGpp (Gentry and Cashel, 1995).

Recently, another enzyme called MazG has been found to be involved in stringent response in bacteria (Zhang and Inouye, 2002). *mazG* is located downstream of *mazF* in the *mazEF* operon. In *E. coli*, *mazEF* is a well-known toxin-antitoxin module that induces programmed cell death as a response to stressful conditions. *mazF* encodes a stable toxin, MazF, while *mazE* encodes a labile antitoxin, MazE. MazG is a nucleoside triphosphate pyrophosphohydrolase that can hydrolyse (p)ppGpp (Gross et al., 2006) and is thought to play a role in allowing bacteria to grow during conditions of starvation. Gross *et al.* noticed that MazG prevented the normal accumulation of (p)ppGpp during the stringent response to amino acid starvation. Gross *et al.* also observed decreased cell survival during nutritional stress when *mazG* was deleted (Gross et al., 2006). This suggests that MazG may be involved in delaying cell death under stringent or stressful conditions.

Furthermore, (p)ppGpp has also been shown to affect virulence and pathogenesis of species such as *Legionella, Vibrio cholera* and *Salmonella* (Hammer and Swanson, 1999; Haralalka et al., 2003; Pizarro-Cerdá and Tedin, 2004). Hammer and Swanson noticed accumulation of ppGpp in response to amino acid starvation in

Legionella pneumophila. This resulted in the conversion of *L. pneumophila* from a replicative to a virulent state (Hammer and Swanson, 1999). Haralalka *et al.* observed significantly reduced production of *V. cholera* virulence factor CT in a *relA* mutant strain which failed to accumulate (p)ppGpp upon amino acid starvation (Haralalka et al., 2003). Pizarro-Cerdá and Tedin found *S. typhimurium* ΔrelA ΔspoT strain, which can not regulate (p)ppGpp levels to be significantly less virulent than wild-type *in vivo* in a mouse model. The strain was also found to be less invasive of colon epithelial cells *in vitro* (Pizarro-Cerdá and Tedin, 2004).

Presence of MazG in Phages

In freshwater cyanobacteria *Anacystis nidulans*, ppGpp was found to accumulate when incident light intensity was reduced, which produced a condition of energy limitation (Mann et al., 1975). An increase in ppGpp was also observed when *A. nidulans* was faced with nitrogen starvation and this was due to increased production of ppGpp (Friga et al., 1981), whereas infection with cyanophage AS-1 interfered with this accumulation (Borbely et al., 1980).

There have been reports of *mazG* homologues present in marine phages. A recent genome analysis of the marine cyanomyovirus S-PM2, which infects marine cyanobacteria *Synechococcus,* showed the presence of an open reading frame encoding a protein with a MazG nucleotide pyrophosphohydrolase domain (Mann et al., 2005). Sullivan *et al.* found *mazG* homologues in the genomes of two cyanomyoviruses P-SSM2 and P-SSM4 that infect strains of another marine cyanobacteria *Prochlorococcus* (Sullivan et al., 2005). Bryan *et al.* (2008) tested

Synechococcus phages for the presence of *mazG* homologues and found there was horizontal transfer of *mazG* between the phages. They also observed a high prevalence and conservation of the *mazG* gene among phage isolates from diverse geographical locations (Bryan et al., 2008). A more recent study showed the presence of *mazG* among all of the 16 cyanophages tested (Sullivan et al., 2010). As MazG has been shown to degrade (p)ppGpp (Gross et al., 2006), this suggests that the phage-encoded MazG functions to decrease the (p)ppGpp pool in phage-infected marine cynaobacteria cells (Clokie and Mann, 2006). This reduction in the (p)ppGpp levels could possibly modify the physiology of an infected bacterial cell making it more similar to a cell in a nutrient rich environment, thus enhancing phage production by promoting expression of genes involved in protein and DNA synthesis. Thus, the presence of *mazG* homologues may impart an advantage to *mazG*-carrying phages by allowing infection of host cells under starving conditions such as those found in nutrient-limiting environment.

Recently, Bcc phages KL1 and AH2 were observed to carry a highly similar *mazG* homologue- gp35 and gp25, respectively (Lynch et al., 2012). KL1 gp35 is similar to MazG homologues from cyanophages such as S-CRM01, S-SM2, S-ShM2 that infect *Synechococcus* and P-HM1, P-HM2, P-SSM2 that infect *Prochlorococcus* (Lynch et al., 2012). AH2 gp25 is similar to *Clostridium* MazG proteins and to the *Burkholderia* phage proteins ϕE255 gp37, BcepMu gp06, and BcepB1A gp71. Since Bcc are found in nutrient-limiting environments such as soil and water, MazG might provide an advantage to these phages enhancing infection and propagation under stressful conditions.

Objective

In this study, we tested MazG encoded by *B. cenocepacia* phages KL1 and AH2 for effects on phage titers. *B. cenocepacia* strains C6433 and K56-2 were transformed with *mazG* from either of the two phages KL1 and AH2. Stationary phase cells were tested for phage production by introducing KS14 phage as a plasmid into the cells and measuring the number of phages produced. We also tested the effect of MazG on phage burst size, which is the number of phages produced from a single cell after infection and completion of one replication cycle. Furthermore, we tested changes in virulence using the *Lemna minor* (duckweed) and the *G. mellonella* (waxworm) larvae models. Duckweed has been established as a model of infection for other human pathogens *P. aeruginosa* and *S. aureus* (Zhang et al., 2010) and more recently *B. cepacia* and *E. coli* (Thomson and Dennis, 2013). We observed increased virulence for both C6433 and K56-2 cells transformed with *mazG* originating from either Bcc phages.

Materials and Methods

Bacterial strains, phages and plasmids

The Bcc bacterial strains used in this study were obtained from the *Burkholderia cepacia* complex experimental strain panel (Mahenthiralingam et al., 2000; Coenye et al., 2003). Bacterial cells were grown overnight aerobically in 10 ml half strength Luria Bertani (½ LB) broth at 30°C and 220 rpm in a gyratory shaker to

an optical density value (OD_{600}) corresponding to stationary phase and a titer of approximately 2 X 10^9 CFUs. Optical density values were measured using a Victor X3 spectrophotometric plate reader (Perkin Elmer, Woodbridge, ON).

Phages KS14, KL1 and AH2 were previously isolated and characterized by members of the Dennis lab, and these results were previously published (Lynch et al., 2010b, 2012). Phages KS14, KL1 and AH2 all contain double-stranded DNA nucleic acids. Phage KS14 belongs to the phage family *Myoviridae* (A1 morphotype) while phages KL1 and AH2 belong to the family *Siphoviridae* as identified by electron microscopy. Phage KS14 (vB_BceM-KS14), isolated from *Dracaena* sp. (dragon tree) soil extract plated on *B. multivorans* C5393, is a *Peduovirinae* subfamily, genus "P2-like" virus (Lynch et al., 2010b) with a genome size of 32,317 base pairs encoding 44 proteins. KS14 can lysogenize host cells as a plasmid (Lynch et al., 2010b). Phage KL1 is a siphovirus isolated from sewage on *B. cenocepacia* K56-2 while phage AH2 was isolated from *Nandina* sp. (heavenly bamboo) soil using *B. cenocepacia* C6433 (Lynch et al., 2012). Both KL1 and AH2 exhibit similar host range and growth characteristics. The KL1 genome is 42,832 base pairs while AH2 genome is slightly larger with 58,065 base pairs (Lynch et al., 2012). Phage stocks were grown using an agar overlay method and the phage stock was filter sterilized using a 0.45 μm filter. Phage titers were determined using the double-layer agar technique (Kropinski et al., 2009). Briefly, 100 μl of phage was added to 100 μl of exponential phase bacterial cells and mixed in 3 ml top agar that was poured onto 20 ml agar plates. Plates were incubated overnight at 30°C, and plaques were identified and examined by visualization.

Table 3-1 Bacterial strains and plasmids used in this study

Strain/ Plasmid	**Characteristics**	**Source**
B. cenocepacia		
C6433	CF isolate	CBCCRRR (Vancouver, BC)
C6433::KL1	C6433 with KL1 *mazG* plasmid	This study
C6433::AH2	C6433 with AH2 *mazG* plasmid	This study
K56-2	CF isolate	CBCCRRR (Vancouver, BC)
K56-2::KL1	K56-2 with KL1 *mazG* plasmid	This study
K56-2::AH2	K56-2 with AH2 *mazG* plasmid	This study
E. coli		
DH5α	F- ϕ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *rec*A1 *end*A1 *hsd*R17(r_k^-, m_k^+) phoA *sup*E44 thi-1 *gyr*A96 relA1 λ^-	Invitrogen (Carlsbad, CA)
Plasmids		
pJET	Cloning vector, Amp^R	ThermoFischer (Burlington, ON)
pSCRhaB2	rhamnose inducible, Tp^R	Cardona et al. (2005)

pSCRha-KL1*mazG*	pSCRha with phage KL1 *mazG,* Tp^R	This study
pSCRha-AH2*mazG*	pSCRha with phage AH2 *mazG*, Tp^R	This study
pDAI-SceI-Cm	pDAI-SceI, Cm resistance cassette, Tet^R Cm^R	Abdu (2013)
pGPI-SceI-MB1	pMB1 ori, TpR , I-SceI recognition site	Abdu (2013)

Media used

Bacterial strains were grown in one-half-strength Luria-Bertani (1/2 LB) broth. For use in double-layer agar (DLA) phage plaquing (Kropinski et al., 2009), this same medium was supplemented with Select agar (Invitrogen, Burlington, ON) at final concentrations of 0.6% and 1.4% in the top and bottom layers, respectively. The growth medium was supplemented with various antibiotics to ensure plasmid maintenance, including 100 μg/ml trimethoprim (Tp), 300 μg/ml chloramphenicol (Cm) and 100 μg/ml tetracyline (Tet) (Sigma-Aldrich, St. Louis, MO). The antibiotic concentrations used for *E. coli* include 100 μg/ml Tp, 25 μg/ml Cm, and 15 μg/ml tetracycline (Tet).

