THE RELATIONSHIP BETWEEN PHENYLACETIC ACID CATABOLISM AND PATHOGENICITY OF *BURKHOLDERIA CENOCEPACIA* K56-2 IN THE *CAENORHABDITIS ELEGANS* HOST MODEL

by

Robyn Jamie Law

A Thesis submitted to the Faculty of Graduate Studies of
The University of Manitoba
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Department of Microbiology
University of Manitoba
Winnipeg

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Of

Master of Science

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Abstract

The *Burkholderia cepacia* complex (Bcc) is a group of genetically similar strains that have been identified as environmental pathogens with the ability to colonize various hosts. *B. cenocepacia* is a member of the Bcc capable of establishing devastating infections associated with severe necrotizing pneumonia and death. Currently, it is unclear as to what virulence factors have contributed to the development of pathogenicity of the Bcc across its diverse host range. In particular, metabolic versatility of these bacteria could be a reason for their ability to colonize diverse hosts. In this work, we characterize gene clusters involved in the phenylacetic acid (PA) degradation pathway of *B. cenocepacia* K56-2 in relation to pathogenicity of the organism in the *Caenorhabditis elegans* infection model. We use *C. elegans* to characterize *B. cenocepacia* infection and the relevance of PA catabolism to this infection. *B. cenocepacia* mutants defective in the activation, ring hydroxylation, ring opening and β-oxidation stages of PA degradation were tested for their ability to infect and kill *C. elegans*. By generating survival curves for *C. elegans* fed on each mutant, we have determined that inhibition of the activation and ring hydroxylation steps of the pathway result in reduced nematode killing. Further analysis of these mutations has confirmed that while activation of PA and a functional ring hydroxylation system are required for full pathogenicity, interruption of β-oxidation and ring opening actually increases the virulence of *B. cenocepacia* in *C. elegans*. Nematode survival was adversely affected by the presence of wild type and β-oxidation mutant metabolites in comparison to that of the ring hydroxylation mutant, which showed no significant effect

on survival. Bacterial titre of the nematode intestine shows no appreciable difference between accumulation of PA pathway mutants and K56-2. Microscopic examination of worms feeding on wild type and mutant strains expressing a fluorescent reporter, reveal similar intestinal distension, characteristic of this type of infection. In addition, nematode innate immune mechanisms employed in response to *B. cenocepacia* infection were investigated using RNA interference. Through specific gene inactivation by ingested double stranded RNA, we have found that worms with compromised immunity due to inhibition of the p38 mitogen-activated protein kinase pathway gene, *pmk-1*, no longer display the reduced killing phenotype; suggesting, that the diminished virulence seen in upper PA pathway mutants is dependent on the p38 MAPK pathway. The requirement for a functional activation and ring hydroxylation complex in relation to *B. cenocepacia* pathogenesis is not completely understood; however, our findings, taken together with other analyses relating similar metabolic pathways to pathogenicity create a potential link between *B. cenocepacia* metabolism and its ability to colonize multiple hosts. Eventually, studies of this nature may provide new insights into Bcc pathogenesis in humans.

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List of Abbreviations

Bcc	-	*Burkholderia cepacia* complex
Bp	-	Base pair
CGD	-	Chronic granulomatous disease
CF	-	Cystic fibrosis
CFTR	-	Cystic fibrosis transmembrane conductance regulator
CFU	-	Colony forming units
Cm	-	Chloramphenicol
DHFR	-	Dihydrofolate reductase
DIC	-	Differential interference contrast
dsRNA	-	Double stranded ribonucleic acid
eGFP	-	Enhanced green fluorescent protein
esp	-	Enhanced susceptibility to pathogens
Gm	-	Gentamicin
IPTG	-	Isopropyl-D-thiogalactopyranoside
Kb	-	Kilo base
Km	-	Kanamycin
L4	-	Larval stage 4
LB	-	Luria Burtani
LPS	-	Lipopolysaccharide
MAPK	-	Mitogen-activated protein kinase
Mb	-	Mega base

NGM	-	Nematode growth medium
NH_4Cl	-	Ammonium chloride
O.D.	-	Optical density
2-OH-PA	-	2-Hydroxy phenylacetic acid
PA	-	Phenylacetic acid
PBS	-	Phosphate buffered saline
PCR	-	Polymerase chain reaction
RNA	-	Ribonucleic acid
RNAi	-	Ribonucleic acid interference
ROS	-	Reactive oxygen species
rpm	-	Revolutions per minute
rRNA	-	Ribosomal ribonucleic acid
Tc	-	Tetracycline
Tp	-	Trimethoprim

1 – Introduction

1.0 The *Burkholderia cepacia* complex (Bcc)

The genus *Burkholderia* is comprised of over 30 species occupying a variety of ecological niches from soil and water to the respiratory tracts of humans (Coenye, Vandamme 2003). The majority of the group is classified as soil microorganisms displaying various non-pathogenic interactions with plants (Coenye, Vandamme 2003). In 1950, W.H. Burkholder described the plant pathogen responsible for soft rot in onions as *Pseudomonas cepacia* (Burkholder 1950), which was later renamed *Burkholderia cepacia* based on 16s rRNA analysis (Yabuuchi, Kosako et al. 1992).

In 1997, 16s rRNA and *recA* sequence homology showed that the organism classified as *B. cepacia* was actually a collection of at least five different species (Vandamme, Holmes et al. 1997). Since that discovery, international collaborative studies have revealed that strains originally designated as *B. cepacia* actually represent a group of closely related species (Mahenthiralingam, Coenye et al. 2000, Vandamme, Holmes et al. 2003, Vandamme, Henry et al. 2002, Coenye, Vandamme et al. 2001). Presently, there are at least nine formally named species that have been identified as members of the *Burkholderia cepacia* complex (Bcc) (Mahenthiralingam, Urban et al. 2005) and an additional seven distinct groups have been added based on multilocus sequence typing (MLST) (Mahenthiralingam, Baldwin et al. 2008).

The Bcc have developed diverse strategies granting them the capacity to inhabit soils, aqueous environments and the rhizosphere. Originally, it was the plant

pathogenic traits of these organisms that lead to their identification in 1950. More recently, the Bcc are being recognized for their ability to cause infection in humans. All members of the Bcc have been isolated from humans, especially immunocompromised individuals and those suffering from cystic fibrosis (CF) and chronic granulomatous disease (CGD) (Isles, Maclusky et al. 1984, Goldmann, Klinger 1986, Bylund, Burgess et al. 2006).

1.1. *B. cenocepacia* K56-2

The *Burkholderia cepacia* complex (Bcc) has been identified as a group of environmental pathogens with the ability to colonize a variety of environments (Mahenthiralingam, Urban et al. 2005). *B. cenocepacia*, is a representative member of the Bcc that has been isolated from Canadian CF patients (Speert, Henry et al. 2002). *B. cenocepacia* K56-2 has been chosen as the principle strain for this research in order to study its interaction with the nematode host.

Although all Bcc bacteria have been cultured from the sputum of CF patients, most infections with this group are caused by *B. cenocepacia* and *B. multivorans* (Mahenthiralingam, Urban et al. 2005). In fact, it has been shown that *B. cenocepacia* is capable of replacing *B. multivorans* during infection (Mahenthiralingam, Vandamme et al. 2001). *B. cenocepacia* K56-2 is a clonal isolate of *B. cenocepacia* J2315, both of which are of the ET-12 lineage (Ortega, Hunt et al. 2005). The highly transmissible ET-12 strain was responsible for the largest CF epidemic in Canada and the United Kingdom (Mahenthiralingam, Urban et al. 2005, Johnson, Tyler et al. 1994). For this reason,

sequencing of the index strain J2315 was initiated as a representative of Bcc opportunistic pathogens. *B. cenocepacia* J2315 and K56-2 are clonal strains sharing comparable macrorestriction patterns (Mahenthiralingam, Coenye et al. 2000) but the genomes of these organisms are not completely similar. *B. cenocepacia* K56-2 does not present the same LPS rough phenotype seen with *B. cenocepacia* J2315 due to lack of an insertional element within the glycosyltransferase gene of the O-antigen gene cluster (Ortega, Hunt et al. 2005). Experimental work was carried out using *B. cenocepacia* strain K56-2 since it more amenable to genetic manipulation than J2315; however, all sequence references and bioinformatic analyses refer to the J2315 genome as sequenced and annotated by the Sanger institute and Holden et al. (Holden, Seth-Smith et al. 2009).

1.1.1 Genome of *B. cenocepacia*

All Bcc bacteria described thus far possess large genomes made up of multiple replicons (Mahenthiralingam, Urban et al. 2005, Lessie, Hendrickson et al. 1996). The genome of *B. cenocepacia* J2315 is comprised of three chromosomes and one plasmid with a total genome size of 8.06 Mb. Chromosome 1 is the largest with an approximate size of 3.87 Mb, followed by chromosome 2 (3.22 Mb), chromosome 3 (0.88 Mb) and a 92 Kb plasmid. Most housekeeping genes are found on chromosome 1, however, ribosomal RNA operons and other essential genes have been found on chromosomes 2 and 3, leading to their designation as chromosomes rather than megaplasmids (Holden, Seth-Smith et al. 2009). In addition to a relatively large genome size, *B. cenocepacia*

J2315 also has a reasonably high GC content of about 66.9 % (Mahenthiralingam, Urban et al. 2005). A number of insertion sequences and genomic islands have also been found in the J2315 genome which may contribute to genome plasticity and virulence. It has been reported that genomic islands make up approximately 10 % of the total genome (Holden, Seth-Smith et al. 2009).

1.1.2 Ecology of *B. cenocepacia*

B. cenocepacia occupies many niches and has been isolated from the environment as well as human clinical sources (Coenye, Vandamme 2003). Isolates have been recovered from soil, fresh water and seawater, as well as the rhizosphere of plants, animals and the human lung (Coenye, Vandamme 2003, Mahenthiralingam, Urban et al. 2005). The Bcc are notorious contaminants of disposable hospital equipment and sterile solutions such as disinfectants and anaesthetics, leading to nosocomial infections (Coenye, LiPuma 2003). In addition, many species have been recovered from pharmaceutical and cosmetic products (Pankhurst, Philpott-Howard 1996, Jimenez 2001). The large genome and metabolic versatility of *B. cenocepacia* reflects the adaptability of this opportunistic pathogen to colonize multiple environments.

Most *Burkholderia* are soil microorganisms exhibiting different pathogenic and symbiotic relationships with plants. The soil environment may offer a rich variety of nutrients for microorganisms but may also contain harmful waste products due to the use of chemical fertilizers that can have an adverse effect on soil microbiota. Evolution

of a relatively large genome and metabolic versatility of Bcc bacteria has likely contributed to the group's ability to survive contaminated environments through metabolism of crude oils, herbicides and other pollutants (Mahenthiralingam, Urban et al. 2005).

Although *B. cenocepacia* is known to cause soft rot of onion bulbs (Burkholder 1950) it is not an important pathogen of other commercial crops. In fact, *B. cenocepacia* has been recognized for its environmentally beneficial effects. In addition to degradation of hazardous pollutants, *B. cenocepacia* is capable of protecting plants against fungal disease and promoting growth through production of antimicrobial compounds (Parke, Gurian-Sherman 2001).

1.1.3 Biotechnological Potential

The metabolic capacity of the Bcc confers significant potential for biotechnological applications. Bcc bacteria are abundant in the soil and rhizosphere and have been shown to produce powerful anti-fungal agents protecting commercially useful crops from seedling damage and root rot (Roitman, Mahoney et al. 1990, Zaki, Misaghi et al. 1998). In addition, these strains have been recognized for use in bioremediation based on their ability to degrade chlorinated compounds (Chiarini, Bevivino et al. 2006, Steffan, Sperry et al. 1999, Krumme, Timmis et al. 1993) and herbicides (Sangodkar, Chapman et al. 1988) that contaminate soil and groundwater supplies. These protective and ecologically beneficial properties of the Bcc lead to registration of certain members of the complex for commercial use as biological control

agents in the United States (Mahenthiralingam, Urban et al. 2005). However, due to concern over the opportunistic nature of these pathogens, Bcc bacteria are no longer in use for these applications.

1.2 Pathogenesis of the Bcc

The pathogenic potential of Bcc bacteria is in marked contrast to their ecological significance. Bcc strains are capable of infecting a variety of hosts ranging from single celled amoeba to multicellular eukaryotes, and are particularly pathogenic to individuals with compromised immunity (Speert D.P. 2002). Due to their natural resistance to many antibiotics as well as their ability to survive in disinfectant solutions, Bcc bacteria have become problematic hospital pathogens capable of causing human nosocomial infections (Mahenthiralingam, Vandamme 2005) however, the predominant class of patients affected by these organisms are those with impaired immunity as a result of underlying disease (Speert D.P. 2002). The prevalence of Bcc infection among CF patients in the 1980's (Isles, Maclusky et al. 1984) provoked major interest in the exploration of virulence mechanisms employed by these strains, and the reasons behind their ability to thrive in certain CF lung environments.

1.2.1 Virulence Factors of *B. cenocepacia*

B. cenocepacia is associated with a wide number of virulence factors involved in initiating disease and host immune response. Production of a variety of virulence factors allows this organism to better establish infection thereby enhancing its disease causing potential.

B. cenocepacia is inherently resistant to a broad range of antibiotics. Specific drug efflux pumps confer resistance to chloramphenicol and quinolones (Burns, Hedin et al. 1989, Burns, Wadsworth et al. 1996) while the reduced size of outer membrane porins (Parr, Moore et al. 1987) inhibits entrance of larger antibiotics to the cell. Production of a trimethoprim-resistant dihydrofolate reductase enzyme (Burns, Lien et al. 1989), as well as β-lactamases (Chiesa, Labrozzi et al. 1986) and penicillin binding proteins also contribute to the multiple resistance mechanisms of this microorganism. In addition, structure of the *B .cenocepacia* LPS imparts resistance to cationic antimicrobial peptides and polymyxin (Shimomura, Matsuura et al. 2003, Vinion-Dubiel, Goldberg 2003). The lack of antibiotic sensitivity of this organism limits the choice of genetic selection markers thereby adding to the difficulty in characterizing pathogenic traits of *B. cenocepacia* and Bcc bacteria.

B. cenocepacia also possesses a type IV secretion system that has been shown to be involved in release of cytotoxic factors (Engledow, Medrano et al. 2004). Production of exotoxins, proteases, siderophores, lipases and a type III secretion system have all been implicated in virulence (Mahenthiralingam, Vandamme 2005). In addition, multiple quorum sensing systems have been identified as being required for full pathogenicity in both worm and rodent infection models (Kothe, Antl et al. 2003, Sokol, Sajjan et al. 2003). *B. cenocepacia* is capable of forming biofilms for increased resistance (Desai, Bühler et al. 1998), and in some cases has been shown to form mixed biofilms with other CF lung colonizers such as *Pseudomonas aeruginosa* (Tomlin, Coll et al. 2001). Cable pili have been identified on epidemic strains (Sun, Jiang et al. 1995) and

appear to be required for adhesion to host epithelia (Sajjan, Wu et al. 2000, Sajjan, Sylvester et al. 2000). In addition, production of catalases and superoxide dismutases likely contribute to intracellular survival (Valvano, Keith et al. 2005, Lefebre, Valvano 2001).

1.2.2 Cystic Fibrosis

Cystic fibrosis (CF) is a genetic multi-system disease affecting approximately one in three thousand childbirths (Dupuis, Hamilton et al. 2005) and in most cases progresses to chronic pulmonary infections (Lyczak, Cannon et al. 2002). CF is an autosomal recessive disorder reflecting loss of function mutations in each allele of the *cftr* gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR). Variation in severity of symptoms amongst affected individuals is the result of several different point mutations in the *cftr* locus that can lead to CF. The most common is the so called *ΔF508* (Lyczak, Cannon et al. 2002) mutation that results in loss of the phenylalanine at position 508 in the CFTR protein. This mutation inevitably leads to the most severe symptoms.

CFTR is a cAMP regulated ion channel mediating transport of chloride (Cl^-) across the epithelia. The *ΔF508* mutation causes improper folding of the CFTR protein, leading to degradation before reaching the epithelial cell membrane (Lyczak, Cannon et al. 2002). Critical loss of Cl^- transport indirectly leads to hyper absorption of Na^+ and reduced water flow resulting in thick viscous mucous and impaired ciliary function.

This abnormally thick mucous can affect epithelial function of the lungs, liver, digestive system, pancreas and reproductive organs. Mucous build up can lead to diminished secretion of pancreatic enzymes hindering transport to the digestive system and malabsorption of nutrients. Obstruction of the vas deferens may result in male sterility, and mucous in the alveoli and air passages can make breathing increasingly difficult. In addition, mucous build up in the lungs usually leads to cycles of infection and inflammation resulting in permanent pulmonary damage and often disability and death.

1.2.3 Cystic Fibrosis Lung Infections

A substantial portion of morbidity and mortality among CF patients is related to pulmonary disease (Goss, Burns 2007). The altered surface liquid of lung epithelia in CF patients predisposes them to chronic bacterial infections of the respiratory tract (Dinwiddie 2000). Lung infections are often polymicrobial and eradication of established bacterial populations with antibiotic therapy is rarely effective. Infection of the airways leads to neutrophil recruitment and inflammation due to release of pro-inflammatory cytokines (Goss, Burns 2007). The cycle of chronic infection and inflammation that leads to respiratory tract injury and progressive deterioration of lung function is the leading cause of death in CF patients (Coenye, LiPuma 2003, Liou, Adler et al. 2001).

The primary colonizer of the CF lung is *Pseudomonas aeruginosa* (Ramphal, Vishwanath 1987), however, infections with *Staphylococcus aureus* (Armstrong,

Grimwood et al. 1996), *Haemophilus influenzae* (Armstrong, Grimwood et al. 1996, Rosenfeld, Gibson et al. 2001) and the Bcc (Isles, Maclusky et al. 1984, Thomassen, Demko et al. 1985, Kidd, Bell et al. 2003) have also been reported. Infections are commonly acquired in an age-dependent manner (Goss, Burns 2007) usually with *S. aureus* and *H. influenzae* in early childhood, followed by the Bcc during early adolescence and *P. aeruginosa* which can be present throughout.

1.2.4 'Cepacia Syndrome' in CF Patients

In comparison to *P. aeruginosa*, only a small proportion of the CF population become infected with Bcc strains, however, their impact on survival is significant (Coenye, LiPuma 2003, Liou, Adler et al. 2001, Snell, de Hoyos et al. 1993). Of the CF patients colonized by *B. cenocepacia* and the Bcc, some exhibit asymptomatic carriage or chronic infection, while others display a rapid and uncontrollable decline in lung function (Mahenthiralingam, Urban et al. 2005). This rapid deterioration combined with necrotizing pneumonia and septicaemia is known as 'Cepacia Syndrome' (Jones, Dodd et al. 2001, Valvano, Maloney et al. 2007). The basis for the differential outcome of CF patients colonized with Bcc is not well understood. Cepacia Syndrome is a unique characteristic of Bcc infection (Valvano, Maloney et al. 2007) and risk factors associated with acquisition of these organisms may include the severity of underlying disease, multiple hospital stays, age of the patient and family members colonized by Bcc strains (Coenye, Vandamme 2003).

1.3 Host Models for the Bcc

Several infection models are currently available for the study of Bcc pathogenesis. The rat agar bead model has been used to study chronic Bcc infection (Hunt, Kooi et al. 2004) while macrophage (Valvano, Keith et al. 2005, Saini, Galsworthy et al. 1999) and epithelial cell lines (Sajjan, Keshavjee et al. 2004) have been used to follow microbial interactions with host cells. Invertebrate hosts such as the fruit fly (*Drosophila melanogaster*) and the nematode (*Caenorhabditis elegans*) have gained interest for studying evolutionarily conserved microbial virulence strategies and innate immunity (Schulenburg, Kurz et al. 2004, Hilbi, Weber et al. 2007). Surrogate hosts, like *C. elegans*, provide a straightforward model in which to follow the infection process and have the potential to reveal multiple facets of the host-pathogen interaction (Steinert, Leippe et al. 2003).

1.3.1 *Caenorhabditis elegans*

C. elegans is a small, free living roundworm that is approximately 1 mm in length. It has a simple body plan consisting of two concentric cylindrical tubes that make up the nematode cuticle and digestive system (Sifri, Begun et al. 2005). The collagenous cuticle and digestive system are separated by the pseudocoelom, which provides a hydrostatic skeleton (Sifri, Begun et al. 2005). Traditionally, the nematode has been used to study development, neurobiology and aging (Brenner 1974, Larsen, Albert et al. 1995); however, since *C. elegans* is a microbivore in its natural soil environment, it has become a valuable tool for the study of environmental pathogens.

The nematode host model is inexpensive and relatively easy to maintain in a laboratory setting (Schulenburg, Kurz et al. 2004, Ahringer 1997). These animals reproduce quickly and possess a relatively small genome which can be easily manipulated, making them excellent candidates for genomic study (Schulenburg, Kurz et al. 2004). The *C. elegans* genome has been completely sequenced and determined to have a length of approximately 100 Mb which is considerably smaller than the genome size of other eukaryotic organisms (Stein, Bao et al. 2003). The length of the human genome, for example, has been estimated at about 2900 Mb, roughly thirty times larger than the genome of *C. elegans* (Mouse Genome Sequencing Consortium, Waterston et al. 2002).

1.3.2 *C. elegans* as an Infection Model

C. elegans feeds naturally on bacteria making the nematode an ideal model for feeding based pathogenicity studies. The use of *C. elegans* as a host model to study bacterial pathogenesis was first reported for the opportunistic pathogen, *P. aeruginosa*, by Tan et al in 1999 (Tan, Mahajan-Miklos et al. 1999). Since then, an increasing number of human and animal pathogens have been shown to kill nematodes by means of five key mechanisms (Sifri, Begun et al. 2005).

Most human pathogens infect and colonize the nematode intestine as is seen with *Staphylococcus aureus* (Sifri, Begun et al. 2003), *P. aeruginosa* (Tan, Mahajan-Miklos et al. 1999) and members of the Bcc (Kothe, Antl et al. 2003, Cardona, Wopperer et al. 2005). Others are capable of intestinal colonization with persistent infection, as

seen with *Salmonella enterica* (Aballay, Yorgey et al. 2000) and *Serratia marcescens* (Kurz, Chauvet et al. 2003). Natural fungal pathogens of *C. elegans* kill via invasion (Jansson 1994) while *Yersinia* species form obstructive biofilms on the worm cuticle (Joshua, Karlyshev et al. 2003). Lastly, production of diffusible toxins has proven to be effective in worm killing for *P. aeruginosa* (Darby, Cosma et al. 1999) as well as several *Burkholderia* species (Kothe, Antl et al. 2003, O'Quinn, Wiegand et al. 2001, Gan, Chua et al. 2002).

When cultured in the laboratory, *C. elegans* is propagated on the relatively benign, auxotrophic strain *Escherichia coli* OP50, and exhibits a lifespan of approximately two weeks at room temperature (Sifri, Begun et al. 2005). The pathogenic effects of various bacterial species can be easily studied by replacing *E. coli* OP50 with the pathogen of interest such that nematode health and survival can be monitored. When fed on pathogenic bacteria such as *B. cenocepacia*, *C. elegans* is prone to a wide range of shorter life spans (Sifri, Begun et al. 2005, Cardona, Wopperer et al. 2005).

C. elegans has emerged as a convenient and relatively facile model for the study of bacterial pathogenesis. A great deal of overlap exists between microbial virulence factors required for full pathogenicity across various hosts. The use of *C. elegans* as a host model for bacterial pathogenesis is validated by the fact that mutation-based screens have identified several virulence genes similarly required for maximum

pathogenicity in both mammalian and nematode systems (Sifri, Begun et al. 2005, Tan, Ausubel 2000, Garsin, Sifri et al. 2001, Tenor, McCormick et al. 2004).

