

**Regulation of Phenylacetic Acid Degradation in
Burkholderia Cenocepacia K56-2**

by

Jason Hamlin

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

MASTER OF SCIENCE

Department of Microbiology
University of Manitoba
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A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of
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ABSTRACT

Infections by *Burkholderia cenocepacia*, a member of the *B. cepacia* complex (Bcc) are prevalent in patients with Cystic Fibrosis. Bcc members are resistant to antimicrobial therapies, transmissible between patients, and often result in increased mortality. Previously, we have demonstrated the necessity for a functional phenylacetic acid (PA) degradation pathway in *B. cenocepacia* for full virulence when infecting the host model *Caenorhabditis elegans*. Here we demonstrate the regulation of PA degradation genes of *B. cenocepacia* K56-2 with plasmid based fluorescent reporters. To achieve this, promoter-*eGFP* translational reporter plasmids containing the promoter region from the first gene of each of the three PA clusters: *paaA*, *paaZ* and *paaH* were constructed. Measuring *eGFP* expression by spectrofluorometry, we observed fluorescence when *B. cenocepacia* is grown in minimal media containing PA and other compounds expected to proceed through the PA pathway, such as phenylalanine, and no fluorescence when grown in media containing succinate, glycerol, or glucose. Fluorescence is significantly reduced when grown in a mixture of PA and succinate or other carbon sources, suggesting the PA genes are subject to catabolic repression. To determine whether PA genes are upregulated during infection of *C. elegans*, a worm infection reporter assay was designed. An increase in fluorescence of the system is observed compared to *in vitro* controls, suggesting that the *paaABCDE* genes are activated in response to *C. elegans* infection. *BCAL0210* encodes a putative TetR-type protein, located downstream of the *paaABCDE* gene cluster. To show that *BCAL0210* encodes a regulator for the PA genes in *B. cenocepacia*, we developed strain JNRH1, a *BCAL0210* mutant. Fluorescence is observed in strain JNRH1 containing PA reporter plasmids regardless of the carbon source of the medium, demonstrating *BCAL0210* functions to negatively regulate *paaA*, *paaH* and *paaZ* genes. Each promoter contains a semi-conserved 15bp inverted repeat (IR). To show that the semi-conserved IR is an operator of PA regulation, mutations of the *paaA* reporter

were created. Cells harbouring the mutated operator exhibit fluorescence regardless of the carbon source in the media, demonstrating that the sequence is required for control of *paaA*. Altogether, our results demonstrate the PA genes of the opportunistic environmental pathogen *B. cenocepacia* are subject to specific and global regulatory signals and are activated in a host model of infection.

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LIST OF ABBREVIATIONS

°C	-	Degrees Celsius
ABC	-	ATP-binding cassette
BCC	-	<i>Burkholderia cepacia</i> Complex
Bp	-	Base pair
CaCl ₂	-	Calcium chloride
cAMP	-	Cyclic adenosine monophosphate
CCR	-	Carbon catabolite repression
cDNA	-	Copy deoxyribonucleic acid
CF	-	Cystic Fibrosis
CFTR	-	Cystic fibrosis transmembrane regulator
CoA	-	Coenzyme-A
DDT	-	Dichloro-Diphenyl-Trichloroethane
DEPC	-	Diethyl pyrocarbonate
DHFR	-	Dihydrofolate reductase
DIC	-	Differential interference contrast
DNA	-	Deoxyribonucleic acid
EDTA	-	ethylenediaminetetraacetic acid
eGFP	-	Enhanced green fluorescent protein
gfp	-	Green fluorescent protein
Gm	-	Gentamicin
HCN	-	Hydrogen cyanide
HPLC	-	High Performance Liquid Chromatography

IR	-	Inverted repeat
kb	-	kilobase
KEGG	-	Kyoto Encyclopedia of Genes and Genomes
Km	-	Kanamycin
KPO ₄	-	Potassium Phosphate
Lac	-	lactose
LB	-	Luria Burtani
Mb	-	Megabase
MgSO ₄	-	Magnesium Sulfate
MLST	-	Multi Locus Sequence Typing
mM	-	millimolar
Na ₂ PO ₄	-	Bisodium Phosphate
NaCl	-	Sodium chloride
NH ₄ Cl	-	Ammonium chloride
nm	-	nanometre
O.D.	-	Optical density
PA	-	Phenylacetic acid
PBS	-	Phosphate Buffered Saline
PCB	-	Polychloronated biphenyl
PCR	-	Polymerase chain reaction
PIA	-	Pseudomonas isolation agar
PWM	-	Position weight matrix
RNA	-	Ribonucleic acid
RNAP	-	Ribonucleic acid polymerase

rRNA	-	Ribosomal ribonucleic acid
RT	-	Reverse Transcription
SCFM	-	Synthetic cystic fibrosis sputum
TAE	-	Tris acetate EDTA
TE	-	Tris EDTA
TetR	-	Tetracycline repressor
Tp	-	Trimethoprim
UV	-	ultraviolet
μM	-	micromolar

1 – INTRODUCTION

1.0 *Burkholderia cenocepacia* strain K56-2

This work studies gene regulation of the opportunistic pathogenic bacteria *Burkholderia cenocepacia* strain K56-2. *B. cenocepacia* is a member of the *Burkholderia cepacia* Complex (BCC), an important group of Cystic Fibrosis (CF) pathogens.

In 1950, Walter Burkholder described the causative agent for bacterial rot of onion bulbs (Burkholder 1950), *Pseudomonas cepacia* (latin ‘onion’ = ‘*cepacia*’). In the early 1970’s *P. cepacia* is described in the literature as a cause of pneumonia (Weinstein et al. 1973), and that infection was acquired by contaminated anesthetics (Schaffner et al. 1973) and these infections were common in the hospital setting (Speller et al. 1971).