Freezer Stocks

Stock suspensions of cells were prepared by growing the bacteria overnight on solid agar (with or without antibiotics) and resuspending the cells in 2 ml of LB broth supplemented with 20% glycerol. Stocks were stored at -80°C.

Isolating phage DNA and cloning *mazG*

Phage genomic DNA was isolated from bacteriophage lysate using the Wizard Lambda DNA purification system (Promega Corp., Madison, WI) with a modified protocol (Lynch et al., 2013; Peters et al., 2015). *mazG* was amplified from phage AH2 genomic DNA using primers AH2MazGF and AH2MazGR and from KL1 genomic DNA using KL1MazGF and KL1MazGR. These were cloned into pJET to allow easy selection and then sub-cloned into pSCRhaB2. The oligonucleotides used in this study (Sigma-Aldrich) are listed in Table 3-2.

Table 3-2 Oligonucleotides used in this study

Primer name	**Sequence 5'-3'**	**Purpose**
AH2 F	ATCTGGTCGAGGCGCTGTAC	Amplify AH2
AH2 R	AAAATCCCCGAGAGGCCGTAT	Amplify AH2
KL1 F	GCGTCGGAACAAATCAGCGTTTC	Amplify KL1
KL1 R	GGCATCGAAGTTATTTCCGGCAATAC	Amplify KL1
AH2MazGF	CATATGAGCAATGCAAAGGAGCAG	Amplify mazG from AH2

AH2MazGR	TCTAGATCAGATCGACTTGCCGC3	Amplify mazG from AH2
KL1MazGF	CATATGAACTACATTGAAGAAGCGCAC	Amplify mazG from KL1
KL1MazGR	TCTAGATCAAGCCTTCTTTTCGAGAATTTC	Amplify mazG from KL1
AH2 UF F	GCTTCTAGAACGACGATCTCTAACCCCTC	Amplify upstream region of AH2 *mazG*
AH2 UF R	TTTGGTACCGTCGCCGATCAGTCGTTC	Amplify upstream flanking region of AH2 *mazG*
AH2 DF F	TTTGGTACCGCCATCGTCGAAAAGCTG	Amplify downstream flanking region of AH2 *mazG*
AH2 DF R	TTTGATATCGCACTCAGGGCTTCGATTTC	Amplify downstream flanking region of AH2 *mazG*
KL1 UF F	GACATATTGCCGTATGCC	Amplify upstream region of AH2 *mazG*
KL1 UF R	GCGAACGCGAAATTCTTC	Amplify upstream flanking region of AH2 *mazG*

KL1 DF F	CTTCTTCAATGTAGTTCATAC	Amplify downstream flanking region of AH2 *mazG*
KL1 DF R	TTGCGATTGGTACATGC	Amplify downstream flanking region of AH2 *mazG*

Plasmid isolation

Plasmids were isolated in this study using the GeneJET™ Plasmid Miniprep kit (Thermo Scientific, Waltham, MA). Plasmid and genomic DNA were quantified using a NanoDrop 2000c spectrophotometer (Thermo Scientific) and stored at 4°C or -20°C.

Restriction digest and PCR

Plasmid DNAs were digested with desired restriction enzymes (Thermo Scientific, Burlington, ON) according to the manufacturer's instructions. PCR was performed using a T100™ Thermal Cycler (Bio-Rad, Hercules, CA). PCR was conducted using phage genomic DNA as the template and was performed according to the manufacturer's standard protocol.

Agarose gel electrophoresis

The separation of DNA was performed using a 0.7% (w/v) agarose gel on a wide Mini-Sub cell GT cell (Bio-Rad) and stained with GelGreen (Phenix Research

Products, Candler, NC). A ChemiDoc MP imaging System (Bio-Rad) was then used to visualize and capture images of the agarose gel.

DNA purification and ligation

Purification of PCR products or DNA fragments separated on 0.8% agarose gels was performed using a GeneJET Gel Extraction Kit (ThemoFischer Scientific, Burlington, ON) or MinElute PCR Purification Kit (Qiagen, Hilden, Germany). DNA used in ligations was eluted with 15 µl of sterile water. Ligation reactions composed of 12.5 µl of DNA, 1.5 µl of ligase buffer, and 1.0 µl of T4 DNA ligase (Promega Corporation, Madison, WI) were incubated at 16°C overnight.

Electroporation and titration of phages

A modified electroporation protocol (Sambrook, Fritsch and Maniatis, 1989) was used to transform *B. cenocepacia.* Overnight cells of the strain were washed with glycerol to make competent cells. 3 ml of the overnight cells were centrifuged at 5000g for 5 minutes and the supernatant was removed. The pelleted cells were washed with 400 µl of 10% glycerol. The wash was repeated twice and the cells were resuspended in 70 µl of 10% glycerol. 0.5 µg of Phage KS14 plasmid was added and allowed to incubate at room temperature for 5 minutes. Electroporation was performed using Bio-Rad MicroPulser (Bio-Rad) and recovered immediately with 500 µl of water. These were allowed to grow for 1 hour at 30°C and 220 rpm. The phages were plated using the double-layer agar overlay (DLA). Briefly, 100 µl of phage was added to 100 µl of exponential phase bacterial cells and mixed in 3 ml top

agar that was poured on 20 ml agar plate. The plates were incubated overnight at 30°C.

Phage burst size measurement

Cells were grown to stationary phase and infected with phage KS14 for burst size measurement. KS14 was added to cells at a multiplicity of infection (MOI) of 1. The unadsorbed phages were diluted away and the infective centers were counted to calculate the burst size. The phage were plated using DLA and the plates were incubated overnight at 30°C. The plaques were counted and titers were determined at t=0 and t=60 mins. Burst size was calculated as phage titers at t=60 divided by the infective centers at t=0.

Virulence assays using duckweed and waxworm larvae model of infection

Both C6433 and K56-2 transformed with pSCRha-KL1*mazG* were tested for virulence using the *L. minor* (duckweed) and the *G. mellonella* (wax-worm larva) infection models respectively. C6433 transformed with pSCRha-AH2*mazG* were also tested in the duckweed model. Duckweed plants were obtained from the greenhouse in the Biological Sciences building (University of Alberta) and were grown in Schenk- Hildebrandt medium supplemented with 1% w/v sucrose (SHS) under conditions developed in the lab (Thomson and Dennis, 2013). Briefly, each duckweed plant was placed in a well filled with 180 µl of SHS media in a 96-well plate. These were infected with varying amounts of C6433 (pSCRha-KL1*mazG*) ranging from 10^5 to 10^2 CFU. Plates were wrapped in cellophane and incubated at 30°C in the dark.

Plant survivors were counted at 96 hours post infection. Plants that displayed >90% loss of green pigmentation were considered dead.

G. mellonella larvae were purchased from Recorp Inc (Georgetown, ON). Larvae were stored in wood chips at 4°C. A 250 µl Hamilton syringe was used to inject 5 µl aliquots of *B. cenocepacia* K56-2 into *G. mellonella* larvae, as previously described (Seed and Dennis, 2008). *B. cenocepacia* K56-2 suspended in 10 mM $MgSO_4$ plus 1.2 mg/ml ampicillin was injected into larvae via the last left proleg*G. mellonella* were injected with $9x10^3$ CFU of K56-2 (pSCRha-KL1*mazG*) or K56-2 and the larvae were kept at 30°C in the dark as described earlier (Seed and Dennis, 2008). Ten larvae were injected for each treatment group and these were scored for death at 48 hours and 72 hours post infection.

Isolating and confirming KL1 lysogen

Strain C6433 was grown with phage KL1 to isolate lysogens. 1ml ($2x10^9$ CFU) of overnight culture of C6433 was mixed with 1ml ($2x10^9$ PFU) of phage KL1 and incubated overnight at 30°C and 220 rpm. Next, 300 µl of the culture was spread on ½ LB plate and incubated overnight at 30°C for two days. The colonies observed were tested for the presence of phage KL1 by growing overnight cultures and infecting with phage KL1. Lack of superinfection suggested the presence of a prophage in the lysogen. This prophage presence was confirmed by PCR specific for KL1 using 5' and 3' primers. The lysogen was confirmed by genome and plasmid preparation. Genome preps were performed using a modified protocol. Briefly, overnight cultures of the strain carrying phage KL1 were grown and the DNA was

isolated using phenol-chloroform extraction. Plasmid preps were performed using ThermoFischer plasmid isolation kit. A 7% agarose gel was run to confirm KL1 in the genome.

