University of Alberta

Characterization of lipooligosaccharide as a phage receptor and fitness factor in *Burkholderia cenocepacia*

by

Ashraf Hussein Abdu

A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of

Master of Science

in

Microbiology and Biotechnology

Biological Sciences

Fall 2013

Edmonton, Alberta

Abstract

The *Burkholderia cepacia* complex (Bcc) are ubiquitous in nature and can be isolated from soil, water, plants and more recently in hospitals. The Bcc comprises at least 17 Gram-negative bacterial species that are notorious for their pathogenic effects in immunocompromised individuals. Host colonization by these bacteria causes problems due to their innate resistance to antibiotics. Because of the difficulties in treating Bcc infections, researchers are reevaluating bacteriophages as an alternative therapy to antibiotics. My objective was to confirm the receptor for the Bcc-specific bacteriophage KS10, as this knowledge will allow for the construction of effective multi-receptor phage cocktails to treat Bcc infections. Through the use of an updated I-SceI deletion system, we were able to construct LOS mutants to verify previous findings that KS10 interacts with LOS as a receptor. In addition to KS10 resistance, both *wabO* and *waaC* mutations displayed reduced fitness in the form of decreased growth and swimming motility when compared to wildtype PC184. These results suggest that phage-resistant mutations can be detrimental to the bacteria.

Acknowledgements

First and foremost, I would like to thank my supervisor Dr. Jonathan Dennis. Without your support, guidance and patience this degree would not have been possible. I would also like to thank the Dennis lab for making it an awesome place to work and for putting up with my shenanigans. A big thanks goes out to Euan Thomson, Karen Lithgow, Erin Dockery, Diana Semler, Bryan Frobb and Fatima Kamal for your support and technical advice. A special thanks goes to my mentor, friend and editor Dr. Lynch.

I would like to thank all my friends in Biological Sciences especially Corey Wenzel, Jeremy Iwashkiw and the Raivio lab for all the experimental advice that you guys have given me over the years.

I would like to thank Bridget, Emily, Karine, Yetayale, Joe and the rest of my friends who provided emotional support throughout my degree. Thanks to my soccer team Looking to Score for making me get out and do physical activity.

Finally I'd like to thank my family for encouraging me to do my best and for supporting me throughout my MSc. And last but not least, I'd like to thank my sister for listening to me rant about things she did not understand and for being an awesome roommate!

Table of Contents

List of Tables

List of Figures

List of Abbreviations

ACC	1-aminocyclopropane-1-carboxylate
Ara4N	4-amino-4-deoxy-4-amino-arabinopyranose
aphI	Aminoglycoside phosphotransferase
Bcc	*Burkholderia cepacia* complex
BLAST	Basic Local Alignment Search Tool
bp	Base pair
cat	Chloramphenicol acetyltransferase
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
CFU	Colony forming units
CGD	Chronic granulomatous disease
Cm	Chloramphenicol
DNA	Deoxyribonucleic acid
EOP	Efficiency of plating
EPS	Exopolysaccharide
ET12	Electrophoretic Type 12
gp	Gene product
Glc	Glucose
h	Hour
Hep	Heptose
IPTG	Isopropyl β-D-1-thiogalactopyranoside
K	Growth rate constant

Kbp	Kilo base pair
Km	Kanamycin
Ko	D-glycero-α-D-talo-oct-2-ulopyranosylonic acid
LB	Luria-Bertani
LOS	Lipooligosaccharide
LPS	Lipopolysaccharide
Max	Maximum
MIC	Minimum inhibitory concentrations
Min	Minute
MOI	Multiplicity of infection
Mot	Motor complex
NADPH	Reduced nicotinamide dinucleotide phosphate
NCBI	National Center for Biotechnology Information
OD	Optical density
OM	Outer membrane
oriR	Origin of replication
PCR	Polymerase chain reaction
PFU	Plaque forming units
PG	Peptidoglycan
PHDC	Philadelphia-District of Columbia
RFLP	Restriction fragment length polymorphism
RND	Resistance-Nodulation-Division
SAR	Signal-arrest-release

SM	Suspension medium
Tet	Tetracycline
Tp	Trimethoprim
v	Volume
w	Weight
WT	Wildtype

Introduction

***Burkholderia cepacia* complex (Bcc)**

William Burkholder discovered *Burkholderia cepacia* because of the bacteria's pathogenic ability to cause sour skin onion rot. After his discovery Burkholder reported the Gram-negative bacterium as *Pseudomonas cepacia* (Burkholder, 1950). During the 1990s, molecular taxonomic analysis of *Pseudomonas* homology group II was performed and a reclassification was in order. 16S rRNA analysis allowed for a new genus, *Burkholderia,* to be proposed for *Pseudomonas* homology group II which contained *Pseudomonas cepacia* (Yabuuchi et al., 1992). In the latter half of the 1990s, additional phenotypic and genotypic analysis resulted in the identification of five phenotypically similar but genotypically distinct species that were referred to as the *Burkholderia cepacia* complex (Bcc) (Vandamme et al., 1997). Currently the number of species included in the Bcc has expanded to include twelve new species which brings the total to at least seventeen (Vanlaere et al., 2009).

Environmental role

Burkholderia species are ubiquitous in nature and can be isolated from the soil, water, plants and more recently in hospitals (Mahenthiralingam et al., 2005). Bcc bacteria are plant pathogens known to cause a variety of diseases such as stripe, wilt, rot, and blight. The phytopathogenic species of the *Burkholderia* group includes: *B. andropogonis, B. caryophylli, B. cepacia, B. gladioli, B. glumae* and *B.*

plantarii (Coenye and Vandamme, 2007). Despite their initial isolation and characterization as plant pathogens, the Bcc bacteria do serve a beneficial role in the environment. Certain Bcc strains are capable of promoting plant growth by fixing nitrogen or by producing 1-aminocyclopropane-1-carboxylate (ACC) deaminase which assist plant root development (Sessitsch et al., 2005). Bcc strains are also capable of producing antimicrobial and antifungal compounds that have an antagonistic effect on bacterial and fungal plant pathogens. An example includes *Burkholderia pyrocinia* which is capable of producing the compound pyrrolnitrin, a compound that inhibits fungal growth (Hammer and Evensen, 1993; Burkhead et al., 1994). Despite some beneficial characteristics as a biological control agent and phytostimulant, the use of Bcc species in agricultural settings has been banned since they are also a human pathogen (Gautam et al., 2011).

Cystic fibrosis

Although members of the Bcc complex can promote healthy plant development, they are more commonly known as opportunistic human pathogens. Bcc bacteria rarely infect healthy individuals, instead disproportionately impacting immunocompromised patients including individuals diagnosed with cystic fibrosis (CF). CF is an autosomal recessive disease that is most common within Caucasian populations affecting 1 in 3000 (O'Sullivan and Freedman, 2009). The prevalence of

CF drops significantly when examining other ethnic backgrounds; the reported frequency for African or Asian descendants is 1 in 20,000 or 1 in 350,000 respectively (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 inhibiting sodium transport. In the absence of a functional CFTR, abnormalities in the transport of electrolytes are found across epithelial cells within the airways, pancreas, sweat duct and the intestinal tract (Robinson and Bye, 2002). The lungs of CF individuals provide favorable conditions for colonization by bacteria due to the accumulation of thick mucus which inhibits mucociliary clearance (Robinson and Bye, 2002). Due to the decline in lung function, respiratory infection by the Bcc bacteria and other microbes plays an instrumental role in morbidity and mortality of CF patients.

Chronic granulomatous disease

Patients with chronic granulomatous disease (CGD) are also susceptible to infection by Bcc organisms (Speert et al., 1994; Winkelstein et al., 2000). This immunodeficiency is due to a mutation in one of the four subunits of reduced nicotinamide dinucleotide phosphate (NADPH) oxidase (van den Berg et al., 2009). This enzyme is responsible for the production of an oxygen free-radical (superoxide), which in turn can be used to synthesize reactive oxygen species (H_2O_2, HOCl, OH•) (Babior,

2004; Van den Berg et al., 2009). Without the microbicidal activity of these derivatives, CGD patients are highly susceptible to Bcc that can result in pneumonia, sepsis and eventually death (Speert et al., 1994).

Bcc infection

Due to the severity and difficulties in treating Bcc infections, infection-control strategies have been established for CF patients to limit the potential spread of virulent bacteria (Saiman and Siegel, 2003). In the early 1980s, the first documented case of “cepacia syndrome” was reported in infected CF patients. Cepacia syndrome is characterized by high fever, bacteremia and rapid lung deterioration that results in a reduced life expectancy (Isles et al., 1984). The acquisition of infections by immunocompromised individuals is thought to occur by contact with an aerosol droplet, contaminated surface or through an infected individual (Mahenthiralingam et al., 2005). After discovering that inter-patient spread of Bcc infection was possible in nosocomial and social settings, infected individuals are expected to follow stringent infection control practices to reduce transmission (Govan et al., 1993; LiPuma et al., 1990; Saiman and Siegel, 2003). The negative outcome of these infections illustrates the importance of additional research to examine the pathogenicity of the Bcc. More information is required to develop new therapeutic strategies to combat the infection of immunocompromised patients.

Bcc virulence factors

Antibiotic resistance

Bcc bacteria demonstrate multiple antibiotic resistance that complicates the efficacy of treatment. The intrinsic resistance to antibiotics of the Bcc has a similar mechanism observed in other Gram-negative species. Resistance can be attributed to the bacterial cell membrane structure, enzymatic modification, alteration of drug targets and activation of efflux pumps (Coenye and Vandamme, 2007). The bacterial outer membrane structure, namely the lipopolysaccharide (LPS), is important for its toxicity and resistance towards antimicrobials. The unique structure of Bcc LPS has been proven to increase resistance of Bcc to a variety of antibiotics. The addition of 4-amino-4-deoxy-4-amino-arabinopyranose (Ara4N) on lipid A and the presence of the unusual sugar D-glycero-α-D-talo-oct-2-ulopyranosylonic acid (Ko) in the inner core contribute to the reduction of the overall net charge of the outer membrane which confers resistance to cationic antimicrobial peptides (Leitão et al., 2010; Silipo et al., 2007). In addition, Bcc bacteria are capable of producing a copious amount of exopolysaccharide (EPS), which allows them to live in organized communities called biofilms. These biofilms have the potential to impede the activity of immune cells and antibiotics (Hughes et al., 2008). The susceptibility of biofilm- and planktonic- grown Bcc bacteria to antibiotics was investigated by Desai et al. (1998). Their findings revealed that biofilm grown Bcc cells showed an increased resistance towards the

antibiotics ciprofloxacin and ceftazidime during exponential phase growth than their planktonic counterparts (Desai et al., 1998). These findings show that Bcc EPS is capable of forming a protective barrier that contributes to their multi-drug resistance.

Antibiotic resistance within the Bcc can also be attributed to the modification of drug targets or incoming antibiotics. Alterations to essential structural components that antibiotics target have been reported in the Bcc. For the latter, chromosomally encoded β-lactamases have been discovered in the genome of Bcc species and shown to confer resistance to β-lactams (Cheung et al., 2002; Prince et al., 1988). Mutations in the structural component of DNA gyrase, *gyrA*, showed an increased resistance to quinolone in *B. vietnamiensis* (Miché and Balandreau, 2001).

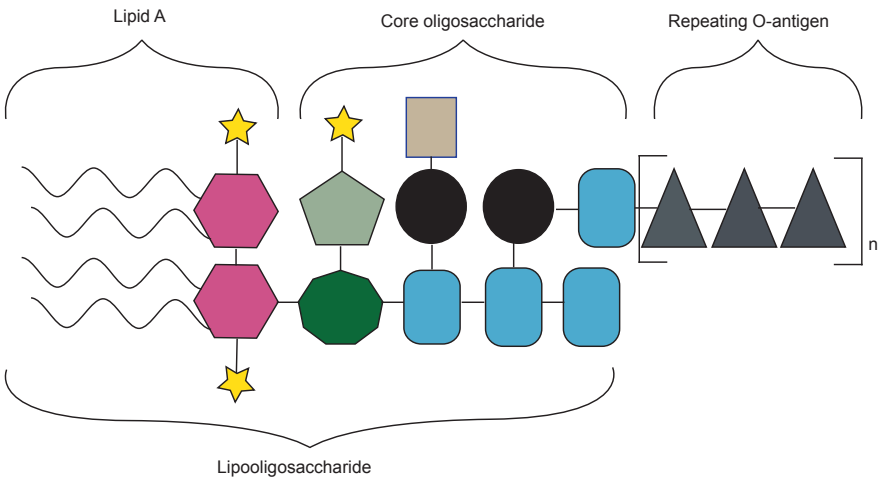
One of the main resistance mechanisms in Gram-negative species is the active efflux of antibiotics out of the cell. *B. cenocepacia* uses a major family of efflux pumps known as Resistance-Nodulation-Division (RND). RND efflux pumps are tripartite complexes composed of an outer membrane channel protein, a periplasmic membrane fusion protein and an inner membrane transporter protein (Bazzini et al., 2011). The advancement of technology in the past decade has allowed the clinical isolate *B. cenocepacia* J2315 to be sequenced and annotated. These recent advancements have resulted in the identification of 16 putative RND efflux systems throughout the *B. cenocepacia* genome (Bazzini et al., 2011). The identification of efflux pumps in J2315 is not surprising as

this clinical isolate is highly antibiotic resistant. The functionality of some of these efflux pumps has been shown experimentally: transfer of the *ceo* operon (RND-10) to an antibiotic susceptible strain conferred resistance to chloramphenicol, trimethoprim, and ciprofloxacin (Nair et al., 2004). Additionally, deleting RND-4 in J2315 revealed an increased sensitivity to aztreonam, chloramphenicol, gentamicin and various fluoroquinolones, suggesting that RND-4 contributes significantly to the resistance of this bacterium (Buroni et al., 2009). To fully understand the intrinsic antibiotic resistance of *B. cenocepacia*, further characterizations of the remaining RND pumps is required.

Lipopolysaccharide

Bcc LPS is a virulence factor that contributes to antibiotic resistance, toxicity, structural integrity and modulation of the immune response (De Soyza et al., 2008). Electrophoretic Type 12 (ET12) is a highly transmissible epidemic lineage of *B. cenocepacia* that spread from Canada to the United Kingdom and was associated with mortalities in CF patients (Govan et al., 1996). I will be concentrating my thesis discussion on *B. cenocepacia* due to the bacterium's virulent and antibiotic resistant nature. The LPS structure of Bcc is typical of other Gram-negative bacteria; it is composed of lipid A, a core oligosaccharide and a repetitive glycan structure referred to as the O-antigen. The presence or absence of the O-antigen results in two distinct phenotypes. Bacteria with a complete

O-antigen have a smooth morphology and cells that contain no O-antigen (with a lipooligosaccharide [LOS] structure instead of LPS) have a rough morphology (Fig 1) (Mahenthiralingam et al., 2005).



Legend:

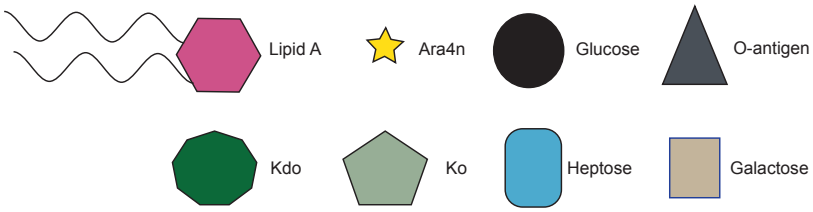


Figure 1: Simplified structure of *B. cenocepacia* lipopolysaccharide.

Currently three *B. cenocepacia* LPS structures have been elucidated. Lipid A is part of the outer membrane *of B. cenocepacia* and like other Gram-negative LPS structures is composed of a phosphorylated glucosamine disaccharide that is decorated with fatty acids. Alternatively, Ara4N residues can be attached to the phosphate groups of the lipid A backbone (Vinion-Dubiel and Goldberg, 2003). The core oligosaccharide of the LPS is comprised of an inner core and outer core region. Often the inner core has a trisaccharide of Ara4N-(1→8)-Ko-(2→4)-Kdo directly attached to the lipid A (Vinion-Dubiel and Goldberg, 2003). Therefore, up to three Ara4N residues can be found in the inner core. As previously mentioned, Ara4N residues confer resistance to cationic antimicrobial peptides by lowering the ionic charge of the outer membrane. A heptosyl trisaccharide is then attached to the O-5 of Kdo and the remainder of the core region can then be assembled with galactose, glucose, and heptose residues (Fig 2) (De Soyza et al., 2004; Ortega et al., 2009). The O-antigen is the outermost structure of the LPS and it covalently links to the core oligosaccharide. The O-antigen is a repetitive glycan structure that has a high degree of variability from strain to strain (De Soyza et al., 2008).

The role of Bcc LPS in disease is well characterized. *Pseudomonas aeruginosa* is another opportunistic bacterial pathogen that infects CF patients. LPS from *P. aeruginosa* was examined and compared to Bcc LPS and it was concluded that Bcc LPS was at least five times more toxic

than its counterpart (Shaw et al., 1995). Both environmental and clinical Bcc isolates revealed the same levels of LPS toxicity. Inner core truncations of Bcc LPS have been shown to decrease resistance and pathogenicity in *in vivo* and *in vitro* studies. Without a complete core oligosaccharide, *B. cenocepacia* was shown to have an increased sensitivity to antimicrobial peptides and attenuated virulence in rat agar bead, *Caenorhabditis elegans* and *Galleria mellonella* infection models (Uehlinger et al., 2009).

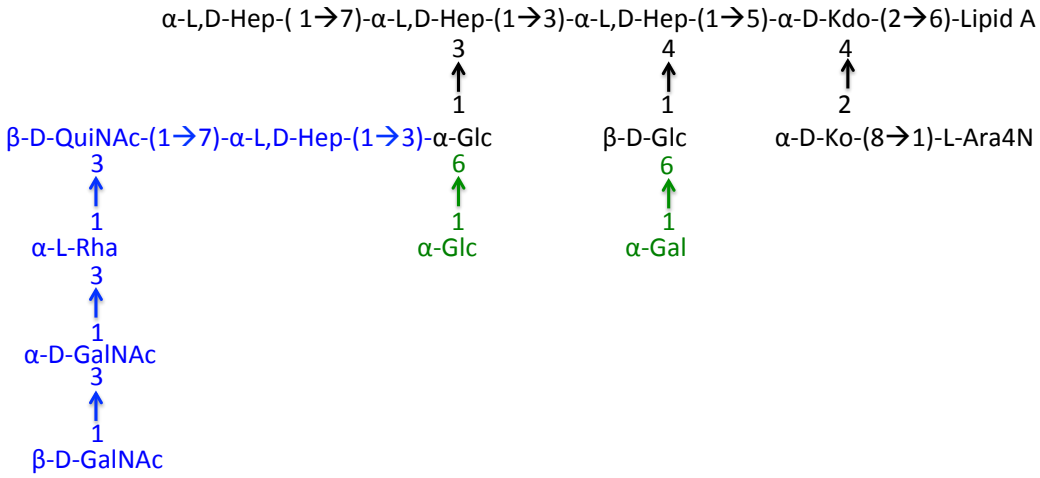


Figure 2: Structural representation of lipopolysaccharide and lipooligosaccharide from two *B. cenocepacia* ET12 strains. Residues shown in black are relevant for both K56-2 and an unnamed ET12 isolate. Blue residues represent LPS structure from *B. cenocepacia* K56-2. Green residues represent LOS structure from the unnamed ET12 isolate.

Exopolysaccharide

Bacteria belonging to the Bcc have been shown to produce EPS (Rich et al., 1990). The secretion of polysaccharides allows the bacterium to adapt to different environments and provides protection from the host immune system. The genus *Burkholderia* is capable of producing at least seven different kinds of EPS. However, only four EPS types have been found in the Bcc to date (Ferreira et al., 2011). These four are PSI, cepacian (PSII), levan and an unnamed polymer (Chiarini et al., 2004). By growing Bcc bacteria in agar containing a mannitol-based medium, it can be quickly determined if a strain produces EPS based on the development of a mucoid colony morphology. A majority of Bcc isolates have been found to produce the EPS cepacian. (Chiarini et al., 2004). As cepacian synthesis genes were found to be well conserved among *Burkholderia*, it was not unexpected to find that both clinical and environmental Bcc isolates produce cepacian.

Cepacian consists of repeating units of branched heptasaccharides that are formed from galactose, glucose, rhamnose, mannose, and glucuronic acid residues in a 3:1:1:1:1 ratio (Cescutti et al., 2000). Two gene clusters called *bce-I* and *bce-II* were found to be responsible for cepacian production, however regulation of *bce-I* and *bce-II* has yet to be determined in Bcc (Moreira et al., 2003; Ferreira et al., 2010). Bartholdson et al. (2008) tested *B. cenocepacia* strains K56-2, J2315, BC7, BTS2 and PC184 for their ability to produce EPS (Bartholdson et al., 2008). It was

shown that only two out of the five strains tested (PC184 and BTS2) were able to produce EPS. The first three strains (K56-2, J2315 and BC7) all contain an 11 base pair (bp) deletion within the *bceB* gene that renders them unable to produce cepacian.

The contribution of Bcc EPS to pathogenicity has been shown in both *in vitro* and *in vivo* studies. The EPS of Bcc was shown to inhibit the neutrophil-mediated host defense of the innate immune system. The secretion of EPS reduced chemotaxis, production of reactive oxygen species and phagocytosis by human neutrophils (Conway et al., 2004; Bylund et al., 2006). Another important role associated with cepacian production was seen in the BALB/c mouse pulmonary infection model. Mucoid and non-mucoid clonal isolates of *B. cenocepacia* were recovered from a CF patient and used to infect BALB/c mice. Greater persistence of *B. cenocepacia* in the BALB/c mouse lung was seen with the mucoid isolate than its non-mucoid counterpart (Conway et al., 2004). A pair of mucoid and non-mucoid clonal isolates belonging to *B. multivorans* was recently recovered by Silva et al. (2011). This group discovered that the EPS-producing isolate had an increased growth rate under low oxygen conditions and was more virulent in the *Galleria mellonella* infection model (Silva et al., 2011). However, the relationship between mucoidy and virulence requires further examination since it was recently discovered that a rapid decline in pulmonary function can be associated with non-mucoid isolates (Zlosnik et al., 2011). There is also contradicting evidence as to

whether the production of EPS positively or negatively impacts the formation of Bcc biofilms (Cunha et al., 2004).

Flagella

Flagella are commonly known for their role in bacterial locomotion, but they also have an important role in virulence for a number of bacterial species including *P. aeruginosa*, *Campylobacter jejuni* and *Helicobacter pylori* (Feldman et al., 1998; Morooka et al., 1985; Eaton et al., 1992). Flagella assembly is composed of over 50 structural and regulatory genes that have been studied extensively in *Escherichia coli* and *Salmonella enterica* serovar *Typhimurium* (White et al., 2011). The flagella is made up of three parts: the basal body, the hook, and the filament. These parts are assembled in an ordered fashion beginning with the components closest to the inner membrane (Fig 3) (Jarrell and McBride, 2008).

Bcc cells have one polar flagellum that mediates motility. Recently a link between flagella and pathogenicity has been shown by Tomich et al. (2002) in *Burkholderia cenocepacia* J2315. A mutation in a component of the basal body encoded by the *fliG* gene rendered the bacterium non-motile. The mutant also showed a reduction in the invasion of human lung cells resulting in attenuated virulence (Tomich et al., 2002). The contribution of flagella to virulence was also shown in the murine agar bead model of lung infection. The inactivation of the major flagellin gene *fliCII in B. cenocepacia* K56-2 resulted in a loss of the polar flagellum and

motility of the bacterium. Mice that were infected with wildtype (WT) K56-2 had only a 60% survival rate, while all mice infected with the flagellar mutant survived (Urban et al., 2004).

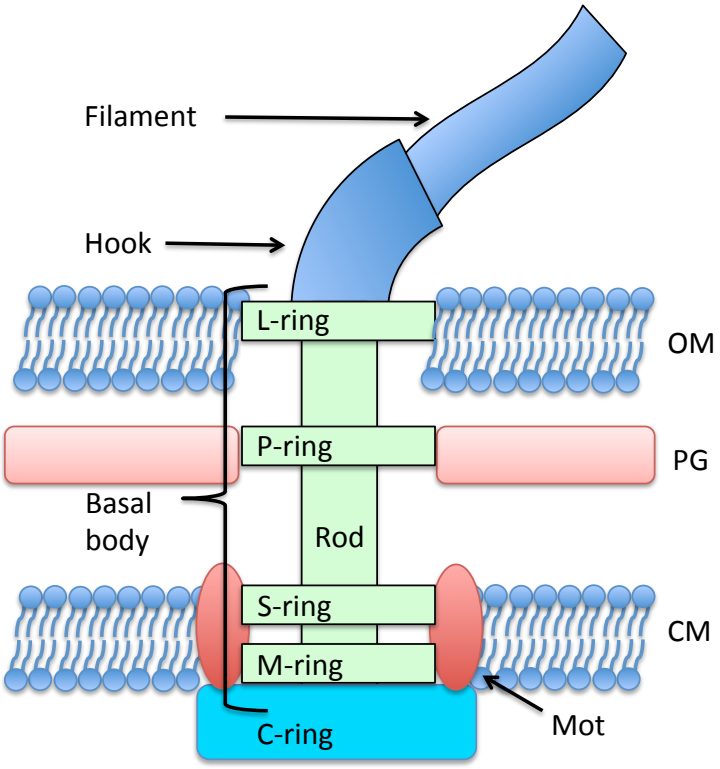


Figure 3: General structure of a bacterial flagellum. The basal body of the flagellum consists of four rings L, P, S and M that are connected by a central rod. The hook and filament protrude from the bacterial cell membrane. Abbreviations: OM, outer membrane; PG, peptidoglycan; CM, cytoplasmic membrane; Mot, motor complex.

Molecular tools in *B. cenocepacia*

Markerless mutagenesis

Burkholderia species are intrinsically resistant to a wide range of antibiotics that make them a difficult pathogen to work with in a laboratory setting. The characterization of *Burkholderia* genes has been hampered by the limited availability of molecular tools. Therefore, researchers have invested time and money to develop mutagenesis systems and expression vectors to genetically manipulate *Burkholderia*.

Over the past decade, multiple unmarked deletion systems for *Burkholderia* have been published (Choi et al., 2008; Lopez et al., 2009; Hamad et al., 2009; Barrett et al., 2008; Norris et al., 2009) but only one was specifically made in *B. cenocepacia* (Flannagan et al., 2008). Flannagan et al. (2008) created a mutagenesis system that elegantly uses I-SceI endonuclease to obtain markerless gene deletions in *B. cenocepacia*. The strategy involves cloning the upstream and downstream elements surrounding the targeted gene for deletion into the suicide plasmid pGPI-SceI (Fig 4). The plasmid carries an 18 bp recognition site for the I-SceI endonuclease. The pGPI-SceI plasmid is then inserted into *B. cenocepacia* K56-2 where it recombines into the genome by homologous recombination (Fig 4). A second plasmid named pDAI-SceI is then introduced into the bacterium, where it constitutively expresses the I-SceI endonuclease. I-SceI expression will cause a double stranded break at the I-SceI recognition site that is then repaired by intramolecular

recombination within the cell. The surviving recombinants will either carry the intact (WT) or deleted (mutant) gene (Flannagan et al., 2008). This system allows researchers to create multiple unmarked gene deletions in the same strain. However, it is only effective if the target genome does not carry an I-SceI recognition site and if the bacterium is sensitive to trimethoprim and tetracycline, the antibiotics used for plasmid selection.

Expression vectors

In 2002, Lefebre and Valvano constructed constitutive and inducible expression vectors for *B. cenocepacia* (Lefebre and Valvano, 2002). Plasmid pMLS7 contains the promoter from the *Burkholderia xenovorans* LB400 S7 ribosomal protein gene that allows for constitutive expression. The authors also created pMLBAD, an arabinose-inducible plasmid that tightly regulates gene expression. However, the concentrations of arabinose required for expression from pMLBAD were sufficiently high to cause a change in cell volume typical of osmotic stress (Cardona and Valvano, 2005; Sousa et al., 2011). Since inducible expression vectors are powerful genetic tools, the Valvano laboratory constructed a rhamnose-inducible plasmid named pSCrhaB2 that would allow for maximum gene expression in the presence of minimal concentration of inducer (Cardona and Valvano, 2005).

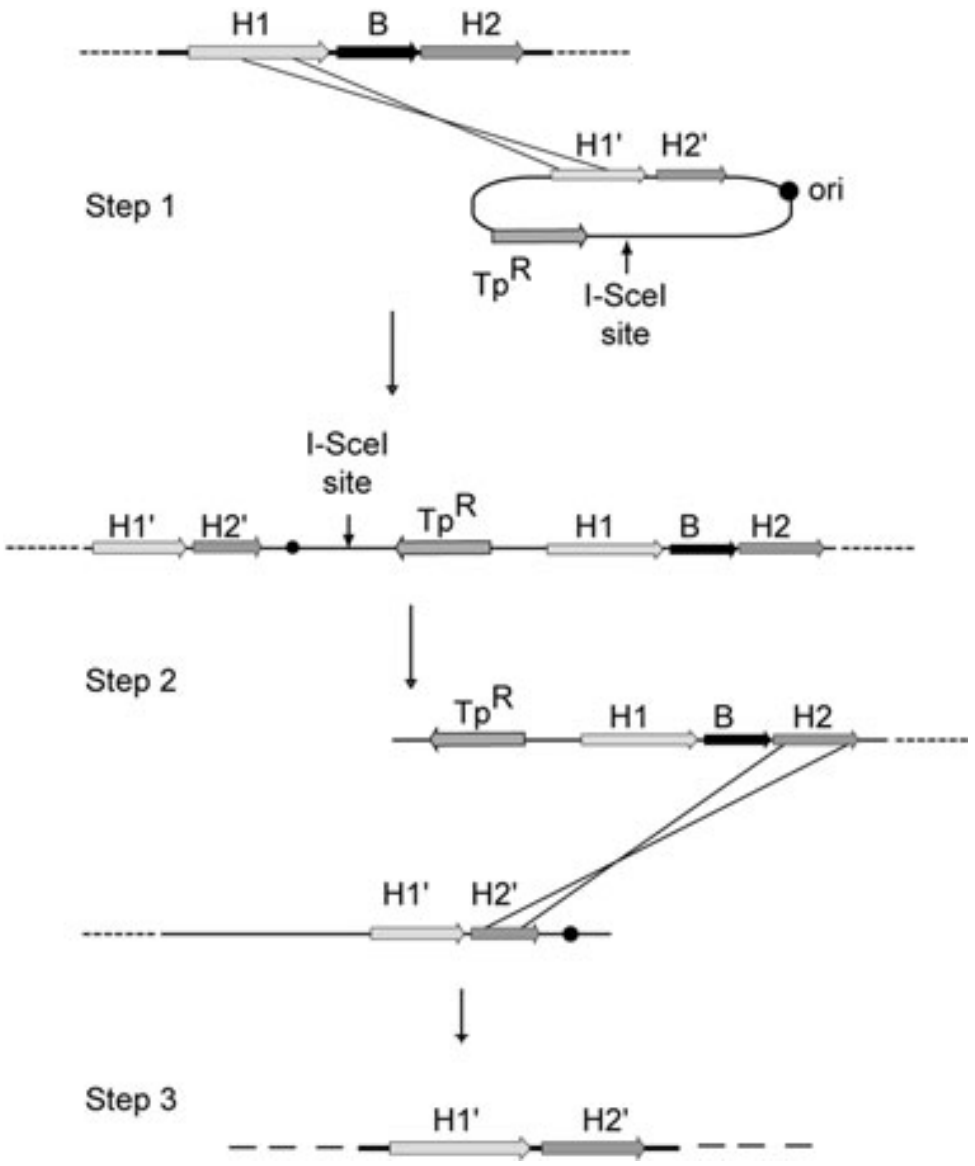


Figure 4: Targeted unmarked deletion system. Step 1: Genes flanking the gene of interest to be deleted (B) are cloned into the suicide vector pGPI-SceI. The plasmid is introduced into the bacterium and allowed to integrate into the genome. Step 2: pDAI-SceI is introduced into the cell and I-SceI expression creates a double strand break at the I-SceI recognition site within pGPI-SceI. This break stimulates host recombination and the gene of interest is deleted when a recombination event occurs between H2 and H2' Step 3: PCR is used to confirm the presence of the deletion in recovered colonies. Taken from Flannagan et al., (2008), with permission.

