University of Alberta

DC1, a podoviridae with a putative cepacian depolymerase enzyme

by

Sarah J.M. Routier

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 2010
Edmonton, Alberta

Examining Committee

Jonathan Dennis, Department of Biological Sciences

Christine Szymanski, Deparment of Biological Sciences

Mario Feldman, Department of Biological Sciences

Stefan Pukatzki, Department of Medical Microboilogy and Immunology

DEDICATION

I would like to dedicate my research efforts to Esther, a woman who lived with Cystic Fibrosis and had a profound impact on my life.

ABSTRACT

Plaques formed by DC1 on *B. cepacia* LMG 18821 and *B. cenocepacia* PC184 are surrounded by large and expanding halos when production of the exopolysaccharide (EPS) cepacian is induced. This plaque morphology indicates that DC1 putatively carries an EPS depolymerase enzyme. Plaque halos were absent when DC1 infected a PC184 cepacian knockout mutant and a non-mucoid LMG 18821 mutant, constructed using plasposon mutagenesis. The virulence of these mutants compared to wildtype PC184 and LMG 18821 was determined using the *Galleria mellonella* infection model. No major changes to virulence were observed for the LMG 18821 mutant. But, the PC184 cepacian knockout mutant was attenuated for virulence suggesting that this carbohydrate pathway may play a role in pathogenesis. The gene(s) involved in halo formation remain unknown although attempts were made to determine the gene(s) involved by cloning and expressing DC1 fragments in *E. coli* and assaying for EPS degradation.

ACKNOWLEDGEMENT

I would like to acknowledge the people who have supported me throughout my Masters degree. Firstly, my supervisor Dr. J. Dennis, without you, this would not have been possible. Thank you for taking me into your lab and taking a chance on someone with a Bachelor of Education Degree.

Secondly, I want to thank my family. Everyone in the Tappenden, Routier and Soprovich families, thank you for your support and belief in my abilities. My parents in particular have worked so hard, have supported me in every way possible and have made countless sacrifices so that I could follow my heart. I am forever grateful to both of you. To my husband, Jesse, thank you for everything. You always let me choose my path and never put limitations on my choices. It is because of you that I strive to be a better person and achieve success.

Lastly, I want to thank the two most influential teachers in my life. Without these individuals, most certainly, I would not have made it this far. Mr. S. Young and Ms. Pat Baker.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

α	alpha
β	beta
ϕ	phi
Amp	ampicillin
BCC	*Burkholderia cepacia* complex
BCSA	*Burkholderia cepacia* selection agar
BLAST	Basic local alignment search tool
BLASTN	Basic nucleotide local alignment search tool
bp	base pair
CGD	Chronic Granulomatus Disease
CF	Cystic Fibrosis
CFU	colony forming units
DNA	deoxyribonucleic acid
dTDP	deoxythymidine diphosphate
EndoE	endosialidase E
EPS	exopolysaccharide
ET12	enzyme electrophoretic type 12
gp	gene product
IPTG	isopropyl β-D-1-thiogalactopyranoside
GDP	guanosine diphosphate
IR	inverted repeats
Kdo	3-Deoxy-D-manno-oct-2-ulosonic acid
kb	kilo base pair
LB	Luria-Bertani media
MIC	minimum inhibitory concentration
MOI	multiplicity of infection
NCBI	National Center for Biotechnology Information
OD	optical density
OM	outer membrane
ORF	open reading frame
oriT	origin of transfer
PCR	polymerase chain reaction
PFU	plaque forming units
pi	post-infection
PIA	*Pseudomonas* isolation agar
RNA	ribonucleic acid
STM	signature-tagged mutagenesis
T4SS	type IV secretion system

TEM	transmission electron micrograph
Tn*5*	Tn*5* transposase
Tp	trimethoprim
UDP	uridine diphosphate
UndPGPT	undecaprenyl-phosphate-glycosyl-1-phosphate transferase
YEM	yeast extract mannitol media
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

INTRODUCTION

***Burkholderia cepacia* complex**

A group of genetically distinct yet phenotypically similar Gram negative opportunistic pathogens called the BCC are able to form infections associated with high levels of morbidity and mortality in individuals with Cystic Fibrosis (CF) and Chronic Granulomatus Disease (CGD) (Courtney et al. 2007; Mahenthiralingam et al. 2005). Nine species; *Burkholderia cepacia, Burkholderia multivorans, Burkholderia cenocepacia, Burkholderia stabilis, Burkholderia vietnamiensis, Burkholderia dolosa, Burkholderia ambifaria, Burkholderia anthina* and *Burkholderia pyrrocinia*, make up the core BCC and have all been isolated from the lungs of individuals with CF (Coneye et al. 2001; Vandamme et al. 1997, 2000; Vermis et al. 2004). Alarmingly, up to 20% of CF individuals infected with the BCC will develop 'cepacia syndrome', which is characterized by a decrease in pulmonary function, necrotizing pneumonia and speticaemia resulting in death (Frangolias et al. 1999; Isles et al. 1984).

Members of the BCC exhibit tremendous intrinsic resistance to a wide variety of antibiotics (Mahenthiralingam et al. 2005). For this reason, the treatment of BCC infections is extremely difficult and contributes to the poor prognosis of BCC infected individuals. Examination into alternative treatment methods such as phage therapy is required and extremely important as it could lead to an increase in the quality of life and potentially increase life expectancy of individuals with CF and CGD.

Contributions towards development of phage therapy for treatment of BCC infections and characterization of novel phages are presented in this

document. Specifically, a novel phage, SR1, was identified and shown to be an expanded host range variant of the well characterized phage KS10 (Goudie et al. 2008). Another important contribution presented herein is the finding that phage DC1 putatively carries a phage depolymerase enzyme that is active against cepacian, an exopolysaccharide (EPS) of the BCC. This work is significant because to date, no phage depolymerase enzymes have been described for BCC specific phages. Such an enzyme could potentially increase the efficacy of phage therapy, be used as a biofilm dispersion tool and could also be used to characterize EPSs of the BCC.

Noteworthy environmental roles of the BCC

The BCC are ubiquitously found in the environment and as a result members of the complex perform a variety of interesting ecological roles. This includes the prevention of damping off disease in seedlings, the prevention of molding of fruit and the promotion of plant growth and crop production through the nodulation of roots where BCC members perform nitrogen fixation. Further, members of the BCC take part in the degradation of ground water contaminants and causation of disease in plants, namely, onion soft rot (Mahenthiralingam et al. 2005). The ability of the BCC to cause disease in mammals and plants, perform beneficial roles in the environment via their impressive metabolic capabilities has led to a large and active research community based upon these opportunistic pathogens.

CF, CGD and the BCC

Among Caucasians, the most commonly inherited lethal autosomal recessive genetic disorder is CF (Govan and Deretic 1996), affecting one in every 2,500 individuals. Mutations in the cystic fibrosis transmembrane conductance regulator (CFTR), a chloride ion channel, result in the production of dehydrated mucus by the sex ducts, pancreatic ducts and lungs, causing a wide range of problems for individuals with CF. In the lungs, the thick mucus is not cleared efficiently due to low mucociliary action, thus providing an environment that is routinely colonized by opportunistic pathogens including the BCC and *Pseudomonas aeruginosa*. Lung infections result in the death of the majority of CF individuals. In the United States the median life expectancy age is alarmingly low at only 37.4 years (www.cff.org) and the quality of life for individuals with CF is affected by the continual exacerbations of low pulmonary function resulting in hospitalization and, if infected with the BCC, social limitations (Speert et al. 2002).

Members of the BCC also infect and cause disease in individuals with CGD (Winkelstein et al. 2000). This genetically inherited disease results in the inability of phagocytic cells to reduce molecular oxygen and synthesize reactive oxygen species. Members of the BCC are among the most virulent bacteria that infect these immunocomprimised individuals and are associated with pneumonia, sepsis and death (Macdonald and Speert 2008; Winkelstein et al. 2000).

BCC infections and antibiotic resistance mechanisms

Infection rates and mortality

Approximately 20% of individuals with CF will become infected with the BCC during the course of their lifetime (Harrison 2007; Hutchison and Govan 1999). Acquisition of the BCC can occur from environmental sources or nosocomially through patient-to-patient transmission (LiPuma et al. 1990; Mahenthiralingam et al. 2001). Of those BCC infected individuals, approximately 45% will be infected with *B. cenocepacia* and as many as 35% will be infected with *B. multivorans* (Reik et al. 2005). Patients infected with either of these two species have died as a result of cepacia syndrome (Jones et al. 2003). The large proportion of individuals infected with *B. cenocepacia* is alarming as this species is associated with high rates of transmissibility, mortality and morbidity (Speert et al. 2002). *B. cenocepacia* strains of particular clinical importance include those from ET12 (enzyme electrophoretic type 12) and Midwest lineages, as these strains have been shown to be highly transmissible and virulent (Mahenthiralingam et al. 2005). *B. multivorans, B. dolosa* and *B. cepacia* have also been shown to be transmissible between patients, but at levels that are much lower than *B. cenocepacia* strains.

Antibiotic resistance abilities of the BCC

Members of the BCC are intrinsically resistant to many antibiotics. This is clearly demonstrated by their ability to use penicillin G as a sole carbon source (Beckman and Lessie 1979). Small porins and decreased permeability across the outer membrane confers resistance to many antibiotics including chloramphenicol

(Arnoff 1988; Burns et al. 1989*a*; Parr et al. 1987). Additionally, the BCC members synthesize β-lactamases (Chiesa et al. 1986) and trimethoprim-resistant dihydrofolate reductases (Burns et al. 1989) that result in resistance to β-lactams and trimethoprim, respectively. As well, spontaneous changes to the lipopolysaccharide (LPS) of the BCC have been associated with resistance to trimethoprim/sulphamethoxazole and chloramphenicol (Vinion-Dubiel and Goldberg 2003).

The unique nature of the LPS also provides intrinsic resistance to cationic peptides (Vinion-Dubiel and Goldberg 2003). Specifically, modifications to the lipid A backbone and a decreased amount of phosphate and/or 3-deoxy-D-manno-oct-2-ulsonic acid (Kdo) result in a relatively neutral LPS that does not attract cationic peptides. *Burkholderia cepacia* selection agar (BCSA) developed by Henry et al. 1997 contains 600,000 U/L polymixin B and 10 mg/L gentamicin. Neither of these two antibiotics were found to limit BCC growth at these concentrations. To the same antibiotics, *P. aeruginosa* was found to have minimum inhibitory concentrations (MICs) of 0.5 – 1 mg/L and 8.6 mg/L, respectively (Tam et al. 2005; Woolfrey et al. 1984).

Adoption of a biofilm lifestyle has also been found to increase antibiotic resistance abilities by members of the BCC (Desai et al. 1998). Cells growing in a biofilm were found to be 15 times more resistant to ciprofloxacin and ceftazidime than their planktonic counterparts. This finding is significant and concerning as strains from all members of the BCC have been shown to be able to form biofilms

in vitro (Conway et al. 2002). Further, it is hypothesized that the BCC adopts a biofilm lifestyle in the CF lung (Berlutti et al. 2005).

In vivo, BCC antibiotic resistance is strengthened by their ability to invade and survive within human cells including epithelial cells (Burns et al. 1996; Caraher et al. 2007) and macrophages (Lamothe et al. 2007). Living within human cells allows these organisms to evade antibody and antibiotic attack, thereby contributing to the persistence of infections and clearance difficulties.

The EPS of the BCC

EPS production capabilities by members of the BCC

In the past decade, the field of research on the EPSs of the BCC has expanded. This is because a large number of BCC strains both clinically and environmentally isolated have been shown to produce EPS (Bartholdson et al. 2008; Chiarini et al. 2004; Ferreira et al. 2010; Lagatolla et al. 2002; Linker et al. 2001; Moreira et al. 2003; Richau et al. 2000; Zlosnik et al. 2008). EPS production is easily determined by examining growth on media containing mannitol or glycerol. Strains that produce EPS will appear mucoid on solid media or will result in a large increase in broth viscosity if grown in liquid medium. Many research groups have reported on the EPS production capabilities of both clinical and environmental isolates, from a variety of regions including Portugal, the United Kingdom and the United States. Richau et al. (2000) found 70% of clinical isolates to be mucoid, Moreira et al. (2003) reported that as many as 80% of clinical isolates are mucoid, Bartholdson et al. (2008) found that 83% of isolates collected from both the environment and the CF lung are mucoid and

lastly, Zlosnik et al. (2008) found 77% of BCC members to be capable of EPS production. While the percentages of EPS production capabilities by members of the BCC vary, it can be hypothesized that a large portion of BCC members are capable of producing EPS. Thus, the impact of EPS on pathogenesis needs to be assessed.

The role of EPS in biofilm formation is unclear. Some strains producing EPS have been found to form biofilms (Cunha et al. 2004; Ferreira et al. 2007) and some mucoid BCC strains have been found to adopt only a planktonic lifestyle *in vitro* (Conway et al. 2004). Cuhna et al. (2004) looked at the EPS production and biofilm formation capabilities of 94 BCC CF isolates and found that there was no correlation between EPS and biofilm formation. The ability for a member of the BCC to form biofilms is strain specific and it is apparently not related to EPS production capabilities. However, *in vitro,* EPS-producing strains of the BCC have been associated with thicker and more mature biofilms than their non-mucoid counterparts (Cunha et al. 2004; Ferreira et al. 2007; Sousa et al. 2007*a*).

Protection from desiccation was also conferred by EPS, as cells covered with 2.5 g/L crude EPS could survive for at least seven days when left exposed to the air. Cells not covered with EPS could survive to a maximum of three days, after which minimal numbers of viable cells could be recovered (Ferreira et al. 2010).

EPS composition

Four different carbohydrate polymers have been isolated from the EPSs of BCC members (Cérantola et al. 1996, Cescutti et al. 2000, Cescutti et al. 2003 and Nimtz et al. 1997), Figure 1. To date, there has been no observed correlation between species and EPS structure. Three of the four EPS polymers have names; PSI, cepacian (PSII) and levan. The final polysaccharide has not been given a formal name and in this document will be described as the Kdo-galactan polymer, as it contains D-galactose and Kdo in a 3:1 ratio (Nimtz et al. 1997).

Cepacian is the most commonly produced EPS among the BCC and for the majority of determined EPS structures, it has been found as the sole polymer (Chiarini et al. 2006; Ferreira et al. 2010; Lagatolla et al. 2002). This was well demonstrated by Linker et al. (2001) who determined the structure of EPSs from eight clinical isolates and found all of them to produce solely cepacian. Additionally, Chiarini et al. (2004) found that ten out of 11 *B. cenocepacia* clinical isolates produced cepacian as the only polysaccharide in their EPS. The eleventh isolate was found to have a heterogeneous EPS as it produced both the Kdo-galactan polymer and cepacian. Cepacian has also been found to be co-produced with PSI by the *B. cenocepacia* strain C9343 isolated from the CF lung (Conway et al. 2004). Rarely, mucoid BCC members not producing cepacian are found. An example of this is *B. cepacia* BTS13, which produces a heterogeneous EPS comprised of the Kdo-galactan polymer and levan (Cescutti et al. 2003).

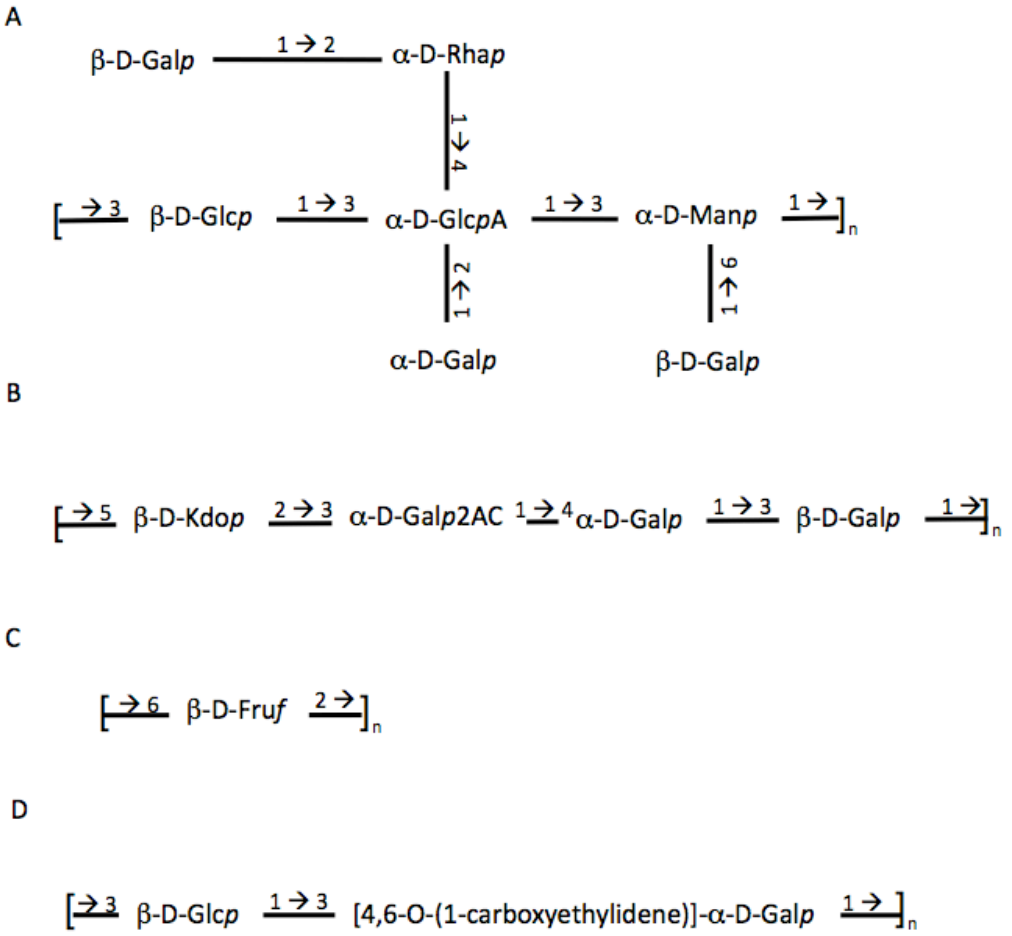

Figure 1 EPS polymers isolated from BCC EPSs. Legend. (A) cepacian (Cescutti et al. 2000); (B) Kdo-galactan polymer (Nimtz et al. 1997); (C) levan (Cescutti et al. 2003); (D) PSI (Cérantola et al. 1996). β-D-Galp, β-D-galactose; α-D-Rhap, α-D-rhamnose; β-D-Glcp, β-D-glucose; α-D-GlcpA, α-D-glucuronic acid; α-D-Galp, α-D-galactose; α-D-Manp, α-D-mannose, β-D-Kdop, β-Kdo (3-Deoxy-D-manno-oct-2-ulosonic acid); β-D-Galp2Ac, β-D-galactose o-acetylated at position 2, β-D-Fruf, β-D-fructofuranosyl, [4,6-O-(1-carboxyethylidene)]- α-D-Galp, [4,6-O-(1-carboxyethylidene)]-a α-D-galactose. Linkages between sugars are written beside the line connecting each monosaccharide. Adapted from Chiarini et al. (2004).

Cepacian biosynthesis

As cepacian is the most abundantly produced EPS among mucoid BCC members, there has been much attention given to its biosynthesis. Cepacian is composed of repeating units of a unique heptasaccharide that contains D-galactose, D-glucose, D-mannose, D-rhamnose and D-glucuronic acid in a 3:1:1:1:1 ratio. It is known that each repeating unit of cepacian is acetylated twice, but the placement of the acetyl groups remains unknown (Cescutti et al. 2000).

Two biosynthetic gene clusters, *bce-I* and *bce-II*, have been identified and encode for almost all of the enzymes predicted to be involved in cepacian biosynthesis, Figure 2 (Ferreira et al. 2010; Moreira et al. 2003). The *bce-I* gene cluster was identified by Moreira et al. (2003) using plasposon insertion mutagenesis of the mucoid *B. cepacia* strain IST408. Plasposon insertion mutants were screened for the absence of EPS by growing strains on EPS inducing media and staining colonies with Sudan Black B dye. After the initial screen, EPS quantification following growth in liquid S media (EPS inducing media) was performed on colonies that could not maintain the Sudan Black B dye. This lead to identification of three mutants that had plasposons inserted into genes that were extremely close together and putatively involved in polysaccharide biosynthesis. Two mutants had the plasposon inserted into a gene putatively involved in the co-polymerization of repeat units and the third had the plasposon just downstream in an open reading frame (ORF) encoding a putative polymerase. These ORFs were later given the names *bceF* and *bceI*, respectively. It must be noted that the genome of IST408 is not sequenced so the genome and sequencing analysis was

done using the fully sequenced and annotated genome of *B. cenocepacia* J2315 (Holden et al. 2009).

Moreira et al. (2003) also examined the region surrounding the plasposon insertion sites and found that the plasposon had inserted into a cluster of 12 genes putatively involved in cepacian biosynthesis, Table 1. This gene cluster was called the *bce* gene cluster and is located on chromosome 2 of J2315 from base pairs (bp) 940,538 and 956,686. Since the discovery of the second *bce* gene cluster, it is now referred to as the *bce-I* gene cluster.

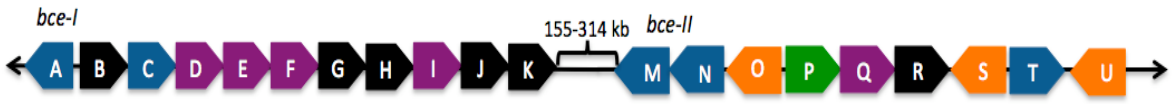

Figure 2 *bce-I* and *bce-II* gene clusters. Legend. blue, putative nucleotide sugar biosynthesis genes, black, putative glycosyl transferase genes, purple, putative polymerization and export genes, orange, putative acyltransferase genes, green, gene with unknown function. Adapted from Ferreira et al. (2010).

Table 1 Genes and their putative function of the *bce-I* and *bce-II* clusters

Gene	Putative function of the gene product	Requirement for cepacian biosynthesis
***bce-I* cluster** (Moreira et al. 2003)		
bceA	Phosphomannose isomerase/ GDP-D-mannose pyrophosphorylase[a,]	-[a]
bceB	Undecaprenyl-phosphate-glycosyl-1-phosphate-transferase[b]	++[d,e]
bceC	UDP-glucose dehydrogenase	ND
bceD	Low-molecular mass phosphotyrosine protein phosphatase[c]	+/-[c]
bceE	Outer-membrane lipoprotein	ND
bceF	Putative copolymerase with protein tyrosine kinase activity[c]	++[c,d]
bceG	Glycosyl transferase	ND
bceH	Glycosyl transferase	ND
bceI	Polymerase	++[d]
bceJ	Glycosyl transferase	ND
bceK	Glycosyl transferase	++*
***bce-II* cluster** (Ferreira et al. 2010)		
bceM	GDP-6-deoxy-D-xylo-4-hexulose reductase	ND
bceN	GDP-D-mannose 4,6-dehydratase	ND
bceO	Acyltransferase	ND
bceP	Unknown	ND
bceQ	Repeat unit flippase	++ [g]
bceR	Glycosyl transferase	++ [g]
bceS	Acyltransferase	+[g]
bceT	UDP-glucose phosphorylase	ND
bceU	Acyltransferase	ND

Legend. [a] confirmed protein function (Sousa et al. 2007*a*); [b], confirmed protein function (Videira et al. 2005), [c], confirmed protein function (Ferreira et al. 2007), [d], Moreira et al. (2003), [e], Bartholdson et al. (2008), [g] , Ferreira et al. (2010), *, this study, ND, not determined, ++, required for cepacian biosynthesis, +, required for full acetylation of cepacian, +/-, mutations in this protein result in decreased EPS production, -, mutations in this protein do not abolish EPS production.

One of the most significant findings of Moreira et al. (2003) was that J2315 contains an 11 bp deletion in *bceB*. *bceB* encodes for undecaprenyl-phosphate-glycosyl-1-phosphate transferase (UndPGPT) that is responsible for incorporating glucose-1-phosphate (G1P) onto the undecaprenyl-phosphate lipid carrier in the inner membrane (Videira et al. 2005). The attachment of G1P to the lipid carrier is the first step in cepacian biosynthesis and without it, the EPS cannot be made. *B. cenocepacia* J2315 is an ET12 strain, and it is hypothesized that this 11 bp deletion is found in the *bceB* of all ET12 strains as all ET12 strains examined have been found to be non-mucoid (Johnson et al. 1994; Moreira et al. 2003).

Ferreira et al. (2010) noted that genes encoding essential enzymes required for cepacian biosynthesis were missing from the *bce-I* cluster. Missing enzymes included those involved in the production of D-rhamnose and D-glucose as well as enzymes involved in the transport and assemblage of cepacian. Bioinformatics were used by Ferreira et al. (2010) to scan the genomes of seven BCC members and eight species not belonging to the complex but still belonging to *Burkholderia*, in order to identify putative ORFs for these genes. In all of the examined genomes, eight additional genes putatively involved in carbohydrate biosynthesis were found clustered together just downstream from the *bce-I* cluster. This second cluster of genes was called *bce-II* and putatively encoded genes for the production of D-rhamnose and UDP-D-glucose among others, Table 1. The *bce-II* gene cluster was shown to be involved in the biosynthesis of cepacian through targeted mutagenesis of three genes contained within the cluster.

A number of studies have investigated the requirement of *bce* genes for the production of cepacian. *bceB, bceF, bceI, bceQ* and *bceR* have been shown to be required for cepacian biosynthesis (Bartholdson et al. 2008; Ferreira et al. 2007; Ferreira et al. 2010; Moreira et al. 2003; Videira et al. 2005). Mutations in any one of these genes result in the abolishment of the mucoid phenotype and the production of cepacian. Mutations to *bceS* and *bceD* result in a cepacian that is acetylated 20% less than normal and decreased production of EPS, respectively (Ferreira et al. 2010). Lastly, mutations to *bceA*, which encodes for a protein with dual function that is responsible for the production of GDP-D-mannose, do not affect EPS synthesis and the mucoid phenotype (Sousa et al. 2007*a*). Analysis of published BCC genomes showed that complex members contain as many as 5 BceA orthologues and it is hypothesized by Sousa et al. (2007*a*) that these genes may compensate for the *bceA* mutation, allowing for the production of cepacian.

Interestingly, mutations to glucose dehydrogenase (Gcd) result in an increased amount of EPS (cepacian) production when Gcd^- strains are grown on media containing an excess amount of glucose (Sage et al. 1990). Gcd mutations did not result in an increase to EPS production when strains were grown on media containing mannitol or glycerol. Gcd^- strains do not rapidly convert glucose into glucuronic acid and 2-ketogluconic acid. Instead, Gcd^- strains convert glucose into glucose-6-phosphate (G6P), which can be used to synthesize GDP-D-glucose, a nucleotide sugar precursor of cepacian (Moreira et al. 2003). The increased production of G6P by Gcd^- strains is likely the reason for the increased cepacian production.

Richau et al. (2000) examined crude cell-free extracts for the activity of ten enzymes involved in nucleotide sugar precursor production. The majority of the enzymes were found to have the greatest activity during late exponential phase or early stationary phase. GDP-D-mannose pyrophosphorylase (GMP) activity was found to be independent of growth. Of the ten enzymes examined, six genes (*bceT*, *bceC*, *bceM*, *bceN* and *bceA*) are found within the two *bce* gene clusters. The genes encoding the four remaining enzymes; phosphoglucose isomerase (PGI), phosphoglucomutase (PGM), UDP-glucose epimerase (UGE) and phosphomannomutase (PMM), have not been elucidated.

The standing model for cepacian biosynthesis is that it is made using the Wzy-dependant pathway, Figure 3 (Ferreira et al. 2010). BceQ, a flippase with 48% similarity to Wzx of *Escherichia coli* is predicted to transport the heptasaccharide repeat unit across the inner membrane. The BceI polymerase is hypothesized to assemble the heptasaccharides together into a polymer in the periplasm with the assistance of BceF a putative polysaccharide co-polymerase with tyrosine kinase activity. It is predicted that the resulting cepacian polymer is shuttled by BceE from the periplasm into the environment. Further work needs to be done in order to verify the production of cepacian by a Wzy-dependant pathway.

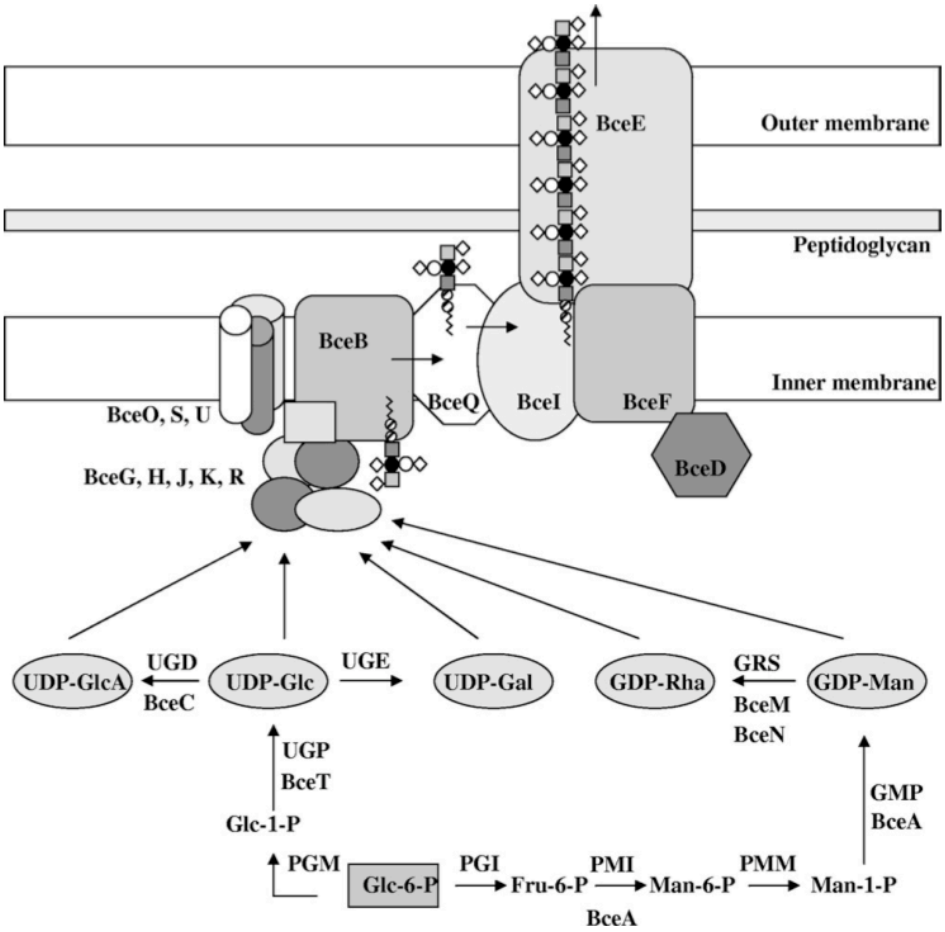

Figure 3 Model for cepacian biosynthesis proposed by Ferreira et al. (2010). Legend. Cepacian biosynthesis is predicted to occur in a Wzy-dependant manner. The UndPGPT, BceB, transfers activated glucose onto the inner membrane lipid carrier. The heptasaccharide repeat unit is then constructed by adding the remaining sugars onto the glucose residue. This is done by the putative glycosyl transferases, BceG, H, J, K and R. BceQ, a putative flippase then translocates the heptasaccharide across the inner membrane where polymerization occurs by the putative polymerase BceI and the putative copolymerase BceF. BceD is a low molecular mass acid phosphatase that putatively acts to deposphorylate BceF, in a manner similar to Wzb in *E. coli*. Finally, the export of cepacian is putatively done by BceE. Abbreviations, GlcA, glucuronic acid, Glc, glucose, Gal, galacatose, Rha, rhamnose, Man, mannose, Glc-6-P, glucose-6-phosphate, Fru-6-P, frucoste-6-phosphate, Man-6-P, mannose-6-phosphate, Man-1-P, mannose-1-phosphate, UDP, uridine-5'-diphosphate, GDP, guanosine-5'-diphosphate, PGM, phosphomannomutase, UGP, UDP-glucose pyrophosphorylase, UGE, UDP-glucose epimerase, PMM, phosphomannomutase, PGI, phosphoglucose isomerase, GMP, GDP-D-mannose pyrophosphorylase, UGD, UDP-glucose dehydrogenase, PMI, phosphomannose isomerase, GRS, GDP-D-rhamnose synthetase Taken from Ferreira et al. (2010), with permission.