Deleting *mazG* and producing KL1 phage lacking *mazG*

The lysogen strains C6433::KL1 and C6433::AH2 were developed in an attempt to delete *mazG* from phages KL1 and AH2 using a previously described system for clean deletions in *B. cenocepacia* (Flannagan, Linn and Valvano, 2008). Briefly, flanking regions of *mazG* were cloned into plasmid pGPI-SceI-MB1-Tp and attempts were made to insert this plasmid into the prophage genome via recombination. Single colony inserts (SCI) were selected on 100Tp plates. A second recombination event was anticipated to be induced by mutagenesis plasmid pDAI-SceI-Tet-Cm.

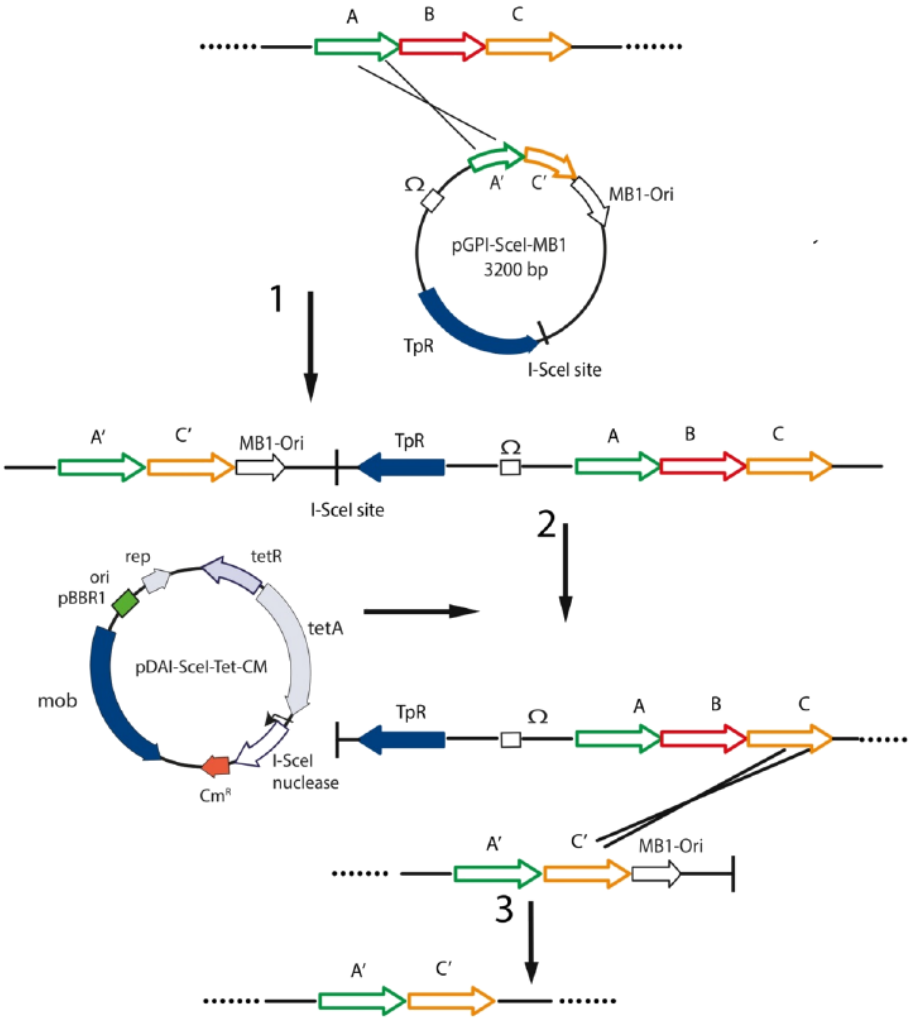


Figure 3-1: Scheme for deleting a gene in *B. cenocepacia* using the clean deletion system (Flannagan et al., 2008). In step 1, flanking regions of the gene to be removed are cloned into a suicide vector. In step 2, suicide vector is introduced into the cells where the flanking regions are incorporated into the genome by homologous recombination. In step 3, a mutagenesis vector is introduced which induces double-stranded break resulting in a second recombination event leading to deletion of the gene.

Adapted from Erin Dockery's Biol 499 report.

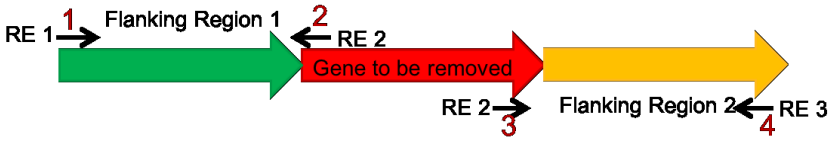


Figure 3-2: Cloning flanking regions of the *mazG* gene to be deleted. The two flanking regions were amplified with primers 1 and 2 for the upstream region and primers 3 and 4 for the downstream region with restriction enzyme cut sites the same on regions 2 and 3 for cloning into the suicide vector.

PAS in *mazG* strains

C6433 (pSCRha-KL1*mazG*) and K56-2 (pSCRha-KL1*mazG*) were tested for Phage Antibiotic Synergy (PAS) as described before (Kamal and Dennis, 2015). Briefly, phage KS14 or KS12 were allowed to infect overnight cultures of C6433 (pSCRha-KL1*maz*) and K56-2 (pSCRha-KL1*mazG*) in the presence 20µg/ml of meropenem (2X MIC) in the top agar and the plates were titered using DLA technique. Plates were incubated overnight at 30°C. Plaque size was measured using Tresna digital calipers under the magnifying glass of New Brunswick colony counter (C110). 5 ml of ½ LB broth was added to the plates and the top layer was scraped and added to 15 ml tubes. Phage counts were measured using DLA.

Phage T4 burst size in *E. coli mazG* and *mazF* mutants

E. coli strains BW25113 *mazG::kn* and BW25113 *mazF::kn* were obtained from Keio collection (Baba et al., 2006). These were tested for production of T4 phage. Cells

were grown to stationary phase overnight at 37°C. Phage T4 was added to cells at a multiplicity of infection (MOI) of 1. Burst size was calculated as explained above.

Results

Phage KS14 production in the Bcc *mazG* strains

To test the effect of phage-encoded MazG homologs *mazG* genes from phages KL1 and AH2 were cloned separately into plasmid pSCRha and used to transform *B. cenocepacia* strains C6433 and K56-2. The transformed strains C6433 (pSCRha-KL1*mazG*), C6433 (pSCRha-AH2*mazG*), K56-2 (pSCRha-KL1*mazG*) and K56-2 (pSCRha-AH2*mazG*) were tested for phage production. Phage KS14 (as a plasmid) was introduced into the cells and the number of phages produced was measured after one hour of incubation at 30°C. As seen in Figure 3-3, C6433 (pSCRha-KL1*mazG*) and C6433 (pSCRha-AH2*mazG*) produced significantly more KS14 phage than the C6433 (pSCRha) control cells. K56-2 (pSCRha-KL1*mazG*) and K56-2 (pSCRha-AH2*mazG*) also showed an increase in KS14 phage production as compared to the K56-2 (pSCRha) control without *mazG*.

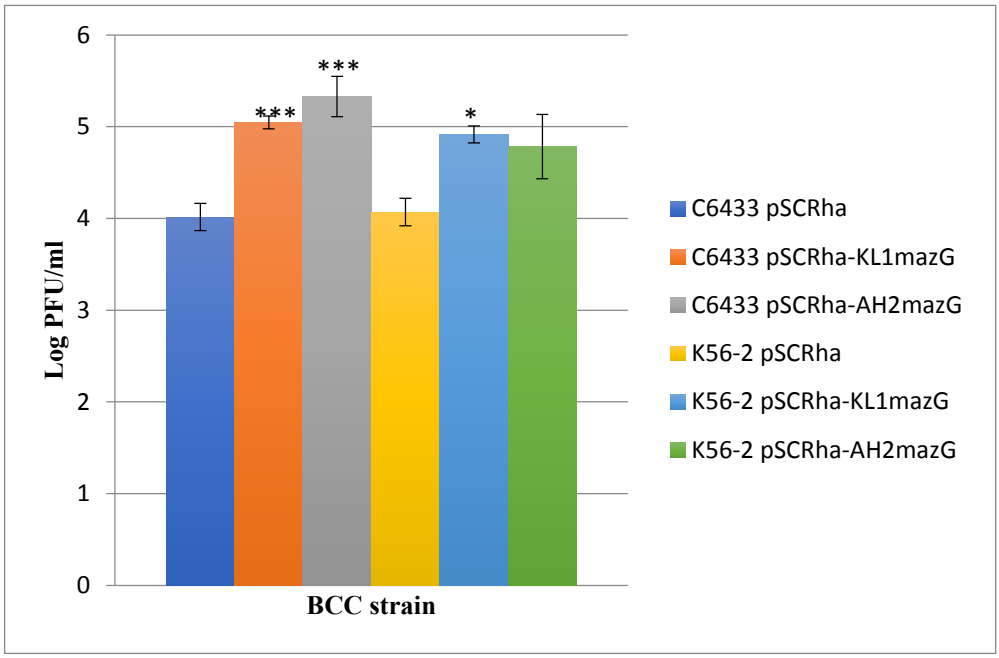


Figure 3-3: Production of phage KS14 in *B. cenocepacia* strains C6433 and K56-2 strains transformed with pSCRha *mazG* from phage KL1 or AH2.