Bacteriophages

History

Bacterial viruses (bacteriophage or phage) were co-discovered by Frederick w. Twort and Felix d'Herelle in 1915 and 1917, respectively (Carlton, 2001). These bacterial eaters were shown to lyse liquid cultures and form plaques (clear zones) on bacterial lawns. Due to limitations in technology at that time, there was a great deal of skepticism, about the existence of these microscopic viruses. However the prospect of using bacteriophages to treat or prevent infectious diseases was apparent to d'Herelle (Sulakvelidze et al., 2001; Summers, 2001). Early studies by d'Herelle that used bacteriophages as a tool to clear infections were reported as being successful in human and animal trials (Merril et al., 2003). However, most of the data collected were anecdotal and there was uncertainty towards the efficacy of phage therapy. During the 1940s two important discoveries were made that would impact the direction of bacteriophage research. First, the invention of the electron microscope allowed the visualization of phages, thus ending the debate over whether viruses that infect bacteria truly exist (Summers, 2001). Second, the development of inexpensive, broad-spectrum antibiotics made bacterial infections readily treatable. After the discovery of antibiotics, the inconsistency of bacteriophage therapy as a treatment option led to its abandonment in the U.S and Western Europe (Merril et al., 2003). Currently, as a result of 80 years of antibiotic misuse, the number of

multidrug resistant pathogens has increased. The emergence of such strains now has researchers reevaluating bacteriophages as an alternative therapy to antibiotics.

Phage therapy

Bacteriophages are ubiquitous and are usually found in the same environments where bacteria thrive. Due to their host specificity, bacteriophages are good candidates for antibacterial therapy. Before phage therapy can be used as a clinical tool, its efficacy must be shown in animal models. Since the number of multidrug resistant pathogenic bacteria has increased, scientists and clinicians have started to see the potential of phage therapy as an effective treatment for antibiotic resistant pathogens. Due to the emergence of vancomycin-resistant *Enterococcus faecium* (VRE), Biswas et al. (2002) began to evaluate the ability of bacteriophages to rescue mice infected with this deadly pathogen. One hundred percent survival was reported when a single intraperitoneal dose of bacteriophage ENB6 was administered 45 minutes post-infection. When the administration of ENB6 was delayed until the mice were in a near-death state, only 50% of the mice survived the treatment (Biswas et al., 2002). This study revealed that one of the most important advantages of phage therapy over antibiotics is their ability to kill antibiotic resistant pathogens.

Even with the introduction of broad-spectrum antibiotics, research regarding the therapeutic use of bacteriophages has continued in Eastern Europe, particularly at the Eliava Institute. This centre is located in Tbilisi, Georgia and was founded in 1923 by Giorgi Eliava and Felix d'Herelle (Sulakvelidze et al., 2001). In 2005, the effective use of bacteriophages on subcutaneous tissues was reported by the institute in *Clinical and Experimental Dermatology* (Jikia et al., 2005). The two patients featured in the study had severe radiation burns on their backs. The patients' wounds were infected with *Staphylococcus aureus* and the isolates were highly resistant to antibiotics including penicillin, streptomycin, chloramphenicol, ampicillin, oxacilin, gentamicin, erythromycin, doxycycline, ciprofloxacin, ceftraixone and cefotaxime. After a month of aggressive antibiotic treatment there was no success in eliminating the infection. After the failure of antibiotic treatment to cure the infection, the patients were treated with *S. aureus*-specific lytic bacteriophages. No detectable *S. aureus* was found in either patient after placing a biodegradable polymer saturated with bacteriophages onto the wounds (Jikia et al., 2005). Although this report is anecdotal, it suggests that phage therapy may be effective in treating human infections.

The specificity of bacteriophages can be seen as both an advantage and a challenge to phage therapy. An advantage to using bacteriophages instead of antibiotics is that bacteriophages will only target a specific host, therefore a patients normal microflora will not be greatly

impacted. The specificity of phages can also be a disadvantage because lysis only occurs in a limited number of bacterial species and bacteria can gain resistance to phage infection (Carlton, 2001). A cocktail of bacteriophages that target different receptors will decrease the probability of the bacterium becoming resistant to phage infection. The efficacy of phage cocktails has been shown in multiple studies (McVay et al., 2007; Hooton et al., 2011; Tanji et al., 2004) and has been proposed as a solution to overcome this challenge.

Clinical trials

One of the most important issues regarding phage therapy is its safety in human use. Concerns have been raised regarding the potential of bacteriophages to induce a strong immune response (Merril et al., 2003). However, humans have been exposed to phages either by consuming them in the food supply or through injections of phage contaminated vaccines (Merril, 1975). Bruttin et al. (2005) administered one of the first safety trials of orally ingested phages in humans. The study determined that no adverse reactions were reported when healthy adult volunteers were administered drinking water spiked with *E.coli* phage T4 (Bruttin and Brüssow, 2005).

Clinical trials have recently examined the ability of bacteriophages to treat highly antibiotic resistant infections (Bruttin and Brüssow, 2005; Rhoads et al., 2009; Wright et al., 2009; Merabishvili et al., 2009). The

study by Wright et al. (2009) has progressed the furthest with respect to clinical trials, having completed phase I/II. This study evaluated the efficacy and safety of Biophage-PA, a cocktail composed of six bacteriophages, as a treatment for chronic ear infections caused by *P. aeruginosa*. Patients were administered a single dose of Biophage-PA containing 6×10^5 plaque forming units (PFU) and evaluated for 42 days. Patients who received treatment reported no observable side effects from the administration of the cocktail but saw significant clinical improvement. The ability of bacteriophages to replicate at the site of an infection is one of the advantages of this treatment. This trait was evident in the study as the number of bacteriophage isolated from the treated patients increased to 1.27×10^8 PFU (Wright et al., 2009). Successful clinical trials such as that of Wright et al. (2009) show that bacteriophages are a viable alternative to antibiotics.

Mechanism of infection

Bacteriophages are obligate intracellular parasites that utilize the host molecular machinery for replication. Tailed phages constitute over 96% of bacteriophages and belong to the order *Caudovirales* (Ackermann and Kropinski, 2007). Based on tail morphology, a bacteriophage can belong to the *Siphoviridae*, *Myoviridae*, or *Podoviridae* family. Bacteriophages can also be classified as virulent (lytic) or temperate depending on their lifestyle. Prior to lytic replication, a bacteriophage will

bind to a receptor on the surface of a host cell. LPS, proteins and polysaccharides are all possible bacteriophage receptors (discussed below). After attachment, the phage transfers its genome directly into the cell. The production of early proteins allows the virus to take control of the host biosynthetic machinery, facilitating the synthesis and assembly of new virions. Lysis of the host cell then releases the newly formed progeny. Temperate phages can also undergo a second mode of replication. After transfer of the phage genomic material, temperate phages can integrate into the host genome and replicate passively as the bacterium divides. Such prophages stay in a dormant state until they are induced to enter the lytic lifecycle (Kropinski, 2006).

Phage depolymerase

Over time, bacteria have evolved phage resistance mechanisms to overcome the threat of infection. The production of exopolysaccharide (EPS) is one strategy that bacteria use to block the adsorption of phages to host receptors (Labrie et al., 2010). As previously mentioned, bacterial EPS can both protect the bacteria from harmful conditions but also provide a physical barrier to prevent bacteriophages from reaching the host cell membrane. However, in the evolutionary war between bacteria and phages, bacteriophages have successfully evolved to counteract EPS by utilizing polysaccharide-degrading enzymes to reach their host receptor (Labrie et al., 2010). These enzymes are usually found on the tail fibers,

tail spikes or base plates of a bacteriophage and they allow the phage to penetrate through the polysaccharide layer.

Evidence of a bacteriophage-borne polysaccharide depolymerase was first reported over 55 years ago (Adams and Park, 1956). The presence of a large and expanding halo surrounding a clear plaque on EPS inducing media may indicate a bacteriophage with a polysaccharide depolymerase enzyme (Glonti et al., 2010; Hughes et al., 1998; Sutherland and Wilkinson, 1965). This enzyme may be required to allow phages to penetrate the EPS layer and make contact with the host cell membrane. Phage depolymerase enzymes are specific to a host EPS and subtle changes to the EPS may render the enzyme ineffective (Hughes et al., 2008).

In 2010, Glonti et al. screened twenty-one *Pseudomonas aeruginosa* specific bacteriophages by inspecting plaque morphology for halo formation indicative of polysaccharide depolymerase expression. Four of the phages tested (PT-1, PT-6, PT-7, PT-12) exhibited a large expanding halo, presumably degrading the alginate EPS of *P. aeruginosa*. All four phages demonstrated the ability to degrade purified alginate from four different sources. This work demonstrates the activity of phage-borne polysaccharide depolymerase enzymes against clinically relevant pathogens.

Polysaccharide depolymerase enzymes may also have significant therapeutic relevance as they have been shown to increase phagocytosis

by macrophages and disrupt biofilm formation, reducing the ability of a pathogen to successfully colonize a host. (Eftekhar and Speert, 1988; Donlan, 2009). Continued research in identifying and purifying these bacteriophage enzymes could assist in the development of efficient phage cocktails or lytic phage with biofilm-degrading properties.

Bacteriophage receptors

Bacteriophages of the order *Caudovirales* are able to attach to hosts using their tail fibers. Since no motility structures have been found on bacteriophages and they cannot move independently, locating cells and their receptors is purely a random event. Gram-negative bacteria have a cell envelope that contains an outer membrane, a cytoplasmic membrane and a peptidoglycan layer that lies between these structure Viral DNA must cross these two membranes and the peptidoglycan layer to enter the host cytoplasm. Bacteriophages must first bind to a receptor on the outer membrane before DNA ejection can occur. As noted above, structures on the outer membrane that can act as receptors include LPS, porins, enzymes, and structural proteins (Rakhuba et al., 2010). Host recognition and attachment is generally divided into two stages. Bacteriophages first attach to their primary receptor in a reversible manner. This weak interaction preserves cell association while the bacteriophage locates and attaches irreversibly to a secondary receptor. This irreversible attachment commits the bacteriophage to infection, as

this step is usually associated with ejection of bacteriophage DNA. After the initial adsorption, a bacteriophage may use the primary receptor for irreversible attachment by interacting in a new way with the primary receptor (Rossmann and Rao, 2012).

Binding of phages to the host cell surface has been studied extensively. Within the *Myoviridae* family, the bacteriophage T4 virion is composed of an icosahedral head, core, contractile sheath, baseplate and tail fibers (Leiman et al., 2003). T4 adsorption is mediated by long (reversible binding) and short (irreversible binding) tail fibers . T4 initiates infection by binding to the outer membrane porin protein C (OmpC) or LPS with at least three or more long tail fibers (Yu and Mizushima, 1982; Bartual et al., 2010). A recognition signal is then sent from the long tail fibers to the baseplate which changes conformation from a hexagon to a star shape that repositions six short tail fibers (Leiman et al., 2003). These short tail fibers bind irreversibly to the heptose residue in the LPS core and commits the bacteriophage to the infection process (Rakhuba et al., 2010; Miller et al., 2003).

Research Objective

Three dominant and highly epidemic lineages of *B. cenocepacia* that have caused significant outbreaks in Europe and North America are Electrophoretic Type 12 (ET12), Midwest and the Philadelphia-District of Columbia (PHDC). Despite the clinical relevance of all three linages, there

is a deficiency in the literature regarding mechanisms of pathogenicity for the Midwest and PHDC lineages. Bcc virulence research has been skewed towards ET12 partially as a result of the availability of resources and tools for manipulation of strains from this lineage.

The objective of my M.Sc. project was to investigate the receptor of KS10, a myovirus that infects *B. cenocepacia* Midwest isolate PC184. Identifying and characterizing bacteriophage receptors allows for the development of efficient phage cocktails. Preliminary data (Fig 5) suggested that KS10 may use LPS as a receptor to infect PC184 (Juarez Lara, 2013). Previous attempts to genetically manipulate this bacterium using insertional mutagenesis were unsuccessful (Routier, 2010). Therefore, in order to locate and confirm the receptor, unmarked clean deletions in the LPS biosynthetic pathway of *B. cenocepacia* PC184 were required. Due to the difficulty in genetically manipulating PC184, there is currently no published literature describing the LPS structure of this strain.

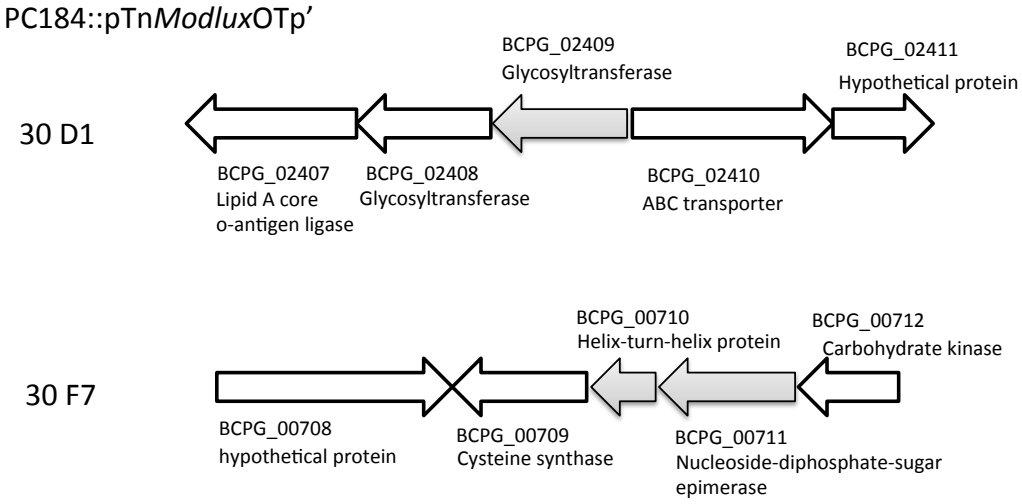


Figure 5: Genetic locations of plasposon insertion in KS10 resistant PC184. Genes disrupted by Tn*Mod*-OTp' are shown in grey.

Materials and Methods

Bacterial strains and culture conditions

The bacterial strains and plasmids used throughout this study are described in Table 1. *Burkholderia cenocepacia* PC184 is part of the Bcc experimental strain panel (Mahenthiralingam et al., 2000) and was provided by the Canadian *Burkholderia cepacia* complex Research and Referral Repository (CBCCRRR). All strains were grown aerobically in half strength Luria-Bertani (½ LB) broth (with shaking at 225 rpm) or on ½ LB agar and incubated at 30^{o}C or 37°C overnight. The growth medium was supplemented with various antibiotics to ensure plasmid maintenance, including 100 mg/l trimethoprim (Tp), 300 mg/l chloramphenicol (Cm) and 200 mg/l kanamycin (Km) (Sigma-Aldrich, St. Louis, MO). The antibiotic concentrations used for *E.coli* include 100 mg/l Tp, 25 mg/l Cm, 50 mg/l Km, and 15 mg/l tetracycline (Tet). Gene expression from pSCrhaB2 was induced by supplementing with 0.02% L-rhamnose (Sigma-Aldrich).

Table 1: Bacterial strains and plasmids used in this study.

Strain/Plasmid	Characteristics	Source or reference
B. cenocepacia		
PC184	Midwest CF clinical isolate	CBCCRRR
PC184Δ*waaC*	*waaC* deletion	This study
PC184Δ*waaC*-S/O	PC184 with pGPI-SceI-*ΔwaaC* integrated, Tp^R	This study
PC184Δ*wabO*	*wabO* deletion	This study
PC184Δ*wabO*-S/O	PC184 with pGPI-SceI-*ΔwabO* integrated, Tp^R	This study
PC184Δ*waaF*	*waaF* deletion	This study
PC184Δ*waaF*-S/O	PC184 with pGPI-SceI-*ΔwaaF* integrated, Tp^R	This study
PC184 (pSCrhaB2)	PC184 with plasmid pSCrhaB2, Tp^R	This study
PC184Δ*waaC* (pSCrhaB2)	*waaC* deletion with pSCrhaB2	This study
PC184Δ*waaC* (pSCrha*waaC*)	*waaC* deletion complemented with pSCrha-*waaC*	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^+) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1 λ^-	Invitrogen (Carlsbad, CA)
JM109	e14$^-$ (McrA$^-$) *rec*A1 *end*A1 *gyr*A96 *thi*-1 *hsd*R17 (r^-m^+) supE44 *rel*A1 Δ(*lac-pro*AB) [F' *tra*D36 *pro*AB *lacI^q*ZΔM15]	Dennis and Zylstra (1998)
SY327	*araD* Δ(*lac pro*) *rec*A56 *rif^R nal*A λ*pir arg*E(Am)	Miller and Mekalanos (1988)
HB101	F− *hsd*S20(r_B^- m_B^-) *sup*E44 *leu*B6 *ara*14 *gal*K *lac*Y1 *pro*A2 *rps*L20 *xyl5 mtl*1 *rec*A13	Boyer and Roulland-Dussoix (1969)
Plasmids		

pGPI-SceI	$ori_{R6K,}$ Tp^R , *mob*$^+$, I-SceI recognition site	Flannagan et al. (2007)
pDA17	ori_{pBBR1}, Tet^R, *mob*$^+$, P_{dhfr}, FLAG tag	Flannagan et al. (2007)
pDAI-SceI	pDA17 expressing I-SceI nuclease	Flannagan et al. (2007)
pRK2013	ori_{colE1}, RK2 derivative, Km^R, *mob*$^+$,*tra*$^+$	Figurski and Helinski (1979)
pSCrhaB2	rhamnose inducible plasmid, Tp^R	Cardona et al. (2005)
p34S-Cm	Source of Cm cassette, Cm^R	Dennis and Zylstra (1998)
p34S-Tp	Source of Tp cassette, Tp^R	Dennis and Zylstra (1998)
p34S-Km	Source of Km cassette, Km^R	Dennis and Zylstra (1998)
pTn*Mod*OTp'	Tp^R plasposon, source of pMB1 *ori*	Dennis and Zylstra (1998)
pGPI-SceI-MB1	pMB1 *ori*, Tp^R , I-SceI recognition site	This study
pDA17-Cm	pDA17, Cm resistance cassette, Tet^R Cm^R	This study
pDAI-SceI-Cm	pDAI-SceI, Cm resistance cassette, Tet^R Cm^R	This study
pDA17-Tp	pDAI-SceI, Tp resistance cassette, Tet^R Tp^R	This study
pDAI-SceI-Tp	pDAI-SceI, Tp resistance cassette, Tet^R Tp^R	This study
pDA17-Km	pDAI-SceI, Km resistance cassette, Tet^R Km^R	This study
pDAI-SceI-Km	pDAI-SceI, Km resistance cassette, Tet^R Km^R	This study
pGPI-SceI-Δ*waaC*	pGPI-SceI with genes flanking *waaC*	This study
pGPI-SceI-Δ*wabO*	pGPI-SceI with genes flanking *wabO*	This study
pGPI-SceI-Δ*waaF*	pGPI-SceI with genes flanking *waaF*	This study
pSCrha*waaC*	pSCrhaB2, *B. cenocepacia* K56-2 *waaC*	This study

Freezer Stocks

Stock suspensions of cells were prepared by growing the bacterium overnight on solid agar (with or without antibiotics) and resuspending the cells in 3 mL of LB broth supplemented with 20% glycerol. Stocks were stored at -80^{o}C.

Bacteriophage KS10 propagation

To propagate KS10, 100 µl of phage stock and 100 µl of PC184 broth culture were mixed together and incubated for 20 min at room temperature. After incubation, 3 ml of top agar (0.7% [w/v] ½ LB agar) was added to the mixture and immediately poured onto the surface of a ½ LB plate. The plate solidified and was placed into a 30^{o}C incubator overnight. The following day the plate was removed and overlaid with 3 ml of modified suspension medium (SM; Lynch et al., submitted). The plate was incubated at room temperature on a rocking platform for 4 h. The supernatant was recovered and centrifuged to pellet bacterial debris. Lastly, the solution was filter sterilized to eliminate any remaining bacteria using a 0.45 µm syringe driven filter (Millipore, Billerica, MA), and stored at 4^{o}C.

Bacterial DNA and plasmid isolation

All genomic and plasmid DNA extractions were from fresh bacterial plates grown overnight. The bacterial genomic DNA was isolated following

the procedure outlined by Wilson (2001). 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^{o}C or -20^{o}C.

General molecular techniques

Restriction digest

Chromosomal and plasmid DNA were digested with desired restriction enzymes (Thermo Scientific) according to manufacturers instructions. If necessary, 1 µl of FastAP Thermosensitive Alkaline Phosphatase (Thermo Scientific) was added to the reaction mixture after 15 min.

PCR

The oligonucleotides used in this study (Sigma-Aldrich) are listed in Table 2. PCR was performed using a T100™ Thermal Cycler (Bio-Rad, Hercules, CA). KAPA HiFi HotStart ReadyMix (Kapa Biosystems, Woburn, MA) was used to amplify PCR products. PCR was conducted using bacterial genomic DNA as the template and was performed according to manufacturer's standard protocol.

To confirm that the unmarked deletions were constructed successfully, colony PCR was performed using Top Taq DNA Polymerase

(Qiagen, Hilden, Germany) with *B. cenocepacia* PC184 colonies as the template. The appropriate reaction composition set out by the manufacturer was followed. All the components except DNA polymerase (dNTPs, buffer, sterile water, and template colonies) were combined and boiled for 5 min then cooled on ice for 5 min. DNA polymerase was added to the mixture and incubated at appropriate conditions in the thermocycler.

Table 2: Oligonucleotides.

Primer Name	Sequence (5' to 3')	Purpose/Comments
pGPI-SceI-BglII	TTTAGATCTATGTGGAATT GTGAGCGGATAAC	Construction of pGPI-SceI-MB1
pGPI-SceI-SpeI	TTTACTAGTCTACGCCGGA CGCATCG	Construction of pGPI-SceI-MB1
F-pMB1-*ori*	CAAGGATCCTACCAGGAA AGAACATGTG	Construction of pGPI-SceI-MB1
R-pMB1-*ori*	AACTCTAGACGATTTCGTT CCACTGAGC	Construction of pGPI-SceI-MB1
FSeqpGPIMB1	TTACTAAGCTGATCCGGTG	pGPI-SceI-MB1 forward sequencing primer
RSeqpGPIMB1	GGGGAAACGCCTGGTATC	pGPI-SceI-MB1 reverse sequencing primer
F-BCPG_00858	GATATCTAGACCATGCACG CAACTGAGAGA	Amplify BCPG_00858
R-BCPG_00858	TTTTGGTACCTTCGAGATA AACACGGCCGAC	Amplify BCPG_00858
F-BCPG_00856	TTTTGGTACCTCGTCAATC TCGGCACGGCGGG	Amplify BCPG_00856
R-BCPG_00856	TTTTGATATCCGACGTGGC CGAAGCGCTCGC	Amplify BCPG_00856
F-Flank-BCPG_00857	CAGGTTGTGTCCGCTCCA G	Confirm deletion of BCPG_00857
R-Flank-BCPG_00857	GAAGGGCTCAAGCGCATC	Confirm deletion of BCPG_00857
F-*waaC*	CAATTAACATATGCATCAT CACCATCACCATCATCACC	Amplify *waaC* from K56-2

	ATCACCAAAAGATCCTGATCGTGC	
R-*waaC*	TATCTAGATTAGTGATGGTGATGATGGTGATGGTGATGATGCAGGAGGCCGAAGCCCG	Amplify *waaC* from K56-2
pSCrha R1	GCTTCTGCGTTCTGA	pSCrha reverse sequencing primer
pSCrha F2	GGCCCATTTTCCTGTC	pSCrha forward sequencing primer
F-BCPG_02410	TAAGGTACCTGGGTTCAGCCATGAAATCGTC	Amplify BCPG_02410
R-BCPG_02410	TAAGAATTCGTCACGCCGCCTGCTGCTG	Amplify BCPG_02410
F-BCPG_02408	TTTGGTACCCTGTTCACGCGAATGACG	Amplify BCPG_02408
R-BCPG_02408	CACTCTAGAGACGAACGGCGCAGTATAC	Amplify BCPG_02408
F-Flank-BCPG_02409	CATGGCAGATGCCGATGTTGGC	Confirm deletion of BCPG_02409
R-Flank-BCPG_02409	ACCGCGATCGGCACGTACC	Confirm deletion of BCPG_02409
F-BCPG_02407	ATAGGTACCCGTGCCCTTCTTCGTCAGCC	Amplify BCPG_02407
R-BCPG_02407	TTAGATATCCTGTTGCGCTCGCGGATCAAG	Amplify BCPG_02407
F-BCPG_02405	TTTGGTACCGAACAAGGCTGGCAATG	Amplify BCPG_02405
R-BCPG_02405	TTTTCTAGAGATCAGGATGCCGTGGAAC	Amplify BCPG_02405
F-Flank-BCPG_02406	CATTGCCAGCCTTGTTC	Confirm deletion of BCPG_02406
R-Flank-BCPG_02406	AGTGCAGCAATATCGGCAG	Confirm deletion of BCPG_02406

Agarose gel electrophoresis

The separation of DNA was performed using a 0.8% (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 GENECLEAN® III kit (MP Biomedicals, Santa Ana, CA). 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.

Sequencing

Sequencing of DNA was carried out by the University of Alberta Department of Biological Sciences Molecular Biology Service Unit (MBSU) using a 3730 DNA Analyzer (Applied Biosystems, Carlsbad, CA). Sequences were then edited using 4Peaks (http://nucleobytes.com/index.php/4peaks) and analyzed using NCBI Blast (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Bacterial transformation strategies

Chemical competence

Chemically competent *E.coli* DH5α (Invitrogen, Carlsbad, CA) were used for routine cloning. Five microliters of ligation mixture was added to a

50 µl aliquot of DH5α and incubated on ice for 30 min. This mixture was then subjected to heat shock (42^{o}C for 30 seconds) and placed on ice for 30 sec. Five hundred microliters of SOC was added to the suspension and placed into a 37^{o}C orbital shaker (225 rpm). After 1 h of recovery, the cells were plated onto the appropriate antibiotic medium and incubated overnight at 37^{o}C.

Preparation of alternative chemically competent cells was performed using a standard protocol (Silhavy et al., 1984). Briefly, 5 ml of broth culture was subcultured (1:50) into fresh medium and grown for 2.5 h. Cultures were pelleted by centrifugation at 1,500 rcf for 10 min. The supernatant was discarded and the pellet was resuspended in 1 ml of Magic Formula (in 100 ml: 80 ml sterile water, 10 ml 1M $CaCl_2$, 10 ml 1M MOPS pH 6.5) and incubated on ice for 30 min. Two microliters of plasmid DNA was then added to the cells and allowed to incubate for 10 min. The cells were then subjected to heat shock (42^{o}C for 30 seconds). One milliliter of LB broth was immediately added to the cell suspension and incubated with shaking at 37^{o}C for 1 h. Following the recovery, cells were plated onto the appropriate selective medium and incubated overnight at 37^{o}C.

Electroporation

A modified electroporation protocol (Sambrook et al., 1989) was used to transform *B. cenocepacia*. To make electrocompetent cells, 3 ml

of broth culture was pelleted at 10,000 x rcf for 30 sec. The cells were then washed with 400 µl of 10% glycerol and pelleted by centrifugation at 5,000 x rcf for 4 min. This wash step was repeated 2 additional times. After the final wash, the cells were resuspended in 70 µl of 10% glycerol and 2-5 µl of plasmid DNA was added. The mixture was incubated at room temperature for 5 minutes and transferred to a 0.1 cm cuvette. Electroporation was performed on a Bio-Rad MicroPulser (Bio-Rad). Immediately following electroporation, 500 µl recovery medium (SOC) was added to the cells. Cells were placed in a 37^{o}C shaking incubator for 1 h and then plated on selective medium.

Triparental mating

Triparental mating was also used to introduce plasmids into bacteria as described by Dennis and Zylstra (1998). Recipient cells (PC184 and derivatives), helper cells (*E.coli* HB101 containing plasmid pRK2013) and donor cells (*E.coli* cells carrying the plasmid to be transferred) were combined in 1 ml of 10% glycerol and centrifuged at 1,500 rcf for 4 min at room temperature. Following removal of the supernatant, the pellet was resuspended in 100 µl of 10% glycerol, deposited onto a ½ LB plate, and incubated at 30^{o}C for 4-6 h. Following incubation, the cells were scraped off the plate and resuspended in 1 ml of 10% glycerol. Cells were plated on the appropriate antibiotic medium that

would select for the *B. cenocepacia* recipients while eliminating the *E.coli* helper and donor strains.