EPS and virulence

The EPS of the BCC has been suggested to act as virulence factor for several reasons. EPS has been shown to interfere with neutrophil phagocytosis, inhibit neutrophil chemotaxis and production of reactive oxygen species (Bylund et al. 2006; Conway et al. 2004). Also, EPS has been shown to increase BCC persistence in BALB/c mice compared to non-mucoid strains of the BCC (Chung et al. 2003; Conway et al. 2004). Using $gp91^{phox-/-}$ mice, the murine model that mimics CGD, cepacian was shown to be associated with increased virulence. *B. cepacia* IST408 infection was associated with 100% mortality six days post infection (pi). When cepacian biosynthesis was inhibited by mutation to *bceF* or *bceI* mortality dropped to 0% at 11 days and 50% at 12 days pi, respectively (Sousa et al. 2007*b*).

To date, no correlation between EPS production and persistence in the CF lung has been elucidated. Cuhna et al. (2004) investigated EPS production capabilities of BCC isolates from 21 CF patients over a seven-year period and were unable to find a correlation between EPS and persistent long-term infection. Interspecies communications and interactions could affect the relationship between EPS and persistent infection as *P. aeruginosa* (Govan and Deretic 1996), *Staphylococcus aureus*, *Stenotrophomonas maltophia*, *Achromobacter xylosoxidans* and *Haemophilus influenzae* can also colonize the CF lung (Harrison 2007). Continued work needs to be done to elucidate the role of EPS in the CF and CGD lung.

Phage Depolymerases

The fact that the EPS of the BCC can act as a virulence factor and that its presence is correlated with mature biofilms, requires that it be considered when developing treatment methods for BCC infections. Specifically, when considering phage therapy, the ability of phages to infect and kill strains producing EPS must be considered. Studies conducted on other bacteria suggest that sometimes EPSs can act as a barrier to phage infection (Durlu-Özkaya et al. 2007). This likely occurs because of a diffusional barrier that is created by the EPS. Other times, EPS does not seem to provide a physical barrier to phage infection. This is possibly due to an enzymatic activity inherent in the viron. Often, phage that infect EPS-producing bacteria carry a carbohydrate depolymerase enzyme on their tail fibers, tail spikes or base plates (Sutherland 1999). These enzymes, generally called phage depolymerase enzymes, degrade the carbohydrate polymer and allow the phage to penetrate through the polysaccharide and reach their primary receptor on the bacterial outer membrane (OM), Figure 4. In some cases, the EPS acts as a receptor for the phage and without it, phage infection cannot occur (Billing 1960).

The ability of EPS to protect the BCC from phage infection has not been elucidated. In this work, the ability of phages to infect EPS-producing BCC members was examined. At the same time, phages were screened for carriage of a depolymerase enzyme that could potentially degrade the BCC EPS. This work is important because the elucidation of a phage depolymerase for the EPS of the BCC, specifically cepacian, could assist in the advancement and efficacy of phage therapy.

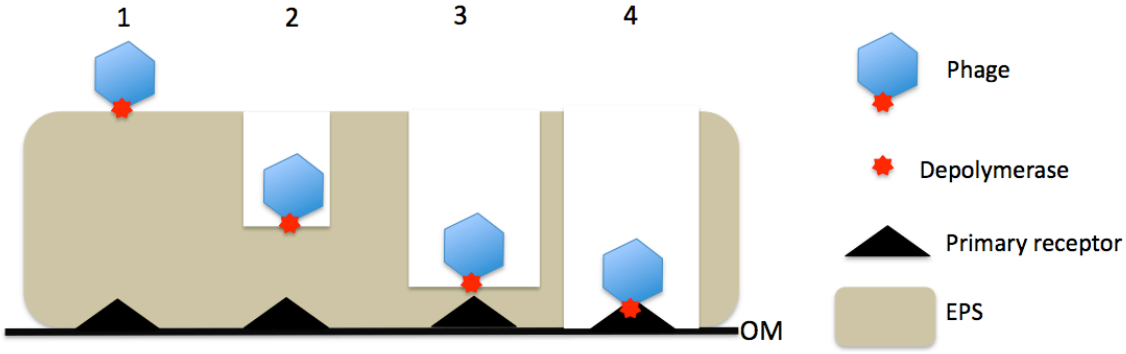

Figure 4 Phage depolymerase assisted penetration of bacterial EPS. Legend. 1, phage binds to EPS, 2 and 3, phage depolymerase hydrolyzes the EPS to allow phage to penetrate the carbohydrate polymer, 4, phage reaches primary receptor on bacterial outer membrane (OM). Adapted from Hughes et al. (1998).

Since the first report of phage depolymerase enzymes by Adams and Park (1956), phages carrying such enzymes have been found for many bacteria including: *P. aeruginosa*, *Pseudomonas putida*, *E. coli*, *Rhizobium trifolii*, *Klebsiella pneumonia*, *Klebsiella aerogenes*, *Erwinia amylovora*, *Enterobacter agglomerans* and *Salmonella typhimurium* (Adams and Park 1956; Bartell et al. 1966; Billing 1960; Eriksson et al. 1979; Higashi and Abe 1978; Hughes et al. 1998b; Sutherland 1967). Phage depolymerases have been found for EPS, capsular polysaccharides (CPS) and LPS, demonstrating that phages have developed efficient methods to reach their bacterial OM receptors.

Phages can be easily screened for the presence of a depolymerase enzyme by infecting the susceptible EPS-producing or CPS-producing host with the phage and observing plaque morphology. In all of the literature reviewed, plaques formed by phages carrying EPS or CPS depolymerase enzymes were invariably surrounded by large halos that expand over time. This is a unique plaque

morphology making putative identification of phage carrying depolymerase enzymes possible. Unambiguous determination of the phage depolymerase enzyme is done by assaying for decreased viscosity of the target polymer following incubation of purified phage enzyme with its substrate as well as elucidation of degradation products.

Phage depolymerase enzymes are typically components of the phage tail. This was clearly demonstrated by Castillo and Bartell (1979) who raised antisera against purified *Pseudomonas* phage 2 depolymerase and used it to show that the enzyme was associated with the tip of the long phage tail. The depolymerases of phages ε34 and P22 that are active against the LPS of *Salmonella* are found as a component of the tailspike protein (Eriksson et al. 1979; Zayas and Villafane 2007).

Recently, it was reported that there are no viable phages in the halo surrounding plaques produced by five independently examined *P. putida* phages (Shaburova et al. 2009). This finding suggests that the depolymerase action found in the halo may be from the enzymatic activity of a dissociated phage tail spike, fiber or base plate. This could be due to phage particle degradation, damage or potentially incomplete assembly in the host. This hypothesis is supported by the observation that active enzymes have been found in non-phage bands on a cesium chloride gradient (Sutherland 1999; Tomlinson and Taylor 1985).

Sutherland (1999) reviewed the enzymatic action of many phage depolymerases and showed that they could be categorized as either lyases or endoglycanases. Lyases cleave at a monosaccharide-uronic acid linkage.

Examples include alginate lyase and a polysaccharide lyase specific for the K5 antigen of *Klebsiella*. Endoglycanases act hydrolytically and cleave specific linkages of carbohydrate polymers. Examples include endogalactosidases, endoglucosidases and endofucosidases. Endogalactosidases and endoglucosidases were found to be the most common enzyme type among characterized phage depolymerase enzymes. For a single phage depolymerase, the substrate specificity is usually extremely high (Castillo and Bartell 1976; Eriksson et al. 1979; Long et al. 1995; Shaburova et al. 2009; Sutherland 1967; Sutherland 1999; Tomlinson and Taylor 1985). However, changes to acetylation of the target polymer do not appear to affect enzymatic activity (Nixon et al. 1987).

Phage degradation of CPS

Phage depolymerase degradation of CPS has been well characterized for *E. coli* K (Adams and Park 1956; Gross et al. 1977; Long et al. 1995; Nimmich et al. 1991; Sutherland 1999; Tomlinson and Taylor 1985). Phage A – E, ϕK5 and ϕK20 have all been found to degrade a K CPS of *E. coli* (Gross et al. 1977; Nimmich et al. 1991; Tomlinson and Taylor 1985). Interestingly, phages ϕK5 and ϕK20 were each found to have two polysaccharide depolymerase activities that allowed these phages to degrade two K CPSs (Nimmich et al. 1991). ϕK5 was found to have glycanase and endo-α-*N*-acetylglucosaminidase activity, which allowed it degrade the K20 CPS releasing Kdo and K5 CPS releasing *N*-acteylglucosamine (GlcNac), respectively. Long et al. (1995) determined the sequence of the gene encoding the endosialidase of phage E (EndoE) which is responsible for hydrolyzing the α-2,8 poly-*N*-acetylneuraminic acid present in the

K1 CPS. This gene encodes for a mature 74 kDa tail protein that putatively forms a trimer that displays high substrate specificity.

Sutherland (1967) investigated the depolymerase activity of five phages specific for *K. aerogenes* Type 54[A3(S1)], a bacterium that produces both CPS and EPS. The phage depolymerase enzymes carried by these phages are specific fucosidases only active against the extracellular polysaccharides produced by A3 strains of *K. aerogenes*. The activity of the enzyme was confirmed *in vitro* by observing an increase in reducing power and a decrease in polysaccharide viscosity following incubation with phage enzyme. At the time of phage depolymerase identification it remained unknown if the phage enzymes were active against the EPS or CPS produced by the host as the two polysaccharides contain the same sugar components. To date, the enzymatic action of these phages has not been further characterized.

Additionally, phage A22 was found to be capable of degrading the CPS of its host, *Azotobacter agilis* (Eklund and Wyss 1962) and phage ERA103 was found to carry a depolymerase enzyme for the CPS of its host, *Erwinia amylovora* (Vandenbergh and Cole 1986).

Phage degradation of LPS, P22 as an example

The endorhamnosidase activity performed by the tail of phage P22 allows it to degrade the smooth LPS of *Salmonella typhimurium* (Eriksson et al. 1979; Israel et al. 1972). Cleavage of the O-antigen occurs at the 1,3-α-*O*-glycosidic bond between L-rhamnose and D-galactose (Iwashita and Kanegasaki 1973; Sutherland 1999). As the enzyme degrades the O-antigen, the primary phage

receptor binding sites are lost. This degradation allows the phage to reach the bacterial OM, a suitable site for intimate binding and DNA injection (Byl and Kropinski 2000). The endorhamnosidase activity was shown to be specific to the tail of P22 by independently purifying phage heads and tails and assaying for the component that resulted in the loss of primary phage receptors (Israel et al. 1972). The phage tails were the only component found to degrade the O-antigen resulting in a loss of phage receptors. Gene nine in the P22 genome encodes for a tail protein that is 666 amino acids long, required for phage infection and responsible for endorhamnosidase acitivity (Byl and Kropinski 2000; Sauer et al. 1982). Additional phages expressing endorhamnosidase activity towards the LPS of *Salmonella* include phages ε34 and ε15 (Greenberg et al. 1995; Kanegasaki and Wright 1973).

EPS depolymerase expressing phage

Phages carrying depolymerases specific for *P. aeruginosa* EPS were characterized by Bartell et al. (1966), Bartell and Orr (1969), Castillo and Bartell (1976) and Glonti et al. (2010). Plaques formed by phages on lawns of their susceptible hosts were surrounded by the characteristic large and expanding halos. EPS degradation was found to be highly specific and resulted in a decrease in the viscosity of their substrate polymer when co-incubated *in vitro* with purified enzyme (Bartell et al. 1966; Bartell and Orr 1969). Significantly, phage PT-6 was shown to be active against the EPS produced by *P. aeruginosa* strains isolated from the CF lung (Glonti et al. 2010). This work demonstrates that phages

expressing depolymerase enzymes can be found for clinically important species such as *P. aeruginosa*.

Phages carrying depolymerase enzymes have been shown to decrease the number of viable cells in biofilms (Hanlon et al. 2001; Hughes et al. 1998*a*; Tait et al. 2002). Hanlon et al. (2001) demonstrated that the number of cells living in a *P. aeruginosa* biofilm could be decreased by two logs following addition of depolymerase expressing bacteriophage. While the decrease in CFU/mL observed does not reflect complete eradication of the biofilm, it was noted that alginate viscosity decreased following phage treatment. This is significant because if paired with antibiotic therapy, the decrease in alginate viscosity may increase treatment delivery and therapeutic efficacy.

Similarly, phage biofilm eradication was shown to be effective by Hughes et al. (1998) who demonstrated that combined application of phage and purified phage depolymerase enzyme could significantly disperse a *E. agglomerans* biofilm grown for 24 h, decreasing viable cells by as much as three logs. Tait et al. (2002) further showed that phage expressing depolymerases could result in a significant decrease in viable cells living in a biofilm. When purified phage enzyme was combined with a disinfectant, such as bleach, biofilm eradication was higher than either method alone and virtually complete.

Efficacy of phage depolymerase to treat bacterial infections

Mushtaq et al. (2005) demonstrated the ability for a phage CPS degrading enzyme to decrease virulence of *E. coli* K1 strains *in vivo* using a neonatal rat infection model. The ability of the purified enzyme to prevent the development of

bacteriema and increase host-immune system killing in neonatal rats pi was determined. Significantly, with only one dose of EndoE delivered 24 h pi, the number of neonatal rats killed by the *E. coli* K1 infection decreased from 100% killed without treatment to < 20% killed when 20 μg EndoE was delivered by an intraperitoneal route. Further, higher bacterial loads were observed in host macrophages, suggesting that the degradation of bacterial CPS assisted in macrophage phagocytosis.

Flachowsky et al. (2008) constructed nine transgenic apple plants (*Malus domestica*) that contained and expressed the EPS depolymerase from phage ϕEa1h. Expression of the phage depolymerase by the transgenic apples was determined by reverse transcription (RT)-PCR, quantitative (RT)-PCR and by assaying for EPS degradation abilities of the apple leaves. The ability for the transgenic plants to resist infections caused by *E. amylovora* was assessed *in vitro*. Four of the nine plants were significantly less susceptible to disease caused by *E. amylovora*. A positive correlation was observed between depolymerase activity and disease resistance as the plants found to produce the most EPS depolymerase were also shown to have high levels of resistance towards *E. amylovora*. This work is important, as *E. amylovora* is the causative agent of Fire blight disease, a highly destructive disease that affects pears and apples.

Phage therapy

Phage therapy has been used to prophylatically treat multiply resistant and highly pathogenic bacteria with great success in animal models. This was demonstrated by Biswas et al. (2002), who used phage therapy to treat a

vancomycin-resistant *Enterococcus faecium* (VRE) gastrointestinal tract infection in mice. When phage treatment was delivered 45 minutes pi, 100% of the infected animals survived as compared to untreated mice where 100% of animals died. When the administration was delayed until the animals were moribund, 50% were rescued by phage therapy. This demonstrates the ability for phages to act as a therapeutic and neutralize clinically relevant infections caused by antibiotic resistant bacteria with success.

S. aureus has been shown to be susceptible to prophylactic action of phage therapy as well. Matsuzaki et al. (2003) demonstrated the ability of phage ϕMR11 to rescue mice from infection with a single lethal dose of a methicillin-sensitive *S. aureus* (MSSA) when administered immediately after infection. ϕMR11 prevented death in 100% of *S. aureus* infected mice for at least seven days pi whereas over 80% of mice not receiving phage therapy died within 24 h pi.

McVay et al. (2007) determined the efficacy of a phage cocktail comprised of three phages to treat a *P. aeruginosa* infection in compromised mice subjected to a single lethal dose of *P. aeruginosa*. Different infection clearing efficacies were observed when phages were delivered by an intramuscular, subcutaneous or intraperitoneal injection, with the latter being the most effective as it prevented death in 87% of test subjects. When delivered by intraperitoneal injection, phages were present at the site of infection faster and with higher abundance than when delivered by the other two routes. The reason for this is unknown.

Significantly, phage therapy has been shown to be effective against the intracellular pathogens *Mycobacterium tuberculosis* and *Mycobacterium avium*. These pathogens are able to evade antibiotic treatment and the immune system by living within macrophages (Broxmeyer et al. 2002). An increase in the prevalence of *M. tuberculosis* and *M. avium* infections in recent years is very alarming as chemical treatment methods are quickly becoming ineffective. Broxmeyer et al. (2002) successfully delivered lytic phage to the intracellular site of infection *in vitro*, by using non-virulent *Mycobacterium smegmatis* as a delivery vector for the lytic phage TM4. Within 48 hours of administering phage therapy, T4M significantly reduced the amount of *M. tuberculosis* and *M. avium* found within macrophages when applied 24 hours pi. This shows the ability of an avirulent bacteria to act as a delivery vehicle for phages to intracellular sites of infection, which has the potential to be adapted for other intracellular pathogens like the BCC.

Phage therapy concerns

As with any therapy method, there are specific concerns and disadvantages inherent to the method, but careful considerations can result in effective solutions to significantly prevent or decrease therapy challenges. The potential to elicit a strong immune response, a narrow host range and perhaps limited application to a diverse group of human pathogens, the ability of phage to act as vehicle for genetic transfer and the low infection clearance efficiency observed with delayed therapy are all included in the concerns of phage therapy. Fortunately, each of the outlined concerns can be avoided through the implementation of simple solutions.

Upon administration, phages may elicit a very strong immune response since they are foreign and act as neo-antigens when first given to their recipient (Merril et al. 2003). However, no adverse effects have been reported following phage treatment in murine models of infection. Matsuzaki et al. (2003) showed that administration of phages to the peritoneal cavity of healthy mice had no adverse effects. Mice receiving phage preparations were as healthy as control mice for at least one month post injection. Additionally, Debarbieux et al. (2010) demonstrated that tumor necrosis factor α (TNFα) and interleukin 6 (IL-6) were both significantly decreased following phage treatment of lung infections formed by *P. aeruginosa* in murine lung compared to non-treated mice.

The potential for phage to cause an adverse reaction in humans was investigated by Ochs et al. (1971). The human immune response of healthy individuals as well as immunocomprimised individuals was determined following injection of phage ϕX174. ϕX174 did elicit an immune response marked by the production of antibodies within two weeks of phage exposure in either test group. Significantly, volunteers remained healthy through the course of the trial and reported no toxic or adverse effects. The elicited immune response was shown to be involved in clearing the phage from the body. Immunodeficient individuals maintained phages for at least 11 days whereas individuals producing antibodies cleared the phage within four days. Future work involves the development of long circulating phages as persistence in the body may increase treatment success (Merril et al. 2003).

Alarmingly, Gram negative bacterial lysis resulting from phage infection will release lipid A (endotoxin), a potent antigen that is able to elicit a strong and adverse immune response (Madigan and Martinko 2006). Studies conducted using phage therapy in animal models demonstrate the ability of phage to clear infections and prevent death in the recipient without further exacerbating the disease or symptoms (Biswas et al. 2002; Matsuzaki et al. 2005; McVay et al. 2007). In these studies, the authors did not report on the immune response elicited towards endotoxin A, but the full recovery of the recipients suggests that endotoxin A does not worsen the health of those individuals receiving phage therapy. Nonetheless, advances are being made to prevent the lysis of the bacterial host cell and subsequent release of endotoxin A. For example, the *E. coli* phage T4 has been modified in such a way that it will not lyse its host during the infection cycle (Thiel 2004). The modified T4 kills its host without lysis by degrading the bacterial genome and shutting down essential cellular processes. This modification has wide applications for phages chosen for therapy.

As phages are very specific and can generally infect only one or very few species of bacteria, the realistic application of phage therapy to treat infections caused by a wide range of pathogens is questionable. But, the specificity of phages can be seen as a benefit, preventing the destruction and disruption of the host's normal bacterial flora. Moreover, there are phages able to infect and kill every bacterial pathogen known on earth, and presumably not all of these phages have been isolated and characterized yet (Thiel 2004). Isolation procedures are

simple and easily employed so it is possible to isolate phages for any organism of interest.

Transduction is a process by which bacterial genes are transferred from one bacterium to another through a phage (Kutter and Sulakvelidze 2005). In this process, bacterial DNA is packaged into a phage head and the resulting phage is active and able to infect its host, delivering bacterial DNA instead of phage DNA. No phage progeny would be produced from this event, but the potential for transfer of virulence genes remains. Transduction during phage therapy can be decreased by using only lytic and non-transducing phages (Skurnik et al. 2007).

Phages can also carry virulence determinants on their genomes. Advances in technology now allow the genomes of phages to be easily sequenced and annotated, permitting therapy developers to know all of the genes carried by the phage. In this way, developers can ensure the genomes of phages chosen for therapy do not contain toxin genes or other genetic elements that will increase the virulence of the bacterium they are targeting.

Phages have been used with great success as prophylatic tools in animal infection models for a number of pathogens. However, the ability of phages to treat infections several hours or even days pi has been scarcely reported in the literature. Most phage therapy studies use bacterial doses that greatly exceed the lethal dose resulting in mortality within 48 h pi. The high bacterial loads used in animal experiments likely decrease the efficacy of delayed treatment. Very recently, this was demonstrated by Capparelli et al. (2010) who showed that delayed phage therapy can be effective when less than lethal doses are used to

establish host infections. Two weeks pi, phage ϕ1 was shown to completely clear an infection caused in the murine host when a sublethal dose of *Salmonella enterica* was used to cause infection. Unfortunately, the researchers did not report on the bacterial load found in the gastrointestinal tract (GI) or in the liver at the time of the phage treatment. The number of pathogenic bacteria found two weeks pi is extremely important when assessing the efficacy of phage therapy. However, these researchers demonstrate a positive move towards the elucidation of the effectiveness of phage therapy in a way that is clinically relevant. Further, it was demonstrated that intravenous delivery of phages did not result in the production of neutralizing antibodies and that phage resistant strains of *S. enterica* were completely avirulent in the murine model. These findings are significant as they indicate that previously noted concerns regarding phage therapy can be overcome and may not limit the applicability of this treatment strategy.

Potential barriers to phage delivery in the CF lung

The healthy lung is coated with mucus, which acts to trap bacteria and inhaled debris and is cleared readily through mucociliary action (Ali and Pearson 2007). In the CF lung, mucus secretions become 30-60 times more viscous than normal mucus and are not cleared efficiently leaving the CF lung an inviting and excellent environment for bacterial proliferation. Mucin, a negatively charged glycoprotein (Lafitte et al. 2007) is the gel-forming component in mucus that makes mucus sticky and creates a heterogeneous mesh that may decrease the diffusion of many macromolecules and therapy vehicles (Olmsted et al. 2001). The inability to clear mucin from the lung stimulates bacterial colonization by the

BCC (Sajjan et al. 1992) and *P. aeruginosa* (Vishwanath and Ramphal 1984) as these pathogens exploit the secreted mucin and use it as a binding receptor. The efficacy of phage therapy in the CF lung will be directly affected by the ability of phages to diffuse through the thick mucus layer and infect their hosts embedded in mucin.

Unfortunately, little research has been done in this area and only recently has the ability for phage infection to occur in mucin containing environments been performed. In an *in vitro* plaque assay, KH1 and SH1 phages were shown to infect their *E. coli* hosts when in the presence of either porcine gastric mucin or mucin taken from the lower gastrointestinal tract of steers (Sheng et al. 2006). This work demonstrates that mucin may not provide a barrier to phage infection. The ability to successfully deliver phages to bacterial colonization sites in the CF lung is unknown. To date, no investigations have been done in order to ascertain the ability of phages to diffuse through mucin collected from CF patients. But, the ability of phages to infect cells when in an environment containing mucin suggests that phage therapy may be effective in the CF lung.

Phage therapy for treatment of BCC infections

The efficacy of phage therapy to treat BCC infections *in vivo* has been investigated by Carmody et al. (2010) and Seed and Dennis (2009). Using the murine model of infection, Carmody et al. (2010) determined the ability of phage BcepIL02 to treat lung infections caused by *B. cenocepacia* AU0728. Phage treatment was associated with significantly less numbers of viable AU0728 cells in the lung when phage were delivered by an intraperitoneal route. When phage

were delivered by intranasal inhalation, bacterial numbers in the lungs decreased as well, however the change was not statistically significant. Surprisingly, phage titers were higher in the mice that received phage treatment via intranasal inhalation than in mice that received phage via intraperitoneal injection. The reason for this is unknown. Significantly, bacterial killing was shown to be phage specific as treatment with UV-inactivated phage did not result in a decrease in the bacterial load compared to infected untreated mice.

Seed and Dennis (2009) used the *Galleria mellonella* model of infection to investigate the efficacy of phages KS4M, KS12, KS14 and DC1 to treat BCC infections. When delivered simultaneously with bacteria, KS4M, KS12 and KS14 were all found to reduce the number of larvae killed 48 h pi compared to untreated infected controls. Interestingly, delaying treatment with KS4M by 12 hours was found to have the greatest infection clearance ability and was associated with the highest survival rates. KS12 was also able to clear infections when applied at 12 h pi. It is unknown why delayed treatment was so effective. However, no phages were found to increase larvae survival when administered 24 h pi. At any treatment time and multiplicity of infection (MOI), DC1 was unable to decrease larvae killing by *B. cenocepacia* C6433 48 h pi, even though a high phage titer could be achieved. C6433, an ET12 strain, was the only one of the five strains susceptible to DC1 infection that was investigated for phage treatment. The ability of DC1 to clear infections formed by other susceptible BCC strains, as well as its ability to act in a phage cocktail will be of extreme interest, as phage DC1 is shown in this study to putatively possess a cepacian depolymerase enzyme.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions

Burkholderia cepacia complex, Escherichia coli strains and plasmids

The *Burkholderia cepacia* complex strain panel (Coenye et al. 2001; Mahenthiralingam et al. 2000) and the Canadian *Burkholderia cepacia* complex Research and Referral Repository provided the sources of the BCC strains used in this research, Table 2. Commercially obtained chemically competent *E. coli* DH5α cells (Invitrogen, Carlsbad, CA) were used in all cloning experiments including the construction and maintenance of pBBR*bceB*Tp and p*bceB*Tp.

Table 2 Bacterial species and strains used in this research.

Species	Strain	Source, Location	Reference or strain source
B. cepacia	LMG 18821	CF, Australia	Mahenthiralingam et al. (2000)
	ATCC 17759	Soil, Trinidad	Mahenthiralingam et al. (2000)
B. multivorans	C5393	CF, Canada	Mahenthiralingam et al. (2000)
	C3430	CF, Canada	CRRR
	C5274	CF, Canada	CRRR
	C5568	CF, Canada	CRRR
	ATCC 17616	Soil, United States	Mahenthiralingam et al. (2000)
B. cenocepacia	715J	CF, Canada	McKevitt et al. 1989
	J2315	CF-e, United Kingdom	Mahenthiralingam et al. (2000)
	K56-2	CF-e, Canada	Mahenthiralingam et al. (2000)
	C6433	CF, Canada	Mahenthiralingam et al. (2000)
	C1257	CF, Canada	CRRR
	C5424	CF, Canada	Mahenthiralingam et al. (2000)
	C4455	CF, Canada	CRRR
	CEP511	CF, Australia	Mahenthiralingam et al. (2000)
	PC184	CF-e, United States	Mahenthiralingam et al. (2000)
B. stabilis	LMG 14294	CF, Belgium	Mahenthiralingam et al. (2000)
	LMG 18870	CF, Canada	Mahenthiralingam et al. (2000)
B. vietnamiensis	DB01	Soil, United States	Walsh and Ballou (1983)
	LMG 10929	Soil, Vietnam	Mahenthiralingam et al. (2000)

Species	Strain	Source, Location	Reference or strain source
	LMG 18835	CF, United States	Mahenthiralingam et al. (2000)
B. dolosa	LMG 18943	CF, United States	Coenye et al. (2001)
	LMG 21443	Soil, Sengal	Coenye et al. (2001)
B. ambifaria	LMG 19467	CF, Australia	Coenye et al. (2001)
	LMG 17828	Soil, United States	Mahenthiralingam et al. (2000)
	LMG 19182	Soil, United States	Mahenthiralingam et al. (2000)
B. anthina	LMG 16670	Soil, United Kingdom	Coenye et al. (2003)
B. pyrrocinia	LMG 14191	Soil, Japan	Coenye et al. (2003)
E. coli	HB101 (pRK2013)	NA	Figurski and Helinski (1979)
E. coli	JM109 (pTn*Mod*OTp')	NA	Dennis and Zylstra (1998)
E. coli	BL21(DE3) (pLysS)	NA	Invitrogen

Legend, abbreviations, CF, cystic fibrosis isolate, CF-e CF epidemic isolate, NA, not applicable, CRRR, Canadian *Burkholderia cepacia* complex Research and Referral Repository.