Statistical analysis was performed using One-way ANOVA with Dunnett's post-test. *$p < 0.05$, ***$p < 0.001$ as compared with control. Values given are averages ± standard deviations for three trials.

Phage KS14 replication in the C6433 transformed with phage *mazG*

Phage replication was also tested by measuring a single burst size. Bcc phage KS14 was allowed to infect stationary phase cells of C6433 (pSCRha-KL1*mazG*) at a multiplicity of infection of 5 and phage produced were enumerated by titration plating after one round of replication. As seen in Figure 3-4, a three-fold increase in phage titers was observed for the *mazG* strain over the control. The average burst size

of 100 PFU/cell is significantly higher ($p < 0.001$) when compared to the average burst size of 30 PFU/cell for control C6433 with the blank plasmid.

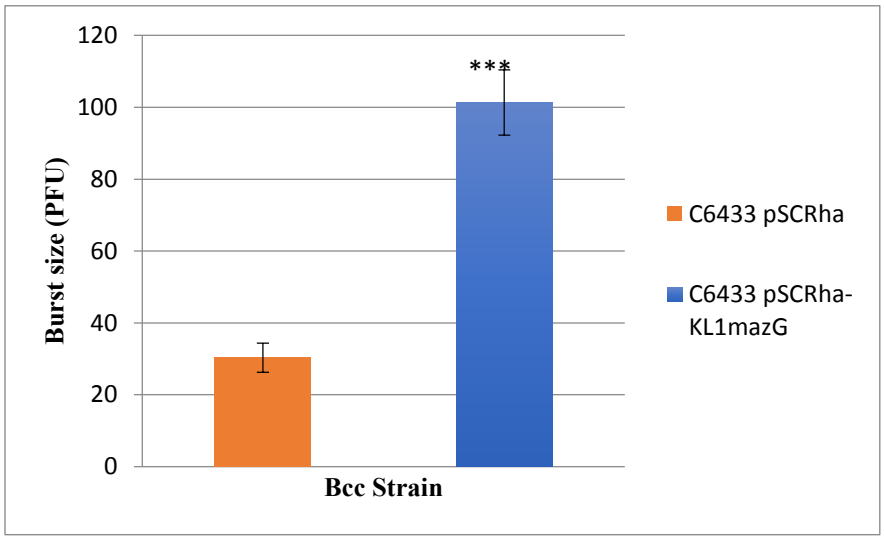


Figure 3-4: Replication of phage KS14 in *B. cenocepacia* C6433 transformed with pSCRha-KL1*mazG*. Six times more phage were produced in overnight cells that carried KL1 encoded MazG compared to control.

Statistical analysis was performed using a unpaired t-test. ***$p < 0.001$ as compared with control. Values given are averages ± standard deviations for three trials.

Galleria mellonella infection with K56-2 (pSCRha-KL1*mazG*)

As (p)pGpp has been implicated in affecting virulence, we tested the virulence of cells transformed with phage *mazG*. *G. mellonella* larvae were injected with K56-2 (pSCRha-KL1*mazG*) and were scored for death after 48 hours and 72 hours post infection. Larvae injected with K56-2 (pSCRha-KL1*mazG*) showed increased death

after both 48 hours and 72 hours as seen in Figure 3-5. Larval death increased from 40% for the control strain to 55% for the strains expressing KL1*mazG*.

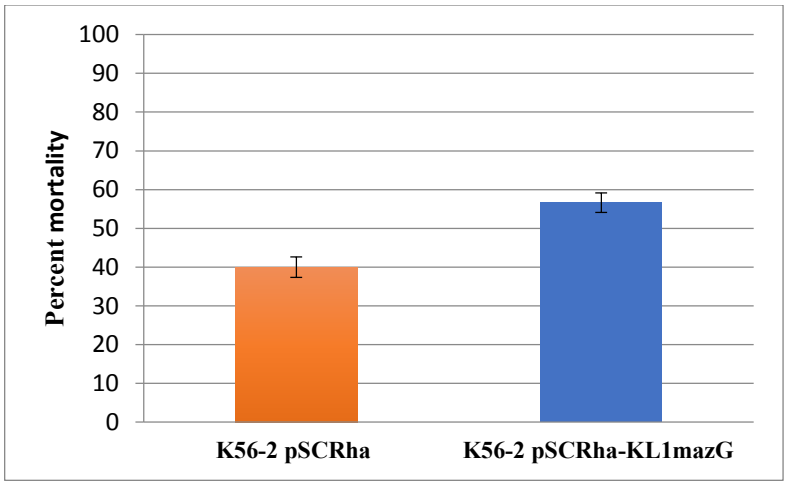


Figure 3-5: Mortality of *G. mellonella* larvae infected with *B. cenocepacia* K56-2 transformed with pscRha-KL1*mazG*. Larvae infected with K56-2 pscRha-KL1*mazG* show increased death 48 hours post infection.

Values given are averages ± standard deviations for three trials (n = 10 larvae per trial). Larvae were infected with 9×10^3 CFU.

Lemna minor infection with C6433 (pSCRha-KL1*mazG*)

L. minor were infected with varying amounts of C6433 (pSCRha-KL1*mazG*) ranging from 10^2 to 10^5 CFU and were scored for death 96 hours post infection. As seen in Figure 3-6, an increase in death was observed as indicated by loss of green colour even at the lowest concentration of 10^3 CFU of C6433 (pSCRha-KL1*mazG*) as compared to the control, which appeared healthy even at the highest concentration of cells (10^5 CFU). At 10^3 CFU of C6433 (pSCRha-KL1*mazG*), only 2/6 plants

appeared to be green and healthy and at higher CFU concentrations of 10^3-10^5, all 6 out of 6 plants appear yellow/unhealthy. This indicates a marked increase in virulence from $>10^5$ CFU to $<10^3$ CFU when *mazG* is present. As seen in Figure 3-6A, 100 times fewer cells of C6433 (pSCRha-KL1*mazG*) were needed to reach LD_{50} as compared to control C6433 (pSCRha). This is a statistically significant lower number with p-value <0.0001 for the t-test. The plants were infected with 10^3 CFU and percent mortality was compared for different strains with or without *mazG*: C6433 (pSCRha-KL1*mazG*) versus C6433 (pSCRha) and lysogen C6433::KL1 versus C6433. Twice the number of plants were killed by C6433::KL1 as compared to C6433 and a six fold increase in death was observed for C6433 (pSCRha-KL1*mazG*) over control C6433 (pSCRha) as seen in Figure 3-7.

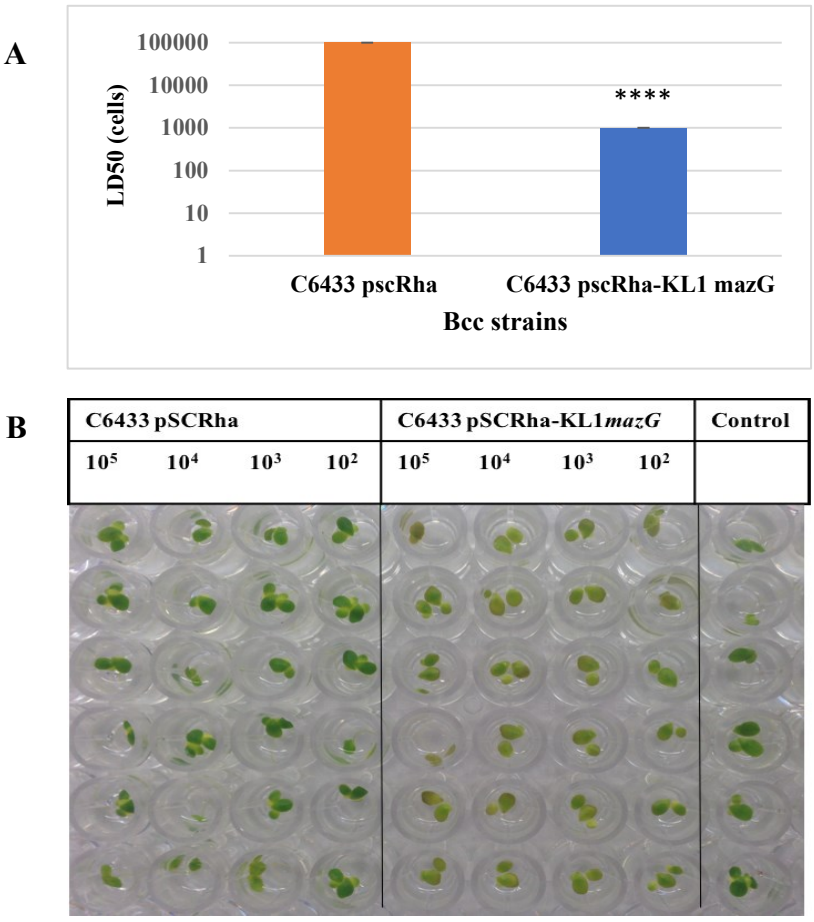


Figure 3-6: *Lemna minor* infected with *B. cenocepacia* C6433 transformed with pSCRha-KL1*mazG*. Plants were infected with 10^5, 10^4, 10^3 or 10^2 CFU of overnight cultures. A. LD_{50} was compared to control. B. Loss of green colour indicates death. Results shown are 96 hours post infection.