The term ‘*cepacia* syndrome’ was first used to describe a devastating lung infection prevalent in CF patients (Isles et al. 1984). In 1992 *P. cepacia* was renamed *Burkholderia cepacia* based on 16s rRNA sequence analysis, and a 7 species were transferred to the new *Burkholderia* genus. (Yabuuchi et al. 1992). Subsequently, it was learned that *B. cepacia* was actually a phenotypically similar but genetically distinct group of at least 5 species sharing high 16s rRNA sequence similarity, moderate 90-95% *recA* similarity and moderate (30-50%) levels of DNA-DNA hybridization (Vandamme et al. 1997). Currently, the BCC contains 10 formally named species plus seven additional distinct groups as defined by the current standard, multilocus sequence typing (MLST) (Mahenthiralingam et al. 2008).

1.1 Classification

Organism **Lineage:** **Bacteria** (eubacteria); **Proteobacteria;** **Betaproteobacteria;**
Burkholderiaceae; *Burkholderia cenocepacia*

B. cenocepacia strain K56-2 is a clonal isolate of *B. cenocepacia* J2315 which is a strain of the ET12 lineage, and is the index strain for transmission among CF patients in the UK, which was epidemic in the late 1990's (Holden et al. 2008). *B. cenocepacia* K56-2 lacks an insertional element located within a putative glycosyltransferase gene, but is otherwise isogenic to the J2315 index strain (Ortega et al. 2005).

1.2 Genome

All references in sequence analyses refer to the J2315 sequenced by the Sanger institute with the annotated version recently made public (Holden et al. 2008). The multi-replicon 8.056 Mb genome contains three chromosomes, sized 3.870 Mb, 3.217 Mb and 0.876 Mb and a 92 kb megaplasmid. The GC content of the DNA is 66.9%. There are multiple insertion sequences, suggesting genomic plasticity, and multiple genomic islands of lower GC content (Holden et al. 2008), including a virulence island necessary for successful infection and colonization in a rat agar bead model of infection (Baldwin et al. 2004). The majority of the housekeeping genes are located on Chromosome 1 but rRNA genes are also located on Chromosome 2 suggesting that at least both are true chromosomes (Holden et al. 2008).

1.3 Virulence Factors

B. cenocepacia has been found to contain numerous genes necessary for successful infection and colonization in models of infection (Mahenthiralingam et al. 2005). It appears that there is a diverse range of pathogenic phenotypes of *B. cenocepacia* (Cardona et al. 2005), thus leading to complexity in characterizing the mode and process of bacterial pathogenesis.

Virulence factors include intrinsic resistance to multiple antibiotics (McGowan 2006), which is due at least in part to altered lipopolysaccharide (LPS) of the gram negative outer membrane envelope, multiple drug efflux systems (Wigfield et al. 2002), beta-lactamases (Beckman and Lessie 1979) and reduced size of outer membrane porins (Parr et al. 1987) compared to other gram negative genera members, which imparts resistance to larger antibiotics such as gentamicin (Allan and Beveridge 2003).

B. cenocepacia has the ability to form biofilms, forms adhesion pili, and contains multiple quorum sensing systems (Subsin et al. 2007). *B. cenocepacia* survives intracellularly by catalase and superoxide dismutase systems, thus avoiding oxidative killing by host macrophage (and in fact surviving intracellularly), (Valvano et al. 2005). An exopolysaccharide is produced during murine models of infection (Chung et al. 2003). Extracellular proteases, siderophores and type III and type VI secretion systems are also shown to be virulence factors.

B. cenocepacia also releases HCN as a method of killing infected tissue (Ryall et al. 2008).

1.4 Ecology

B. cenocepacia has been isolated from a wide range of diverse habitats. *B. cenocepacia* has been isolated from soil, seawater, plant rhizosphere and the human lung (Mahenthiralingam et al. 2005). The large genome of *B. cenocepacia* is thought to equip the bacteria with the ability to persist in such a wide range of habitats. Indeed, *Burkholderia* isolates have also been found as contaminants in detergents, disinfectants, and the animal extract gelatin (Hutchinson et al. 2004; Mahenthiralingam et al. 2005). Importantly, understanding the ecology in which *B. cenocepacia* is found may provide clues as to how this bacteria functions as an opportunistic pathogen.

The majority of *Burkholderia* species are known as soil bacteria. Given the complex mixture of soil, it is not surprising that *Burkholderia* has evolved a large genome, in order to deal with i) a variety of potential nutrient sources, ii) predation and iii) competition. *Burkholderia* shares a large genome with a related soil dwelling proteobacteria – *Pseudomonas*. This is mentioned because it should be considered that *P. aeruginosa* is also an important CF pathogen, and unfortunately infection with either species is a strong predictor of CF mortality (Courtney et al. 2007).

B. cenocepacia is also known to participate in beneficial plant – bacteria interactions. It is one of the more common BCC isolates in the plant rhizosphere (Lou MiaoMiao et al. 2007). *B. cenocepacia* carries genes for nitrogen fixation (Caballero-Mellado et al. 2007). Other species in the genus are important plant pathogens, primarily of commercial rice crops.

1.5 Biotechnology Applications

Given the capability of *Burkholderia* to promote the growth of certain plant species (Mahenthiralingam et al. 2008), it has been previously applied for rhizosphere fertilization purposes. *Burkholderia* is also a potent anti-fungal bacterium and has been applied to crops to prevent fungal contamination. Finally *Burkholderia* has been studied because of the ability to degrade extremely recalcitrant chlorinated aromatic compounds (Chiarini et al. 2006), such that it may be used for bioremediation of contaminated environments. Unfortunately, due to the fact that members that are pathogenic to humans have been isolated from both the environment and clinical sources, FDA approval for use of *Burkholderia* for these applications was withdrawn in 2003.

1.6 Cystic Fibrosis

Cystic fibrosis (CF) is an autosomal recessive disorder affecting approximately 1 in 2570 live births, with Caucasian populations having the highest incidence of the disease (Davis 2006). CF is caused by a mutated cystic fibrosis transmembrane regulator (*CFTR*) gene which encodes for a cAMP dependent trans-epithelial chloride channel. This gene, located on chromosome 7, was first identified in 1989 (Kerem et al. 1989). The protein normally functions to transport chloride out of the cell, sodium follows by a subsequent mechanism, and water follows by an osmotic gradient. Thus, dysfunction results in increased thick mucus and decreased clearance of mucoid membranes of the lungs, pancreas and other organs.