Mutagenesis

Construction of the pGPI-SceI-MB1 vector

Plasmid pGPI-SceI-MB1 was created by amplifying a 2,575 bp product that contained the multiple cloning site dihydrofolate reductase (*dhfr*) and the I-SceI recognition sequence from pGPI-SceI using the primer pair pGPI-SceI-BglII and pGPI-SceI-SpeI. This amplicon was digested with BglII and SpeI then ligated to a 714 bp pMB1 *oriR* fragment that was previously digested with BamHI and XbaI. The pMB1 *oriR* was PCR-amplified from pTn*Mod*OTp' using the primer pair F-pMB1-*ori* and R-pMB1-*ori*. The resulting plasmid was named pGPI-SceI-MB1 (3,289 bp) and was used throughout this study to create clean deletions.

Construction of pDA17 and pDAI-SceI vectors derivatives

To construct derivatives pDA17 and pDAI-SceI vectors, the *dhfr*, *cat*, and *aphI* antibiotic resistance cassettes were removed by digesting their corresponding p34S plasmid with PstI. The cassettes were then cloned into pDA17 and pDAI-SceI previously digested with PstI, giving rise to pDA17-Tp, pDAI-SceI-Tp, pDA17-Cm, pDAI-SceI-Cm, pDA17-Km, and pDAI-SceI-Km.

Mutagenesis procedure

The gene knockout procedure used in this study has been described by Flannagan et al. (2008). Modifications to this protocol have been made to allow for genetic manipulations in PC184. Before using this protocol, it is essential to confirm that the target bacterium lacks an I-SceI recognition sequence (5'-TAGGGATAACAGGGTAAT-3') within its genome. Protocols for such confirmation include screening for lethality following transfer of the I-SceI expression plasmid into the host or examining the sequenced genome for an I-SceI recognition site. It is also vital that the target bacterium is sensitive to at least two different antibiotics with corresponding resistance cassettes. If the strain lacks an I-SceI site and is antibiotic sensitive, one can start cloning the surrounding regions flanking the gene to be deleted into pGPI-SceI-MB1, the suicide vector that carries the I-SceI recognition site. The plasmid is transferred into PC184 via electroporation (described above) where it recombines into the host genome and confers resistance to Tp. After integration, the mutagenesis plasmid pDAI-SceI-Cm (constitutively expressing the I-SceI nuclease) is introduced into the cell by triparental mating or electroporation and a double strand break is created at the I-SceI recognition site. Resulting Cm resistant Tp sensitive colonies are then PCR screened for the absence of the target gene. Confirmed mutants are subsequently passaged in ½ LB broth for three consecutive days to remove pDAI-SceI-Cm.

Construction of mutagenesis plasmids

To knockout *waaC* in PC184 using the modified clean deletion system, the upstream and downstream regions of BCPG_00857 were cloned into pGPI-SceI-MB1. This was accomplished by using the PCR primers F-BCPG_00858 and R-BCPG_00858 to amplify BCPG_00858, located upstream of BCPG_00857. Primer pair F-BCPG_00856 and R-BCPG_00856 were used to amplify BCPG_00856 the gene downstream of BCPG_00857. BCPG_00858 was digested with XbaI and KpnI while BCPG_00856 was digested with KpnI and EcoRV. These amplicons were then inserted sequentially into pGPI-SceI-MB1 to make pGPI-SceI-Δ*waaC.*

To delete *wabO* in PC184, the upstream and downstream regions of BCPG_02409 were cloned into pGPI-SceI-MB1. This was achieved by amplifying the adjacent gene BCPG_02410 with the primers F-BCPG_02410 and R-BCPG_02410, then digesting the product with KpnI and EcoRI. BCPG_02408 was amplified using the primer pair F-BCPG_02408 and R-BCPG_02408 and subsequently digesting the PCR product with KpnI and XbaI. Both products were then inserted into pGPI-SceI-MB1 to make pGPI-SceI-Δ*wabO.*

To delete *wabP* in PC184, the adjacent genes surrounding BCPG_02406 were cloned into pGPI-SceI-MB1. Primer pair F-BCPG_02407 and R-BCPG_02407 were used to amplify BCPG_02407. This amplicon was subsequently digested with KpnI and EcoRV then

cloned into pGPI-SceI-MB1. To clone the downstream gene BCPG_02405, the primers F-BCPG_02405 and R-BCPG_02405 were used and the resulting product was digested with XbaI and KpnI. This amplicon was then cloned into pGPI-SceI-MB1-O2407 resulting in the plasmid pGPI-SceI-Δ*wabP.*

Construction of the waaC complementation vector

Complementation experiments in PC184 were accomplished using the rhamnose-inducible plasmid pSCrhaB2. A cross-complementation approach was undertaken to complement PC184Δ*waaC*. To amplify *waaC*, *Burkholderia cenocepacia* K56-2 genomic DNA and the primer pair F-*waaC*, R-*waaC* were used. The PCR product was digested with NdeI and XbaI and ligated to pSCrha previously digested with NdeI and XbaI resulting in the plasmid pSCrha-*waaC*.

Assays

LPS isolation and SDS polyacrylamide gel electrophoresis

LPS was extracted using the method described by Marolda et al. (2006) with some modifications. Briefly, 1.5 ml of broth culture was pelleted by centrifugation at 9,300 rcf for 1 min. Pelleted cells were then resuspended in 150 µl of modified lysis buffer (2% [w/v] sodium dodecyl sulphate, 10% glycerol, 1M Tris-HCl pH 6.8). 4 µl of 20mg/mL proteinase K was added to the mixture and incubated at 55^{o}C until the following

morning. Six microliters of β-mercaptoethanol was added and allowed to boil for 10 min at 100^{o}C. An additional 2 µl of proteinase K was added and allowed to incubate at 60^{o}C for 1 h. Following this incubation, 150 µl of 95% phenol was added and tubes were incubated at 70^{o}C for 15 minutes (with inversion at 5 min intervals). Samples were placed on ice for 10 min and then centrifuged for 10 min at 18,400 rcf. The aqueous phase was transferred to a tube containing 380 µl of ethanol and the LPS was pelleted by centrifugation for 20 min at 21,100 rcf. LPS was resuspended in 50 µl of sterile water and separated by electrophoresis on a 14% SDS-polyacrylamide gel (Sambrook et al., 1989). LPS was detected using the silver-stain protocol outlined by Tsai et al. (1982).

Motility assay

The swimming motility of PC184 WT and mutants was examined on 0.3% (w/v) agar ½ LB plates (Rashid and Kornberg, 2000). Swim plates were inoculated with 2 µl of broth culture and incubated 48 h at 37^{o}C. Colony diameter was measured and pictures were taken with the ColonyDoc-It Imaging Station (UVP, Upland, Ca). Swimming assays were performed in three separate trials in quadruplicates. Averages and standard deviations were calculated using Microsoft Excel.

Growth curve

A growth assay of PC184 WT and mutants was performed by subculturing 10 ml of a broth overnight culture (in triplicate) into three wells of a 96 well plate adding 10 µl of culture to 190 µl of ½ LB broth. The plates were covered with plastic wrap to prevent dehydration and incubated with shaking at 37^{o}C. Optical density measurements at 600 nm were measured at 1 h intervals for 12 hours with a Wallac 1420 VICTOR^{2} multilabel counter (PerkinElmer, Waltham, MA). Measurements were also taken at 24 and 48 h. Averages and standard deviations of three separate trials were calculated using Microsoft Excel.

Liquid phage infection assay

To assay bacteriophage killing in PC184, 10 ml of broth culture (in triplicate) was subcultured into six wells of a 96 well plate by adding 10 µl of culture to 140 µl of ½ LB broth. Half of the wells were then inoculated with 50 µl of high titer phage stock and the other half with 50 µl of modified SM. The plates were covered with plastic wrap and incubated with shaking at 37^{o}C. Optical density measurements at 600 nm were measured at 2 h intervals for 8 h with a Wallac 1420 VICTOR^{2} multilabel counter (PerkinElmer). Averages and standard deviations of three separate trials were calculated using Microsoft Excel.

Transmission electron microscopy

The presence of flagella was examined by transmission electron microscopy (TEM). One milliliter of broth overnight culture was pelleted by centrifugation for 1 min at 18,400 rcf. The pellet was then resuspended in 500 µl of 4% paraformaldehyde. Cells were placed on top of a carbon-coated copper grid and incubated for 5 min at room temperature. Excess liquid was removed by blotting onto a filter paper. The grid was subsequently stained with 2% phosphotungstic acid for 30 seconds. Images were captured using a Philips/FEI (Morgagni) transmission electron microscope with a charge-coupled device camera (University of Alberta Department of Biological Sciences Advanced Microscopy Facility).

Results

Identification of PC184 endotoxin

The three endotoxins currently characterized in *B. cenocepacia* belong to the ET-12 strains J2315, K56-2, and a third unnamed isolate. No detectable O-antigen was observed in J2315 or the unnamed *B. cenocepacia* isolate, both of which contain LOS in their outer membrane (Silipo et al., 2007; Ieranò et al., 2010). In contrast, the LPS banding pattern of *B. cenocepacia* K56-2 revealed an O-antigen ladder, indicating that this bacterium contains LPS instead of LOS in the outer membrane (Loutet et al., 2006; Ortega et al., 2009). Prior to this project, the LPS or LOS structure of PC184 was unknown. In order to identify which type of endotoxin PC184 produces, LPS was extracted as described in Materials and Methods and subjected to SDS-PAGE. The gel revealed no detectable O-antigen after staining with silver nitrate (Fig 6). Due to the absence of the polymeric O-antigen pattern, we can conclude that the Midwest clinical isolate PC184 produces LOS.

Figure 6: LOS phenotype of *B. cenocepacia* PC184. LOS extracted from PC184 was separated on a 14% SDS-PAGE gel and stained using silver nitrate. LOS recovered from PC184 revealed that the bacterium contains no O-antigen.

Putative LOS structure

Since the LOS structure of *B. cenocepacia* PC184 has yet to be elucidated, a putative structure will be referenced throughout this thesis. Comparing the three published LPS and LOS structures of *B. cenocepacia* J2315, K56-2 and the unnamed ET12 strain, several commonalities within the core polysaccharide were observed (Fig 7). First, all three strains contain a trisaccharide of Ara4N-Ko-Kdo that is directly attached to the lipid A. Second, the inner core contains a heptosyl trisaccharide that is attached to the Kdo. Finally, a glucose residue is attached to the first heptose residue in the inner core. Regions of dissimilarity among the three isolates occurred at residues branching from the second heptose or from the glucose sugar in the inner core (question marks in Fig 7).

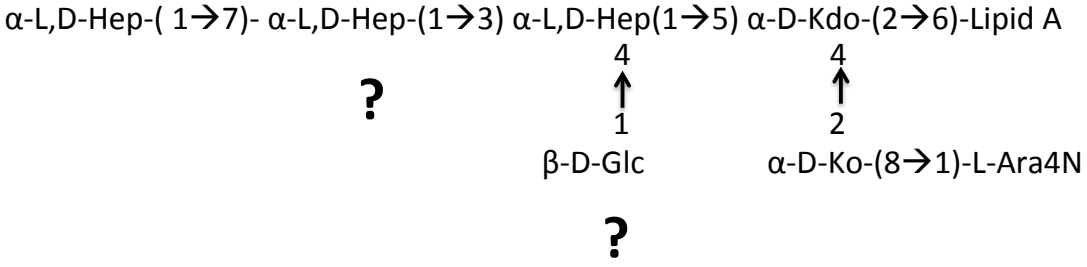
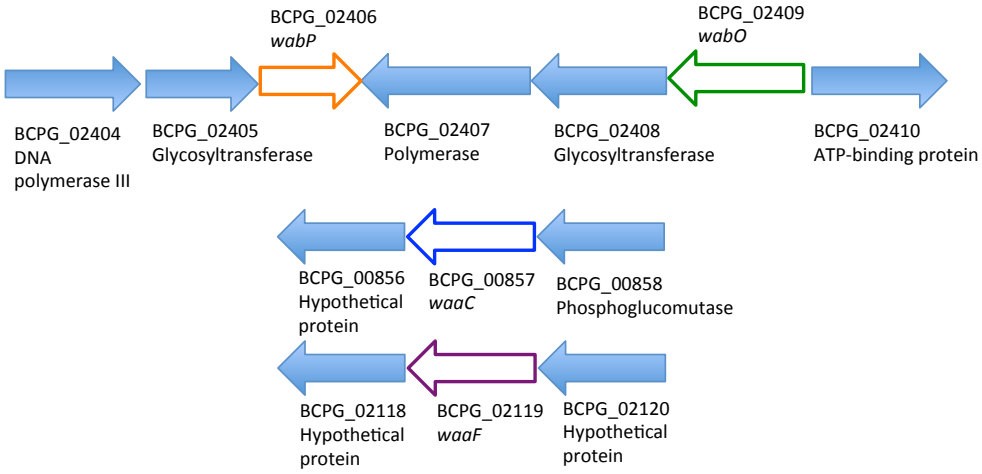


Figure 7: Putative structure of PC184 LOS. Question marks represent regions of ambiguity.

Identifying and locating LOS synthesis genes

The majority of the genes involved in K56-2 core oligosaccharide synthesis have been identified (Ortega et al., 2009). Genes required for the biosynthesis of the LPS core were found in three different regions within the genome. Only the genes similar to those involved in synthesizing the putative LOS structure of PC184 (Fig 7) will be discussed in this thesis. In K56-2 the addition of the first heptose sugar onto Kdo was found to be established by the heptosyltransferase I gene *waaC* (BCAL3112). It is hypothesized that the addition of the second heptose sugar is catalyzed by the heptosyltransferase II gene *waaF* (BCAL0967). The heptosyltransferase *waaP* (BCAL2403) is predicted to add the last heptose sugar in the heptosyl trisaccharide. The glycosyltransferase *wabO* (BCAL2402) catalyzes the addition of glucose onto the first heptose residue. Each of these genes was located in PC184 using NCBI BLASTX (Fig 8). Similar to K56-2, PC184 core oligosaccharide genes were found in three different locations in chromosome 1. Throughout this thesis, PC184 BCPG_00857, BCPG_02409, BCPG_02119 and BCPG_02406 will be referred to as *waaC*, *wabO*, *waaF*, and *wabP* respectively.

PC184 LPS gene cluster



Gene	K56-2	PC184	Query Coverage	E-value	Max. identity
waaC	BCAL3112	BCPG_00857	75%	3×10^{-154}	100%
wabO	BCAL2402	BCPG_02409	96%	1×10^{-121}	80%
waaF	BCAL0967	BCPG_02119	99%	0	99%
wabP	BCAL2403	BCPG_02406	97%	0	67%

Figure 8: LOS biosynthesis genes of PC184. Genes were located using NCBI BLASTX. No homologs were located in K56-2 for the putative PC184 biosynthesis genes BCPG_02405, BCPG_02407, and BCPG_02408.

Creating clean deletion mutants in PC184

Modification of pDAI-SceI

Two specific conditions are required for functionality of the *Burkholderia* I-SceI clean deletion system developed by Flannagan et al. (2008). The first requirement is bacterial sensitivity to the selection markers of pGPI-SceI and pDAI-SceI (trimethoprim and tetracycline, respectively). In 2002, Nzula et al. tested 65 Bcc strains for their susceptibility to antimicrobials. The authors determined that PC184 was sensitive to both trimethoprim and tetracycline at minimum inhibitory

concentrations (MIC) of 0.5 mg/l and 64 mg/l, respectively. Recently, the tetracycline MIC for PC184 was determined in our laboratory in liquid culture and a result of 63 mg/l confirms the findings by Nzula et al. (Kamal, unpublished data). However, in contrast to the results in liquid culture, growth was observed when PC184 was inoculated onto solid medium containing tetracycline at concentrations of 100 mg/l to 600 mg/l. Therefore, in order to satisfy one of the two conditions of this deletion system, the original pDAI-SceI plasmid requires an alternative antibiotic resistance cassette for use in PC184 (discussed below).

The second condition involves determining whether the bacterium must lacks an I-SceI restriction site within its genome. To address this condition, a BLASTN search was used to screen for the 18 bp I-SceI restriction site in PC184. This analysis revealed no site in the PC184 genome. To confirm that the bacterium tolerates endonuclease expression, pDAI-SceI (the I-SceI expression vector) was modified to carry a *dhfrbII* cassette, resulting in the plasmid pDAI-SceI-Tp (Fig 9). Transfer of pDAI-SceI-Tp into PC184 resulted in Tp^R exconjugants that contained the plasmid (data not shown). As nuclease expression was tolerated, this result confirms that there is no I-SceI restriction site within the genome of PC184.

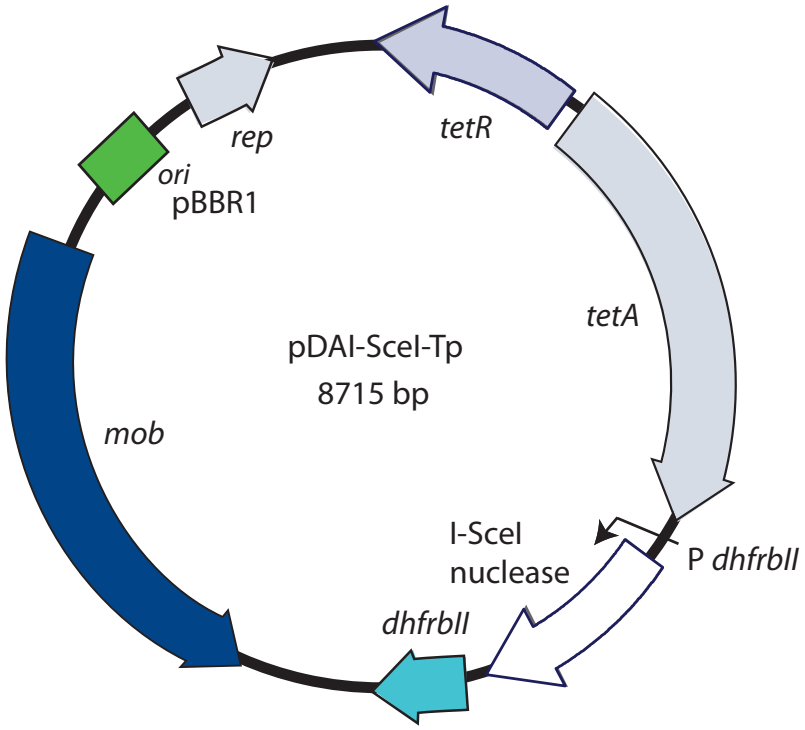


Figure 9: Plasmid map of pDAI-SceI-Tp. The *dhfrbII* antibiotic resistance cassette was removed from p34S-Tp and inserted into the PstI site of pDAI-SceI.

As discussed above, the clean deletion system requires bacterial sensitivity to a minimum of two different antibiotics for selection of pGPI-SceI and pDAI-SceI. As pGPI-SceI requires trimethoprim selection pDAI-SceI-Tp (Fig 9) cannot be used for mutant construction and an alternative resistance cassette must be inserted into pDAI-SceI. As PC184 is sensitive to chloramphenicol and kanamycin on solid medium (data not shown), two additional pDAI-SceI plasmids were constructed containing either the *cat* or *aphI* antibiotic resistance cassette. The cassettes were excised from their corresponding p34S plasmid and inserted into pDAI-SceI resulting in the plasmids pDAI-SceI-Cm and pDAI-SceI-Km (Fig 10).

These two plasmids were then transformed by electroporation into PC184 and selected on the appropriate antibiotic medium. Selection on kanamycin resulted in both small and large colony morphologies. Plasmid DNA isolation was performed on both colony types to confirm that pDAI-SceI-Km was carried by these cells. The plasmid could not be recovered from Km^R colonies, potentially due to induced aminoglycoside resistance in PC184. In contrast, pDAI-SceI-Cm was recovered from transformed Cm^R PC184 indicating that this plasmid pDAI-SceI-Cm can be used to construct clean deletions in PC184.

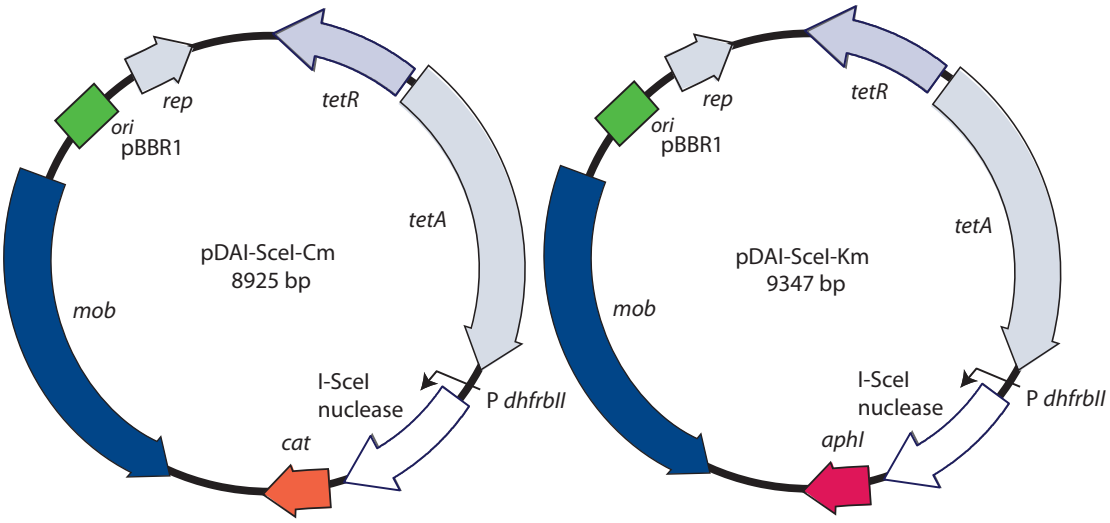


Figure 10: Plasmid maps of pDAI-SceI-Cm and pDAI-SceI-Km. *cat* and *aphI* antibiotic resistance cassettes were removed from their respective p34S plasmid and inserted into the PstI site of pDAI-SceI.

Modification of pGPI-SceI

The suicide vector pGPI-SceI is a derivative of pGPΩTp that contains the I-SceI recognition site (Flannagan et al., 2008). As pGPI-SceI replication is dependent on pir, this plasmid cannot replicate in *Burkholderia*. The *pir* gene encodes the π protein, which is required for DNA replication of plasmids that carry the R6K origin of replication (*oriR*). To construct clean deletions using the I-SceI system, the flanking regions of the targeted gene are cloned into pGPI-SceI and maintained in *E.coli* SY327 (which expresses the π protein). Preliminary attempts to transform such pGPI-SceI constructs or blank vector into SY327 (by either electroporation or heat shock) were unsuccessful. As colonies could not be recovered in the *pir*-dependent system, the R6K *oriR* was replaced with the pTn*Mod*OTp' pMB1 *oriR*, resulting in the plasmid pGPI-SceI-MB1 (Fig 11). Because this plasmid contains a pMB1 *oriR*, it acts as a suicide in *B. cenocepacia* PC184. Manipulation and cloning of pGPI-SceI-MB1 was successful and is comparatively straightforward (as this plasmid can be maintained in *E.coli* DH5α as opposed to a *pir* strain).

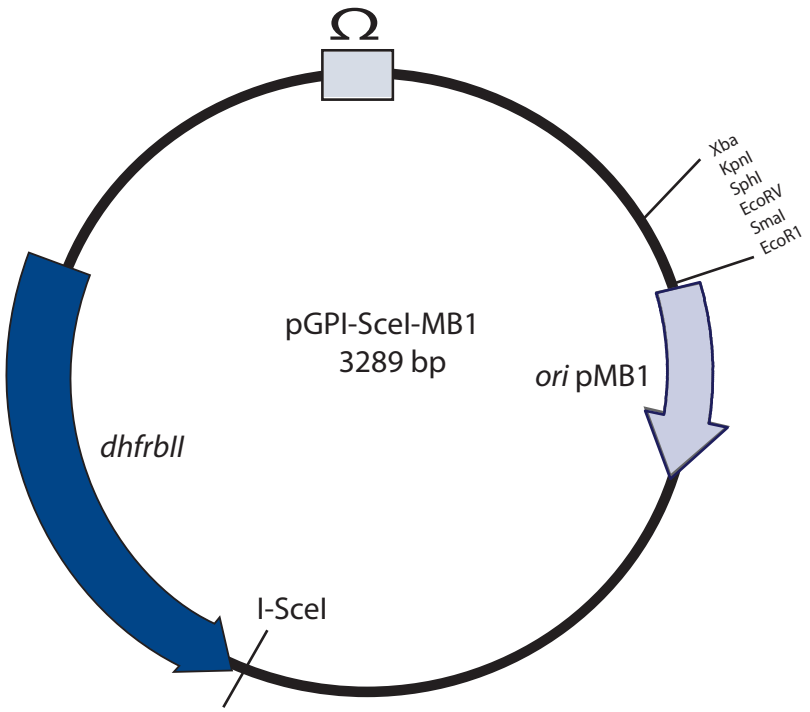


Figure 11: Plasmid map of pGPI-SceI-MB1. A 2575 bp fragment was amplified from pGPI-SceI using primer pair pGPI-SceI-BglII and pGPI-SceI-SpeI and subsequently digested with BglII and SpeI. The pMB1 origin of replication (*oriR)* was amplified from pTn*Mod*-OTp' using the primer pair F-pMB1-*ori* and R-pMB1-*ori*. This amplicon was digested with XbaI and BamHI and ligated to the 2575 pGPI-SceI fragment resulting in pGPI-SceI-MB1.

Construction of PC184ΔwaaC

Manipulation of the I-SceI system (described above) allowed for the construction of clean deletions in PC184. To confirm that LOS is the receptor for bacteriophage KS10, the *waaC* gene was targeted for deletion in order to make a deep inner core truncation in PC184. According to BLASTX analysis, PC184 BCPG_00857 shows homology to the *waaC* heptosyl transferase of K56-2 (BCAL3112). To delete *waaC* in PC184, the flanking genes BCPG_00856 and BCPG_00858 were cloned into the suicide vector pGPI-SceI-MB1, resulting in the plasmid pGPI-SceI-Δ*waaC*

(Fig 12). Transformation of pGPI-SceI-Δ*waaC* by electroporation into PC184 produced Tp^R colonies generated by recombination of the plasmid into the genome (PC184Δ*waaC*-S/O).

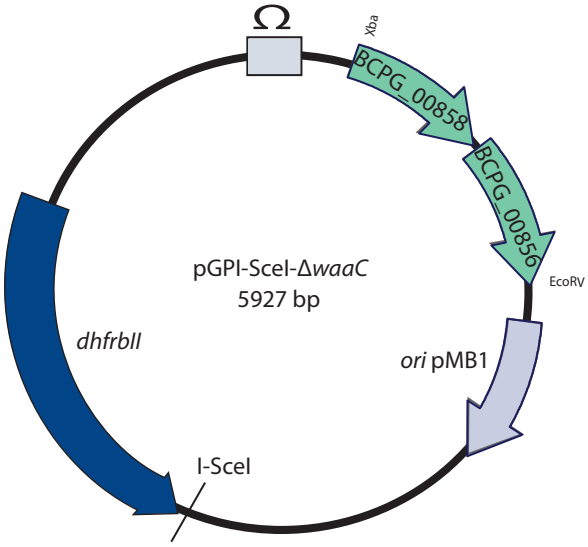


Figure 12: Plasmid map of pGPI-SceI-Δ*waaC*. The genes flanking *waaC* (BCPG_00857) were amplified using PCR and cloned into pGPI-SceI-MB1.

The I-SceI expression vector pDAI-SceI-Cm was transformed by electroporation into PC184Δ*waaC*-S/O. Resulting Cm^R transformants were screened for trimethoprim sensitivity indicative of a second homologous recombination event and loss of pGPI-SceI-MB1. Cm^RTp^S colonies were PCR screened for the *waaC* deletion using the flanking primers F-Flank-BCPG_00857 and R-Flank-BCPG_00857 (Fig 13A). A ~1,330 bp PCR product was amplified from wild-type PC184, whereas a ~300 bp product was amplified from the PC184Δ*waaC* mutant (Fig 13B). PCR-confirmed *waaC* mutants produced a shift in LOS migration compared to wild-type

PC184 on the SDS PAGE gel (Fig 13C). $Cm^{R}Tp^{S}$ PC184Δ*waaC* colonies were then passaged in 10 ml ½ LB broth for three consecutive days to cure PC184Δ*waaC* of pDAI-SceI-Cm. After the third passage, the broth culture was plated to obtain single colonies that were then screened for Cm sensitivity. Different profiles of antibiotic resistance observed during the construction of mutants using the I-SceI system are illustrated in Fig 14. In soft-agar overlays, KS10 was unable to infect PC184Δ*waaC* (data not shown), confirming that this phage uses LOS as a receptor.