Statistical analysis was performed using a unpaired t-test. ****$p < 0.0001$ as compared with control.

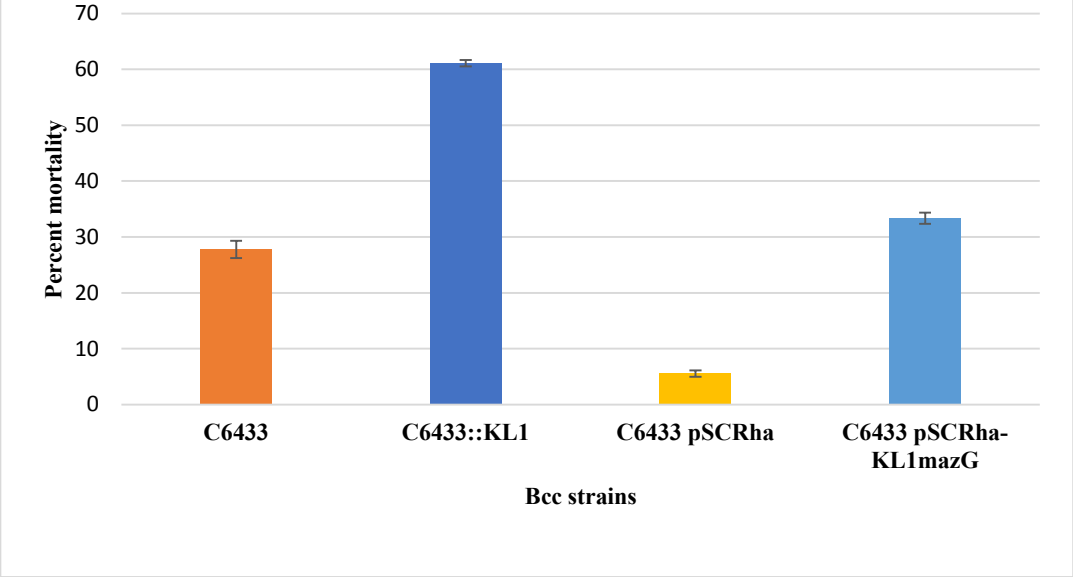


Figure 3-7: *Lemna minor* infected with different strains of *B. cenocepacia* C6433. Plants were infected with 10^3 CFU of overnight cultures of C6433, C6433::KL1, C6433 (pSCRha) or C6433 (pSCRha-KL1*mazG*). Plants infected with C6433::KL1 and C6433 (pSCRha-KL1*mazG*) show increased death 96 hours post infection.

Values given are averages ± standard deviations for three trials (n = 6 duckweed per trial).

Attempted deletion of *mazG* from the AH2 and KL1 genomes

Phage AH2 and KL1were lysogenized into C6433 and confirmed by PCR (Figure 3-8). Since the two phage genomes were found to be capable of being isolated by plasmid prep, they were isolated and propagated in C6433 to produce phage plaques (Figure 3-9). Flanking regions were cloned into the suicide vector (Figure 3-10) but recombination into the genome failed to take place to produce Tp^R single crossovers.

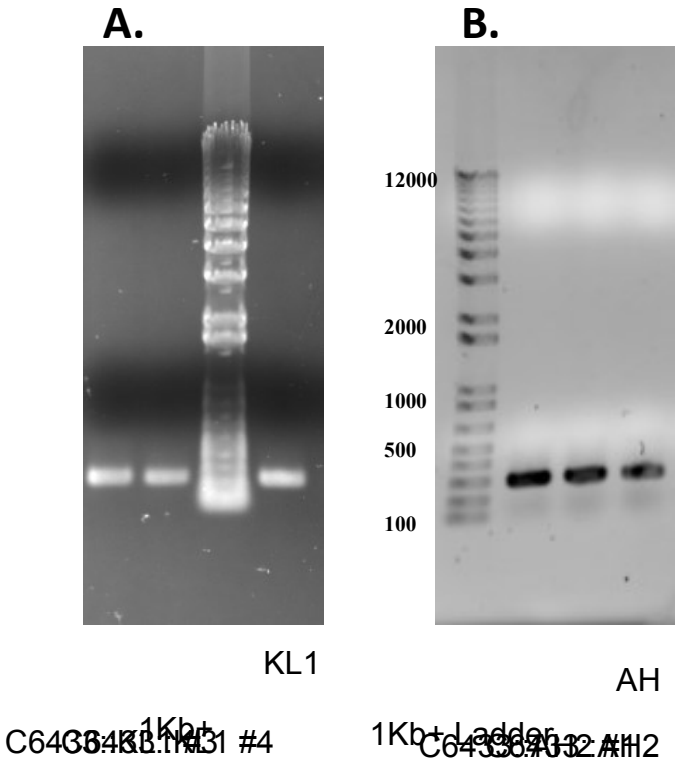


Figure 3-8: PCR confirmation of A. C6433::KL1 and B. C6433::AH2 on a 0.7% agarose gel. C6433::KL1 and C6433::AH2 lysogens were tested using specific primers for presence of KL1 and AH2 respectively.

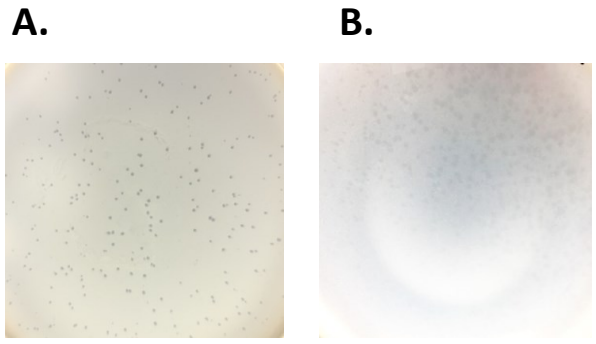
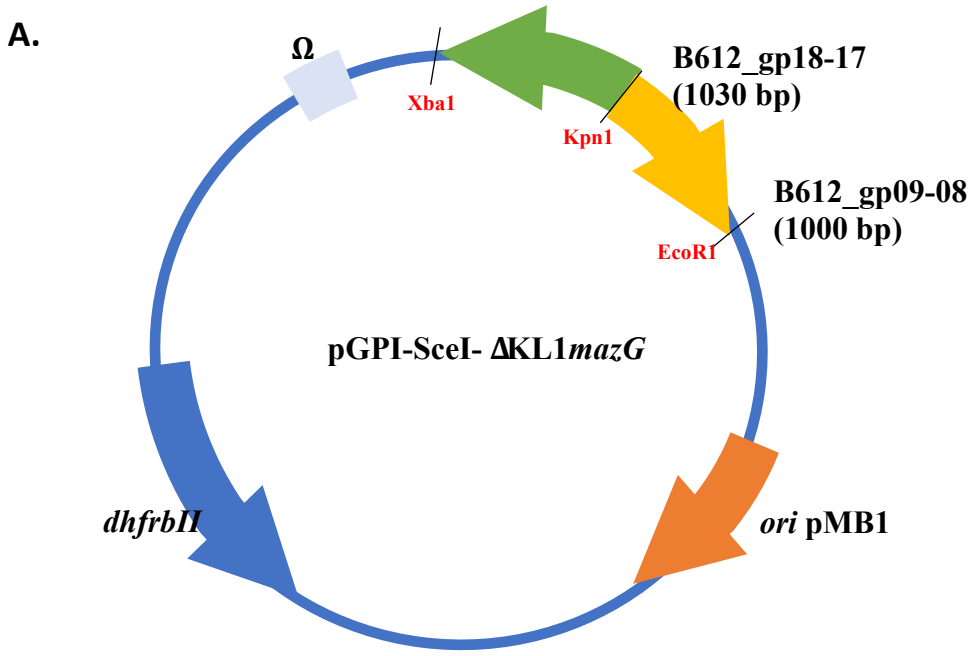


Figure 3-9: Plaques produced from phage plasmid A. pKL1 and B. pAH2 on *B. cenocepacia* C6433. Phage plasmids were electroporated into C6433 and recovered at

30°C for one hour. These were then plated in agar overlays with overnight cells of C6433. Plates were incubated at 30°C overnight.