CF was first separately identified in 1938 as “cystic fibrosis of the pancreas” (Davis 2006). The discovery in 1948 that CF children had about a five fold excess of sodium and

chloride in their sweat led to better diagnosis and treatment of the disease, mainly with digestive enzymes for nutrient deficiency and antibiotics for respiratory care.

Pulmonary failure caused by bacterial infection and intense inflammatory response is the major cause of morbidity and mortality in CF (Berger 2002; Heijerman 2005). Initial bacterial infections, while not more common in CF children than normal children, are more symptomatic. Initial infection is usually by *Staphylococcus aureus* and *Haemophilus influenzae*, these are subsequently replaced with *P. aeruginosa*. Eventually, a persistent bacterial infection resistant to antibiotic therapy is established. These persistent infections are characterized by successive acute pulmonary exacerbations and deterioration (Ferkol et al. 2006). As of 2002 the median age for CF patients was 37 years.

Current treatment programs for CF recommend aggressive and early antibiotic therapy upon *Pseudomonas* infection in the hopes to eradicate early colonizers, in a less dense planktonic form than during a more resistant biofilm stage and this approach has been met with some success (Lebecque et al. 2006).

1.7 “Cepacia Syndrome”

Colonization by *B. cenocepacia* or another BCC member is not sufficient to be called infection with BCC as some patients do not manifest symptoms when colonized. A small subset of those colonized develops “Cepacia Syndrome”, a necrotizing pneumonia and septicemia which results in early death. It is currently thought that a combination of host and bacterial factors result in this outcome (Chiarini et al. 2006).

BCC isolates are highly transmissible and this has led to stringent infection control measures. While most CF isolates are *B. cenocepacia*, all BCC members have been isolated from various “Cepacia Syndrome” patients, therefore it is not species dependent (Mahenthiralingam et al. 2005). BCC isolates are highly resistant to antibiotic treatment and new targets of antimicrobial treatment are required.

1.7.1 CF Lung Environment

The reduced water content of the CF mucus is thought to result in decreased clearance, which traps bacteria. Infection begins with a persistent neutrophilic inflammatory response (Bonfield et al. 1995) and then macrophages plus bacterial products ultimately destroy the airway wall (Berger 2002).

CF sputum is known to contain a high amount of amino acids (Barth and Pitt 1996). Additionally, induction of virulence factors in *Pseudomonas aeruginosa* has been shown to be dependent upon the presence of aromatic amino acids in synthetic media based on cystic fibrosis sputum components (SCFM) (Palmer et al. 2007). This demonstrates a strong link between the host environment and pathogenesis (Brown et al. 2008), a theme that is re-emerging in the analysis of chronic bacterial infection.

1.8 *Caenorhabditis elegans* as a Host Model

Many types of infection models have been used with varying degrees of success for BCC bacteria (Mylonakis and Aballay 2005). Environmental predators have been shown to be good models for bacterial pathogenesis as they are naturally more resistant to infection (Hilbi et al. 2007). BCC bacteria show diverse pathogenic phenotypes in the *C. elegans* model of infection (Cardona et al. 2005), a natural predator of bacteria in the soil. Studies show that some aspects of the innate immune system are conserved between *C. elegans* and vertebrate models (Aballay and Ausubel 2002; Alegado et al. 2003; Ewbank 2003; Gallagher and Manoil 2001), and therefore *C. elegans* serves as an appropriate host model for preliminary pathogenesis studies.

C. elegans natural environment is the soil. The soil is a complicated environment consisting of a colossal array of different types of nutrient sources and predation factors. Soil dwelling gram negative non-spore forming bacteria that have evolved in these conditions have developed numerous mechanisms to thrive in these conditions. Chemicals encountered in the environment may include signals and nutrients from plants or rhizosphere fungi as well as antibiotics or chemical warfare agents from competing bacteria. The decomposition of plant compounds includes numerous aromatic derivatives containing energy that can be mineralized aerobically to provide energy for the growing bacteria.

1.9 Phenylacetic Acid (PA) – Properties

Phenylacetic acid (PA) is a white crystalline compound at room temperature synonymous with α -Tolylic acid, Benzeneacetic acid, PAA and PA. The molecular formula of PA is $C_6H_5CH_2CO_2H$. PA has a boiling point of 265°C a melting point of 76-78°C and a pKA of 4.76 (acetic acid). PA is moderately soluble in water.

PA is a common downstream intermediate during the biological degradation of many aromatic compounds. Therefore, PA degradation should be viewed in the context that it is a part of a hierarchical degradation scheme, and not as an isolated compound. Upstream compounds include aromatic compounds with aliphatic R-groups containing N+1 carbon atoms. (aromatic R-group containing N carbon atoms proceed to benzoic acid degradative pathways). Assumedly, these R groups are degraded in a fatty acid beta-oxidation type manner until PA is reached and then the compound enters the PA pathway.

Early on, PA was recognized as a byproduct of penicillin G degradation (Moyer and Coghill 1947). The CF lung is rich in amino acids, including aromatic amino acids. Because aromatic amino acids such as phenylalanine also proceed via the PA pathway in other bacteria (Navarro-Llorens et al. 2005), this is an important biological source of PA. Man made aromatic compounds may also proceed through the PA pathway. For example it has been demonstrated that PA is a product of DDT degradation in *Pseudomonas* (Skriabin et al. 1977).

1.10 Bacterial Degradation of Phenylacetic Acid

Much research has been done on the bacterial mineralization of PA. Bacterial species including representatives from phyla diverse as Proteobacteria, Firmicutes, Actinobacteria and Bacteroidetes were isolated from various soils and all were capable of mineralizing PA suggesting that the ability to metabolize PA is widely distributed among bacteria (Abe-Yoshizumi et al. 2004; Erb et al. 2008).