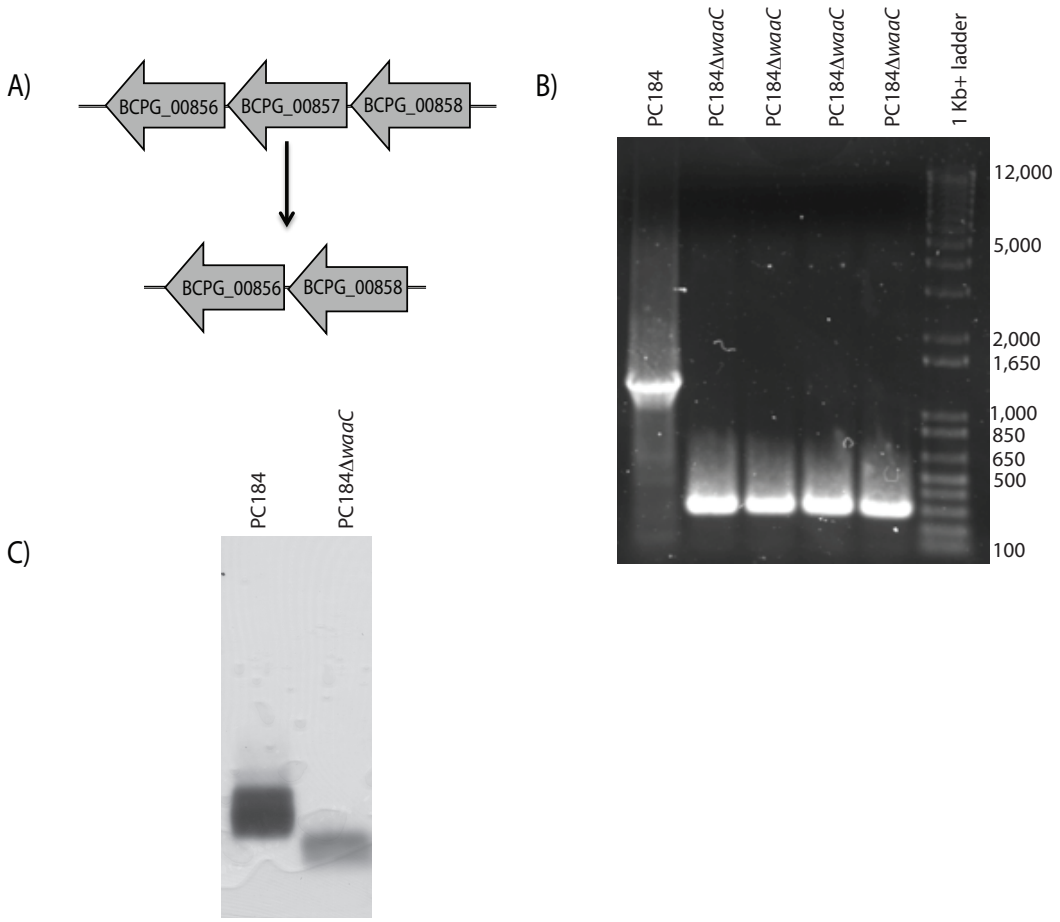


Figure 13: Construction of PC184Δ*waaC*. A) Schematic representation of the LOS biosynthesis locus before (above) and after (below) deletion of *waaC* (BCPG_00857). B) PCR screening for PC184Δ*waaC* on a 0.8% agarose gel. PC184Δ*waaC* produces a ~300 bp amplicon while wildtype PC184 produces a ~1,330 bp amplicon. C) LOS stained with silver nitrate. Deletion of *waaC* results in a truncated core oligosaccharide that migrates faster than wildtype.

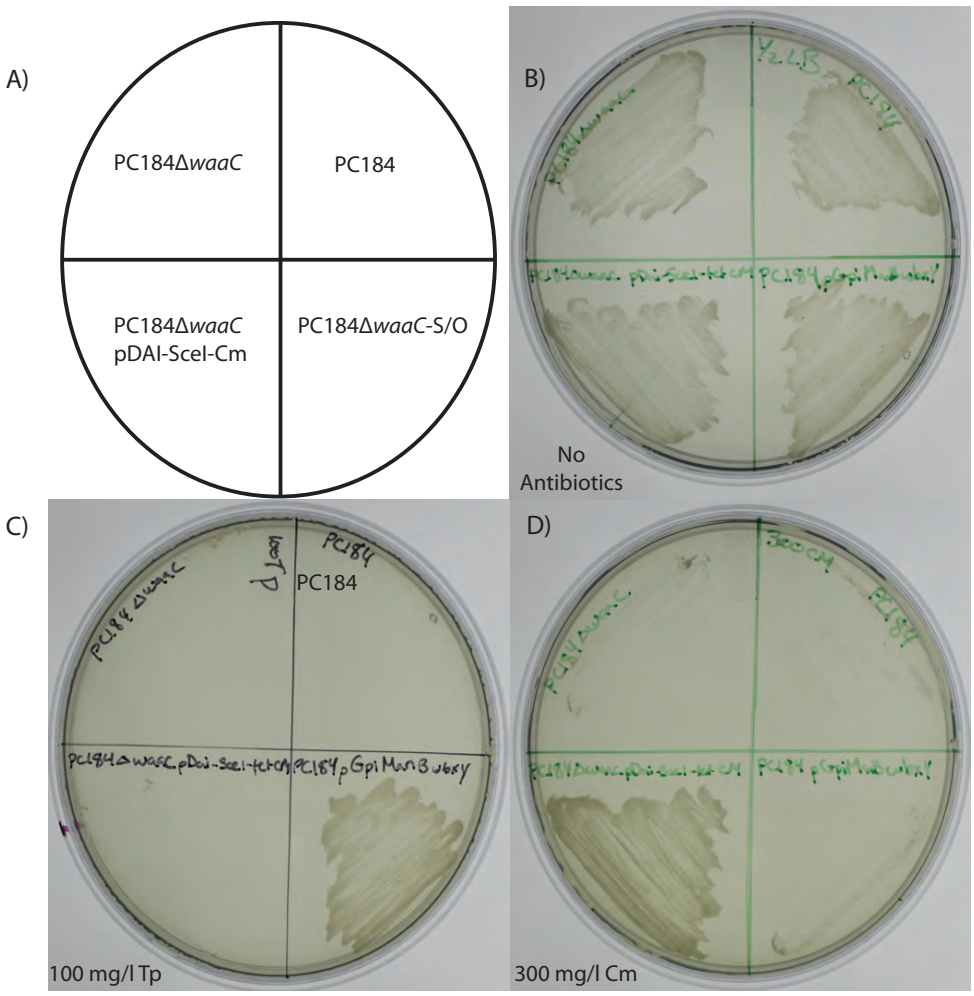


Figure 14: Various antibiotic resistance profiles observed during the construction of PC184Δ*waaC*. A) Schematic representation PC184 isolates plated on each quadrant. B) ½ LB agar; growth is observed for all isolates. C) ½ LB agar supplemented with 100 mg/l of Tp; growth is only observed for PC184Δ*waaC*-S/O (bottom right quadrant) D) ½ LB agar supplemented with 300 mg/l of Cm; growth is only observed for PC184Δ*waaC* pDAI-SceI-Cm (bottom left quadrant).

Construction of PC184ΔwabO and PC184ΔwabP

Two additional mutants were constructed in order to determine the specific core oligosaccharide residue used for KS10 attachment: the glycosyltransferase *wabO* (BCPG_02409) and the heptosyl transferase *wabP* (BCPG_02406). Both mutants were constructed in a similar fashion to PC184Δ*waaC.* To construct PC184Δ*wabO*, the genes flanking *wabO* were amplified and cloned into pGPI-SceI-MB1 resulting in the plasmid pGPI-SceI-Δ*wabO* (Fig 15A). This plasmid was transformed by electroporation into PC184. Tp^R isolates were transformed with pDAI-SceI-Cm and Cm^R Tp^S isolates were then PCR screened for deletion of *wabO* (Fig 15C). LOS was extracted from PC184Δ*wabO* and a shift in LOS was observed due to deletion of the glycosyltransferase (Fig 15D).

Similarly, PC184Δ*wabP* was constructed by PCR amplifying BCPG_02405 and BCPG_02407. These amplicons were then cloned into pGPI-SceI-MB1 resulting in pGPI-SceI-Δ*wabP* (Fig 16A). This plasmid was transformed by electroporation into PC184. Tp^R isolates were transformed with pDAI-SceI-Cm and Cm^R Tp^S isolates were then PCR screened for deletion of *wabP* (Fig 16C). LOS was extracted from PC184Δ*wabP* and no visible shift in LOS was observed (Fig 16D). Putative LOS structures of PC184, PC184Δ*wabP,* PC184Δ*wabO,* and PC184Δ*waaC* (based on previously determined K56-2 LPS structure [Ortega et al., 2009]) are shown in Figure 17.

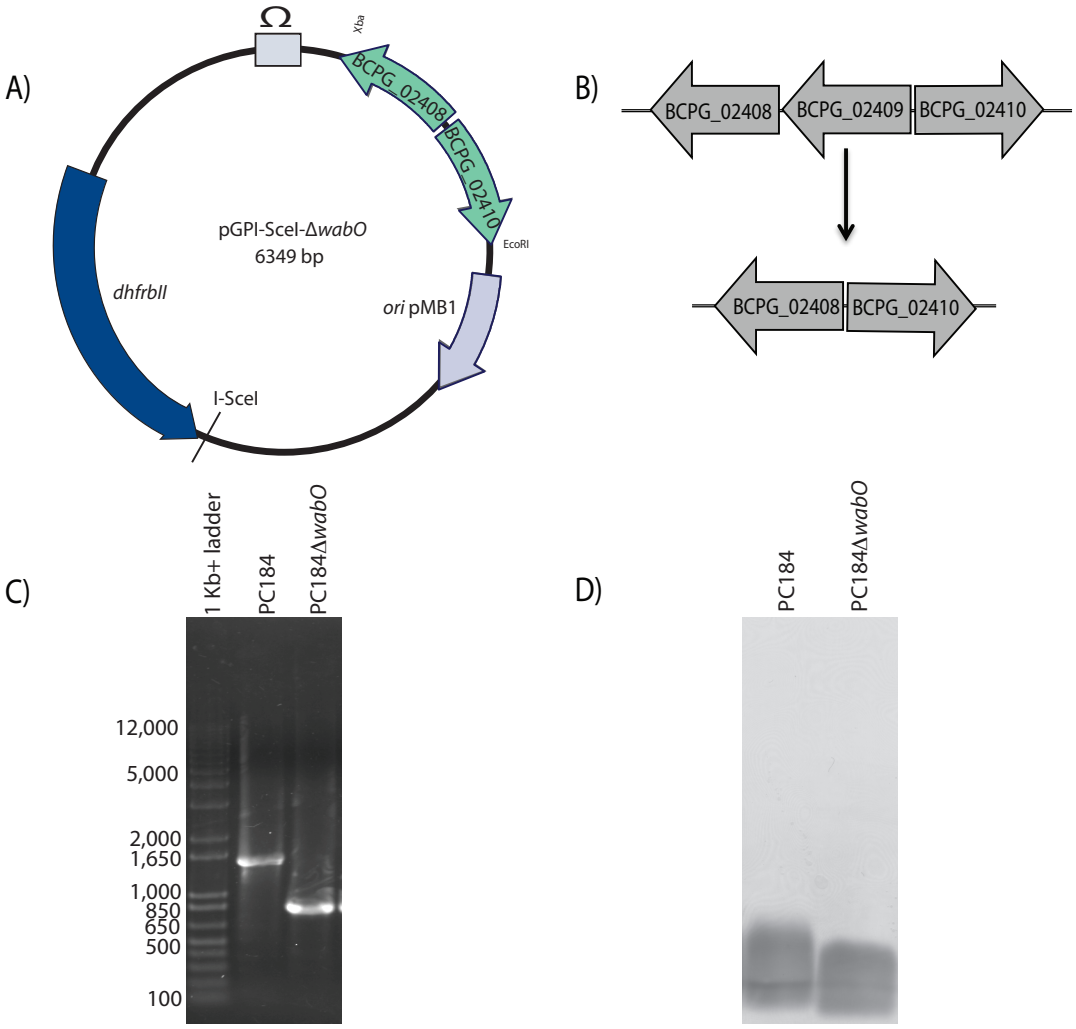


Figure 15: Construction of PC184Δ*wabO*. A) Plasmid map of pGPI-SceI-Δ*wabO.* B) Schematic representation of the LOS biosynthesis locus before (above) and after (below) deletion of *wabO* (BCPG_02409). C) PCR screening for PC184Δ*wabO* on a 0.8% agarose gel. PC184Δ*wabO* produces a ~770 bp amplicon while wildtype PC184 produces a ~1,520 bp amplicon. D) LOS stained with silver nitrate. Deletion of *wabO* results in a truncated core oligosaccharide that migrates faster than wildtype.

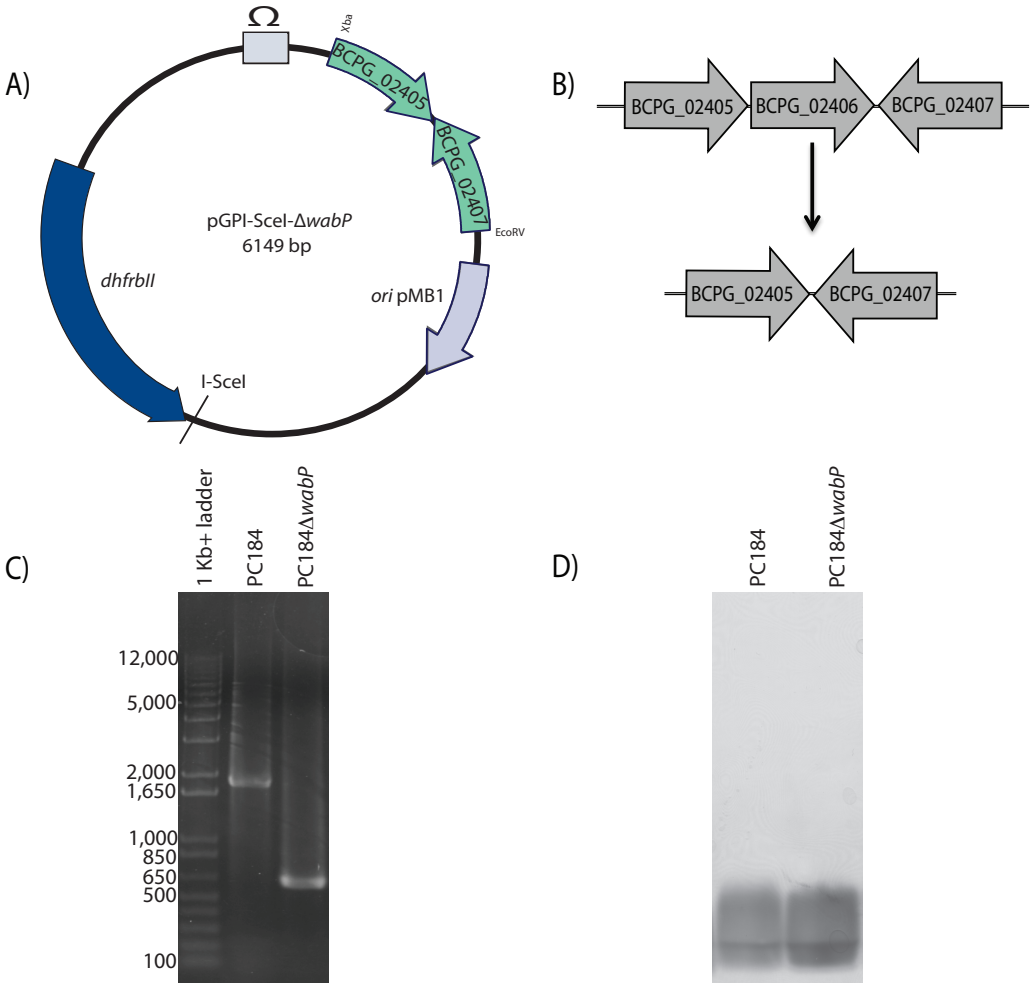


Figure 16: Construction of PC184Δ*wabP*. A) Plasmid map of pGPI-SceI-Δ*wabP.* B) Schematic representation of the LOS biosynthesis locus before (above) and after (below) deletion of *wabP* (BCPG_02406). C) PCR screening for PC184Δ*wabP* on a 0.8% agarose gel. PC184Δ*wabP* produces a ~640 bp amplicon whereas wildtype PC184 produces a ~1,820 bp amplicon. D) LOS stained with silver nitrate. Deletion of *wabP* results in no observable shift in LOS.

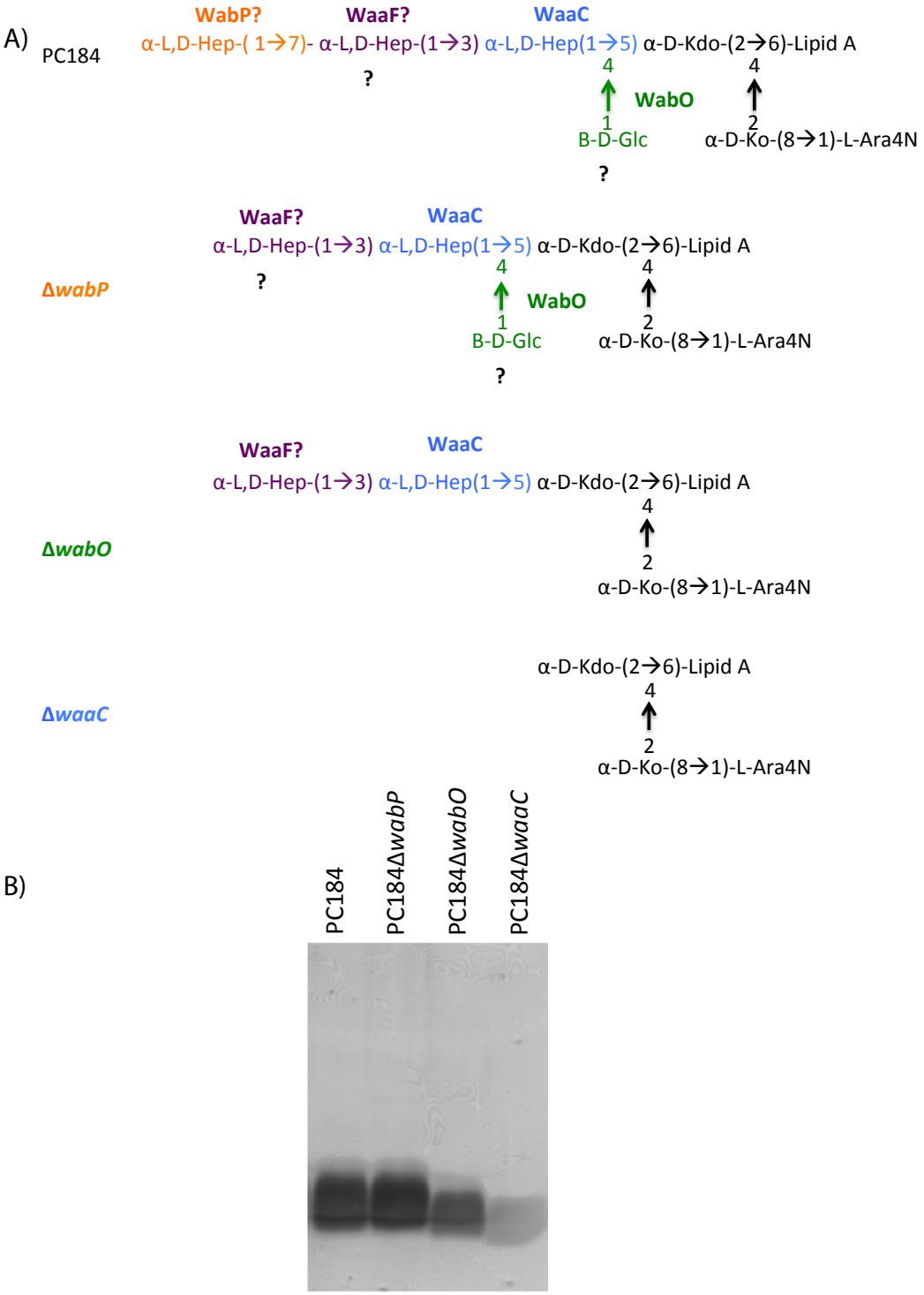


Figure 17: Putative structural profiles of LOS from PC184, PC184Δ*wabP*, PC184Δ*wabO*, and PC184Δ*waaC*. A) Structural predictions for PC184 and LOS mutants. B) LOS fractions from PC184 and LOS mutants separated by SDS-PAGE and stained with silver nitrate. Structural predictions were based on the previously determined K56-2 LPS structure (Ortega et al., 2009).

Biological activity of PC184 and LOS mutants

Growth pattern of PC184 and LOS mutants

To examine if LOS truncations have an effect on bacterial growth, a 12 hour growth curve was performed on the constructed mutants. The results from the 12 hour time course revealed an increase in lag phase growth for two of the three mutants when compared to wildtype (Fig 18). Abnormal growth patterns were observed for PC184Δ*waaC and* PC184Δ*wabO,* with the latter showing the greatest increase in lag phase growth. It is unclear why the *wabO* mutant spends a greater amount of time in lag phase than the *waaC* mutant, as the latter strain has the deepest LOS truncation. Both *wabO* and *wabP* mutants (k=0.41 $hour^{-1}$) contain a reduced growth rate when compared to wildtype (k=0.54 $hour^{-1}$). No reduction in growth rate was observed in the heptosyltransferase *wabP* mutant (k=0.53 $hour^{-1}$). Optical density readings taken at 24 and 48 hours revealed no difference in turbidity between PC184 and the LOS mutants, indicating that the *waaC* and *wabP* mutants eventually reached the same density as wildtype in stationary phase (data not shown).

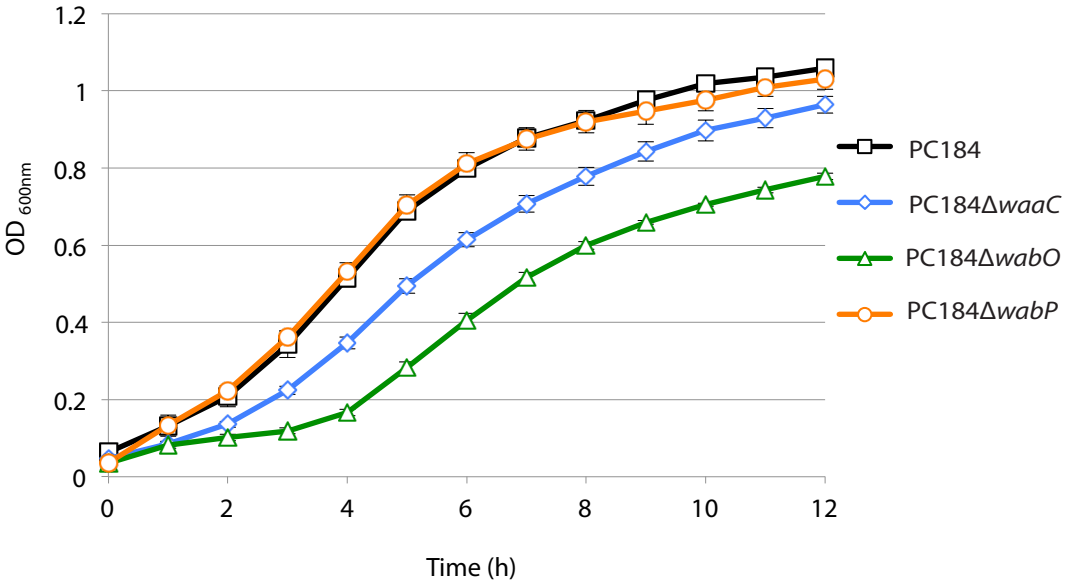


Figure 18: Growth curves of PC184, PC184Δ*waaC*, PC184Δ*wabO,* and PC184Δ*wabP*. Broth cultures were diluted twenty-fold in ½ LB broth and incubated at 37°C with shaking at 225 rpm. Optical density readings were taken at 600 nm every hour for 12 hours.

Swimming motility of PC184 and LOS mutants

Swimming motility of PC184, PC184Δ*waaC*, PC184Δ*wabO,* and PC184Δ*wabP* was examined on 0.3% agar. Wildtype PC184 maintained a swim diameter of 2.40 ± 0.11 cm (Fig 19). No difference was observed for PC184Δ*wabP*, as its swim diameter was 2.41± 0.20 cm. In contrast, both PC184Δ*waaC* and PC184Δ*wabO* demonstrated significantly smaller swim zones compared to wildtype (student t-test $p < 0.001$). Swimming motility of PC184Δ*wabO* was reduced to 1.5 ± 0.08 cm, while the average swim zone of PC184Δ*waaC* was 1.25 ± 0.13 cm. Deletion of *wabP* did not affect LOS gel mobility (Fig 17), growth (Fig 18) or swimming motility (Fig 19) compared to wildtype PC184. This result may be attributed to its relatively

minor role in LOS biosynthesis. Deletion of *wabP* removes a single heptose moiety and therefore does not significantly compromise the structure of the core-oligosaccharide (Fig 17).

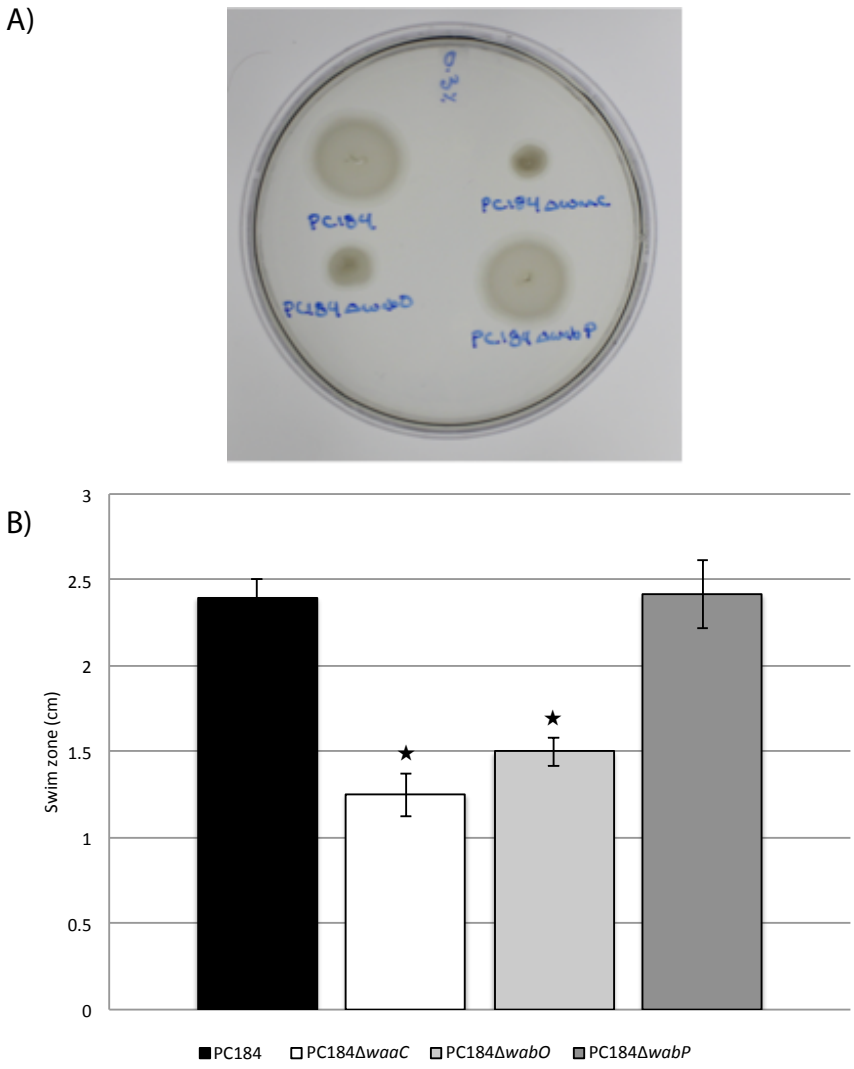


Figure 19: Swimming motility assay for PC184 and LOS mutants. A) A representative image of colony diameters on 0.3% agar after 48 hours. Clockwise, starting from top left: PC184, PC184Δ*waaC*, PC184Δ*wabP*, PC184Δ*waaO*. B) Average swim zones of PC184 and LOS mutants plated on 0.3% agar. Stars denotes statistically significant differences compared to wildtype (student t-test $p < 0.001$).

Transmission electron microscopy of PC184 and LOS mutants

The motility assay (Fig 19) revealed that LOS truncations significantly affect bacterial swimming. To determine if flagellar biosynthesis was disrupted by *waaC* and *wabO* mutations, transmission electron micrographs of PC184 and the mutants were taken. As shown in Fig 20, PC184, PC184Δ*waaC*, PC184Δ*wabO,* and PC184Δ*wabP* all expressed polar flagella. Therefore, a defect in the flagellar biosynthetic machinery of PC184Δ*waaC* and PC184Δ*wabO* did not cause the loss of motility in these mutants.

Previous studies have shown that LPS truncations significantly affect bacterial motility in *E. coli*, *Pseudomonas*, and *Stenotrophomonas* (Genevaux et al., 1999; Abeyrathne et al., 2005; Huang et al., 2006) due to the loss of flagella. However, as Lindhout et al. (2009) determined that LPS truncations did not always inhibit flagellar synthesis (as observed here for PC184 [Fig 20]), loss of motility cannot always be attributed to this mechanism. Although the factors causing motility defects in PC184Δ*waaC* and PC184Δ*wabO* have not yet been identified, this effect may be attributable to increased cell-to-cell interaction caused by loss of the core oligosaccharide.

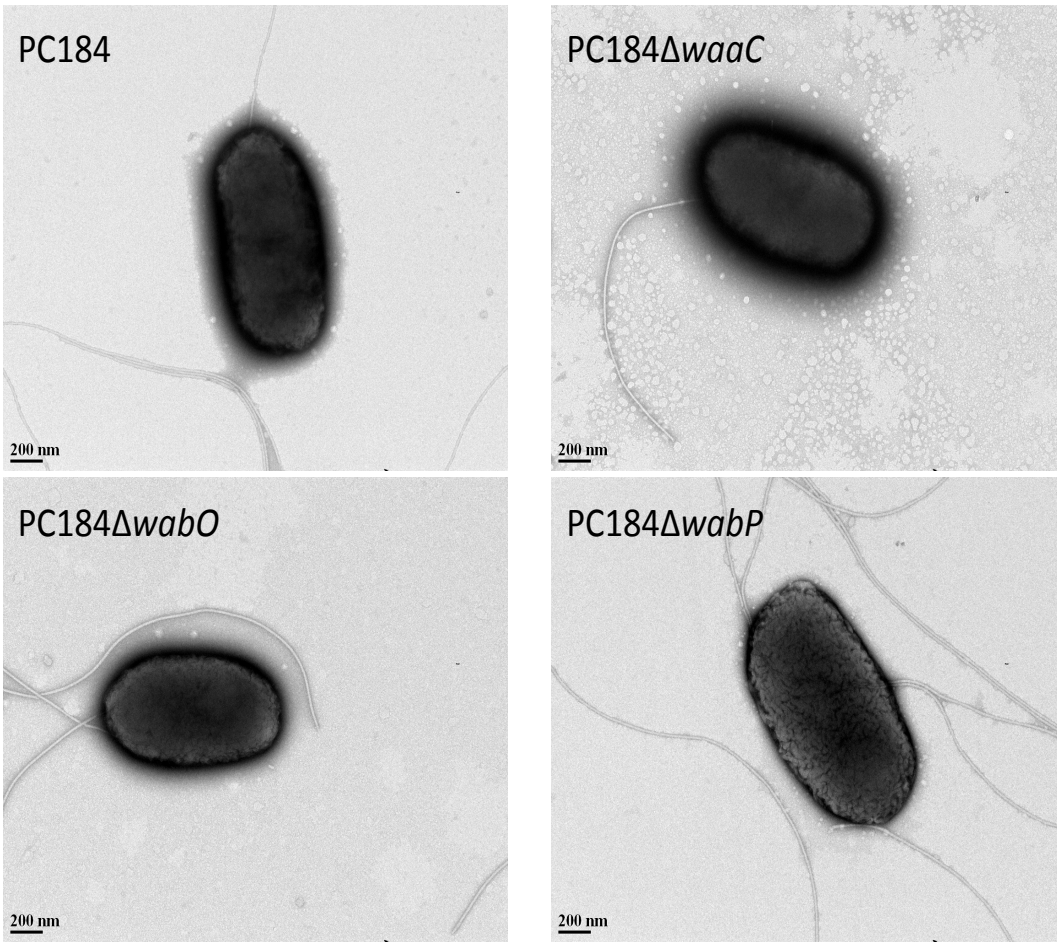


Figure 20: Transmission electron micrographs of PC184 and LOS mutants. A polar flagella was present on the surface of all strains.