1.3.3 *C. elegans* Innate Immunity

Infection by pathogenic bacteria represents a primary risk to the survival of most living organisms. As a soil dwelling nematode frequently found in decaying material, *C. elegans* is likely to encounter a variety of microorganisms on a daily basis and exposure to pathogens is expected (Schulenburg, Kurz et al. 2004). Despite the simplicity of the nematode in relation to mammalian models, *C. elegans* possesses an innate immune system that partially resembles those of higher organisms (Kurz, Ewbank 2003). Innate immunity is a natural barrier to infection that has not arisen from previous challenge from an infecting organism. In humans, these barriers may include the unbroken skin and skin oils, lysozymes and amylases in tears and saliva, as well as stomach acids, mucous and the cough reflex. In addition to physical and chemical barriers, the mammalian innate immune response also relies on a variety of cells involved in pathogen clearance; these include natural killer cells, phagocytic cells, basophils, eosinophils and mast cells. Several homologous features of the innate immune system are conserved across phyla, suggesting conservation over years of evolution (Schulenburg, Kurz et al. 2004). The innate immune response provides immediate and non-specific defense against invading pathogens and, in vertebrates, is involved in initiating adaptive immunity (Medzhitov, Janeway 1998). The facility of the nematode

host provides a fast and efficient means of elucidating virulence mechanisms of pathogenic bacteria and host factors involved in pathogen response.

The defensive strategies employed by *C. elegans* in response to pathogenic organisms can be organized into three main divisions: behavioral responses; physical barriers; and physiological defenses (Schulenburg, Kurz et al. 2004). It has been well documented that *C. elegans* is capable of recognizing and responding to a wide assortment of chemical cues resulting in coordinated behavioral responses (Troemel 1999, Bargmann 2006) including pathogen evasion (Pradel, Zhang et al. 2007, Pujol, Link et al. 2001). Physical barriers protecting the nematode from potentially harmful organisms include a multilayered collagenous cuticle (Kramer 1994, Peixoto, Kramer et al. 1997), a chintinous membrane lining the intestinal epithelium (Borgonie, Claeys et al. 1995), and a pharyngeal grinder (Avery, Thomas 1997). The function of the grinder is to crush bacterial cells before they enter the intestinal lumen. Studies have shown that defects in the pharyngeal grinder can produce worms that are hypersusceptible to pathogens such as *B. cepacia* (Kothe, Antl et al. 2003).

A number of physiological responses and conserved signalling pathways have been shown to play a significant role in worm defence. Three mitogen activated protein kinase (MAPK) signalling cascades have been identified for their involvement in response to various stressors. In particular, the *C. elegans* p38 MAPK pathway is required for resistance to bacterial pathogens such as *P. aeruginosa* (Troemel, Chu et al. 2006, Kim, Feinbaum et al. 2002) and *S. typhimurium* (Aballay, Drenkard et al. 2003). As

seen with macrophages (Cross, Segal 2004), plants (Apel, Hirt 2004) and flies (Ha, Oh et al. 2005), *C. elegans* is known to produce reactive oxygen species (ROS) in response to pathogens (Chavez, Mohri-Shiomi et al. 2007). Several other defence mechanisms have also been reported as contributing to *C. elegans* innate immunity, including production of antimicrobial factors and detoxifying enzymes (Schulenburg, Kurz et al. 2004), programmed cell death (Aballay, Ausubel 2001), and an insulin-like signalling pathway involved in stress response and longevity (Kurz, Ewbank 2003, Chavez, Mohri-Shiomi et al. 2007) to name just a few.

1.3.4 The p38 Mitogen Activated Protein Kinase (MAPK) Pathway

Eukaryotic signal transduction cascades involving three main groups of MAPKs have been identified as evolutionarily conserved stress response pathways that are capable of responding to a number of extracellular stimuli (Inoue, Tateno et al. 2001). These include the p38 MAPKs, c-JUN N-terminal kinases (JNK) and the extracellular signal-regulated protein kinases (ERK) (Kurz, Ewbank 2003, Inoue, Tateno et al. 2001) and have been found to be involved in cellular stress and innate immune responses of plants (Zhang, Klessig 2001), insects (Inoue, Tateno et al. 2001), nematodes (Kim, Feinbaum et al. 2002), and mammals (Kyriakis, Avruch 2001).

The p38 MAPK and JNK cascades are initiated most strongly in response to cellular stress and inflammatory cytokines (Inoue, Tateno et al. 2001). In the nematode, stress signals such as infection, starvation and heavy metals have been shown to activate the p38 MAPKs (Kurz, Ewbank 2003), while osmotic shock, inflammatory

cytokines and endotoxins have been shown to be involved in activation of mammalian p38 MAPKs (Kyriakis, Avruch 2001). In addition, two p38 MAPK pathways found in *D. melanogaster* can be activated by inflammatory stimuli such as LPS (Inoue, Tateno et al. 2001).

Three classes of protein kinase make up the ERK, JNK and p38 MAPK cascades. These include the MAP kinases (MAPK), MAPK kinases (MAP2K) and MAPKK kinases (MAP3K) (Inoue, Tateno et al. 2001). The *C. elegans* p38 MAPK homologue, PMK-1, is activated by the MAP2K, SEK-1, and the MAP3K, NSY-1, in a phosphorylation dependent manner to mediate pathogen resistance and stress responses (Troemel, Chu et al. 2006, Kim, Feinbaum et al. 2002). It has been proposed that PMK-1 influences nematode intestinal immunity by controlling expression of genes encoding secreted antimicrobials or cell to cell communication factors to promote immunity (Troemel, Chu et al. 2006). It has been shown that inhibition of the *pmk-1* gene through RNA interference (RNAi) renders *C. elegans* hypersusceptible to *S. typhimurium* (Aballay, Drenkard et al. 2003) and *P. aeruginosa* (Kim, Feinbaum et al. 2002). Thus, the p38 MAPK signal cascade is a vital component of *C. elegans* physiological defence against pathogens and is required for worm immunity.

1.3.5 Inactivation of *C. elegans* Genes by RNA Interference

RNA interference (RNAi) pathways in eukaryotic cells are involved in controlling the level of expression of a variety of genes and can be initiated by endogenously or exogenously produced dsRNA molecules. Inhibition of mRNA can therefore be used as a

defense mechanism in response to exogenous RNA or as a modulator of endogenous protein expression. As in higher organisms, *C. elegans* possesses an RNAi pathway involved in the control of specific gene expression (Boisvert, Simard 2008). Inactivation of foreign genetic material, such as viral RNA, is an integral part of innate immunity and although no natural viral pathogens have been identified for *C. elegans*, it has been shown that RNAi pathway components are upregulated in response to viral infection (Wilkins, Dishongh et al. 2005). In the nematode, inhibition of particular genes by RNAi is one of the quickest and easiest methods for generation of knockdown strains and has been accepted as a potent tool for reverse genetic studies of host-pathogen interactions.

Initially, knockdown of *C. elegans* genes was achieved through injection of dsRNA into the nematode body cavity to induce RNAi (Fire, Xu et al. 1998). The point of injection is not crucial for gene inactivation (Timmons, Fire 1998), thus making it possible to initiate RNAi through ingestion of dsRNA. Successful gene inhibition has been accomplished by soaking nematodes in a solution of dsRNA (Tabara, Grishok et al. 1998) as well as by feeding worms with dsRNA-expressing *E. coli* strains (Timmons, Fire 1998, Kamath, Martinez-Campos et al. 2001).

C. elegans is naturally bacterivorous and consequently, delivery of dsRNA by feeding confers several advantages. The feeding process involves a limited amount of labour to supply dsRNA to a range of population sizes and thus is convenient for working with a large number of worms or a large number of genes (Kamath, Martinez-Campos et

al. 2001). In addition, *E. coli* specifically engineered to produce high levels of dsRNA are ingested in the same manner as regular laboratory food strains. Bacterial cells are ground in the pharynx and the contents absorbed in the intestine; dsRNA is then distributed to somatic and germ line tissue of the nematode (Timmons, Fire 1998, Kamath, Martinez-Campos et al. 2001).

The effects of RNAi through ingestion of dsRNA are reversible and do not reflect stable genetic changes (Timmons, Fire 1998) but can evoke strong gene specific phenotypes in *C. elegans* (Timmons, Court et al. 2001). For detection of post-embryonic phenotypes, such as enhanced susceptibility to pathogens, ingestion of dsRNA by feeding is as effective as micro injection for knockdown of specific genes (Kamath, Martinez-Campos et al. 2001), and the phenotypes produced are comparable to their corresponding loss of function mutations in *C. elegans* (Timmons, Court et al. 2001). Decreasing expression of genes through RNAi permits the study of gene function in various nematode backgrounds, and conveniently illustrates the physiological roles of the associated gene products.

1.4 Metabolism and Pathogenesis in the Cystic Fibrosis Lung Environment

Recently, attention has been directed towards the relationship between successful pathogenesis of infecting microorganisms and in vivo carbon sources necessary for growth and survival (Brown, Palmer et al. 2008). Understanding the importance of microbial physiology and the significance of nutrient availability in relation to bacterial pathogenesis has been described for a number of organisms. For

example, lactic acid metabolism in *Neisseria meningitidis* has been linked to immune evasion and colonization of host tissue (Exley, Goodwin et al. 2005, Exley, Shaw et al. 2005). Additionally, it has been proposed that pathogenic bacteria are capable of responding to nutritional cues within the host, as is the case for *P. aeruginosa* in the CF lung environment. CF sputum contains sugars and is rich in amino acids such as phenylalanine, tyrosine and tryptophan (Palmer, Aye et al. 2007) that can be used as carbon and nitrogen sources by CF lung pathogens. It has been shown that *P. aeruginosa* is capable of growing to high densities in CF sputum (Ohman, Chakrabarty 1982, Palmer, Mashburn et al. 2005) and that aromatic amino acids present within the sputum may serve as cues to modulate virulence factor production (Brown, Palmer et al. 2008, Palmer, Aye et al. 2007). Given that the Bcc are CF lung pathogens and phylogenetically related to *P. aeruginosa*, it may be reasonable to assume that these strains are also capable of responding to nutritional cues within CF sputum.

1.4.1 Metabolism of Aromatic Compounds

Compounds containing benzene rings are found widely distributed in nature (Diaz, Ferrandez et al. 2001) and represent suitable carbon and energy sources for organisms capable of degrading aromatics. The catabolism of aromatic compounds is carried out largely by microorganisms (Harayama, Timmis 1992) and the ability to metabolize a variety of structurally complex carbon sources provides a significant advantage over competing bacterial strains. In the context of the host environment, proteins are a major source of aromatic carbon in the form of amino acids. Tyrosine and

phenylalanine can be degraded by bacteria for carbon and nitrogen via homogentisic (Arias-Barrau, Olivera et al. 2004) and phenylacetate (Luengo, Garcia et al. 2001) catabolism.

1.4.2 Properties of Phenylacetic acid (PA)

Phenylacetic acid is an organic compound formed in the environment from an array of natural and synthetic sources (El-Said Mohamed 2000). PA has a molecular formula of $C_8H_8O_2$ and contains both a phenyl and an acetic acid functional group. At room temperature, PA exists as a white crystalline solid with a melting temperature range of 77 – 78.5^{o}C, a boiling point of 265^{o}C and moderate water solubility. PA is also known as benzeneacetic acid, phenylethanoic acid, α-toluic acid and 2-phenylacetic acid.

PA is used industrially as an ingredient in perfume, possessing a honey-like odour, as well as in biosynthesis of penicillin G (Hillenga, Versantvoort et al. 1995). PA is also a precursor of 1-phenyl-2-propanone (P-2-P), which is involved in the production of amphetamines and methamphetamines (United Nations Economic and Social Council 2009) and is therefore a controlled substance in the United States.

In nature, PA has been identified as an active auxin in higher plants (Wightman, Lighty 1982). It has been reported to have anaesthetic effects on growing plant parts as well as stimulatory effects on adventitious roots (Leuba, LeTourneau 1990). As a biocontrol agent, PA has been shown to hinder growth and germination of pathogenic

fungi (Hwang, Lim et al. 2001) and displays in vitro toxic effects against the pinewood nematode, *Bursaphelenchus xylophilus* (Kawazu, Zhang et al. 1996).

The effects of PA and related compounds on eukaryotic cells are not well understood at the molecular level. However, PA has been shown to have anti-inflammatory effects on cells involved in vertebrate immunity. Inhibition of TNF-α, IL-6 and inducible nitric oxide synthase (iNOS) expression has been observed in murine macrophages as well as repression of the DNA-binding and transcriptional activities of NF-κβ (Park, Lee et al. 2007). In addition, PA has been shown to inhibit LPS-induced production of cytokines as well as LPS and cytokine-mediated expression of iNOS in rat microglia, primary astrocytes and macrophage cells (Pahan, Sheikh et al. 1997).

In terms of biological availability, PA is formed as a catabolite of phenylalanine and other structurally related aromatic compounds and should therefore be considered as part of a degradative hierarchy rather than an isolated compound. For the purposes of this study, PA should be viewed as a product of biological degradation that can be used as a carbon source for certain types of bacteria.

1.4.3 Phenylacetic acid Degradation in Bacteria

Phenylacetic acid catabolism is central to the degradation of aromatic compounds such as styrene, phenylalanine, phenylacetyl esters, phenylacetylamides, phenylacetaldehydes, trans-styryl acetic acid and 2-phenylethylamine (Luengo, Garcia et al. 2001). The term "catabolon" has been used to describe the route of convergence of

these structurally related compounds through PA into general metabolites of the Krebs cycle (Luengo, Garcia et al. 2001).

Bacterial strains with the ability to metabolize PA have been found throughout the eubacteria (Abe-Yoshizumi, Kamei et al. 2004) and several microbial genomes have been found to contain genes thought to be involved in the degradation of PA. However, experimental evidence for functional PA degradative pathways has only been established for a few organisms; *Escherichia coli* (Diaz, Ferrandez et al. 2001, Ferrandez, Minambres et al. 1998, Ismail, El-Said Mohamed et al. 2003), *Pseudomonas putida* (Jimenez, Minambres et al. 2002, Kim, Cho et al. 2006, Olivera, Minambres et al. 1998), *Azoarcus evansii* (Mohamed, Ismail et al. 2002) and *Rhodococcus* sp. (Navarro-Llorens, Patrauchan et al. 2005).

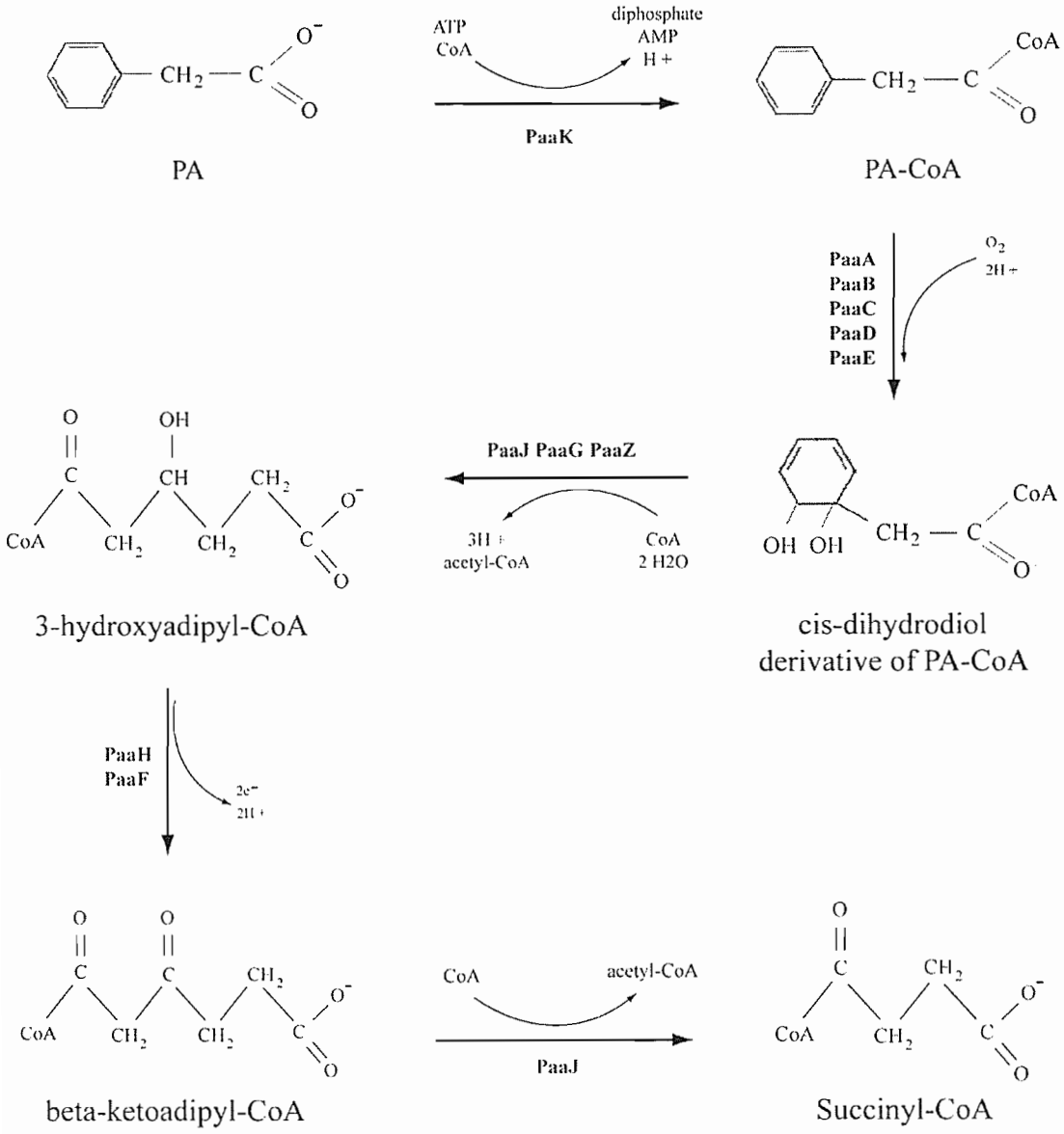
Organization of PA catabolic gene clusters varies among bacterial species. The *P. putida* cluster is composed of fifteen genes arranged in five adjacent operons (Luengo, Garcia et al. 2001, Olivera, Minambres et al. 1998) while *E. coli* W possesses fourteen PA degradation genes organized into three transcriptional units (Ferrandez, Minambres et al. 1998). Recently, we determined that the *B. cenocepacia* J2315 genome contains all genes necessary for a functional PA catabolic pathway and that these genes are organized into three separate clusters on two chromosomes (Law, Hamlin et al. 2008).

Despite the differences in genetic organization of PA catabolic genes, the route of degradation for each experimentally determined PA-metabolizing species seems to proceed through a related series of functional units. The first step of PA degradation

involves the activation of PA to phenylacetyl-CoA (PA-CoA) by the enzyme phenylacetyl-coenzyme A ligase (PaaK) (Ferrandez, Minambres et al. 1998). The next step involves ring hydroxylation of PA-CoA to yield 2-hydroxy-PA-CoA (2-OH-PA-CoA) by a complex of enzymes; PaaA, PaaB, PaaC, PaaD, PaaE (Ferrandez, Minambres et al. 1998, Ismail, El-Said Mohamed et al. 2003). The last two steps entail aromatic ring opening, which may be performed by PaaZ (Ferrandez, Minambres et al. 1998) or PaaZ, PaaG and PaaJ (Ismail, El-Said Mohamed et al. 2003), and β-oxidation of the resulting aliphatic compound by PaaF, PaaH (Ismail, El-Said Mohamed et al. 2003) and PaaJ (Nogales, Macchi et al. 2007). Figure 1 shows the proposed enzymes and intermediate compounds for each step of PA catabolism in *E. coli* (Keseler, Bonavides-Martinez et al. 2009).

Although these enzymes have been functionally assigned to each step of PA degradation, not all have been experimentally confirmed. The activation and ligation of PA to PA-CoA as the first stage of PA catabolism has been established for *E. coli* (Ferrandez, Minambres et al. 1998), *P. putida* (Olivera, Minambres et al. 1998, Schleissner, Olivera et al. 1994) and *A. evansii* (El-Said Mohamed 2000). In addition, mutation of each component of the PaaABCDE multi-enzyme complex has been shown to be required for efficient hydroxylation of PA-CoA to produce 2-OH-PA-CoA (Fernandez, Ferrandez et al. 2006). Lastly, disruption of the ring opening enzyme, *paaZ* (Ferrandez, Minambres et al. 1998, Olivera, Minambres et al. 1998), and the enoyl-CoA hydratase/isomerase, *paaF* (Olivera, Minambres et al. 1998), have been shown to result in accumulation of 2-OH-PA in the external medium. However, it should be noted that

Figure 1. Proposed pathway for degradation of PA in *E. coli*. PA catabolic enzymes and intermediates are in accordance with those found in the EcoCyc database (Keseler, Bonavides-Martinez et al. 2009) and (Diaz, Ferrandez et al. 2001).


PA
ATP
CoA
diphosphate
AMP
H +
PaaK
PA-CoA
PaaA
PaaB
PaaC
PaaD
PaaE
O2
2H+
3-hydroxyadipyl-CoA
PaaJ PaaG PaaZ
3H +
acetyl-CoA
CoA
2 H2O
cis-dihydrodiol
derivative of PA-CoA
PaaH
PaaF
2e-
2H +
beta-ketoadipyl-CoA
CoA
acetyl-CoA
PaaJ
Succinyl-CoA

2-OH-PA is likely derived from a hydroxylated PA-CoA intermediate (Ferrandez, Minambres et al. 1998) and is not a true metabolite of the pathway, but rather a stable dead end product (Olivera, Minambres et al. 1998, Fernandez, Ferrandez et al. 2006).

Analysis of PA growth-defective mutant supernatants suggests that while PA-CoA accumulates within PA-degrading cells (Mohamed, Ismail et al. 2002), 2-OH-PA (Ferrandez, Minambres et al. 1998, Fernandez, Ferrandez et al. 2006) and several other PA metabolites (Ismail, El-Said Mohamed et al. 2003, Mohamed, Ismail et al. 2002) are secreted to the external medium. It has been proposed that the actual hydroxylated product of the PaaABCDE enztmatic complex is a cis-dihydrodiol derivative of PA-CoA (Ismail, El-Said Mohamed et al. 2003) that readily hydrolyzes to end products such as 2-OH-PA, 1,2-dihydroxy-1,2-diphenylacetyl lactone and conjugated cyclohexadiene (Ismail, El-Said Mohamed et al. 2003) in ring cleavage mutants.

1.4.4 Phenylacetyl-Coenzyme A Ligase

The most well characterized step of PA catabolism involves conversion of PA to PA-CoA through the action of the PA-CoA ligase enzyme. This reaction constitutes the first step in aerobic metabolism of PA (Abe-Yoshizumi, Kamei et al. 2004) and has been described and characterized for species of the eubacteria as well as archaea (El-Said Mohamed 2000, Ferrandez, Minambres et al. 1998, Olivera, Minambres et al. 1998, Martinez-Blanco, Reglero et al. 1990, Erb, Ismail et al. 2008). PA-CoA is the first common intermediate for many peripheral pathways proceeding through the PA degradation pathway to general metabolites of the tri-carboxylic acid cycle.

The PA catabolic pathway is often referred to as a hybrid pathway. This is because aerobic degradation of PA involves aryl-CoA derivative intermediates thought to be specific for catabolism in anaerobes (Luengo, Garcia et al. 2001). Aerobic catabolism of aromatic rings often involves reduction by insertion of molecular oxygen, thereby destabilizing the ring before fission (Martinez-Blanco, Reglero et al. 1990, Dagley, Fewster et al. 1952, Sparnins, Dagley 1975). Conversely, under anaerobic conditions, a CoA thioesterification step is often required to activate aromatic compounds prior to destabilization of the aromatic ring (El-Said Mohamed 2000, Heider, Fuchs 1997).

Experimental evidence for *P. putida* (Martinez-Blanco, Reglero et al. 1990), *E. coli* (Ferrandez, Garcia et al. 2000), and *A. evansii* (El-Said Mohamed 2000) has determined that PA-CoA is the likely inducer of PA catabolism and that degradation of PA is repressed in the presence of glucose (Martinez-Blanco, Reglero et al. 1990, Ferrandez, Garcia et al. 2000). PA-CoA is the first common intermediate in the degradation of a variety of aromatic compounds (Garcia, Olivera et al. 2000) and it has been determined that catabolism of PA is subject to negative transcriptional regulation in *E. coli* (Ferrandez, Garcia et al. 2000), *P. putida* U (del Peso-Santos, Bartolome-Martin et al. 2006, del Peso-Santos, Shingler et al. 2008) and *B. cenocepacia* K56-2 (Hamlin 2008). Mutation of the *P. putida* PA-CoA ligase renders mutant strains capable of growth on carbon sources producing PA-CoA as an intermediate, but incapable of growth on those producing PA (Martinez-Blanco, Reglero et al. 1990, Garcia, Olivera et al. 2000). In *E. coli*, PA-CoA was found to specifically inhibit repressor binding to target DNA (Ferrandez,

Garcia et al. 2000) and, in *A. evansii*, the PA-CoA ligase was specifically induced in the presence of PA and phenylalanine (El-Said Mohamed 2000).