PA degradation was reported for *Vibro* spp. (Dagley et al. 1952) and describes the accumulation of in order of decreasing concentration: malic, fumaric and succinic acids in the growth media, suggesting complete degradation to tricarboxylic acid (TCA) intermediates. Transport of PA into the cell is not well conserved among studied bacteria. PA uptake is accomplished by a permease encoded by *phaJ* and a porin encoded by *phaK* in *Pseudomonas putida* U (Luengo et al. 2001).

In *E. coli* the PA catabolic gene cluster is organized as a single operon encoding complexes involved in four enzymatic steps. The PA activating enzyme, phenylacetyl-coenzyme A (PA-CoA) ligase, (PaaK) (Ferrandez et al. 1998) creates a thiolester bond between coenzyme A and the carboxyl group of PA. For the next step the PA-CoA ring-hydroxylation system is comprised of PaaA, PaaB PaaC, PaaD, and PaaE (Ferrandez et al. 1998; Ismail et al. 2003) and the resultant product being 2-hydroxy-PA-CoA (2-OH-PA-CoA).

The third step, the opening of the aromatic ring, may be performed by PaaZ (Ferrandez et al. 1998) or by PaaZ, PaaG and PaaJ (Ismail et al. 2003). This results in a ketoadipyl-CoA

compound which proceeds through a β -oxidation like pathway complex, by PaaF, PaaH (Ismail et al. 2003) and PaaJ (Nogales et al. 2007).

Recently, the gene *paaI* has been found to encode a putative thioesterase (Song et al. 2006). It is speculated that the purpose of PaaI is to release coenzyme A from 2-hydroxy-PA-coA after the hydroxylation step although the reason for this is not clear.

1.11 Bacterial Gene Regulation Overview

Soil dwelling bacteria are exposed to a plethora of environments and have evolved highly efficient mechanisms for controlling gene expression on the transcriptional, post-transcriptional and translational level. For the obvious reasons of economy, the first step, transcriptional regulation is the primary method by which bacteria regulate gene expression (Browning and Busby 2004). This is accomplished by controlling if and when gene transcription may begin at promoters.

Prokaryotic promoters are simpler than their eukaryotic counterparts, but this should not belie their ability to stringently and efficiently control gene transcription. The primary elements of a promoter are described in relation to their location from the first bases of DNA that is transcribed into RNA. For most promoters these are called the -10 and -35 elements and form the 'core promoter'. These elements are the DNA contact points for proteins called sigma factors which, when combined with RNA polymerase (RNAP) is termed the RNAP holoenzyme. The RNAP holoenzyme recognizes promoters to begin the transcription process.

Bacteria have evolved numerous methods to control gene expression at the transcriptional level. Usually this involves proteins called transcription factors which bind the promoter region to assist or hinder the beginning of transcription. The simplest form of regulation of a catabolic pathway is steric hinderance of RNAP open complex formation by a repressor binding the core promoter DNA (Beckett 2001).

Additional levels of regulation are superimposed upon specific positive or negative regulation in the promoter region. These signals may alter the availability of the sigma factors and RNAP, or bind and alter the DNA structure making it more or less favourable for transcription to proceed. Such factors control the expression of many genes depending upon factors such as extracellular stimuli, or the general energy level of the cell.

1.12 Reporter Systems for Studying Bacterial Gene Regulation

In order to characterize the regulation of a bacterial gene of interest, a reporter system is commonly used. A reporter system consists of a piece of DNA from the gene of interest that is linked to a “reporter” gene. The piece of DNA is usually copied from an upstream region of the gene of interest. In prokaryotes, this segment is small, usually a few hundred base pairs – 1 kb. The reporter gene encodes for an easily assayable product, such as an enzyme that produces readily identifiable colour change upon addition of a specific substrate such as the lac system, or encodes for a fluorophore that can be detected with fluorometry.

In 2008 the Nobel prize was awarded to three scientists, Martin Chalfie, Osamu Shimomura and Roger Y. Tsien who characterized and exploited the use of green fluorescent protein. The green fluorescent protein (gfp) was originally isolated from the jellyfish *Aequorea*

(Prendergast and Mann 1978). The significance of this finding was not understood until it was demonstrated that the protein was a powerful tool for reporting gene expression (Chalfie et al. 1994), due to its unique properties. Remarkably, gfp does not require any additional cofactors or substrates to function, enabling it to be expressed in heterologous systems. This has made gfp one of the most useful reporter proteins for gene expression studies.

1.13 The Position Weight Matrix (PWM)

The small size and variations in consensus sequences for transcription factor binding sites makes predicting their location difficult. Frequently, aligning known transcription factor binding sites and formulating a consensus sequence based on the most common residue in each position is insufficient to find binding sites which may be physiologically relevant but vary from the consensus sequence (Wasserman and Sandelin 2004). For example, many prokaryotic genes have the sequence GTG instead of ATG encoding the first amino acid, methionine, at the start of a gene. To account for these variations, a Position Weight Matrix (PWM) may be used instead of a consensus sequence.

A PWM is calculated as a log of a fraction. The numerator contains the number of instances a nucleotide appears in the set and the denominator contains the total number of sequences. This fraction is adjusted for %GC content and small numbers of sequences. The GC content is important for high GC content species like *B. cenocepacia* (66.9%), because that makes guanine or cytosine residues less informative (as they appear more often) than adenine or thymine residues. When only small numbers of sequences are used to generate a set, a pseudocount function (Fig. 5 c) is used to lower the overall informative score of positions.

1.14 Regulation of Aromatic Compound Degradation

Many metabolic pathways for naturally occurring aromatic compounds are widespread among microbial species. These include pathways for aromatic amino acids, hydroxylated benzoates, phenylpropionic acids, aromatic amines and ubiquinones (Diaz et al. 2001). Many of these compounds are likely the product of the breakdown of more complex aromatics found in the natural environment.