Table 3 Plasmids and plasposons used in this research.

Plasmid/Plasposon	Description	Reference
Plasmids		
pBBR1Tp	Broad-host-range cloning vector, derivative of pBBR1MCS	Kovach et al. 1995
pBBR*bceB*Tp	pBBR1Tp derivative containing *bceB* amplified from LMG 18821 chromosome inserted into MCS with *Kpn*I and *Cla*I	This study
p*bceB*Tp	pMB1oriR, trimethoprim resistance cassette and *bceB* amplified from LMG 18821	This study
Plasposons		
pTn*Mod*OTp'	Plasposon, trimethoprim resistance cassette	Dennis and Zylstra (1998)

Growth conditions

BCC strains were grown aerobically at 30°C in ½ Luria-Bertani (LB) broth for approximately 16 hours with shaking at 250 rpm or at 30°C on ½ LB solid agar (Sambrook et al. 1989). All *E. coli* strains were grown at 37°C on LB solid agar (when appropriate, supplemented with antibiotics), or in LB broth at 37°C with shaking at 250 rpm.

Experiments involving BCC EPS production were performed on yeast extract mannitol (YEM) media (0.45% wt/vol mannitol, 0.05% wt/vol yeast extract and 1.5% wt/vol agar) (Chung et al. 2003; Sage et al. 1990) with incubation at 37°C.

Bacteriophages were propagated on an appropriate BCC strain and stored at 4°C in suspension media (SM) (50 mM Tris/HCL, pH 7.5, 100 mM NaCl, 10 mM $MgSO_4$ and 0.01% gelatin solution) (Sambrook et al. 1989).

Determination of minimum inhibitory concentrations

Minimum inhibitory concentrations (MICs) were determined by subculturing 1 mL of liquid overnight cultures into 5 mL fresh broth and growing cells to mid-logarithmic phase (approximately 4 – 5 h). 10 μL aliquots of the mid-log phase culture was then transferred into the wells of a 96 well plate containing 100 μL LB broth supplemented with varying concentrations of the antibiotic being investigated. The 96 well plate was then incubated at 30°C overnight with shaking following which, the OD_{600} of each subculture was taken. Cells grown in LB broth containing no antibiotics provided a positive control.

Preparation of frozen bacterial stocks

For long-term storage, cells were grown overnight on solid LB media (supplemented with selecting antibiotics, when appropriate) and suspended in 2 mL of LB broth + 20% glycerol then transferred to a 2 mL Nalgene cryovial and stored at -80°C.

DNA isolation, manipulation and cloning

Isolation of bacterial chromosomal DNA and plasmid DNA

Genomic DNA was isolated from bacteria as previously described (Ausubel et al. 1999) and stored at -20°C. Plasmids were isolated using GeneJET^{TM} Plasmid Miniprep Kit (Fermentas, Burlington, ON) according to the manufacturers directions and stored at 4°C.

Isolation of phage genomic DNA

Phage genomic DNA was isolated using a modified version of the Cold Spring Harbor Protocol for isolation of lambda genomic DNA (Cold Spring Harbor Protocol; 2006; doi:10.1001/pdb.prot3972). Briefly, 10 mL of high titer phage lysate (~10^9 PFU/mL) was centrifuged at 10,000 rcf for 10 min at 4°C to remove bacterial debris. The supernatant was then filter sterilized using a 0.45 μm filter. The sterilized lysate was incubated at 37°C for 1 hour with 10 μL DNase I (Fermentas), 10 μL DNase buffer and 6 μL RNase (Fermentas). 0.5 M EDTA (ethylenediaminetetraacetic acid) (pH 8.0) was then added to a final concentration of 20 mM, 20 mg/mL proteinase K was added to a final concentration of 50 μg/mL and 500 μL of 10% SDS was added. The lysate was then mixed by

inversion and incubated for 1 hour at 37°C. Following the incubation, the mixture was cooled to room temperature and an equal volume of equilibrated phenol was added and inverted several times. The aqueous and non-aqueous phases were separated by centrifugation at 5,000 rpm for 5 min at room temperature. The aqueous phase was recovered, centrifuged again at 5,000 rpm for 5 min and the supernatant was kept. The extraction was repeated by adding an equal volume of chloroform, followed by centrifugation. The resulting aqueous phase was mixed with 0.1 volumes of 3 M NaOAC (pH 5.2) and 2.5 volumes of cold 100% ethanol, incubated for 20 minutes at -20°C and then centrifuged for 10 min at 12,000 rpm. The pellet was then washed with 1 mL of 70% ethanol, pelleted again by centrifugation and allowed to dry overnight. The dry pellet was resuspended in 200 μL TE (pH 8.0) and the final genomic DNA concentration (ng/μL) was determined using NanoDrop ND-1000 Spectrophotometer and software.

Purification of DNA following agarose gel electrophoresis and restriction enzyme digestion

DNA fragments that had been separated and visualized using agarose gel electrophoresis were recovered from the approximately 0.07% agarose gel using the Geneclean III Kit (Q-Biogene, Irvine, CA) or the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) (each used according to the manufacturers directions). Additionally, these kits were used according to the manufacturers directions to purify DNA that had been digested with restriction enzymes.

Polymerase chain reaction and DNA sequencing

Top Taq (Qiagen) was used to perform polymerase chain reactions (PCR) according to the manufacturers directions. The extension time for each PCR was set so that 1 min was given for each 1 kb of product to be amplified. The annealing temperature for each primer set and the primer sequences can be found in Table 4.

PCR performed using phage lysate as a template was done in the same manner as described above except, the template was sterilized phage lysate instead of prepared bacterial genomic DNA.

Colony PCR is a technique that is used to amplify DNA without having to first isolate genomic DNA from the clone of interest. Before the PCR reaction is allowed to run through the gradient cycler, the colony of interest is suspended in solution containing the appropriate PCR buffer, dNTPs, primers and mQH_2O. This is incubated for 5 minutes at 99.9°C, cooled to room temperature and an appropriate amount of Taq DNA polymerase is added. The PCR reaction is then carried out using a thermocylcer set to the desired PCR specifications.

PCR reactions were cleaned using QIAquick PCR Purification Kit (Qiagen) according to the product specifications.

Table 4 PCR specifications and primers

Primer set and sequence	Template (Amplified region)	Annealing temperature (°C)	Amplification product size (bp)
PCR: Top Taq DNA polymerase (Qiagen)			
F3: CCGATTCCCACATCACGATCC R3: TGCGGGCATTTCAGCTTTCG (Goudie 2008)	KS10 lysate or K56-2	70	~2000
bcebF-4: TCGCGGCAAGAATTTCCGATTATTTTTC bcebR4: AATTCCGCATTCGACACGACGACCGAGAAC	LMG 18821* or PC184*	60	~4850
TpPlas1: AAGGATCCATGCATGTCGACGGT TpPlas2: AAGGATCCGAATTCAGAGCTCGCTTAG	pTn*Mod*OTp'	55	~1400
EcobceB-F1: AA**GAATTC**AAACGCCTGTTCAAAGAA KpnbceB-R1: AA**GGTACC**GATTCGACACGACCGAGA	LMG 18821 or J2315	56	1846
DC1F: GTGATGGTGAACGTCGATAAGA DC1R: ATCAGGAAAGAGGCAGAAAGTG	DC1 lysate (bp: 59670-60378)	55	707
D5049F: AAATACTCGTGCGGCAGCAT D5049R: TGAAGAACGTCGACGGC	DC1 lysate (bp: 1492-6726)	U	6726
D3893F: CAGTCACCGTCGCAAATAAA D3893R: GATTGTTCCTGTGCCGTCTT	DC1 lysate (bp: 14135-18148)	U	4013
DC1AF: AAGGAGCAAAGAGCAT DC1AR: GACTCGATGACCAGAATG	DC1 lysate (bp: 20479-24502)	50	4023
DC1BF: ACGCTCATCTGCATCATCT DC1BR: GCCGGTAGTAGGAGAGGA	DC1 lysate (bp: 24228-26622)	50	2394
DC1CF: GAATGACAGCGACAGCA DC1CR: CAATCTCCTTCAGCAACG	DC1 lysate (bp: 26175-29159)	50	2984
DC1DF: ATGAAGCAGCAGCAGGAGAT DC1DR: GCAGATGTACGAGACGAGCA	DC1 lysate (bp: 29032-33140)	50	4108
DC1EF: GGTCGAAACGCTCCATTC DC1ER: CCGAACCATTGAGCCAGAT	DC1 lysate (bp: 32958-36188)	50	3230
DC1FF: TCAGGGCGGCAATCTT DC1FR: ACCGAGCTGAAATGACCAAT	DC1 lysate (bp: 828-1980)	52	1300
DC1GF: CGTCAAGGTGTGGCTCGT DC1GR: ACAGTGGCTCCATCTTTTGC	DC1 lysate (bp: 35286-36911)	52	1700
DC1HF: ACCATGTCGCAGGAAAAG DC1HR: AGGTGCTCGTCGAACTTCAG	DC1 lysate (bp: 40838-42408)	52	1570
DC1gp22-27F: GCATCAACTTCTGAGGACGC DC1gp22-27R: GCTTTTTGGTCGGTGGACAT	DC1 lysate (bp: 14653-18023)	50	3370
DC1GP51F: AGACGGAAATCGTGCTC DC1GP51R: AGTGTGCAGCGACAGAAACG	DC1 lysate (bp: 32345-33935)	50	1590
DC1GP56F: CAAGACCGCATCCATCATC DC1GP56R: ACGTGAGACAGTCAGCGTGT	DC1 lysate (bp: 35380-35800)	50	1589
DC1GP44F: AGCACATCCAGAAGCAGACC DC1GP44R: CGTAGTTGAACACCGAGCAC	DC1 lysate (bp: 28153-29707)	50	1554
DC1gp5F: GGAAAGCGGTGTTCATCTG DC1gp5R: TTCGGGAGGACAGTCAACTT	DC1 lysate (bp: 3544-4968)	50	1424
Sequencing Reactions			
$M13^{-40}$: GTTTTCCCAGTCACGAC (Fermentas)	V	NA	NA
$M13^{REV}$: CAGGAAACAGCTATCAC (Fermentas)	V	NA	NA
JDoriR: GGGGAAACGCCTGGTATC (Dennis and Zylstra 1998)	V	NA	NA

Primer set and sequence	Template (Amplified region)	Annealing temperature (°C)	Amplification product size (bp)
JDTpR: TTTATCCTGTGGCTGC (Dennis and Zylstra 1998)	V	NA	NA
BBRF: GTGGAATTGTGAGCGGATAAC	V	NA	NA
BBRR: CCAGGGTTTTCCCAGTCACGA	V	NA	NA

Legend. Unless otherwise specified, all primers were designed in this study. Bold face type indicates restriction enzyme recognition sequences. *, putative transformant colony from electroporation with p*bceB*Tp, NA, not applicable and V, various templates.

DNA sequencing was performed using the DYEnamic ET kit (Amersham Biosciences, Piscataway, NJ). Briefly, 11 μL sequencing buffer (20 mM Tris, 5 mM $MgCl_2 \bullet 6H_2O$, pH 9.0), 6 μL DNA template, 2 μL ET and 1 μL primer were combined on ice and the standard sequencing reaction was performed. This included 35 cycles of a 20 second denaturation at 95°C, a 15 second primer annealing step at 50°C and a 1 minute extension at 60°C. Following this, the sequencing reaction was maintained at 4°C. To stop and clean up the sequencing reactions, 2 μL of sodium acetate/EDTA and 80 μL of 95% ethanol were added and the mixture was incubated at –20°C for 15 minutes to precipitate DNA. DNA was pelleted by centrifugation at 13,200 rpm for 10 minutes following which, the supernatant was removed and the pellet was dried in a vacuum for 25 minutes. The Biological Sciences Molecular Biology Service Unit then used an ABI 377 Gene Analyzer to sequence the DNA. DNA sequences were analyzed using the software 4peaks and the NCBI BLASTN database (blast.ncbi.nlm.nih.gov/Blast.cgi). If required, sequence alignment was done using lalign server (http://www.ch.embnet.org/software/LALIGN_form.html).

DNA restriction enzyme digestions and ligations

Restriction enzymes and their cognate buffers from either Invitrogen or Fermentas were used to digest chromosomal and plasmid DNA according to the manufacturers directions. Phage genomic DNA digestions were done using Invitrogen enzymes and were allowed to incubate at 37°C overnight.

To ligate DNA, 15 μL of DNA, 1.5 μL T4 DNA ligase and 1.5 μL ligase buffer (Promega, Madison WI) were mixed together and incubated for 4 hours at 16°C or 22°C. Ligations involving only one restriction enzyme were performed at the lower temperature and ligations involving two restriction enzyme sites were performed at the latter temperature.

Transformation of chemically competent E. coli

Chemically competent subcloning efficiency *E. coli* DH5α (Invitrogen) cells were used in all transformations, unless otherwise specified. Transformations were performed according to the manufacturers directions, except, 500 μL of SOC (in 990 mL dH_2O: 20 g Bactotryptone, 5 g yeast extract, 0.5g NaCl, 2.5 mL 1 M KCl, 10 mL M $MgCl_2$ and 20 mL 1M glucose, pH 9.0 using 10 M NaOH) (Sambrook et al. 1989) was used instead of 950 μL and cells were incubated on ice for 30 seconds following the heat shock instead of 2 minutes. 5 μL of DNA was used in transformations.

Triparental Matings

Triparental matings were performed to mobilize a plasposon from *E. coli* to members of the BCC as described previously (Dennis and Zylstra 1998). Specifically, *B. cepacia* LMG 18821 and *B. cenocepacia* PC184 were mated with

E. coli harboring pTn*Mod*OTp'. Briefly, matings were conducted by combining the helper strain, *E. coli* HB101 pRK2013 (Figurski and Helinski 1979), with *E. coli* pTn*Mod*OTp' and the BCC strain of interest in 1 mL of 10% glycerol. The cell mixture was then pelleted by gentle centrifugation at 4,000 rcf for 4 minutes. The cells were resuspended in 100 μL of 10% glycerol, pooled on ½ LB solid agar and incubated at 30°C for approximately 16 h. Following this incubation, cells were resuspended in 1 mL of 10% glycerol and appropriate dilutions were spread onto *Pseudomonas* isolation agar (PIA) (Becton, Dickson and Company, Sparks, MD) supplemented with 300 mg/L Tp.

Construction of pbceBTp

In attempts to knockout *bceB* using targeted mutagenesis of *B. cenocepacia* PC184 and *B. cepacia* LMG 18821, p*bceB*Tp was constructed. To do this, *bceB* was amplified from LMG 18821 chromosome using the primers EcobceB-F1 and KpnbceB-R1. The trimethoprim (Tp) resistant cassette along with pMB1 origin of replication were amplified from pTn*Mod*OTp' (Dennis and Zylstra 1998) using the primers TpPlas1 and TpPlas2. Following PCR, the DNA fragments were visualized and purified using agarose gel electrophoresis. The recovered DNA was digested at 37°C with Fast Digest *Kpn*I and *Eco*RI (Fermentas), cleaned and ligated together.

The ligation mixture was used to transform *E. coli* DH5α using LB media supplemented with 100 mg/L Tp to select for clones. Plasmids isolated from the resulting clones were analyzed using restriction digestion profile analysis and

DNA sequencing using the primers TpPlas1, TpPlas2, EcobceB-F1 or KpnbceB-R1.

Construction of pBBRbceBTp

To construct a plasmid that could supply a functional copy of *bceB* in *trans* in *B. cenocepacia* K56-2, the *bceB* gene from *B. cepacia* LMG 18821 was amplified and inserted into the multiple cloning site of pBBR1Tp (Kovach et al. 1995) using *Cla*I and *Kpn*I. Briefly, *bceB* amplified from LMG 18821 chromosome using the primer pair EcobceB-F1/ KpnbceB-R1 was digested with *Cla*I and *Kpn*I (Invitrogen) and ligated to purified pBBR1Tp digested with *Cla*I and *Kpn*I (Invitrogen).

The ligation mixture was used to transform *E. coli* DH5α using LB media supplemented with 100 mg/L Tp to select for clones carrying pBBR*bceB*Tp. Plasmids isolated from recovered clones were digested with *Cla*I, *Kpn*I or *Kpn*I and *Cla*I and visualized using agarose gel electrophoresis. Plasmids with appropriately sized fragments were confirmed to be pBBR*bceB*Tp by sequencing using BBRF or BBRR.

Isolation and characterization of bacteriophages

Isolation of phages from soil rhizosphere

Soil obtained from the rhizosphere of a domestic vegetable Garden outside of Calmar, Alberta was used as a potential reservoir for bacteriophages of the BCC. Briefly, approximately 10 g of soil was incubated with 10 mL of ½ LB broth, 1 mL SM media and 100 μL of a 16 h overnight culture of K56-2 or PC184

for 24 h with shaking at 250 rpm. After incubation, soil debris was removed by centrifugation at 10,000 rcf for 10 minutes and the supernatant was sterilized by passage through a 0.45 μm filter and subsequent addition of $CHCl_3$ (Seed and Dennis 2005). To screen for phage, 400 μL of supernatant and 100 μL of bacterial culture were incubated together for 20 minutes at room temperature followed by the addition of 3 mL of molten top agar maintained at 55°C. This bacteria/phage/media mixture was then poured onto ½ LB plates and incubated at 30°C overnight.

Preparation of high titer stocks

Putative phages SR1, SR2 and SR3 were isolated by picking individual plaques from lawns of PC184 infected with supernatant from soil + K56-2 using a sterilized glass Pasteur pipette and placing the plugs into 1 mL of SM media followed by sterilization. This was incubated at room temperature for 1 h and then stored at 4°C. These phage stocks were then used to create individual high titer stocks by flooding confluent lysis plates with 3 mL of SM and incubating these at 4°C with gentle rocking for 6 h. SM recovered after 6 h was sterilized and subsequently stored at 4°C. DC1 (Carpentier and Dennis, unpublished) stocks were made and stored in the same way except LMG 18821 or K56-2 were used as hosts to propagate the phages.

Determination of SR1, SR2 and SR3 host ranges

SR1, SR2 and SR3 host ranges were determined by testing the ability of the phages to infect each of the 28 BCC strains listed in Table 2. To do this, the soft agar overlay technique was used to examine the ability of each phage to

infect a particular bacterium. Briefly, 100 μL of each strain (grown overnight at 30°C with shaking in ½ LB broth) was added to 100 μL of high titer phage stock and incubated for 20 minutes at room temperature. 3 mL of molten top agar was added after incubation and this mixture was poured over ½ LB solid agar plates and incubated at 30°C for two overnights, checking for plaques after one overnight incubation.

PCR analysis of SR1, SR2 and SR3 using KS10 specific primers

Single SR1, SR2 and SR3 plaques were picked into SM from PC184 lawns and PCR was performed on each using the KS10 specific primers F3 and R3 (Goudie et al. 2008). K56-2 chromosome and PC184 chromosome provided the positive and negative controls, respectively. The amplified products were visualized using agarose gel electrophoresis.

Towards the identification of a phage depolymerase enzyme for cepacian

Determination of EPS production by members of the BCC

Each of the 28 strains of the BCC listed in Table 2 were assayed for their ability to produce EPS by streaking each out on YEM media and incubating at 30°C or 37°C for 24 h. Strains were scored for EPS production using the following scale: ++ copious amounts of EPS; +/- moderate EPS produced; - no EPS produced.

Screening of bacteriophages for depolymerase activity

KS1, KS5, KS6, SR1, KS12, KS14, DC1 and DC2 (Seed and Dennis 2005, Goudie et al. 2008, Lynch and Dennis, unpublished, Carpentier and Dennis,

unpublished) were screened for the presence of a depolymerase enzyme. Using the soft agar overlay technique and a very low MOI, each phage was tested against its host range for phage depolymerase activity, as previously described by Billing (1960) and Hughes et al. (1998). A diluted ½ LB top agar, containing 1 g select agar in 250 mL, was used in phage depolymerase experiments. YEM plates were used to assay for depolymerase activity and ½ LB solid agar plates were used as a control. The plates were incubated at 37°C and 30°C for up to one week.

Towards construction of LMG 18821 and PC184 bceB knockout mutants

Attempts to knockout *bceB* in LMG 18821 and PC184 using targeted mutagenesis were done using the newly created p*bceB*Tp. In attempts to recover a *bceB* insertion mutant in either of these two strains, a variety of electroporation strategies were employed and are described below.

Firstly, the standard electroporation protocol used to transform bacteria in this research was a modified version of the protocol outlined by Sambrook et al. (1989). Briefly, cells were resuspended in 1 mL of 10% glycerol and then pelleted by centrifugation at 5,000 rcf for 4 minutes. The cells were then washed 3 times with 450 μL of 10% glycerol. After the final wash, the pellet was resuspended in 70 μL of 10% glycerol and 5 μl of plasmid DNA (maintained in mQH_2O) was added. The mixture was allowed to incubate at room temperature for 5 minutes, following which, the cells were transferred to a 1.8 cm cuvette and exposed to a 1.8 kV electric field for 2.5 milliseconds using a MicroPulse^{TM} electroporation apparatus (Bio-Rad). Immediately following the electric pulse, 500 μL of SOC was added and the cells were allowed to recover for 1 hour at 30°C with shaking

at 250 rpm. After the recovery period appropriate dilutions of the culture was spread onto ½ LB supplemented with 300 mg/L Tp and incubated for two nights at 30°C or 37°C.

The standard electroporation protocol was altered in several ways to try and increase recovery of a *bceB* knockout in LMG 18821 and PC184. The changes included: increasing the amount of DNA added by adding 7, 9, 10, 11, 13, 15, 19, 10 or 25 μL of DNA; using 5 mL of cells (pelleted and resuspended in 1 mL of 10% glycerol) from a 16 h overnight culture; growing cells at 42°C with shaking for 5 h prior to electroporation; using 37°C as a recovery temperature; increasing the incubation time following exposure to an electric field; using 42°C as a recovery temperature; addition of Type One™ Restriction Inhibitor (Epicenter, Madison, WI) according to the manufacturers directions; increasing the electric field to 2.5 kV; and, chilling cells and reagents to 4°C during the electroporation protocol. Electroporations of LMG 18821 and PC184 with pTn*Mod*OTp' (Dennis and Zylstra 1998) and pBBR-1Tp (Kovach et al. 1995) served as positive controls in this set of experiments. Colonies recovered from plates were tested for the *bceB* knockout using colony PCR and the primers bcebF-4 and bcebR-4 as described in Table 4. In addition, the ability of recovered colonies to produce EPS on YEM media was examined.

Plasposon mutagenesis of PC184 and LMG 18821 to obtain EPS deficient mutant

pTn*Mod*OTp' (Dennis and Zylstra 1998) was electroporated into PC184 and LMG 18821 and subsequent transformants were screened for their phenotype on YEM media. The loss of the mucoid phenotype on YEM was used to choose

mutants to investigate for disruption of genes involved in cepacian biosynthesis. The plasposon was recovered from appropriate transformants as previously described, (Dennis and Zylstra 1998) and the insertion site was determined by sequencing using JDoriR and JDTpR primers. DC1 plaque morphology on ½ YEM and ½ LB media was then determined for each mutant.

Analysis of exopolysaccharide using magic angle spinning ^{1}H HR-MAS-NMR

Bacteria were prepared for magic angle spinning ^{1}H- HR-MAS-NMR as previously described (Szymanski et al. 2003). Briefly, PC184, LMG 18821, PC184bceK::Tn*Mod*OTp', PC184rml::Tn*Mod*OTp' and LMG 18821dry1 were grown on YEM media and harvested in 1 mL of D_2O-PBS. Cells were washed twice with D_2O-PBS and killed by incubating them for 1 h at room temperature in 10% sodium azide prepared in D_2O-PBS. Following the incubation, the cells were washed once again with D_2O PBS, pelleted and stored at -20°C. The samples were analyzed by Lieke van Alphen, a post-doctoral member of Dr. Szymanski's lab using a Varian Inova four channel 500 mHz NMR spectrometer.

Virulence assays using the Galleria mellonella model of infection

The *G. mellonella* killing assays were performed as previously described (Seed and Dennis 2008), except, a 250 μL Hamilton syringe with a reproducibility adapter was used to deliver bacteria into the larvae instead of a 10 μL Hamilton syringe. Additionally, mannitol (10 g/L) was added to the 10 mM $MgSO_4$ + 1.2 mg/mL ampicillin buffer for one round of killing experiments in attempts to see if the EPS stimulant could affect virulence. Also, virulence of PC184 and LMG 18821 grown overnight in S media (in 1 L, 12.5 g Na_2HPO_4, 2.0 g KH_2PO_4, 1.0 g

K_2SO_4, 1.0 g NaCl, 0.2g $MgSO_4 \bullet 7H_2O$, 1.0 g yeast extract, 1.0 g Casamino acid (Difco), 0.01 g $CaCl_2 \bullet 2H_2O$, 0.001 g $FeSO_4 \bullet 7H_2O$, 11.0 mL 1 M glucose) (Richau et al. 2000) was determined.

Construction of K56-2 bceB complementation strain

To provide K56-2 with a functional copy of *bceB* in *trans*, pBBR*bceB*Tp was introduced into K56-2 using electroporation. Transformants were selected using ½ LB supplemented with 300 mg/L Tp. Plasmid DNA was isolated from transformants, digested with *Cla*I and *Kpn*I (Invitrogen) and the resulting DNA fragments were analyzed using agarose gel electrophoresis. Further, complementation was confirmed using sequencing analysis of isolated plasmid DNA using the primers BBRR or BBRF.

Characterization of bceB complemented K56-2

Three K56-2 transformants carrying the pBBR*bceB*Tp were tested for the phenotype on YEM media. The plaque morphology of DC1 on the K56-2 *bceB* complemented transformants was tested on YEM and ½ LB using the soft agar overlay technique and a DC1 lysate stock containing either 2880 PFU/mL or 410 PFU/mL. Plates were incubated at 37°C for up to 72 h.

Construction of a DC1 library in *E. coli*

DC1 cloning with pUC19

Six replicates of 4 µL of DC1 genome (717 ng/µL) were digested with *Eco*RI (Fermentas) at 37°C overnight. DNA fragments visualized and isolated from a 0.07% agarose gel were individually isolated and ligated to pUC19 (also

digested with *Eco*RI) in a 6:1 ratio. Plasmids that had taken up a DC1 genome insert were selected for using X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) (Invitrogen) on LB + 100 mg/L Amp. Appropriate clones were chosen and plasmid DNA was analyzed using *Eco*RI restriction digestion and sequencing analysis using $M13^{-40}$ and $M13^{REV}$ primers.

DC1 cloning with pJET1.2/blunt

DC1 fragments that could not be cloned into pUC19 were obtained using PCR. PCR amplification products were ligated to pJET1.2/blunt (Fermentas) using the sticky end protocol provided by the manufacturer. The resulting plasmids were then transformed into *E. coli* DH5α as previously described using LB + 100 mg/L Amp to select for clones. Plasmid DNA isolated from resulting clones was digested with Fast Digest *Bgl*II (Fermentas) and sequenced using the pJET1.2/blunt forward and reverse primers provided by the manufacturer.

Testing E. coli DC1 clones for EPS degrading abilities

Clones that harbored a DC1 insert in pUC19, or pJET1.2/blunt were assayed for putative EPS clearing activity of *B. cepacia* LMG 18821 and *B. cenocepacia* PC184 lawns grown on YEM media. To do this, 100 μL of an overnight culture of LMG 18821 or PC184 was combined with 3 mL of molten top agar containing 100 μg/mL Amp and poured over YEM medium. Once the top agar was solidified, 5 μL of an overnight culture of *E. coli* DC1 clones were spotted onto the top agar. The plates were then incubated at 37°C for 72 hours.

pJET1.2/blunt clones that had the DC1 fragment orientated such that the ORFs were running 5'-3' out of PT7 promoter were electroporated into *E. coli*

BL21(DE3)pLysS (Invitrogen) and then tested for their ability to degrade EPS. In this assay, IPTG (Isopropyl β-D-1-thiogalactopyranoside) was used to induce the T7 RNA polymerase. To do this, an overnight culture of the desired clone was diluted 1:100 into 2 mL LB broth + 100 mg/L amp + 25 mg/L chloramphenicol (Cm) and allowed to grow for 1 h with shaking at 37°C. Two μL of 0.1 M IPTG was then added and the culture was allowed to grow an addition 3 h following which the clones were tested for possible EPS degradation as previously described.

Obtaining transmission electron microscope images of DC1

A high titer DC1 stock ($4.7X10^9$ PFU/mL) was used to obtain a transmission electron microscope (TEM) image of DC1. The phage lysate was spotted onto a copper grid and stained with 2% phosphotungstic acid. The images were taken by a member of the Biological Sciences Microscopy unit using a Philips/FEI (Morgagni) Transmission Electron Microscope with CCD camera.

Isolation of a putative DC1 prophage in LMG 18821

A putative DC1 LMG 18821 prophage was isolated by taking a colony from the centre of a plaque formed on ½ LB media after the plates had been incubated at 30°C for one week. Two resulting clones (LMG 18821R1 and LMG 18821R2) were chosen to investigate for the presence of a DC1 prophage. To do this, the clones were grown in liquid culture and then tested for DC1 susceptibility using the soft gel agar overlay technique. Additionally, colony PCR was performed on LMG 18821R1 and LMG 18821R2 using the primers DC1F and DC1R.

RESULTS AND DISCUSSION

Isolation of the bacteriophage SR1

Isolation of a novel bacteriophage

Three potentially novel bacteriophages, SR1, SR2 and SR3, were isolated from *B. cenocepacia* K56-2 culture supernatants collected from cultures grown with soil planted to onions, carrots and chives. The three phages were each isolated from single plaques formed on *B. cenocepacia* PC184 lawns. SR1, SR2 and SR3 were each propagated to high titer and their host ranges were determined against the BCC strains listed in Table 2. The three phages were found to have the same host range, plaque morphology and stability when stored at 4°C. The host range included *B. cenocepacia* PC184, *B. multivorans* ATCC 17616, *B. stabilis* LMG 18870 and *B. ambifaria* LMG 19467. As no differences were seen between SR1, SR2 and SR3 and because they were isolated from the same culture supernatant in a single soft-gel overlay plaque assay, they were deemed to be the same phage. The phage isolated in this work was subsequently called SR1. Interestingly, it was the first phage isolated in the Dennis lab that was found to infect a *B. multivorans* strain.