Many microbial genomes contain genes encoding putative homologues of experimentally confirmed PA-CoA ligases that are broadly distributed across phyla. Evolution of the PA-CoA ligase does not correlate within bacterial phyla based on 16s rRNA gene sequences (Abe-Yoshizumi, Kamei et al. 2004), indicating that PA-CoA ligase genes have been subject to horizontal transfer within and across species.

1.4.5 Properties of Experimentally Confirmed PA-CoA Ligases

PA-CoA ligase is an activating enzyme involved in the conversion of PA to PA-CoA in an ATP driven process. The chemical reaction requires PA, ATP, CoA and Mg^{2+} for activity (El-Said Mohamed 2000, Martinez-Blanco, Reglero et al. 1990) and is inhibited by sulfhydyl reagents (El-Said Mohamed 2000). The PA-CoA ligases of *P. putida* (Martinez-Blanco, Reglero et al. 1990), *E. coli* (Ferrandez, Minambres et al. 1998), and *A. evansii* (El-Said Mohamed 2000) have the same approximate mass, ranging from 48 to 52 kDa, and are present as monomeric proteins made up of one polypeptide chain (El-Said Mohamed 2000, Martinez-Blanco, Reglero et al. 1990). In addition, putative AMP-binding consensus motifs have been identified in the protein sequence of the PA-CoA ligases for all three organisms (El-Said Mohamed 2000, Ferrandez, Minambres et al. 1998).

2 - Rationale and Hypothesis

The ability of Bcc bacteria to infect and cause disease in immunocompromised individuals is well documented (Coenye, Vandamme 2003, Mahenthiralingam, Urban et al. 2005, Isles, Maclusky et al. 1984, Liou, Adler et al. 2001, LiPuma, Dasen et al. 1990). Although a great deal of information exists about virulence factors that contribute to Bcc infection (Mahenthiralingam, Urban et al. 2005), little is known about how the environmental capabilities of these bacteria may enhance the disease process. Our laboratory is interested in studying metabolism in *B. cenocepacia*, a representative member of the Bcc, as it relates to pathogenicity in the nematode host. Preliminary research determined that disruption of a gene involved in PA catabolism resulted in survival-defective *B. cenocepacia* in the rat model of infection (Hunt, Kooi et al. 2004) and decreased virulence in *C. elegans* (Cardona, Wopperer et al. 2005). The goal of this research was to characterize the pathogenic phenotype of *B. cenocepacia* PA degradation mutants using a *C. elegans* model of infection. **Our working hypothesis is that interruption of genes involved in the ring opening and β-oxidation steps of PA degradation will lead to a build up of metabolites with deleterious effects on *C. elegans* survival.** The specific objectives of this research are to investigate the virulence phenotypes of several PA pathway mutants using the nematode host; to identify genes required for maximum pathogenicity of *B. cenocepacia*; and to examine the role of a specific *C. elegans* innate immune pathway as it relates to pathogen susceptibility.

3 - Materials and Methods

3.0 Bacterial Strains, Nematode Strains and Culture Conditions

Table 1 lists all bacterial strains, nematode strains and plasmids used in this study. *B. cenocepacia* strains were cultured at 37°C with Luria-Burtani (LB) or M9 minimal media (Na_2PO_4 33.9 g l^{-1} KPO 415.0 g l^{-1} NaCl 2.5 g l^{-1} and NH_4Cl 5.0 g l^{-1}, 2 ml 1M $MgSO_4$, 0.1 ml $CaCl_2$) plus the indicated carbon source, supplemented as required, with trimethoprim 100 µg ml^{-1}, gentamicin 50 µg ml^{-1}, chloramphenicol 200 µg ml^{-1} and tetracycline 100 µg ml^{-1}. *E. coli* strains were grown at 37°C in LB medium supplemented with trimethoprim 50 µg ml^{-1}, tetracycline 20 µg ml^{-1}, chloramphenicol 20 µg ml^{-1} or kanamycin 40 µg ml^{-1}. The nematode strain DH26 was obtained from the Caenorhabditis Genetics Centre (CGC) and maintained according to standard protocols as outlined by Stiernagle (Stiernagle 1999).

3.1 Bacterial Growth

Growth curves were performed as previously described (Law, Hamlin et al. 2008). Briefly, ninety-six-well microplates containing 150 µl of M9 plus 0.2% glucose or 5 mM PA were inoculated with 3 µl of stationary phase culture. Bacterial cultures were grown overnight in LB broth, washed, resuspended in M9, and adjusted to a final OD_{600} of 2.0. Microplates were incubated at 37°C with shaking at 200 rpm while OD_{600} measurements were taken every hour for 30 hours using a Biotek Synergy 2 plate reader. Optical density values were converted to a 1 cm path length OD_{600} by prior calibration with an Ultraspec 3000 spectrophotometer.

Table 1. Bacterial strains and plasmids

Strain or plasmid	Features	Reference or source
B. cenocepacia strains		
K56-2 (LMG18863)	ET12 clone related to J2315, CF clinical isolate	Mahenthiralingam et al. 2000
STC155-*paaE*	K56-2 *paaE*::pSC152, Tp^r	Law et al. 2008
STC179-*paaA*	K56-2 *paaA*::SC175, Tp^r	Law et al. 2008
STC183-*paaZ*	K56-2 *paaZ*::pSC177, Tp^r	Law et al. 2008
STC199-*paaF*	K56-2 *paaF*::pSC186, Tp^r	Law et al. 2008
RJL1	K56-2 Δ*paaK1*, Tc^r	This study
RJL2	K56-2 Δ*paaK2*, Tc^r	This study
RJL3	K56-2 Δ*paaK1*, Δ*paaK2*, Tc^r	This study
E. coli strains		
DH5α	F^-, ϕ 80 *lacZ*⊿M15 *endA1 recA1 hsdR*17($r_K^- m_K^+$) *supE*44 *thi*-1 ⊿*gyrA*96 (⊿*lacZYA-arg*F)U169 *relA*1	Invitrogen
SY327	*araD* Δ(*lac pro*) *argE* (Am) *recA*56 Rif^r *nalA* λ *pir*	Miller et al. 1988
Plasmids		
pGPΩTp	*ori* $_{r6K}$, ΩTp^r *mob*+	Flannagan et al. 2007
pRK2013	*ori* $_{colE1}$, RK2 derivative, Km^r *mob*$^+$ *tra*$^+$	Figurski et al. 1979
pAP20	*ori* $_{PBBR1}$, P_{DHFR} Cm^r Cm duplicated region deleted	Law et al. 2008
pAS1	pAP20, *paaE*, Cm^r	Law et al. 2008
pRL1	pAP20, *paaF*, Cm^r	This study
pJH1	pAP20, eGFP Cm^r	Law et al. 2008
pGPI-SceI	*ori* $_{r6K}$, P_{DHFR}, *mob*$^+$, Tp^r, carries I-SceI cut site	Flannagan et al. 2008
pDAI-SceI	*ori* $_{pBBR1}$, P_{DHFR}, *mob*$^+$, carries I-SceI gene, Tc^r	Flannagan et al. 2008
pRL5	pGPI-SceI, *paaK1* flanking regions	This study
pRL6	pGPI-SceI, *paaK2* flanking regions	This study

Cm, chloramphenicol; Km, kanamycin; Tc, tetracycline; Tp, trimethoprim.

3.2 Oxidative Stress Assays

The effect of reactive oxygen species (ROS) on viability of *B. cenocepacia* was assayed essentially as described elsewhere (Katsuwon, Anderson 1989). Bacterial cultures were grown overnight in LB broth with shaking at 37°C. Following incubation, a 10^{-2} dilution of each culture was made in 50 ml of fresh LB and grown at 37°C with shaking to an OD_{600} of 2.0. Cells were spun down and resuspended in 5 ml aliquots to yield six 5 ml tubes for each *B. cenocepacia* strain tested. Hydrogen peroxide (H_2O_2 30% w/w) was added to each of three tubes at concentrations of 2.5 mM, 5.0 mM, and 10 mM. Sterile water was added to control tubes in place of H_2O_2. All tubes were incubated with shaking at 25°C for 30 min, serially diluted and plated on LB plus gentamicin 50 μg ml^{-1}. Plates were incubated at 37°C for 48 hr and the number of colony forming units per ml (CFU ml^{-1}) was calculated. Protocols to determine the effects of H_2O_2 on *B. cenocepacia* pre-treated with PA were carried out as described above with the exception that overnight cultures were diluted in 50 ml of LB plus 5 mM PA.

3.3 Hypochlorite Synchronization of *C. elegans* DH26 L4 Larvae

One to two fresh NGM plates containing many gravid worms and eggs were washed with 5 ml of sterile demineralized water and collected in a 16 ml tube. A 1.5 ml toxic solution (0.6 ml dH_2O, 0.5 ml 10-12% NaOCl, 0.4 ml 5 N NaOH) was added to the worm/egg suspension and vortexed intermittently for 8 minutes. The liquid suspension was then transferred to four microcentrifuge tubes and pelleted at 3500 rpm for 30

seconds. Supernatant was removed from each tube and the pellet resuspended in 1 ml sterile dH_2O. Suspensions were pelleted and resuspended twice more and brought to a final volume of 200 µl in one microcentrifuge tube. The resulting liquid egg suspension was then spotted onto fresh NGMI plates and incubated at 25°C for 48 hrs to allow development of L4 stage nematodes. It was desirable to use a synchronous population of worms since it has been shown that *C. elegans* displays differential susceptibilities to pathogens at various stages of development (Kurz, Ewbank 2003).

3.4 Nematode Infection Assays

C. elegans slow killing assays were performed as previously described (Kothe, Antl et al. 2003, Cardona, Wopperer et al. 2005, Law, Hamlin et al. 2008). Thirty five millimetre plates filled with modified nematode growth (NG) media (3 g l^{-1} NaCl, 17 g l^{-1} agar, 3.5 g l^{-1} peptone, 1 ml 5 mg ml^{-1} cholesterol, 1 ml 1 M $CaCl_2$, 1 ml 1 M $MgSO_4$, 12.5 ml phosphate buffer) were seeded with 50 µl of stationary phase culture adjusted to an OD_{600} of 1.7. Plates were incubated overnight at 37°C to allow formation of a bacterial lawn before 20 to 40 hypochlorite-synchronized L4 larvae of *C. elegans* DH26 were inoculated to each plate, plates were then stored at 25°C for the duration of the experiment. Plates were scored for live worms at the time of inoculation and every 24 hours subsequently for five to six days using a stereo-master dissecting microscope. Worms were considered dead when unresponsive to touch with a sterile wire pick. Assays were performed in triplicate and analyzed using survival curves generated by the Kaplan-Meier statistical method. The log rank test was used to compare survival

differences for statistical significance using GraphPad Prism, version 4.0. A *P* value <0.05 was considered to be significant. Worm pictures were taken 48 hours post-infection with a Nikon SMZ 1500 stereomicroscope equipped with a Nikon coolpix 8400 digital camera.

3.5 Quantification of Nematode Intestinal Colonization

Bacterial colonization of the *C. elegans* intestine was quantified as described elsewhere (Law, Hamlin et al. 2008, Moy, Ball et al. 2006). *C. elegans* L4 hypochlorite-synchronized nematodes were allowed to feed on 35 mm NG agar plates seeded with *B. cenocepacia* strain K56-2 or STC155-*paaE* for up to 48 hours. At 8, 24, and 48 hr post-infection, approximately 10 to 15 nematodes were manually transferred to a 1.5 ml Eppendorf tube of M9 buffer containing 1 mM NaN_3, washed three times, and brought to a final volume of 250 µl. One millimolar NaN_3 was used to prevent expulsion of *B. cenocepacia* from the *C. elegans* intestine. A 50 µl aliquot was removed from each Eppendorf tube, serially diluted, and plated to determine viable external CFU/worm. To the remaining 200 µl, 400 mg of 1.0 mm silicon carbide particles were added. Tubes were then vortexed intermittently at 2,000 rpm for a total of 90 seconds to disrupt worms. The resulting suspension was serially diluted and plated on LB plus 50 µg ml^{-1} gentamicin to determine viable internal CFU/worm. An unpaired *t* test was used to measure statistical significance of colonization differences where *P* values <0.05 were considered to be statistically significant.

3.6 Fluorescent Microscopy

Nematodes exposed to *B. cenocepacia* K56-2 or STC155-*paaE* containing the constitutive eGFP expression vector, pJH1 (Hamlin 2008), were examined by differential interference contrast (DIC) and fluorescent microscopy using a Zeiss Fluorescent microscope with Axiovision software. Nematode growth plates were seeded and incubated as previously described. Colonization of the *C. elegans* intestine with eGFP expressing bacteria was observed at 24 and 48 hr post-infection on 2% agarose slides at 400 X and 1000 X magnification. Images represent what was typically observed upon inspection of approximately 10 worms per condition.

3.7 Metabolite Filter Diffusion Assays

Hypochlorite-synchronized L4 larvae of *C. elegans* DH26 were obtained as described for nematode infection assays. Filter diffusion assays were performed using methods similar to those of Kothe et al. (Kothe, Antl et al. 2003). Sterile 0.45 µm nitrocellulose filters were placed over the surface of modified NG plates and spread with 50 µl of overnight K56-2, STC179-*paaA* or STC199-*paaF* cultures adjusted to an OD_{600} of 1.7. After 24 hr incubation at 37°C, filters were removed and plates were spread with *B. cenocepacia* K56-2 or STC179-*paaA* as previously stated. Plates were incubated overnight at 37°C to promote formation of bacterial lawns before inoculation with *C. elegans* larvae. Nematode infection assays were carried out as described above.

3.8 RNAi Knockdown and Survival

RNA interference and survival were assayed as explained elsewhere (Timmons, Fire 1998, Fraser, Kamath et al. 2000, Kerry, TeKippe et al. 2006, Shapira, Hamlin et al. 2006). Briefly, *pmk-1* (RNAi) immunocompromised worms were obtained by allowing *C. elegans* DH26 hypochlorite-acquired eggs to hatch on NG media containing 1 mM isopropyl-D-thiogalactopyranoside (IPTG), 100 μg ml^{-1} ampicillin and inoculated with overnight bacterial cultures of *E. coli* HTT115 carrying the L4440 expression vector for the targeted gene. After 48 hour incubation at 25°C, 20 to 40 L4 synchronized nematodes were transferred to NG plates pre-seeded with overnight cultures of test strains and slow killing assays were carried out as described above.

3.9 Molecular Biology Techniques

E. coli DH5α and SY327 cells were transformed using the calcium chloride protocol (Cohen, Chang et al. 1972). DNA amplification conditions were optimized for each primer pair and DNA was amplified using an Eppendorf Mastercycler ep gradient S thermal cycler with either *Taq* DNA polymerase (Qiagen), Proofstart DNA polymerase (Qiagen), or the Phusion high-fidelity PCR kit (New England Biolabs). PCR products and plasmids were purified using the QIAquick purification kit (Qiagen) and QIAprep Miniprep and QIAquick gel extraction kits (Qiagen), respectively. DNA ligases (New England Biolabs and Invitrogen) were used as recommended by the manufacturers.

3.10 Tri-parental Mating with *B. cenocepacia*

Delivery of plasmids to *B. cenocepacia* was achieved by tri-parental mating (Craig, Coote et al. 1989) with *E. coli* DH5α carrying the helper plasmid pRK2013 (Figurski, Helinski 1979). Donor and helper *E. coli* strains were streaked onto LB plates containing appropriate antibiotics and incubated overnight at 37°C. On the same day, *B. cenocepacia* recipient strains were inoculated into liquid LB plus appropriate antibiotics and incubated with shaking at 37°C. The following day, a 10^{-2} dilution of recipient culture was made in fresh LB broth and incubated with shaking until an OD_{600} of 0.3 – 0.6 was reached. Optical densities of PBS diluted helper and donor cultures were determined and mixed as if to prepare a suspension containing 1.5 ml of donor with an OD_{600} of 0.3, 1.5ml of helper with an OD_{600} of 0.3 and 0.5 ml of recipient with an OD_{600} of 1.0. The mixed cell suspension was spun down to remove supernatant, resuspended in 100 µl LB broth, dropped in the centre of an LB plate and incubated lid side up overnight at 37°C. Cells were collected the following day with a sterile loop into 2 ml LB broth. For homologous recombination, 100 – 200 µl of undiluted cell suspension was spread onto LB agar containing selective antibiotics and incubated for 48 hrs. For conjugation without recombination, 100 µl of 10^{-1} to 10^{-2} dilutions were used.

3.11 Colony PCR Screening

Isolated colonies were streaked onto appropriate LB plates with a sterile pick and incubated overnight. Cell suspensions were prepared for each clone in 100 µl sterile distilled water and a 2 µl volume of each suspension was added to an

appropriately labelled PCR tube on ice. A master PCR mix was prepared according to the enzyme manufacturer's instructions, and 8 µl was added to each 2 µl cell volume. Thermal cycling conditions were adjusted according to the enzyme manufacturer's instructions as well as annealing temperature of the primers used. PCR reactions were analyzed using a 2 µl sample in agarose gel.

3.12 Complementation of STC199-*paaF*

The plasmid pAP20 was constructed as previously described (Law, Hamlin et al. 2008) and used to clone *paaF* under the control of the constitutive *dhfr* promoter. The complete coding sequence of the *paaF* gene was PCR amplified with primers SC036 and SC037 (Table 2), digested with NdeI and XbaI, ligated into NdeI/XbaI-digested pAP20 and transformed into *E.coli* DH5α. The resulting plasmid, pRL1, was introduced into *B. cenocepacia* STC199-*paaF* by triparental mating. Positive clones were confirmed with colony PCR using primers SC003 and SC004. As a negative control for complementation experiments, the empty pAP20 vector was introduced into the *paaF* mutant strain.

3.13 Construction of *B. cenocepacia* Deletion Mutants

Mutagenesis plasmids and general mutagenesis procedures were carried out according to methods outlined by Flannagan and Valvano (Flannagan, Linn et al. 2008). DNA fragments immediately upstream and downstream from the start and stop codons of the *paaK1* and *paaK2* genes were amplified from purified *B. cenocepacia* K56-2 chromosome using the specified primer pairs (Table 2). Purified upstream regions were then digested with XbaI, ligated into XbaI-digested pGPI-SceI and transformed into

Table 2. Primers used in this study

name	Oligonucleotide sequence*	Purpose or location
SC003	GGCGTAGAGGATCTGCTCATGTTTG	External to pAP20 multi-cloning site, 5'-3'
SC004	GCTACTGCCGCCAGGCAAATTCTGT	Extenal to pAP20 multi-cloning site, 3'-5'
SC036	GGAGGAGCATATGGCTTACGAGAACATCCTG	Amplification of *paaF*, 5'-3'
SC037	ACACCTCTAGATCAGCGGTGCTTGAAGACCG	Amplification of *paaF*, 3'-5'
SC137	CCAGTTCTAGACGTTCGAGCAGTTTCATCCA	Amplification of *paaK1* upstream, 5'-3'
SC138	ATTGTTCTAGACTCCAGTGTTTGTTATGGGC	Amplification of *paaK1* upstream, 3'-5'
SC139	ATCATGGTACCTGTACGTCGCCCAACGG	Amplification of *paaK1* downstream, 5'-3'
SC140	CATTTGATATCGAGGCGTTCTTCCTGCA	Amplification of *paaK1* downstream, 3'-5'
SC141	AACTCTCTAGAATCCAGCAGGAACTCGTCAA	Amplification of *paaK2* upstream, 5'-3'
SC142	CTTTGTCTAGAATGGCGTGATGTCGTTATC	Amplification of *paaK2* upstream, 3'-5'
SC143	CAGAATGGTACCGCCGTTCTACCCGAGGAAAC	Amplification of *paaK2* downstream, 5'-3'
SC144	GCAGTGATATCTTCGAGCGTGTCGTCGAGCG	Amplification of *paaK2* downstream, 3'-5'
SC153	GTGGATGACCTTTTGAATGACCTTT	External to pGPI-SceI multi-cloning site, 5'-3'
SC154	ACAGGAACACTTAACGGCTGACATG	External to pGPI-SceI multi-cloning site, 3'-5'
SC157	TTTGAGTGACACAGGAACACTTAAC	pRL5 sequencing primer, 5'-3'
SC158	CTTTTGAATGACCTTTAATAGATTA	pRL5 sequencing primer, 3'-5'
SC159	CGCACTGAGAAGCCCTTAGAGCCTC	pRL6 sequencing primer, 5'-3'
SC160	CGGTGGATGACCTTTTGAATGACCT	pRL6 sequencing primer, 3'-5'
SC162	AACATCCCGAACGGATAGCTGTC	5' end of *paaK1* (check pRL5 recombinants)
SC163	TCAGATCGTGCTGACGAAGGAAGG	3' end of *paaK1* (check pRL5 recombinants)
SC164	CGCAGGTCGTTCTTGGTCGAGAA	5' end of *paaK2* (check pRL6 recombinants)
SC165	GCCAGTTCCAGATCACGCTGTCG	3' end of *paaK2* (check pRL6 recombinants)
SC166	GCGTGCAACTCGTACAACCTGAGTG	Check deletion of *paaK1*, 5'-3'
SC167	CGGATGTGGCTGCTGTTCCCGAATT	Check deletion of *paaK1*, 3'-5'
SC168	ATGGAGAAGGGCGTGAACTATCCGT	Check deletion of *paaK2*, 5'-3'
SC169	ATCGCTGGCTATCACGATGTCGCAG	Check deletion of *paaK2*, 3'-5'

* Restriction sites are underlined

E. coli SY327 to generate plasmids pRL3 and pRL4. Directional insertion of upstream fragments was determined by colony PCR using the indicated primers (Table 2). Next, purified downstream regions were digested with KpnI and EcoRV, ligated into KpnI/EcoRV-digested pRL3 and pRL4, and transformed into *E. coli* SY327 to yield pRL5 and pRL6, respectively. Direction of insertion was confirmed by sequencing of mutagenesis plasmids using primers SC157 and SC158 (pRL5) and SC159 and SC160 (pRL6). Conjugative transfer was used to introduce the mutagenesis plasmids into *B. cenocepacia* K56-2. At least ten trimethoprim resistant colonies were screened by colony PCR using specified primers (Table 2) to confirm single cross-over insertion of mutagenic plasmids into the K56-2 genome. Next, the homing endonuclease expression vector, pDAI-SceI, was introduced into strains harbouring the integrated mutagenic plasmids by conjugation. Exconjugants were selected based on tetracycline resistance (presence of pDAI-SceI) and trimethoprim sensitivity (loss of integrated plasmid). Clones were screened for gene deletions by colony PCR and sequencing using primer pairs SC166 and SC167 (*paaK1*) and SC168 and SC169 (*paaK2*).

3.14 Phylogenetic Analysis of the Putative *B. cenocepacia* PA-CoA Ligases

The amino acid sequence of the putative *B. cenocepacia* PA-CoA ligase encoded by *paaK1* was used as the query in a BLASTP search of the non-redundant (NR) protein sequence database (National Centre for Biotechnology Information, NIH, Bethesda). Five representatives of the Bcc were chosen for analysis, each having two different putative *paaK* genes in their sequenced genomes. In addition, *B. thailandensis*,

Ralstonia eutropha, and *Cupravidus taiwanensis* were selected based on evidence of two putative PA-CoA ligase encoding genes. Protein sequences from *Pseudomonas putida*, *Escherichia coli*, and *Azoarcus evansii* were included since experimental evidence for functional PA degradation pathways exists for each of these organisms (Ferrandez, Minambres et al. 1998, Ismail, El-Said Mohamed et al. 2003, Olivera, Minambres et al. 1998, Mohamed, Ismail et al. 2002). All other sequences were selected based on classification within or close relation to groups already chosen. All sequences belong to either β-proteobacteria or γ-proteobacteria except *Manetospirrium magneticum*, an α-proteobacteria, which was chosen as the outgroup for the data set. All sequences used for phylogenetic analysis had E values less than or equal to $2e^{-164}$. ClustalX2.0.3 (Thompson, Gibson et al. 1997) software was used for complete alignment of protein sequences while the GeneDoc (Nicholas, Nicholas et al. 1997) sequence editor program was used for removal of gap columns and non homologous regions. The Phylip 3.67 (Felsenstein 1989) program package was used to generate a phylogenetic tree of the sequence alignment. Briefly, a Jones-Taylor-Thornton distance matrix (Jones, Taylor et al. 1992) was generated with Protdist and the matrix was used to construct a neighbor-joining tree using the Neighbor program. Seqboot was used to generate bootstrap replicas which could then be analyzed by the parsimony method using Protpars. Finally, the Consense program was used to summarize the Protpars output based on analyzing 1000 bootstrap replicates and the resulting majority rule consensus tree shows the level of confidence for the nodes in the tree. Sequence alignment of the *B. cenocepacia* PaaK proteins was generated using Boxshade 3.21 (Hofman, Baron 2006) with default settings

and percent GC content was calculated using OligoCalc (Kibbe 2007). In addition, the AMP binding site sequence logo was generated using GeneDoc alignment data on the WebLogo site, University of California, Berkeley (Crooks, Hon et al. 2004).