Bacteriophage KS10 infection of PC184 and LOS mutants

A liquid clearing assay was performed on wildtype PC184 and the LOS mutants to identify phage-susceptible strains and determine the specific LOS residue used by KS10 as a receptor. The results obtained from this experiment are shown in Fig 21. PC184Δ*wabP* was sensitive to KS10 infection, indicating that KS10 does not bind to the third heptose in the inner core heptosyl trisaccharide. In contrast, PC184Δ*wabO* and PC184Δ*waaC* were resistant to KS10 infection both in liquid culture (Fig 21) and on soft-agar overlays (data not shown). As PC184Δ*wabO* truncated LOS contains a heptosyl disaccharide (encoded by *waaF* and *waaC*) attached to the Kdo (Fig 17), this disaccharide is not the KS10 receptor. Furthermore, as PC184Δ*waaC* LOS contains the Kdo,Ko, and Ara4N residues, we can also conclude that none of these moieties act as the phage receptor. By process of elimination, the KS10 receptor must be within the branched residues attached to the second heptose or the residues branching from the first heptose (including β–D-Glc [Fig 17]). Further mutagenesis and structural analysis will be required to pinpoint the exact residue used as the KS10 receptor.

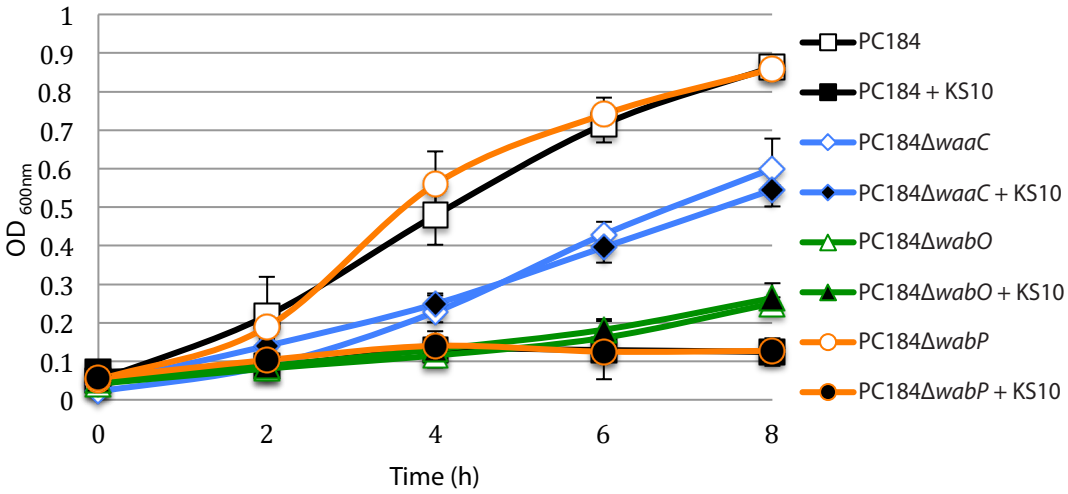


Figure 21: Optical density at 600 nm (OD_{600}) of cultures grown with and without bacteriophage KS10. Broth cultures were diluted twenty-fold and incubated at 37°C with shaking at 225 rpm. Optical density readings were taken at 600 nm every 2 hours for 8 hours. Deletion of *waaC* and *wabO* genes provided resistance to KS10 infection.

Complementation of PC184Δ*waaC*

A cross-complementation approach was used to restore PC184Δ*waaC* LOS biosynthesis. The K56-2 *waaC* gene (BCAL3112) was PCR amplified with the primer pair F-*waaC* and R-*waaC*. This amplicon was inserted into the rhamnose-inducible expression vector pSCrhaB2 using the restriction enzymes NdeI and XbaI, resulting pSCrha*waaC*. This plasmid was then transformed by electroporation into PC184Δ*waaC*. As a control, pSCrhaB2 was transformed into both PC184 and PC184Δ*waaC*. As expression from pSCrhaB2 lacks tight control and has been shown to be leaky (unpublished data), experiments using this plasmid and

derivatives thereof were performed in both the presence and absence of rhamnose.

A series of experiments was performed using PC184 (pSCrhaB2), PC184Δ*waaC (*pSCrhaB2), and PC184Δ*waaC* (pSCrha*waaC)* to determine if BCAL3112 could complement PC184Δ*waaC* mutation with respect to LOS biosynthesis, growth, swimming motility, and phage sensitivity. LOS was isolated from these three strains and separated by SDS-PAGE. Cross-complementation restored PC184Δ*waaC* LOS biosynthesis to wildtype levels under both induction conditions (Fig 22).

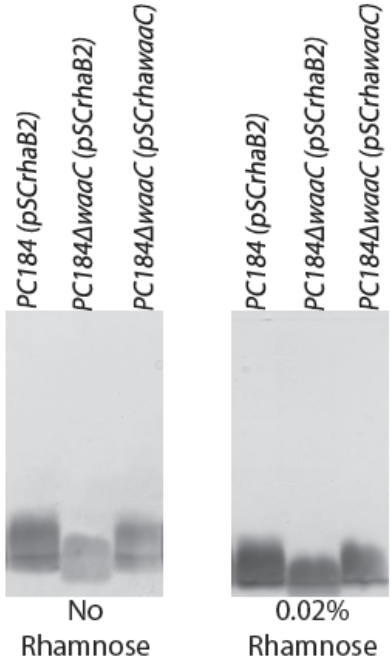


Figure 22: LOS profile of PC184 (pSCrhaB2), PC184Δ*waaC* (pSCrhaB2), and PC184Δ*waaC* (pSCrha*waaC*). LOS was extracted from broth cultures supplemented with or without rhamnose.

Deletion of *waaC* inhibited growth compared to wildtype PC184 (Fig 18). A 12 hour growth curve was performed to determine if the growth rate could be restored by trans-complementation (Figure 22). When no rhamnose was added, the PC184Δ*waaC* (pSCrha*waaC*) growth rate returned to wildtype levels. However, induction of expression with 0.02% rhamnose resulted in a reduced growth rate for the complemented strain, suggesting that overexpression of *waaC* is detrimental to bacterial growth.

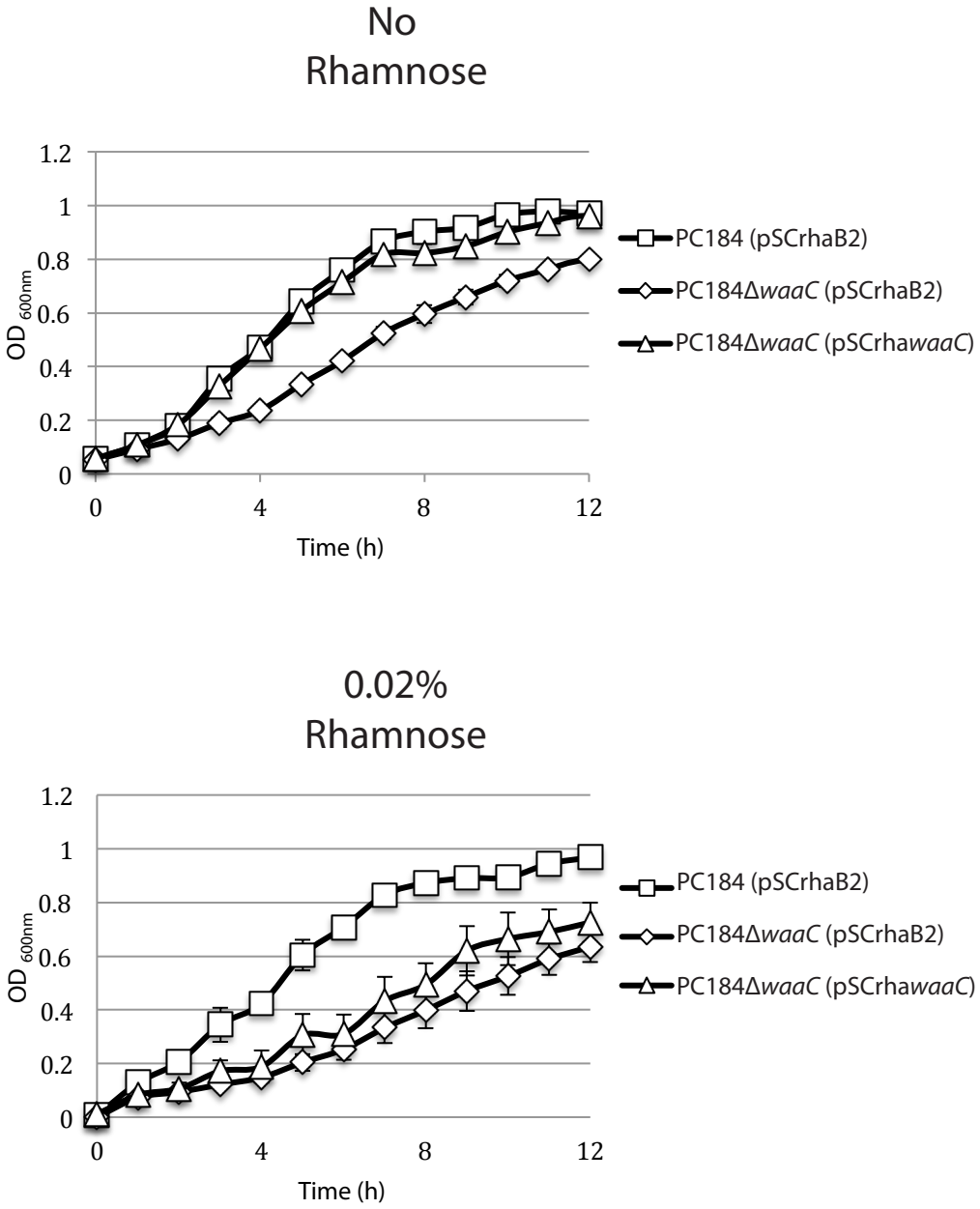


Figure 23: Growth curves of PC184 (pSCrhaB2), PC184Δ*waaC* (pSCrhaB2), and PC184Δ*waaC* (pSCrha*waaC*). Broth cultures were diluted twenty-fold in ½ LB 100 Tp broth (with or without rhamnose) and incubated at 37°C with shaking at 225 rpm. Optical density readings were taken at 600 nm every hour for 12 hours.

As shown in Fig 19, deep inner core LOS truncations were associated with a loss of swimming motility. A swimming assay was performed on PC184 (pSCrhaB2), PC184Δ*waaC* (pSCrhaB2), and PC184Δ*waaC* (pSCrha*waaC*) to determine if trans-complementation could restore motility (Figure 24). As expected based on the data in Fig. 19, non-induced vector control swim zones diameters were greater for PC184 (pSCrhaB2) than for PC184Δ*waaC* (pSCrhaB2) (2.68 ± 0.29 cm and 1.31 ± 0.123 cm, respectively). When PC184Δ*waaC* was cross-complemented with BCAL3112, swimming motility was restored to wildtype levels (2.60 ± 0.25 cm [Figure 24]). Only partial restoration of motility was observed with 0.02% rhamnose induction, providing further evidence that *waaC* overexpression is detrimental to the bacterium.

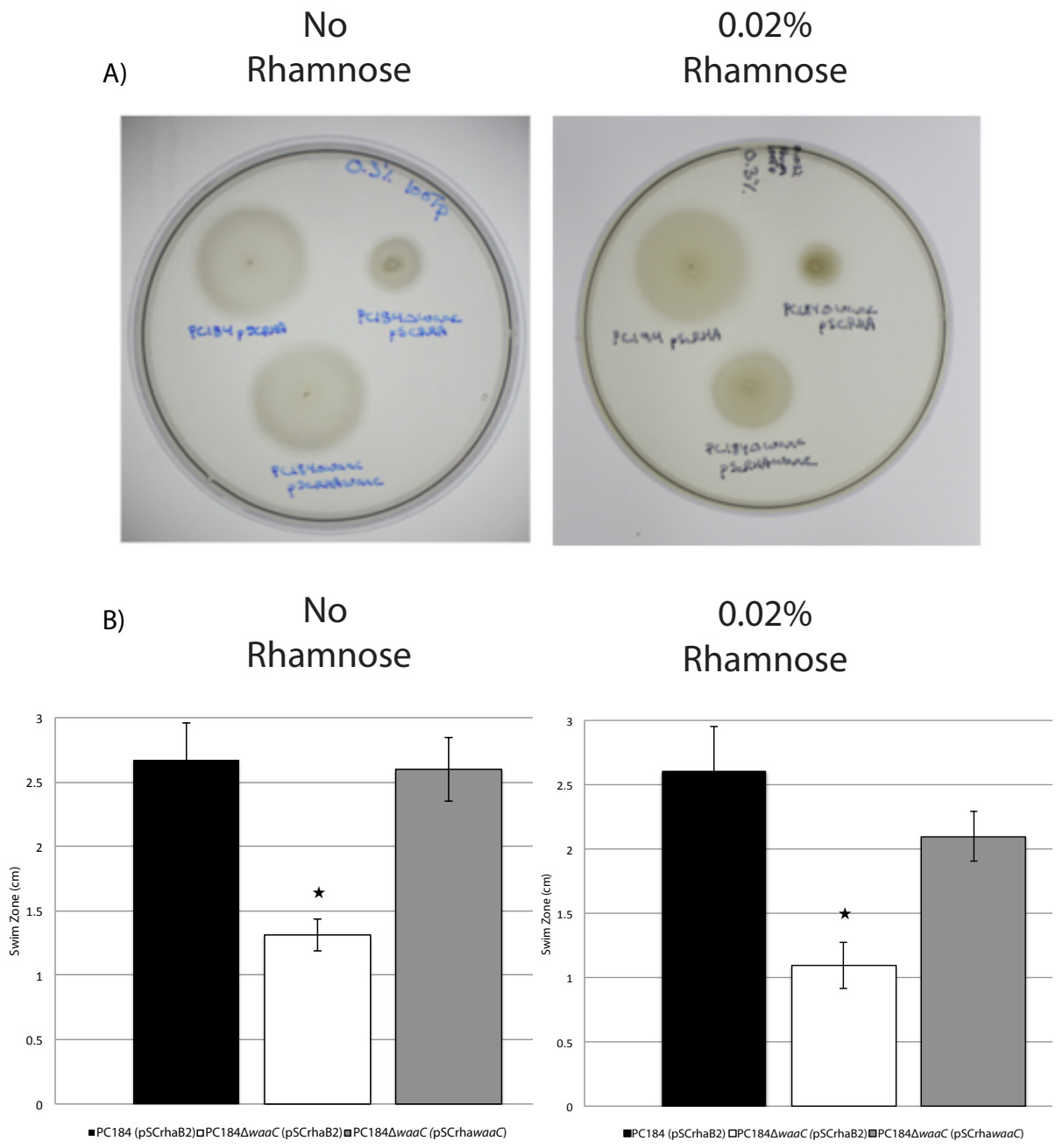


Figure 24: Motility assay for PC184 (pSCrhaB2), PC184Δ*waaC* (pSCrhaB2), and PC184Δ*waaC* (pSCrha*waaC*). A) Representative image of colony diameters on 0.3% 100 Tp agar supplemented with or without rhamnose after 48 hours. Plate colonies (clockwise, starting from top left): PC184 (pSCrhaB2), PC184Δ*waaC* (pSCrhaB2), and PC184Δ*waaC* (pSCrha*waaC*). B) Average swim zones of isolates plated on 0.3% 100 Tp agar supplemented with or without rhamnose after 48 hours. Stars denotes statistically significant differences compared to wildtype (student t-test $p < 0.01$).

Cross-complementation of PC184Δ*waaC* LOS biosynthesis should restore both the KS10 receptor and sensitivity to phage infection. To confirm this hypothesis, a KS10 liquid clearing assay was performed with PC184 (pSCrhaB2), PC184Δ*waaC* (pSCrhaB2), and PC184Δ*waaC* (pSCrha*waaC*). PC184Δ*waaC* sensitivity to KS10 was fully restored when trans-complemented, both in liquid culture (Figure 25) and on soft-agar overlays (data not shown). Although PC184Δ*waaC* (pSCrha*waaC*) does not grow (Fig 23) or swim (Fig 24) at wildtype levels when induced with rhamnose, bacteriophage sensitivity was almost fully restored post-induction (Fig 25).

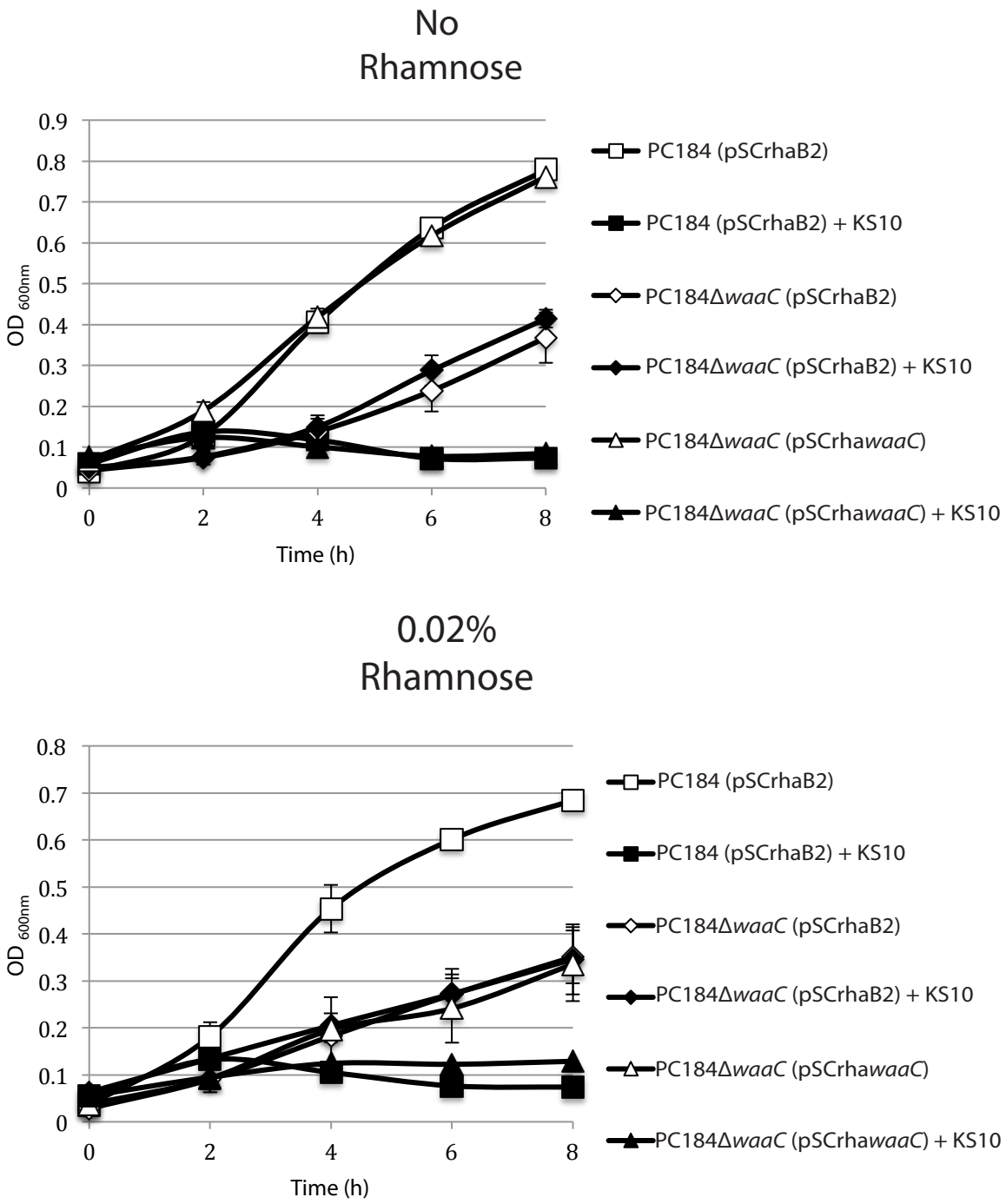


Figure 25: Optical density at 600 nm (OD_{600}) of PC184 (pSCrhaB2), PC184Δ*waaC* (pSCrhaB2), and PC184Δ*waaC* (pSCrha*waaC*) cultures grown with or without bacteriophage KS10. Broth cultures were diluted twenty-fold and incubated at 37°C with shaking at 225 rpm. Optical density readings were taken at 600 nm every 2 hours for 8 hours.

Discussion

Bcc research has advanced considerably over the past decade due in part to the development of novel tools for genetic manipulation and analysis of these species. One of the most intensively studied Bcc organisms is the highly virulent and broadly antibiotic-resistant clinical isolate *B. cenocepacia* K56-2. Many studies have focused on this strain as it can be genetically manipulated more readily than many other Bcc strains (Dubarry et al., 2010). As a consequence, our knowledge of *B. cenocepacia* and of the Bcc in general has been disproportionately skewed towards this bacterium.

In 2008, a targeted clean deletion system was developed that facilitates the construction of unmarked mutations in *B. cenocepacia* strains (Flannagan et al. 2008). While the original published protocol is effective for making deletions in K56-2, the project described here has significantly improved this system with respect to both ease of use and versatility. The first key modification was the replacement of the R6K *oriR* with a pMB1 *oriR* in the suicide vector pGPI-SceI. Plasmids with the R6K origin can be difficult to manipulate and maintain because they must be transformed into the specialized *E. coli* strain SY327. With the pMB1 *oriR*, cloning can be performed using standard competent DH5α cells. The second key modification to the system was the addition of a chloramphenicol-resistance cassette in the nuclease expression vector pDAI-SceI. Selection for this plasmid as originally designed is effective in tetracycline-sensitive strains such as K56-2. However, an alternative

cassette was required for mutagenesis of Bcc strains such as PC184 that are tetracycline-resistant when grown on solid medium. The two modifications to the I-SceI clean deletion system described here will facilitate rapid and effective genetic analysis of both PC184 and other Bcc strains.

The updated I-SceI system was used to construct targeted deletions in three PC184 putative LOS biosynthesis genes: heptosyltransferase gene *wabP*, glycosyltransferase gene *wabO*, and heptosyltransferase gene *waaC*. These mutants were used to confirm previous findings that KS10 interacted with LOS as a receptor (Juarez Lara, 2013) and to further localize the site of KS10 attachment. The KS10 sensitivity of PC184Δ*wabP* and KS10 insensitivity of PC184Δ*wabO and* PC184Δ*waaC* suggest that this phage interacts with branched moieties attached to the second heptose or with moieties branching from the first heptose in the LOS core oligosaccharide. As little is known about the LOS structure of PC184, the exact residue within the core oligosaccharide that is used for phage attachment has yet to be determined. In order to definitively identify this receptor, we will use 2D NMR spectroscopy and mass spectrometry to determine the complete LOS structure of PC184 and the LOS mutants. Structural elucidation of the LOS will play a fundamental role in understanding how this biological compound can induce a strong inflammatory response.

In addition to developing KS10 resistance, both PC184Δ*wabO* and PC184Δ*waaC* displayed reduced fitness in the form of decreased growth and swimming motility when compared to wildtype PC184. Although previous studies have shown that LPS truncation can prevent flagellar biosynthesis (Abeyrathne et al., 2005; Genevaux et al., 1999; Huang et al., 2006), our observations were dissimilar as both wildtype and LOS mutant PC184 produced flagella (Fig 20). Motility loss may be caused by the repression of flagellar genes, aggregation of cells, and increased attachment to the agar matrix (Lindhout et al., 2009; Girgis et al., 2007). Although bacteriophage resistance has always been considered as a disadvantage of phage therapy, this study revealed that phage-resistant mutations can concomitantly make the cell less virulent. Further experiments will be conducted to identify the association between LOS truncations, growth and swimming motility.

Although not addressed in this study, it is likely that LOS truncation in PC184 has other pleiotropic effects based on previous analysis of K56-2 LPS mutants. Such phenotypes may include increased susceptibility to antimicrobial peptides, decreased virulence, and various secondary changes to the structure and composition of the outer membrane (Loutet et al., 2006; Ortega et al., 2009). Further experiments to assay for these phenotypes may include determination of minimum inhibitory concentrations, virulence assays in infection models such as wax moth larvae, duckweed, and mice (Seed and Dennis, 2009; Zhang et al., 2010;

Carmody et al., 2010), and identification of proteins in outer membrane fractions. Analysis of secondary structural effects is particularly important with respect to identification of the KS10 receptor, as it remains possible that KS10 also uses a protein primary or secondary receptor that is misfolded or absent in the outer membrane of PC184Δ*wabO* and PC184Δ*waaC*.

The overall significance of this project to Bcc research is two-fold. First, the improvements made to the I-SceI clean deletion system allow for characterization of genes in potentially every Bcc organism, thus substantially broadening our knowledge of this complex beyond our current focus on *B. cenocepacia* K56-2. Second, mutagenesis of PC184 LOS biosynthesis genes revealed that this structure is both a phage receptor and a fitness factor in this strain. Future use of the modified I-SceI system for receptor identification is important for phage therapy development, as it will allow for the construction of effective multi-receptor phage cocktails to treat Bcc infections.

Literature cited

Abeyrathne, P. D., Daniels, C., Poon, K. K. H., Matewish, M. J., and Lam, J. S. (2005). Functional characterization of WaaL, a ligase associated with linking O-antigen polysaccharide to the core of *Pseudomonas aeruginosa* lipopolysaccharide. *J. Bacteriol.* 187, 3002–3012.

Ackermann, H. W. H., and Kropinski, A. M. A. (2007). Curated list of prokaryote viruses with fully sequenced genomes. *Res. Microbiol.* 158, 12–12.

Adams, M. H. M., and Park, B. H. B. (1956). An enzyme produced by a phage-host cell system. II. The properties of the polysaccharide depolymerase. *Virology* 2, 719–736.

Babior, B. M. B. (2004). NADPH oxidase. *Curr. Opin. Immunol.* 16, 6–6.

Barrett, A. R., Kang, Y., Inamasu, K. S., Son, M. S., Vukovich, J. M., and Hoang, T. T. (2008). Genetic Tools for Allelic Replacement in *Burkholderia* Species. *Appl. Environ. Microbiol.* 74, 4498–4508.

Bartholdson, S. J., Brown, A. R., Mewburn, B. R., Clarke, D. J., Fry, S. C., Campopiano, D. J., and Govan, J. R. W. (2008). Plant host and sugar alcohol induced exopolysaccharide biosynthesis in the *Burkholderia cepacia* complex. *Microbiology* 154, 2513–2521.

Bartual, S. G., Otero, J. M., Garcia-Doval, C., Llamas-Saiz, A. L., Kahn, R., Fox, G. C., and van Raaij, M. J. (2010). Structure of the bacteriophage T4 long tail fiber receptor-binding tip. *Proc. Natl. Acad. Sci. U.S.A.* 107, 20287–20292.

Bazzini, S. S., Udine, C. C., and Riccardi, G. G. (2011). Molecular approaches to pathogenesis study of *Burkholderia cenocepacia*, an important cystic fibrosis opportunistic bacterium. *Appl. Microbiol. Biotechnol.* 92, 887–895.

Biswas, B. B., Adhya, S. S., Washart, P. P., Paul, B. B., Trostel, A. N. A., Powell, B. B., Carlton, R. R., and Merril, C. R. C. (2002). Bacteriophage therapy rescues mice bacteremic from a clinical isolate of vancomycin-resistant *Enterococcus faecium*. *Infect. Imm.* 70, 204–210.

Bruttin, A. A., and Brüssow, H. H. (2005). Human volunteers receiving *Escherichia coli* phage T4 orally: a safety test of phage therapy. *Antimicrob. Agents Chemother.* 49, 2874–2878.

Burkhead, K. D., Schisler, D. A., and Slininger, P. J. (1994). Pyrrolnitrin Production by Biological Control Agent *Pseudomonas cepacia* B37w in Culture and in Colonized Wounds of Potatoes. *Appl. Environ. Microbiol.* 60, 2031–2039.

Burkholder, W. H. (1950). Sour skin, a bacterial rot of Onion bulbs. *Phytopathology* 40, 115–117 .

Buroni, S. S., Pasca, M. R. M., Flannagan, R. S. R., Bazzini, S. S., Milano, A. A., Bertani, I. I., Venturi, V. V., Valvano, M. A. M., and Riccardi, G. G. (2009). Assessment of three Resistance-Nodulation-Cell Division drug efflux transporters of *Burkholderia cenocepacia* in intrinsic antibiotic resistance. *BMC Microbiol.* 9, 200–200.

Bylund, J., Burgess, L. A., Cescutti, P., Ernst, R. K., and Speert, D. P. (2006). Exopolysaccharides from *Burkholderia cenocepacia* inhibit neutrophil chemotaxis and scavenge reactive oxygen species. *J. Biol. Chem.* 281, 2526–2532.

Cardona, S. T., and Valvano, M. A. (2005). An expression vector containing a rhamnose-inducible promoter provides tightly regulated gene expression in *Burkholderia cenocepacia*. *Plasmid* 54, 219–228.

Carlton, R. M. R. (2001). Phage therapy: past history and future prospects. *Arch. Immunol. Ther. Exp.* 47, 267–274.

Carmody, L. A., Gill, J. J., Summer, E. J., Sajjan, U. S., Gonzalez, C. F., Young, R. F., and LiPuma, J. J. (2010). Efficacy of bacteriophage therapy in a model of *Burkholderia cenocepacia* pulmonary infection. *J. Infect. Dis.* 201, 264–271.

Cescutti, P. P., Bosco, M. M., Picotti, F. F., Impallomeni, G. G., Leitão, J. H. J., Richau, J. A. J., and Sá-Correia, I. I. (2000). Structural study of the exopolysaccharide produced by a clinical isolate of Burkholderia cepacia. *Biochem. Biophys. Res. Commun.* 273, 1088–1094.