A significant amount of study has also been focused on the degradation of substituted aromatics such as chlorobenzoates, PCBs and other man made recalcitrant compounds with the goal of engineering or utilizing microbes for bioremediation (Diaz and Prieto 2000). In *P. putida* U, 2-phenylethylamine, phenylacetaldehyde, styrene and other phenylakanoic acids with an even number of carbon atoms activate the PA pathway (Olivera et al. 1998).

1.14.1 TetR-type Regulators of Pathways for Aromatic compounds

TetR-type regulators regulate their cognate gene products typically by binding to a consensus inverted repeat within the promoter of the genes they are regulating, and usually block RNAP holoenzyme from initiating transcription and are thus negative regulators (Ramos et al. 2005). In all instances where a regulatory protein has been identified as a TetR-type, the effector molecule of the protein is normally a metabolite or target of the genes under regulation. Notably, TetR-type regulators bind consensus sequences that are inverted repeats which contain little or no space between each half of the sequence (Ramos et al. 2005).

While many TetR-type regulators have been described for systems similar to their namesake, Tetracycline efflux pump repressor (Saenger et al. 2000), few have been implicated as regulators of catabolic degradation of aromatics. CymR is a repressor for the expression of p-cymene and p-cumate in *P. putida* F1 (Eaton 1997). Interestingly, a TetR-type negative regulator for an aromatic pathway in a high %GC soil dwelling gram positive bacteria, *Corynebacterium glutamicum* has also been described (Brinkrolf et al. 2006). Recently a TetR-type regulator has been found next to a set of genes for PA degradation in *Arthrobacter oxydans* CECT386 (Navarro-Llorens et al. 2008), although a genetic analysis has not been performed.

AcrR is a TetR-type protein that regulates a multi-drug efflux pump of *E. coli* (Su et al. 2007). AcrR has also been shown to be upregulated during elevated stress levels (Ma et al. 1996). Thus far, no AcrR-type proteins have been implicated in phenylacetate regulation.

1.14.2 Bacterial Regulation of Phenylacetic Acid Degradation

Regulation of PA degradation has been described in detail in *E. coli* (Ferrandez et al. 2000) and *P. putida* U (del Peso-Santos et al. 2006; del Peso-Santos et al. 2008). In these systems, the PA genes are negatively regulated by the transcriptional regulator PaaX. Transcriptional repression is relieved in the presence of the effector molecule phenylacetyl-Coenzyme A (PA-CoA), a product of the first step of PA degradation mediated by PaaK (Ferrandez et al. 2000). In both of these organisms, PaaX has been classified as a GntR-type regulators, the type regulator originally characterized as a repressor for gluconate metabolism (Miwa and Fujita 1988).

In 2007, a PA responsive promoter – gfp construct was developed, termed a ‘whole cell bioreporter’ (Kim et al. 2007) in *P. putida* KT2440 for the purpose of detecting PA produced by other microorganisms. The authors show a quantitative increase in reporter activity in response to growth in different levels of PA, with induction between 25µm and 50µm of PA. Interestingly the *paaA* reporter was repressed when added to exponential phase cells growing at the expense of glucose, but not succinate or pyruvate.

PaaX has also been shown to bind the styrene operon in *P. putida* (del Peso-Santos et al. 2008), and interestingly PaaX has been shown to negatively regulate penicillin G acylase expression in *E. coli* (Kim et al. 2004). In that case, PA is a byproduct of the acylase cleavage and proceeds assumedly down the PA degradation pathway.

Thus the PaaX regulator controls several upstream pathways that proceed to the PA metabolic pathway. Overall, PA degradation in these organisms has been termed the PA ‘catabolon’ (Luengo et al. 2001), a functional unit which integrates upstream peripheral metabolic pathways.

1.15 Carbon Catabolite Repression

The selective generation of enzymes to degrade preferential carbon sources when faced with multiple potential sources of carbon is termed carbon catabolic repression (CCR) and has been extensively studied in many bacteria (Aranda-Olmedo et al. 2005; Bruckner and Titgemeyer 2002; Deutscher 2008; Gorke and Stulke 2008; Martinez-Blanco et al. 1990). Given that bacteria are faced with a diverse range of potentially energy yielding compounds in the soil, we would expect to find CCR present in this environment.

The PA-CoA ligase (PaaK) has been reported to be under catabolite control in *P. putida* U (Martinez-Blanco et al. 1990). It was later shown that protein Crc is a broad catabolic repressor for aromatic substrates in *Pseudomonas* (Morales et al. 2004). Crc functions post-transcriptionally, by binding mRNA transcript and preventing translation (Moreno et al. 2007). A conserved RNA target structure has not been identified for this process.

CCR has been reported for *Burkholderia* for the degradation of alkanes in the presence of organic acids (Marin et al. 2001) and CCR has also been shown for the protocatechuate dioxygenase enzyme (Zylstra et al. 1989). Mechanisms of CCR in *Burkholderia* have not been established.

2 – RATIONALE AND HYPOTHESES

2.0 Rationale

Our lab has recently reported that a functional PA catabolic pathway is required for full pathogenesis in a *C. elegans* model of infection (Law et al. 2008). Originally, a transposon mutant was isolated that was defective in a rat lung model of infection and in a *C. elegans* model (Cardona et al. 2005). The mutant was defective for growth on PA as the sole carbon source. This prompted us to characterize the PA pathogenic phenotype and demonstrate that the PaaABCDE ring hydroxylation complex is necessary for full virulence, for reasons that remain unknown.

2.1 Hypotheses

Based on the available information we hypothesized:

- The PA gene promoters controlling the PA degradation genes of *B. cenocepacia* K56-2 are induced in the presence of PA.
- A proximal TetR-type gene is necessary for regulation of the PA genes of *B. cenocepacia* K56-2
- A conserved DNA sequence located in the core promoter of *paaA*, *paaZ* and *paaH* is a *cis* element required for regulation.
- The PA ring hydroxylation complex gene operon *paaABCDE* is induced during infection of *C. elegans*.