4 – Results

Downstream components of PA catabolism and their contribution to the pathogenicity of *B. cenocepacia*

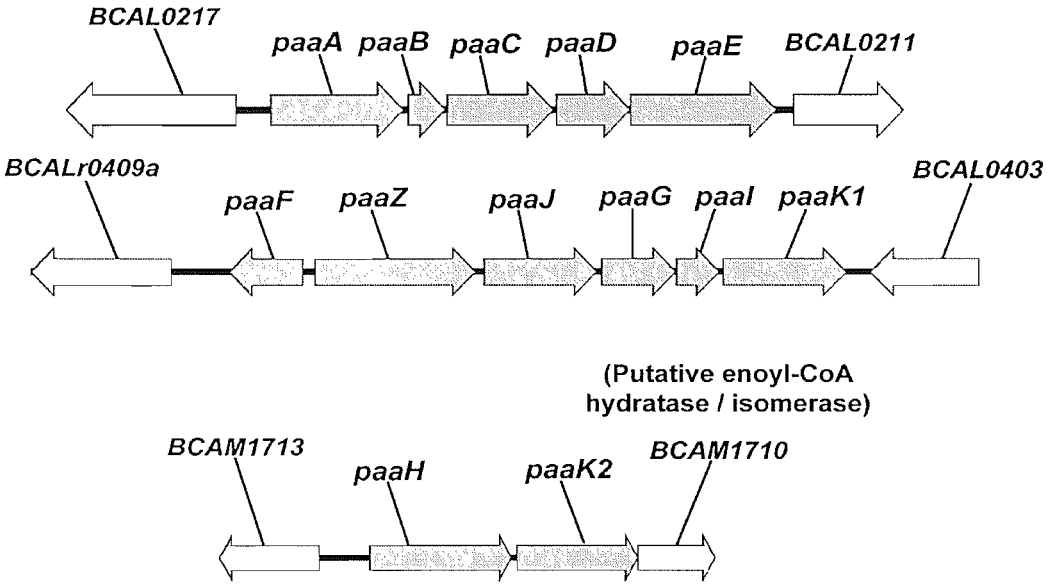
4.0 Mutants in the ring-hydroxylation step of the *B. cenocepacia* K56-2 PA catabolic pathway display diminished virulence in comparison to mutants of the ring opening and β-oxidation steps in the *C. elegans* model of infection.

Mutations in the *B. cenocepacia* PA degradation pathway have been shown to produce strains defective for growth in the rat model of chronic infection (Hunt, Kooi et al. 2004). In addition, the *B. cenocepacia* 4A7 transposon mutant, which contains an interruption in the *paaE* gene, has shown a non-pathogenic phenotype using the nematode model (Cardona, Wopperer et al. 2005). Figure 2 shows the genetic arrangement of the PA catabolic gene clusters in *B. cenocepacia* and the putative metabolic intermediates produced at each step of the pathway. To further explore the pathogenic effects of PA catabolism-defective strains, various insertional mutants of the *B. cenocepacia* PA catabolic pathway were studied in vivo for pathogenic phenotypes in the nematode.

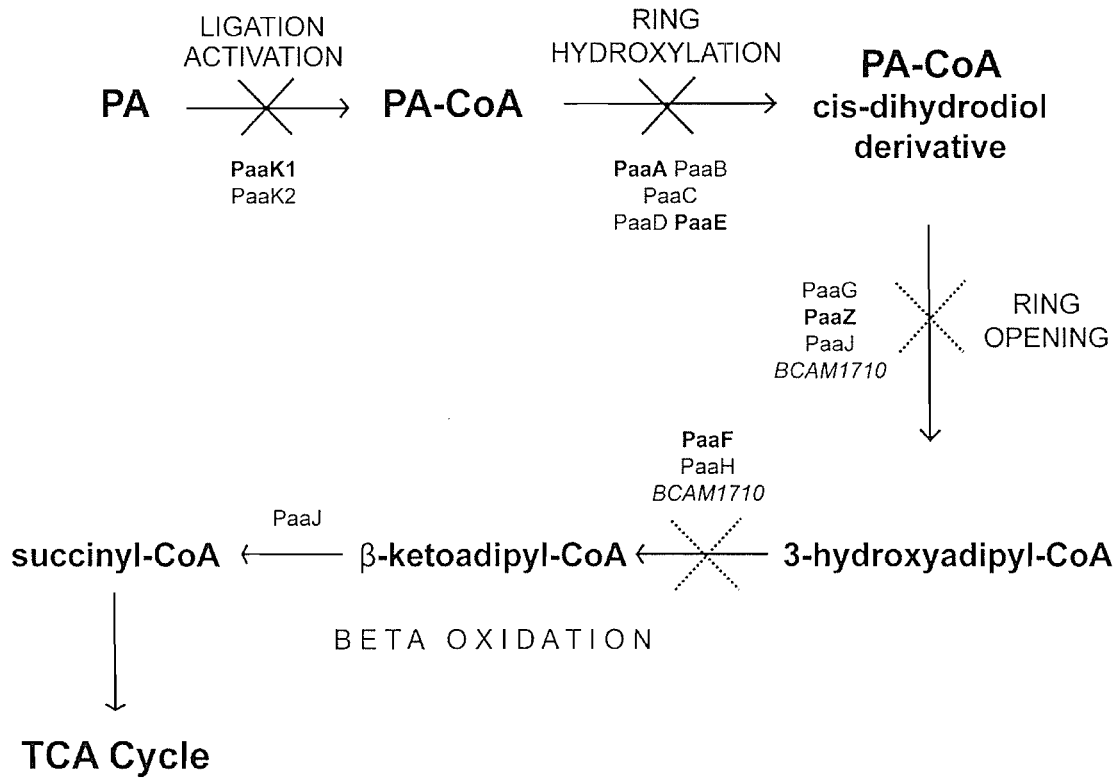
C. elegans is naturally bacterivorous and *B. cenocepacia* has been shown to be capable of causing a persistent intestinal infection in *C. elegans* (Kothe, Antl et al. 2003). When fed pathogenic bacteria such as *B. cenocepacia*, *C. elegans* is prone to a wide range of shorter life spans relative to what is seen when fed with the non-infectious laboratory strain, *E. coli* OP50 (Sifri, Begun et al. 2005). Nematode infection assays were

Figure 2. Proposed PA catabolic pathway of *B. cenocepacia* J2315. (A) Genetic organization of the PA catabolic gene clusters in *B. cenocepacia* J2315. (B) PA catabolic enzymes and putative intermediates of the PA catabolic pathway. Genes deleted or disrupted by insertional mutagenesis are shown in bold; genes with unidentified involvement are italicized. Disrupted steps are marked with an "X" and the observed pathogenic phenotype summarized as follows: solid lines, attenuated pathogenicity; dashed lines, increased pathogenicity. Gene names are in accordance with those listed in reference (Diaz, Ferrandez et al. 2001).

A


BCAL0217
paaA
paaB
paaC
paaD
paaE
BCAL0211
BCALr0409a
paaF
paaZ
paaJ
paaG
paaI
paaK1
BCAL0403
(Putative enoyl-CoA hydratase / isomerase)
BCAM1713
paaH
paaK2
BCAM1710

B


LIGATION ACTIVATION
PA
PA-CoA
PaaK1
PaaK2
RING HYDROXYLATION
PaaA PaaB
PaaC
PaaD PaaE
PA-CoA
cis-dihydrodiol derivative
PaaG
PaaZ
PaaJ
BCAM1710
RING OPENING
PaaF
PaaH
BCAM1710
PaaJ
succinyl-CoA
β-ketoadipyl-CoA
3-hydroxyadipyl-CoA
BETA OXIDATION
TCA Cycle

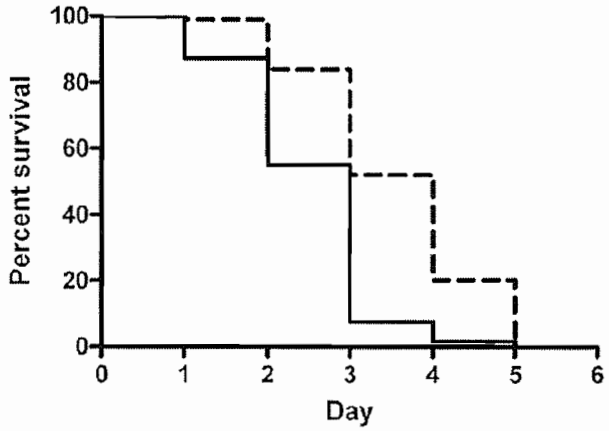
used to compare the ability of wild type K56-2 and the *paaA* and *paaE* ring hydroxylation mutants to kill *C. elegans*.

The *C. elegans* strain DH26 was used for survival assays due to a temperature-sensitive mutation in the *fer-15* spermatogenesis gene which renders worms sterile above 25°C and facilitates the scoring of worms without the interference of progeny. Slow killing assays performed in triplicate suggest that these mutations result in a decreased pathogenic phenotype in *C. elegans*. Figure 3 shows a statistically significant reduction in survival of nematodes fed on wild type K56-2 in comparison to that of STC179-*paaA* and STC155-*paaE*. Additionally, this apparent attenuation of pathogenicity was visually evident after 48 hours of infection with K56-2 fed *C. elegans* appearing under developed and less active than worms exposed to either of the ring-hydroxylation mutants, (Figure 3C). It should be noted, however, that immobile K56-2 exposed nematodes were still scored as "live" since they were able to respond to a mechanical stimulus.

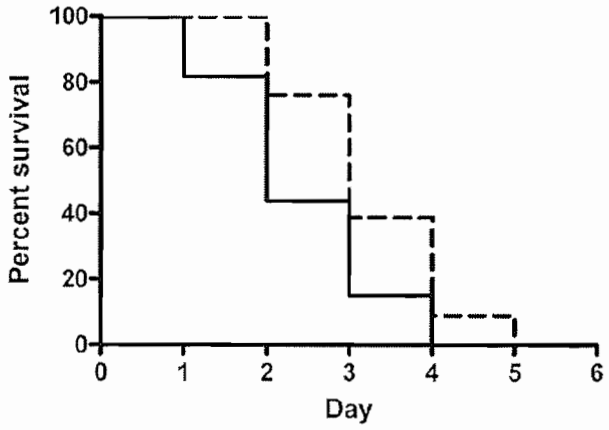
To test the virulence of strains with mutations in the lower steps of the PA catabolic pathway, assays were conducted using strains identified as mutants in ring opening (STC183-*paaZ*) and β-oxidation (STC199-*paaF*) (Law, Hamlin et al. 2008). Contrary to what was found with the *paaA* and *paaE* mutants, STC183-*paaZ* and STC199-*paaF* did not present an attenuation phenotype. As shown in Figure 4, the *paaF* and *paaZ* mutants displayed a slightly but significantly more pathogenic phenotype in *C. elegans* than wild type K56-2.

Figure 3. The virulence of STC179-*paaA* and STC155-*paaE* is diminished in the *C. elegans* infection model. Kaplan-Meier survival plots for DH26 worms fed with *paaA* and *paaE* mutant strains. (A) The killing ability of wild type *B. cenocepacia* strain K56-2 (n = 66) was compared to that of STC179-*paaA* (n = 113; $P < 0.0001$) in slow killing assays using *C. elegans* strain DH26. Solid lines, K56-2; dashed lines, STC179-*paaA*. (B) The killing ability of K56-2 compared to that of STC155-*paaE* (n = 67; $P < 0.0001$). Solid lines, K56-2; dashed lines, STC155-*paaE*. (C) Appearance of worms after two days exposure to bacterial strains. Worms exposed to the non-pathogenic *E. coli* OP50 or *B .cenocepacia* strains were randomly chosen and photographed at 80 X magnification.

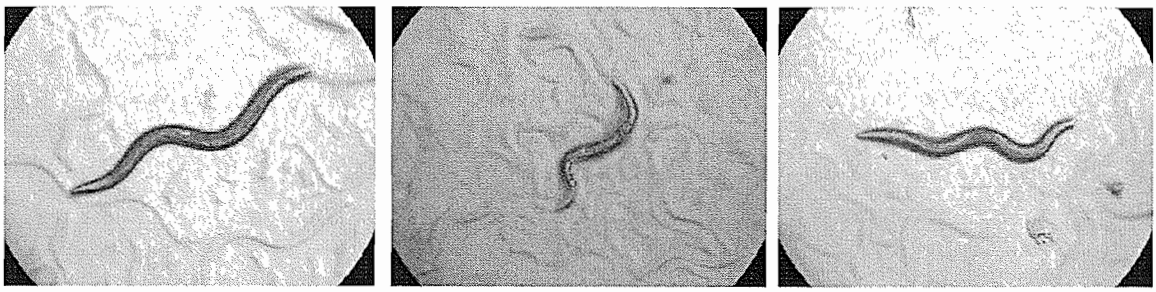
A



B



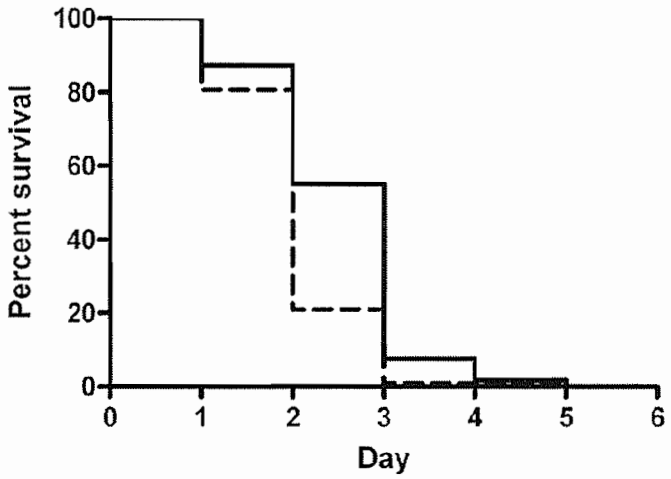
C



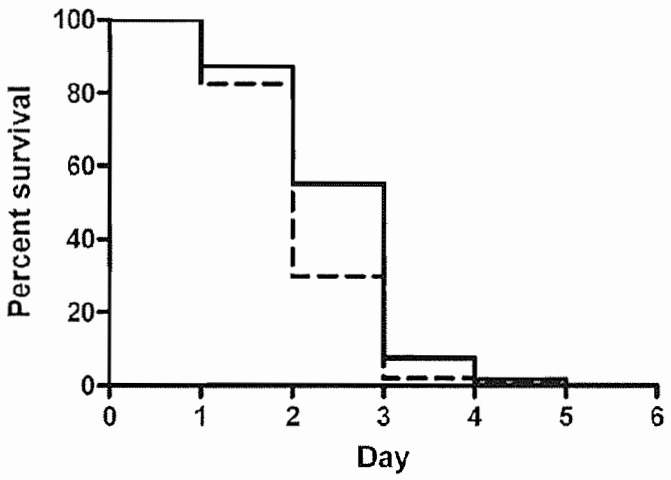
OP50 K56-2 STC155-*paaE*

Figure 4. The virulence of STC199-*paaF* and STC183-*paaZ* is increased in the *C. elegans* infection model. (A) Kaplan-Meier survival plot for DH26 worms fed with the *paaF* mutant (n = 114) compared to worms fed with wild type *B. cenocepacia* strain K56-2 (n = 118; $P < 0.0001$). Solid line, K56-2; dashed line, STC199-*paaF*. (B) Kaplan-Meier survival plot for DH26 worms fed with the *paaZ* mutant (n = 97) compared to worms fed with wild type *B. cenocepacia* strain K56-2 (n = 118; $P = 0.0006$). Solid line, K56-2; dashed line, STC199-*paaZ*.

A


Percent survival
100
80
60
40
20
0
0
1
2
3
4
5
6
Day

B


Percent survival
100
80
60
40
20
0
0
1
2
3
4
5
6
Day

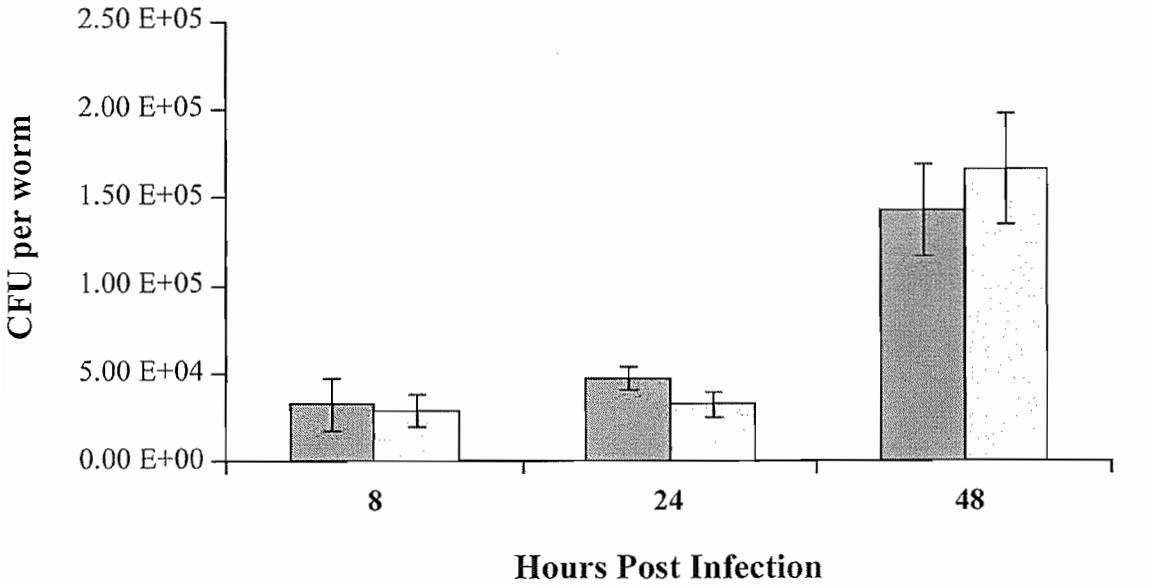
Previous work has shown that insertional mutation of the ring-hydroxylation, ring opening and β-oxidation stages of PA degradation result in *B. cenocepacia* strains that are unable to grow using PA as a sole carbon source (Law, Hamlin et al. 2008). Taken together, results indicate that the observed increase in nematode survival seen with STC179-*paaA* and STC155-*paaE* is not due to reduced growth in the presence of PA but more likely, is related to interruption of the ring-hydroxylation complex itself.

4.1 Wild type *Burkholderia cenocepacia* K56-2 and the ring-hydroxylation mutant STC155-*paaE* accumulate to similar levels in the *C. elegans* intestine

Our work has shown that mutation of the ring-hydroxylation complex, involved in phenylacetic acid degradation, results in bacteria that are less pathogenic to *C. elegans* than wild type K56-2 (Law, Hamlin et al. 2008). For this reason, it was hypothesized that accumulation of the *paaA* and *paaE* mutants may be reduced in comparison to wild type bacteria in the nematode intestine.

To determine whether intestinal bacterial load differs between K56-2 and STC155-*paaE* fed nematodes, worms were removed from infection plates at pre-determined time intervals, washed, and disrupted by vortexing to recover cells residing in the intestine. As shown in Figure 5, the amount of bacteria occupying the *C. elegans* intestine accumulates over the 48 hour testing period reaching approximately 10^5 CFU per worm. The average CFU per worm at 8, 24 and 48 hours post-infection from five independent experiments was approximately equal for nematodes exposed to either K56-2 or STC155-*paaE*.

Figure 5. Wild type *B. cenocepacia* K56-2 and the mutant STC155-*paaE* accumulate to similar levels in the *C. elegans* intestine. Data represent the mean number of CFU per worm from five independent experiments, where error bars signify standard error of measurement (SEM). Gray bars, K56-2; light blue bars, STC155-*paaE*. *P* values for 8, 24 and 48 hrs were 0.8766, 0.1666 and 0.5745, respectively.

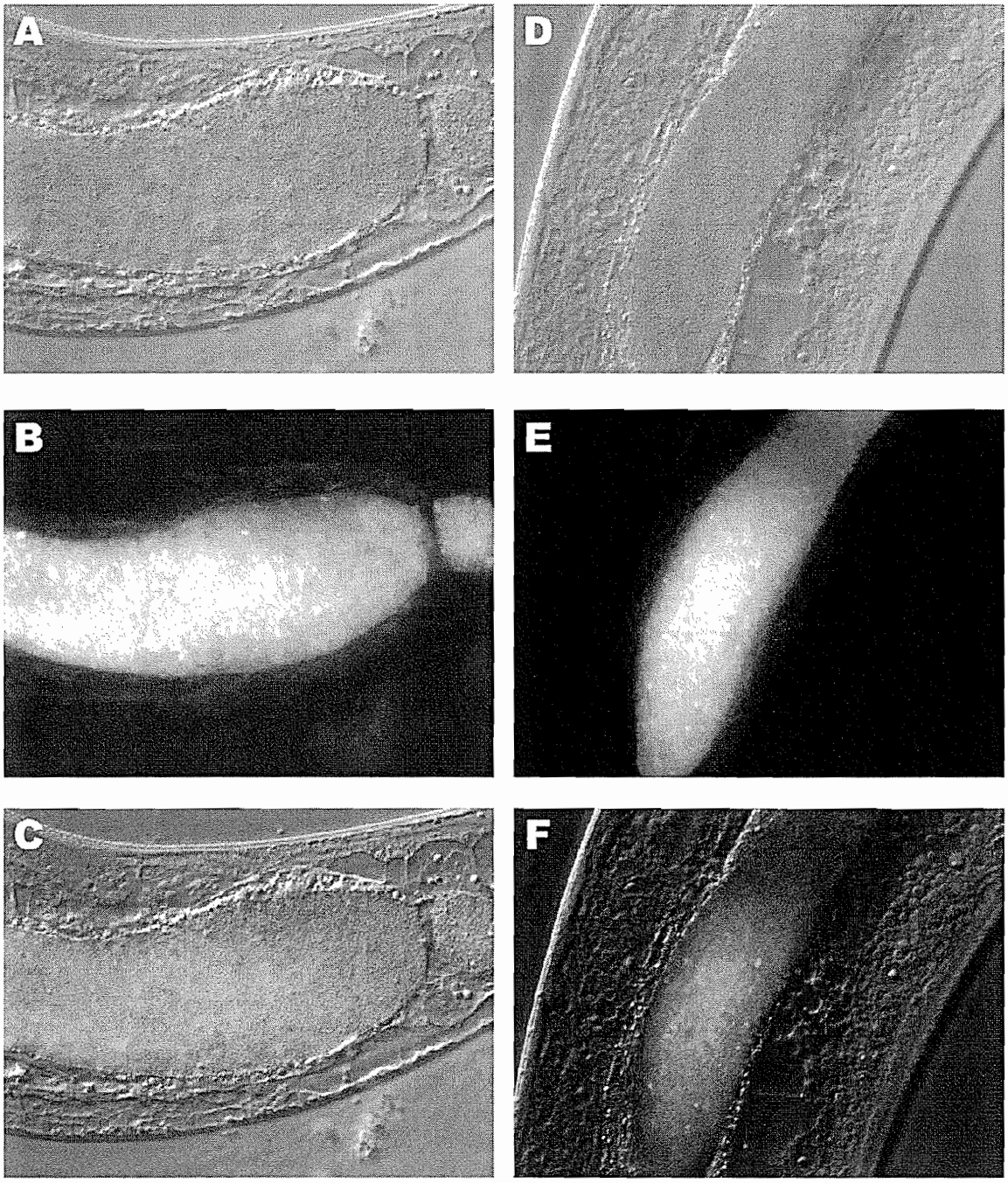

2.50 E+05
2.00 E+05
1.50 E+05
1.00 E+05
5.00 E+04
0.00 E+00
CFU per worm
8
24
48
Hours Post Infection

It should be noted that bacterial viability was not affected by this procedure. Bacterial cultures were vortexed in the presence and absence of disruptive silicon carbide particles, serially diluted and plated to assess any differences in survival. No significant difference was detectable (data not shown).