Cheung, T. K. M., Ho, P. L., Woo, P. C. Y., Yuen, K. Y., and Chau, P. Y. (2002). Cloning and expression of class A beta-lactamase gene blaA (BPS) in *Burkholderia pseudomallei*. *Antimicrob. Agents Chemother.* 46, 1132–1135.

Chiarini, L., Cescutti, P., Drigo, L., Impallomeni, G., Herasimenka, Y., Bevivino, A., Dalmastri, C., Tabacchioni, S., Manno, G., Zanetti, F., et al. (2004). Exopolysaccharides produced by *Burkholderia cenocepacia* recA lineages IIIA and IIIB. *J. Cystic Fibrosis* 3, 165–172.

Choi, K.-H., Mima, T., Casart, Y., Rholl, D., Kumar, A., Beacham, I. R., and Schweizer, H. P. (2008). Genetic Tools for Select-Agent-Compliant Manipulation of *Burkholderia pseudomallei*. *Appl. Environ. Microbiol.* 74, 5265–5265.

Coenye, T., and Vandamme, P. (2007). *Burkholderia*: molecular microbiology and genomics. Wymondham: Horizon Bioscience.

Conway, B. A. D., Chu, K. K., Bylund, J., Altman, E., and Speert, D. P. (2004). Production of exopolysaccharide by *Burkholderia cenocepacia* results in altered cell-surface interactions and altered bacterial clearance in mice. *J. Infect. Dis.* 190, 957–966.

Cunha, M. V. M., Sousa, S. A. S., Leitão, J. H. J., Moreira, L. M. L., Videira, P. A. P., and Sá-Correia, I. I. (2004). Studies on the involvement of the exopolysaccharide produced by cystic fibrosis-associated isolates of the *Burkholderia cepacia* complex in biofilm formation and in persistence of respiratory infections. *J. Clin. Microbiol.* 42, 3052–3058.

De Soyza, A., Ellis, C. D., Khan, C. M. A., Corris, P. A., and de Hormaeche, R. D. (2004). *Burkholderia cenocepacia* Lipopolysaccharide, Lipid A, and Proinflammatory Activity. *Am. J. Respir. Crit. Care Med.* 170, 70–77.

De Soyza, A., Silipo, A., Lanzetta, R., Govan, J. R., and Molinaro, A. (2008). Chemical and biological features of *Burkholderia cepacia* complex lipopolysaccharides. *Innate Immunity* 14, 127–144.

Dennis, J. J., and Zylstra, G. J. (1998). Plasposons: modular self-cloning minitransposon derivatives for rapid genetic analysis of gram-negative bacterial genomes. *Appl. Environ. Microbiol.* 64, 2710–2715.

Desai, M. M., Bühler, T. T., Weller, P. H. P., and Brown, M. R. M. (1998). Increasing resistance of planktonic and biofilm cultures of *Burkholderia cepacia* to ciprofloxacin and ceftazidime during exponential growth. *J. Antimicrob. Chemother.* 42, 153–160.

Donlan, R. M. (2009). Preventing biofilms of clinically relevant organisms using bacteriophage. *Trends in Microbiol.* 17, 66–72.

Dubarry, N., Du, W., Lane, D., and Pasta, F. (2010). Improved electrotransformation and decreased antibiotic resistance of the cystic fibrosis pathogen *Burkholderia cenocepacia* strain J2315. *Appl. Environ. Microbiol.* 76, 1095–1102.

Eaton, K. A., Morgan, D. R., and Krakowka, S. (1992). Motility as a factor in the colonisation of gnotobiotic piglets by *Helicobacter pylori*. *J. Med. Microbiol.* 37, 123–127.

Eftekhar, F. F., and Speert, D. P. D. (1988). Alginase treatment of mucoid *Pseudomonas aeruginosa* enhances phagocytosis by human monocyte-derived macrophages. *Infect. Immun.* 56, 2788–2793.

Feldman, M., Bryan, R., Rajan, S., Scheffler, L., Brunnert, S., Tang, H., and Prince, A. (1998). Role of flagella in pathogenesis of

Pseudomonas aeruginosa pulmonary infection. *Infect. Immun.* 66, 43–51.

Ferreira, A. S., Leitao, J. H., Silva, I. N., Pinheiro, P. F., Sousa, S. A., Ramos, C. G., and Moreira, L. M. (2010). Distribution of Cepacian Biosynthesis Genes among Environmental and Clinical *Burkholderia* Strains and Role of Cepacian Exopolysaccharide in Resistance to Stress Conditions. *Appl. Environ. Microbiol.* 76, 441–450.

Ferreira, A. S. A., Silva, I. N. I., Oliveira, V. H. V., Cunha, R. R., and Moreira, L. M. L. (2011). Insights into the role of extracellular polysaccharides in *Burkholderia* adaptation to different environments. *Front. Cell. Inf. Microbio.* 1:16.

Flannagan, R. S., Linn, T., and Valvano, M. A. (2008). A system for the construction of targeted unmarked gene deletions in the genus *Burkholderia*. *Environ. Microbiol.* 10, 1652–1660.

Gautam, V., Singhal, L., and Ray, P. (2011). *Burkholderia cepacia* complex: beyond *Pseudomonas* and *Acinetobacter*. *Indian J. Med. Microbiol.* 29, 4–12.

Genevaux, P. P., Bauda, P. P., DuBow, M. S. M., and Oudega, B. B. (1999). Identification of Tn10 insertions in the rfaG, rfaP, and galU genes involved in lipopolysaccharide core biosynthesis that affect *Escherichia coli* adhesion. *Arch. Microbiol.* 172, 1–8.

Girgis, H. S., Liu, Y., Ryu, W. S., and Tavazoie, S. (2007). A Comprehensive Genetic Characterization of Bacterial Motility. *PLoS Genet* 3, e154.

Glonti, T., Chanishvili, N., and Taylor, P. W. (2010). Bacteriophage-derived enzyme that depolymerizes the alginic acid capsule associated with cystic fibrosis isolates of *Pseudomonas aeruginosa*. *J. Appl. Microbiol.* 108, 695–702.

Govan, J. R. J., Hughes, J. E. J., and Vandamme, P. P. (1996). *Burkholderia cepacia*: medical, taxonomic and ecological issues. *J. Med. Microbiol.* 45, 395–407.

Govan, J. R., Brown, P. H., Maddison, J., Doherty, C. J., Nelson, J. W., Dodd, M., Greening, A. P., and Webb, A. K. (1993). Evidence for transmission of *Pseudomonas cepacia* by social contact in cystic fibrosis. *Lancet* 342, 15–19.

Hamad, M. A. M., Zajdowicz, S. L. S., Holmes, R. K. R., and Voskuil, M. I. M. (2009). An allelic exchange system for compliant genetic manipulation of the select agents *Burkholderia pseudomallei* and

Burkholderia mallei. *Gene* 430, 123–131.

Hammer, P. E., and Evensen, K. B. (1993). Postharvest control of *Botrytis cinerea* on cut rose flowers with pyrrolnitrin. *Plant Disease* 77, 283–286.

Hooton, S. P. T. S., Atterbury, R. J. R., and Connerton, I. F. I. (2011). Application of a bacteriophage cocktail to reduce *Salmonella Typhimurium* U288 contamination on pig skin. *Int. J. Food Microbiol.* 151, 157–163.

Huang, T.-P. T., Somers, E. B. E., and Wong, A. C. L. A. (2006). Differential biofilm formation and motility associated with lipopolysaccharide/exopolysaccharide-coupled biosynthetic genes in *Stenotrophomonas maltophilia*. *J. Bacteriol.* 188, 3116–3120.

Hughes, K. A., Sutherland, I. W., and Jones, M. V. (2008). Biofilm susceptibility to bacteriophage attack: the role of phage-borne polysaccharide depolymerase. *Microbiology* 144, 3039–3047.

Hughes, K. A., Sutherland, I. W., Clark, J., and Jones, M. V. (1998). Bacteriophage and associated polysaccharide depolymerases--novel tools for study of bacterial biofilms. *J. of Appl. Microbiol.* 85, 583–590.

Ieranò, T., Silipo, A., Sturiale, L., Garozzo, D., Corris, P., Perry, J., Lanzetta, R., Parrilli, M., De Soyza, A., and Molinaro, A. (2010). Structural Elucidation of a Novel *B. cenocepacia* ET-12 Lipooligosaccharide Isolated from a Cystic Fibrosis Patient after Lung Transplantation. *Eur. J. Org. Chem.* 2010, 1299–1306.

Isles, A., Maclusky, I., Corey, M., Gold, R., Prober, C., Fleming, P., and Levison, H. (1984). *Pseudomonas cepacia* infection in cystic fibrosis: an emerging problem. *J. Pediatr.* 104, 206–210.

Jarrell, K. F., and McBride, M. J. (2008). The surprisingly diverse ways that prokaryotes move. *Nat. Review. Microbiol.* 6, 466–476.

Jikia, D. D., Chkhaidze, N. N., Imedashvili, E. E., Mgaloblishvili, I. I., Tsitlanadze, G. G., Katsarava, R. R., Morris, J. J. G., and Sulakvelidze, A. A. (2005). The use of a novel biodegradable preparation capable of the sustained release of bacteriophages and ciprofloxacin, in the complex treatment of multidrug-resistant *Staphylococcus aureus*-infected local radiation injuries caused by exposure to Sr90. *Clin. Exp. Dermatol.* 30, 23–26.

Juarez Lara, G. R. (2013). MSc. Thesis University of Alberta, Edmonton, AB. Characterization of bacteriophage receptors in the *Burkholderia cepacia* complex.

Kropinski, A. M. (2006). Phage Therapy - Everything Old is New Again. *Can. J. Infect. Dis.* 17, 297–306.

Labrie, S. J., Samson, J. E., and Moineau, S. (2010). Bacteriophage resistance mechanisms. *Nat. Rev. Microbiol.* 8, 317–327.

Lefebre, M. D., and Valvano, M. A. (2002). Construction and Evaluation of Plasmid Vectors Optimized for Constitutive and Regulated Gene Expression in *Burkholderia cepacia* Complex Isolates. *Appl. Environ. Microbiol.* 68, 5956–5964.

Leiman, P. G. P., Kanamaru, S. S., Mesyanzhinov, V. V. V., Arisaka, F. F., and Rossmann, M. G. M. (2003). Structure and morphogenesis of bacteriophage T4. *Cell. Mol. Life Sci.* 60, 2356–2370.

Leitão, J. H. J., Sousa, S. A. S., Ferreira, A. S. A., Ramos, C. G. C., Silva, I. N. I., and Moreira, L. M. L. (2010). Pathogenicity, virulence factors, and strategies to fight against *Burkholderia cepacia* complex pathogens and related species. *Appl. Microbiol. Biotechnol.* 87, 31–40.

Lindhout, T., Lau, P. C. Y., Brewer, D., and Lam, J. S. (2009). Truncation in the core oligosaccharide of lipopolysaccharide affects flagella-mediated motility in *Pseudomonas aeruginosa* PAO1 via modulation of cell surface attachment. *Microbiology* 155, 3449–3460.

LiPuma, J. J., Dasen, S. E., Nielson, D. W., Stern, R. C., and Stull, T. L. (1990). Person-to-person transmission of *Pseudomonas cepacia* between patients with cystic fibrosis. *Lancet* 336, 1094–1096.

Lopez, C. M., Rholl, D. A., Trunck, L. A., and Schweizer, H. P. (2009). Versatile Dual-Technology System for Markerless Allele Replacement in *Burkholderia pseudomallei*. *Appl. Environ. Microbiol.* 75, 6496–6503.

Loutet, S. A., Flannagan, R. S., Kooi, C., Sokol, P. A., and Valvano, M. A. (2006). A complete lipopolysaccharide inner core oligosaccharide is required for resistance of *Burkholderia cenocepacia* to antimicrobial peptides and bacterial survival *in vivo*. *J. Bacteriol.* 188, 2073–2080.

Lynch, K. H., Abdu A. H., Schobert M., and Dennis J. J. (Submitted) Genomic characterization of JG068, a novel lytic podovirus active against *Burkholderia cenocepacia. BMC Genomics*

Mahenthiralingam, E. E., Urban, T. A. T., and Goldberg, J. B. J. (2005). The multifarious, multireplicon *Burkholderia cepacia* complex. *Nat. Rev. Microbiol.* 3, 144–156.

Mahenthiralingam, E., Coenye, T., Chung, J. W., Speert, D. P., Govan, J. R., Taylor, P., and Vandamme, P. (2000). Diagnostically and experimentally useful panel of strains from the *Burkholderia cepacia* complex. *J. Clin. Microbiol.* 38, 910–913.

Marolda, C. L., Lahiry, P., Vinés, E., Saldías, S., and Valvano, M. A. (2006). Micromethods for the characterization of lipid A-core and O-antigen lipopolysaccharide. *Methods Mol. Biol.* 347, 237–252.

McVay, C. S., Velásquez, M., and Fralick, J. A. (2007). Phage therapy of *Pseudomonas aeruginosa* infection in a mouse burn wound model. *Antimicrob. Agents Chemother.* 51, 1934–1938.

Merabishvili, M., Pirnay, J.-P., Verbeken, G., Chanishvili, N., Tediashvili, M., Lashkhi, N., Glonti, T., Krylov, V., Mast, J., Van Parys, L., et al. (2009). Quality-controlled small-scale production of a well-defined bacteriophage cocktail for use in human clinical trials. *PLoS ONE* 4(3), e4944.

Merril, C. R. (1975). Phage in Human Vaccines. *Science* 188, 8.

Merril, C. R., Scholl, D., and Adhya, S. L. (2003). The prospect for bacteriophage therapy in Western medicine. *Nat. Rev. Drug Discov.* 2, 489–497.

Miché, L. L., and Balandreau, J. J. (2001). Effects of rice seed surface sterilization with hypochlorite on inoculated *Burkholderia vietnamiensis*. *Appl. Environ. Microbiol.* 67, 3046–3052.

Miller, E. S. E., Kutter, E. E., Mosig, G. G., Arisaka, F. F., Kunisawa, T. T., and Rüger, W. W. (2003). Bacteriophage T4 genome. *Microbiol. Mol. Biol. Rev.* 67(1), 86–156.

Moreira, L. M., Videira, P. A., Sousa, S. A., Leitão, J. H., Cunha, M. V., and Sá-Correia, I. (2003). Identification and physical organization of the gene cluster involved in the biosynthesis of *Burkholderia cepacia* complex exopolysaccharide. *Biochem. Biophys. Res. Commun.* 312, 323–333.

Morooka, T., Umeda, A., and Amako, K. (1985). Motility as an intestinal colonization factor for *Campylobacter jejuni*. *J. Gen. Microbiol.* 131, 1973–1980.

Nair, B. M. B., Cheung, K.-J. K., Griffith, A. A., and Burns, J. L. J. (2004). Salicylate induces an antibiotic efflux pump in *Burkholderia cepacia* complex genomovar III (B. cenocepacia). *J. Clin. Invest.* 113, 464–473.

Norris, M. H., Kang, Y., Lu, D., Wilcox, B. A., and Hoang, T. T. (2009). Glyphosate Resistance as a Novel Select-Agent-Compliant, Non-Antibiotic-Selectable Marker in Chromosomal Mutagenesis of the Essential Genes *asd* and *dapB* of *Burkholderia pseudomallei*. *Appl. Environ. Microbiol.* 75, 6062–6075.

O'Sullivan, B. P., and Freedman, S. D. (2009). Cystic fibrosis. *Lancet* 373, 1891–1904.

Ortega, X., Silipo, A., Saldias, M. S., Bates, C. C., Molinaro, A., and Valvano, M. A. (2009). Biosynthesis and structure of the *Burkholderia cenocepacia* K56-2 lipopolysaccharide core oligosaccharide: truncation of the core oligosaccharide leads to increased binding and sensitivity to polymyxin B. *J. Biol. Chem.* 284, 21738–21751.

Prince, A., Wood, M. S., Cacalano, G. S., and Chin, N. X. (1988). Isolation and characterization of a penicillinase from *Pseudomonas cepacia* 249. *Antimicrob. Agents Chemother.* 32, 838–843.

Rakhuba, D. V. D., Kolomiets, E. I. E., Dey, E. S. E., and Novik, G. I. G. (2010). Bacteriophage receptors, mechanisms of phage adsorption and penetration into host cell. *Pol. J. Microbiol.* 59, 145–155.

Rashid, M. H. M., and Kornberg, A. A. (2000). Inorganic polyphosphate is needed for swimming, swarming, and twitching motilities of *Pseudomonas aeruginosa*. *Proc. Natl. Acad. Sci. U.S.A.* 97, 4885–4890.

Rhoads, D. D. D., Wolcott, R. D. R., Kuskowski, M. A. M., Wolcott, B. M. B., Ward, L. S. L., and Sulakvelidze, A. A. (2009). Bacteriophage therapy of venous leg ulcers in humans: results of a phase I safety trial. *J. Wound Care* 18, 237–233.

Rich, D. P. D., Anderson, M. P. M., Gregory, R. J. R., Cheng, S. H. S., Paul, S. S., Jefferson, D. M. D., McCann, J. D. J., Klinger, K. W. K., Smith, A. E. A., and Welsh, M. J. M. (1990). Expression of cystic fibrosis transmembrane conductance regulator corrects defective chloride channel regulation in cystic fibrosis airway epithelial cells. *Nature* 347, 358–363.

Robinson, M. M., and Bye, P. T. B. P. (2002). Mucociliary clearance in cystic fibrosis. *Pediatr. Pulmonol.* 33, 293–306.

Rossmann, M. M. G., and Rao, V. B. (2012). *Viral molecular machines*. New York: Springer.

Routier, S. (2010). MSc. Thesis University of Alberta, Edmonton, AB. DC1, a Podoviridae with a putative cepacian depolymerase enzyme.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). *Molecular cloning: a laboratory manual*. New York; Cold Springs Harbor Laboratory Press.

Saiman, L. L., and Siegel, J. J. (2003). Infection control recommendations for patients with cystic fibrosis: Microbiology, important pathogens, and infection control practices to prevent patient-to-patient transmission. Am. J. Infect. Control 31, S1–S52.

Seed, K. D., and Dennis, J. J. (2009). Experimental Bacteriophage Therapy Increases Survival of *Galleria mellonella* Larvae Infected with Clinically Relevant Strains of the *Burkholderia cepacia* Complex. *Antimicrob. Agents Chemother.* 53, 2205–2208.

Sessitsch, A., Coenye, T., Sturz, A. V., Vandamme, P., Barka, E. A., Salles, J. F., Van Elsas, J. D., Faure, D., Reiter, B., Glick, B. R., et al. (2005). *Burkholderia* phytofirmans sp. nov., a novel plant-associated bacterium with plant-beneficial properties. *Int. J. Syst. Evol. Microbiol.* 55, 1187–1192.

Shaw, D. D., Poxton, I. R. I., and Govan, J. R. J. (1995). Biological activity of *Burkholderia* (*Pseudomonas*) *cepacia* lipopolysaccharide. *FEMS Immunol. Med. Microbiol.* 11, 99–106.

Silhavy, T.J., Berman, M.L., Enquist, L.W. (1984) *Experiments with Gene Fusions.* Cold Spring Harbor: Cold Spring Harbor Laboratory.

Silipo, A. A., Molinaro, A. A., Ieranò, T. T., De Soyza, A. A., Sturiale, L. L., Garozzo, D. D., Aldridge, C. C., Corris, P. A. P., Khan, C. M. A. C., Lanzetta, R. R., et al. (2007). The complete structure and pro-inflammatory activity of the lipooligosaccharide of the highly epidemic and virulent gram-negative bacterium *Burkholderia cenocepacia* ET-12 (strain J2315). *Chemistry* 13, 3501–3511.

Silva, I. N., Ferreira, A. S., Becker, J. D., Zlosnik, J. E. A., Speert, D. P., He, J., Mil-Homens, D., and Moreira, L. M. (2011). Mucoid morphotype variation of *Burkholderia multivorans* during chronic cystic fibrosis lung infection is correlated with changes in metabolism, motility, biofilm formation and virulence. *Microbiology* 157, 3124–3137.

Sousa, S. A., Ramos, C. G., and Leitão, J. H. (2011). *Burkholderia cepacia* Complex: Emerging Multihost Pathogens Equipped with a Wide Range of Virulence Factors and Determinants. *Int. J. Microbiol.* 2011, 1–9.

Speert, D. P. D., Bond, M. M., Woodman, R. C. R., and Curnutte, J. T. J. (1994). Infection with Pseudomonas cepacia in chronic granulomatous

disease: role of nonoxidative killing by neutrophils in host defense. *J. Infect. Dis.* 170, 1524–1531.

Sulakvelidze, A. A., Alavidze, Z. Z., and Morris, J. G. J. (2001). Bacteriophage therapy. *Antimicrob. Agents Chemother.* 45, 649–659.

Summers, W. C. (2001). Bacteriophage therapy. *Microbiology* 55, 437–451.

Sutherland, I. W., and Wilkinson, J. F. (1965). Depolymerases for bacterial exopolysaccharides obtained from phage-infected bacteria. *J. Gen. Microbiol.* 39, 373–383.

Tanji, Y. Y., Shimada, T. T., Yoichi, M. M., Miyanaga, K. K., Hori, K. K., and Unno, H. H. (2004). Toward rational control of *Escherichia coli* O157:H7 by a phage cocktail. *Appl. Microbiol. Biotechnol.* 64, 270–274.

Tomich, M., Herfst, C. A., Golden, J. W., and Mohr, C. D. (2002). Role of flagella in host cell invasion by *Burkholderia cepacia*. *Infect. Immun.* 70, 1799–1806.

Tsai, C. M., and Frasch, C. E. (1982). A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. *Anal. Biochem.* 119, 115–119.

Uehlinger, S., Schwager, S., Bernier, S. P., Riedel, K., Nguyen, D. T., Sokol, P. A., and Eberl, L. (2009). Identification of specific and universal virulence factors in *Burkholderia cenocepacia* strains by using multiple infection hosts. *Infect. Immun.* 77, 4102–4110.

Urban, T. A. T., Griffith, A. A., Torok, A. M. A., Smolkin, M. E. M., Burns, J. L. J., and Goldberg, J. B. J. (2004). Contribution of *Burkholderia cenocepacia* flagella to infectivity and inflammation. *Infect. Immun.* 72, 5126–5134.

Van den Berg, J. M., van Koppen, E., Ahlin, A., Belohradsky, B. H., Bernatowska, E., Corbeel, L., Español, T., Fischer, A., Kurenko-Deptuch, M., Mouy, R., et al. (2009). Chronic granulomatous disease: the European experience. *PLoS ONE* 4(4), e5234.

Vandamme, P., Holmes, B., Vancanneyt, M., Coenye, T., Hoste, B., Coopman, R., Revets, H., Lauwers, S., Gillis, M., Kersters, K., et al. (1997). Occurrence of multiple genomovars of *Burkholderia cepacia* in cystic fibrosis patients and proposal of Burkholderia multivorans sp. nov. *Int. J. Syst. Bacteriol.* 47, 1188–1200.

Vanlaere, E., Baldwin, A., Gevers, D., Henry, D., De Brandt, E., LiPuma, J.

J., Mahenthiralingam, E., Speert, D. P., Dowson, C., and Vandamme, P. (2009). Taxon K, a complex within the *Burkholderia cepacia* complex, comprises at least two novel species, *Burkholderia contaminans* sp. nov. and *Burkholderia lata* sp. nov. *Int. J. Syst. Evol. Microbiol.* 59, 102–111.

Vinion-Dubiel, A. D. A., and Goldberg, J. B. J. (2003). Lipopolysaccharide of *Burkholderia cepacia* complex. *J. Endotoxin Res* 9, 201–213.

White, D., Drummond, J. T., and Fuqua, C. (2011). *The Physiology and Biochemistry of Prokaryotes.* New York; Oxford University Press.

Wilson, K. (2001). Preparation of genomic DNA from bacteria. *Curr. Protoc. Mol. Biol.* Chapter 2, Unit 2.4.

Winkelstein, J. A. J., Marino, M. C. M., Johnston, R. B. R., Boyle, J. J., Curnutte, J. J., Gallin, J. I. J., Malech, H. L. H., Holland, S. M. S., Ochs, H. H., Quie, P. P., et al. (2000). Chronic granulomatous disease. Report on a national registry of 368 patients. *Medicine (Abingdon)* 79, 155–169.

Wright, A. A., Hawkins, C. H. C., Anggård, E. E. E., and Harper, D. R. D. (2009). A controlled clinical trial of a therapeutic bacteriophage preparation in chronic otitis due to antibiotic-resistant *Pseudomonas aeruginosa*; a preliminary report of efficacy. *Clin. Otolaryngol.* 34, 349–357.

Yabuuchi, E., Kosako, Y., Oyaizu, H., Yano, I., Hotta, H., Hashimoto, Y., Ezaki, T., and Arakawa, M. (1992). Proposal of *Burkholderia* gen. nov. and transfer of seven species of the genus *Pseudomonas* homology group II to the new genus, with the type species *Burkholderia cepacia* (Palleroni and Holmes 1981) comb. nov. *Microbiol. Immunol.* 36, 1251–1275.

Yu, F., and Mizushima, S. (1982). Roles of lipopolysaccharide and outer membrane protein OmpC of *Escherichia coli* K-12 in the receptor function for bacteriophage T4. *J. Bacteriol.* 151, 718–722.

Zhang, Y., Hu, Y., Yang, B., Ma, F., Lu, P., Li, L., and Wan, C. (2010). Duckweed (*Lemna minor*) as a model plant system for the study of human microbial pathogenesis. PLoS ONE 5(10) e13527.

Zlosnik, J. E. A., Costa, P. S., Brant, R., Mori, P. Y. B., Hird, T. J., Fraenkel, M. C., Wilcox, P. G., Davidson, A. G. F., and Speert, D. P. (2011). Mucoid and Nonmucoid *Burkholderia cepacia* Complex Bacteria in Cystic Fibrosis Infections. *Am. J. Respir. Crit. Care Med.* 183, 67–72.

Appendix I

Characterization of the bacteriophage DC1 halo plaque phenotype

Introduction

To date, no phage polysaccharide depolymerase enzyme has been described for the Bcc. Previously, Bcc-specific bacteriophages were screened on EPS-inducing media (½ YS or YEM) for development of a plaque phenotype with a large, expanding halo characteristic of either polysaccharide depolymerase activity or, in rare cases, LPS degradation (Hughest et al. 1998; Sutherland, 1999; Pleteneva et al., 2011, Cornelissen, 2011). As the podovirus DC1 produced such plaques on both *B. cepacia* LMG18821 and *B. cenocepacia* PC184, this phage was predicted to encode a polysaccharide depolymerase enzyme (Routier, 2010). It was hypothesized that this enzyme targets cepacian, a well-characterized and abundant EPS produced by many Bcc strains (Cescutti et al., 2003).

Although the EPS profile of PC184 has never been characterized, the mucoid phenotype of this strain on EPS-inducing media is predicted to be caused by cepacian production. PC184 encodes numerous cepacian biosynthesis genes including *bceB* (Routier 2010). Previous studies have shown that inactivation of this gene results in the loss of cepacian production (Bartholdson et al., 2008; Moreira et al., 2003). The objective of this study was to identify the DC1 protein responsible for halo formation on EPS-inducing media through the use of random mutagenesis, protein expression, and targeted cepacian and LOS mutagenesis.

Materials and Methods

Strains and growth conditions

B. cepacia LMG 18821 and *B. cenocepacia* PC184 are part of the Bcc experimental strain panel (Mahenthiralingam et al., 2000) and were provided by the Canadian *Burkholderia cepacia* complex Research and Referral Repository (CBCCRRR). Both strains were grown aerobically overnight at 30^{o}C in half strength Luria-Bertani (½ LB) broth (with shaking at 225 rpm) or on ½ LB agar. The growth medium was supplemented with various antibiotics to ensure plasmid maintenance, including 100 mg/l trimethoprim (Tp) and 300 mg/l chloramphenicol (Cm). For EPS induction, strains were grown on ½ YS (0.125 g yeast extract, 1.125g mannitol, 7.5 g agar in 500 ml distilled water) or yeast extract medium (YEM; 0.25 g yeast extract, 2 g mannitol, and 7.5 g agar in 500 ml distilled water).

Bacteriophage DC1 propagation

To propagate DC1, 100 µl of phage stock and 100 µl of PC184 broth culture were mixed and incubated for 20 min at room temperature. After incubation, 3 ml of top agar (0.7% [w/v] ½ LB agar) was added to the mixture and immediately poured onto the surface of a ½ LB plate. The overlay was allowed to solidify and incubated overnight at 30^{o}C. The plate was overlaid with 3 ml of suspension medium (SM; 1.45 g NaCl, 0.5 g $MgSO_4$, 12.5 ml Tris-HCl [pH 7.5], 1.25 ml 2% gelatin and 250 ml of distilled water) and incubated for 4 h at room temperature on a rocking

platform. The supernatant was recovered, centrifuged to pellet bacterial debris, filter sterilized using a 0.45 µm syringe-driven filter (Millipore, Billerica, MA), and stored at 4^{o}C.

Depolymerase activity screen

To assay for halo formation, DC1 and either PC184 or LMG 18821 were plated in agar overlays on EPS-inducing media and incubated overnight at 30°C. Plates were then incubated at room temperature for up to a week. All halo assays were was performed with 0.3% (w/v) ½ LB top agar.

Bacterial DNA and plasmid isolation

All genomic and plasmid DNA extractions were performed using plates grown overnight at 30°C. Genomic DNA was isolated following the procedure outlined by Wilson (2001). Plasmid DNA was isolated using a GeneJET™ Plasmid Miniprep kit (Thermo Scientific, Waltham, MA). DNA was quantified using a NanoDrop 2000c spectrophotometer (Thermo Scientific) and stored at 4^{o}C or -20^{o}C.

General molecular techniques such as mutagenesis, restriction digestion, PCR, DNA purification, ligation and sequencing were performed as previously described in this thesis. The oligonucleotides used in this study are listed in Table 3.

Random mutagenesis

DC1 DNA was subjected to mechanical shearing (nebulization), producing ~3000 bp fragments. Fragmented DNA was then randomly mutagenized with *in vitro* transposition using an EZ-Tn5 insertion kit (Epicentre, Madison, WI). This DNA was transformed by electroporation into putative PC184 DC1 lysogens, allowing for recombination and subsequent growth of antibiotic resistant colonies (Fig 26). These lysogens could then be induced in order to isolate viable randomly mutagenized DC1.

UV mutagenesis was performed on DC1 by exposing 500 µl of lysate (as noted above) to UV (Thermo Scientific, Waltham, MA). Phage lysate was mutagenized for up to 8 min and plated with either LMG 18821 or PC184 on EPS-inducing media to screen for a halo-negative plaque.