3 - MATERIALS AND METHODS

3.0 Bacterial Strains, Nematode Strains and Growth Conditions

Bacterial strains and plasmids are listed in Table 1. *B. cenocepacia* K56-2 was grown at 37 °C in Luria Bertani (LB) or M9 minimal media (Na₂PO₄ 33.9g/l KPO₄15.0g/L NaCl 2.5g and NH₄Cl 5.0g, 2 mL 1.0 M MgSO₄, 0.1 mL CaCl₂) plus indicated carbon source, supplemented as required, with 100 µg/ml trimethoprim (Tp), 50 µg/ml gentamicin (Gm) and 200 µg/ml chloramphenicol (Cm). *E. coli* strains were grown at 37 °C in LB medium supplemented with 50 µg/ml Tp, 40 µg/ml kanamycin (Km) or 20 µg/ml Cm. The nematode *C. elegans*, strain DH26 and *E. coli* OP50 were obtained from the Caenorhabditis Genetics Center (CGC), University of Minnesota, Minneapolis. *C. elegans* strains were maintained on nematode growth media according to standard practices at the CGC.

3.1 Reporter Activity Assays

96-well microplates containing 150 µl of M9 minimal media supplemented with indicated carbon source(s) were inoculated with 2 µl from an overnight culture grown in LB, washed with PBS and adjusted to an O.D. 600 of 2.0 with M9 minimal salts. Corning #3904 microplates were incubated at 37 °C with shaking at 200 rpm. eGFP protein has excitation/emission wavelengths of 488/509 (Cormack et al. 1996). Fluorescence and O.D. 600 was measured with a Biotek Synergy 2 plate reader during late log phase or early stationary phase (18-24h), using excitation 485/20 and emission 528/20 filter sets.

3.2 *Caenorhabditis elegans* Infection Reporter Assay

The nematode *C. elegans*, strain DH26, which contains a temperature sensitive mutation and does not replicate at 25C was used as the host for the in vivo induction assay. Hypochlorite-synchronized worms were raised on NGM with *E. coli* as a food source for two days then transferred to plates containing M9 minimal media + 25 mM glycerol (infection reporter assay plates) with a lawn of *B. cenocepacia* wild type or containing promoter-eGFP reporter plasmid, and infection allowed to proceed for 48 hours. To prepare the in vivo induction assay plates, *B. cenocepacia* strains were grown overnight in LB, washed in PBS then 50µl of O.D. 600 1.7 cells were used to inoculate 90mm plates containing M9 minimal media + 25 mM glycerol, grown at 25C for 2 days then used immediately. For a negative control, additional *B. cenocepacia* plates without worms were likewise incubated. Specimens were collected by rinsing with sterile PBS and 25 µl was plated on 1.5% agarose coated microscope slide and covered with a #1 glass coverslip. Slides were examined with a Zeiss Axiovision Z1 microscope with filters 450-490 for excitation and 500-550 for emission. An apotome (Zeiss) was used to obtain fluorescent optical sections. Fluorescent images were exposed for various lengths (ms) in all conditions and averaged. Differential interference contrast (DIC) microscopy was used adjust focus along various lengths of the worm to where the gut width appears greatest, and then fluorescent and DIC images were obtained. Fluorescence intensity was quantified using ImageJ (NIH) software. To achieve this, a 150 x 150 square pixel selection (22500 pixels) were analyzed for overall average pixel intensity. Results are the average of 5 separate samples.

3.3 Bioinformatics Analysis

BLAST searches of the genome sequence of *B. cenocepacia* strain J2315 were performed with the *B. cenocepacia* Blast Server at Sanger Institute (http://www.sanger.ac.uk/cgi-bin/blast/submitblast/b_cenocepacia). J2315 belongs to the same clonal lineage as strain K56-2 (Darling et al. 1998). Gene clusters were visualized with Artemis software (Rutherford et al. 2000) and VectorNTI software (Invitrogen). PWM scores were calculated manually (Wasserman and Sandelin 2004) (Fig. 5) as described by Hertz and Stormo (Hertz and Stormo 1999) and Schnieder and Stephens (Schneider and Stephens 1990). This PWM was then re-entered into the Target Explorer (Sosinsky et al. 2003) and used via the PATSER program to scan each of the *B. cenocepacia* chromosomes and the plasmid. Identification of binding sites was also achieved using the Target Explorer. For TCOFFEE analysis the default substitution matrix was used, with a gap opening penalty of -10 and a gap extension penalty of -1.

3.4 Molecular Biology Techniques

Recombinant DNA techniques were carried out as previously described (Sambrook and Russell 2001). DNA ligase (New England Biolabs) was used as recommended by the manufacturers. *E. coli* DH5 α cells were transformed using the calcium chloride protocol (Cohen et al. 1972) and electroporation was used for transformation of *E. coli* SY327 cells (Miller and Mekalanos 1988). Reporter plasmids were constructed in *E. coli* and conjugation into *B. cenocepacia* K56-2 was accomplished by triparental mating (Craig et al. 1989) with *E. coli* DH5 α carrying the helper plasmid pRK2013 (Figurski and Helinski 1979). DNA was amplified using a PTC-221 DNA engine (MJ Research) or an Eppendorf Mastercycler ep gradient S

thermal cycler with either Taq DNA polymerase, Phusion High-Fidelity PCR Kit or Proofstart DNA polymerase (Qiagen) (New England Biolabs). Amplification conditions were optimized for each primer pair. PCR products and plasmids were purified with QIAquick purification kit (Qiagen) and QIAprep Miniprep kit (Qiagen), respectively.

3.5 RNA Isolation Methods and RT-PCR Analysis

For RNA isolation, bacteria were grown in LB supplemented with 1mM PA. Cells were harvested and lysed in TE buffer pH 8.0 containing 400 µl/ml lysozyme for 5 minutes at room temperature. RNA was recovered with the RNeasy Mini kit (Qiagen), and samples eluted into (Diethyl Pyrocarbonate) DEPC treated water. Total RNA was visualized by running in a 1% agarose gel in TAE buffer. Residual DNA was removed by on column treatment with DNase I (15 min, room temperature), in DNase buffer (Qiagen). The RNA was then used as a template in reverse transcriptase (RT) or stored at -20°C until use. Reverse transcription was performed by SuperScript RT First-Strand synthesis using relevant gene specific primers (Table 2). The resultant cDNA was PCR amplified using gene specific primers (Table 2), and the conditions optimized for each reaction. For every PCR, the appropriate controls with water and RNA in the absence of RT were included to ensure that the amplicons obtained were a result of cDNA and not of contaminating genomic DNA.