Identification of SR1 as a variant of KS10

KS10, a bacteriophage isolated previously (Seed and Dennis 2005) has many similarities to SR1. KS10 is a prophage in K56-2 and since SR1 was isolated from K56-2 culture supernatants, it was required that SR1 be investigated as a potential variant of KS10. Additionally, both phages infect all the same BCC strains with the exception of *B. multivorans* ATCC 17616, which is only

susceptible to SR1 infection. Further, KS10 and SR1 both do not infect *B. cenocepacia* K56-2, a strain that exhibits a very phage susceptible phenotype.

PCR analysis using primers specific for a unique KS10 sequence showed that SR1 is a variant of KS10, Figure 5. In a PCR reaction using the primer pair F3/R3 a 1.0 kb product was amplified from SR1 lysate and K56-2 chromosomal DNA, positively identifying SR1 as a variant of KS10. PC184 chromosomal DNA provided a negative control for PCR, as it does not harbor a KS10 prophage.

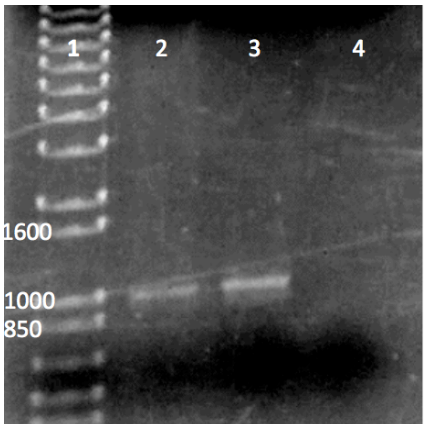

Figure 5 PCR analysis of SR1 lysate using F3/R3 primers. Legend. 1, 1 kb+ ladder (Invitrogen), 2, SR1 lysate, 3, K56-2 chromosomal DNA, 4, PC184 chromosomal DNA. All PCRs were performed using TopTaq, a 1 minute extension time and an annealing temperature of 70°C.

SR1 was probably not present in the soil samples used during attempts to isolate phage from the environment. It is hypothesized that the conditions used did not enrich BCC specific phages or, did not allow for the detection of BCC phages. SR1 is a competitive mutant of KS10 that likely arose from spontaneous mutations that occurred to KS10 as it was propagating lytically in K56-2. Such a mutation could allow this phage to have an expanded host range and explains the only observable difference between these two phages. This hypothesis was

recently confirmed by Lynch and Dennis (unpublished) who sequenced the SR1 genome and compared it to the sequence of KS10 published previously (Goudie et al. 2008). SR1 was found to contain more than one base pair change from KS10, but only one base pair change was found at a position of interest. At base 35,918 there is a single base change in SR1 that is predicted to shorten the tail fiber by 205 amino acids. Lynch and Dennis (unpublished) hypothesize that this may result in the expanded host range observed in SR1.

Towards the identification of a BCC specific phage carrying a depolymerase enzyme

Exopolysaccharide production by members of the BCC

Members of the BCC were assayed for their ability to produce EPS on YEM media. Of the 28 strains examined, 20 appeared mucoid on YEM media, which showed that they were able to produce EPS (Richau et al. 2000), Table 5. Strains were scored qualitatively for EPS production as being copious EPS producers (++), moderate EPS producers (+/-) or non-EPS producers (-), Figure 6 provides an example of each.

In the current study, 71% of strains tested were mucoid. Compared to results published by Moreira et al. (2003) and Zlosnik et al. (2008), this is a decrease of approximately 10% in the number of strains that produce EPS. This decrease could be due to the fact that three of the tested *B. cenocepacia* strains are from the ET-12 lineage, which has been shown to carry a 11 bp deletion in *bceB*, the gene encoding UndPGPT (Moreira et al. 2003). This deletion results in a frameshift mutation that prevents cepacian biosynthesis by rendering the cells

unable to transfer glucose onto the inner membrane lipid carrier during cepacian biosynthesis.

Table 5 Exopolysaccharide production by members of the BCC

Species	Strain	EPS production on YEM
B. cepacia	LMG 18821	++
	ATCC 17759	++
B. multivorans	C5393	++
	C3430	++
	C5274	+/-
	C5568	++
	ATCC 17616	+/-
B. cenocepacia	715J	++
	J2315*	-
	K56-2*	-
	C6433	-
	C1257	++
	C5424*	-
	C4455	++
	CEP511	+/-
	PC184	++
B. stabilis	LMG 14294	-
	LMG 18870	-
B. vietnamiensis	DB01	+/-
	LMG 10929	++
	LMG 18835	++
B. dolosa	LMG 18943	++
	LMG 21443	++
B. ambifaria	LMG 19467	-
	LMG 17828	++
	LMG 19182	-
B. anthina	LMG 16670	++
B. pyrrocinia	LMG 14191	++

Legend. BCC EPS production when grown on YEM, ++, copious amounts of EPS produced, +/-, moderate amounts of EPS produced, -, no EPS produced. * denotes ET12 lineage.

The results presented in the current study are corroborated by Bartholdson et al. (2008) and Zlosnik et al. (2008) who reported on the EPS production of many of the strains tested here. Strains tested by all three studies included ATCC 17616, J2315, K56-2, PC184, LMG 10929 and LMG 14294. The results were the same for all the strains except for LMG 14294, which was reported as mucoid when grown in the presence of mannitol or fructose by Bartholdson et al. (2008), but as non-mucoid when mannitol is provided in the growth medium by Zlosnik et al. (2008), and in this study, Figure 6. The reason for this discrepancy is not known.

The EPS production capabilities of C5424, C6433, C5393 and ATCC 17759 reported here are supported by Zlosnik et al. (2008). However, in this study CEP511 was found to be more mucoid than Zlosnik et al. (2008), Figure 6. The reason for this difference is not known. It is important to note that all reports on EPS production in this study and in the other two studies discussed were done using the same YEM media recipe and scored qualitatively. Quantitative measurements may be required to obtain the most accurate account of EPS production by members of the BCC.

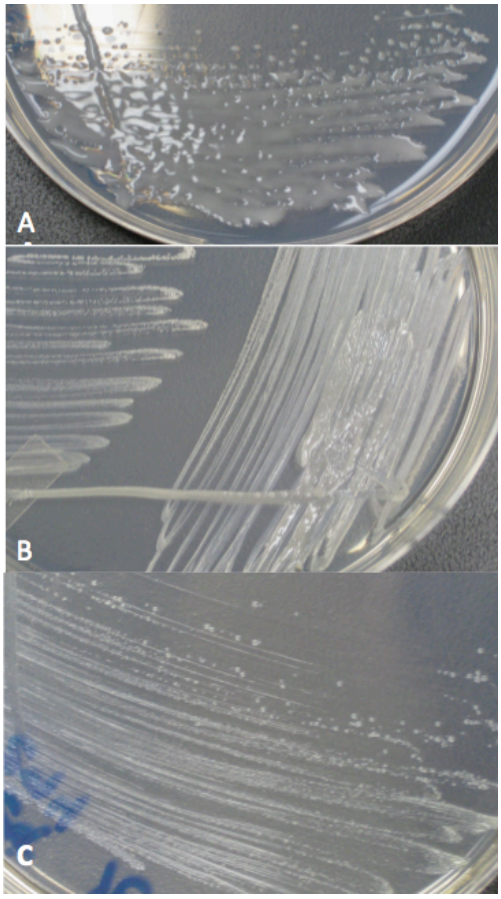

Figure 6 Copious, moderate and non-EPS production capabilities of the BCC. Legend. Photographs showing representative strains demonstrating copious (++), moderate (+/-) and non-EPS (-) production capabilities. A, *B. cenocepacia* 715J (++), B, *B. cenocepacia* CEP511 (+/-), C, *B. stabilis* LMG 14294 (-).

Mannitol is widely accepted as an inducer of EPS production by members of the BCC and is currently one of the most common sugar alcohols used to promote EPS production during studies involving EPS (Bartholdson et al. 2008; Herasimenka et al. 2007; Moreira et al. 2003; Sage et al. 1990; Zlosnik et al. 2008). Alarmingly, mannitol is used in the treatment of CF patients because it can act as an osmolyte that is able to hydrate the mucus in the CF lung and has been found to improve mucus clearance from the respiratory tract (Bartholdson et al. 2008; Minasian et al. 2007). The effect that inhaled mannitol has on the

phenotype adopted by BCC strains *in vivo* has not been elucidated and deserves further investigation since many BCC clinical isolates have been found to produce EPS.

Identification of a phage expressing a putative phage depolymerase

Eight bacteriophages, KS1, KS5, KS6, SR1, KS12, KS14, DC1 and DC2 were investigated for expression of a phage depolymerase that would be active against the EPS produced when strains are grown on YEM media, Table 6. The presence of a depolymerase enzyme was assayed for by looking for large and expanding halos surrounding phage plaques when host strains were grown on EPS inducing media (Bartholdson et al. 2008; Herasimenka et al. 2007; Hughes et al. 1998*a*; Moreira et al. 2003; Sage et al. 1990; Zlosnik et al. 2008). All phages were able to infect their hosts under EPS inducing conditions, suggesting that for these phages, EPS does not provide a barrier to phage infection. But, only plaques formed by DC1 when infecting LMG 18821 and PC184 were surrounded by halos. The halos were visible after one overnight incubation at 30°C or 37°C and increased in size for up to one week. The large and expanding halos were absent from infection on non-EPS inducing media. In all cases, all phages were found to be able to infect their hosts while producing EPS, suggesting that at least for the phages examined, EPS does not block phage infection. Transmission electron micrograph examination of DC1 showed that it is a podoviridae, Figure 7.

Table 6 Phages tested for depolymerase activity and their EPS-producing hosts

Phage	Host range	Reference
KS1	*B. cenocepacia* 715J	Seed and Dennis (2005)
KS5	*B. cepacia* LMG 18821 ATCC 17759 *B. cenocepacia* 715J	Seed and Dennis (2005)
KS6	*B. cepacia* LMG 18821 *B. multivorans* C5393 C5274 *B. cenocepacia* 715J	Seed and Dennis (2005)
SR1	*B. cenocepacia* PC184 *B. multivorans* ATCC 17616	This study
KS12	*B. cenocepacia* C5274	Goudie et al. (2008)
KS14	*B. multivorans* C5393 *B. cenocepacia* 715J C5274 PC184 *B. dolosa* LMG 21443 *B. ambifaria* LMG 178283	Goudie et al. (2008)
DC1	*B. cepacia* LMG 18821 *B. cenocepacia* PC184 CEP511	Carpentier and Dennis (unpublished)
DC2	*B. cenocepacia* C5274	Carpentier and Dennis (unpublished)

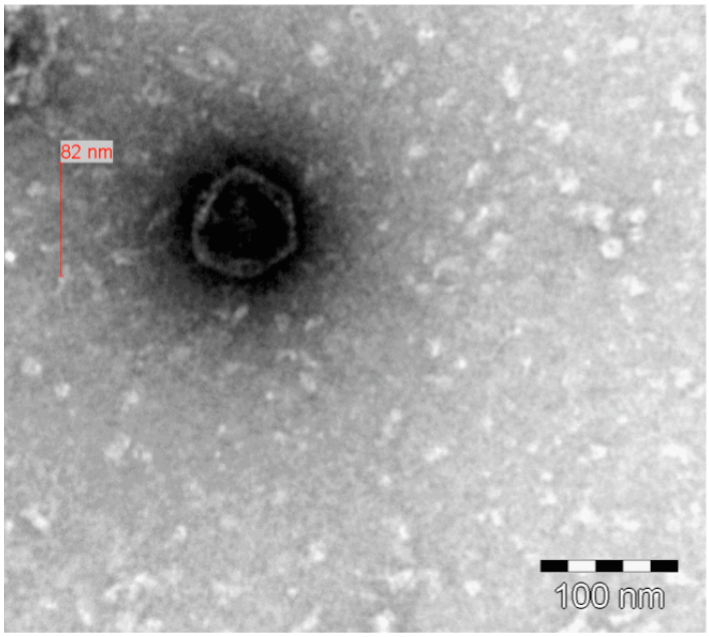

Figure 7 Transmission electron micrograph of DC1. Image taken at 140,000X magnification using a Philips/FEI (Morgagni) Transmission Electron Microscope with CCD camera in the University of Alberta Biological Sciences Department's Microscopy Unit.

Halo size was variable between the two strains, between plaques and had the greatest diameter when bacteria and phage were plated together on ½ YEM. The diameter of the halos observed on LMG 18821 lawns grown on ½ YEM ranged from 5 mm to 12 mm. DC1 plaques observed on LMG 18821 lawns grown on ½ LB media ranged from 2 mm to 4 mm and had no visible halos, Figure 8. The halos observed around DC1 plaques on PC184 lawns grown on ½ YEM were also very large, and ranged from 4 mm to approximately 10 mm.

DC1 plaques on PC184 lawns grown on ½ LB media had a different morphology than those on ½ YEM. DC1 plaques formed on PC184 when grown on non-EPS inducing media had small halos or turbid areas surrounding them. These were not large and, unlike the halos formed on EPS inducing media, did not

increase in size with time. This plaque morphology is attributed to the fact that DC1 is a temperate phage capable of entering the lysogenic life cycle. It is hypothesized that the small halos seen around plaques on ½ LB are from DC1 replicating as a prophage in PC184. These halos are not related to the putative depolymerase action of DC1 as they are observed under non-EPS inducing conditions.

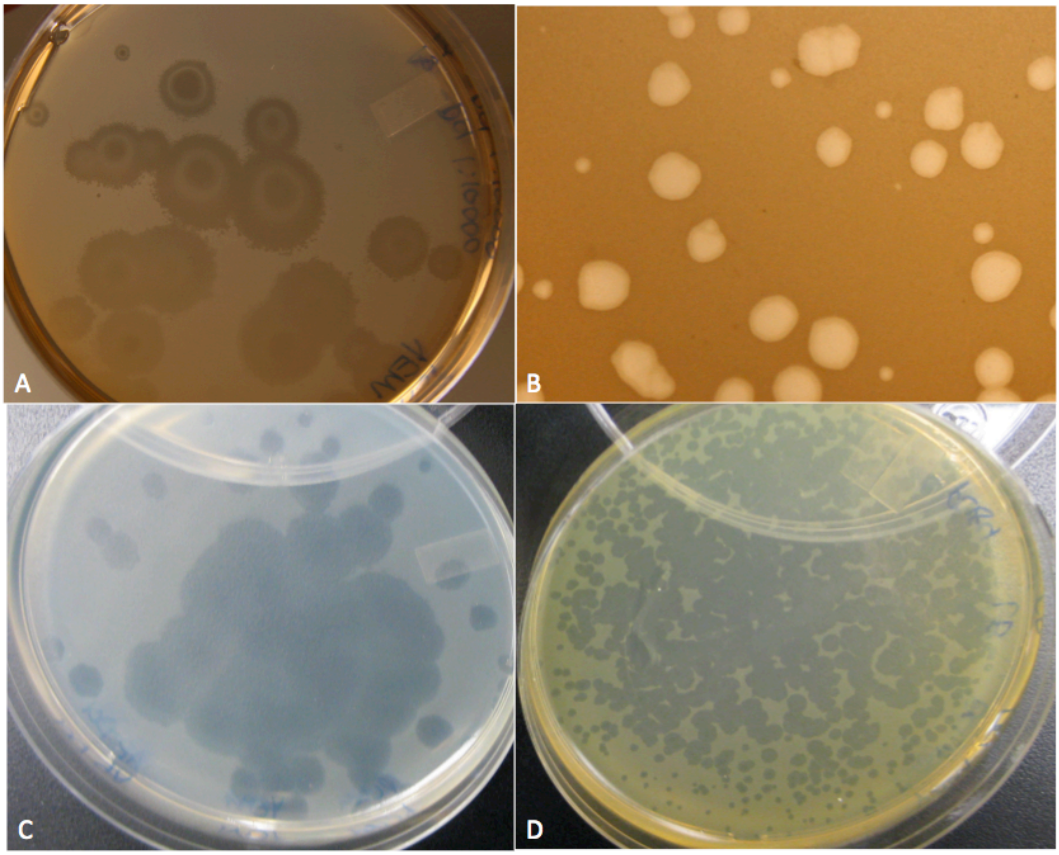

Figure 8 DC1 plaques on LMG 18821 and PC184. Legend. A, DC1 plated on LMG 18821 on ½ YEM media, B, DC1 plated on LMG 18821 on ½ LB media approximately 1X magnification, C, DC1 plated on PC184 on ½ YEM media. D, DC1 plated on PC184 on ½ LB.

No halos were seen when DC1 was allowed to infect the susceptible non-EPS-producing strains, K56-2, C6433 and 18870 and the minor EPS-producing strain CEP511 when the plaque assay was performed on either YEM, ½ YEM or ½ LB. This suggests that EPS is not required for DC1 infection.

Historically, the presence of halos surrounding plaques is used as the key and first identifying factor of phage depolymerases (Bartell et al. 1966; Bartell and Orr 1969; Billing 1960; Castillo and Bartell 1976; Higashi and Abe 1978; Nimmich et al. 1991; Shaburova et al. 2009; Sutherland 1967; Tomlinson and Taylor 1985). The presence of halos surrounding DC1 plaques when plated with EPS-producing bacteria and the absence of halos surrounding plaques on non-EPS-producing bacteria or under non-EPS inducing conditions suggest that DC1 carries a putative phage depolymerase enzyme that is active against the EPS induced by manntiol.

Preliminary experiments done by a collaborator, Cescutti, show that the EPSs produced by LMG 18821 and PC184 both contain cepacian and that LMG 18821 also co-produces the Kdo-galactan polymer. Interestingly, Cescutti predicts that there is a difference in cepacian acetylation between these two strains. The significance of this is not known, but, previous work has shown that acetylation differences may not affect depolymerase enzyme activity (Nixon et al. 1987). To date, no literature has been published on acetylation differences in cepacian polymers between BCC strains. It is hypothesized that cepacian is putatively degraded by DC1 because it is the only EPS that is produced by both strains

(Cescutti, unpublished), and its production is thought to be induced by mannitol (Bartholdson et al. 2003, Cescutti, unpublished).

Previous studies on phages predicted to have depolymerase enzymes have been expanded to definitively show the activity of such an enzyme by purifying active enzyme from phage lysate and showing its ability to reduce the viscosity of its target polymer. Our collaborator, Cescutti, has attempted to assay for cepacian degradation by looking for a loss in EPS viscosity following incubation with phage and/or phage lysate supernatants. No EPS degradation products could be identified using ESI-MS analysis of EPS extracts incubated with phage and/or phage lysate. Further, the viscosity of cepacian was not changed by phage and/or phage lysate, suggesting that by this method, no phage depolymerase activity could be observed or defined. It has been extremely difficult to purify active putative phage depolymerase from phage DC1 even though the phage can be propagated at titers as high as 10^{10} PFU/mL. Cescutti predicts that 10^{11} PFU/mL may be required to purify sufficient amounts of DC1 enzyme for *in vitro* degradation assays.

Construction of EPS deficient mutants in B. cepacia and B. cenocepacia

An alternative approach was employed to provide further support to the hypothesis that cepacian degradation by DC1 was responsible for the formation of halos surrounding plaques. This included attempts to construct EPS deficient mutants in *B. cepacia* LMG 18821 and *B. cenocepacia* PC184 using targeted mutagenesis and random plasposon mutagenesis. If cepacian deficient mutants

could be constructed in these strains, then, DC1 plaque morphology on ½ YEM could be assessed in attempts to see if cepacian was required for halo formation.

In the targeted mutagenesis approach, the *bceB* gene was chosen for insertional inactivation because it had been shown that ET12 strains, which contain a frame shift mutation in this gene, are unable to synthesize cepacian (Moreira et al. 2003). Additionally, Bartholdoson et al. (2008) showed that inactivation of *bceB* in *B. ambifaria* AMMD resulted in a loss of EPS production.

A new plasmid, p*bceB*Tp, constructed in this study, was used to electroporate LMG 18821 and PC184 in attempts to knockout *bceB* in these strains through insertion of the plasmid at the *bceB* locus, Figure 9. Over 30 standard electroporations were conducted with each strain and resulted in the isolation of many (>200) PC184 colonies and very few LMG 18821 colonies (<20). All LMG 18821 colonies and approximately 50-100 PC184 colonies were tested for EPS production as well as insertional inactivation at the *bceB* locus. None of the tested colonies were EPS deficient or had inserted p*bceB*Tp into *bceB*, as determined by PCR.

The reason for the unusually high number of recovered PC184 colonies is unclear as the MIC of this strain is approximately 100 mg/L Tp and 300 mg/L Tp was used to select clones. It is possible that non-homologous recombination was occurring elsewhere in the genome to allow the clones to gain trimethoprim resistance. The colonies isolated were not contaminants as they were all able to grow on BCSA, a feature unique to members of the BCC (Henry et al. 1997).

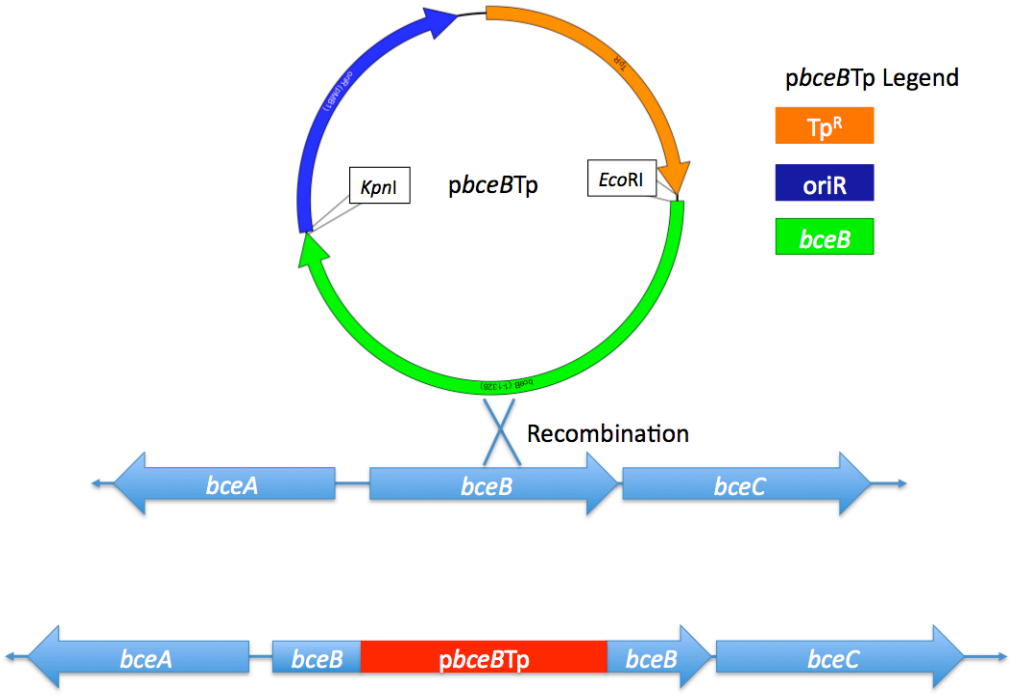

Figure 9 Schematic of p*bceB*Tp insertional inactivation of *bceB*. Legend. p*bceB*Tp (2809bp) constructed in this study using *Eco*RI and *Kpn*I to ligate *bceB* amplified from LMG 18821 chromosome to pMB1 and oriR amplified from pTn*Mod*OTp'. p*bceB*Tp was used in attempts to knockout *bceB* through insertion into the ORF of *bceB* by homologous recombination. Orange, trimethoprim resistance cassette, dark blue, pMB1 origin of replication, green, *bce*B, light blue, *bce* genes located on chromosome, red, insertion of p*bceB*Tp into chromosomal *bceB*.

The electroporation protocol was altered in many ways, as previously described in this document, to try and optimize for the construction of *bceB* mutants. An additional 50 electroporations were done, and a *bceB* insertional mutant was never recovered for either strain. The transformability of each strain was confirmed through successful electroporations with pBBR1Tp and pTn*Mod*OTp'. The reason for the inability to construct a *bceB* mutant using targeted mutagenesis is not known. It is unlikely that it is due to degradation of exogenous DNA because electroporations with pBBR1Tp and pTn*Mod*OTp' were

successful and a type I restriction inhibitor was not found to increase transformation efficiency. It could be due to potentially low amounts of RecA assisted recombination in either of these two strains.

Since all attempts at targeted mutagenesis of LMG 18821 and PC184 were unsuccessful, random plasposon mutagenesis of these strains was undertaken to obtain EPS deficient mutants. In this method, the strains were electroporated with pTn*Mod*OTp' and transformants were screened for loss of EPS production by streaking them on YEM media, and selecting those that were dry and non-mucoid. A variation of this method was used by Moreira et al. (2003) with great success and resulted in the identification of the *bce-I* gene cluster.

PC184 exhibits higher transformation efficiencies than LMG 18821 and as a result, the EPS production capabilities of approximately 3000 PC184 transformants and approximately 100 LMG 18821 transformants was screened. Two PC184 EPS deficient mutants and one LMG 18821 EPS deficient mutant were isolated.

Sequence analysis of the plasposon insertion site in the PC184 mutants showed that the plasposon had inserted in *bceK*, a putative glycosyl transferase (Moreira et al. 2003) and in the *rml* region, which encodes the genes involved in dTDP-L-rhamnose biosynthesis (Vinion-Dubiel and Goldberg 2003). The mutants were renamed PC184bceK::Tn*Mod*OTp' and PC184rml::Tn*Mod*OTp', respectively. Sequence analysis of the plasposon insertion site in the LMG 18821 non-mucoid mutant was less informative. Sequences with homology were found to encode for hypothetical proteins with unknown function from a variety of

species. Further, protein domain searches did not provide any functional information about the plasposon insertion site. This mutant was called LMG18821dry1 and interestingly, its pigmentation is produced earlier and it is more yellow on YEM media than wildtype LMG 18821.

DC1 plaque morphology on non-mucoid B. cepacia and B. cenocepacia mutants

DC1 plaque morphology on the non-mucoid strains was investigated by infecting each with DC1 at a low MOI on EPS inducing (½ YEM) and non-inducing media. DC1 plaques observed on PC184bceK::Tn*Mod*OTp' lawns plated on ½ LB and ½ YEM were void of the large and expanding halos, but the small halos observed during infection of wildtype PC184 on ½ LB were present, Figure 10. This small halo phenotype is attributed to the fact that DC1 is a temperate phage, and likely some lysogeny is occurring in the bacteria surrounding the clear zones of lysis. *bceK* is the eleventh gene in the *bce* gene cluster and its product is a putative glycosyl transferase (Moreira et al. 2003). The exact process performed by BceK has not been elucidated, but it is likely that it is involved directly in the synthesis of cepacian because it is located in the *bce* cluster. It is reasonable to hypothesize that the dry and non-mucoid phenotype of PC184bceK::Tn*Mod*OTp' observed on YEM media is due to an inability to synthesize cepacian. The lack of the large and expanding halos surrounding DC1 plaques on YEM media support the hypothesis that cepacian degradation by DC1 is responsible for halo formation.

When plated on ½ YEM media, DC1 plaques on PC184rml::Tn*Mod*OTp' retained the large and expanding halos, Figure 11. The *rml* genes *rmlA*, *rmlB*,

rmlC and *rmlD* are commonly clustered together in Gram negative bacteria and are responsible for synthesizing dTDP-L-Rhamnose, a component of the O-antigen (Li et al. 2006; Vinion-Dubiel and Goldberg 2003). Interestingly, cepacian also contains rhamnose, but, it contains D-rhamnose instead of L-rhamnose (Cescutti et al. 2000). The enzymes involved in carbohydrate synthesis have been investigated and it is proposed that the GDP-D-rhamnose synthetase (GRS) is involved in the production of D-rhamnose for cepacian (Richau et al. 2000) and that the *rml* genes are involved in the production of L-rhamnose for the O-antigen (Vinion-Dubiel and Goldberg 2003). Genes *bceM* and *bceN* located in the second *bce* gene cluster putatively encode the enzymes comprising the GRS synthetase (Ferreira et al. 2010).

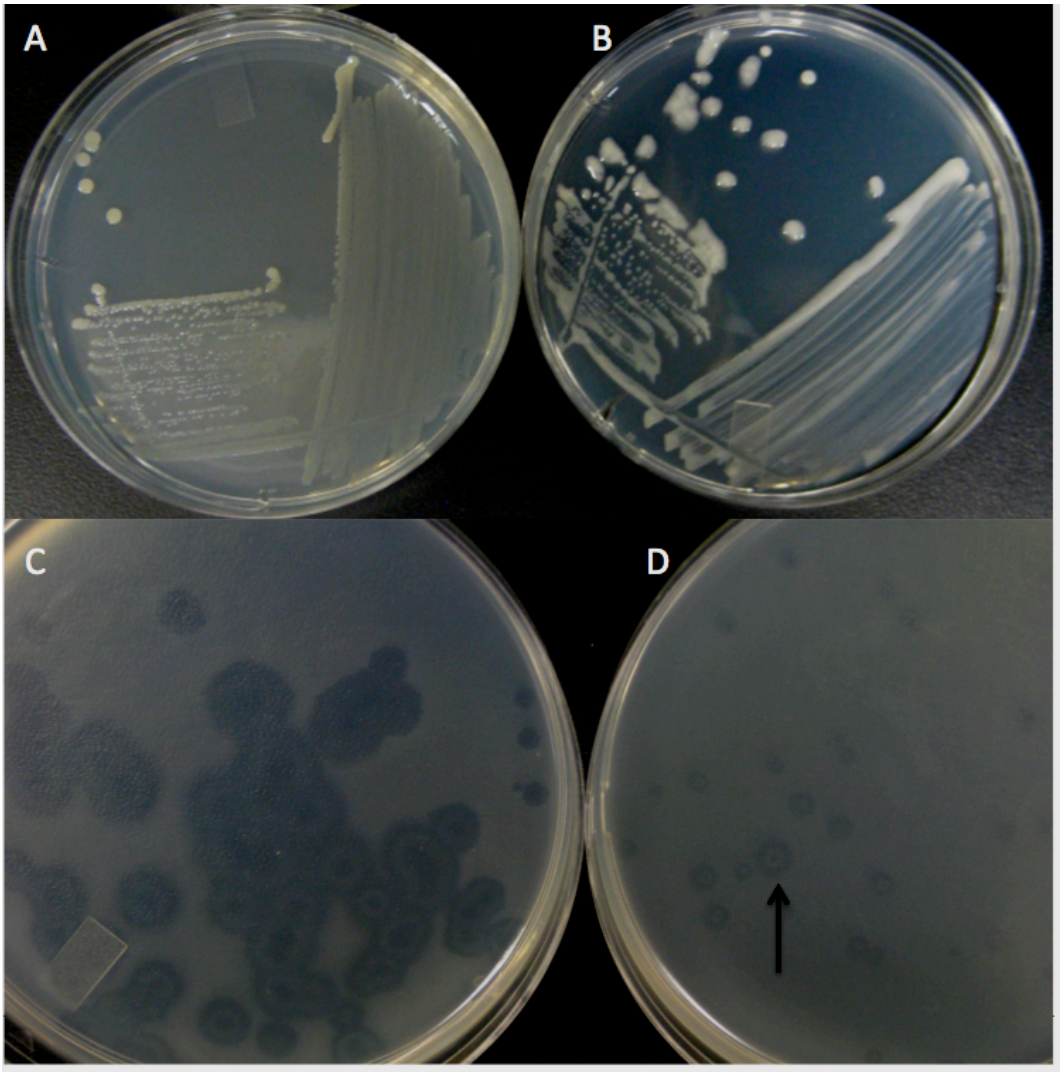

Figure 10 EPS production of and DC1 plaque morphology on PC184bceK::Tn*Mod*OTp'. Legend. A, PC184bceK::Tn*Mod*OTp' plated on YEM media; B, PC184 plated on YEM; C, DC1 plaques on PC184 grown on ½ YEM; D, DC1 plaques on PC184bceK::Tn*Mod*OTp' grown on ½ YEM. Black arrow, points to the small and turbid halo surrounding a DC1 plaque that does not increase in size with time.