Table 3: Primers used in this study

Primer Name	Sequence (5' to 3')	Purpose/Comments
F-DC1GP5	ACAGGTACCCTAAGCATTAGGTAAGGC	Amplify gene *5*
R-DC1GP5	GTAGGTACCACCAGAGTTGATGCAGGC	Amplify gene *5*
F-DC1GP10-11	ATAGGTACCTTCGAATCTCACCGCCTCC	Amplify gene *10-11*
R-DC1GP10-11	ATAGGTACCTCGTGCGGCCGATTTAAGCG	Amplify gene *10-11*
F-DC1GP14-18	ATAGGTACCTCGACGAAATGGACGACG	Amplify gene *14-18*
R-DC1GP14-18	TAAGGTACCTCCAGCAGATGCTCGGACG	Amplify gene *14-18*
F-DC1GP54-55	ATCGGTACCTTCGACGTCGGTTCGCTG	Amplify gene *54-55*
R-DC1GP54-55	ATTGGTACCTGCTCACGG	Amplify gene *54-55*

	CGTTGTTGG	
F-DC1GP54-58	ATTGGTACCCTCAACACGC CGCGCACGC	Amplify gene *54-58*
R-DC1GP54-58	TCTGGTACCGGTTGGTCAT CATGGGTG	Amplify gene *54-58*
F-DC1GP54-55	ATCGGTACCTTCGACGTC GGTTCGCTG	Amplify gene *54-60*
R-DC1GP57-60	CTAGGTACCGTCAGCCTG TCAAGATGCC	Amplify gene *54-60*
F-DC1GP57-60	TTAGGTACCCGTCGCAATC CCGATCATCCGC	Amplify gene *57-60*
R-DC1GP57-60	CTAGGTACCGTCAGCCTG TCAAGATGCC	Amplify gene *57-60*
F-DC1GP57-60	TTAGGTACCCGTCGCAATC CCGATCATCCGC	Amplify gene *57*
R-DC1GP57	ATAGGATCCAGGTCGAGC CCTCCAGAC	Amplify gene *57*
F-DC1GP67	TCAGGTACCATAGAATCGA ACCACTGC	Amplify gene *67*
R-DC1GP67	GCTGGATCCCTACTGGTC GAGCTGCG	Amplify gene *67*
F-DC1GP68-69	AATGGTACCCAGTTTGTGC GGCGTAGAGC	Amplify gene *68-69*
R-DC1GP68-69	AGAGGTACCGAGGAAACG CTGTATCGC	Amplify gene *68-69*
F-DC1GP70-73	AGCGGTACCTTCTGATACC TAACCGTGG	Amplify gene *70-73*
R-DC1GP70-73	TATGGTACCTCGCGCACG TAGCTTTGC	Amplify gene *70-73*
F-BCPG_03685	TTTTTCTAGAGGCGTGTTG TCGGAAAGCCG	Amplify BCPG_03685
R-BCPG_03685	TTTTGGTACCTGTTTCCTC TTCTTTGAACAGGCG	Amplify BCPG_03685
F-BCPG_03683	TTTTGGTACCAGGCTTCAC CGGCAGCAACGC	Amplify BCPG_03683
R-BCPG_03683	GCCAGCAGTCGCCGCATC ACCG	Amplify BCPG_03683
F-Flank-BCPG_03684	GTGACGAGCCCTACGTAA CCGCTG	Confirm deletion of BCPG_03684
R-Flank-BCPG_03684	CTCCACGGTTGAAGTCAGT GCCTG	Confirm deletion of BCPG_03684

Construction of pGPI-SceI-ΔbceB and PC184ΔbceB

To knockout *bceB* (BCPG_03684) in PC184, the regions upstream (BCPG_03685) and downstream (BCPG_03683) of BCPG_03684 were cloned into pGPI-SceI-MB1. BCPG_036865 was PCR amplified using primer pair F-BCPG_03685/R-BCPG_03685 and digested with XbaI and KpnI. BCPG_03683 was PCR amplified using F-BCPG_03683/R-BCPG_03683 and digested with KpnI. These amplicons were then inserted sequentially into pGPI-SceI-MB1 to make pGPI-SceI-Δ*bceB.* This plasmid was transformed by electroporation into PC184. Tp^R isolates were transformed with pDAI-SceI-Cm and Cm^RTp^S isolates were PCR screened for deletion of *bceB* (Fig 28B).

Results and Discussion

Mutagenesis

To identify the DC1 protein responsible for halo production, phage DNA was randomly mutagenized and plaques were screened for loss of halo formation. The first method of random mutagenesis used was *in vitro* transposition followed by recombination (Fig 26). This method was unsuccessful as no antibiotic resistant colonies were obtained following transformation. As DC1 lysogeny is thought to be unstable, it is likely that the putative lysogens no longer contained DC1 DNA and therefore could not recombine with the mutagenized fragments (Lynch et al., 2012; Gill et al., 2011). The second method of random mutagenesis used was UV

exposure. The optimal UV dose for induction of mutations corresponds with 10-50% survival (Lawrence, 1991). When DC1 was exposed to UV light for 150 seconds, 50% of the phages remained viable (Fig 27). Over 200 of these phages were screened for loss of halo formation, but no mutants were identified.

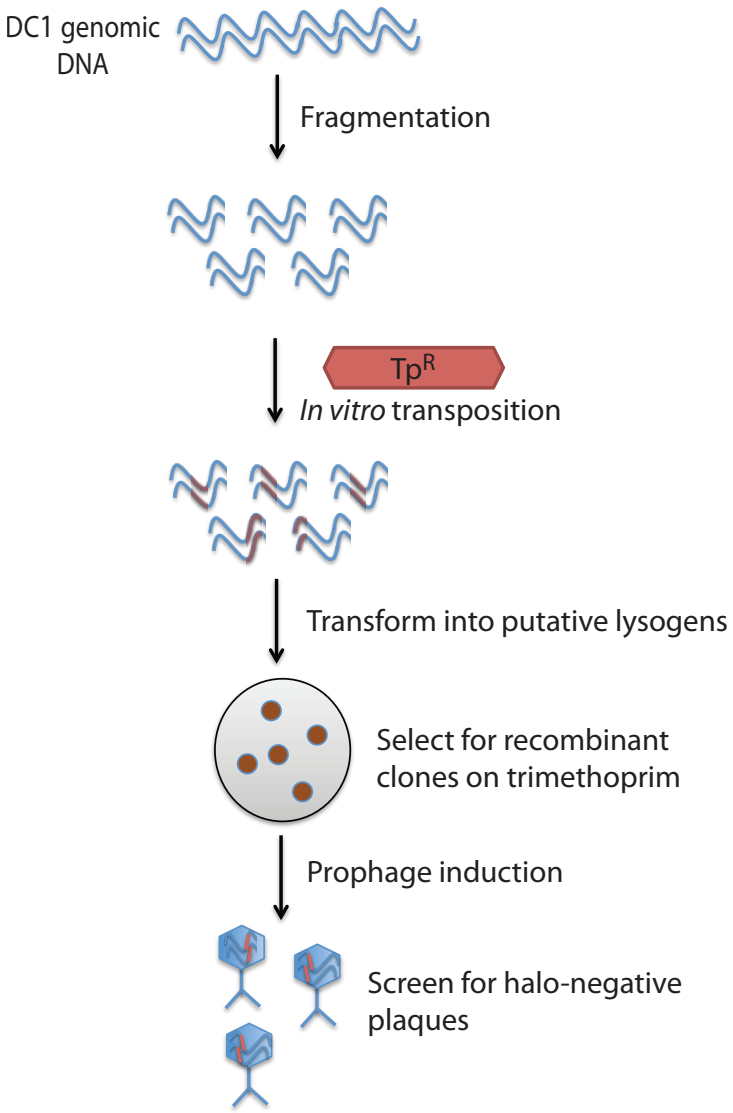


Figure 26: Strategy for halo-negative plaque isolation using *In vitro* mutagenesis of DC1 DNA.

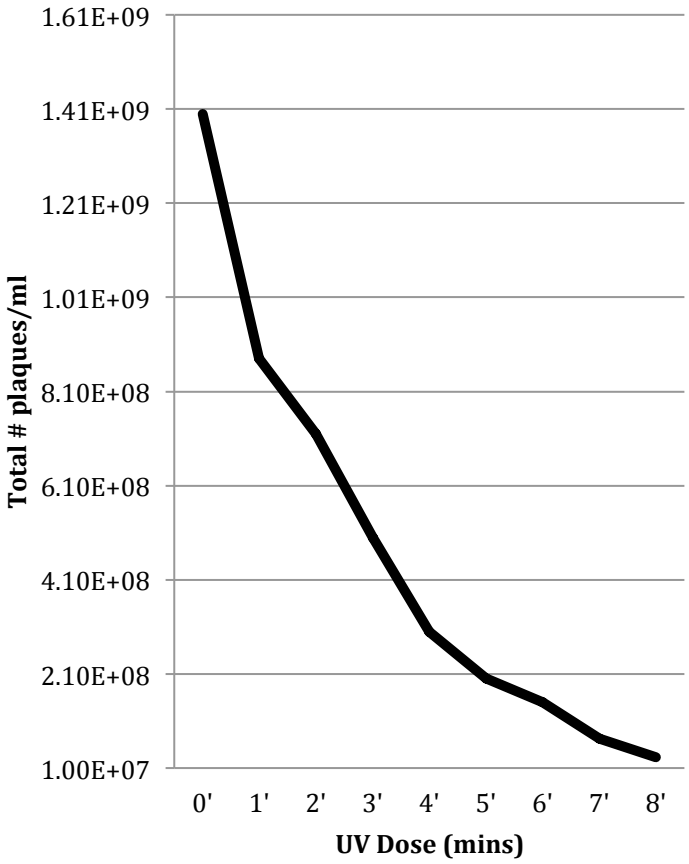


Figure 27: DC1 viability following exposure to UV light.

As random mutagenesis was unsuccessful in knocking out halo production, an alternative expression-based strategy was developed to assay for enzymatic activity against cepacian or LOS. Here, a selection of DC1 genes (out of 73 in total) were amplified and cloned into pBBR1Tp (Table 4; DeShazer and Woods 1996, Lynch et al. 2012). Each clone was then transformed into both *E. coli* DH5α and PC184. *E. coli* transformants were sonicated and spotted onto lawns of PC184, but no clearing (indicative of enzyme activity) was observed. PC184 transformants were screened for loss of mucoidy (suggestive of depolymerase activity) and/or

increased halo size following phage infection (due to an increase in enzyme expression), but neither phenotype was observed.

Table 4: DC1 genes cloned into pBBR1Tp

Gene(s)	Function	Size (Kbp)
5	Unknown	1.1
10-11	Unknown	1.4
14-18	Unknown	1.9
54-55	Unknown	1.2
54-58	Unknown, tail fibers	3.7
54-60	Unknown, tail fibers	5.6
57-60	Unknown, tail fibers	3.5
57	Unknown	1.1
67	Lytic transglycosylase	1.0
68-69	Unknown	0.7
70-73	Holin, lysine, Rz	1.5

Cepacian knockout

Cepacian (Δ*bceB*) and LOS (Δ*waaC*, discussed above) knockouts in PC184 were constructed to determine the target of the putative halo-producing enzyme. When plated on ½ YS, PC184 has a mucoid phenotype, while the cepacian knockout becomes non-mucoid (Fig 28C,D). DC1 plaques were surrounded by halos following infection of PC184Δ*bceB*, indicating that degradation of cepacian does not cause this phenomenon (Fig 29B). Similarly, DC1 infection of PC184Δ*waaC* produced smaller halos surrounding clear plaques (Figure 29C). Based on these experiments, degradation of neither cepacian nor LOS is responsible for halo formation on EPS-inducing media.

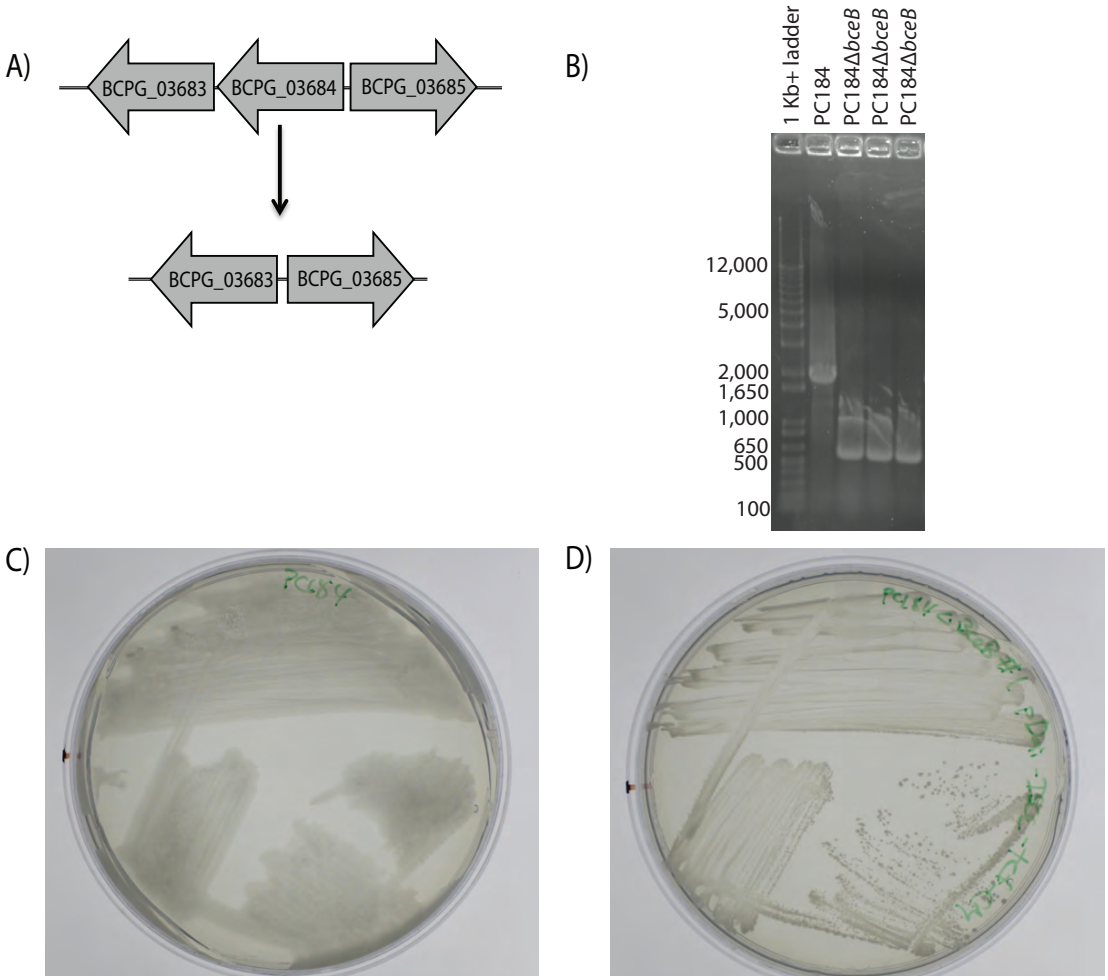


Figure 28: Construction of PC184Δ*bceB*. A) Schematic representation of DNA before (above) and after (below) deletion of *bceB* (BCPG_03684). B) PCR verification of PC184Δ*bceB* on a 0.8% agarose gel. PC184Δ*bceB* contains a ~550bp product while wildtype PC184 contains a ~2,000bp fragment C) PC184 grown on YEM. D) PC184Δ*bceB* grown on YEM.

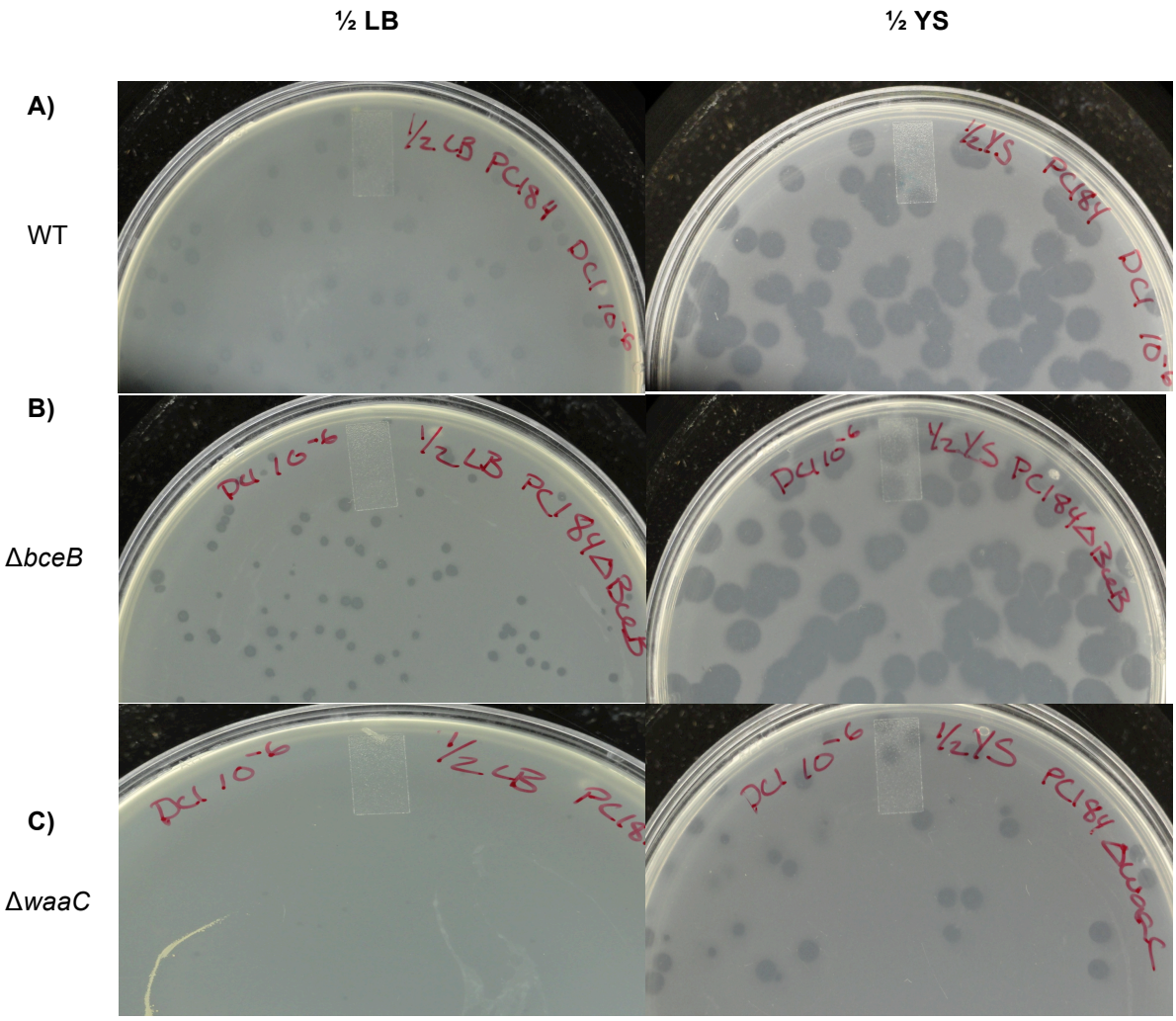


Figure 29: DC1 plaque morphology. A) PC184; B) PC184Δ*bceB*; C) PC184Δ*waaC*. DC1 was mixed with host cells, plated on ½ LB or ½ YS with 0.3% ½ LB top agar and incubated at 30°C.

Conclusion

Previous work suggested that DC1 expresses a polysaccharide or LOS-degrading enzyme that is responsible for its unique plaque phenotype on mannitol-rich medium (Routier, 2010). However, the results here show that halo formation continues in the absence of both cepacian and core LOS, thus excluding these moieties as enzymatic targets. Continued research is required to determine the cause of this phenotype. Since the EPS profile of PC184 has yet to be determined, the hypothesis of DC1 expressing a polysaccharide depolymerase is still valid. Further work could include determining the EPS profile of PC184 and their corresponding genes to construct PC184 mutants that could be screened for a halo-negative phenotype. Alternatively, DC1 can be screened against the PC184 plasposon library to identify halo-negative plaques. The latter would allow for an unbiased approach in determining the halo formation of DC1.

Literature cited

Bartholdson, S. J., Brown, A. R., Mewburn, B. R., Clarke, D. J., Fry, S. C., Campopiano, D. J., and Govan, J. R. W. (2008). Plant host and sugar alcohol induced exopolysaccharide biosynthesis in the *Burkholderia cepacia* complex. *Microbiology* 154, 2513–2521.

Cescutti, P., Impallomeni, G., Garozzo, D., Sturiale, L., Herasimenka, Y., Lagatolla, C., and Rizzo, R. (2003). Exopolysaccharides produced by a clinical strain of *Burkholderia cepacia* isolated from a cystic fibrosis patient. *Carbohydr. Res.* 338, 2687–2695.

Cornelissen, A. (2011). PhD. Thesis University of Leuven, Leuven, Belgium. Characterization of *Pseudomonas*-infecting bacteriophages: the search for virion-associated biofilm-degrading enzymes.

Gill, J. J., Summer, E. J., Russell, W. K., Cologna, S. M., Carlile, T. M., Fuller, A. C., Kitsopoulos, K., Mebane, L. M., Parkinson, B. N., Sullivan, D., et al. (2011). Genomes and Characterization of Phages Bcep22 and BcepIL02, Founders of a Novel Phage Type in *Burkholderia cenocepacia*. *J. of Bacteriol.* 193, 5300–5313.

Hughes, K. A., Sutherland, I. W., and Jones, M. V. (2008). Biofilm susceptibility to bacteriophage attack: the role of phage-borne polysaccharide depolymerase. *Microbiology* 144, 3039–3047.

Hughes, K. A., Sutherland, I. W., Clark, J., and Jones, M. V. (1998). Bacteriophage and associated polysaccharide depolymerases--novel tools for study of bacterial biofilms. *J. Appl. Microbiol.* 85, 583–590.

Lawrence, C. 1991. Classical mutagenesis techniques. *Methods Enzymol.* 194, 273–281.

Lynch, K. H., Stothard, P., and Dennis, J. J. (2012). Characterization of DC1, a Broad-Host-Range Bcep22-Like Podovirus. *Appl. Environ. Microbiol.* 78, 889–891.

Mahenthiralingam, E., Coenye, T., Chung, J. W., Speert, D. P., Govan, J. R., Taylor, P., and Vandamme, P. (2000). Diagnostically and experimentally useful panel of strains from the *Burkholderia cepacia* complex. *J. Clin. Microbiol.* 38, 910–913.

Moreira, L. M., Videira, P. A., Sousa, S. A., Leitão, J. H., Cunha, M. V., and Sá-Correia, I. (2003). Identification and physical organization of the gene cluster involved in the biosynthesis of *Burkholderia cepacia* complex exopolysaccharide. *Biochem. Biophys. Res. Commun.* 312, 323–333.

Pleteneva, E. A., Burkal'tseva, M. V., Shaburova, O. V., Krylov, S. V., Pechnikova, E. V., Sokolova, O. S., and Krylov, V. N. (2011). TL, the new bacteriophage of *Pseudomonas aeruginosa* and its application for the search of halo-producing bacteriophages. *Genetika* 47, 5–9.

Routier, S. (2010). MSc. Thesis University of Alberta, Edmonton, AB. DC1, a Podoviridae with a putative cepacian depolymerase enzyme.

Sutherland, I. W. (1999). Polysaccharases for microbial exopolysaccharides. *Carbohydr. Polymers* 38, 319–328.

Wilson, K. (2001). Preparation of genomic DNA from bacteria. *Curr Protoc Mol Biol* Chapter 2, Unit 2.4.

Appendix II

Functional characterization of Bcc bacteriophage JG068

Portions of this chapter have been submitted as:

Lynch, K.H., A.H. Abdu, M. Schobert, and J.J. Dennis. Submitted

Genomic characterization of JG068, a novel lytic podovirus active against *Burkholderia cenocepacia.* BMC Genomics manuscript 1304414502972137

Introduction

Phage therapy has resurfaced in recent years as a result of the increasing prevalence of antibiotic resistant bacteria. Detailed genomic and molecular characterization of bacteriophages must be performed before the introduction of phage therapy into clinical settings**.** One of the most important aspects of phage therapy development is the selection of appropriate therapeutic phage candidates. To be clinically applicable, a phage should have a broad host range (that includes clinical isolates) and a sequenced and characterized genome that lacks genes encoding putative pathogenicity factors and lysogeny-related proteins. A novel Bcc bacteriophage JG068 was acquired and sequenced in-house using the Ion Torrent platform. The objectives of this project was to sequence and functionally characterize the novel Bcc-specific bacteriophage JG068.

Materials and Methods

Bacterial strains and culture conditions

Bcc strains were grown aerobically at 30°C overnight on half-strength Luria-Bertani (½ LB) solid agar or agarose plates or in ½ LB broth. Strains used for host range testing and phage propagation are members of the original and updated Bcc experimental strain panels (Mahenthiralingam et al., 2000; Coenye et al., 2003). K56-2 LPS mutants (Ortega et al., 2009; Loutet et al., 2006) were grown at 30°C on ½ LB plates or in ½ LB broth containing 100 mg/l trimethoprim. *E. coli* DH5α

transformed with pUC19 or pET22b was grown at 37°C on LB plates containing 100 mg/l ampicillin. *E. coli* BL21(DE3)pLysS was grown at 37°C in LB broth containing 25 mg/l chloramphenicol or, if transformed with pET22b, 25 mg/l chloramphenicol and 100 mg/l ampicillin.

Phage propagation, host range analysis, lysogeny screen, and electron microscopy

JG068 was isolated from a sewage plant in Steinhof near Braunschweig in Germany following a protocol previously described (Garbe et al., 2011). For propagation of JG068, 100 μl high-titre phage stock and 100 μl Bcc liquid culture were combined and incubated 20 min at room temperature, mixed with 3 ml 0.7% ½ LB agar or 0.6% ½ LB agarose, overlaid on a ½ LB plate, and incubated at 30°C overnight. High-titre stocks were made in modified suspension medium (modified SM; 50 mM Tris-HCl [pH 7.5], 100 mM NaCl, 10 mM $MgSO_4$). Phage plates were overlaid with 3 ml modified SM and incubated >1 h at room temperature on a platform rocker. The supernatant was recovered, pelleted by centrifugation for 2 min at 10,000 rcf, filter-sterilized using a Millex-HA 0.45 μm syringe-driven filter unit (Millipore, Billerica, MA), and stored at 4°C.

Spot testing was used for preliminary host range analysis. Soft-agar overlays containing 100 μl Bcc liquid culture were allowed to solidify for 10 min at room temperature, spotted with 10 μl drops of high-titer JG068 stock, and assayed for clearing and/or plaque formation after incubation at

30°C overnight. Strains that showed phage susceptibility in this assay were re-tested in soft-agar overlays containing both the strain and JG068. The host ranges for LPS mutants of K56-2 (Ortega et al., 2009; Loutet et al., 2006) and PC184 were tested similarly.

To assay for JG068-lysogenized K56-2, JG068 and K56-2 were plated in agar overlays at a multiplicity of infection (MOI) of 1 and incubated at 30°C overnight. To recover surviving cells, plates were overlaid with 3 ml sterile water and incubated 1 h at room temperature on a platform rocker. Cells were pelleted by centrifugation for 2 min at 10,000 rcf, washed and resuspended in sterile water, and plated to obtain isolated colonies. One hundred colonies were struck out onto ½ LB plates and spotted with separate 10 μl drops of high-titer JG068 stock and high-titer KS12 stock. Isolates were scored as sensitive or insensitive compared to a sensitive wildtype K56-2 control that was lysed by both phage stocks.

For electron microscopy, phage stocks were prepared as described above with the following modifications: agarose plates and soft agarose were used for overlays, sterile water was used in place of modified SM, and a 0.22 μm filter was used for syringe-driven filtration. A carbon-coated copper grid was incubated with lysate for 5 min and stained with 2% phosphotungstic acid for 30 s. Transmission electron micrographs were captured using a Philips/FEI (Morgagni) transmission electron microscope with charge-coupled device camera (University of Alberta Department of Biological Sciences Advanced Microscopy Facility).

Phage DNA isolation, RFLP analysis, and sequencing

Phage DNA was isolated using a modified version of a Wizard DNA Clean-Up protocol (Promega, 2006). Ten milliliters of filter-sterilized JG068 lysate (propagated using K56-2 on agarose medium) was treated with 10 μl DNase I (Thermo Scientific, Waltham, MA), 100 μl 100x DNase I buffer (1 M Tris-HCl, 0.25 M $MgCl_2$, 10 mM $CaCl_2$), and 6 μl RNase (Thermo Scientific) and incubated 1 h at 37°C to degrade the bacterial nucleic acids. Four hundred microliters of 0.5 M EDTA and 25 μl of 20 mg/ml proteinase K (Applied Biosystems, Carlsbad, CA) were added and incubated 1 h at 55°C to inactivate DNase I. After cooling to room temperature, the lysate was added to 8.4 g of guanidine thiocyanate and briefly mixed. One milliliter of warmed, resuspended Wizard DNA Clean-Up Resin (Promega Corporation, Madison, WI) was added to the mixture and mixed by inverting for 15 min to allow for release of the phage DNA and binding to the resin. The mixture was pelleted by centrifugation at room temperature for 10 min at 5,000 rcf and the supernatant was drawn off until ~5 ml remained. This mixture was resuspended by swirling, transferred into an empty syringe barrel attached to a Wizard Minicolumn (Promega Corporation), and pushed into the column. The column was then washed with 2 ml 80% isopropanol and dried by centrifugation for 2 min at 10,000 rcf. JG068 DNA was eluted from the column following addition of 100 μl of 80°C nuclease-free water (Integrated DNA

Technologies, Coralville, IA), incubation for 1 min, and centrifugation for ≥20 s at 10,000 rcf.

Phage and plasmid DNA were quantified using a NanoDrop 2000c spectrophotometer (Thermo Scientific). For restriction fragment length polymorphism (RFLP) analysis, 1 μg JG068 DNA was digested with EcoRI (Thermo Scientific), separated on a 0.8% agarose gel, stained with GelGreen (Phenix Research Products, Candler, NC), and visualized using a ChemiDoc MP Imaging System (Bio-Rad, Hercules, CA). For shotgun cloning, EcoRI fragments were purified using a GENECLEAN III kit (MP Biomedicals, Santa Ana, CA), ligated into pUC19, and transformed into *E. coli* DH5α (Invitrogen, Carlsbad, CA). Plasmids were purified using the GeneJET Plasmid Miniprep kit (Thermo Scientific) and sequenced using a 3730 DNA Analyzer (Applied Biosystems) by the University of Alberta Department of Biological Sciences Molecular Biology Service Unit (MBSU). Sequences were edited and assembled using Geneious.