In addition, fluorescent micrographs of nematodes fed on either K56-2 or STC155-*paaE* for up to 48 hours provide visual evidence of intestinal colonization. As previously shown (Figure 3C), the attenuation phenotype is visually evident in worms at two days post- infection. For this reason, nematodes were exposed to *B. cenocepacia* strains expressing eGFP from the constitutive expression vector, pJH1 (Hamlin 2008), and viewed at 24 and 48 hours post-exposure using DIC and fluorescent microscope channels. Results confirm that bacterial accumulation is restricted to the intestinal lumen and provides visual evidence that wild type and PA pathway mutants are capable of colonizing *C. elegans* to a similar extent (Fig. 6). It should be noted that intestinal fluorescence was not due to build up of auto fluorescent lipofuscin, an age-related pigment that accumulates within intestinal cells and emits yellow-green fluorescence. Very little auto fluorescence was observed for nematodes exposed to non-fluorescent *B. cenocepacia* (data not shown).

Taken together, results indicate that decreased colonization of the *C. elegans* intestine does not appear to be the reason for the observed attenuation of pathogenicity phenotype. More likely, the explanation for this reduction in virulence is

Figure 6. Colonization of *C. elegans* by wild type *B. cenocepacia* K56-2 and the mutant STC155-*paaE* is restricted to the nematode intestinal lumen. Micrographs were taken at 1000 X magnification using differential interference contrast (DIC) and fluorescent microscope channels . (A, B) Worms fed on K56-2 for 24 hr; (D, E) Worms fed on STC155-*paaE* for 48 hr. (C, F) False colour overlay of DIC and fluorescent images.


A
D
B
E
C
F

due either to less-virulent bacteria or to worms that are able to mount a more efficient defence against ring-hydroxylation mutants.

4.2 Complementation of STC155-*paaE* with the *paaE* gene in trans restores wild type pathogenicity of *B. cenocepacia* K56-2, while the complemented strain STC199-*paaF*/pRL1 remains slightly more pathogenic in the *C. elegans* host model

Due to the nature of the insertional mutants used for nematode infection assays, complementation analysis of STC155-*paaE* and STC199-*paaF* was performed with the *paaE* and *paaF* genes in trans. As described elsewhere (Law, Hamlin et al. 2008), the attenuated or pathogenic phenotypes of the *paaA, E, F, Z* mutants could have been the result of polar effects on downstream genes caused by transcriptional terminators inserted into the chromosome by pGPΩTp. The *paaE* and *paaF* mutants were chosen for complementation analysis based on their locations, each at the end of a genetic cluster.

The constitutive expression vector, pAP20, was used to clone the *B. cenocepacia* K56-2 *paaE* gene using similar methods to those described for *paaF* in materials and methods. The resulting plasmids, pAS1 (Law, Hamlin et al. 2008) and pRL1, were then introduced through conjugation into STC155-*paaE* and STC199-*paaF*, respectively. Exconjugants were then assessed for growth in the presence of PA and pathogenic phenotypes in vivo.

With the *paaE* gene provided in trans, the capability to use PA as a sole carbon source (Law, Hamlin et al. 2008) as well as pathogenic phenotype was restored in the

complemented strain, STC155-*paaE*/pAS1. When grown in 10 mM PA, STC155-*paaE*/pAS1 was able to reach an end point growth value of 73% to that of the wild type K56-2 in comparison to 19% seen with the *paaE* mutant, STC155-*paaE* (Law, Hamlin et al. 2008). In addition, STC155-*paaE*/pAS1 shows a virulence phenotype in *C. elegans* equal to that of wild type K56-2, whereas STC155-*paaE* and the plasmid control strain STC155-*paaE*/pAP20 both display an attenuation of pathogenicity, (Figure 7). Thus, the reduced virulence of the *paaE* mutant is the result of interruption of the *paaE* gene and not to secondary site spontaneous mutations or polar effects on downstream genes.

When the *paaF* gene was provided in trans, the ability of STC199-*paaF*/pRL1 to grow on PA as a sole carbon source was restored to 57% of that seen with wild type K56-2 (Law, Hamlin et al. 2008). This was more than double what was observed for the *paaF* mutant, with an end point growth value of 22% of the wild type level (Law, Hamlin et al. 2008). Although in vitro growth could be restored with the *paaF* complement, presence of the *paaF* gene in trans did not restore the pathogenic phenotype of STC199-*paaF* to wild type levels. As shown in Figure 8, a slight but statistically significant difference in nematode survival is observed between *B. cenocepacia* K56-2 and STC199-*paaF*/pRL1. Thus, in contrast to what was seen with complementation of the *paaE* gene, presence of the *paaF* gene in trans could not completely complement STC199-*paaF* to restore wild type pathogenicity.

Figure 7. Complementation of STC155-*paaE* with the *paaE* gene in trans restores full pathogenicity in *C. elegans* DH26. Kaplan-Meier survival plot for DH26 worms fed with K56-2 (n = 67), STC155-*paaE*/pAP20 (n = 91; $P < 0.0001$), or STC155-*paaE*/pAS1 (n = 78; P = 0.05740). Squares and solid lines, K56-2; crosses and solid lines, STC155-*paaE*/pAP20; triangles and dashed lines, STC155-*paaE*/pAS1.

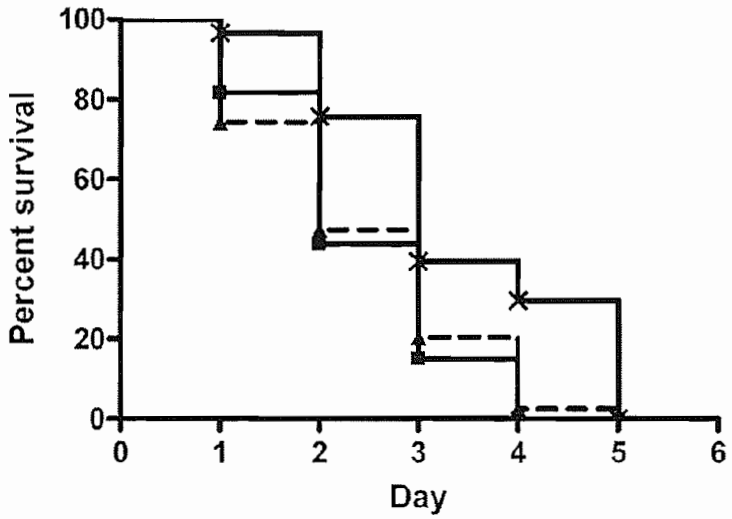
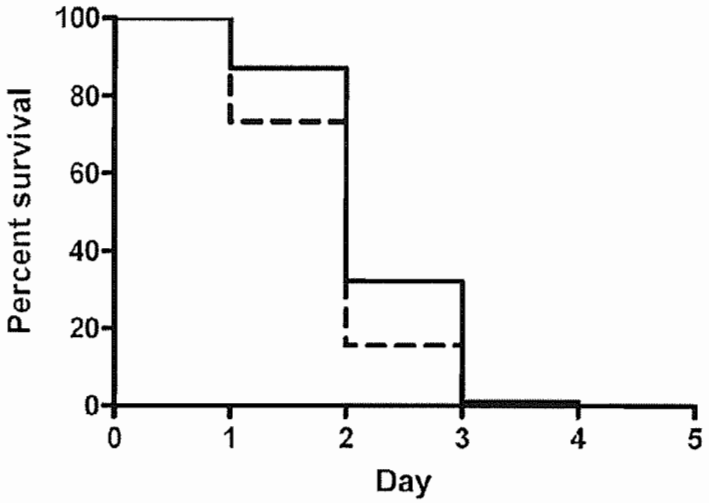
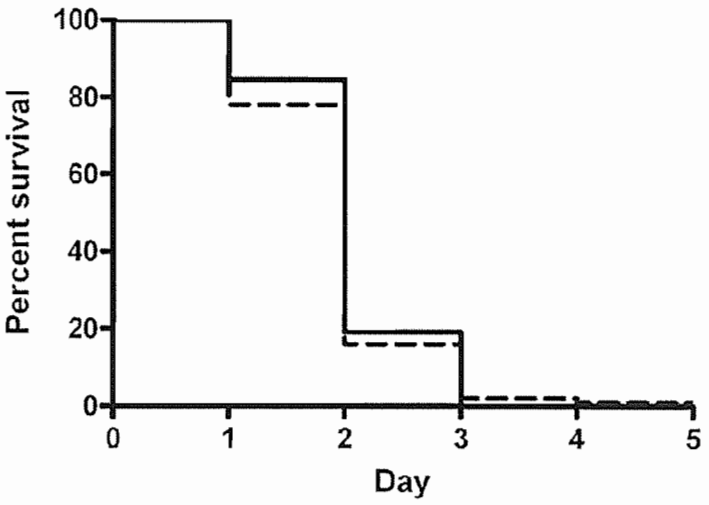

Percent survival
100
80
60
40
20
0
0
1
2
3
4
5
6
Day

Figure 8. Complementation of STC199-*paaF* with the *paaF* gene in trans does not restore pathogenicity to wild type levels in *C. elegans* DH26. (A) Kaplan-Meier survival plot for worms fed with STC199-*paaF* (n = 110) compared to worms fed with STC199-*paaF*/pRL1 (n = 109; *P* = 0.1006). Solid line, K56-2; dashed line, STC199-*paaF*/pRL1. (B) Kaplan-Meier survival plot for worms fed with STC199-*paaF* (n = 110) compared to worms fed with STC199-*paaF*/pAP20 (n = 100; *P* = 0.4232). Solid line, STC199-*paaF*; dashed line, STC199-*paaF*/pAP20.

A


100
80
60
40
20
0
Percent survival
0
1
2
3
4
5
Day

B


100
80
60
40
20
0
Percent survival
0
1
2
3
4
5
Day

4.3 Attenuation of pathogenicity in the *paaA* and *paaE* mutants is dependent on a functional p38 mitogen-activated protein kinase (MAPK) pathway in *C. elegans*

Thus far, our results have shown that mutation of the *paaA* and *paaE* genes results in *B. cenocepacia* strains that are attenuated for virulence in the *C. elegans* host model. However, it is not fully understood how mutation of the ring-hydroxylation system contributes to full pathogenicity. In an effort to uncover the mechanism behind the observed attenuation of pathogenicity phenotype, the response of immunocompromised *C. elegans* to STC179-*paaA* and STC155-*paaE* was examined in comparison to wild type K56-2.

Ingested double stranded RNA was used to specifically interfere with the *C. elegans pmk-1* gene (Timmons, Fire 1998). The *pmk-1* gene is known to code for the p38 MAPK homologue and interference of this gene by ingested dsRNA has been shown to produce worms with an enhanced-susceptibility to pathogens (esp) phenotype (Kim, Feinbaum et al. 2002). Survival results for *pmk-1* (RNAi) *C. elegans* in comparison to DH26 nematodes are consistent with previously reported data showing enhanced bacterially mediated killing (Kim, Feinbaum et al. 2002).

As shown in Figure 9 and Law et al. (2008), *pmk-1* (RNAi) *C. elegans* were hypersusceptible to *B. cenocepacia* K56-2 in comparison to *C. elegans* DH26. Next, it was hypothesized that the attenuation of virulence of the *paaA* and *paaE* mutants could involve an enhanced immune response to these strains in *C. elegans*. For this reason, survival of *pmk-1* (RNAi) *C. elegans* was compared to that of DH26 nematodes exposed

to the attenuated STC179-*paaA* and STC155-*paaE* strains as well as the complemented STC155-*paaE*/pAS1. Figure 9 shows that when exposed to the *paaA* mutant, immunocompromised *C. elegans* are hypersusceptible to killing, which is in contrast to DH26 nematodes that were more resistant to STC179-*paaA*. Kaplan-Meier analysis also shows that the median survival time of *pmk-1* (RNAi) worms is reduced to 1 day in the presence of either K56-2 or the *paaA* mutant.

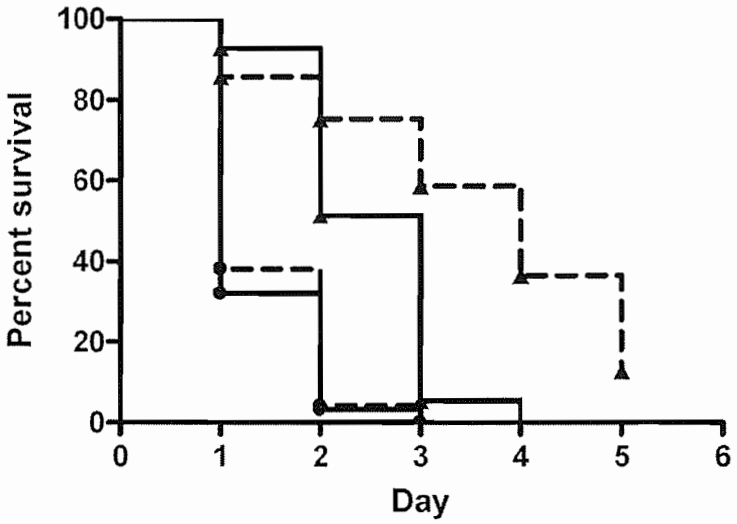
Since the in vivo phenotype of the *paaE* mutant could be complemented, the pathogenicity of STC155-*paaE* and STC155-*paaE*/pAS1 was also assessed using the *pmk-1* (RNAi) model. As already shown (Figure 7), the *paaE* mutant is attenuated for killing in *C. elegans* DH26 and STC155-*paaE*/pAS1 is as virulent as wild type *B. cenocepacia* K56-2. As was seen with immunocompromised *C. elegans* exposed to the *paaA* mutant, Figure 10 shows that *pmk-1* (RNAi) nematodes are similarly hypersusceptible to both STC155-*paaE* and STC155-*paaE*/pAS1. In contrast to what is seen with *C. elegans* DH26, these data show that inhibition of the p38 MAPK pathway by ingested dsRNA results in immunocompromised worms that are equally susceptible to wild type *B. cencoepacia* K56-2 and the *paaA* and *paaE* mutants.

4.4 *Burkholderia cenocepacia* ring-hydroxylation mutants are not more susceptible to reactive oxygen species (ROS) than wild type K56-2

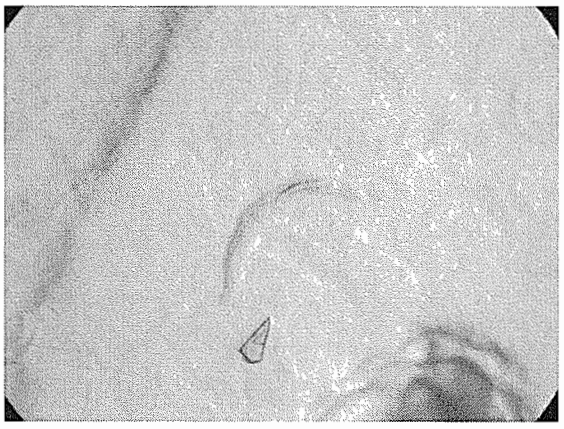
It has been shown that *C. elegans* produce reactive oxygen species as a defense mechanism in response to pathogens and that signs of oxidative stress occur in the intestinal lumen (Chavez, Mohri-Shiomi et al. 2007). It is also known that *B. cenocepacia*

Figure 9. *pmk-1* (RNAi) worms are hypersusceptible to *B. cenocepacia* K56-2 and the *paaA* mutant. (A) Kaplan-Meier survival plots for DH26 and *pmk-1* (RNAi) worms, fed with *B. cenocepacia* K56-2 or STC179-*paaA*. The killing abilities of *B. cenocepacia* K56-2 (solid lines; $P < 0.0001$) and STC179-*paaA* (dashed lines; $P < 0.0001$) were assayed in killing assays using *C. elegans* DH26 (triangles) or *pmk-1*(RNAi) worms (circles). (B) Appearance of *pmk-1* (RNAi) worms exposed to *B. cenocepacia* K56-2 or STC179-*paaA* for 2 days. Five to ten worms were chosen randomly and photographed at 80 X magnification.

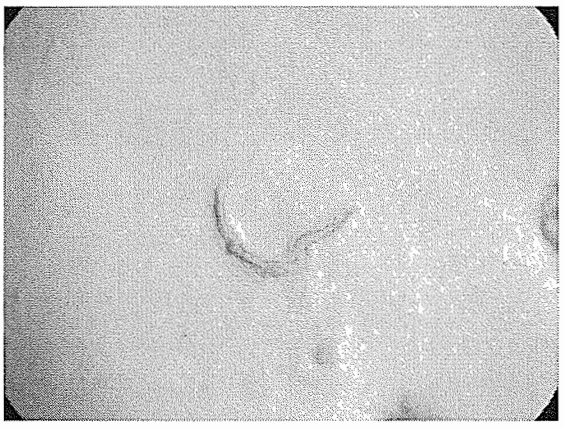
A



B

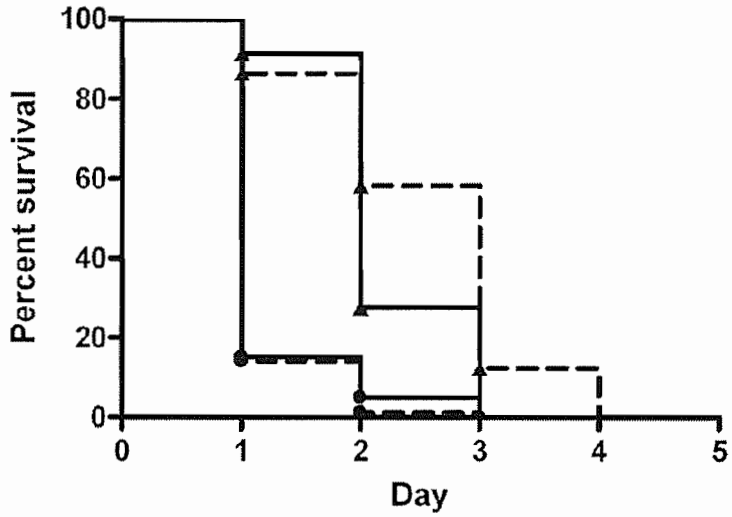


K56-2



STC179-*paaA*

Figure 10. *pmk-1* (RNAi) worms are equally hypersusceptible to STC155-*paaE* and STC155-*paaE*/pAS1. Kaplan-Meier survival plots for DH26 and *pmk-1* (RNAi) worms, fed with *B. cenocepacia* STC155-*paaE* or STC155-*paaE*/pAS1. The killing ability of STC155-*paaE* (dashed lines) was compared to that of STC155-*paaE*/pAS1 (solid lines) in slow-killing assays using *C. elegans* DH26 (triangles) ($P < 0.0016$) or *pmk-1* (RNAi) (circles) ($P = 0.4354$) worms.


Percent survival
100
80
60
40
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0
0
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2
3
4
5
Day

contains two functional catalases and at least one superoxide dismutase for protection against host generated ROS (Lefebre, Flannagan et al. 2005, Keith, Valvano 2007).

To determine whether ROS generated at the site of host-pathogen interaction in the nematode intestine might have a greater effect on PA ring-hydroxylation mutants, bacterial viability was assessed in the presence and absence of hydrogen peroxide (H_2O_2). Using methods similar to those described elsewhere (Katsuwon, Anderson 1989), wild type *B. cenocepacia* K56-2 and the *paaA* mutant were exposed to three different concentrations of H_2O_2 and viability determined based on plating as described in the Materials and Methods.

If STC179-*paaA* and STC155-*paaE* were generating intermediates rendering them more susceptible to *C. elegans*-produced ROS, a reduction in viability would have been expected for strains exposed to H_2O_2 in comparison to wild type K56-2. However, no difference was observed between the mutant and wild type strains, (Table 3). Despite mutation of the PA ring-hydroxylation complex, the *paaA* and *paaE* mutants should still possess genes encoding functional catalases and superoxide dismutase similar to that of K56-2. These data are consistent with production of antioxidant enzymes in both wild type and PA mutant *B. cenocepacia*. All calculations resulted in CFU ml^{-1} within the same order of magnitude for both strains at all concentrations of H_2O_2 (Table 3). It should also be noted that ROS treated cells showed similar results as their respective water treated controls.

In addition, similar protocols were carried out using bacterial cultures pre-treated with phenylacetic acid to determine whether oxidative stress in the presence of PA would result in reduced viability for STC179-*paaA*. As was seen with non-pretreated cultures, CFU ml^{-1} calculations were of similar magnitude for both wild type K56-2 and the *paaA* mutant (Table 4). Taken together, the attenuation phenotype observed for the *paaA* and *paaE* mutants is not likely due to a hypersusceptibilty to ROS produced in the *C. elegans* intestine.

4.5 Survival of *Caenorhabditis elegans* is reduced in the presence of *B. cenocepacia paaF* and *paaZ* mutant released metabolites

The process of PA degradation is a central route through which many aromatic compounds are catabolized and directed to the Krebs cycle (Luengo, Garcia et al. 2001). It is possible that interruption of genes at different stages of this degradative process will lead to an increase of phenolic intermediates with varying effects on *C. elegans*. Based on a previously proposed scheme for PA degradation in *B. cenocepacia* (Law, Hamlin et al. 2008), interruption of the *paaZ* and *paaF* genes should result in a build up of a PA-CoA cis-dihydrodiol derivative or 3-hydroxyadipyl-CoA and possible release of their hydrolyzed products to the media. On the other hand, mutation of *paaA* and *paaE* should result in an increase of PA-CoA and prevent accumulation of the two aforementioned compounds.

As an indirect measure of PA degradation, a general Gibbs assay to assess phenolic content of wild type and mutant supernatants was performed as described

Table 3. Effect of ROS on wild type *B. cenocepacia* K56-2 and the *paaA* mutant

	[H_2O_2] mM	CFU ml^{-1} x 10^8
K56-2	2.5	175
	5.0	225
	10.0	156
STC179-*paaA*	2.5	TNTC
	5.0	243
	10.0	187

Table 4. Effect of ROS on wild type *B. cenocepacia* K56-2 and the *paaA* mutant pre-treated with 5 mM PA

	H_2O (CFU ml^{-1} x 10^8)	10 mM H_2O_2 (CFU ml^{-1} x 10^8)
K56-2	700	780
	850	840
STC179-*paaA*	470	600
	550	630

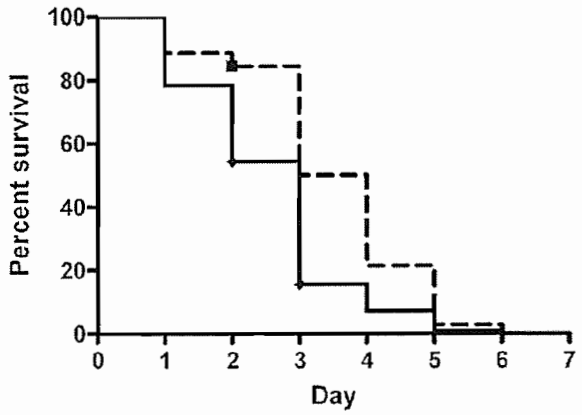
previously (Law, Hamlin et al. 2008). Published results show a dramatic increase in phenolic content of the *paaF* and *paaZ* mutant supernatants when compared to that of wild type K56-2 or the *paaA* and *paaE* mutants (Law, Hamlin et al. 2008). These results are consistent with an accumulation of the proposed dihydrodiol derivative of PA-CoA due to downstream blockage of the degradation of this compound.

To test the hypothesis that presence of excreted PA metabolites could be contributing to different survival phenotypes in *C. elegans*, metabolic products of mutant and wild type cultures were added to plates by filter diffusion in the presence of *B. cenocepacia* K56-2 or STC179-*paaA* as the food source. As shown in Figure 11, the attenuation phenotype observed for the *paaA* mutant is abolished in the presence of either wild type or *paaF* mutant filtrate. Figure 11 shows a statistically significant reduction in nematode survival between worms exposed to STC179-*paaA* alone versus worms exposed to STC179-*paaA* plus the filtrate of more pathogenic strains. As a control, nematodes were also exposed to STC179-*paaA* vs. STC179-*paaA* plus *paaA* mutant filtrate. As shown in Figure 11C, no difference in *C. elegans* survival was observed for control plates.