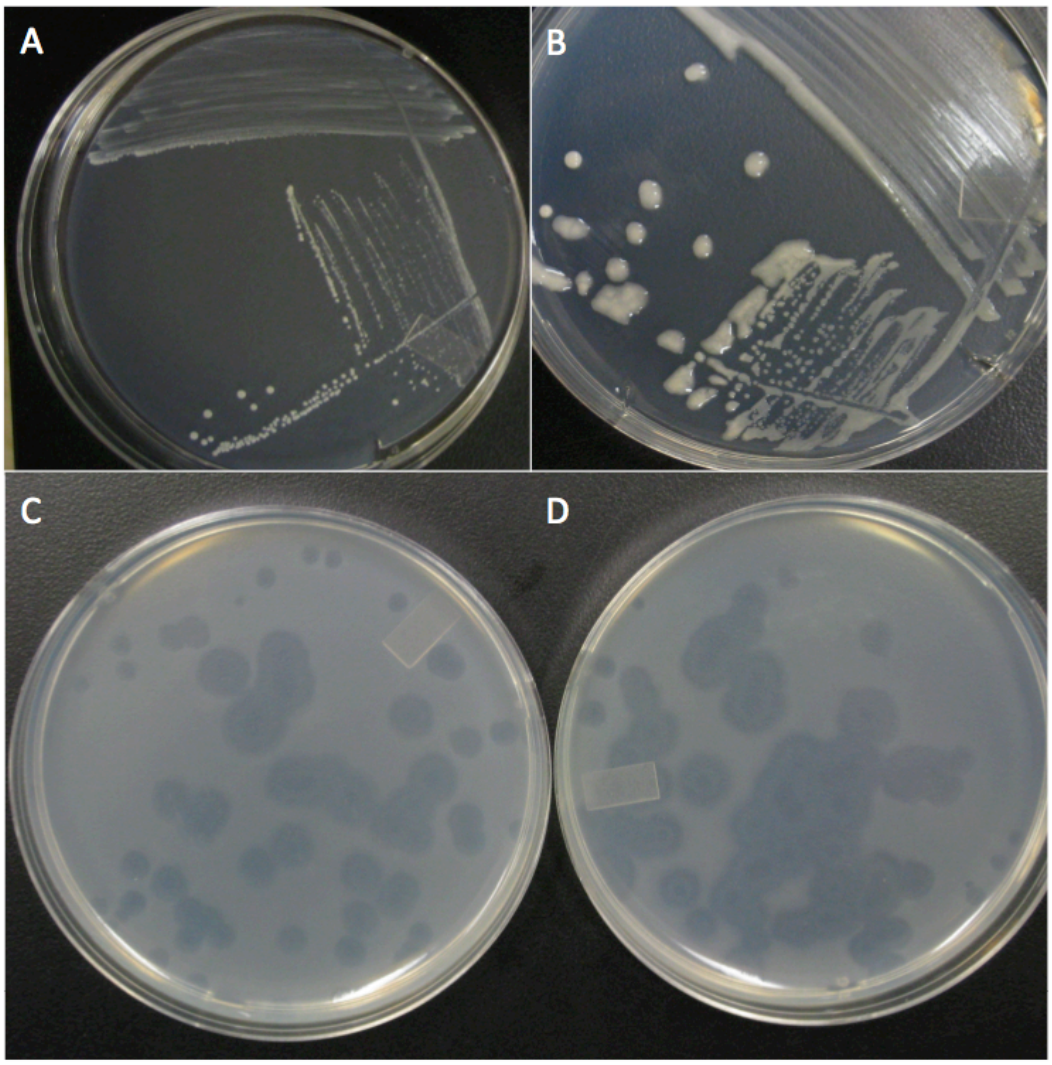

Figure 11 EPS production of and DC1 plaque morphology on PC184rml::Tn*Mod*OTp'. Legend. A, PC184rml::Tn*Mod*OTp' plated on YEM media; B, PC184 plated on YEM; C, DC1 plaques on PC184 grown on ½ YEM; D, DC1 plaques on PC184rml::Tn*Mod*OTp' grown on ½ YEM.

It remains unknown why PC184rml::Tn*Mod*OTp' has lost the mucoid phenotype, but presumably, this strain is still capable of producing D-rhamnose which is required for cepacian biosynthesis. It is possible that by some unknown mechanism, PC184rml::Tn*Mod*OTp' produces very low levels of cepacian such that it appears non-mucoid but is actually still able to provide a substrate for degradation by the putative DC1 depolymerase. The plasposon insertion could have also caused a polar mutation affecting downstream genes like the putative glycosyl transferase encoded by a gene found just downstream from the *rml* genes (Vinion-Dubiel and Goldberg 2003). Future work could include performing a western blot on whole cell lysates using anti-cepacian antisera in order to determine if this strain is indeed capable of producing low levels of cepacian. Additionally, EPS production by PC184rml::Tn*Mod*OTp' could be assayed using Sudan Black B dye (Moreira et al. 2003).

Finally, regardless of the medium used to grow the host lawns, DC1 plaques formed on LMG18821dry1 had no halos surrounding them, Figure 12. It is difficult to hypothesize why DC1 halo formation is lost because sequencing data was not informative when attempting to identify the plasposon insertion site. In attempts to determine if LMG18821dry1 and the two PC184 non-mucoid mutants were able to produce cepacian or if they produced an altered EPS, magic angle spinning ^{1}H-NMR was performed on prepared samples with help from van Alphen and Szymanski. Unfortunately, no conclusive data could be gathered using this technique (^{1}H-NMR spectrum not shown). Whether the inability to observe resonance in extracellular polysaccharides of the BCC strains was because the spectrometry conditions used may not have been optimized correctly for the BCC, or because cepacian is a very rigid and stiff polysaccharide remains unknown. Most likely the latter is the reason for the inconclusive data as magic angle spinning ^{1}H-NMR is most effective for flexible carbohydrate structures.

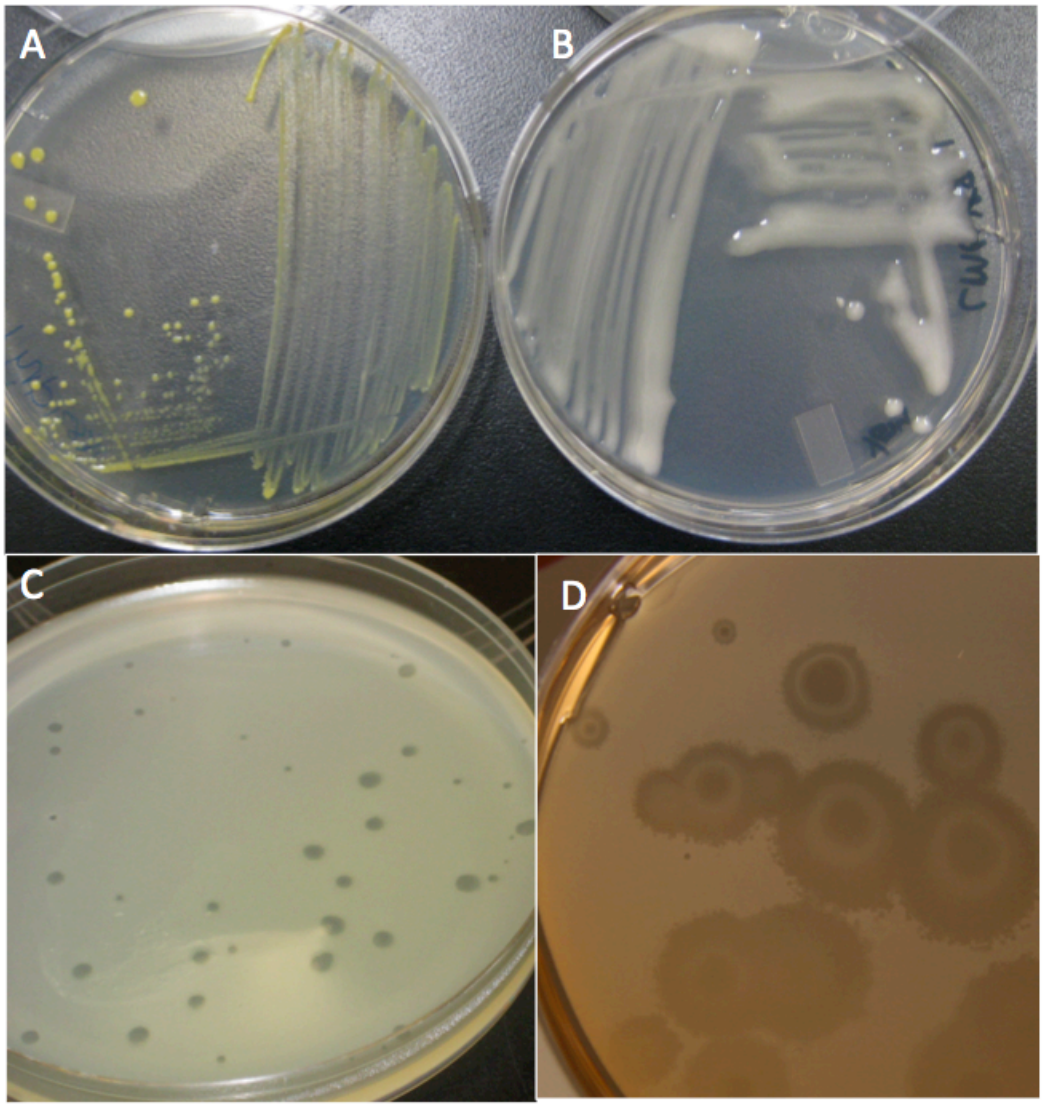

Figure 12 EPS production of and DC1 plaque morphology on LMG18821dry1. Legend. A, LMG18821dry1 plated on YEM media; B, LMG 18821 plated on YEM, C, DC1 plaques on LMG18821dry1 grown on ½ YEM, D, DC1 plaques on LMG 18821 grown on ½ YEM.

Virulence of EPS deficient mutants in Galleria mellonella infection model

The *G. mellonella* infection model was recently shown to be an effective model for studying the virulence of the BCC (Seed and Dennis 2008). Using this model, the virulence of PC184, PC184bceK::Tn*Mod*OTp', PC184rml::Tn*Mod*OTp', LMG 18821 and LMG18821dry1 was determined by observing the abilities of these strains to kill larvae. Larvae were scored as dead or alive 48 h pi as described by Seed and Dennis (2008).

PC184 is able to kill 100% of *G. mellonella* larvae 48 hours pi when 10^6 cells are delivered to the hindmost left proleg of the larvae, Figure 13. When the amount of bacteria used to inoculate the larvae hemolymph was decreased by one log, 75% of larvae were killed. No larvae were killed by PC184 when the infection dose was 10^4 or lower. PC184bceK::Tn*Mod*OTp' and PC184rml::Tn*Mod*OTp' were significantly attenuated for virulence, killing 70% and 10% of larvae when 10^6 cells are delivered to the larvae hemolymph, respectively ($p < 0.005$). At a 10^5 bacterial dose, PC184rml::Tn*Mod*OTp' was unable to kill any larvae and PC184bceK::Tn*Mod*OTp' killed only 40% of infected larvae. These results suggest that these two carbohydrate biosynthesis pathways may be important for the virulence of PC184.

Towards determining whether EPS expression may be important in *G. mellonella* killing by PC184, larvae killing was assayed after growing the cells in S media (liquid media that induces EPS production) and when mannitol was co-injected into the hemolymph with bacteria (grown in ½ LB), Figure 13. Growth in S media significantly decreased virulence at inoculums of 10^5 and 10^6. The reason

for the decreased virulence is not known. It is important to note that PC184 was only grown for 16 h in S media and other researchers commonly grow PC184 in S media for at least three days to allow for high amounts of EPS in the media (Cescutti, unpublished and Ferreira et al. 2010). The S media grown PC184 culture did not appear viscous compared to ½ LB grown PC184 cultures. It is possible that the cells were not producing large amounts of EPS when aliquots were taken to use for the virulence assay.

When mannitol was co-injected with bacteria into the hemolymph, there was no significant change in virulence at any bacterial dose. It remains unknown if the addition of mannitol was sufficient to allow PC184 to adopt a mucoid phenotype in the larvae hemolymph. It can be concluded then, that the integrity of the *rml* genes and the *bce-I* cluster are important for the virulence of PC184. Further, it can be concluded that growth in S media for 16 h prior to infection as well as co-injection with manntiol (1.0 g/L) does not increase virulence.

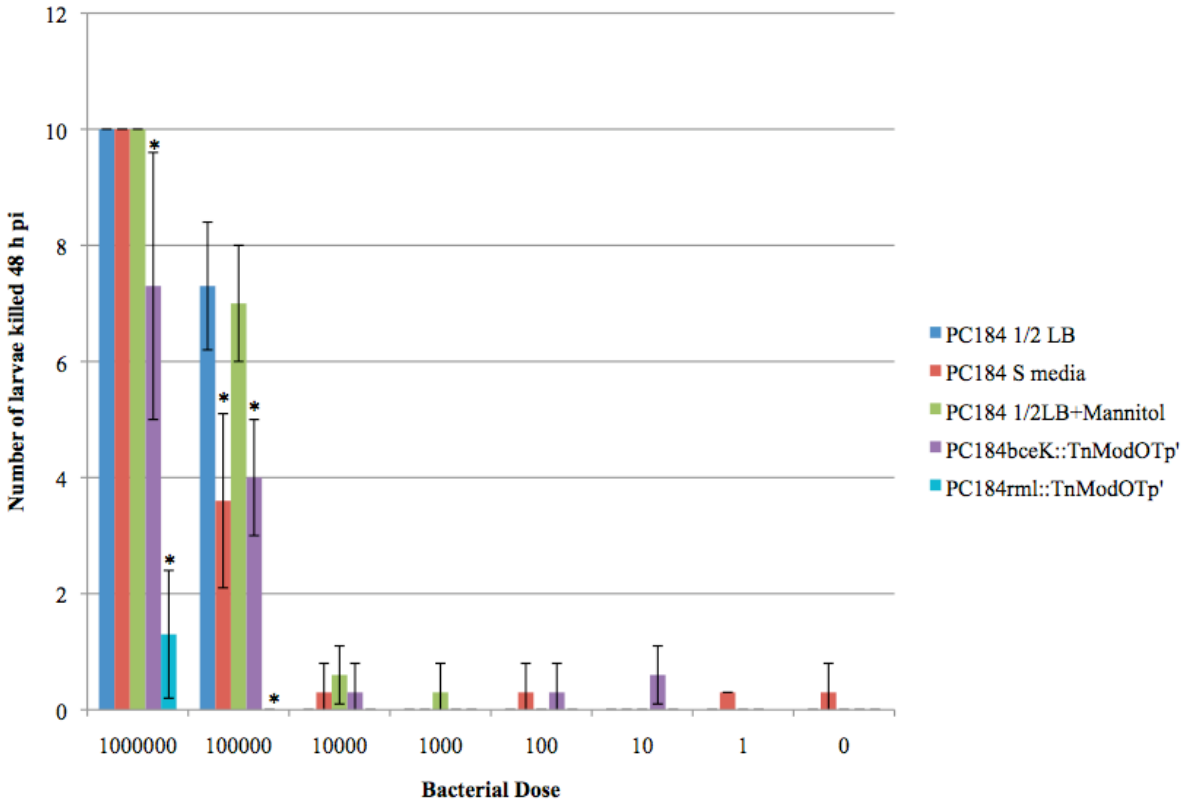

Figure 13 *G. mellonella* larvae killed from PC184 and PC184 non-mucoid mutants 48 h post-infection. Legend. Bacterial inoculums (5 µL) were delivered to the hindmost left proleg of *G. mellonella* larvae. Larvae were scored as dead or alive 48 h pi. Larvae were considered dead if they had melonized or if they did not respond to prodding. Blue, PC184 grown 16 h in ½ LB broth, pink, PC184 grown 16 h in S media, green, PC184 grown 16 h in ½ LB broth and co-infected with mannitol in buffer; purple, PC184bceK::Tn*Mod*OTp' grown 16 h in ½ LB broth, teal, PC184rml::Tn*Mod*OTp' grown 16 h in ½ LB broth. Virulence assays were done in triplicate, experiment groups contained 10 larvae and error bars represent standard deviation *, indicates statistically different from wild type PC184 grown in ½ LB ($p<0.005$).

LMG 18821 was shown by Seed and Dennis (2008) to be highly virulent in the *G. mellonella* model of infection, having an LD_{50} of only 30 CFU. In this work, LMG 18821 was again found to be highly virulent, killing 100% of larvae at inoculums as low as 10^4 and killing just under 50% at inoculums of 10^3, Figure 14. Interestingly, when LMG 18821 was co-injected with mannitol or grown overnight in S media prior to infection, the virulence increased. At inoculums of 10^3, LMG 18821 grown in ½ LB media was found to kill just under 50% of infected larvae, but LMG 18821 grown in EPS inducing conditions or co-injected with mannitol were found to kill almost 100% of infected larvae ($p \leq 0.0003$). LMG18821dry1 had no drastic change in virulence compared to wildtype. At inoculums of 10^4, LMG18821dry1 killed approximately 56% of larvae and wildtype LMG 18821 was found to kill approximately 40% of larvae ($p = 0.0035$). The reason for the increased virulence of LMG18821dry1 at this bacterial dose is not known.

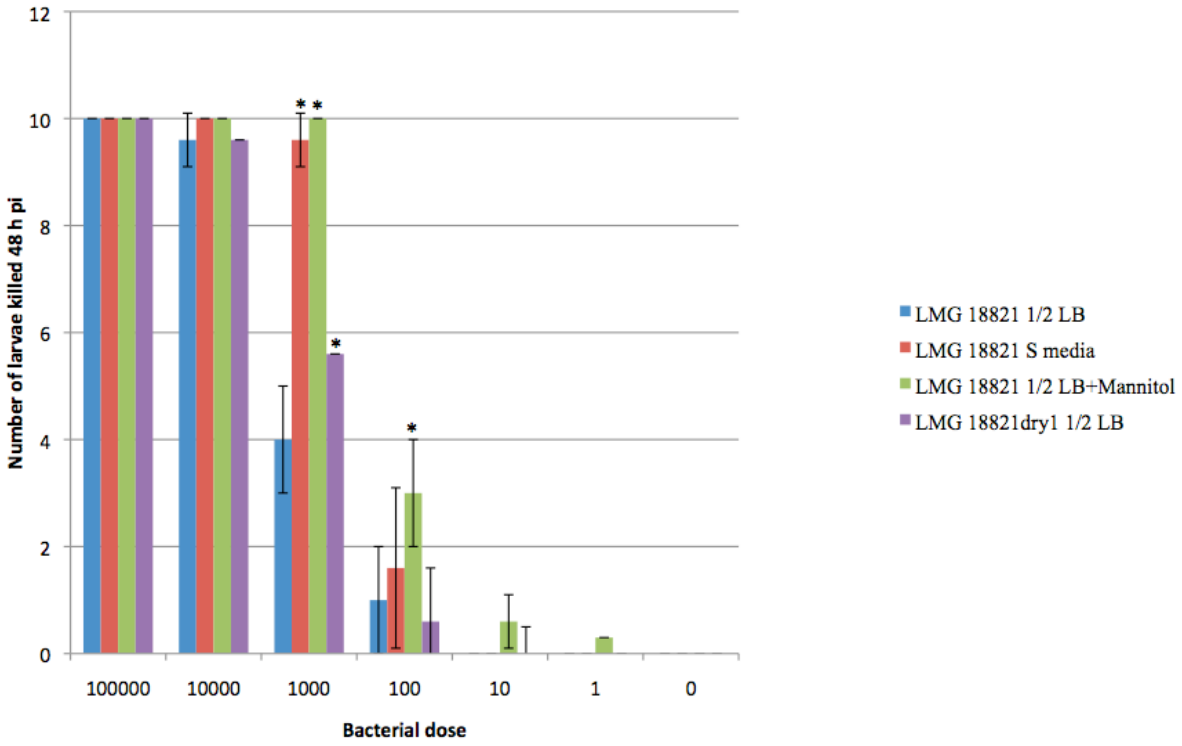

Figure 14 *G. mellonella* larvae killed from LMG 18821 and LMG18821dry1 48 h post-infection. Legend. Bacterial inoculums (5 µL) were delivered to the hindmost left proleg of *G. mellonella* larvae. Larvae were scored as dead or alive 48 h pi as previously described (Seed and Dennis 2008). Blue, LMG 18821 grown 16 h in ½ LB broth, pink, LMG 18821 grown 16 h in S media, green, LMG 18821 grown 16 h in ½ LB broth and co-infected with mannitol in buffer, purple, LMG18821dry1 grown 16 h in ½ LB broth. Virulence assays were done in triplicate, experiment groups contained 10 larvae and error bars represent standard deviation, *, indicates statistically different from wild type LMG 18821 grown in ½ LB ($p \leq 0.005$).

The *G. mellonella* infection model demonstrates that the non-mucoid mutants of PC184 are less virulent than wildtype PC184 when grown in media that does not induce EPS production. This suggests that the non-mucoid phenotype may elicit unobservable changes to PC184 when grown in ½ LB broth. Additionally, it suggests that for PC184, the mucoid phenotype and these carbohydrate pathways are important for virulence. The non-mucoid mutant LMG18821dry1 was not attenuated for virulence. Interestingly, growth for 16 h in EPS inducing media was able to increase the virulence of LMG 18821 and appears to decrease virulence of PC184 at lower bacterial doses. This difference could be due to non-optimized EPS-producing conditions for PC184, such as not providing enough time for cells to produce large quantities of EPS. Growth curves of LMG 18821 and PC184 in S media could be done to determine if there are any growth differences between these two strains and between S media and ½ LB that could explain the observed differences in virulence.

Towards construction of a EPS-producing B. cenocepacia K56-2

B. cenocepacia K56-2, a highly virulent, highly transmissible, non-EPS-producing member of the BCC (Mahenthiralingam et al. 2005; Seed and Dennis, 2008) is susceptible to DC1 infection. As a member of the ET12 lineage, this strain is unable to produce cepacian as it putatively contains the 11 bp deletion in *bceB* (Moreira et al. 2003). When infected by DC1 on EPS inducing or non-inducing media plaques are not surrounded by halos. In attempts to provide further support for the hypothesis that DC1 expresses a depolymerase enzyme that

can degrade cepacian, K56-2 was complemented with *bceB* in order to attempt to restore cepacian biosynthesis.

A functional copy of *bceB* was amplified from the chromosome of LMG 18821 using the primers EcobceB-F1 and KpnbceB-R1 and inserted into the multiple cloning site of pBBR1Tp using *Cla*I and *Kpn*I, Figure 15. The resulting plasmid, pBBR*bceB*Tp was used to transform K56-2 and a subsequent *bceB* complemented K56-2 strain was assessed for EPS production and DC1 plaque morphology.

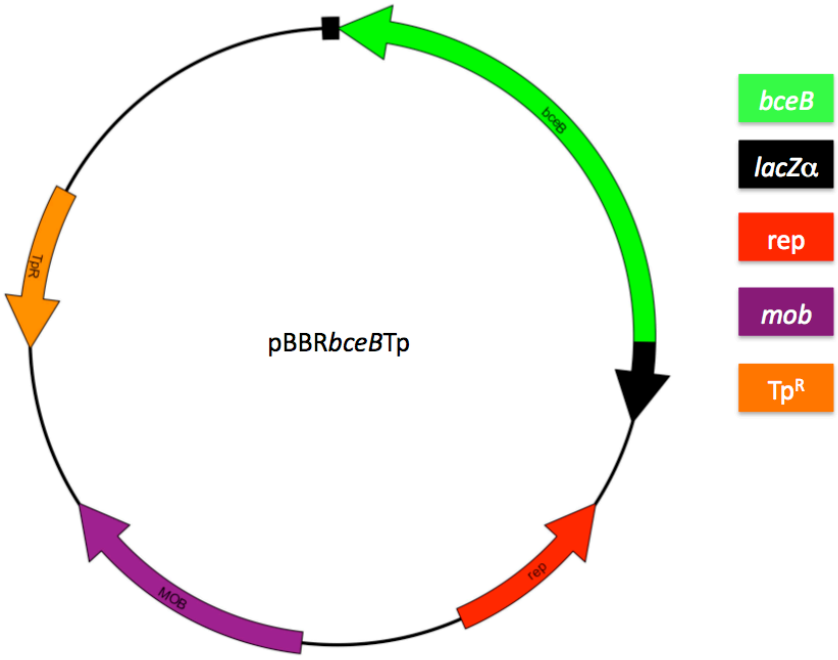

Figure 15 Schematic of pBBR*bceB*Tp. Legend. pBBR*bceB*Tp, 7105 bp. Green, *bceB* amplified from LMG 18821 chromosome, black, Bluescript II *lacZα* and multiple cloning site, red, origin of replication, purple, mobilization locus, orange, trimethoprim resistance cassette.

The K56-2 *bceB* complemented strain did not produce EPS and remained non-mucoid on YEM media. DC1 plaque morphology on K56-2 lawns grown on ½ LB or ½ YEM was determined; no observable differences were observed between the two growth substrates, and no halos were observed surrounding DC1 plaques.

Complementation with *bceB* alone was not enough to restore cepacian biosynthesis in K56-2. It is possible that *bceB* expression from pBBR*bceB*Tp was not sufficient to provide enough BceB to rescue EPS biosynthesis. It is also possible that K56-2 carries additional mutations in the *bce* genes. The 11 bp deletion in *becB* was found to be the only disruption in cepacian biosynthesis (*bce-I* and *bce-II*) genes found in the genome of J2315. The genome of K56-2 is not published and although the genome of J2315 is often used to predict the sequence of K56-2 because the two are related and both ET12 strains, it is possible that K56-2 has obtained additional mutations in any of the *bce* genes. This would explain why complementation with *bceB* alone was not able to restore cepacian biosynthesis. Additionally, Ferreira et al. (2010) showed that in the non-EPS-producing strain *B. multivorans* D2214, there was no induction of *bce* genes when grown in EPS stimulating media, as determined by real-time RT-PCR. However, for the mucoid strain *B. multivorans* D2095, there was a two-fold induction of *bce* genes when grown on EPS stimulating media. Based on this information, it is also possible that there is minimal to no expression from the *bce* gene clusters in K56-2, and so the enzymes required for cepacian biosynthesis may not be present.

Complementation of K56-2 was done in order to try and unambiguously elucidate if cepacian degradation by DC1 was responsible for halos by creating an EPS-producing ET12 strain of the BCC. Complementation of *bceB* was not sufficient to restore cepacian biosynthesis, and so no further conclusions regarding halo formation by DC1 can be made.

Genomic analysis of DC1: Towards identifying the gene or genes involved in halo formation

Analysis of DC1 genome and annotation

The 61,847 bp genome of DC1 was recently sequenced and annotated by Lynch and Dennis (unpublished). The majority of the genes in the DC1 genome share homology with the phages BcepIL02 (GenBank Accession number: FJ937737.1, Gill et al. 2009) and/or Bcep22 (NCBI: NC_005262, Summer et al. 2007). When annotated, no genes were found to show homology to a putative depolymerase enzyme. Many genes were found that have unknown function and six genes were found to have no homology to any phage in the NCBI database. It is hypothesized that the putative phage depolymerase gene is among the genes with unknown function. Analysis of putative protein domains was also performed on each of the 72 ORFs in the DC1 genome and again, nothing relating to a phage depolymerase was found (Lynch and Dennis, unpublished).

Cloning of DC1 genome fragments

Cloning of DC1 genome fragments into plasmid vectors was undertaken in attempts to identify the gene or genes involved in halo formation. This work was

done in order to make a DC1 library that could be used to screen for EPS degradation activity. To do this, the genome of DC1 was analyzed for restriction endonuclease enzyme cut sites using NEBcutter V2.0 (http://tools.neb.com/NEBcutter2/). *Eco*RI was found to be the most appropriate enzyme to use for cloning DC1 into pUC19 as it was found to cut DC1 nine times.

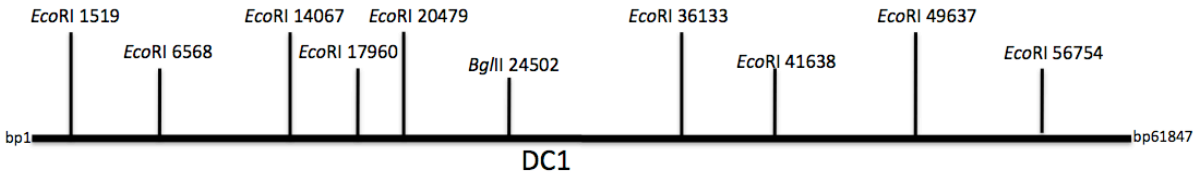

Figure 16 Schematic of the *Eco*RI cut sites in the DC1 genome, determined using NEBcutter V2.0. The position of each cut site is shown with a vertical bar. Additionally, the *Bgl*II cut site used to make clone DC1-BE4-8 is shown.