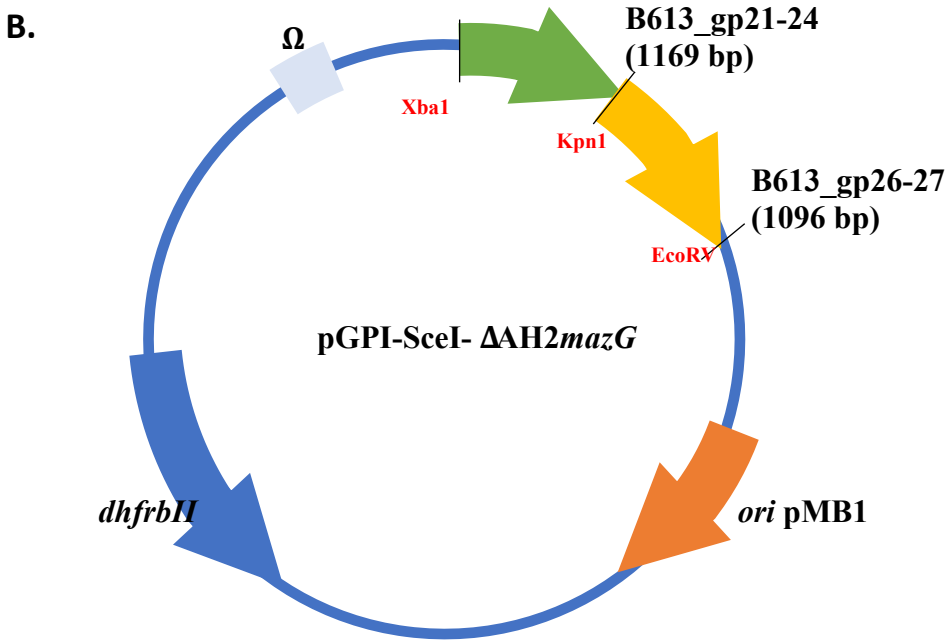


Figure 3-10: Plasmid maps of A. pGPI-SceI-ΔKL1*mazG* and B. pGPI-SceI-ΔAH2*mazG*. Upstream and downstream flanking regions of *mazG* were cloned into suicide vector pGPI-Sce1-MB1 for clean deletion of *mazG*.

Testing C6433 (pSCRha-KL1*mazG*) for Phage-Antibiotic Synergy (PAS)

Bcc phage KS14 were titered on C6433 (pSCRha) and C6433 (pSCRha-KL1*mazG*) in the presence of 20 µg/ml of meropenem (MEM) in the top agar to test for PAS effect. The phage did not show an increased plaque size in the *mazG* strains as compared to control as seen in Figure 3-11.

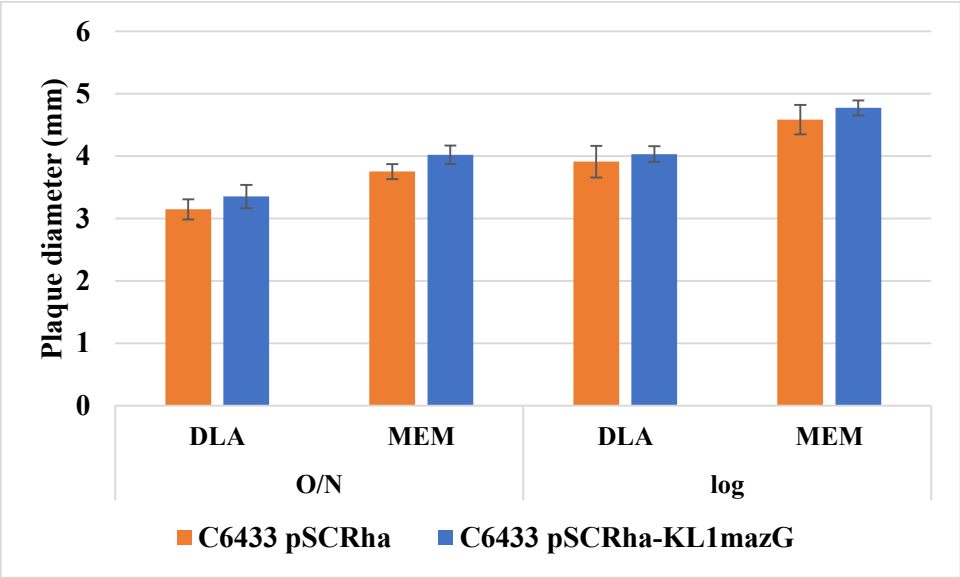


Figure 3-11: Plaque size of phage KS14 in the presence of meropenem (MEM) in the top agar. KS14 was titered on overnight and log-phase cells of C6433 (pSCRha) and C6433 (pSCRha-KL1*mazG*) and plaque size was measured.

Values given are averages ± standard deviations for ten plaques.

Phage T4 replication in *E. coli mazG* mutants

As bacterial *mazG* has been studied in E. coli, we tested its effect on phage production. T4 phage was allowed to infect stationary phase cells of *E. coli* BW25113 *mazG::kn* and BW25113 *mazF::kn* at a multiplicity of infection of 1 and phage produced were enumerated after one round of replication. As seen in Figure 3-12, a two-fold increase in phage titers was observed for the *mazG::kn* and *mazF::kn* mutants over control.

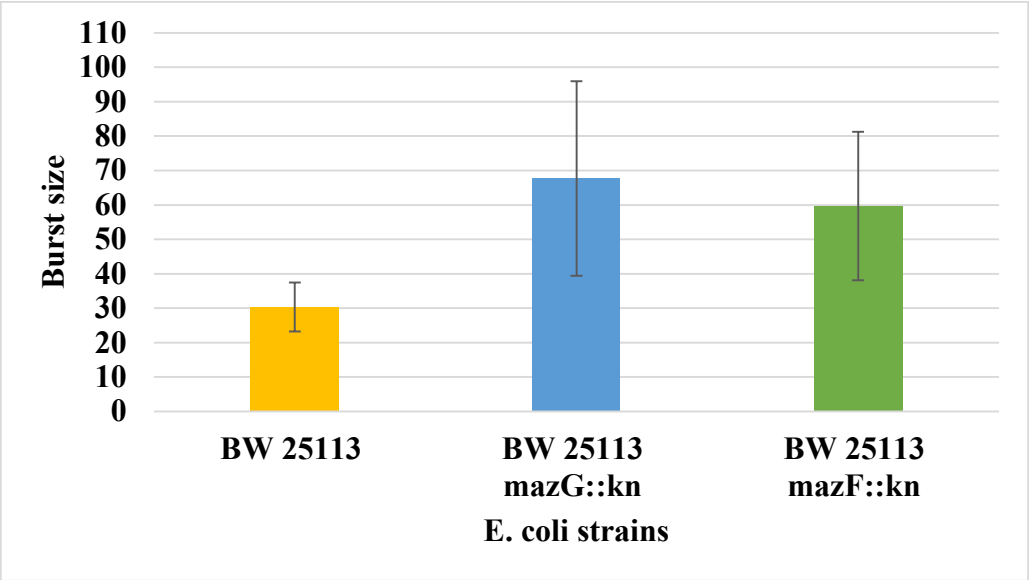


Figure 3-12: Replication of phage T4 in *E. coli* BW25113 *mazG::kn* and BW25113 *mazF::kn.* Twice more phage were produced in cells of BW25113 *mazG::kn* and BW25113 *mazF::kn* compared to control.

Values given are averages ± standard deviations for three trials.

Lemna minor infection with *E. coli*

L. minor were infected with varying amounts of *E. coli* BW25113 ranging from 10^1 to 10^5 CFU and were scored for death 96 hours post infection. The LD_{50} for this strain was determined to be 10^1 under these conditions. The *L. minor* plants were infected with 10^1 CFU *E. coli* BW25113 and percent mortality was compared for the strains BW25113, BW25113 *mazG::kn*, and BW25113 *mazF::kn*. The results show a three-fold increase in plants killed by BW25113 *mazG::kn*, and a four-fold increase in plants killed by BW25113 *mazF::kn* as compared to wild-type BW25113, as shown in Figure 3-13