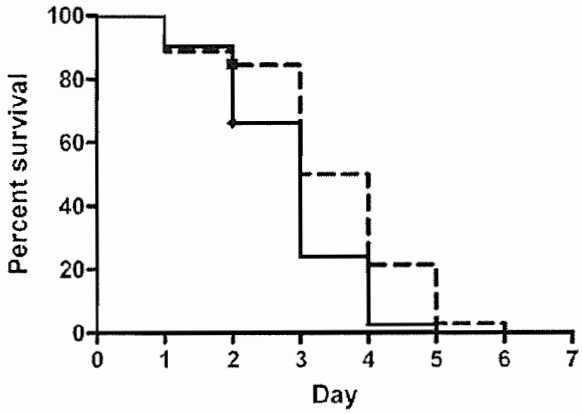
Similarly, nematode survival was also scored using wild type K56-2 as a food source in the presence and absence of wild type, *paaA* and *paaF* mutant filtrate, (Figure 12). Figure 12B shows a statistically significant difference in survival of worms exposed to K56-2 plus *paaF* mutant filtrate in comparison to those exposed to wild type bacteria only. Control plates containing wild type filtrate also show a decrease in nematode

Figure 11. The attenuated pathogenicity of STC179-*paaA* is abolished in the presence of *B. cenocepacia* wild type and *paaF* mutant filtrate but not *paaA* mutant filtrate. Kaplan-Meier survival plots for DH26 worms fed with STC179-*paaA* alone (n = 73; dashed lines) or STC179-*paaA* in the presence of wild type K56-2 or mutant filtrate (solid lines). (A) K56-2 filtrate (n = 112; $P < 0.0001$). (B) STC199-*paaF* filtrate (n = 83; $P < 0.0001$). (C) STC179-*paaA* filtrate (n = 93; $P = 0.6443$).

A


Percent survival
100
80
60
40
20
0
0 1 2 3 4 5 6 7
Day

B


Percent survival
100
80
60
40
20
0
0 1 2 3 4 5 6 7
Day

C

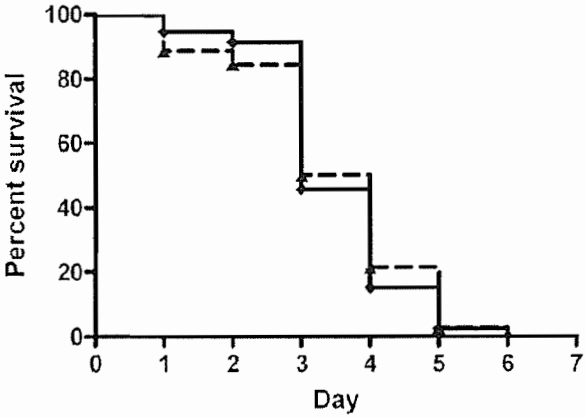
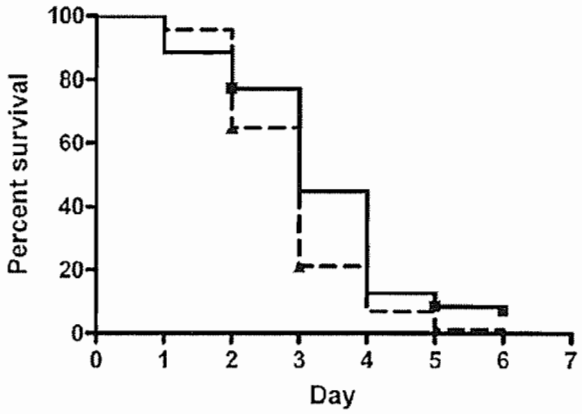
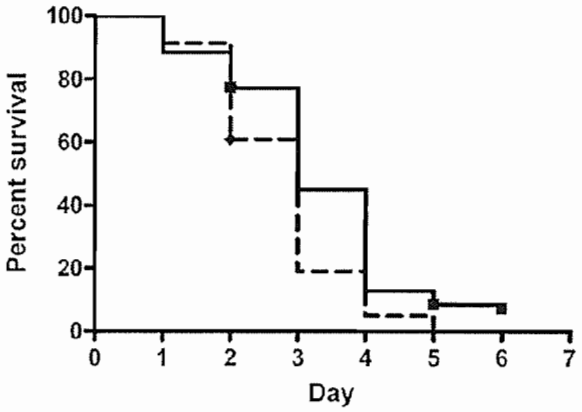

Percent survival
100
80
60
40
20
0
0 1 2 3 4 5 6 7
Day

Figure 12. Pathogenicity of *B. cenocepacia* K56-2 is enhanced in the presence of *B. cenocepacia* K56-2 and *paaF* mutant filtrate. Kaplan-Meier survival plots for DH26 worms fed with wild type K56-2 alone (n = 96; solid lines) or K56-2 in the presence of wild type or mutant filtrate (dashed lines). (A) K56-2 filtrate (n = 91; $P = 0.0016$). (B) STC199-*paaF* filtrate (n = 83; $P = 0.0003$). (C) STC179-*paaA* filtrate (n = 88; $P = 0.0314$).

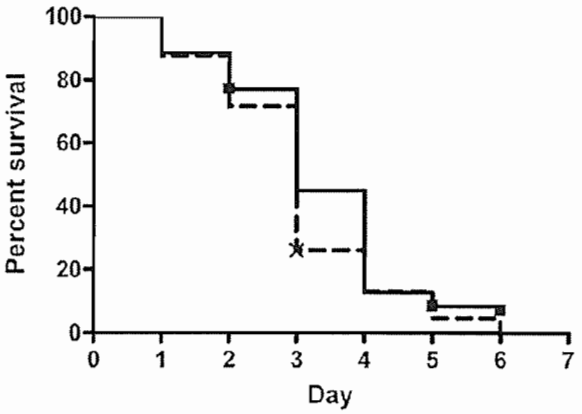
A


Percent survival
100
80
60
40
20
0
0 1 2 3 4 5 6 7
Day

B


Percent survival
100
80
60
40
20
0
0 1 2 3 4 5 6 7
Day

C

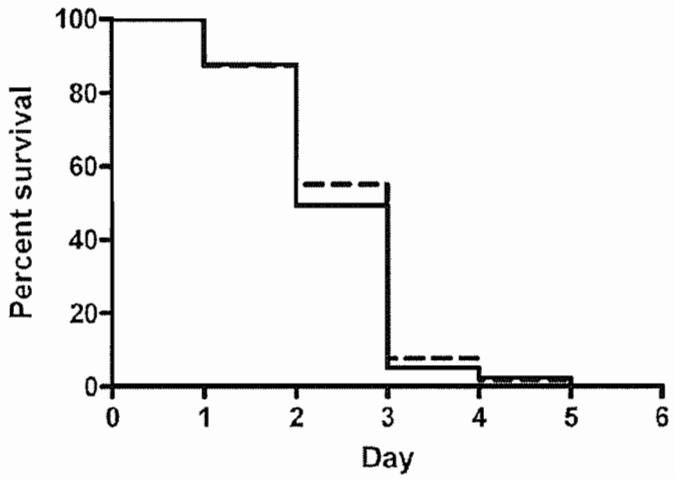

Percent survival
100
80
60
40
20
0
0 1 2 3 4 5 6 7
Day

survival (Figure 12A) which could be explained due to increased levels of wild type metabolite production. Control plates containing *paaA* mutant filtrate also show a statistically significant reduction in survival vs. worms exposed to K56-2 only. Given this difference, it is possible that background metabolite production, irrespective of the PA catabolic pathway, is also contributing to nematode survival phenotypes.

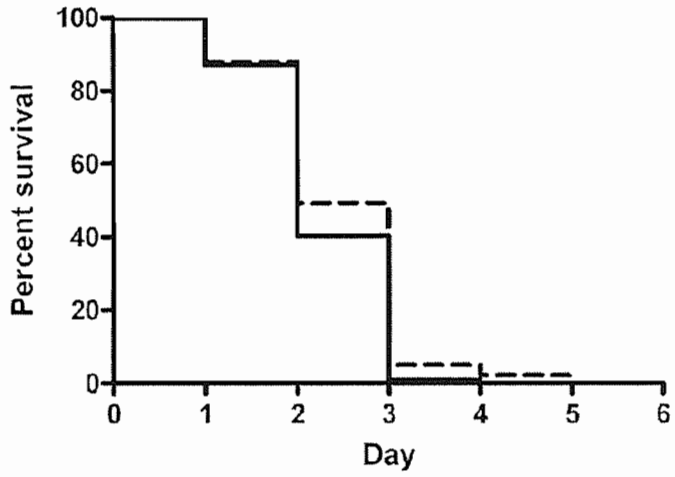
To address whether PA itself would affect nematode survival, worms were exposed to lawns of wild type K56-2 grown on plates with varying concentrations of phenylacetic acid added directly to NG media. *C. elegans* infection assays were performed as discussed previously for PA mutant strains. The expectation was that if exogenous PA was contributing to the attenuation phenotype, an increase in nematode survival would be observed in plates containing PA versus those containing plain NG media. No difference in survival was observed either in the presence or absence of PA, (Figure 13). However, it is still possible that PA may exert some effect within the intestinal lumen at the site of infection. It is not known whether exogenous PA was able to penetrate external barriers of the nematode or if PA-containing agar was ingested with bacterial cells during feeding.

Figure 13. Exogenous PA does not affect nematode survival in the presence of *B. cenocepacia* K56-2. Kaplan-Meier survival plots for DH26 worms fed with *B. cenocepacia* K56-2 on NG plates containing PA. (A) The killing ability of wild type *B. cenocepacia* strain K56-2 with no PA added to plates (n = 118) was compared to that of K56-2 on plates containing 10 μM PA (n = 138; $P = 0.4273$). Dashed line, 0 μM PA; solid line, 10 μM PA. (B) The killing ability of wild type *B. cenocepacia* strain K56-2 with 10 μM PA added to plates was compared to that of K56-2 on plates containing 100 μM PA (n = 94; $P = 0.0702$). Dashed line, 10 μM PA; solid line, 100 μM PA.

A


100
80
60
40
20
0
Percent survival
0
1
2
3
4
5
6
Day

B


100
80
60
40
20
0
Percent survival
0
1
2
3
4
5
6
Day

Activation of PA: The contribution of two PA-CoA ligase enzymes to growth and pathogenicity of *B. cenocepacia*

4.6 In vitro growth analysis of the *B. cenocepacia* PA-CoA ligase mutants

It has been shown that the STC179-*paaA* and STC155-*paaE* mutants of the *B. cenocepacia* PA degradation pathway produce an attenuation of infection phenotype in the *C. elegans* host model, (Figure 3). Based on a search of the sequenced *B. cenocepacia* J2315 genome, two homologues of the *E. coli* PA-CoA ligase, *paaK*, were found in chromosome one and two of the J2315 genome (Law, Hamlin et al. 2008). Due to the site of action of PaaK, upstream of PaaA and PaaE during PA catabolism (Figure 2B), it was desirable to generate deletion strains for the *paaK1* and *paaK2* genes to investigate their in vitro growth and in vivo pathogenic phenotypes. Figure 14 shows the general scheme for *paaK* gene deletion and Figure 15 shows the vector maps for the mutagenesis plasmids used.

Growth experiments were conducted in 96-well microplates with glucose and PA as sole carbon sources. Strains were grown over a 30 hour period and data from the 15 hour time point was selected as representative of growth during exponential phase. Figure 16 shows the wild type K56-2 and all three mutant strains were able to grow well using glucose as a sole source of carbon. In addition, the *paaK1* single deletion mutant, RJL1, was able to grow to similar levels as K56-2 in M9 + 5 mM PA. This result corresponds to previous findings (Law, Hamlin et al. 2008) demonstrating the ability of a *paaK1* insertional mutant to use PA as a sole source of carbon. Figure 16 also shows

Figure 14. Strategy for the I-SceI-dependent deletion of the *paaK1* and *paaK2* genes.

Step 1. Cloning of chromosomal regions, U and D, flanking the targeted gene (*paaK*) into pGPI-SceI, which has an I-SceI recognition site (arrow) and encodes trimethoprim resistance (Tp^R). The mutagenesis plasmid is introduced into *B. cenocepacia* and becomes integrated into the genome by homologous recombination.

Step 2. The I-SceI expression vector, pDAI-SceI (not shown), is introduced into the recombinant strain. I-SceI cleaves at the recognition sequence (5'-TAGGGATAACAGGGTAAT-3', arrow), causing a double strand break in the chromosome that stimulates host recombination/repair machinery. A deletion is obtained if recombination occurs between D' and D while recombination between U and U' causes reversion to wild type. (Copyright © Blackwell Publishing Ltd., Environmental Microbiology, 10(6), 1653, 2008).

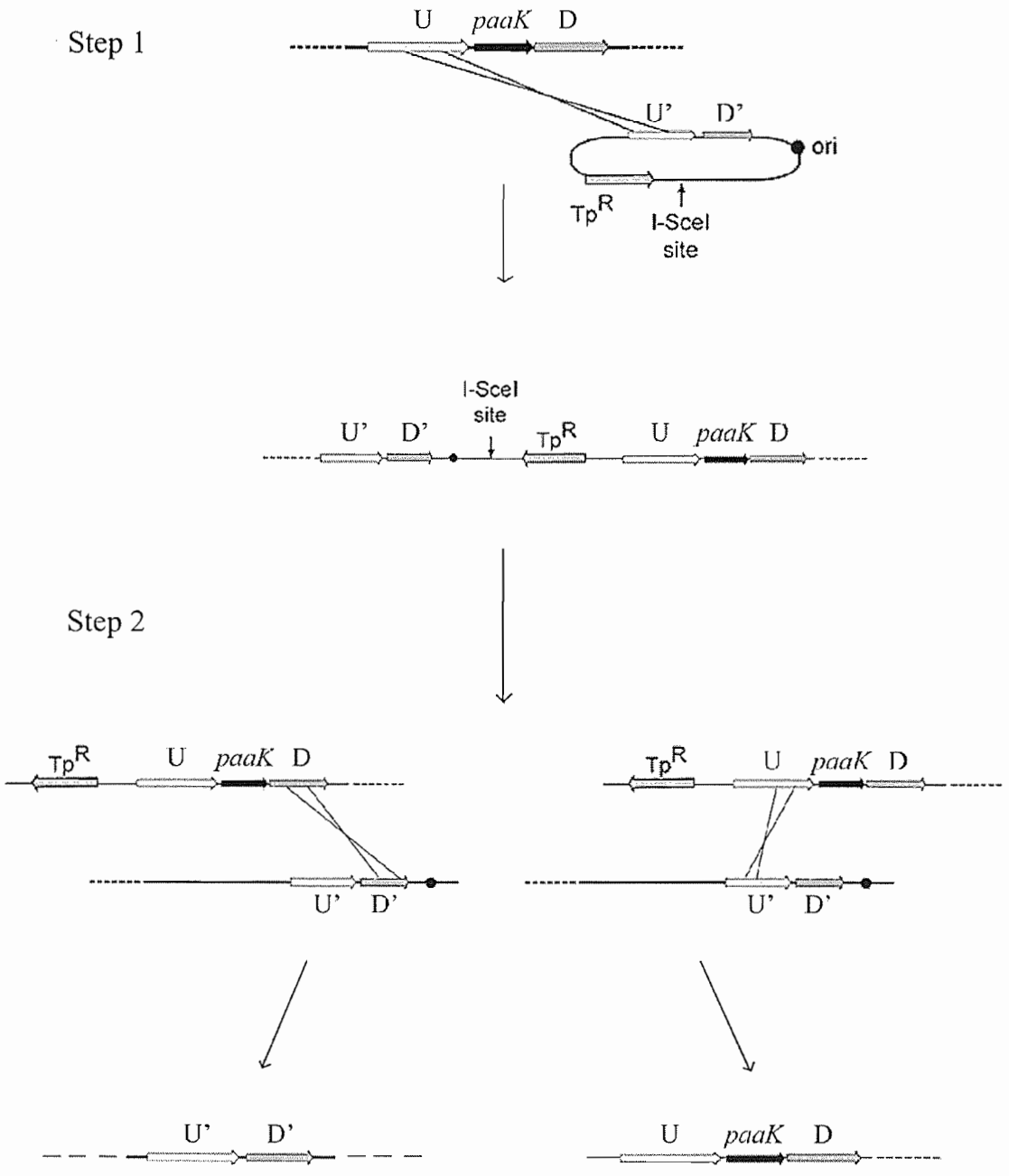
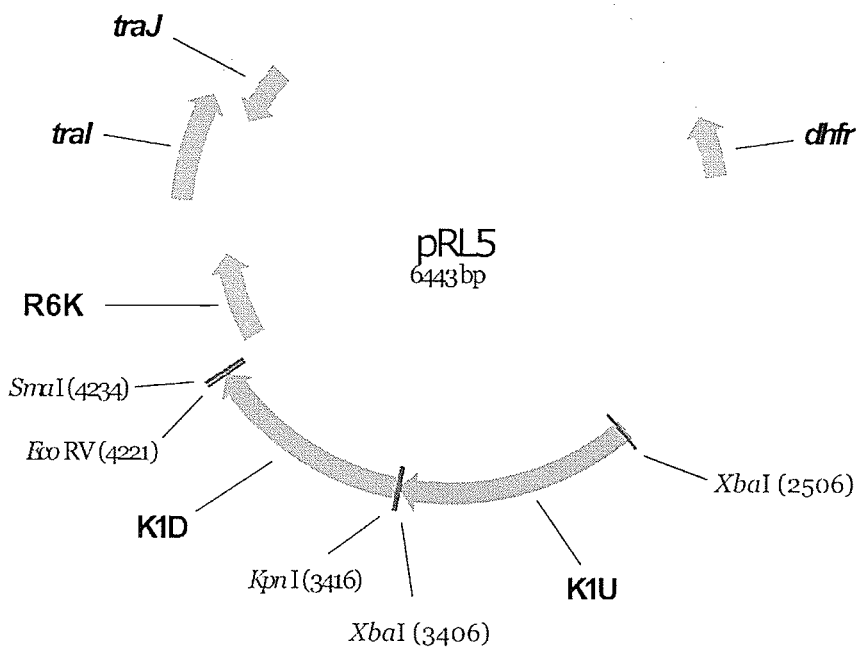
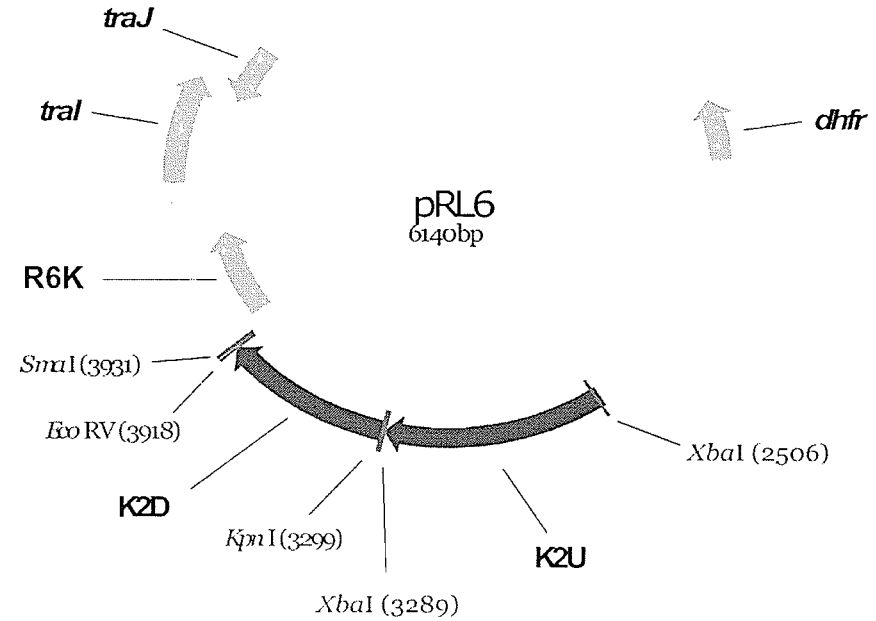

Step 1
U
paaK
D
U'
D'
ori
TpR
I-SceI
site
I-SceI
site
U'
D'
TpR
U
paaK
D
Step 2
TpR
U
paaK
D
U'
D'
TpR
U
paaK
D
U'
D'
U'
D'
U
paaK
D

Figure 15. Plasmid maps of pRL5 and pRL6; mutagenesis plasmids derived from the suicide vector, pGPI-SceI. (A) pRL5. Contains upstream and downstream chromosomal regions of the *paaK1* gene. (B) pRL6. Contains upstream and downstream chromosomal regions of the *paaK2* gene.

A


traJ
traI
dhfr
pRL5
6443bp
R6K
SmaI (4234)
EcoRV (4221)
XbaI (2506)
K1D
KpnI (3416)
K1U
XbaI (3406)

B


traJ
traI
dhfr
pRL6
6140bp
R6K
SmaI (3931)
EcoRV (3918)
XbaI (2506)
K2D
KpnI (3299)
K2U
XbaI (3289)

that the double mutant strain, RJL3, was unable to grow using PA and in contrast to what was seen with RJL1, a complete lack of growth was observed for the *paaK2* single deletion mutant, RJL2.

Sequencing of the chromosomal regions containing *paaK1* and *paaK2* revealed that while deletion of *paaK1* was successfully achieved in both RJL1 and RJL3, deletion of *paaK2* may have resulted in polar effects (Figure 17). Sequence analysis of RJL2 and RJL3 shows that deletion of *paaK2* may have interfered with the start codon of BCAM1710, a putative enoyl-CoA hydratase/isomerase located immediately downstream, (Figure 17B).

Given the growth analysis of the *paaK* mutants, combined with the sequencing data, it is possible that interruption of the putative enoyl-CoA hydratase/isomerase is affecting the ability of *paaK2* mutant strains to grow on PA. In the case of RJL2, functionality of the PA-CoA ligase encoded by *paaK1* is masked by the second site mutation. Inability of the double mutant to grow using PA as a sole carbon source is likely due to the absence of both PA-CoA ligases but mutation of the enoylCoA hydratase/isomerase cannot be ruled out as a contributing factor.

4.7 In vivo analysis of the *B. cenocepacia* PA-CoA ligase mutants

Due to mutation of the PA-CoA ring-hydroxylation system, *B. cenocepacia* strains STC179-*paaA* and STC155-*paaE* present an attenuation of pathogenicity phenotype in the nematode host, (Figure 3). The putative PA-CoA ligase, PaaK, catalyzes the first step of PA catabolism, acting upstream from PaaA and PaaE and involves conversion of PA to

Figure 16. Growth of wild type and PA-CoA ligase-defective *B. cenocepacia* during mid-exponential phase in M9 minimal media supplemented with 0.2% glucose or 5 mM PA. Data represents the average OD_{600} of three independent cultures after 15 hours of growth. Error bars represent the standard deviation of three independent cultures.