Using *Eco*RI digestion of DC1 and ligation into pUC19, six of the nine *Eco*RI fragments could be recovered, Table 7. All of the fragments were recovered in the 5'-3' direction relative to the lacZα promoter on pUC19. Two of the fragments were also recovered in the 3'-5' direction relative to the lacZα promoter. *Eco*RI restriction digestion analysis on representative clones from each *Eco*RI fragment is shown in Figure 17.

DC1 *Eco*RI fragments that could not be obtained were those from bps: 1,519-6,568, 14,067-17,960 and 20,479-36,133, Figure 16. Analysis of these fragments revealed that each of them contained at least one gene of unknown function. PCR was used to amplify the unknown genes from each of these *Eco*RI fragments and the resulting amplicons were ligated into pJET1.2/blunt, Table 7. The unknown gene contained in *Eco*RI fragment bp 1,519-6,568, gp5, was

successfully amplified from DC1 lysate using primers DC1gp5F and DC1gp5R and cloned into pJET1.2/blunt. The subsequent clone was called pJETDC1-gp5-2.

The unknown genes, gp22 – 27, from *Eco*RI fragment bp 14,067-17,960 were successfully amplified from DC1 using the primers DC1gp22-27F and DC1gp22-27R. This amplification product could not be recovered in pJET1.2/blunt. Five transformations were conducted and ten resulting clones were assessed for insertion of unknown gp22 – 27 and no correct clones were found. It is hypothesized that all or some of these unknown fragments may be lethal. Additional work could be done to amplify and recover the individual ORFs in pJET1.2/blunt.

The other DC1 *Eco*RI fragment that could not be recovered in pUC19 spans DC1 bp 20,479-36,133. PCR primers were designed to small segments of this 15,694 bp region. Primers DC1AF and DC1AR were used to successfully amplify bp 20,457-25,694, this fragment was cloned into pJET1.2/blunt and the resulting plasmid is maintained in pJETDC1-A-1. This section of DC1 is also found cloned into pUC19 in the clone DC1-BE-4-8, which was obtained by digesting DC1 chromosome with *Eco*RI and *Bgl*II and ligating the approximately 5 kb fragment to pUC19 digested with *Eco*RI and *Bam*HI.

DC1 bp 24,228-26,622 was amplified using the primer pair DC1BF and DC1BR and is found ligated to pJET1.2/blunt in the clones pJETDC1-B-1 and pJETDC1-B-5 in the forward and reverse direction relative to the lacUV5 promoter, respectively. DC1 bps 26,175-29,159 and bps 29,031-33,139 were both successfully amplified using DC1CF/DC1CR and DC1DF/DC1DR, respectively.

Neither of these fragments could be recovered in pJET1.2/blunt vector. It is hypothesized that one or more of the unknown genes found in this region may be lethal to the *E. coli* host. Finally, the DC1 fragment spanning bps 32,958-36,188 was amplified using the primers DC1EF and DC1ER and successfully cloned into pJET1.2/blunt. The resulting clone is called pJETDC1-E-1.

The final step in constructing a DC1 library in *E. coli* included determining if any of the *Eco*RI cut sites or primer sites interrupted a gene that could potentially be responsible for halo formation. Most of the cut sites and primer sties were found in between genes or in genes that are not predicted to be important for putative depolymerase action, Table 7. But, two of the *Eco*RI cut sites were found to be in genes of unknown function and one was found in tail fiber or collar fiber gene. Two of the PCR primers were found to interrupt ORFs with unknown function. In order to ensure that these genes were represented in the DC1 library, PCR primers were designed to amplify these regions. The two unknown genes cut by *Eco*RI, gp3 and gp62, were successfully amplified by PCR and are cloned into pJETDC1-F-5 and pJETDC1-H-2, respectively. The tail fiber or collar gene cut by *Eco*RI digestion is found in pJETDC1-G-2. The ORFs that were disrupted by PCR, gp44 and gp51, can be found in pJET1.2/blunt in clones pJETDC1-44-2 and pJETDC1-51-2. *Eco*RI restriction digestion analysis of the clones constructed using PCR analysis and pJET1.2/blunt is shown in Figure 17.

Table 7 *E. coli* clones compromising the DC1 library

E. coli clone	Region of DC1 (bp)	Features of region
DC1 clones maintained in pUC19		
DC1-5-4 DC1-5-1*	56754-1519	**SLT/helicase/methylase** Type II holin Lysozyme Rz/RzI Unknown gp1, gp2, **gp3** gp68, gp69
DC1-7.5-3	6568-14067	**Endonuclease** Repressor Transcriptional regulator tRNA-Serine Single stranded DNA binding protein Replication protein O **DnaC** Unknown gp10, gp11, gp12, gp14, gp15, gp16, gp17, gp18
DC1-2500-6	17960-20479	**Terminase small subunit** Unknown gp 29, 30, 31, 32, 33
DC1-BE4-8	20479-24502	BstYI methyltransferase BstYI restriction endonuclease DNA ligase **Terminase Large subunit** Unknown gp34, 35, 36, 37
DC1-10-6 DC1-10-58*	36133-41638	Tail fiber/tail collar **gp56**, gp57, gp59 and gp60 Unknown gp58, gp61, **gp62**
DC1-10-16	41638-49637	Phosphoadenylyl (PAPS) reductase GCN5-related N-acetyltransferase (GNAT) **SLT/helicase/methylase** Unknown gp58, gp61, **gp62**
DC1-10-9	49637-56754	**SLT/helicase/methylase**
DC1 clones maintained in pJET1.2/blunt		
pJETDC1-A-1	20457-24694	BstYI methyltransferase BSTYI restriction endonuclease DNA ligase Terminase Large subunit Unknown gp34, gp35, gp36, gp37
pJETDC1-B-5-1^ pJETDC1-B-1	24228-26622	Unknown gp42
pJETDC1-E-1	32958-36188	Major capsid protein Unknown gp52, 54, 55
pJETDC1-F-5-1^	828-1980	Unknown gp3

E. coli clone	Region of DC1 (bp)	Features of region
pJETDC1-H-2 pJETDC1-H-3-1^	40838-42408	Unknown gp62
pJETDC1-G-2 pJETDC1-G-3^	35386-36911	Tail fiber or collar fiber gp56
pJETDC1-gp56-2	35380-35800	Tail fiber or collar fiber gp56
pJETDC1-51-2 pJETDC1-51-3^	32345-33935	Unknown gp51
pJETDC1-44-3 pJETDC1-44-3-2^	28153-29707	Unknown gp44
pJETDC1-gp5-2^	3544-4968	Unknown gp5
DC1 clones that could not be obtained		
pJETDC1-C	26175-29159	Unknown gp44, 45
pJETDC1-D	29031-33139	Unknown gp47, 48, 49, 50 Carbon Storage Regulator CsrA
pJETDC1-gp22-27		Unknowns gp 22, 23, 24, 25, 26, 27
DC1 1519-6568	1519-6568	Integrase RecT **Endonuclease** Unknwon **gp3**, gp5
DC1 14067-17960	14067-17960	**DnaC** Palmitoyl transferase for lipid A Unknown gp22, gp23, gp24, gp25, gp26, gp27
DC1 24502-36133	24502-36133	Terminase Large subunit Portal protein Carbon storage regulator CsrA Major capsid protein Tail fiber or tail collar Unknown gp42, gp44, gp45, gp47, gp48, gp49, gp50, gp51, gp52, gp54, gp55, **gp56**

Legend. *, DC1 fragment running 3'-5' in multiple cloning site of pUC19; ^, DC1 ORFs running 5'-3' from pT7 promoter in pJET1.2/blunt and additionally found in *E. coli* BL21(DE3)pLysS, bolded text, ORFs that are cut by *Eco*RI digestion.

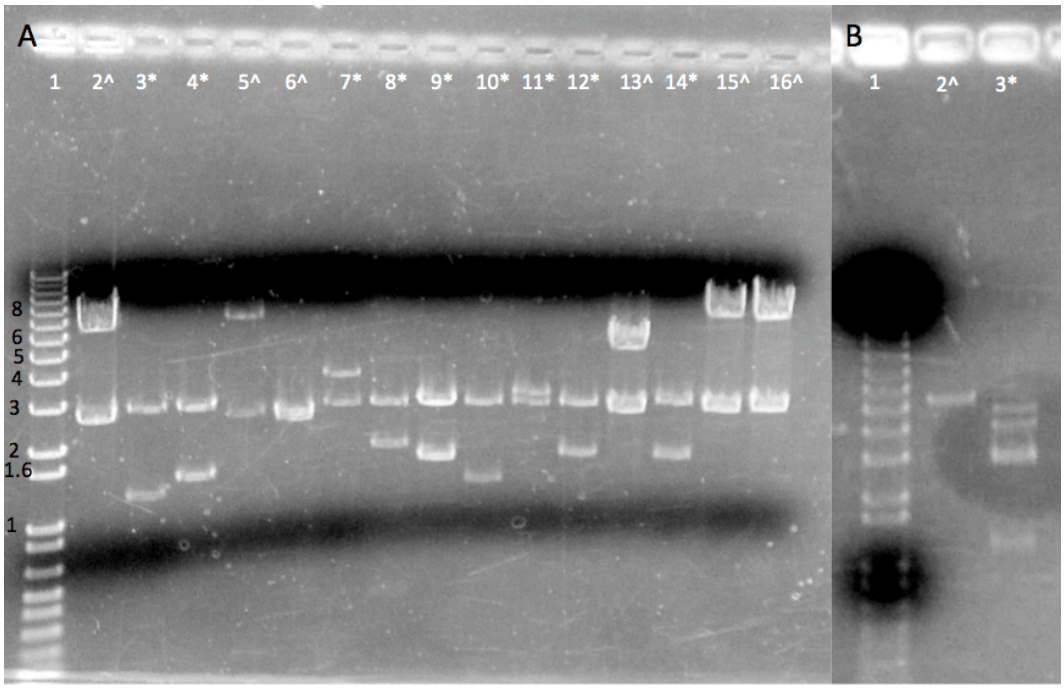

Figure 17 Restriction digestion analysis of DC1 clones. Legend. Agarose gel electrophoresis showing restriction digestion analysis of DC1 clones. (A) 1, 1kb+ ladder (Invitrogen), 2, DC1-5-4, 3, DC1-F-5, 4, DC1-gp5-2, 5, DC1-7.5-3, 6, DC1-2500-3, 7, DC1-A-1, 8, DC1-B-5, 9, DC1-gp44-3, 10, DC1-gp51-2, 11, DC1-E-1, 12, DC1-G-3, 13, DC1-10-6, 14, DC1-H-3, 15, DC1-10-16, 16, DC1-10-9. (B) 1, 1kb+ ladder (Invitrogen), 2, DC1-BE-4-8, 3, DC1-56-2 ^ indicates cut with *Eco*RI and pUC19 vector, * indicates cut with *Bgl*II and pJET1.2/blunt vector.

The MCS of pJET1.2/blunt is flanked by two promoters, lacUV5 and pT7. The latter of the two promoters is able to be induced when the plasmid is found in *E. coli* BL21/DE3(pLysS). The majority of the DC1 clones made using pJET1.2/blunt are found oriented 5'-3' with respect to the lacUV5 promoter. pJETDC1 clones containing ORFs oriented 5'-3' with respect to pT7 are: pJETDC1-B-5-1, pJETDC1-G-3-4, pJETDC1-51-2-4, pJETDC1-44-3-2 and pJETDC1-F-5-1 pJETDC1-gp-5-2. The cloning vector containing the DC1 insert from each of these clones was transformed into *E. coli* BL21/DE3 (pLysS) in order to obtain an inducible clone for each of these fragments.

Preliminary experiments performed to test DC1 E. coli clones for EPS degradation activity

In preliminary experiments, all 24 strains included in the *E. coli* DC1 library were assayed for their ability to degrade the EPS produced by LMG 18821 and PC184. This was done by spotting 5 μL of overnight cultures onto lawns of LMG 18821 or PC184 plated on YEM media. In this experiment it was hypothesized that the *E. coli* clone carrying the putative depolymerase ORF would be able to make this protein and may be able to export it to the surrounding environment, or, may be able to produce the protein in the cytoplasm. In the latter case, it is hypothesized that cells that lyse naturally during growth or death would release the protein into the liquid medium allowing for detection of enzyme activity. Additionally, the *E. coli* BL21/DE3(pLysS) strains harboring a pJET1.2/blunt plasmid with a DC1 insert were induced with IPTG and screened for degradation activity. As a positive control, 5 μL of DC1 lysate was spotted onto bacterial lawns.

None of the 24 DC1 *E. coli* clones showed EPS degradation activity in this assay. This suggests that the putative depolymerase enzyme is not secreted into the environment. The enzyme may be produced by one of the clones in the library and there may not have been enough natural cell lysis to allow for detection of the enzyme. Interestingly, it was observed that the *E. coli* clones were unable to grow on the BCC lawns. This suggests that PC184 and LMG 18821 may express and secrete antimicrobial compounds that are active against *E. coli*.

This work was indeed done on a preliminary basis, and, it is strongly suggested that it be expanded by performing EPS degradation assays of whole cell lysates from the clones constructed. Such work was done on the polysaccharide depolymerases from phages ERA103 and ϕEa1h that are active against *Erwinia amylovora* capsule. Each enzyme was successfully cloned into *E. coli* and shown to retain depolymerase activity when expressed in the foreign host (Kim et al. 2004; Vandenbergh and Cole 1986). This was done by cloning portions of each phages' genome into *E. coli* using a plasmid vector and testing cell lysates obtained using French press or sonication for depolymerization activity. Kim and Geider (2000) demonstrated that the phage depolymerase enzyme of ϕEa1h was found clustered with the ORFs for the lysozyme and holin proteins. The lysozyme (gp71) and type II holin (gp70) of DC1 are found beside 2 ORFs of unknown function, gp68 and gp69, and the phage lysis protein Rz/Rz1 (gp72). Homologues of gp68 and gp69 are found in BcepIL02 and putatively encode a conserved phage protein of unknown function (83% homology) and a conserved phage protein with a helix turn helix motif (73% homology), respectively. Analysis of cell lysate from *E. coli* DC1-5-4 and *E. coli* DC1-5-1 would show whether either of these two ORFs are involved in halo formation. Goudie and Dennis are currently testing lysate from DC1-5-1 and DC1-5-4 as well as the other 22 DC1 clones constructed in this work to determine if any of them display BCC EPS depolymerase activity.

Determining the gene that encodes the putative DC1 depolymerase is important as it could potentially be used to enhance phage therapy. Barnet and Humphrey (1975) showed that a phage EPS depolymerase was able to degrade

EPS from strains it was not lytic towards as long as the structure was similar. This finding suggests that the putative depolymerase from phage DC1, which is hypothesized to target the most abundant EPS produced by the BCC, may be extremely useful when treating BCC infections with phage. When used in conjugation with a phage cocktail, the degradation of cepacian may help other phages to reach their OM receptors. The ability of phage depolymerase enzymes to increase phage infectivity was demonstrated by Bull et al. (2010) who showed that the fitness and infectivity of *E. coli* K specific phages lacking CPS depolymerases was increased by the addition of a phage endosialidase. Further, including a cepacian depolymerase as part of a phage therapy preparation may assist in releasing cells living in biofilms, which would in turn increase their susceptibility to phage, chemical antibiotic, and host immune system attack.

DC1 prophage

Two putative DC1 prophages in LMG 18821, LMG18821R1 and LMG18821R2, were isolated by picking a single very small colony from the center of a DC1 plaque formed on a lawn of LMG 18821. The single colony was struck out for single colonies on ½ LB media, and two colonies were chosen to investigate as putative DC1 prophages. LMG18821R1 and LMG18821R2 were found to be resistant to DC1 infection. Amplification product was obtained when colony PCR was performed on each using the DC1 specific primers, DC1F and DC1R, Figure 18. LMG 18821 chromosome and DC1 lysate provided the negative and positive controls for PCR analysis, respectively. Identification of a DC1 prophage is important because it can be tested to ensure that lysogenization

by DC1 does not act to increase BCC host virulence. Such an assay is extremely important when considering temperate phage for phage therapy. Further, it can allow for elucidation of the phage integration site.

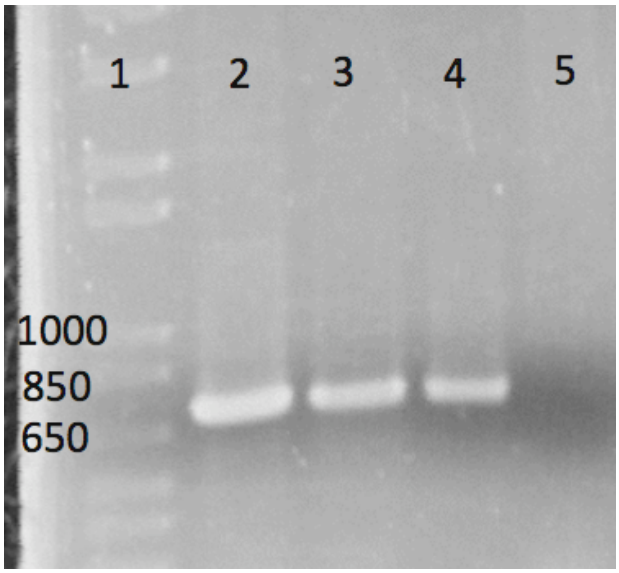

Figure 18 PCR analysis of putative DC1 prophages in LMG 18821. Legend. agarose gel electrophoresis of PCR amplification products obtained using primers DC1F/DC1R, 1, 1Kb+ ladder (Invitrogen), 2, putative DC1 prophage LMG12281R1, 3, putative DC1 prophage LMG18821R2, 4, DC1 lysate, 5, LMG 18821 chromosome.

Conclusion

Proposed DC1 infection model

DC1 plaques, when formed on EPS-producing hosts stimulated to express cepacian, are surrounded by large and expanding halos. This plaque morphology has been well documented as the major identifying factor of a phage depolymerase enzyme. DC1 plaques were not surrounded by large and expanding halos when formed on strains unable to synthesize cepacian, and on strains not stimulated to produce EPS. Additionally, when cepacian biosynthesis was blocked

in the normally mucoid strain PC184 by plasposon insertion in the *bce-I* gene cluster, DC1 plaques were not surrounded by the large and expanding halos. These findings when taken together strongly suggest that DC1 carries a depolymerase enzyme that is specific to cepacian. Degradation products resulting from cepacian depolymerization remain unknown as do the gene or genes involved in enzymatic activity. Interestingly, the PC184 strain mutated in the *rml* region shows a loss of the mucoid phenotype on YEM media. However, DC1 plaques formed on this strain maintain their large and expanding halos. The reason for the non-mucoid phenotype in this strain as well as the formation of halos surrounding DC1 plaques remains unknown.

The hypothesized model for DC1 infection is as follows; a depolymerase enzyme likely found on a DC1 tail spike or fiber recognizes cepacian surrounding its host bacterium and degrades it. This allows DC1 to reach the surface of the host bacterium where it can associate with its receptor, inject its genome and propagate either lytically or lysogenically. The degradation of EPS is not thought to be necessary for phage infection as non-EPS-producing strains can be killed by DC1. Further, DC1 infection can occur when host cells capable of producing EPS are not stimulated to produce EPS. It is hypothesized that the large and expanding halos surrounding DC1 plaques are formed from either intact phage degrading the surrounding EPS, or, from enzymatically active phage tail spikes or fibers that have sheered off the phage and diffused away, Figure 19.

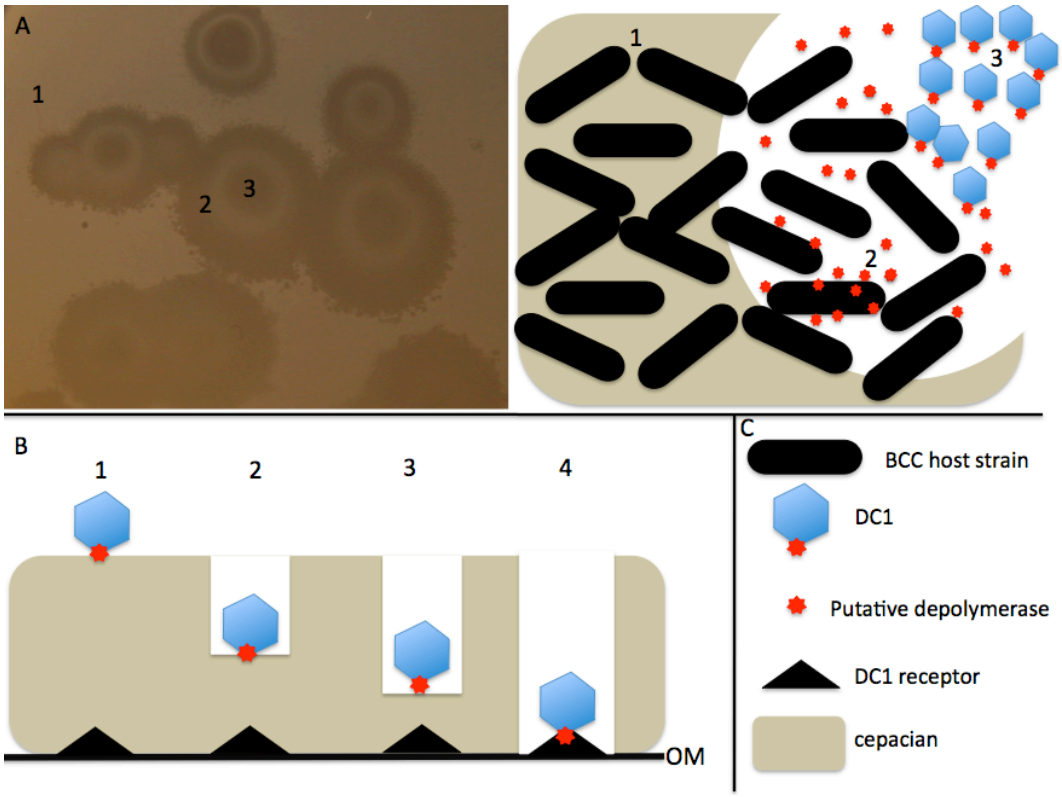

Figure 19 Proposed model of DC1 infection. Legend. (A) 1, DC1 host growing in a lawn on EPS inducing media, 2, halo surrounding plaque where degradation of cepacian is performed by intact phage or by enzymatically active tail spike or fiber that has sheered off the DC1 capsid, viable bacteria can be found here, 3, plaque center formed from confluent lysis of host cells, highest concentration of phage found here. (B) 1, DC1 associates with EPS (cepacian) surrounding host cell, 2 and 3, EPS enzymatically active tail spike or fiber degrades cepacian allowing phage to penetrate the viscous polysaccharide layer (4) phage reaches its receptor on bacterial outer membrane (OM), Adapted from Hughes et al. (1998). (C) legend for symbols used in diagram.

LITERATURE CITED

Adams, M.H., and Park, B.H. 1956. An enzyme produced by a phage-host cell system II. The properties of the polysaccharide depolymerase. Virology **2**(6): 719-736.

Ali, M.S., and Pearson, J.P. 2007. Upper airway mucin gene expression: A review. Laryngoscope **117**(5): 932-938.

Aronoff, S.C. 1988. Outer membrane permeability in *Pseudomonas cepacia*: Diminished porin content in a β-lactam-resistant mutant and in resistant cystic fibrosis isolates. Antimicrob. Agents Chemother. **32**: 1636-1639.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K. (Editors). 1999. Short protocols in molecular biology. John Wiley and Sons, Inc., New York.

Barnet, Y.M., and Humphrey, B. 1975. Exopolysaccharide depolymerases induced by *Rhizobium* bacteriophages. Can. J. Microbiol. **21**: 1647-1650.

Bartell, P.F., and Orr, T.E. 1969. Distinct slime polysaccharide depolymerases of bacteriophage-infected *Pseudomonas aeruginosa*: evidence of close association with the structured bacteriophage particle. J. Virol. **4**(5): 580-584.

Bartell, P.F., Orr, T.E., and Lam, G.K. 1966. Polysaccharide depolymerase associated with bacteriophage infection. J. Bacteriol. **92**(1): 56-62.

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**(8): 2513-2521.

Beckman, W., and Lessie, T.G. 1979. Response of *Pseudomonas cepacia* to β-lactam antibiotics: Utilization of penicillin G as the carbon source. J. Bacteriol. **140**(3): 1126-1128.

Berlutti, F., Morea, C., Battistoni, A., Sarli, S., Cipriani, P., Superti, F., Ammendolia, M.G., and Valenti, P. 2005. Iron availability influences aggregation, biofilm, adhesion and invasion of Pseudomonas aeruginosa and Burkholderia cenocepacia. Int. J. Immunopath. Ph. **18**(4): 661-670.

Billing, E. 1960. An association between capsulation and phage sensitivity in *Erwinia amylovora*. Nature **186**(4727): 819-820.

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

Immun. **70**(1): 204-210.

Broxmeyer, L., Sosnowska, D., Miltner, E., Chacón, O., Wagner, D., McGarvey, J., Barletta, R.G., and Bermudez, L.E. 2002. Killing of *Mycobacterium avium* and *Mycobacterium tuberculosis* by a mycobacteriophage delivered by a nonvirulent *Mycobacterium*: A model for phage therapy of intracellular bacterial pathogens. J. Infect. Dis. **186**(8): 1155-1160.

Burns, J.L., Hedin, L.A., and Lien, D.M. 1989*a*. Chloramphenicol resistance in *Pseudomonas cepacia* because of decreased permeability. Antimicrob. Agents Chemother. **33**(2): 136-141.

Burns, J.L., Lien, D.M., and Hedin, L.A. 1989. Isolation and characterization of dihydrofolate reductase from trimethoprim-susceptible and trimethoprim-resistant *Pseudomonas cepacia*. Antimicrob. Agents Chemother. **33**(8): 1247-1251.

Burns, J.L., Jonas, M., Chi, E.Y., Clark, D.K., Berger, A., and Griffith, A. 1996. Invasion of respiratory epithelial cells by *Burkholderia* (*Pseudomonas*) *cepacia*. Infect. Immun. **64**(10): 4054-4059.

Bull, J.J., Vimr, E.R., and Molineux, I.J. 2010. A tale of tails: Sialidase is key to success in a model of phage therapy against K1-capsulated *Escherichia coli*. Virology **398**(1): 79-86.

Byl, C.V., and Kropinski, A.M. 2000. Sequence of the genome of *Salmonella* bacteriophage P22. J. Bacteriol. **182**(22): 6472-6481.

Bylund, J., Burgess, L., 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**(5): 2526-2532.

Capparelli, R., Nocerino, N., Lannaccone, M., Ercolini, D., Parlato, M., Chiara, M., and Lannelli, D. 2010. Bacteriophage therapy of *Salmonella enterica*: A fresh appraisal of bacteriophage therapy. J. Infect. Dis. **201**(1): 52-61.

Caraher, E., Duff, C., Mullen, T., McKeon, S., Murphy, P., Callaghan, M., and McClean, S. 2007. Invasion and biofilm formation of *Burkholderia dolosa* is comparable with *Burkholderia cenocepacia* and *Burkholderia multivorans*. J. Cyst. Fibros. **6**: 49-56.

Carmody, L., Gill, J. J., Summer, E., Sajjan, U., Gonzalez, C., Young, R., and LiPuma, J.J. 2010. Efficacies of bacteriophage therapy in a model of *Burkholderia cenocepacia* pulmonary infection. J. Infect. Dis. 201: 264-271.

Castillo, F.J., and Bartell, P.F. 1976. Localization and functional role of the *Pseudomonas* bacteriophage 2 depolymerase. J. Virol. **18**(2): 701-708.

Cescutti, P., Bosco, M., Picotti, F., Impallomeni, G., Leitão, J.H., Richau, J.A., and Sá-Correia, I. 2000. Structural study of the exopolysaccharide produced by a clinical isolate of *Burkholderia cepacia*. Biochem. Biophys. Res. Commun. **273**(3): 1088-1094.

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. Carbohyd. Res. **338**(23): 2687-2695.

Chiarini, L., Bevivino, A., Dalmastri, C., Tabacchioni, S., and Visca, P. 2006. *Burkholderia cepacia* complex species: health hazards and biotechnological potential. Trends Microbiol. **14**(6): 277-286.

Cérantola, S., Lemassu-Jacquier, A., and Montrozier, H. 1999. Structural elucidation of a novel exopolysaccharide produced by a mucoid clinical isolate of *Burkholderia cepacia*. Eur. J. Biochem. **260**: 373-383.

Cérantola, S., Mary, N., and Montrozier, H. 1996. Structural studies of the acidic exopolysaccharide produced by a mucoid strains of *Burkholderia cepacia* isolated from cystic fibrosis. Carbohyd. Res. **285**: 59-67.

Chiarini, L., Cescutti, P., Drigo, L., Impallomeni, G., Herasimenka, Y., Bevivino, A., Dalmastri, C., Tabacchioni, S., Manno, G., Zanetti, F., and Rizzo, R. 2004. Exopolysaccharides produced by *Burkholderia cenocepacia rec*A lineages IIIA and IIIB. J. Cyst. Fibros. **3**(3): 165-172.

Chiesa, C., Labrozzi, P.H., and Aronoff, S.C. 1986. Decreased baseline β-lactamase production and inducibility associated with increased piperacillin susceptibility of *Pseudomonas cepacia* isolated from children with cystic fibrosis. Pediatr. Res. **20**(11): 1174-1177.

Chung, J.W., Altman, E., Beveridge, T.J., and Speert, D.P. 2003. Colonial morphology of *Burkholderia cepacia* complex genomovar III: Implications in exopolysaccharide production, pilus expression, and persistence in the mouse. Infect. Immun. **71**(2): 904-909.

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

Coenye, T., Vandamme, P., Govan, J.R.W., and Lipuma, J.J. 2001. Taxonomy and identification of the *Burkholderia cepacia* complex. J. Clin. Microbiol.

39(10): 3427-3436.

Conway, B.D., Venu, V., and Speert, D.P. 2002. Biofilm formation and acyl homoserine lactone production in the *Burkholderia cepacia* complex. J. Bacteriol. **184**(20): 5678-5685.

Conway, B. 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**(5): 957-966.