3.6 Construction of Insertional Mutant *BCAL0210* of *B. cenocepacia* K56-2 (JNRH1)

BCAL0210 was disrupted using single crossover mutagenesis with plasmid pGPΩTp, a derivative of pGP704 that carries the *DHFR* gene flanked by terminator sequences (Flannagan et

al. 2007). Briefly, an internal 300-bp fragment of *BCAL0210* was PCR amplified using appropriate primers (Table 2). The PCR-amplified was digested with *Xba*I and *Eco*RI respectively, cloned into the *Xba*I and *Eco*RI digested vector and maintained in *E. coli* SY327. The resulting plasmids (Table 1) were conjugated into *B. cenocepacia* strain K56-2 by triparental mating. Conjugants that had the plasmid integrated into the K56-2 genome were selected on LB agar plates supplemented with Tp 100 µg/ml and Gm 50 µg/ml. Integration of the suicide plasmids was confirmed by colony PCR, using primer SC025 that anneals to the R6K origin of replication of pGPΩTp, and primers upstream of the expected site of insertion (Table 2). All mutant strains were confirmed by sequencing PCR-amplified DNA fragments containing the insertion site.

3.7 Construction of eGFP Reporter Vectors

To create pJH1, digestion with *Xba*I/*Nde*I of pSCrhaB4 resulted in a 784bp fragment containing *eGFP* which was cloned into the same sites in pAP20 (Law et al. 2008b) such that *eGFP* is under control of the constitutive *DHFR* promoter. *E. coli* transformants were selected with 20µg/ml chloramphenicol. The plasmid was conjugated into *B. cenocepacia* K56-2 by triparental mating with *E. coli* helper strain containing plasmid pRK2013. As *B.cenocepacia* is intrinsically resistant to gentamicin, in all conjugations gentamicin was added to the final transfer to eliminate donor *E. coli*. Fluorescent analysis shows constitutive expression in *E. coli* and *B. cenocepacia* with more effective expression in the latter where colony fluorescence is visible to the naked eye under UV light. This plasmid was then PCR-amplified using divergently oriented primers (Table 2) containing restriction sites on the 5' ends such that the self ligated product of the reaction has a multiple cloning site in place of the original promoter to create pJH2.

3.8 Creation of Translational Reporter Plasmids

Various fragments of *B. cenocepacia* K56-2 chromosomal DNA were amplified and cloned into pJH2. A 510bp fragment of *paaZ* from -420 to +90 was cloned into pJH2 to create plasmid pJH6. A 480bp fragment of *paaA* from -396 to +84 was cloned into pJH2 to create pJH7. A 399bp fragment of *paaH* from -327 to +72 was cloned into pJH2 to create pJH8 (Fig. 3).

3.9 Construction of Site Directed Plasmid Mutants

The plasmids pJH10, pJH11 and pJH12 were constructed by plasmid PCR mutagenesis to contain mutations in the entire, left or right region of the conserved IR in the *paaA* core promoter. Appropriate phosphorelated primers (Table 2) were used to divergently amplify template pJH7 (containing the *paaA* promoter), and each contained mismatch mutations on their 5' ends. Plasmids were self-ligated, transformed into *E. coli* DH5 α and then conjugated into *B. cenocepacia* wild type. All mutations were verified by sequence analysis (The Centre for Applied Genomics, Toronto)

Table 1. Bacterial strains and plasmids

Strain or plasmid	Features	Reference or source
<i>B. cenocepacia</i> strains		
K56-2 (LMG18863)	ET12 clone related to J2315, CF clinical isolate	(Mahenthiralingam et al. 2000)
JNRH1	K56-2 <i>BCAL0210::pJH9</i> , <i>Tp^r</i>	This study
<i>E. coli</i> strains		
DH5 α	F ⁻ , ϕ 80 <i>lacZ</i> Δ M15 <i>endA1 recA1 hsdR17</i> (r _K ⁻ m _K ⁺) <i>supE44 thi-1 ΔgyrA96 (ΔlacZYA-argF)U169 relA1</i>	Invitrogen
SY327	<i>araD Δ(lac pro) argE (Am) recA56 Rif^r nalA λ pir</i>	(Miller and Mekalanos 1988)
Plasmids		
pGP Ω Tp	<i>ori_{r6K}</i> , Ω Tp ^r <i>mob+</i>	(Flannagan et al. 2007)
pRK2013	<i>ori_{colE1}</i> , RK2 derivative, Km ^r <i>mob⁺ tra⁺</i>	(Figurski and Helinski 1979)
pJH9	pGP Ω Tp, internal fragment from <i>BCAL0210</i>	This study
pJH1	<i>pap20, eGFP</i>	(Law et al. 2008b)
pJH2	pJH1, <i>eGFP</i> replaced promoter with multiple cloning site	This study
pJH5	pJH2, <i>BCAL0211</i> promoter region (<i>P_{BCAL0211}</i>)	This study
pJH6	pJH2, <i>paaZ</i> promoter region (<i>P_{paaZ}</i>)	This study
pJH7	pJH2, <i>paaA</i> promoter region (<i>P_{paaA}</i>)	This study
pJH8	pJH2 <i>paaH</i> promoter region (<i>P_{paaH}</i>)	This study
pJH10	pJH7, ACCGACCGGTCGGT → TAGATGT-ATCTCAG	This study
pJH11	pJH7, ACCGACCGGTCGGT → TAGATGT-GGTCGGT	This study
pJH12	pJH7, ACCGACCGGTCGGT → ACCGACC-ATCTCAG	This study

Cm, chloramphenicol; Km, kanamycin; Tp, trimethoprim. Arrow represents mutation in sequence indicated.