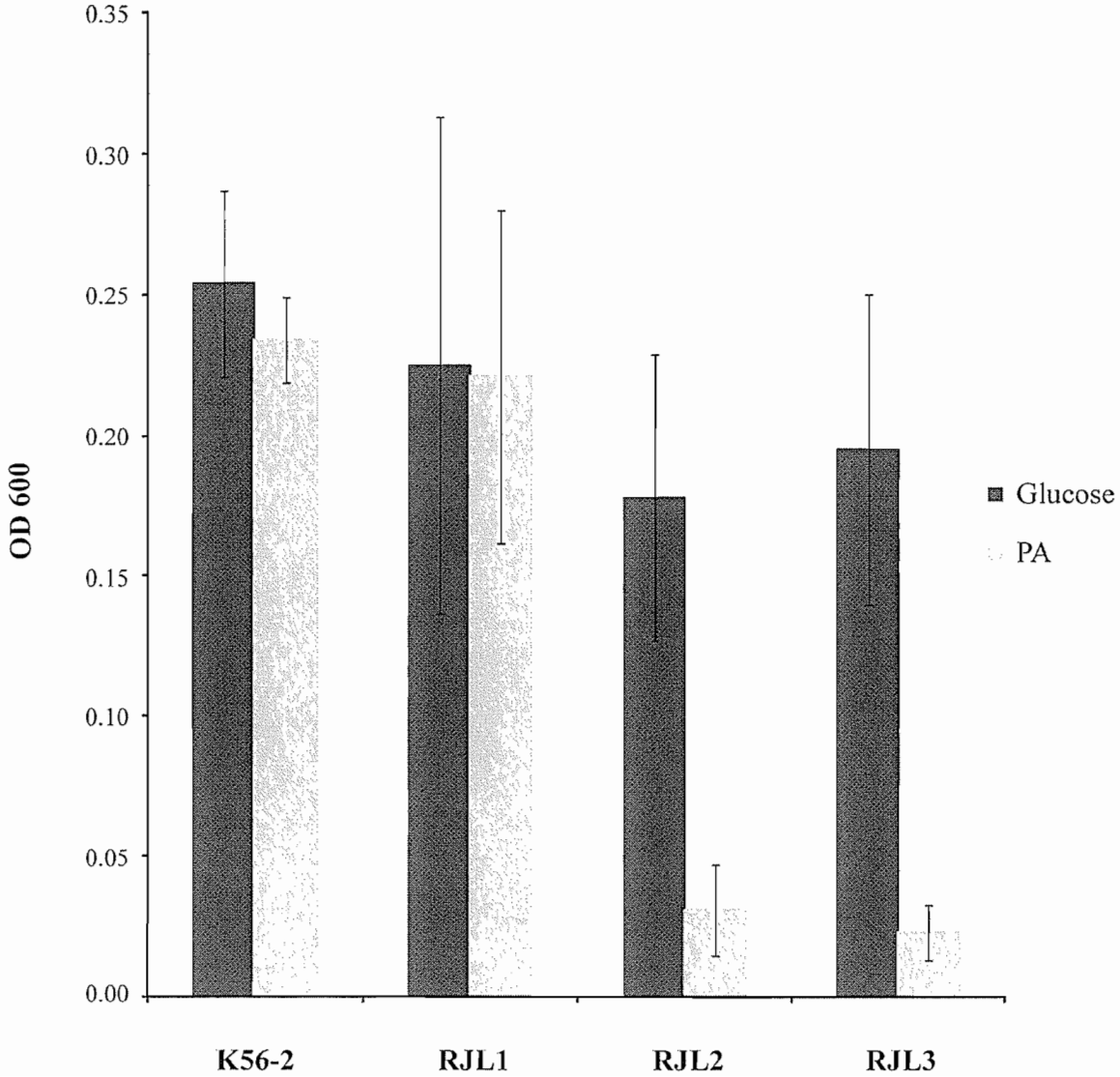

0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
OD 600
Glucose
PA
K56-2
RJL1
RJL2
RJL3

Figure 17. Sequence analysis of wild type and *paaK* mutant chromosomal regions. Start and stop codons of the wild type sequence are highlighted in red; *paaK1* and *paaK2* whole gene sequences have been omitted for clarity and replaced by dashed lines. Differences between mutant and wild type base sequences appear in bold; introduced restriction sites for XbaI (tctaga) and KpnI (ggtacc) are underlined. (A) Chromosomal region surrounding *paaK1*. (B) Chromosomal region surrounding *paaK2* including start codon of BCAM1710 (underlined in green).

A

Wild-type: gccca taacaaacac tggag**acatg ---- tga gggtcgctcg ccacc**tgtac gtcgcccaac

Mutant: gccca taacaaacac tggag**tctag aggtacctgt** tgtacgtcgc ccaacggcga

B

Wild type: cgaca tcacgccatt **ggaggagcac cccgatg ---- tgatg tcgaggtgt**g ccgttctac ccg

Mutant: cgaca tcacgccat**tc tagaggtacc** gccgttctac ccg

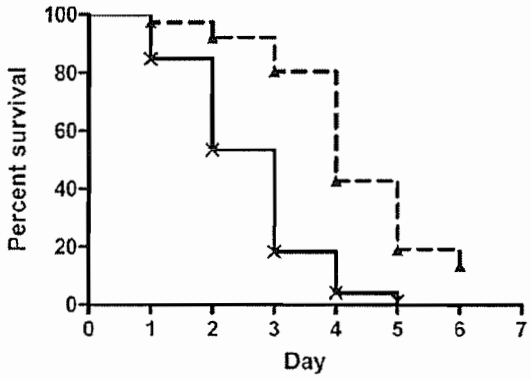
PA-CoA. Based on previously discussed nematode survival data and location of PA-CoA ligase activity, it was hypothesized that RJL3 would show a similar attenuation phenotype as was seen with STC179-*paaA* and STC155-*paaE*.

Figure 18 shows survival data of worms exposed to lawns of wild type K56-2 in comparison to RJL1, RJL2 or RJL3. As seen with the *paaA* and *paaE* mutants, nematodes fed on RJL3 display an increased survival phenotype. Given that RJL1 did not demonstrate visible growth in PA (Figure 16), it is interesting that this mutant exhibits a reduction in virulence similar to what is seen with STC179-*paaA*, STC155-*paaE* and RJL3. This data suggests that PaaK1 is the primary PA-CoA ligase and that removal of its encoding gene results in a dominant attenuation of virulence phenotype in both RJL1 and RJL3, regardless of effects on *paaK2* downstream genes as mentioned above.

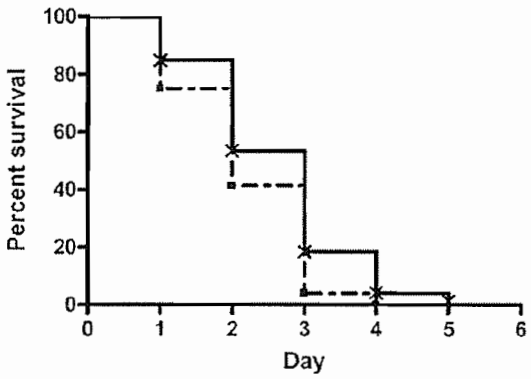
On the other hand, RJL2, which was unable to grow using PA as a sole carbon source has the opposite phenotype in vivo. As shown in Figure 18B, RJL2 displays a slightly enhanced pathogenicity in *C. elegans* in comparison to K56-2. Here, interruption of the enoyl-CoA hydratase/isomerase, downstream of *paaK2*, may be causing an accumulation of phenolic intermediates thereby producing a phenotype similar to that of the *paaZ* and *paaF* mutants. Taken together, these results support the proposed hypothesis that attenuation of virulence is due to mutations preventing accumulation of phenolic metabolites.

Figure 18. The virulence of RJL1 and RJL3, but not RJL2, is diminished in the *C. elegans* infection model. Kaplan-Meier survival plots for DH26 worms fed with *B. cenocepacia* K56-2 (n = 119; solid lines) compared to worms fed with *paaK* mutant strains (dashed lines). (A) RJL1 (n = 80; $P < 0.0001$). (B) RJL2 (n = 104; $P = 0.0014$). (C) RJL3 (n = 119; $P < 0.0001$).

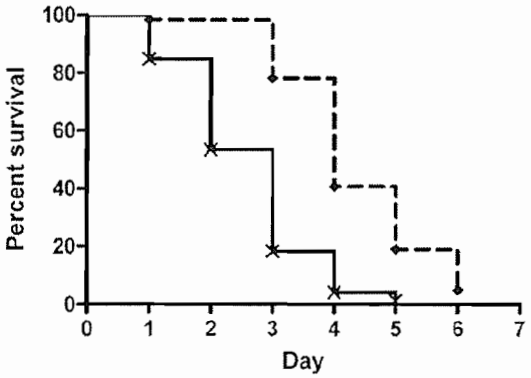
A


Percent survival
100
80
60
40
20
0
0 1 2 3 4 5 6 7
Day

B


Percent survival
100
80
60
40
20
0
0 1 2 3 4 5 6
Day

C


Percent survival
100
80
60
40
20
0
0 1 2 3 4 5 6 7
Day

4.8 Phylogenetic analysis of the putative *B .cenocepacia* PA-CoA ligase enzymes

The aerobic degradation of phenylacetic acid (PA) progresses through a series of steps ultimately leading to intermediates of the Krebs cycle (Luengo, Garcia et al. 2001). It has been shown that the PA-CoA ligase enzyme is involved in the first step of this degradative process and is essential for aerobic catabolism of PA (El-Said Mohamed 2000). Bioinformatic analysis of the sequenced *B. cenocepacia* J2315 genome has revealed two genes encoding putative PA-CoA ligases (Law, Hamlin et al. 2008).

The differences among in vitro growth and in vivo pathogenic phenotypes of the single and double mutants may indicate that PaaK1 and PaaK2 are functional under different conditions. For this reason, phylogenetic analysis of the two proteins was carried out to determine their relatedness to each other and to other PA-CoA ligase proteins. The first step in evaluating sequence relatedness was to generate an alignment of the *B. cenocepacia* PaaK1 and PaaK2 proteins. Figure 19 shows the sequence alignment created using Boxshade 3.21 (Hofman, Baron 2006). The putative *B. cenocepacia* PA-CoA ligases share 69.6 % amino acid sequence similarity and comparable percent GC content; 66 % and 69 % for PaaK1 and PaaK2, respectively.

Two conserved substrate binding motifs and an AMP binding motif previously found in the PA-CoA ligase of *A. evansii* (El-Said Mohamed 2000) were also identified for both *B. cenocepacia* PA-CoA ligases. All three sequences were identified in each member of the data set used for phylogenetic tree construction with almost complete sequence conservation. Figure 20 shows the sequence logo for the putative AMP

Figure 19. Boxshade 3.21 sequence alignment showing 69.6 % amino acid sequence similarity between the *B. cenocepacia* J2315 PaaK1 and PaaK2 proteins. Identical residues are shaded in black, similar residues are shaded in gray and dissimilar residues appear in white. Conserved motifs are underlined in red. HASSGTTGKPTV, AMP binding; DIYGLSE and YRTRD, substrate binding.

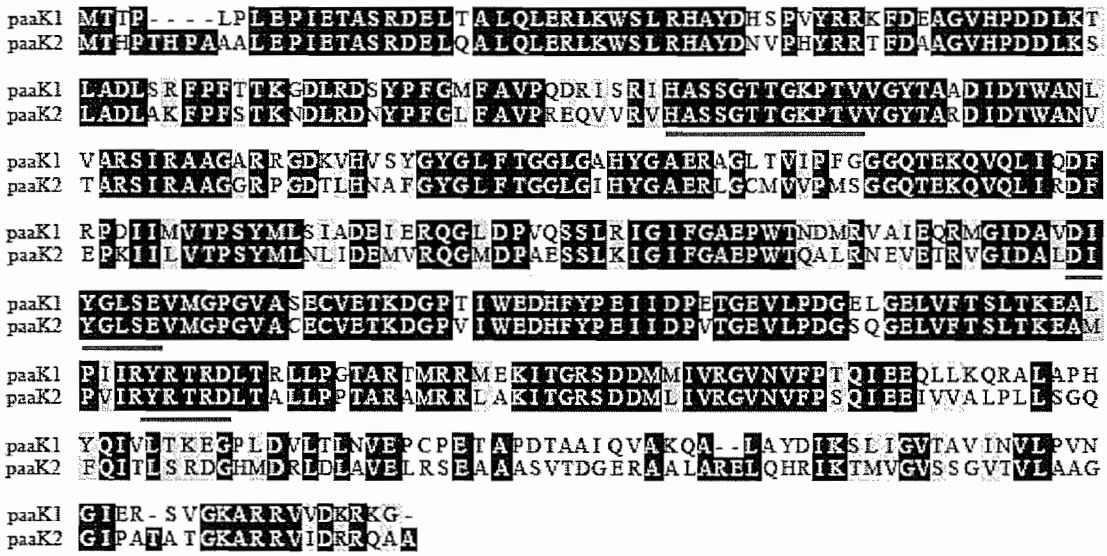
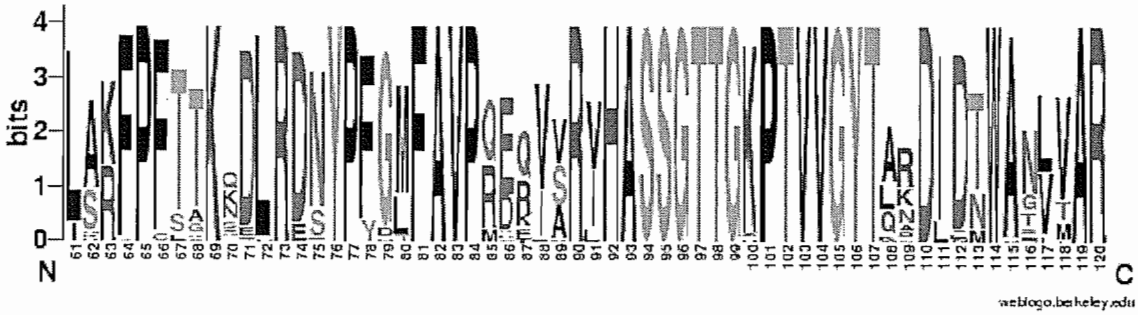

paaK1 MTTP----LPLEPIETASRDELTALQLERLKWSLRHAYDHSPVYRRKFDEAGVHPDDLKT
paaK2 MTHPTHPAAALEPIETASRDELQALQLERLKWSLRHAYDNVPHYRRTFDAAGVHPDDLKS
paaK1 LADLSRFPFTTKGDLRDSYPFGMFAVPQDRISRIHASSGTTGKPTVVGYTAADIDTWANL
paaK2 LADLAKFPFSTKNDLRDNYPFGLFAVPREQVVRVHASSGTTGKPTVVGYTARDIDTWANV
paaK1 VARSIRAAGARRGDKVHVSYGYGLFTGGLGAHYGAERAGLTVIPFGGGQTEKQVQLIQDF
paaK2 TARSIRAAGGRPGDTLHNAFGYGLFTGGLGIHYGAERLGCMVVPMSGGQTEKQVQLIRDF
paaK1 RPDIIMVTPSYMLSIADEIERQGLDPVQSSLRIGIFGAEPWTNDMRVAIEQRMGIDAVDI
paaK2 EPKIILVTPSYMLNLIDEMVRQGMDPAESSLKIGIFGAEPWTQALRNEVETRVGIDALDI
paaK1 YGLSEVMGPGVASECVETKDGPTIWEDHFYPEIIDPETGEVLPDGELGELVFTSLTKEAL
paaK2 YGLSEVMGPGVACECVETKDGPVIWEDHFYPEIIDPVTGEVLPDGSQGELVFTSLTKEAM
paaK1 PIIRYRTRDLTRLLPGTARTMRRMEKITGRSDDMMIVRGVNVFPTQIEEQLLKQRALAPH
paaK2 PVIRYRTRDLTALLPPTARAMRRLAKITGRSDDMLIVRGVNVFPSQIEEIVVALPLLSGQ
paaK1 YQIVLTKEGPLDVLTLNVEPCPETAPDTAAIQVAKQA--LAYDIKSLIGVTAVINVLPVN
paaK2 FQITLSRDGHMDRLDLAVELRSEAAASVTDGERAALARELQHRIKTMVGVSSGVTVLAAG
paaK1 GIER-SVGKARRVVDKRKG-
paaK2 GIPATATGKARRVIDRRQAA

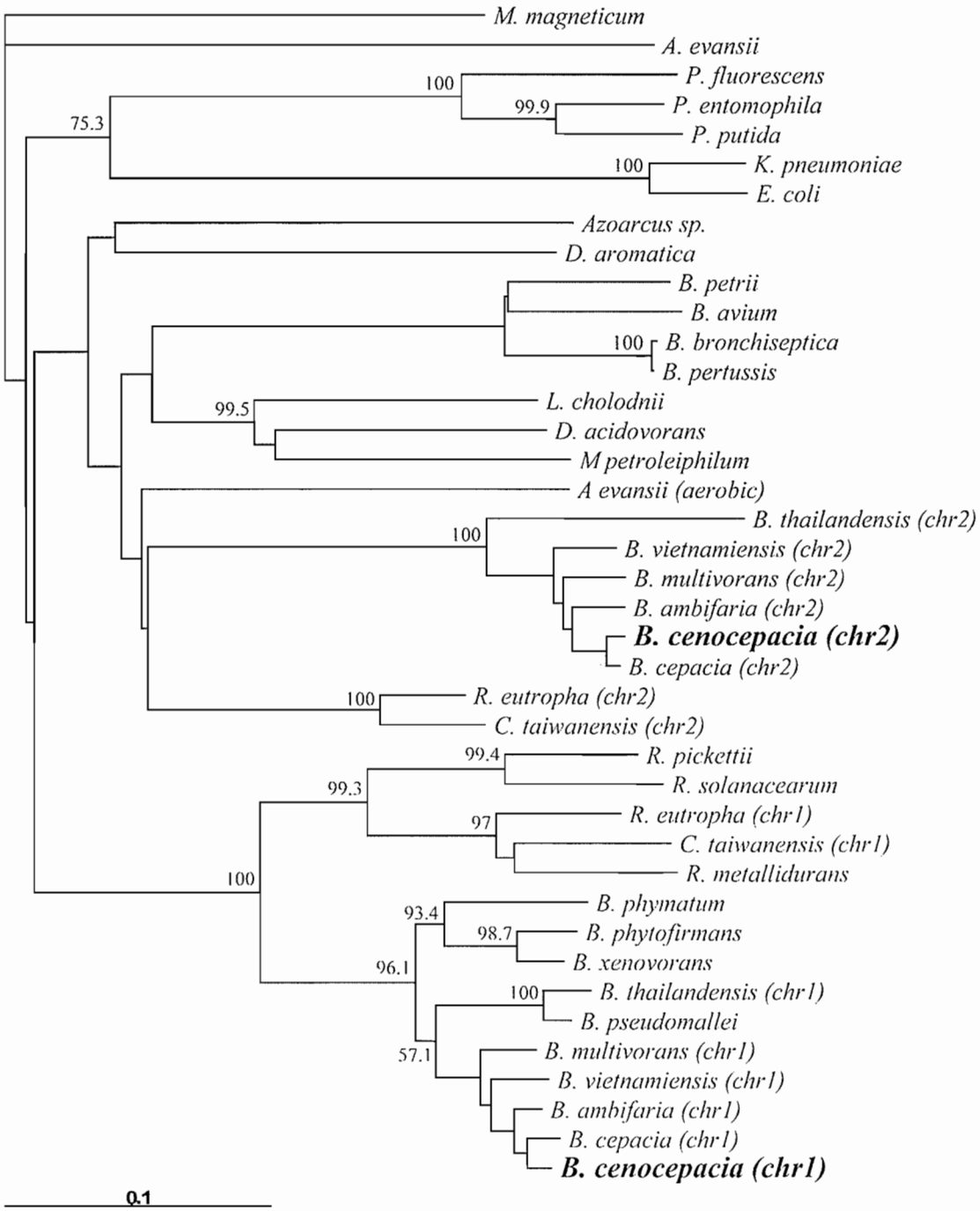
Figure 20. Sequence logo showing a graphical representation of amino acid conservation covering positions 61 to 120 of a forty species protein sequence alignment. The logo includes the putative AMP-binding sequence, HASSGTTGKPTV, from position 92 to 107. Letter height is proportional to frequency of occurrence of the corresponding amino acid, while stack height correlates with sequence conservation at a given site.


bits
4
3
2
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0
N
C
weblogo.berkeley.edu

binding motif, HASSGTTGKPTV. Stack height indicates sequence conservation at a given position within the sequence logo, while letter height relates to the relative frequency of the corresponding amino acid (Crooks, Hon et al. 2004). The presumed AMP binding motif is almost completely conserved across the data alignment, with the exception of two lysine substitutions at position 100. This suggests that this region may have structural or functional significance for the putative protein.

Given the similarity between the protein sequences of PaaK1 and PaaK2, we originally thought that the *paaK1* and *paaK2* genes of *B. cenocepacia* had arisen from a duplication event within the species; however, Figure 21 shows that the PaaK1 and PaaK2 proteins do not branch from a common tree node. Protein sequences originating from genes found on chromosome one for each representative of the Bcc, cluster together with other members of the genus *Burkholderia* and *Ralstonia*. This result is not unexpected since these two genera have been shown to be phylogenetically related based on 16s rRNA and *recA* sequence homology (Coenye, Vandamme 2003, Payne, Vandamme et al. 2005). Similarly, the second putative PA-CoA ligase (PaaK2) seems to share more sequence identity with other second copy ligases of the genus *Burkholderia* as well as *R. eutropha* and *C. taiwanensis*. Confidence estimates based on majority rule consensus data show that the PaaK1 ligases of *Burkholderia* species group together 96% of the time while the PaaK2 ligases group 100% of the time. The PaaK1 and PaaK2 proteins do not appear to share an immediate common ancestral sequence, and in fact, are located several nodes apart on the phylogenetic tree. In addition, the functionally confirmed PA-CoA ligases of *E. coli* and *P. putida* cluster with other members of the

Figure 21. Phylogenetic tree of the PA-CoA ligase sequence alignment of forty Proteobacteria with *M. magneticum* as the outgroup. Protein sequences were aligned using ClustalX software and a Jones-Taylor-Thornton distance matrix was used to construct the neighbour-joining tree. Branch point numbers represent the percentage of times that tree node was supported in 1000 bootstrap replicates. Scale bar indicates an evolutionary distance of 0.1 amino acid substitutions per position.


M. magneticum
A. evansii
P. fluorescens
P. entomophila
P. putida
K. pneumoniae
E. coli
Azoarcus sp.
D. aromatica
B. petrii
B. avium
B. bronchiseptica
B. pertussis
L. cholodnii
D. acidovorans
M petroleiphilum
A evansii (aerobic)
B. thailandensis (chr2)
B. vietnamiensis (chr2)
B. multivorans (chr2)
B. ambifaria (chr2)
B. cenocepacia (chr2)
B. cepacia (chr2)
R. eutropha (chr2)
C. taiwanensis (chr2)
R. pickettii
R. solanacearum
R. eutropha (chr1)
C. taiwanensis (chr1)
R. metallidurans
B. phymatum
B. phytofirmans
B. xenovorans
B. thailandensis (chr1)
B. pseudomallei
B. multivorans (chr1)
B. vietnamiensis (chr1)
B. ambifaria (chr1)
B. cepacia (chr1)
B. cenocepacia (chr1)
100
99.9
75.3
100
100
99.5
100
100
99.4
99.3
97
100
93.4
98.7
96.1
100
57.1
0.1

gamma-proteobacteria several tree nodes away from either *Burkholderia* group. Based on this data, it cannot be stated for certain which of the two ligases is more closely related to those experimentally confirmed as PA-CoA ligases.

Separation of the two protein sequences could indicate that one of the PA-CoA ligase genes may have been acquired through horizontal transfer with a more distantly related organism or that the *paaK1* and *paaK2* genes could be the result of an ancient duplication event that occurred prior to speciation. It is also possible that if PaaK1 and PaaK2 derive from different origins, they may possess unique functions or perform the same function under separate conditions.

5 – Discussion

5.0 Inhibition of phenylacetyl-CoA ring hydroxylation during phenylacetate metabolism results in attenuation of pathogenicity in *B. cenocepacia* K56-2

A link between PA catabolism and pathogenicity was first discovered during a signature-tagged mutagenesis (STM) screen for *B. cenocepacia* isolates incapable of survival in vivo (Hunt, Kooi et al. 2004). In particular, the 4A7 mutant, which had a transposon inserted in the *paaE* gene, was defective for survival in the rat lung and did not kill *C. elegans* (Cardona, Wopperer et al. 2005).

Our results show that interruption of the *paaA* and *paaE* genes does produce an attenuation of pathogenicity in *B. cenocepacia*. The effect, however, is not as powerful as what is seen with the 4A7 mutant. *B. cenocepacia* K56-2, which typically displays rough colony morphology on agar media, is known to spontaneously undergo transition to shiny colony variants. Recently, it was shown that these shiny variants were associated with decreased biofilm formation, extracellular matrix and virulence (Bernier, Nguyen et al. 2007). Inspection of the 4A7 mutant confirmed that it exhibited the shiny morphotype (Law, Hamlin et al. 2008). Therefore, the strong attenuation of virulence seen with the 4A7 mutant is most likely due to mutation of the *paaE* gene in combination with a second site mutation associated with shiny colony morphology.