Courtney, J.M., Bradley, J., Mccaughan, J., O'connor, T.M., Shortt, C., Bredin, C.P., Bradbury, I., and Elborn, J.S. 2007. Predictors of mortality in adults with cystic fibrosis. Pediatr. Pulmonol. **42**(6): 525-532.

Cunha, M.V., Sousa, S.A., Leitão, J.H., Moreira, L.M., Videira, P.A., and Sá-Correia, 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**(7): 3052-3058.

Debarbieux, L., Leduc, D., Maura, D., Morello, E., Criscuolo, A., Grossi, O., Balloy, V., and Touqui, L. 2010. Bacteriophages can treat and prevent *Pseudomonas aeruginosa* lung infections. J. Infect. Dis. **201**(7): 1096-1104.

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. Microb. **64**(7): 2710-2715.

Desai, M., Bühler, T., Weller, P.H., and Brown, M.R.W. 1998. Increasing resistance of planktonic and biofilm cultures of *Burkholderia cepacia* to ciprofloxacin and ceftazidime during exponential growth. J. Antimicrob. Chemother. **42**(2): 153-160.

Durlu-Özkaya, F., Aslim, B., and Taha Ozkaya, M. 2007. Effect of exopolysaccharides (EPSs) produced by *Lactobacillus delbrueckii* subsp. *bulgaricus* strains to bacteriophage and nisin sensitivity of the bacteria. LWT - Food Sci. Technol. **40**(3): 564-568.

Eklund, C., and Wyss, O. 1962. Enzyme associated with bacteriophage infection. J. Bacteriol. **85**: 1209-1215.

Eriksson, U., Svenson, S.B., Lonngren, J., and Lindberg, A.A. 1979. *Salmonella* phage glycanases: Substrate specificity of the phage P22 endo-rhamnosidase. J. Gen. Virol. **43**(3): 503-511.

Ferreira, A.S., Leitão, J.H., Sousa, S.A., Cosme, A.M., Sá-Correia, I., and Moreira, L.M. 2007. Functional analysis of *Burkholderia cepacia* genes *bce*D and *bce*F, encoding a phospholyrosine phosphatase and a tyrosine autokinase, respectively: Role in exopolysaccharide biosynthesis and biofilm formation. Appl. Environ. Microbiol. **73**(2): 524-534.

Ferreira, A.S., Leitão, J.H., Silva, I.N., Pinheiros, 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**(2): 441-450.

Figurski, D.H., and Helinski, D.R. 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. P. Natl. Acad. Sci. USA **76**(4): 1648-1652.

Flachowsky, H., Richter, K., Kim, W., Geider, K., and Hanke, M. 2008. Transgenic expression of a viral EPS-depolymerase is potentially useful to induce fire blight resistance in apple. Ann. Appl. Biol. **153**(3): 345-355.

Frangolias, D.D., Mahenthiralingam, E., Rae, S., Raboud, J.M., Davidson, A.G.F., Wittmann, R., and Wilcox, P.G. 1999. *Burkholderia cepacia* in cystic fibrosis: Variable disease course. Am. J. Respir. Crit. Care Med. **160**(5 I): 1572-1577.

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**(2): 695-702.

Goudie, A., Lynch, K. H., Seed K., Stothard, P., Shirvastava, 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).

Govan, J.R.W., and Deretic, V. 1996. Microbial pathogenesis in cystic fibrosis: mucoid *Pseudomonas aeruginosa* and *Burkholderia cepacia*. Microbiological Reviews **60**(3): 539-574.

Greenberg, M., Dunlap, J., and Villafane, R. 1995. Identification of the tailspike protein from the *Salmonella newington* phage ε34 and partial characterization of its phage-associated properties. J. Struct. Biol. **115**(3): 283-289.

Gross, R.J., Cheasty, T., and Rowe, B. 1977. Isolation of bacteriophages specific for the K1 polysaccharide antigen of *Escherichia coli*. J. Clin. Microbiol. **6**(6): 548-550.

Hanlon, G.W., Denyer, S.P., Olliff, C.J., and Ibrahim, L.J. 2001. Reduction in exopolysaccharide viscosity as an aid to bacteriophage penetration through *Pseudomonas aeruginosa* biofilms. Appl. Environ. Microbiol. **67**(6): 2746-2753.

Harrison, F. 2007. Microbial ecology of the cystic fibrosis lung. Microbiology **153**: 917-923.

Henry, D.A., Campbell, M.E., LiPuma, J.J., and Speert, D.P. 1997. Identification of *Burkholderia cepacia* isolates from patients with cystic fibrosis and use of a simple new selective medium. J. Clin. Microbiol. **35**(3): 614-619.

Herasimenka, Y., Cescutti, P., Impallomeni, G., Campana, S., Taccetti, G., Ravenni, N., Zanetti, F., and Rizzo, R. 2007. Exopolysaccharides produced by clinical strains belonging to the *Burkholderia cepacia* complex. J. Cyst. Fibros. **6**(2): 145-152.

Higashi, S., and Abe, M. 1978. Phage induced depolymerase for exopolysaccharide of *Rhizobiaceae*. J. Gen. Appl. Microbiol. **24**(3): 143-153.

Holden, M.T.G., Seth-Smith, H.M.B., Crossman, L.C., Sebaihia, M., Bentley, S.D., Cerdeño-Tárraga, A.M., Thomson, N.R., Bason, N., Quail, M.A., Sharp, S., Cherevach, I., Churcher, C., Goodhead, I., Hauser, H., Holroyd, N., Mungall, K., Scott, P., Walker, D., White, B., Rose, H., Iversen, P., Mil-Homens, D., Rocha, E.P.C., Fialho, A.M., Baldwin, A., Dowson, C., Barrell, B.G., Govan, J.R., Vandamme, P., Hart, C.A., Mahenthiralingam, E., and Parkhill, J. 2009. The genome of *Burkholderia cenocepacia* J2315, an epidemic pathogen of cystic fibrosis patients. J. Bacteriol. **91**(1): 261-277.

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

Hughes, K.A., Sutherland, I.W., and Jones, M.V. 1998*b*. Biofilm susceptibility to bacteriophage attack: The role of phage-borne polysaccharide depolymerase. Microbiology **144**(11): 3039-3047.

Hutchison, M.L., and Govan, J.R.W. 1999. Pathogenicity of microbes associated with cystic fibrosis. Microb. Infect. **1**: 1005-1014.

Isles, A., Maclusky, I., Corey, M., Gold, R., Prober, C., Flemming, P., and Levison, H. 1984. *Pseudomonas cepacia* infections in cystic fibrosis: an emerging problem. J. Pedatr. **104**: 206-210.

Israel, V., Rosen, H., and Levine, M. 1972. Binding of bacteriophage P22 tail parts to cells. J. Virol. **10**(6): 1152-1158.

Iwashita, S., and Kanegasaki, S. 1973. Smooth specific phage adsorption: Endorhamnosidase activity of tail parts of P22. Biochem. Biophys. Res. Commun. **55**(2): 403-409.

Johnson, W.M., Tyler, S.D., and Rozee, K.R. 1994. Linkage analysis of geographic and clinical clusters in *Pseudomonas cepacia* infections by multilocus enzyme electrophoresis and ribotyping. J. Clin. Microbiol. **32**(4): 924-930.

Jones, A.M., and Webb, A.K. 2003. Recent advances in cross-infection in cystic fibrosis: *Burkholderia cepacia* complex, *Pseudomonas aeruginosa*, MRSA and *Pandoraea spp*. J. R. Soc. Med., Supplement **96**(43): 66-72.

Kanegasaki, S., and Wright, A. 1973. Studies on the mechanism of phage adsorption: Interaction between phage ε15 and its cellular receptor. Virology **52**(1): 160-173.

Kim, W., Salm, H., and Geider, K. 2004. Expression of bacteriophage φEa1h lysozyme in *Escherichia coli* and its activity in growth inhibition of *Erwinia amylovora*. Microbiology **150**(8): 2707-2714.

Kitson, C., Angel, B., Judd, D., Rothery, S., Severs, N.J., Dewar, A., Huang, L., Wadsworth, S.C., Cheng, S.H., Geddes, D.M., and Alton, E.W.F. 1999. The extra- and intracellular barriers to lipid and adenovirus-mediated pulmonary gene transfer in native sheep airway epithelium. Gene Ther. **6**: 534-546.

Kovach, M.E., Elzer, P., Hill, D.S., Robertson, G.T., Farris, M., Roop, R.M., and Peterson, K. 1995. Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. Gene **166**: 175.

Kutter, E., and Sulakvelidze, A. (Editors). 2005. Bacteriophages biology and applications. CRC Press, Boca Raton, Fl.

Lafitte, G., Thuresson, K., Jarwoll, P., and Nydén, M. 2007. Transport properties and aggregation phenomena of polyoxyethylene sorbitane monooleate (polysorbate 80) in pig gastrointestinal mucin and mucus. Langmuir **23**(22): 10933-10939.

Lagatolla, C., Skerlavaj, S., Dolzani, L., Tonin, E.A., Monti Bragadin, C., Bosco, M., Rizzo, R., Giglio, L., and Cescutti, P. 2002. Microbiological characterisation of *Burkholderia cepacia* isolates from cystic fibrosis patients: Investigation of the exopolysaccharides produced. FEMS Microbiol. Lett.

209(1): 99-106.

Lamothe, J., Huynh, K., Grinstein, S., and Valvano, M. 2007. Intracellular survival of *Burkholderia cenocepacia* in macrophages is associated with delay in the maturation of bacteria-containing vacuoles. Cell Microbiol. **9**(1): 40-53.

Li, W., Xin, Y., McNeil, M.R., and Ma, Y. 2006. *rml*B and *rml*C genes are essential for growth of *Mycobacteria*. Biochem. Biophys. Res. Commun. **342**(1): 170.

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**(8723): 1094-1096.

Linker, A., Evans, L.R., and Impallomeni, G. 2001. The structure of a polysaccharide from infectious strains of *Burkholderia cepacia*. Carbohydr. Res. **335**(1): 45-54.

Long, G.S., Bryant, J.M., Taylor, P.W., and Luzio, J.P. 1995. Complete nucleotide sequence of the gene encoding bacteriophage E endosialidase: Implications for K1E endosialidase structure and function. Biochem. J. **309**(2): 543-550.

Macdonald, K.L., and Speert, D.P. 2008. Differential modulation of innate immune cell functions by the *Burkholderia cepacia* complex: *Burkholderia cenocepacia* but not *Burkholderia multivorans* disrupts maturation and induces necrosis in human dendritic cells. Cell. Microbiol. **10**(10): 2138-2149.

Madigan, T. and Martinko, J. 2006. Brock biology of microorganisms. Pearson Prentice Hall, Inc.,Upper Saddle River, NJ. pp. 720-721.

Mahenthiralingam, E., Urban, T.A., and Goldberg, J.B. 2005. The multifarious, multireplicon *Burkholderia cepacia* complex. Nat. Rev. Microbiol. **3**(2): 144-156.

Mahenthiralingam, E., Bischof, J., Byrne, S.K., Radomski, C., Davies, J.E., Av-Gay, Y., and Vandamme, P. 2000. DNA-based diagnostic approaches for identification of *Burkholderia cepacia* complex, *Burkholderia vietnamiensis*, *Burkholderia multivorans*, *Burkholderia stabilis*, and *Burkholderia cepacia* genomovars I and III. J. Clin. Microbiol. **38**(9): 3165-3173.

Matsuzaki, S., Rashel, M., Uchiyama, J., Sakurai, S., Ujihara, T., Kuroda, M., Ikeuchi, M., Tani, T., Fujieda, M., Wakiguchi, H., and Imai, S. 2005. Bacteriophage therapy: A revitalized therapy against bacterial infectious diseases. J. Infect. Chemother. **11**(5): 211-219.

Matsuzaki, S., Yasuda, M., Nishikawa, H., Kuroda, M., Ujihara, T., Shuin, T., Shen, Y., Jin, Z., Fujimoto, S., Nasimuzzaman, M.D., Wakiguchi, H., Sugihara, S., Sugiura, T., Koda, S., Muraoka, A., and Imai, S. 2003. Experimental protection of mice against lethal *Staphylococcus aureus* infection by novel bacteriophage φMR11. J. Infect. Dis. **187**: 613-624.

McKevitt, A.I., Bajaksouzian, S., Klinger, J.D., and Woods, D.E. 1989. Purification and characterization of an extracellular protease from *Pseudomonas cepacia*. Infect. Immun. **57**(3): 771-778.

McVay, C.S., Vela´squez, M., and Fralick, J.A. 2007. Phage therapy of *Pseudomonas aeruginosa* infection in a mouse burn wound model. Antimicrob. Agents Ch. **51**(6): 1934-1938.

Merril, C.R., Scholl, D., and Adhya, S.L. 2003. The prospect for bacteriophage theapy in western medicine. Nat. Rev. Drug Discov. **2**: 489-497.

Minasian, C.C., Wallis, C., and Bush, A. 2007. Mannitol as a mucolytic in cystic fibrosis. J. R. Soc. Med., Supplement **100**(47): 53-56.

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. Bioph. Res. Co. **312**(2): 323-333.

Mushtaq, N., Redpath, M.B., Luzio, J.P., and Taylor, P.W. 2005. Treatment of experimental *Escherichia coli* infection with recombinant bacteriophage-derived capsule depolymerase. J. Antimicrob. Chemother. **56**(1): 160-165.

Nimmich, W., Schmidt, G., and Krallmann-Wenzel, U. 1991. Two different *Escherichia coli* capsular polysaccharide depolymerases each associated with one of the coliphage ΦK5 and ΦK20. FEMS Microbiol. Lett. **82**(2): 137-141.

Nimtz, M., Wray, V., Domke, T., Brenneke, B., Häussler, S., and Steinmetz, I. 1997. Structure of an acidic polysaccharide of *Burkholderia pseudomallei*. Eur. J. Biochem. **250**: 608-616

Nixon, A., Parolis, H., Dutton, G.G.S., and Leek, D.M. 1987. *Klebsiella* serotype K39: Structure of an unusual capsular antigen deduced by use of a viral endoglucosidase. Carbohydr. Res. **167**(C): 279-290.

Ochs, H.D., Davis, S.D., and Wedgwood, R.J. 1971. Immunologic responses to bacteriophage phi-X 174 in immunodeficiency diseases. J. Clin. Invest. **50**: 2559-2568.

Olmsted, S.S., Padgett, J.L., Yudin, A.I., Whaley, K.J., Moench, T.R., and Cone, R.A. 2001. Diffusion of macromolecules and virus-like particles in human cervical mucus. Biophys. J. **81**(4): 1930-1937.

Parr Jr., T.R., Moore, R.A., Moore, L.V., and Hancock, R.E.W. 1987. Role of porins in intrinsic antibiotic resistance of *Pseudomonas cepacia*. Antimicrob. Agents Chemother. **31**: 121-123.

Reik, R., Spilker, T., and LiPuma, J.J. 2005. Distribution of *Burkholderia cepacia* complex species among isolates recovered from persons with or without cystic fibrosis. J. Clin. Microbiol. **43**(6): 2926-2928.

Richau, J.A., Leitão, J.H., and Sá-Correia, I. 2000. Enzymes leading to the nucleotide sugar precursors for exopolysaccharide synthesis in *Burkholderia cepacia*. Biochem. and Bioph. Res. Co. **276**(1): 71-76.

Sage, A., Linker, A., Evans, L.R., and Lessie, T.G. 1990. Hexose phosphate metabolism and exopolysaccharide formation in *Pseudomonas cepacia*. Curr. Microbiol. **20**(3): 191-198.

Sajjan, U., Corey, M., Karmali, M., and Forstner, J. 1992. Binding of *Pseudomonas cepacia* to normal human intestinal mucin and respiratory mucin from patients with cystic fibrosis. J. Clin. Invest. **89**: 648-656.

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

Sauer, R.T., Krovatin, W., Poteete, A.R., and Berget, P.B. 1982. Phage P22 tail protein: Gene and amino acid sequence. Biochemistry-US **21**(23): 5811-5815.

Seed, K.D., and Dennis J.J. 2005. Isolation and characterization of bacteriophages of the *Burkholderia cepacia* complex. FEMS Microbiol. Lett. **251**: 273-280.

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**(3): 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**(5): 2205-2208.

Shaburova, O.V., Krylov, S.V., Veiko, V.P., Pleteneva, E.A., Burkal'Tseva, M.V., Miroshnikov, K.A., Cornelissen, A., Lavigne, R., Sykilinda, N.N., Kadykov, V.A., Mesyanzhinov, V.V., Volckaert, G., and Krylov, V.N. 2009. Search for

destruction factors of bacterial biofilms: Comparison of phage properties in a group of *Pseudomonas putida* bacteriophages and specificity of their halo-formation products. Russ. J. G. **45**(2): 161-170.

Sheng, H., Knecht, H.J., Kudva, I.T., and Hovde, C.J. 2006. Application of bacteriophages to control intestinal *Escherichia coli* O157:H7 levels in ruminants. Appl. Environ. Microbiol. **72**(8): 5359-5366.

Skurnik, M., Pajunen, M., and Kiljunen, S. 2007. Biotechnological challenges of phage therapy. Biotechnol. Lett. **29**(7): 995-1003.

Sousa, S.A., Moreira, L.M., Wopperer, J., Eberl, L., Sá-Correia, I., and Leitão, J.H. 2007*a*. The *Burkholderia cepacia bce*A gene encodes a protein with phosphomannose isomerase and GDP-D-mannose pyrophosphorylase activities. Biochem. Biophys. Res. Commun. **353**(1): 200-206.

Sousa, S.A., Ulrich, M., Bragonzi, A., Burke, M., Worlitzsch, D., Leitão, J.H., Meisner, C., Eberl, L., Sá-correia, I., and Döring, G. 2007*b*. Virulence of *Burkholderia cepacia* complex strains in gp91$^{phox-/-}$ mice. Cell Microbiol. **9**(12): 2817-2825.

Speert, D.P., Henry, D., Vandamme, P., Corey, M., and Mahenthiralingam, E. 2002. Epidemiology of *Burkholderia cepacia* complex in patients with cystic fibrosis, Canada. Emerg. Infect. Dis. **8**(2): 181-187.

Sutherland, I.W. 1999. Polysaccharases for microbial exopolysaccharides. Carbohydr. Polym. **38**(4): 319-328.

Sutherland, I.W. 1967. Phage-induced fucosidases hydrolysing the exopolysaccharide of *Klebsiella arogenes* type 54 [A3(S1)]. Biochem. J. **104**(1): 278-285.

Szymanski, C.M., St. Michael, F., Jarrell, H.C., Li, J., Gilbert, M., Larocque, S., Vinogradov, E., and Brisson, J. 2003. Detection of conserved N-linked glycans and phase-variable lipooligosaccharides and capsules from *Campylobacter* cells by mass spectrometry and high resolution magic angle spinning NMR spectroscopy. J. Biol. Chem. **278**(27): 24509-24520.

Tait, K., Skillman, L.C., and Sutherland, I.W. 2002. The efficacy of bacteriophage as a method of biofilm eradication. Biofouling **18**(4): 305-311.

Tam, V.H., Schilling, A.N., Vo, G., Kabbara, S., Kwa, A.L., Wiederhold, N.P., and Lewis, R.E. 2005. Pharmacodynamics of polymyxin B against *Pseudomonas aeruginosa*. Antimicrob. Agents Ch. **49**(9): 3624-3630.

Thiel, K. 2004. Old dogma, new tricks - 21st Century phage therapy. Nat. Biotechnol. **22**(1): 31-36.

Tomlin, K.L., Coll, O.P., and Ceri, H. 2001. Interspecies biofilms of *Pseudomonas aeruginosa* and *Burkholderia cepacia*. Can. J. Microbiol. **47**(10): 949-954.

Tomlinson, S., and Taylor, P.W. 1985. Neuraminidase associated with coliphage E that specifically depolymerizes the *Escherichia coli* K1 capsular polysaccharide. J. Virol. **55**(2): 374-378.

Vandamme, P., Holmes, B., Vancanneyt, M., Coenye, T., Hoste, B., Coopman, R., Revets, H., Lauwers, S., Gillis, M., Kersters, K., and Govan, J.R. 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.

Vandenbergh, P.A., and Cole, R.L. 1986. Cloning and expression in *Escherichia coli* of the polysaccharide depolymerase associated with bacteriophage-infected *Erwinia amylovora*. Appl. Environ. Microbiol. **51**(4): 862-864.

Vermis, K., Coenye, T., LiPuma, J.J., Mahenthiralingam, E., Nelis, H.J., and Vandamme, P. 2004. Proposal to accommodate *Burkholderia cepacia* genomovar VI as *Burkholderia dolosa* sp. nov. Int. J. Syst. Bacteriol. **54**: 689-691.

Videira, P.A., Garcia, A.P., and Sá-Correia, I. 2005. Functional and topological analysis of the *Burkholderia cenocepacia* priming glucosyltransferase BceB, involved in the biosynthesis of the cepacian exopolysaccharide. J. Bacteriol. **187**(14): 5013-5018.

Vinion-Dubiel, A.D., and Goldberg, J.B. 2003. Lipopolysaccharide of *Burkholderia cepacia* complex. J. Endoxtin Res. **9**(4): 201-213.

Vishwanath, S., and Ramphal, R. 1984. Adherence of *Pseudomonas aeruginosa* to human tracheobronchial mucin. Infect. Immun. **45**(1): 197-202.

Walsh, T. A., and D. P. Ballou. 1983. Halogenated protocatechuates as substrates for protocatechuate dioxygenase from *Pseudomonas cepacia*. J. Biol. Chem. **258**: 14413–14421.

Winkelstein, J.A., Marino, M.C., Johnston Jr., R.B., Boyle, J., Curnutte, J., Gallin, J.I., Malech, H.L., Holland, S.M., Ochs, H., Quie, P., Buckley, R.H., Foster, C.B., Chanock, S.J., and Dickler, H. 2000. Chronic granulomatous disease: Report on a national registry of 368 patients. Medicine **79**(3): 155-169.

Woolfrey, B.F., Lally, R.T., Ederer, M.N., and Quall, C.O. 1984. Evaluation of the automicrobic system for susceptibility testing of *Pseudomonas aeruginosa* to gentamicin, tobramycin and amikacin. J. Clin. Microbiol. **19**(4): 502-505.

Zayas, M., and Villafane, R. 2007. Identification of the *Salmonella* phage ε34 tailspike gene. Gene **386**(1-2): 211-217.

Zlosnik, J.E.A., Hird, T.J., Fraenkel, M.C., Moreira, L.M., Henry, D.A., and Speert, D.P. 2008. Differential mucoid exopolysaccharide production by members of the *Burkholderia cepacia* complex. Journal of Clinical Microbiology **46**(4): 1470-1473.

APPENDIX: Construction of plasposon insertion libraries in *B. cenocepacia*

INTRODUCTION

Plasposons are similar to transposons as they can be used to randomly mutate the genomes of Gram negative bacteria (Dennis and Zylstra 1998). However, plasposons have several advantages as mutation tools over transposons. Specifically, since the transposase is not part of the inserted fragment, the plasposon insertion is stable. Further, plasposons are self-cloning, allow for more than one round of mutagenesis and they are smaller than transposons making them particularly easy to use. To date, no plasposons containing the *lux* operon have been published. Such a plasposon would be an excellent genetic tool as it could be used to randomly mutate the genome of a target bacterium and, it would be able to act as a reporter of gene expression. The construction of such a plasposon, pTn*Modlux*OTp', is discussed below. Additionally, in preliminary experiments, *Dictyostelium discoideum* was investigated as a potential host model system for the BCC that could be used to screen large plasposon insertion libraries.

Plasposon mutagenesis of the BCC

Plasposons have been used with great success to randomly mutate and indentify genes of the BCC. A particularly good example that demonstrates the utility of plasposon mutagenesis in the BCC is the identification of the Ptw type IV secretion system (T4SS). Initially, it was demonstrated that the *ptw* genes carried on a plasmid in *B. cenocepacia* K56-2 were involved in plant tissue watersoaking (Engledow et al. 2004). This was determined by screening plasposon mutants of K56-2 for the loss of the ability to create tissue damage. One such clone was found and sequencing analysis showed that the plasposon had

inserted into a region of the genome that contained genes putatively involved in a T4SS. It was hypothesized that this T4SS was responsible for toxin delivery and plant tissue watersoaking. Future work demonstrated that this region did indeed encode a T4SS, called Ptw T4SS, and that it was important for intracellular survival and replication of K56-2 in monocyte-derived macrophages (Sajjan et al. 2008). Recently, it was shown that the Ptw T4SS is not involved in DNA transfer, and it is predicted to be involved in toxin delivery (Zhang et al. 2009). Collectively, this work identifies and characterizes a second T4SS of the BCC that is important for pathogenesis. The identification of the Ptw T4SS may not have been possible if it were not for the initial screen of K56-2 plasposon mutants.

In recent years, plasposons have been adapted to contain tags allowing them to be used in signature tag mutagenesis (STM) of Gram negative bacteria including the BCC (Hunt et al. 2004; Rakhimova et al. 2008; Wiehlmann et al. 2007). Researchers have used STM plasposons to screen *P. aeruginosa* for attenuated killing of *C. elegans*, quorum sensing deficiencies, alterations in type II secretion effectors and decreased resistance to polymorphonuclear leukocytes (Wiehlmann et al. 2007). Additionally, genes involved in bacterial survival *in vivo* in the rat agar bead model have been investigated using STM plasposon mutagenesis in the BCC (Hunt et al. 2004).

To date, no plasposon containing *luxCDABE* has been reported in the literature. Such a plasposon would be extremely useful in mutagenesis because it would allow one to assay the activity of many promoters under a variety of conditions. Additionally, there are no large plasposon insertion libraries published

for the clinically important *B. cenocepacia* ET12 strain K56-2 or the Midwest strain PC184 (Mahenthiralingam et al. 2005). Plasposon insertion libraries could be shared with collaborators in the BCC research field and used to assay for a number of attributes including but not limited to: phage receptors, antibiotic resistant determinants and virulence factors.

Molecular mechanism of plasposons

Plasposons have been demonstrated to be effective broad host range cloning tools (Dennis and Zylstra 1998). The transposed genetic element of a plasposon contains the pMB1 origin of replication and an antibiotic resistance cassette found between the Tn*5* inverted repeats (IR). The backbone of the plasmid that carries the plasposon contains the RP4 origin of transfer, allowing the plasmid to be mobilized from *E. coli* into the target bacterium. Also found on the backbone is the Tn*5* transposase enzyme, a broad host range transposase.

Tn*5* transposase does not require host proteins to transpose and, other than the specificity requirement of the IR, Tn*5* does not demonstrate high amounts of host specificity (Reznikoff 2008). The mechanism of transposition is cut and paste, resulting in a nine bp duplication in the target DNA. Since the Tn*5* transpoase is not inserted in the genome, plasposon insertion events are final, resulting in mutants that will be stable over time (Dennis and Zylstra 1998).

***Dictyostelium discoideum* as a host model**

The amoebae *D. discoideum* has been shown to be a valid host model to assess bacterial virulence and intracellular survival. It has been used to assay the virulence of *Legionella pneumophila*, *Mycobacterium* spp., *Cryptococcus*

neoformans, *Pseudomonas aeruginosa*, and *Vibrio cholerae* (Pukatzki et al. 2006 and Unal and Steinert, 2006). Specifically, transposon insertion mutants constructed in *Vibrio cholerae* were screened to identify those that were attenuated for virulence as determined by the inability to kill *D. discoideum* (Pukatzki et al. 2006). From this screen, a type VI secretion system was identified in *V. cholerae* that was further shown to be important for cytotoxicity in macrophages. It is hypothesized that the novel type VI secretion system is also important in human pathogenesis. During this work, Pukatzki et al. (2006) showed that *D. discoideum* can be used as a high throughput host model.

D. discoideum provides an excellent alternative infection model for opportunistic human pathogens as its phagocytosis steps are similar to macrophages, it has a known haploid genomic sequence and it is extremely easy to use as it grows at room temperature (Cosson et al. 2008; Unal and Steinert, 2006). Plaque assays are done by plating bacteria and amoebae together on media that does not support the growth of the amoebae. In this way, the amoebae will only survive if it can feed on the bacteria. If bacteria are virulent, they will kill the amoebae resulting in a lawn of bacteria. If the bacteria are avirulent, the amoebae can feed on them and form clear plaques in the bacterial lawn.

The amoebae *Acanthamoeba polyphaga* has been proposed as a reservoir for the BCC and as a potential transmission vehicle as members of the BCC can survive intracellularly in *A. polyphaga* (Lamothe et al. 2007; Marolda et al. 1999). To date, very little work has been done using *D. discoideum* to assay for virulence in the BCC. Aubert et al. (2008) used *D. discoideum* to assay for virulence of a

type VI secretion system mutant in K56-2. However, no work has been done to test the validity of *D. discoideum* as an infection model for the BCC as a whole. In this work, the virulence of members of the BCC in *D. discoideum* is compared to the *G. mellonella* model of infection (Seed and Dennis 2008).

MATERIALS AND METHODS

Bacterial strains and growth conditions

BCC strains listed in Table 1 were grown as previously described in this document. Additional strains used in this work are listed in Table 8. Chemically competent *E. coli* DH5α cells (Invitrogen, Carlsbad, CA) were used in all cloning experiments including the construction and maintenance of pTn*Modlux*OTp'. *E. coli* HB101 pRK2013 (Figurski and Helinski 1979) was used to mobilize plasposons from *E. coli* during triparental matings.

All plasposons (Dennis and Zylstra 1998) and plasmids used in this research are shown in Table 9. *Dictyostelium discoideum* AX3, *Klebsiella aerogenes* and *Vibrio cholerae* V52 (Pukatzki et al. 2006) were provided by Dr. S. Pukatzki.