The complete JG068 genome sequence was determined with the assistance of the MBSU using an Ion Torrent PGM (Applied Biosystems) and assembled using SeqMan NGen (DNASTAR, Madison, WI). Ambiguous regions in the assembly were resequenced using Sanger sequencing from EcoRI clones.

SAR endolysin expression

To assess the activity of the gp47 SAR endolysin, gene *47*

including the signal sequence (bp 40,232 – 40,753; NdeI-SS-F: ATAATAACATATGCACCCCATCGTCAAGCGAG; HindIII-R: AAAAAGCTTCTATCGCCGGATACCAGCAACG) or excluding the signal sequence (bp 40,307 – 40,753; NdeI-ΔSS-F: TAATAACATATGGACGAGGGTATCCGGAACGTC; HindIII-R: as above) was PCR amplified using KAPA HiFi HotStart ReadyMix (Kapa Biosystems, Woburn, MA) and ligated in-frame into pET22b. These constructs were separately transformed into *E. coli* DH5α, sequenced to verify that the inserts were correct, and transformed into *E. coli* BL21(DE3)pLysS using a standard protocol (Silhavy et al.,1984). A pET22b blank control plasmid was isolated from *E. coli* MC4100 and transformed similarly into BL21(DE3)pLysS. To assay endolysin activity in *E. coli*, 5 ml liquid cultures of the three strains (in triplicate) were each subcultured into six wells of a 96 well plate by adding 10 μl culture to 190 μl LB broth containing 25 mg/l chloramphenicol and 100 mg/l ampicillin. The plate was then covered with plastic wrap and incubated with shaking at 37°C. Optical density measurements at 600 nm (OD_{600}) were measured at 1 h intervals with a Wallac 1420 VICTOR^2 multilabel counter (PerkinElmer, Waltham, MA). At 3 h (OD_{600} ~ 0.6), half of the samples were induced with IPTG (1 mM final concentration) and optical density measurements continued at 1 h intervals up to 8 h. Averages and standard deviations were calculated using Microsoft Excel.

Galleria mellonella assays

G. mellonella infection and treatment assays were performed as described previously (Seed and Dennis, 2009) with modifications. One milliliter of a 16 h K56-2 overnight culture was pelleted by centrifugation for 2 min at 10,000 rcf, resuspended in 1 ml $MgSO_4$-ampicillin solution (10 mM $MgSO_4$, 1.2 mg/ml ampicillin), and diluted in this solution to $1:10^4$. High-titre JG068 lysate was passaged through a Detoxi-Gel Endotoxin Removing Column (Thermo Scientific) and supplemented with ampicillin. Larvae (RECORP Inc., Georgetown, ON) were stored at 4°C and warmed to room temperature prior to injection with a 250 μl syringe with repeating dispenser (Hamilton Company, Reno, NV). In the left hindmost proleg, 5 μl of the diluted K56-2 suspension (3.7×10^3 CFU/5 μl) was injected into ten infected/treated larvae and ten infected/untreated larvae and 5 μl of $MgSO_4$-ampicillin solution was injected into ten uninfected/treated larvae. In the adjacent left proleg, 5 μl of the JG068 lysate (1.3×10^6 PFU/5 μl) was injected into ten infected/treated larvae and ten uninfected/treated larvae and 5 μl of $MgSO_4$-ampicillin solution was injected into ten infected/untreated larvae. Larvae were placed into petri dishes, incubated aerobically at 30°C for 72 h, and scored for survival. Experiments were repeated in triplicate. Averages and standard deviations were calculated using Microsoft Excel.

Results and Discussion

Isolation, host range, and morphology

JG068 was isolated from a sewage processing plant using an uncharacterized strain of *Burkholderia dolosa*. Additional JG068 hosts were identified using spot tests and soft-agar overlays of Bcc strains. A total of 30 strains (Table 5) were tested and seven were found to support lytic propagation of JG068: *Burkholderia multivorans* ATCC 17616, *Burkholderia cenocepacia* K56-2, J2315, and PC184, *Burkholderia stabilis* LMG 14294, and *Burkholderia dolosa* AU0158 and CEP021. Excluding the soil isolate ATCC 17616, each of these strains was identified as a CF clinical isolate (Mahenthiralingam et al., 2000; Coenye et al., 2003). Furthermore, the three susceptible *B. cenocepacia* strains and AU0158 have all been linked to epidemic spread among CF patients (Mahenthiralingam et al., 2000). This host range is relatively broad, clinically relevant, and dissimilar to that of any of the Bcc phages that we have previously characterized (Seed and Dennis, 2005; Goudie et al., 2008; Lynch et al., 2010a, 2010b, 2012a, 2012b). On K56-2, JG068 forms large clear plaques, 1-3 mm in diameter, suggesting that this phage may be obligately lytic (discussed below).

Table 5: Host range of Bcc-specific phage JG068

Species	Strain	Lysis
B. cepacia	ATCC 17759	-
	LMG 18821	-
B. multivorans	ATCC 17616	+
	C3430	-
	C5393	-
	C5274	-
B. cenocepacia	K56-2	+
	J2315	+
	PC184	+
	C5424	-
	C6433	-
	715J	-
	CEP511	-
	C1257	-
	C4455	-
B. stabilis	LMG 14294	+
	LMG 18870	-
B.vietnamiensis	DB01	-
	LMG 10929	-
	LMG 18835	-
B. dolosa	AU0158	+
	CEP021	+
	LMG 21443	-
	LMG 18943	-
	LMG 21820	-
B. ambifaria	LMG 17828	-
	LMG 19467	-
	LMG 19182	-
B. anthina	LMG 16670	-
B. pyrrocinia	LMG 14191	-

+: plaque formation

Several Bcc phages – including KS4, KS5, KS9, KS10, and KS12 – have been previously suggested to use LPS as a receptor (Lynch et al., 2010a; Juárez Lara, unpublished data). To assess if JG068 uses a similar receptor, a panel of both K56-2 (Loutet et al., 2006; Ortega et al., 2009) and PC184 LPS mutants were tested with the phage in spot tests and soft-agar overlays. In K56-2, JG068 efficiently infected the wildtype and *wbxE* and *waaL* mutants, inefficiently infected a *wabR* mutant, and did not infect *wabS*, *wabO*, or *waaC* mutants (Table 6). Similarly, in PC184, JG068 efficiently infected the wildtype, inefficiently infected a *wabP* mutant, and did not infect *wabO* or *waaC* mutants (Table 6). As JG068 is able to efficiently infect mutants with full LPS cores (even in the absence of O-antigen), but showed minimal to no lysis of mutants with truncated LPS cores, we predict that the tail of this phage interacts with components in the LPS core of its hosts. As noted in a previous Bcc phage study, further experiments are required to validate this prediction as LPS truncation may also result in secondary changes to the cell surface structure of the mutants (Lynch et al., 2010a; Loutet et al., 2006).

Table 6: JG068 LPS mutant host range

B. cenocepacia strain	Mutant strain	Mutation	Reference	Phenotype	Lysis
K56-2	wildtype	none	Mahenthiralingam et al. (2000)	wildtype	+
	RSF19	*wbxE*	Loutet et al. (2006)	truncated O-antigen	+
	XOA7	*waaL*	Ortega et al. (2009)	no O-antigen	+
	XOA15	*wabR*	Ortega et al. (2009)	truncated outer core	+/-
	XOA17	*wabS*	Ortega et al. (2009)	truncated outer core	-
	XOA8	*wabO*	Ortega et al. (2009)	truncated inner core	-
	CCB1	*waaC*	Ortega et al. (2009)	truncated inner core	-
PC184	wildtype	none	Mahenthiralingam et al. (2000)	wildtype	+
	Δ*wabP*	*wabP*	Abdu	truncated inner core	+/-
	Δ*wabO*	*wabO*	Abdu	truncated inner core	-
	Δ*waaC*	*waaC*	Abdu	truncated inner core	-

+: plaque formation at similar levels compared to wildtype; +/-: plaque formation at reduced levels compared to wildtype; -: no plaque formation.

Electron microscopy of JG068 virions (Fig 30) shows that this phage has a short tail and belongs to the family *Podoviridae* (Ackermann, 2001). This morphology is relatively unique for a Bcc phage as the only *Burkholderia* podoviruses identified to date are the BPP-1-like BcepC6B and the Bcep22-like DC1, Bcep22, BcepIL02, and BcepMigl (Lynch et al., in press). The JG068 capsid is icosahedral and approximately 60 nm in diameter (Fig 30).

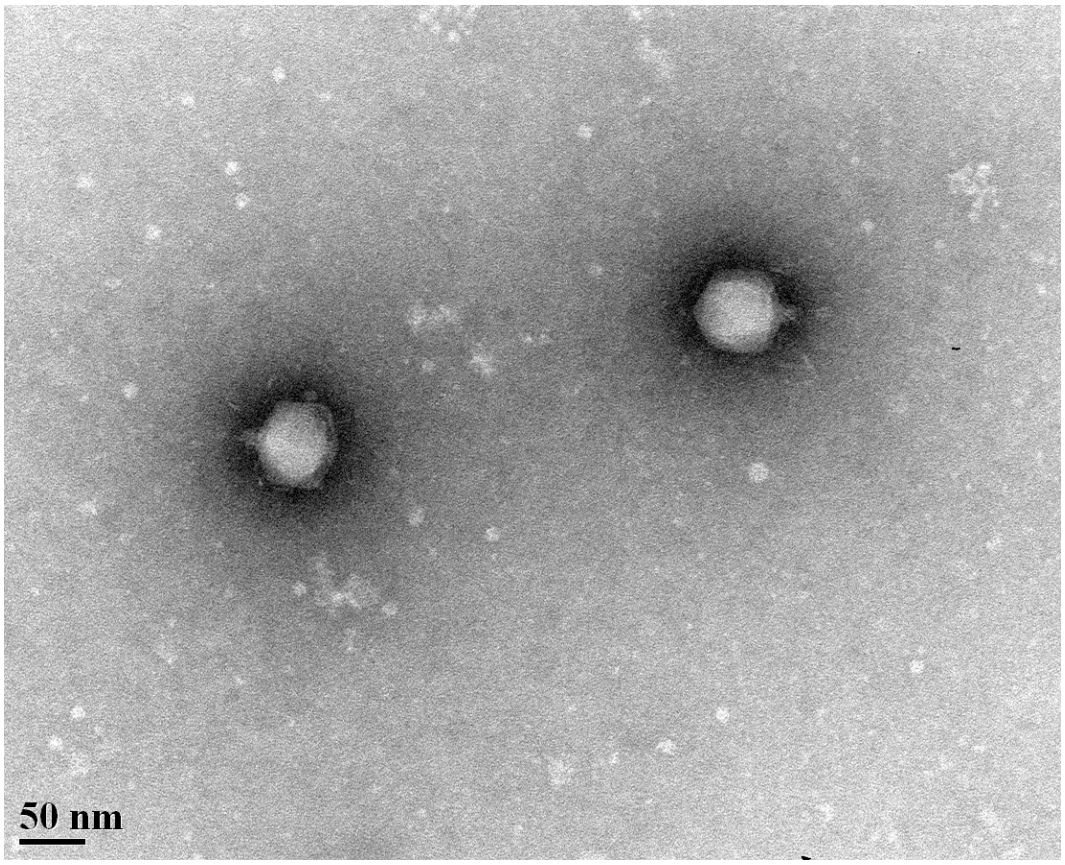


Figure 30: Transmission electron micrograph of phosphotungstic acid-stained JG068 virions at 140,000-fold magnification. Scale bar represents 50 nm.

Genome sequencing and assembly

To initially determine whether JG068 was similar to previously characterized Bcc phages, we performed restriction fragment length polymorphism (RFLP) analysis using the restriction enzyme EcoRI. The RFLP profile of JG068 appeared to be unique, with fragments ranging from 4–8 kilobase pairs (kbp) in length (data not shown). In order to determine if the genome sequence of JG068 was novel, several of these fragments were cloned into pUC19 and analyzed using Sanger

sequencing. Resulting reads were analyzed using BLASTN and found to be novel but similar to a variety of sequences, including those from previously characterized podoviruses. The genome sequence was completed using ion semiconductor technology on the Ion Torrent platform.

Genome annotation

Annotation and bioinformatics analyses for the JG068 genome were performed by K. H. Lynch (data not shown). One of the advantages of using Bcc-specific phages therapeutically is that they do not in general appear to encode virulence factors (Summer et al., 2007). Based on this generalization, we predicted that the same would be true for JG068. Using BTXpred analysis combined with BLASTP and HHpred comparisons (data not shown), no significant similarity to putative virulence proteins was detected, suggesting that JG068 is likely to be a safe candidate for clinical testing.

A cluster of genes commonly identified in other Bcc phages that is notably absent from the JG068 genome is that for lysogeny. JG068 is thought to be a lytic phage as it forms clear plaques and does not encode either an integrase or a repressor. In order to verify experimentally that JG068 was not capable of lysogenizing Bcc strains, we isolated JG068-insensitive K56-2 from a JG068/K56-2 lysate. To determine if these cells were insensitive due to either receptor mutation or superinfection

immunity, we attempted to lyse these isolates using a second putatively lytic Bcc-specific phage, KS12. Previous assays have shown that KS12 uses LPS as a receptor (Juárez Lara, unpublished data). If JG068-insensitive K56-2 isolates are also insensitive to KS12 infection, they are likely LPS mutants, while if they are sensitive to KS12 infection, they are likely JG068 lysogens. One hundred JG068-insensitive isolates were screened and all were found to be insensitive to both phages compared to wildtype K56-2, strongly suggesting that resistance arises due to receptor mutation and not due to JG068 lysogeny. LPS extractions on these JG068-insensitive isolates can be performed to confirm our hypothesis.

SAR endolysin

The lysis proteins of JG068, encoded on the far right end of the genome, include a putative pinholin, signal-arrest-release (SAR) endolysin, Rz, and Rz1. The SAR endolysin of JG068 (gp47) has a very similar organization to that of the closely related phage ϕKMV. SignalP 3.0 predicts an N-terminal signal sequence probability of 0.97 for gp47. The gp47 N-terminus contains two signal sequence regions followed by three predicted catalytic residues of the lysozyme domain (Fig 31). Residues 1-7, including a positively-charged histidine, lysine, and arginine, make up the N-region. Residues 8-25 make up the hydrophobic glycine- and alanine-rich H-region, also predicted to be a transmembrane domain by OCTOPUS analysis. Using CD Search and BLASTP alignment with the

ϕKMV SAR endolysin, the putative lysozyme catalytic residues were identified as 27E, 36D, and 45T, all found immediately downstream of the H-region. As in ϕKMV, because the C-region is absent, the protein will not be cleaved by a signal peptidase upon secretion and will instead remain associated with the inner membrane until release into the periplasm (Briers et al., 2011).

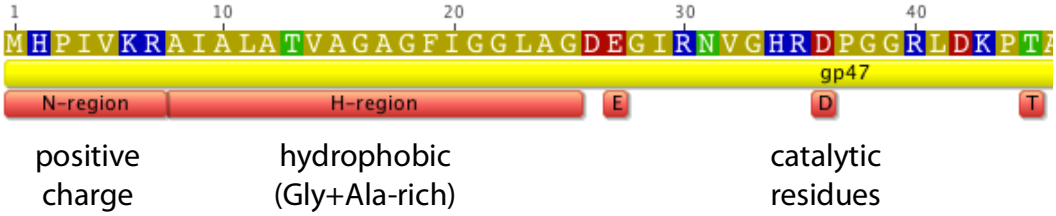


Figure 31: Schematic representation of the N-terminal signal sequence of JG068 gp47.

Using inducible expression of the ϕKMV SAR endolysin in *E. coli*, it was shown that this protein could decrease culture absorbance in the absence of a pinholin, but only if the N-terminal signal sequence of the endolysin was present (Briers et al., 2011). We performed a similar experiment using the JG068 SAR endolysin to further characterize the export mechanism and biological activity of this protein. To allow for tightly controlled inducible expression, we cloned gene *47* into pET22b (with [gp47] or without [gp47ΔSS] the putative signal sequence in the first 25 residues) and transformed these plasmids into *E. coli* BL21(DE3)pLysS. When these cells (or a pET22b blank control) are subcultured, their growth rates prior to IPTG induction are similar based on optical density

measurements at 600 nm (OD_{600}; up to 3 h in Fig 32). However, following induction, expression of gp47 is lethal to the cells as the OD_{600} decreases from ~0.6 at 3 hours to ~0.45 at 8 hours (black squares, Fig 32). A very different trend is observed for the blank control, gp47ΔSS, and uninduced gp47 strains, where the OD_{600} increases from ~0.6 at 3 hours to ~0.9 at 8 hours, double that of the induced gp47 OD_{600} (Fig 32). As the lytic activity is dependent upon expression of not only the lysozyme domain but also the signal sequence, we can conclude that JG068 gp47 acts as a typical SAR endolysin in Gram-negative bacteria.

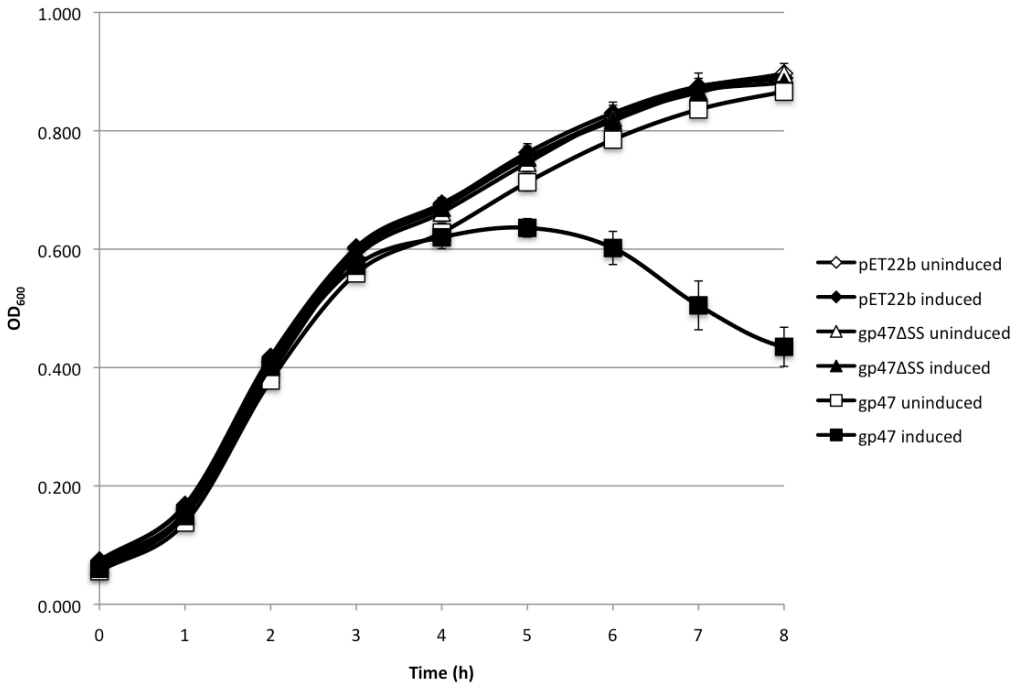


Figure 32: Signal sequence-dependent lytic activity of the gp47 SAR endolysin expressed in *E. coli* BL21(DE3)pLysS. Cells carrying pET22b or expressing either gp47 or gp47 lacking the putative signal sequence (gp47ΔSS) were subcultured and incubated with shaking at 37°C. Cells were induced with 1 mM IPTG (final concentration) at 3 h (OD_{600} ~ 0.6).

In vivo activity

The *Galleria mellonella* (greater wax moth) larvae model is commonly used to assess both strain virulence and phage therapy efficacy for members of the Bcc, particularly *B. cenocepacia* (Seed and Dennis, 2008, 2009; Lynch et al., 2010a). Although this model is less complex than a mammalian system, it shows positive correlation with both mouse and rat models of infection (Seed and Dennis, 2008). To determine if JG068 possesses lytic activity against *B. cenocepacia in vivo*, we infected *G. mellonella* larvae with 3.7×10^3 colony forming units (CFU) of K56-2 and treated with endotoxin-removed JG068 at an MOI of 350 (1.3×10^6 plaque forming units [PFU]). For infected, untreated controls, no larvae survived after 72 hours (Fig 33 and 34, left). Those larvae that received only JG068 remained healthy over the course of the experiment (Fig 33 and 34, centre), suggesting that phage treatment produced no harmful effects. For infected, treated larvae, JG068 administration significantly increased survival to an average of 77% (from 0% in untreated controls) (Fig 33 and 34, right). In a previous study, the putatively lytic phage KS12 was the most effective for immediate treatment of K56-2 infection in the *G. mellonella* model, resulting in 93±12% survival (MOI=5000) or 57±6% survival (MOI=500) (Seed and Dennis, 2009). Based on the *G. mellonella* data presented in Figure 7, JG068 is almost as effective as a significantly larger dose of KS12, suggesting that JG068 is highly active *in vivo*.

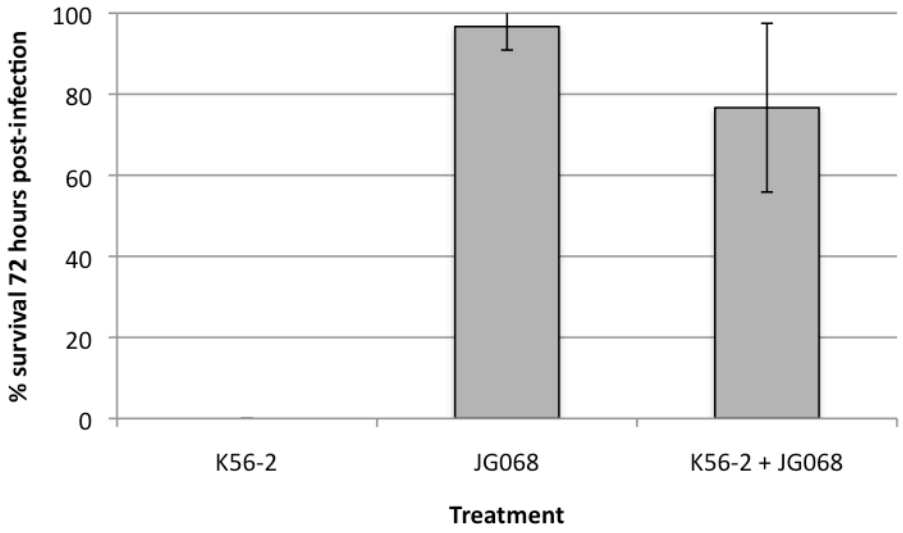


Figure 33: *In vivo* activity of JG068 against *B. cenocepacia* K56-2. *G. mellonella* larvae were infected with K56-2 and treated with endotoxin-removed JG068 at a multiplicity of infection (MOI) of 350. Survival was assessed 72 hours post-infection.

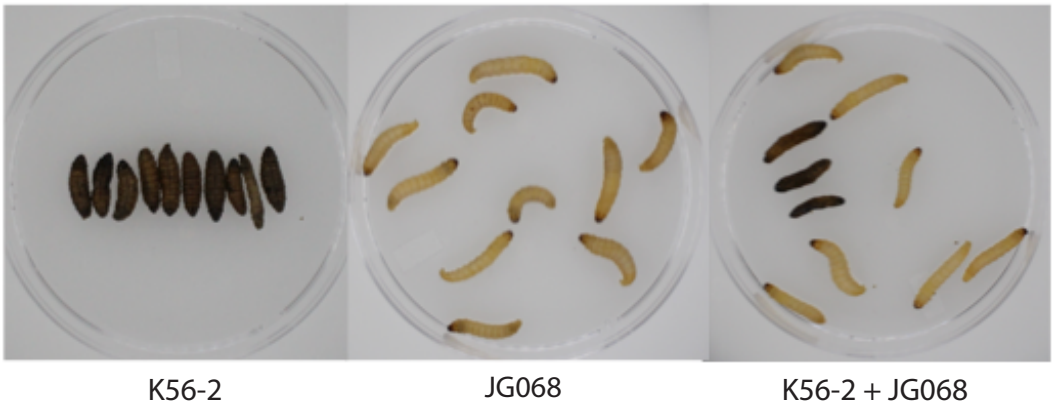


Figure 34: Visual representation of JG068 activity against *B. cenocepacia* K56-2 in the *G. mellonella* infection model. Larvae were infected with K56-2 and treated with endotoxin-removed JG068 at a multiplicity of infection (MOI) of 350. Images were taken 72 hours post-infection.

Conclusions

One of the greatest challenges in Bcc phage therapy development is the identification of phages with a broad, clinically relevant host range that are free of virulence genes, obligately lytic, and active *in vivo*. Our characterization of the podovirus JG068 suggests that it is a rare example of a Bcc phage that satisfies each of these requirements. JG068 interacts with LPS as a receptor to infect strains of *B. multivorans*, *B. cenocepacia*, *B. stabilis*, and *B. dolosa*. JG068 lacks virulence genes, is obligately lytic, and encodes a functional SAR endolysin, the first to be experimentally verified for the Bcc. Administration of JG068 to *B. cenocepacia* K56-2-infected *G. mellonella* larvae significantly decreases larval mortality, providing the first evidence that sequenced, obligately lytic Bcc phages are active against *B. cenocepacia in vivo*. Given these characteristics, further study is warranted regarding the development of JG068 into an active antimicrobial for use in CF patients.

Literature cited

Ackermann, H. W. H. (2001). Frequency of morphological phage descriptions in the year 2000. Brief review. *Arch. Virol.* 146, 843–857.

Briers, Y., Peeters, L. M., Volckaert, G., and Lavigne, R. (2011). The lysis cassette of bacteriophage ϕKMV encodes a signal-arrest-release endolysin and a pinholin. *Bacteriophage* 1, 25–30.

Coenye, T. T., Vandamme, P. P., LiPuma, J. J. J., Govan, J. R. W. J., and Mahenthiralingam, E. E. (2003). Updated version of the *Burkholderia cepacia* complex experimental strain panel. *J. Clin. Microbiol.* 41, 2797–2798.

Garbe, J., Bunk, B., Rohde, M., and Schobert, M. (2011). Sequencing and Characterization of *Pseudomonas aeruginosa* phage JG004. *BMC Microbiology* 11, 102.

Goudie, A. D., Lynch, K. H., Seed, K. D., Stothard, P., Shrivastava, S., Wishart, D. S., and Dennis, J. J. (2008). Genomic sequence and activity of KS10, a transposable phage of the *Burkholderia cepacia* complex. *BMC Genomics* 9, 615.

Loutet, S. A., Flannagan, R. S., Kooi, C., Sokol, P. A., and Valvano, M. A. (2006). A complete lipopolysaccharide inner core oligosaccharide is required for resistance of *Burkholderia cenocepacia* to antimicrobial peptides and bacterial survival *in vivo*. *J. Bacteriol.* 188, 2073–2080.

Lynch K.H., Dennis, J.J. (In press). Genomics of *Burkholderia* phages. In *Burkholderia:* From Genomes to Function. Edited by Coenye T., Mahenthiralingam E. Hethersett: Horizon Scientific Press.

Lynch, K. H., Seed, K. D., Stothard, P., and Dennis, J. J. (2010a). Inactivation of *Burkholderia cepacia* Complex Phage KS9 gp41 Identifies the Phage Repressor and Generates Lytic Virions. *J. Virol.* 84, 1276–1288.

Lynch, K. H., Stothard, P., and Dennis, J. J. (2012a). Characterization of DC1, a Broad-Host-Range Bcep22-Like Podovirus. *Appl. Environ. Microbiol.* 78, 889–891.

Lynch, K. H., Stothard, P., and Dennis, J. J. (2012b). Comparative analysis of two phenotypically-similar but genomically-distinct *Burkholderia cenocepacia*-specific bacteriophages. *BMC Genomics* 13, 223.

Lynch, K. H., Stothard, P., and Dennis, J. J. (2010b). Genomic analysis

and relatedness of P2-like phages of the *Burkholderia cepacia* complex. *BMC Genomics* 11, 599.

Mahenthiralingam, E., Coenye, T., Chung, J. W., Speert, D. P., Govan, J. R., Taylor, P., and Vandamme, P. (2000). Diagnostically and experimentally useful panel of strains from the *Burkholderia cepacia* complex. *J. Clin. Microbiol.* 38, 910–913.

Ortega, X., Silipo, A., Saldias, M. S., Bates, C. C., Molinaro, A., and Valvano, M. A. (2009). Biosynthesis and structure of the *Burkholderia cenocepacia* K56-2 lipopolysaccharide core oligosaccharide: truncation of the core oligosaccharide leads to increased binding and sensitivity to polymyxin B. *J. Biol. Chem.* 284, 21738–21751.

Promega Corporation. (2006). DNA Isolation From Lambda Lysates Using the Wizard® DNA Clean-Up System. [cited: 2013, June, 11]. Available from: http://www.promega.ca/resources/articles/pubhub/enotes/dna-isolation-from-lambda-lysates-using-the-wizard-dna-cleanup-system

Seed, K. D. K., and Dennis, J. J. J. (2005). Isolation and characterization of bacteriophages of the *Burkholderia cepacia* complex. *FEMS Microbiol. Letters* 251, 8–8.

Seed, K. D., and Dennis, J. J. (2008). Development of *Galleria mellonella* as an Alternative Infection Model for the *Burkholderia cepacia* Complex. *Infect. Immun.* 76, 1267–1275.

Seed, K. D., and Dennis, J. J. (2009). Experimental Bacteriophage Therapy Increases Survival of *Galleria mellonella Larvae* Infected with Clinically Relevant Strains of the *Burkholderia cepacia* Complex. *Antimicrob. Agents Chemother.* 53, 2205–2208.

Silhavy, T. J., Berman, M. L., Enquist, L. W. (1984) *Experiments with Gene Fusions.* Cold Spring Harbor: Cold Spring Harbor Laboratory.

Summer, E. J. E., Gill, J. J. J., Upton, C. C., Gonzalez, C. F. C., and Young, R. R. (2007). Role of phages in the pathogenesis of *Burkholderia*, or 'Where are the toxin genes in *Burkholderia* phages?'. *Curr. Opin. Microbiol.* 10, 410–417.