Despite the influence of secondary mutations in the 4A7 mutant, our results show that interruption of genes involved in the ring hydroxylation step of PA degradation does contribute to attenuation of pathogenicity in *B. cenocepacia* K56-2.

STC179-*paaA* and STC155-*paaE* present a rough phenotype on agar media and are attenuated for virulence in *C. elegans* when compared to wild type K56-2.

The *paaA* and *paaE* insertional mutants were tested for pathogenicity in the nematode based on their locations at the beginning and end of the *paaABCDE* cluster. As stated previously, insertional mutants were generated through integration of the suicide plasmid, pGPΩTp, into the *B. cenocepacia* chromosome (Law, Hamlin et al. 2008). Transcriptional terminators introduced by the plasmid are believed to affect transcription of genes downstream from the insertion site (Flannagan, Aubert et al. 2007). For this reason, it was expected that interruption of *paaA* would prevent expression of the entire operon, while disruption of *paaE* would only affect transcription of *paaE* itself, based on its location at the end of the cluster.

It is possible that insertion of transcriptional terminator sequences could have affected expression of genes downstream of the *paaABCDE* operon. However, our results show that complementation of the *paaE* gene in trans not only restores wild type pathogenicity, but also re-establishes the ability of the mutant to use both PA and phenylalanine as a sole source of carbon (Law, Hamlin et al. 2008). Thus, our findings indicate that the ring hydroxylation complex is necessary for full virulence of *B. cenocepacia* in the nematode host; and the observed attenuation phenotype is a direct result of the *paaE* mutation and not likely due to downstream effects or secondary mutations.

5.1 Colonization of the *C. elegans* intestine by *B. cenocepacia* K56-2 is not affected by inhibition of PA-CoA ring hydroxylation

Many bacterial pathogens are capable of colonizing the *C. elegans* intestine, which consequently leads to a lethal infection. A number of studies have shown that mutation of bacterial genes associated with diminished virulence in the nematode model correlates with reduced intestinal accumulation (Garsin, Sifri et al. 2001, Begun, Gaiani et al. 2007, Aballay, Ausubel 2002). Conversely, it has been shown that nematodes with mutations that give rise to defects in the pharyngeal grinder, result in improved colonization by otherwise attenuated bacterial pathogens (Kothe, Antl et al. 2003).

Our results show that the observed attenuation of virulence of STC179-*paaA* and STC155-*paaE* is not likely due to a diminished capacity to colonize *C. elegans* or to effects on the grinder. Fluorescent micrographs of nematodes infected with *B. cenocepacia* K56-2 or the *paaE* mutant support quantification data of intestinal colonization, showing that the mutant and wild type strains are capable of accumulating to similar levels over a 48 hour infection period. It should be noted that while intestinal colonization is a hallmark of bacterial pathogenesis in the nematode, in some cases, it does not necessarily result in killing. For example, when grown aerobically, *Enterococcus faecium* proliferates in the *C. elegans* intestinal lumen without affecting worm survival (Garsin, Sifri et al. 2001).

Another possible explanation for the attenuation of pathogenicity seen with the *paaA* and *paaE* mutants was that defects in the PA pathway itself could lead to reduced growth in *C. elegans*. In addition to STC179-*paaA* and STC155-*paaE*, strains with gene mutations further down the PA pathway, lacked the capacity to use PA as a sole carbon source (Law, Hamlin et al. 2008); however, not all mutations resulted in an attenuated pathogenic phenotype. It is not likely then, that interruption of the pathway itself leads to decreased bacterial growth inside the nematode, followed by attenuation of virulence.

Therefore, disruption of the ring hydroxylation complex results in bacterial strains capable of colonizing *C. elegans* to a similar extent as *B. cenocepacia* K56-2, but with reduced killing ability.

5.2 Virulence of *B. cenocepacia* K56-2 is increased as a result of impaired ring cleavage and oxidative degradation during phenylacetate metabolism

The mutant strains STC183-*paaZ* and STC199-*paaF* were generated using the plasmid pGPΩTp, as described above for the *paaA* and *paaE* mutants. Presumably, introduction of transcriptional terminators would affect expression of *paaZ* as well as genes downstream from the *paaZ* insertion site; however, given that *paaF* is located at the end of a cluster, it is likely that only the *paaF* gene itself was affected following plasmid insertion.

Unlike what was seen with *B. cenocepacia* mutants of the ring hydroxylation complex, our results show that STC183-*paaZ* and STC199-*paaF* are slightly but

significantly more pathogenic to *C. elegans*. Based on the expectation that insertional inactivation of *paaF* was not affecting multiple downstream targets, complementation analysis was carried out with the *paaF* gene in trans to verify the enhanced pathogenic phenotype. Although the ability of STC199-*paaF* to use PA and phenylalanine as a carbon source was restored when *paaF* was provided in trans (Law, Hamlin et al. 2008), the in vivo pathogenic character of the strain did not return to wild type levels. In addition, total phenolic content of STC199-*paaF*/pRL1 supernatant was reduced in comparison to STC199-*paaF*, but was still greater than that found in wild type supernatant (Law, Hamlin et al. 2008).

It is possible that this lack of complementation could be due to differences in regulation of *paaF* expression. In the complemented strain, *paaF* is constitutively expressed under the control of P_{DHFR}, and thus is not regulated as the native enzyme would be. Furthermore, when *paaF* is provided in trans it is present on a multi-copy number plasmid; therefore, it is also possible that over production of the enzyme may be having an unidentified effect on the PA pathway.

Given that the *paaF* and *paaZ* mutants impart a more pathogenic phenotype in *C. elegans*, which is in contrast to what is seen with the *paaA* and *paaE* mutants, it is plausible to consider that the reason for this phenotypic variation could be due to effects caused by different metabolic intermediates. In *E. coli*, the proposed product of the PaaABCDE complex is 1,2-dihydroxy-1,2-dihydro-PA-CoA (Ismail, El-Said Mohamed et al. 2003, Fernandez, Ferrandez et al. 2006). If the same is true for *B. cenocepacia*,

blockage of the ring opening step would result in an increased presence of this compound or a hydrolyzed derivative allowing for phenolic detection through a general Gibbs assay. This would explain the high levels of phenolic compounds encountered in the *paaZ* mutant supernatant (Law, Hamlin et al. 2008); however, disruption of PaaF, thought to be involved in the β-oxidation step of PA degradation, should result in an increase of a hydroxylated adipyl-CoA compound.

PaaF and PaaG have both been identified as putative enoyl-CoA hydratase/isomerases acting at the β-oxidation and ring opening steps of PA catabolism, respectively (Law, Hamlin et al. 2008). Given the high phenolic content of the *paaF* mutant supernatant, it is probable that PaaF acts during ring opening while PaaG is involved in β-oxidation (Figure 2B). This proposition is additionally supported by the in vivo phenotypes observed for the *paaF* and *paaZ* mutants, which could be the result of increased amounts of a cis-dihydrodiol derivative of PA-CoA.

5.3 Enhanced production of phenolic intermediates during phenylacetate catabolism corresponds to a decline in *C. elegans* survival

In accordance with our hypothesis that accumulated metabolites of different PA pathway mutants would have alternative effects on *C. elegans* survival, filter diffusion slow killing assays were performed with attenuated and more pathogenic *B. cenocepacia* strains. Our results show that nematode survival was adversely affected by the presence of wild type and *paaF* mutant metabolites in comparison to those of STC179-*paaA*.

If our hypothesis is true, it is not surprising that strains producing PA-CoA derivatives are capable of enhanced virulence in *C. elegans*. We show that addition of *paaA* mutant filtrate does not affect virulence attenuation of STC179-*paaA*, while wild type and *paaF* mutant filtrate eliminate *paaA* mutant attenuation. STC179-*paaA* and STC155-*paaE* show an extremely low level of total phenolic content when compared to the *paaZ* and *paaF* mutants (Law, Hamlin et al. 2008); the wild type strain, however, shows higher amounts of phenolic intermediates than the attenuated mutants, but much less than those of STC183-*paaZ* and STC199-*paaF* (Law, Hamlin et al. 2008).

Given that the differences in phenolic production of the wild type and mutant strains correspond to varying degrees of pathogenicity, it is possible that certain metabolic intermediates have an effect on virulence phenotypes. Nevertheless, it should be noted that background metabolites unrelated to PA catabolism could also have an effect on nematode survival. Figure 12C shows a slight increase in pathogenicity of K56-2 in the presence of *paaA* mutant filtrate. It is possible that background toxins involved in fast killing may supersede the attenuated effects of *paaA* mutant related metabolites since wild type *B. cenocepacia* is already more pathogenic than STC179-*paaA*. In addition, the stability of these as yet unidentified intermediates has not been determined, and it is not known whether their effects on *C. elegans* are altered based on occurrence inside the intestinal lumen or in the external medium. Further analysis involving forced ingestion of PA or related metabolites will need to be explored using liquid media in order to assess the internal effects of these compounds on nematode survival.

5.4 The pathogenic phenotypes of three *B. cenocepacia* PA-CoA ligase mutants correlate with differences in production of hydrolyzed dihydro-diol derivatives of phenylacetyl-CoA

Two putative homologues of the PA-activating enzyme, PA-CoA ligase, have been identified in the *B. cenocepacia* genome (Law, Hamlin et al. 2008). Based on our previous findings, STC181-*paaK1* was the only insertional mutant tested that did not present a reduced-growth phenotype in PA and phenylalanine. For this reason, we predicted that both putative PA-CoA ligases must be functional and the expectation was that a double *paaK* mutation would result in PA deficient growth as well as attenuation of pathogenicity.

In agreement with our previous findings, the *paaK1* single deletion mutant, RJL1, was able to use PA as a sole carbon source and grow to a similar extent as wild type *B. cenocepacia* K56-2. This was in contrast to the PA-deficient growth observed for both the double *paaK* mutant and the single *paaK2* mutant. The inability of RJL3 to grow using PA is not surprising given that both putative PA-CoA ligase genes have been deleted. Based on the prediction that both *paaK1* and *paaK2* encode functional PA-CoA ligases, the lack of growth observed for RJL2 was unanticipated. However, these results could be explained if PaaK2 was the dominant *B. cenocepacia* PA-CoA ligase or if the two ligases are functional under different growth conditions. Evidence for separate PA-CoA ligases, induced under aerobic and anaerobic conditions, has been reported in *A. evansii* (El-Said Mohamed 2000). It is therefore possible that PaaK2 is the dominant aerobic

ligase and that PaaK1 is functional under different oxic or temperature conditions. This could explain the growth phenotypes of STC181-*paaK1* and RJL1 under the conditions used. However, complementation analysis of the *paaK1* and *paaK2* genes will need to be performed in order to exclude the possibility that the observed phenotypes are due to secondary mutations that are unrelated to either gene deletion.

Alternatively, sequencing of the chromosomal region surrounding the *paaK2* deletion has revealed a probable interruption of the enoyl-CoA hydratase/isomerase (BCAM1710) directly downstream. It is likely that interruption of the second enzyme is contributing to the observed phenotype. It has been previously shown that catabolism of PA in *E. coli* involves two enoyl-CoA hydratase/isomerase enzymes active at the ring-opening and β-oxidation stages of degradation (Luengo, Garcia et al. 2001). It is possible that interruption of this enzyme in RJL2 could produce a growth phenotype similar to what was seen with STC183-*paaZ* and STC199-*paaF* regardless of a functional PaaK1.

Additionally, in vivo analysis of the virulence phenotypes associated with the PA-CoA ligase mutants seems to support a *paaZ*/*paaF* mutant-like phenotype for RJL2. Like STC183-*paaZ* and STC199-*paaF*, RJL2 displays increased pathogenicity in *C. elegans*. This is consistent with production of harmful metabolites due to disruption of the downstream enoyl-CoA hydratase/isomerase. The opposite effect for RJL1 suggests that PaaK1 may in fact be the dominant PA-CoA ligase in the in vivo condition; if PaaK2 was fully functional in this strain, a pathogenic phenotype similar to that of the wild type would be expected. Since deletion of the *paaK1* gene alone results in attenuation of

virulence analogous to what is seen with STC179-*paaA* and STC155-*paaE*, it is reasonable to assume that it may be the principal ligase in the nematode condition. In support of this suggestion is the attenuation of virulence seen in the double *paaK* mutant, RJL3. Even though RJL3 likely contains a disruption in the enoyl-CoA hydratase/isomerase downstream of the *paaK2* deletion, the prevailing phenotype corresponds to the attenuation seen in RJL1, STC179-*paaA* and STC155-*paaE*. Overall, these results reinforce the hypothesis that PA pathway intermediates produced during the ring hydroxylation and ring opening steps of PA degradation cause an increased virulence phenotype in *C. elegans*.

Although these results are in support of our hypothesis, the contribution of the BCAM1710 mutation to PA-dependent growth and virulence phenotypes cannot be overlooked. In order to fully analyze the effects of a *paaK2* deletion, mutants will need to be created that do not interfere with expression of BCAM1710. The *paaK2* chromosomal region consists of *paaH*, *paaK2* and BCAM1710. While deletion of *paaK2* does not appear to have interfered with *paaH*, there are approximately 60 bp between the *paaH* stop codon and the *paaK2* start codon. This may mean that although the two genes appear to share the same promoter, P_{paaH}, they may not be co-translated. The intergenic region between *paaH* and *paaK2* will have to be examined to identify potential ribosomal binding sites before a new deletion can be made. In addition, the stop codon of *paaK2* and the start codon of BCAM1710 seem to share an adenine. This being the case, a small 3' portion of *paaK2* will have to remain in the chromosome after deletion so as not to interfere with expression of BCAM1710.

5.5 Phylogenic and functional evidence for two *B. cenocepacia* phenylacetate-CoA ligases

Thus far, the majority of our results support the general hypothesis that intermediates produced during the third and fourth steps of PA degradation have adverse effects on *C. elegans* survival. Based on the assumption that both the *paaK1* and *paaK2* genes encode functional PA-CoA ligases, our prediction was that mutation of both genes would produce an in vivo phenotype similar to what was seen for the *paaA* and *paaE* mutants. On the contrary we expected that single mutations in each gene would yield a phenotype similar to that of *B. cenocepacia* K56-2. However, conflicting data for PA-dependent growth and in vivo pathogenic phenotypes of the *paaK* mutants may imply functionality of the two ligases under separate growth conditions.

The genomes of many microorganisms contain putative PA-CoA ligase gene sequences, however, experimental evidence for functional enzymes exists for only a few organisms (El-Said Mohamed 2000, Schleissner, Olivera et al. 1994). Phylogenetic analysis of the *B. cenocepacia* PA-CoA ligases was carried out in order to establish the relatedness of their amino acid sequences to each other and to those of other Proteobacteria.

Given the sequence similarity between the *B. cenocepacia* PaaK1 and PaaK2 proteins, we originally thought that the *paaK1* and *paaK2* genes of *B. cenocepacia* were paralogues. However, based on our results, the *B. cenocepacia* PaaK1 and PaaK2 proteins share only 70% sequence homology and do not appear to be related by an

immediate common ancestral sequence on the phylogenetic tree. In addition, each sequenced member of the Bcc appears to contain two copies of a PA-CoA ligase encoding gene. It is therefore possible that duplication of one of the *paaK* genes occurred prior to speciation of the Bcc bacteria. It is also possible that *paaK1* and *paaK2* are the result of lateral gene transfer between *B. cenocepacia* and a more distant relative which resulted in more than one copy of a PA-CoA ligase encoding gene in the J2315 genome. Genes acquired through horizontal transfer are retained in the recipient's genome when the gene product confers an advantage or useful metabolic property to the receiving organism (Abe-Yoshizumi, Kamei et al. 2004). Maintaining two functional PaaK proteins could be an advantage to an organism such as *B. cenocepacia* that is ubiquitous in many different environments. *A. evansii* is known to produce an isoenzyme of its aerobically grown PA-CoA ligase when cultured under anaerobic conditions (El-Said Mohamed 2000).

The reaction catalyzed by PA-CoA ligase is the conversion of PA to PA-CoA in the presence of ATP. We have identified a putative AMP-binding motif in both PaaK1 and PaaK2, suggesting this region of the protein may be involved in hydrolysis of ATP to AMP and inorganic phosphate. In addition, two postulated substrate binding motifs that have been identified in all proven or putative PA-CoA ligases, are also present in PaaK1 and PaaK2; however, these sequences may not contribute to specific substrate binding affinity of the PA-CoA ligases (El-Said Mohamed 2000).

It is not yet known whether both the *paaK1* and *paaK2* genes encode functional PA activating enzymes. Complementation analysis of the *paaK* genes in trans will be required to confirm functionality of both PA-CoA ligase enzymes as well as the PA-dependent growth and pathogenicity phenotypes of the mutants. Thus far, in vitro growth analysis of the *paaK1* single mutant implies that a second functional ligase is produced in the presence of PA. On the other hand, the dominant attenuation phenotype of RJL1 in vivo suggests that *paaK1* also encodes a functional protein which may be the dominant ligase in the in vivo condition.

5.6 The p38 MAPK pathway contributes to pathogen resistance in *C. elegans* regardless of a functional phenylacetate catabolic pathway in *B. cenocepacia* K56-2

The reasons behind the requirement for a functional ring hydroxylation system are not well understood. Our hypothesis centres on the presumption that certain PA pathway intermediates confer a negative effect on *C. elegans* survival; however, it is not known what role the nematode immune system may play in relation to the various pathogenic phenotypes observed for wild type *B. cenocepacia* and the PA pathway mutants.

For this reason, we decided to target a known *C. elegans* innate immune effector, PMK-1, through RNA interference. Inhibition of the *pmk-1* gene has been reported to produce worms that are hypersusceptible to pathogens such as *P. aeruginosa*, in a manner that is independent of feeding, fitness or defecation (Kim,

Feinbaum et al. 2002). Our results show a similar trend in susceptibility for *pmk-1* (RNAi) *C. elegans* exposed to both attenuated and wild type *B. cenocepacia* strains.

We rationalized that attenuation of pathogenicity in STC179-*paaA* and STC155-*paaE* could be the result of an enhanced immune reaction in *C. elegans* in response to these mutants. However, little difference in susceptibility was observed between worms fed on attenuated mutants versus wild type and complemented pathogenic strains. If *C. elegans* were mounting a more efficient defence against the *paaA* and *paaE* mutants, we would have expected to see a loss of virulence attenuation for these strains in comparison to K56-2 or STC155-*paaE*/pAS1. The median survival of *pmk-1* inhibited *C. elegans* was reduced to one day when exposed to STC179-*paaA*, STC155-*paaE*, K56-2 or STC155-*paaE*/pAS1. Since the *pmk-1* (RNAi) nematodes display such an enhanced susceptibility to all *B. cenocepacia* tested, it cannot be said for certain whether attenuation is actually lost in the *paaA* and *paaE* mutants or if the effects of *pmk-1* inhibition are too strong for it to be seen. In addition, analysis of transcript levels by reverse transcriptase PCR could be used to quantitatively asses the level of *pmk-1* inhibition in worms exposed to wild type and PA mutant strains.

To further investigate the sensitivity of the *paaA* and *paaE* mutants to *C. elegans* physiological defences, the effects of ROS on *B. cenocepacia* viability were assessed. Our findings show that STC179-*paaA* is not more sensitive to H_2O_2 than K56-2, even after pre-treatment with PA. If metabolites formed due to blockage of the PaaABCDE enzymatic complex were causing the *paaA* and *paaE* mutants to become more sensitive

to oxidative stress in the intestinal lumen, we would expect to see a reduction in viability of the mutants in vitro upon exposure to ROS. *B. cenocepacia* is an aerobic microorganism and as such, produces reactive oxygen by-products during cellular respiration. In order to avoid ROS attack on cellular targets such as DNA, RNA, lipids and proteins, specific antioxidant enzymes are used to decrease the amount of ROS. The *B. cenocepacia* genome is known to encode both catalases and superoxide dismutases for protection against these potentially damaging species (Lefebre, Flannagan et al. 2005, Keith, Valvano 2007).

The interaction of *C. elegans* innate immune system and the PA degradation pathway of *B. cenocepacia* remains to be determined. Based on these results, it can be said that *C. elegans*-produced ROS is not likely involved in the attenuation of pathogenicity observed for the *paaA* and *paaE* mutants, and that a functional p38 MAPK pathway is absolutely required for nematode defence against *B. cenocepacia* wild type and PA mutant strains.

5.7 Carbon metabolic pathways as novel anti-microbial drug targets

The increasing prevalence of infections caused by antibiotic resistant microorganisms has become a serious health concern and novel therapeutic strategies are needed to combat these infections. The fate of a pathogen rests with its ability to acquire all nutrients necessary for replication within host cells. It has been shown that *Neisseria meningitidis* strains, defective for the transport of lactose, are incapable of colonizing certain tissues (Exley, Goodwin et al. 2005). The opportunistic *B. cenocepacia*

is known to possess many proteases which may be involved in expanding the pool of available carbon sources by releasing aromatic amino acids such as phenylalanine for degradation through the PA pathway. Targeting carbon metabolic pathways for development of new antibiotics may offer a means to disarm pathogens by preventing the exploitation of accessible nutrients. *B. cenocepacia* is capable of metabolizing a considerable number of carbon substrates and decreasing the pool of available carbon could affect the ability of *B. cenocepacia* to successfully colonize certain hosts. However, given that virulence attenuated mutants of the *B. cenocepacia* PA catabolic pathway are still pathogenic to *C. elegans*, pursuing the PA pathway directly as a carbon metabolic target may not be a practical option.

On the other hand, PA has been identified as an inhibitor of NF-κβ activity as well as iNOS and cytokine induction (Park, Lee et al. 2007, Pahan, Sheikh et al. 1997) and it is not known whether this molecule could be used by the bacterium to suppress the host inflammatory response during infection. It does not appear as though the genome of *C. elegans* harbors any homologues of iNOS or NF-κβ; however, several *C. elegans* nuclear hormone receptors display high sequence homology with the eukaryotic receptor PPARγ (peroxisome proliferator-activated receptor γ) (Law, Hamlin et al. 2008), which has been shown to bind PA (Samid, Wells et al. 2000). We have found evidence that mutations affecting production of putative hydroxylated and reduced PA-CoA intermediate compounds result in changes to *B. cenocepacia* pathogenicity. Over production of these metabolites yields an increase in bacterial virulence whereas their blockage leads to an attenuated pathogenic phenotype. It is possible that these PA-CoA hydrolyzed

intermediates may be acting as signal molecules or signal precursors in an alternate virulence pathway rather than initiating a toxic effect directly toward *C. elegans*. The effects of PA or hydrolyzed derivatives of PA-CoA on eukaryotic cells and their involvement in virulence related signaling pathways of *B. cenocepacia* remains to be determined.

6 – Conclusions

Little is known about the connection between pathogenicity and the metabolic capabilities of *B. cenocepacia*. Our interest in exploring this relationship developed from a preliminary observation that linked attenuation of virulence in the *C. elegans* host to disruption of the *B. cenocepacia* PA degradation pathway.

We show that inhibition of PA-CoA ring hydroxylation, due to insertional inactivation of the *paaA* and *paaE* genes, results in reduced virulence of *B. cenocepacia* in the nematode. Interestingly, the reduction in virulence exhibited by STC155-*paaE* is not attributable to decreased intestinal colonization. We demonstrate that interruption of genes involved in the ring opening and β-oxidation stages of PA catabolism give rise to an increased pathogenicity phenotype in *C. elegans*. Using RNAi technology, we have determined that the virulence attenuation phenotype is dependent on a functional *C. elegans* p38 MAPK pathway.

We also show that deletion of *paaK1*, thought to be involved in the activation stage of PA catabolism, results in bacterial strains that are equally as attenuated for virulence as those defective in PA-CoA ring hydroxylation. In addition, the single deletion mutant RJL1, does not present a growth-deficient phenotype in the presence of PA which implies expression of a second functional enzyme responsible for PA-activation.

Currently, it is unclear as to what virulence factors have contributed to the development of the pathogenicity of *B. cenocepacia* and the Bcc across such a diverse

host range. What is undeniable, however, is the urgent need for antibiotics to treat infections caused by these organisms due to their deadly and highly transmissible nature. The first step in understanding how best to treat patients with *B. cenocepacia* infection is to recognize how this organism interacts with its host. The information gathered from the study of the interaction between *C. elegans* and *B. cenocepacia* may well prove beneficial for understanding how *B. cenocepacia* interacts with a host model and may provide new insights into Bcc pathogenesis in humans.

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