Table 8 Bacterial strains used in this research

Species	Strain	Reference
Escherichia coli	HB101 pRK2013	Figurski and Helinski (1979)
Dictyostelium discoideum	AX3	(Pukatzki et al. 2006 and dictyBase)
Klebsiella aerogenes		(Pukatzki et al. 2006)
Vibrio cholerae	V52	(Pukatzki et al. 2006)

Table 9 Plasmids and plasposons used in this research

Plasmid/Plasposon	Description	Reference
Plasmids		
pNLp10	pSC26 derivative containing promoterless *luxCDABE*	(Price and Raivio 2009)
Plasposons		
pTn*Mod*OTp'	Plasposon, trimethoprim resistance cassette	Dennis and Zylstra (1998)
pTn*Modlux*OTp'	Plasposon, promoterless *luxCDABE* reporter, trimethoprim resistance cassette	This study

D. discoideum AX3 was grown aerobically in 25 mL of HL5 broth (in 1L; 10 g glucose, 5 g yeast extract, 5 g bactopeptone, 5 g thiotone peptone, 0.67 g $Na_2HPO_4 \bullet 7H_2O$, 0.34 g KH_2PO_4 and 0.05 g dihydrostreptomycin-sulfate, pH 6.5) (Sussman 1987) at 22°C with shaking at 200 rpm and passaged every 96 h. AX3 predation experiments were done on SM/5 (in 1 L; 2 g glucose, 2 g bactopeptone, 0.2 g yeast extract, 0.1 g $MgSO_4$, 1.9 g KH_2PO_4 and 1.0 g K_2HPO_4, pH 6.5) (Pukatzki et al. 2006) supplemented with 2.92 g/L NaCl and incubated at 22°C for 72 h.

Construction of a plasposon insertion library *B. cenocepacia*

Construction of pTnModluxOTp'

To construct pTn*Modlux*OTp', pNLp10 and pTn*Mod*OTp' were individually digested with *Not*I (Invitrogen) and ligated together at 16°C for four hours. 5 μL of the ligation mixture was then used to transform *E. coli* DH5α using 100 mg/L Tp to select for a clone carrying pTn*Modlux*OTp'. Isolation of plasmid DNA from clones followed by digestion with *Not*I was done on approximately 20 clones and those with the correctly sized insertion fragment were sequenced to determine the orientation of *luxCDABE*. Sequencing primers included: Lux5'seq (GCACTAAATCATCACTTTCG), Lux3'seq (CGTCGCGTCAATACGAGGG) and JDTpR. A clone, *E. coli* pTn*Modlux*OTp' (*E.coli* Modlux429) harboring the desired plasposon was isolated and is stored at -80°C.

Determining the ability of pTnModluxOTp' to act as a reporter of gene expression

To show that pTn*Modlux*OTp' can be used as a reporter of promoter activity, K56-2::*Modlux*OTp' exconjugates were assayed for light production over 24 hours using a Wallac VICTOR^2 1420 multilabel counter. To do this, 25 clones were grown ON at 37°C in liquid following which, 20 μL was subcultured into 200 μL of ½ LB broth and incubated at 37°C with shaking. Luminescence readings (CPS) and OD_{600} measurements were taken of the overnight culture and of the subcultures at 1.5 h, 3 h, 4 h, 6 h and 24 h.

Using pTnModluxOTp' to create insertion mutants in B. cenocepacia

Triparental matings, were performed using *B. cenocepacia* K56-2 and *B. cenocepacia* PC184. *Pseudomonas* isolation agar (PIA) + 300 mg/L Tp was used to select for exconjugates. Additionally, electroporation was used to transform K56-2 and PC184 with pTn*Modlux*OTp', with selection using LB + 300 mg/L Tp.

Chromosomal DNA was isolated from 20 K56-2::Tn*Modlux*OTp' exconjugates and used to determine the plasposon insertion sites as previously described, (Dennis and Zylstra 1998). The following enzymes were used individually to digest the chromosome: *Pst*I, *Xba*I, *Sal*I and *Hind*III. *PstI* was found to give the best recovery of recombinant plasmids. The insertion site of the plasposon was determined by isolating the recombinant plasmid from *E. coli* clones and sequencing with Lux5'seq and/or JDoriR. Additionally, the sequencing protocol described by Leveau et al. (2006) for plasposon insertion determination was performed on all 20 isolated chromosomes in attempts to recover insertion sequences.

Library construction using pTnModluxOTp' and pTnModOTp'

All plasposon insertion libraries were made using the Genetix Q-Pix machine and software. Plasposon insertion clones were collected into 240 μL of LB broth + 10% glycerol. Cells were allowed to grow at 37°C with gentle shaking for 24 hours before storing at -80°C. For construction of the K56-2::Tn*Modlux*OTp' library, triparental mating was used to deliver the plasposon into K56-2. Electroporation was used to deliver pTn*Mod*OTp', and pTn*Modlux*OTp' into K56-2 and PC184, respectively, for the construction of plasposon insertion libraries in these strains.

Toward development of Dictyostelium discoideum as a high throughput screening method for BCC virulence

Dictyostelium discoideum AX3 plaque assay

Virulence of BCC members was assayed for using the *D. discoideum* AX3 plaque assay (Pukatzki et al. 2006). Briefly, 10 μL of an anexic *D. discoideum* AX3 culture, grown for four days was collected and the amoebae concentration was determined using a hemacytometer (Hausser Scientific, Horsham, PA). The AX3 culture was then washed once with SorC (16.7 mM Na_2H, 16.7 mM KH_2PO_4, 50 μM $CaCl_2$, pH 6.0) (Pukatzki et al. 2006) and diluted to contain 5 x 10^3 cells/mL. 100 μL of the 5 x 10^3 cells/mL AX3 culture was combined with 100 μL of bacterial overnight culture on SM/5 plates and incubated for three days at 21°C. Each BCC strain assayed for virulence was done in triplicate. An avirulent strain of *Klebsiella aerogenes* and the virulent *Vibrio cholerae* V52 provided controls for these experiments, maintained at -80°C.

RESULTS AND DISCUSSION

Plasposon insertion libraries in *B. cenocepacia*

Construction of plasposon containing lux reporter

A novel plasposon, pTn*Modlux*OTp' was constructed by inserting the *lux* operon from *Photorabdus luminescens* (contained on pNLp10) into the plasposon backbone of pTn*Mod*OTp', figures P and Q. pTn*Modlux*OTp' is maintained in *E. coli* DH5α.

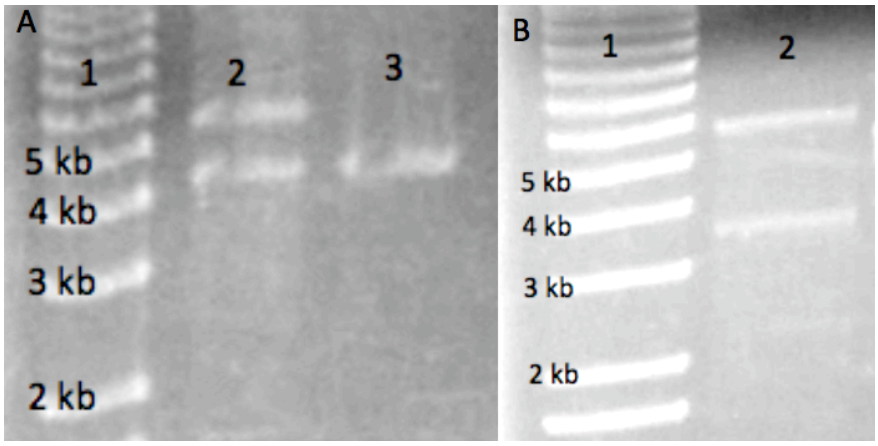

Figure 20 Restriction digestion analysis of pTn*Modlux*OTp' using agarose gel electrophoresis. Legend. (A) 1, 1 kb+ ladder (Invitrogen), 2, pTn*Modlux*OTp', 3, pTn*Mod*OTp'. (B) 1, 1 kb+ ladder (Invitrogen), 2, pNLp10. All plasmids are cut with *Not*I (Invitrogen).

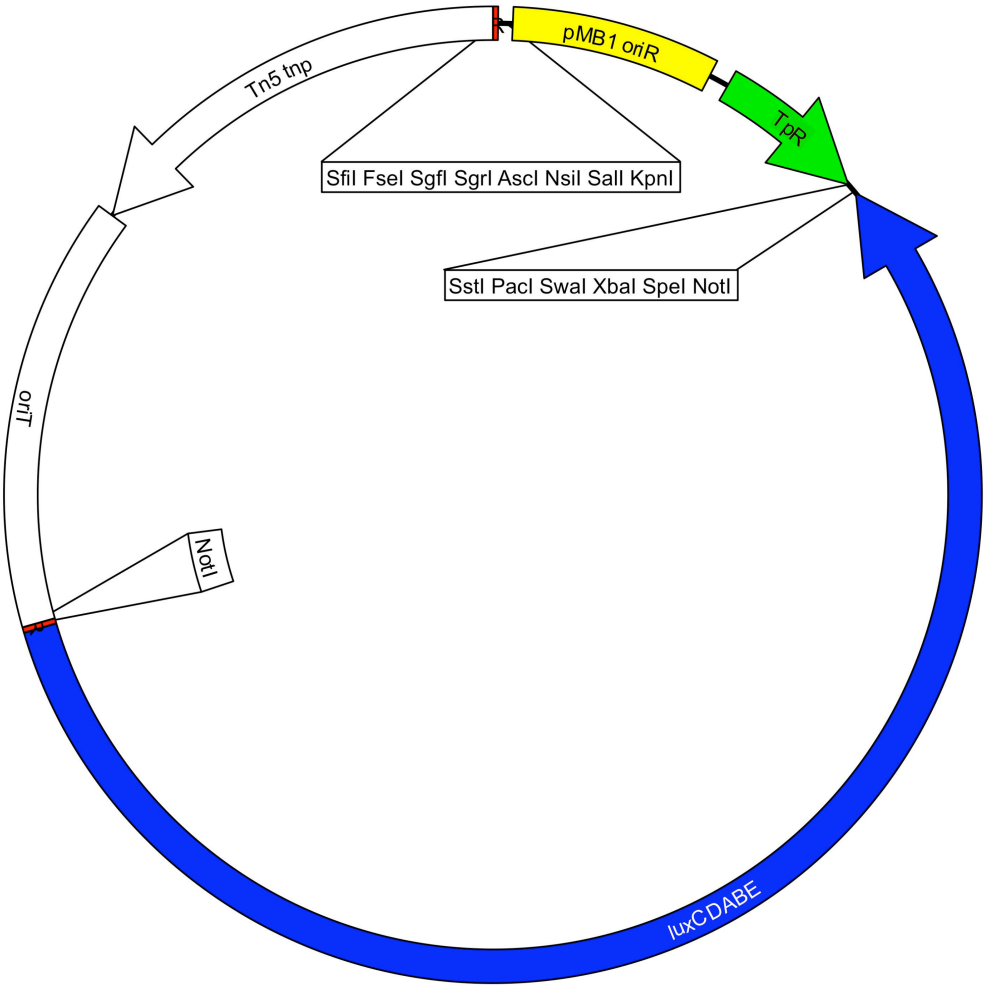

Figure 21 Schematic of pTn*Modlux*OTp'. Legend. Blue, *luxCDABE*, green, trimethoprim resistance cassette, yellow, pMB1 origin of replication, red, inverted repeats, Tn*5* tnp, Tn*5* transposase, oriT, origin of transfer. The colored pieces red, yellow, green and blue are included in the inserted fragment.

The ability of pTn*Modlux*OTp' to act as a reporter of gene expression was determined by assaying for light production by 25 *B. cenocepacia* K56-2::Tn*Modlux*OTp' plasposon insertion mutants. Ten representatives are shown in Figure 22. Light production was not only variable among insertion mutants but also changed over time. This demonstrated that the promoterless *lux* operon is functional and can serve as a reporter of promoter expression. Wildtype K56-2, containing no Tn*Modlux*OTp', provided the negative control.

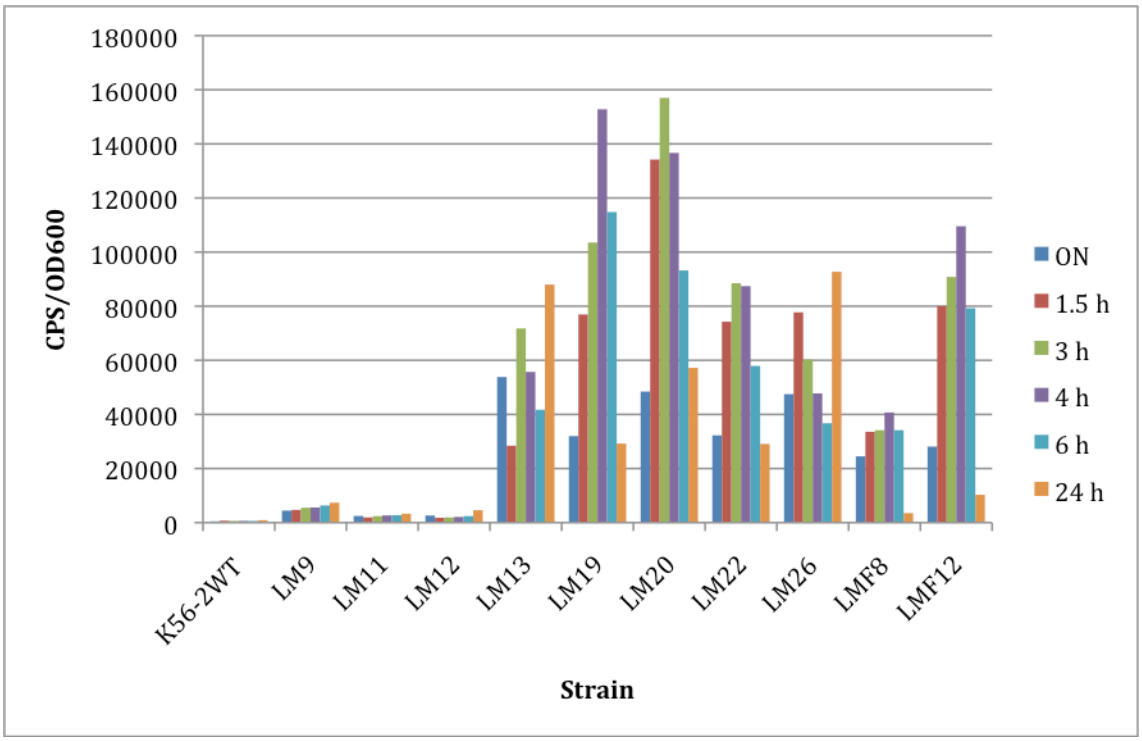

Figure 22 Luminescence of ten K56-2::Tn*Modlux*OTp' mutants. Legend. Luminescence reading taken from a culture grown; blue, overnight (ON), red, 1.5 h, green, 3 h, purple, 4 h, teal, 6 h, orange, 24 h.

To confirm that pTn*Modlux*OTp' can insert correctly into the genome of a Gram negative bacterium, chromosomal DNA from 20 K56-2::Tn*Modlux*OTp' was isolated and attempts were made to determine the plasposon insertion site in each mutant. To do this, chromosomal DNA was digested with a single enzyme then ligated to create a recombinant plasmid that could be recovered using *E. coli* and sequenced for determination of the plasposon insertion site (Dennis and Zylstra 1998). Over 50 attempts using a variety of restriction enzymes were done in order to determine the insertion site of all 20 K56-2 clones. But, only six recombinant plasmids could be recovered, Table 10. The size of the plasposon (approximately 7400 bp) made it difficult to find unique restriction enzymes to use for chromosomal digestion and also made recombinant plasmids very large and difficult to recover. Using restriction enzymes that cut in the *lux* operon did not increase recombinant plasmid recovery.

Five of the seven K56-2::Tn*Modlux*OTp' exconjugates sequenced demonstrated that pTn*Modlux*OTp' could insert correctly and randomly in the chromosome. Sequence analysis from two of the recombinant plasmids showed that the DNA flanking the plasposon was the pTn*Mod*OTp' backbone (oriT and Tn*5*). This result was unexpected and is not completely understood as these genes should not be included in the inserted plasposon. Similar sequencing results have been obtained when sequencing pTn*Mod*OTp' insertion sites in K56-2, though at a much lower frequency. Dennis and Zylstra (1998) who invented plasposons did not observe this when recovering plasposons.

Table 10 pTn*Modlux*OTp' insertion sites in K56-2

K56-2::Tn*Modlux*OTp' exconjugate	Homologue, as determined using NCBI BLASTN analysis of sequence flanking plasposon
LM13	*B. cenocepacia* J2315 putative membrane protein *B. cenocepacia* MC0-3 ZipA-FtsZ-binding region
LM9	*B. cepacia* 383 HAD-superfamily phosphate subfamily IIIC/FkbH
LM12	*B. cenocepacia* J2315 2 sensor kinase protein: 2 component system and putative cation efflux pump *B. cenocepacia* MC0-3 heavy metal sensor signal transduction major super family MFS1
LM F8	*B. cenocepacia* J2315 putative capsule polysaccharide export protein
LM19	*B. cenocepacia* J2315 glucosyl transferase
LM1	pTn*Mod*OTp'*
LM5	pTn*Mod*OTp'*

Legend. Insertion sites of Tn*Modlux*OTp' in K56-2 exconjugates obtained from triparental matings. Determined by sequencing with primers JDoriR and Lux5'seq. *, sequenced into the oriT and Tn*5* of pTn*Mod*OTp'.

In attempts to increase the number of sequenced K56-2::Tn*Modlux*OTp' insertion mutants, a protocol for plasposon insertion sequencing described by Leveau et al. (2006) was employed. This protocol involved sequencing directly from the isolated chromosome and was not effective in elucidation of plasposon insertion sites in this study. Leveau et al. (2006) did report the elucidation of a plasposon insertion site in a member of the BCC, *B. multivorans* ATCC 17616. However, sequencing data obtained from K56-2::Tn*Modlux*OTp' exconjugates using the described protocol was not useable.

Library construction in B. cenocepacia

pTn*Modlux*OTp' and pTn*Mod*OTp' were used to construct plasposon insertion libraries in *B. cenocepacia*. Creating such libraries is of great importance because they can be used to screen for virulence factors, phage receptors,

antibiotic resistance determinants, and much more. Select members of the BCC including *B. multivorans* strains C5393, C3430, C5274 and ATCC 17616 as well as *B. cenocepacia* PC184 and K56-2 were examined for their utility in creating a plasposon insertion library. To do this, each strain was assayed for the ability to take up and incorporate either pTn*Modlux*OTp' or pTn*Mod*OTp' by electroporation or through triparental mating. These two species of the BCC were chosen for investigation because of their prevalence in CF patients (Reik et al. 2005).

The *B. multivorans* strains were unable to be mutagenized by either of the two plasposons for one of two reasons. Either, the trimethoprim MIC of the strain was very high leading to the recovery of thousands of colonies following transformation, or, no colonies were recovered from multiple electroporations and triparental matings. ATCC 17616, C3430, C5393 were found to have MICs $\geq$ 400 mg/L Tp and therefore are not good candidates for library construction using a plasposon with the trimethoprim selection marker. The last *B. multivorans* strain tested, C5274, had an acceptable MIC of approximately 100 mg/L Tp, however, transformations with either plasposon were not successful in generating clones. The reason for the inability to mutagenize C5274 with either pTn*Modlux*OTp' or pTn*Mod*OTp' is not known. Continued work should to be done in order to create a plasposon insertion library in the clinically relevant species *B. multivorans*.

Both *B. cenocepacia* PC814 and K56-2 could be mutagenized with either plasposon. When pTn*Modlux*OTp' was delivered into K56-2 by electroporation, the luminescence ability was lost. The reason for this is not known. But, as shown

earlier in this work, triparental mating is an effective way to deliver the reporter plasposon into K56-2. pTn*Modlux*OTp' reporter ability was not lost when it was delivered into PC184 via electroporation. Luminescence readings of PC184::Tn*Modlux*OTp' ranged from <1,000 to >150,000 CPS/OD_{600} when plasmid was delivered by electroporation. The transformation efficiency was very low when triparental mating was used to introduce pTn*Modlux*OTp' into PC184, so, electroporation was used to deliver the plasposon.

Large plasposon insertion libraries were constructed in both *B. cenocepacia* PC184 and K56-2. pTn*Modlux*OTp' was used to create an approximately 9600 clone library in PC184 and an approximately 2500 clone library in K56-2. Luminescence readings of PC184::Tn*Modlux*OTp' and K56-2::Tn*Modlux*OTp' transformants grown for 24 h in LB + 10% glycerol demonstrated that the *lux* operon was functional and could act as a reporter. Light production by transformants was found to range from <1,000 CPS/OD_{600} to >400,000 CPS/OD_{600}, showing that *lux* is acting as a reporter of promoter activity.

Finally, an approximately 9600 clone library was made in K56-2 using pTn*Mod*OTp'. Construction of this library was important because K56-2 is the most phage susceptible strain in the BCC strain panel used in this work. This library can be used along with the reporter library to screen for phage receptors. To ensure that pTn*Mod*OTp' can be effective as a mutagen in K56-2, the insertion site of three K56-2 clones was determined. NCBI BLASTN analysis of the plasposon insertion site in each clone showed that clone K56-2::Tn*Mod*OTp'-6 had taken up the plasposon in a putative sodium/hydrogen exchanger gene, clone

K56-2::Tn*Mod*OTp'-3 had the plasposon inserted in a putative peptidoglycan glycosyltransferase and finally, clone K56-2::Tn*Mod*OTp'-21-4 had the plasposon inserted in a putative guanine specific ribonuclease.

All libraries were constructed using the Genetix Q-Pix machine and software. There was one major disadvantage to using this machine; the machine often missed colonies meaning that there are blank wells in almost every 96 well plate. Future work could include reviewing the library for missed wells and either inoculating them by hand or combining plates to make completely full plates. But, the machine did provide several advantages that largely outweighed the disadvantage. These include, being able to set a minimum size and shape of colony to select as well as the rapid speed with which clones are collected. Without the speed of the machine gathering the > 20,000 colonies would have taken an extremely long time and it is likely that contamination by human error could have occurred which would be more deleterious to the library than vacant wells in the plates.

Investigation of *Dictyostelium discoideum* as an infection model for the BCC

The *Dictyostelium discoideum* AX3 killing assay was investigated as a potential model for screening virulence of the BCC, Table 11. The premise of this work was to identify if the amoebae killing assay results were comparable to those reported for other infection models such as the *G. mellonella* model presented by Seed and Dennis (2008).

The virulence of the BCC strains tested here are generally comparable to the LD_{50}s reported for *G. mellonella* model of infection (Seed and Dennis 2008),

with the exception of *B. cenocepacia* strains. Strains found to have LD_{50}s > 300,000 in *G. mellonella* were all found to be avirulent in the AX3 predation studies. And, strains with LD_{50}s < 300,000 were found to be virulent in the AX3 model except for *B. dolosa* LMG 21443 and *B. cenocepacia* strains CEP511, C1257, C4455 and C5424. CEP511 was associated with a low percentage of lung pathology in the rat agar bead model (Bernier et al. 2003) and was found to be avirulent towards *D. discoideum*. However, in the alfalfa model and in *G. mellonella*, CEP511 was found to be virulent. Strains C4455 and C1257 were delayed for *G. mellonella* killing and had LD_{50}s of 100,000 and 40,000 72 h pi, respectively. The delay in larvae killing by C4455 and C1257 may be the reason for the observed difference in virulence between *G. mellonella* and *D. discoideum*.

Variability was seen in the virulence of *B. cenocepacia* K56-2. During the initial killing assay, K56-2 was found to be avirulent. This result was unexpected since Aubert et al. (2008) had reported this strain as virulent in this model. The AX3 plaque assay was performed an additional five times (in triplicate each time, with positive and negative controls) for this strain to determine if this was a one time unexplainable event. K56-2 was found to be virulent only 66% of the time. The reason for this variability is not known. During the identification of the Type VI secretion system of K56-2, Aubert et al. (2008) used *D. discoideum* AX3 killing as a screen for virulence of K56-2 and K56-2 type VI secretion mutants. There was no discussion by Aubert et al. (2008) on K56-2 virulence variability in this model. Further, there are no major differences in the *D. discoideum* AX3

killing assay performed in this study and the protocol employed by Aubert et al. (2008). In this study, approximately 500 amoebae were combined with 100 μL of bacteria grown to approximately 10^9 CFU/mL instead of approximately 100 amoebae combined with 100 μL of bacteria grown to approximately 10^7 CFU/mL. This minor difference is not thought to be the reason for the variability in the virulence observed for K56-2.

Interestingly, the growth of the individual BCC strains on SM/5 plates used in the plaque assay was highly variable, Table 11. As such, the bacterial growth of each strain was recorded when determining the outcome of the killing assay. Bacterial growth did not appear to correlate to virulence.

Table 11 Virulence of BCC in *Dictyostelium discoideum* AX3

Species	Strain	Virulent or avirulent	LD_{50} (CFU) in *G. mellonella*[a]	Growth
B. cepacia	LMG 18821	V	30	EG
	ATCC17759	V	1	EG
B. multivorans	C5393	A	>3,000,000	G
	C3430	A	>3,000,000	G-EG
	C5274	A	1,000,000	G-MG
	C5568	A	>3,000,000	G-EG
	ATCC 17616	A	ND	G-EG
B. cenocepacia	K56-2	A/V*	900	G-EG
	J2315	V	100,00	G-EG
	715J	V	4,000	EG
	C6433	V	30,000	G-MG
	C1257	A	100,000	EG
	C5424	A	30,000	G
	C4455	A	200,000	G
	Cep511	A	80,000	MG
	PC184	V	ND	G-EG
B. stabilis	LMG 14294	A	2,000,000	G
	LMG 18870	A	>2,000,000	G-MG
B. vietnamiensis	LMG 10929	V	ND	G
	DB01	V	200,000	EG
B. dolosa	LMG 18943	A	>4,000,000	G
	LMG 21443	A	40,000	G-MG
B. ambifaria	LMG 19467	V	800,000	G
	LMG 17828	V	ND	EG
	LMG 19182	A	ND	G-MG
B. anthina	LMG16670	A	300,000	EG
B. pyrrocinia	LMG14191	V	300	EG

Legend. BCC strains assayed for virulence in *Dictyostelium discoideum* AX3, V, virulent – ability of BCC strain to resist killing by amoebae, A, avirulent – ability of amoebae to kill BCC strain, *, inconsistent results with regard to virulence. BCC strains were additionally scored for growth on SM/5 medium; EG, excellent growth, G, growth, MG, minimal growth. [a], data from Seed and Dennis (2008).

Significantly, *B. vietnamiensis* DB01 was found to be virulent in this model. This finding is important as this strain is very easy to work with and genetically manipulate. Goudie and Dennis (unpublished) have constructed a plasposon insertion library containing approximately 2500 clones in DB01. Future work could include adapting *D. discoideum* as a high throughput model for screening virulence of DB01. This work would be extremely interesting and could lead to the identification of novel virulence factors or genes involved in the intracellular survival of the BCC.

Conclusions

In this work, a novel plasposon, pTn*Modlux*OTp', was constructed. This plasposon was shown to work as a reporter of gene expression and as a genetic tool capable to mutagenizing the genomes of *B. cenocepacia* K56-2 and PC184. This reporter plasposon as well as the traditional plasposon pTn*Mod*OTp' were used to construct large insertion libraries in K56-2 and PC184. These libraries contribute to the BCC field of research as libraries have wide spread application and these strains are both clinically important.

Additionally, the amoebae *D. discoideum* AX3 was investigated as an additional model of infection to investigate the virulence of the BCC. With the exception of a few strains of *B. cenocepacia*, the preliminary experiments done here show that the overall virulence seen in *D. discoideum* are similar to those seen in the *G. mellonella* model of infection (Seed and Dennis 2008). Additionally, this work has shown that *D. discoideum* can work as an infection

model for *B. vietnamiensis* DB01. This is important because DB01 is a strain that is commonly used in genetic studies in the BCC. It is suggested that future work include screening the constructed plasposon insertion libraries for phage receptors, antibiotic resistant determinants and/or virulence factors.

LITERATURE CITED

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. Microb. **64**(7): 2710-2715.

Engledow, A.S., Medrano, E.G., Mahenthiralingam, E., LiPuma, J.J., and Gonzalez, C.F. 2004. Involvement of a plasmid-encoded type IV secretion system in the plant tissue watersoaking phenotype of *Burkholderia cenocepacia*. J. Bacteriol. **186**(18): 6015-6024.

Figurski, D.H., and Helinski, D.R. 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in *trans*. P. Natl. Acad. Sci. USA **76**(4): 1648-1652.

Hunt, T.A., Kooi, C., Sokol, P.A., and Valvano, M.A. 2004. Identification of *Burkholderia cenocepacia* genes required for bacterial survival *in vivo*. Infect. Immun. **72**(7): 4010-4022.

Lamothe, J., Huynh, K., Grinstein, S., and Valvano, M. 2007. Intracellular survival of *Burkholderia cenocepacia* in macrophages is associated with delay in the maturation of bacteria-containing vacuoles. Cell Microbiol. **9**(1): 40-53.

Marolda, C.L., Hauröder, B., John, M.A., Michel, R., and Valvano, M.A. 1999. Intracellular survival and saprophytic growth of isolates from the *Burkholderia cepacia* complex in free-living amoebae. Microbiology **145**(7): 1509-1517.

Price, N.L., and Raivio, T.L. 2009. Characterization of the Cpx regulon in *Escherichia coli* strain MC4100. J. Bacteriol. **191**(6): 1798-1815.

Pukatzki, S., Ma, A.T., Sturtevant, D., Krastins, B., Sarracino, D., Nelson, W.C., Heidelberg, J.F., and Mekalanos, J.J. 2006. Identification of a conserved bacterial protein secretion system in *Vibrio cholerae* using the *Dictyostelium* host model system. P. Natl. Acad. Sci. USA **103**(5): 1528-1533.

Rakhimova, E., Munder, A., Wiehlmann, L., Bredenbruch, F., and Tümmler, B. 2008. Fitness of isogenic colony morphology variants of *Pseudomonas aeruginosa* in murine airway infection. PLoS ONE **3**(2).

Reik, R., Spilker, T., and LiPuma, J.J. 2005. Distribution of *Burkholderia cepacia* complex species among isolates recovered from persons with or without cystic fibrosis. J. Clin. Microbiol. **43**(6): 2926-2928.

Reznikoff, W. 2008. Transposon Tn*5*. Annu. Rev. Genet. **42**: 269-286.

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**(3): 1267-1275.

Sussman, M. 1987. Cultivation and synchronous morphogenesis of *Dictyostelium* under controlled experimental conditions. Methods Cell Biolo. **29**: 9-29.

Wiehlmann, L., Munder, A., Adams, T., Juhas, M., Kolmar, H., Salunkhe, P., and Tümmler, B. 2007. Functional genomics of *Pseudomonas aeruginosa* to identify habitat-specific determinants of pathogenicity. Int. J. Med. Microbiol. **297**(7-8): 615-623.

Zhang, R., LiPuma, J., and Gonzalez, C. 2009. Two type IV secretion systems with different functions in *Burkholderia cenocepacia* K56-2. Microbiology **155**: 4005-4013.