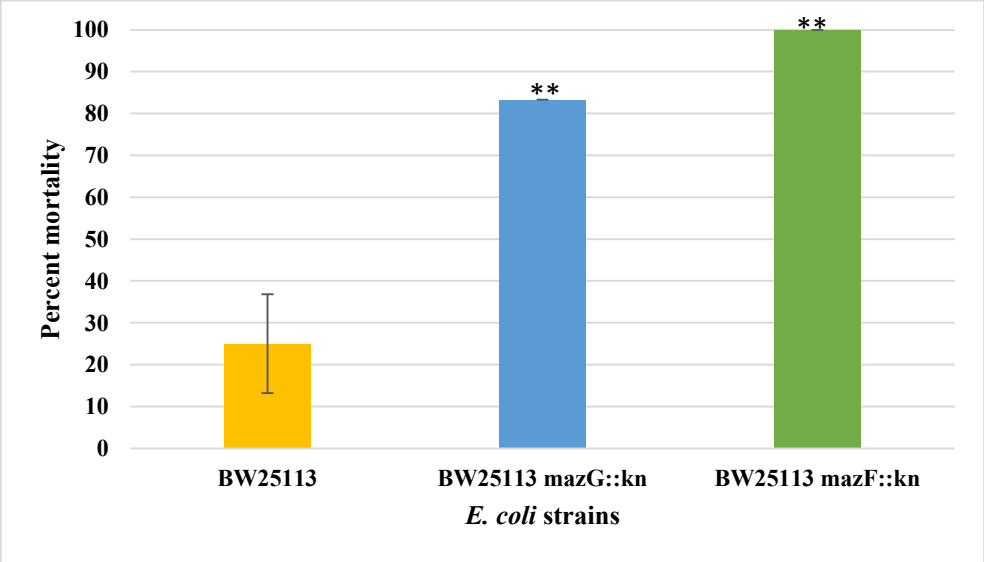


Figure 3-13: *Lemna minor* infected with different strains of *E. coli* BW25113. Plants were infected with 15 CFU of overnight cultures of BW25113, BW25113 *mazG::kn*, BW25113 *mazF::kn*. Plants infected with BW25113 *mazG::kn* and BW25113 *mazF::kn* show increased death 96 hours post infection.

Statistical analysis was performed using One-way ANOVA with Dunnett's post-test. **$p < 0.01$ as compared with control Values given are averages ± standard deviations for three trials ($n = 6$ duckweed per trial).

Discussion

Certain marine phages have been discovered to carry a MazG homolog (Mann et al., 2005; Sullivan et al., 2010). This gene has also been found to be conserved in phages from different geographical locations. Since MazG has been associated with the control of the stringent response in bacteria, phages possessing a mazG copy in their genomes are thought to have an advantage when infecting slow-growing host

bacteria found in nutrient-limiting environments (Clokie and Mann, 2006; Lynch et al., 2012).

In this study, we have examined the MazG homologs from two Bcc phages KL1 and AH2. The two homologs are highly similar to *E. coli* MazG (Lynch et al., 2012). Since Bcc are found ubiquitously in soil and water, which can be low-nutrient environments, the *mazG* gene possessed by these phages may enhance phage infection and production of progeny. We tested the effects of the KL1 and AH2 encoded MazG on production of Bcc phage KS14 in *B. cenocepacia* C6433. The cells were recovered in water, a low-nutrient environment, after introduction of phage KS14 as a plasmid. We observed increased phage particles produced from cells which contained MazG. This effect was also observed when KS14 burst size was measured in the presence of MazG in the stationary cells. There was no difference observed when log phase cells were used for burst sizes. This suggests that MazG primarily assists with phage production once host cells exhaust nutrients and reach stationary phase.

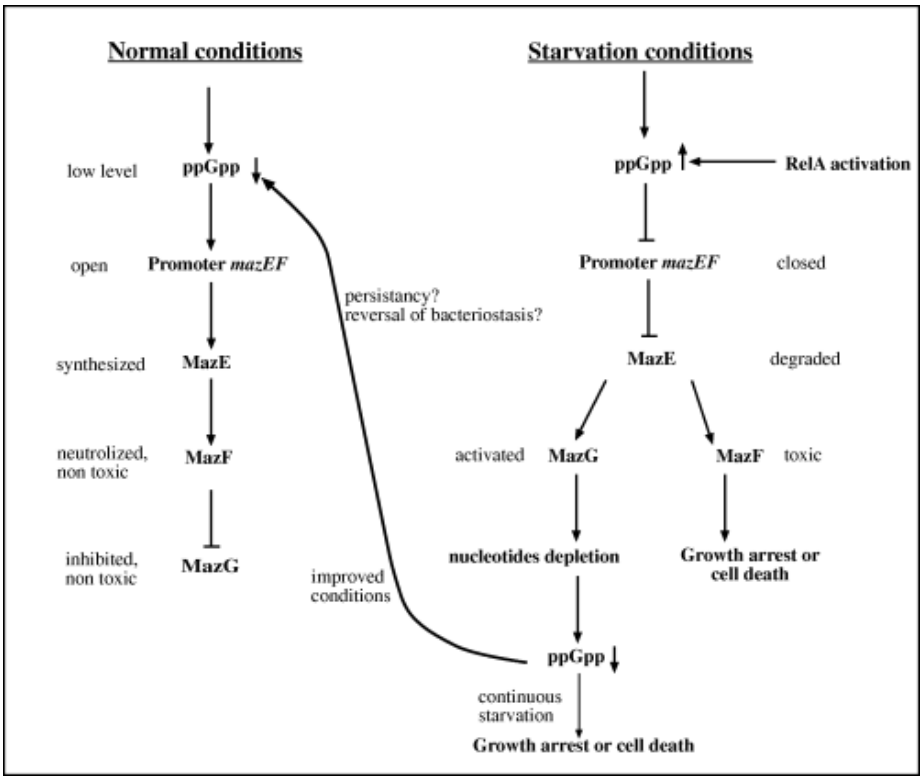


Figure 3-14: Model of MazG function in the stringent response in bacteria as proposed by Gross *et al,* 2006.

As seen in figure 3-14, MazG acts on (p)ppGpp which is a main effector of the stringent response and works by preventing the accumulation of (p)ppGpp under stressful conditions (Gross et al., 2006). Since cellular stress and virulence factor elaboration are intimately connected in bacteria, (p)ppGpp also affects the virulence of bacteria. Therefore, we tested the effect of phage encoded MazG on the virulence of transformed Bcc cells. We noted a statistically significant 1000-fold lower LD_{50} for C6433 (pSCRha-KL1*mazG*) in a duckweed infection model. This effect was observed as increased plant death for both the C6433::KL1 lysogen as well as C6433 with the *mazG* gene cloned into plasmid pSCRha-KL1*mazG.* The infection titers for C6433 (pSCRha) and C6433 (pSCRha-KL1*mazG*) were slightly lower than those of

the lysogen, which may explain the reduced killing observed when compared to C6433 and C6433::KL1. But in both cases [C6433::KL1 versus C6433 and C6433 (pSCRha-KL1*mazG*) versus C6433 (pSCRha)], increased virulence was observed when *mazG* was present. Although we were not measuring ppGpp directly, these results are in contrast to earlier studies where reduced virulence was observed in *V. cholera* and *S. typhimurium* as a result of reduced (p)ppGpp pools (Haralalka et al., 2003; Pizarro-Cerdá and Tedin, 2004). Conversely, other studies in certain bacteria have also observed increased virulence when the pool of (p)pGpp is increased (Jain, Kumar and Chatterji, 2006). To ensure this effect was not related to the plant-based infection model, we also tested K56-2 (pSCRha-KL1*mazG*) in a *G. mellonella* model and similarly observed an increase in waxworm larval mortality over controls, again suggesting that expressed *mazG* was reducing ppGpp, thus enhancing *B. cenocepacia* virulence. This could be because Bcc virulence factors are regulated differently to those in other tested bacteria, or perhaps because phage-encoded MazG plays a different role to bacterial MazG in virulence regulation.

Besides examining phage encoded *mazG* homologs, we also extended our study to the effect of bacterial *mazG* specifically *E. coli*. We tested the virulence of *E. coli mazG* and *mazF* deletion mutants in the duckweed model. MazF is the toxin in the MazEF TA module that governs programmed cell death in *E. coli*. *mazG* is found downstream of *mazF* and has been implicated to play a possible role allowing bacteria to grow under starvation condition. A significant increase in plant death was observed for both the mutant strains. We expected an increase in virulence when *mazG* was deleted as this would result in increased (p)ppGpp levels in the cells. We

also observed increased virulence for *mazF* deletion mutant. This suggests that deletion of *mazF* has the same effect on the cells.

Since KL1 encoded MazG increased phage production, we also wanted to test *mazG* in *E. coli* with available *E. coli* phages. We examined phage T4 burst size upon infecting stationary cells of *mazG* deletion mutant BW25113 *mazG:kn* and mazF deletion mutant BW25113 *mazF:kn.* Interestingly, we observed increased phage production in cells lacking *mazG* or *mazF* with two times more T4 phage produced in both conditions, as compared to controls. As the wild-type cells could enter programmed cell death but the not the *mazF::kn* mutants, this would explain why T4 could infect more *mazF*::kn as compared to wild-type cells. This has been observed in a previous study where more P1 phages were produced from Δ*mazEF* mutant than wild-type (Engelberg-Kulka et al., 2006). This is also supported by the model proposed by Gross *et al.* (2006) where presence of the toxin MazF results in growth arrest or cell dealth under starvation conditions. The absence of MazF would allow cells to escape death and allow phages to replicate which is what we observed for *mazF::kn* cells. It is interesting that *mazG::kn* produced a similar effect.

We also attempted to delete *mazG* from the two phages to examine the effect on phage infection and propagation. We used a system for making unmarked deletions in the Bcc (Flannagan et al., 2008). This clean deletion system has been used successfully in the lab with *B. cenocepacia* strain K56-2. This is the first attempt to use it for deleting a phage gene. For this purpose, we generated lysogens of both the phages in *B. cenocepacia* C6433. However, we had problems recovering a single crossover in the cell where the flanking regions of the *mazG* would have integrated

into the phage genome. Since we have had success with this system in K56-2 previously, we transformed K56-2 with plasmids KL1 and AH2 to produce K56-2 pKL1 and K56-2 pAH2. However, attempts at deleting *mazG* resulted in the same results as C6433. This could indicate that *mazG* might be required by phage and deleting it results in loss of phage from the cells or else there is some other problem with recombination between the phage genome as plasmid and the suicide vector.

Lastly, we also used 20 µg/ml (2XMIC) meropenem in the top agar of the plate to test *mazG* transformed C6433 for PAS. Although, an increase in KS14 plaque size was observed for both C6433 (pSCRha-KL1*mazG*) and the control C6433 (pSCRha), no significant difference was observed between the two.

Acknowledgement

We would like to thank Karlene Lynch for initial experiments with *mazG* from KL1 and AH2 on Bcc phage production and Carly Davis for isolating C6433::AH2 lysogen and attempting to clean delete *mazG* from AH2.

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Chapter 4 : Conclusions

Research Goals

Bacteria of the Bcc cause chronic infections in CF patients which are difficult to eradicate because of their extremely high resistance to antimicrobials (Isles et al., 1984; Lewin et al., 1993; LiPuma, 1998). The Bcc bacteria are also especially problematic due to their ability to spread from person to person (LiPuma et al., 1990; Govan et al., 1993) and their ability to convert to an acute infection, causing rapid death by necrotizing pneumonia which has been observed to occur in approximately 10% of cases (LiPuma, 1998). The resistance to antibiotics involves both acquired as well as intrinsic mechanisms, and some reports describe extreme antibiotic resistance to 50% of all antibiotics tested (Aaron et al., 2000). Since resistance appears to occur readily, the clinical treatment of Bcc-infected patients typically involves the use of three antibiotic combinations, such as ceftazidime, meropenem and minocycline, and recently trimethoprim-sulfamethoxazole has emerged as a major therapeutic option (Aaron et al., 2000; Zhou et al., 2007; Avgeri et al., 2009). Despite this, multi-drug resistant Bcc isolates are not uncommon (Horsley et al., 2012).

This high level of drug resistance in the Bcc demands better and alternative treatment methods. One of the most promising alternative strategies available is the use of bacteriophages to kill pathogenic bacteria. With the rise of multi-drug resistant bacteria and a renewed interest in phage therapy, several research groups are exploring the use of phages in the treatment of bacterial infections (Roach and Debarbieux, 2017). For phage therapy to be most effective, however, we need to consider methods that enhance its treatment efficacy.

With a focus on enhancing efficacy of phage therapy against the Bcc, one of the strategies we explored was testing the synergistic effect of chemical antibiotics on phages, as described in Chapter 2. Towards this end, phages KS12 and KS14 were tested against *B. cenocepacia* strains K56-2 and C6433, respectively. The phages were combined with a number of antibiotics initially, and three of the antibiotics (ciprofloxacin, meropenem and tetracycline) were chosen for further Phage-Antibiotic Synergy (PAS) studies. PAS was observed to increase phage plaque sizes and phage titers for both KS12 and KS14. We also observed a significant increase in survival of Bcc-infected *G. mellonella* larvae when treated with meropenem or minocycline, in combination with phage as compared to phage or antibiotics alone. These results show that traditional antibiotics can be used along with phages to enhance treatment efficacy.

Another strategy we explored was to test the effects of phage-encoded MazG on Bcc phage production. In Chapter 3 we describe the impact of phages KL1 and AH2 MazG on the enhancement of phage production in overnight cultures of *B. cenocepacia* C6433 and K56-2. Bcc transformed with KL1 or AH2 *mazG* produced significantly higher number of phages upon the infection of stationary phase cells. Because Bcc bacteria during chronic infections are likely to be at least in part in stationary phase, we have demonstrated that MazG can advantage attacking phages to produce more phages in slow-growing cells. However, we also noted potentially increased virulence for Bcc hosts in the presence of phage MazG.

Future directions

Bcc are excellent candidates for phage therapy given their high level resistance to antibiotics. One of the mechanisms that contributes to this high resistance among Bcc isolates is the ability to form biofilms (Conway et al., 2004). Bcc cells embedded within biofilms are protected from therapeutic agents, making it more difficult to clear bacterial infections. There have been previous reports of phages being used in combination with antibiotics to eradicate biofilms formed by *E. coli* and *P. aeruginosa* (Ryan et al., 2012; Nouraldin et al., 2016). It would be interesting to observe whether PAS can be used against Bcc biofilms. Herein, we have provided some preliminary data that suggests that PAS against biofilm-grown cells can be extended to the Bcc. Also, we observed a change in Bcc cell morphology in the presence of ciprofloxacin and meropenem resulting in elongated filamentous cells, similar to previous reports (Comeau et al., 2007; Spratt, 1975; Diver and Wise, 1986). However, we observed cell clustering instead of filamentation when tetracycline was present rather than β-lactams. Real-time imaging of cell growth in the presence of these antibiotics and phage infection, and replication and release of progeny phage would confirm our hypothesis that increased accessibility to host cells permits enhanced multiplication of phages. One approach that could be used for this would be atomic force microscopy, as described in a study for visualizing phage infection in *E. coli* (Dubrovin et al., 2008). In this report, cell surface changes associated with phage interaction were observed.

We noticed differences in the effect of minocycline when compared to the other antibiotics that exhibit PAS. Although phage plaque size was increased in the presence of minocycline, phage titers were not significantly increased, and much higher doses of minocycline were required to rescue Bcc-infected *G. mellonella* as compared to meropenem. These differences could be due to a different PAS mechanism of action for the two antibiotics. Further investigation of these differences might help us to better understand and describe PAS activity.

In Chapter 3, we tested the role of MazG in phage production. Cloning the *mazG* gene from phages KL1 and AH2 into *B. cenocepacia* did increase production of KS14 upon infection of the transformed cells. However, we encountered difficulty when attempting to remove the *mazG* gene from the genomes of the two phages. We attempted to perform a clean deletion of the gene using a system that has worked well previously for *Burkholderia* species in our lab (Flannagan et al., 2007). However, failure to obtain a deleted gene from the prophage genomes suggests that it may not be as effective for manipulating phage genomes. Alternatively, there are other systems available, such as BRED, that have been reported in the successful recombineering of mycobacteriophages (Marinelli et al., 2008). Although this system has not yet been adapted for use in *Burkholderia,* this possibility exists. BRED makes use of a phage recombinase to induce homologous recombination between a phage genome and a template electroporated into the cell (Marinelli et al., 2008). Another promising approach is the use of CRISPR/Cas, which has been recently adapted as a tool for genome engineering of phages (Martel and Moineau, 2014). This report demonstrated the use of CRISPR/Cas to engineer point mutations and large deletions

in the genome of a phage that infects *Streptococcus thermophilus*. This system may be the most promising approach that can be adapted for manipulating Bcc phage genomes.

There are several questions left unanswered from Chapter 3. Once *mazG* is deleted from the phage genomes, we can study the specific differences in bacterial infection by comparing Δ*mazG* phages to the wild-type versions. We expect wild-type phages to possess an advantage over Δ*mazG* phages in infecting cells, especially those growing slowly in stationary phase. Recently, persister cells have also been found to occur in Bcc biofilms (Van Acker et al., 2013). These cells are thought to remain dormant in a population under stress, such as that caused by antibiotic treatment (Shah et al., 2006). It may be that *mazG* phages will also be effective against persister cells in a Bcc biofilm population, inducing them back to actively growing cells and thus supporting phage propagation. *E. coli* MazG has been shown to hydrolyze nucleoside triphosphates (Zhang and Inouye, 2002). It is hypothesized to reduce the pool of (p)ppGpp, the alarmone which acts an effector of stringent response, thus allowing cells to avoid programmed cell death (Gross et al., 2006). Phage-encoded MazG should be tested in a biochemical assay for this nucleoside triphosphate pyrophosphohydrolase activity, since both KL1 and AH2 MazG exhibit significant homology to *E. coli* MazG (Lynch et al., 2012).

Significance

We have shown that PAS can be used to increase the efficacy of treatment by allowing phages and antibiotics to work together, as observed in the *G. mellonella*

infection model. With the mucous buildup in the lungs of CF patents and the occurrence of complex multi-species biofilms, the dose of antibiotics that actually reaches the bacterial cells is sub-inhibitory. As PAS permits phages to work with increased efficacy in the presence of (sub-inhibitory concentrations of) antibiotics, this PAS alternative treatment could enhance therapeutic success. We have also shown a potential role for phage-encoded MazG in allowing enhanced phage production from cells in stationary phase. However, more work is required to better understand how phage encoded MazG improves phage infection and production, and how it may affect other cellular traits including virulence.

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