TABLE 2. Primers used in this study

Name	Sequence	Purpose
SC1	AACACCCGGGATGGTGAGCAAGGGCGAG	forward primer for amplifying <i>eGFP</i>
SC 2	CCGCTCTAGATTACTTGTACAGCTCGTCCA	reverse primer for amplifying <i>eGFP</i>
SC 3	GGCGTAGAGGATCTGCTCATGTTTG	forward external primer to check insertion in pAP20 / pJH2
SC 4	GCTACTGCCGCCAGGCAAATTCTGT	reverse external primer to check insertion in pAP20 / pJH2
SC 11	GCGCAGATCTCCCGGGCGTACTCATATGCCATGGCTCG AGTAAGCTGTCAAACA	pJH2 forward
SC 12	GCGCAGATCTGGTACCCGTACTGGATCCGAATTCGTG AGCAAGGGCGAGGAGCT	pJH2 reverse
SC 23	CCGTAGGTGGCATCGCCCTC	external promoter sequencing primer
SC 30	GCGTCAGATCTTCGAGATCGTGCTCGACGGCAAG	P _{BCAL0211} forward
SC 31	GCGGTGAATTCGACGACGCGCTCGTCGCGTC	P _{BCAL0211} reverse
SC 38	AGTTAGAATTCGGCGAACGGGCTCCAGTACCCG	P _{paaZ} forward
SC 39	AGTCTGAAGATCTCGGCTTGCGGATCTCGCGGAC	P _{paaZ} reverse
SC 43	AGTCTGAAGATCTAGACGATCTGACGCACGGTTCG	P _{paaA} forward
SC 44	AGTTAGAATTCATCGAACC GCGTCTGCTCGGG	P _{paaA} reverse
SC 45	AGTCTGAAGATCTAGCGCACGTGACGACGCGTG	P _{paaH} forward
SC 46	AGTTAGAATTCGCCGATCACGCCGACGACGG	P _{paaH} reverse
SC 51	ATTATTCTAGACGAGCACGTGCGATGTCCGATCTC	BCAL0210 internal fragment forward
SC 52	ATTATGAATTCCGAGCACGATTTACGCTGCGC	BCAL0210 internal fragment reverse
SC 53	TAACGGTTGTGGACAACAAGCCAGGG	Colony screening vector primer
SC 66	ACATCTATGGTTTATACTTCGCACACACTCAACCG	pGPomegaTP
SC 67	ATCTCAGTAATAAATTTGTTGTGGATTTGCGGCGGG	Forward divergent mutagenic primer (HPLC pure 5' P)
SC 68	CGGCTGAAGCACTGCACGCC	Reverse divergent mutagenic primer (HPLC pure 5' P)
SC 72	GGTCGGTTGGTTTATACTTCGCACAC	sequencing primer for pJH series plasmids
SC 73	GGTCGGTTAATAAATTTGTTGTGG	Forward divergent NON mutagenic primer match with p67
SC 108	ACCCCGACCTGAAATGGAACGAAG	Reverse divergent NON mutagenic primer match with p66
SC 109	GTCGGATGACGGTAGATCTTGTCG	RT PCR <i>paaA</i> -> <i>paaB</i>
SC 110	CCTCGACCACAAGCATTGCGGC	RT PCR <i>paaB</i> -> <i>paaA</i>
SC 111	GTGCGGCAGTTCGGCGAGCG	RT PCR <i>paaB</i> -> <i>paaC</i>
SC 112	GACTACCTGATGCCGTACACACG	RT PCR <i>paaC</i> -> <i>paaB</i>
SC 113	TCTCGATCCGGTGCGGCGGC	RT PCR <i>paaD</i> -> <i>paaC</i>
SC 114	GGACGACCGACTGGATTACGCAG	RT PCR <i>paaD</i> -> <i>paaE</i>
SC 115	ACGCGAAGTTCGAGAAGCGGCC	RT PCR <i>paaE</i> -> <i>paaD</i>
SC 123	CGTGCAAGGGCGGCGTGTGC	RT PCR <i>paaE</i> -> BCAL0211
SC 124	GCGTTCCAGTCGGGAATCTGGTC	RT PCR BCAL0211 -> <i>paaE</i>
SC 125	GGGTCAGCAACACGATCAAGAGC	RT PCR BCAL0211-> BCAL0210
SC 126	CACCCCTCGACCTCGGCGATG	RT PCR BCAL0210-> BCAL0211

4 - RESULTS

4.0 Detection of a Conserved DNA Sequence Located in the Promoters of the Phenylacetic Acid Degradation Genes of *B. cenocepacia*

The PA degradation genes are arranged in three separate clusters in *B. cenocepacia*, namely *paaABCDE*, *paaFZJGIJK1* and *paaHK2* (Fig. 1) (Law et al. 2008b). Our first step in determining regulatory aspects of these genes was to align the upstream sequence of *paaA*, *paaZ* and *paaH*, corresponding respectively to the first gene in each of the clusters. This allowed us to determine if there were any sequences similar between the genes that may signify some function in similar between them. The *paaF* gene is divergently transcribed from the *paaZJGIJK1* cluster and the upstream region is mostly the reverse complement of the *paaZ* gene.

A 500bp region from the +90 to -410 from the predicted transcription start site of the genes *paaA*, *paaZ*, *paaH*, was aligned using TCOFFEE (Notredame et al. 2000). Each promoter contains a near perfect 15bp inverted repeat (IR) sequence – ACCRACC-GGTCGGTT (Fig. 2), (R = A or G) located between the putative -10 and -35 core promoter signals (Fig. 3 b). The location and symmetry of the sequence strongly suggest that it may be a transcription factor binding site, and possibly a *cis* operator of PA regulation.

The IR observed is highly similar to the DNA operator sequence of QacR, a TetR-type regulatory protein (Ramos et al. 2005). QacR is a multidrug efflux pump first described in *Staphylococcus aureus* that is responsible for the efflux of Quaternary Ammonium Compounds

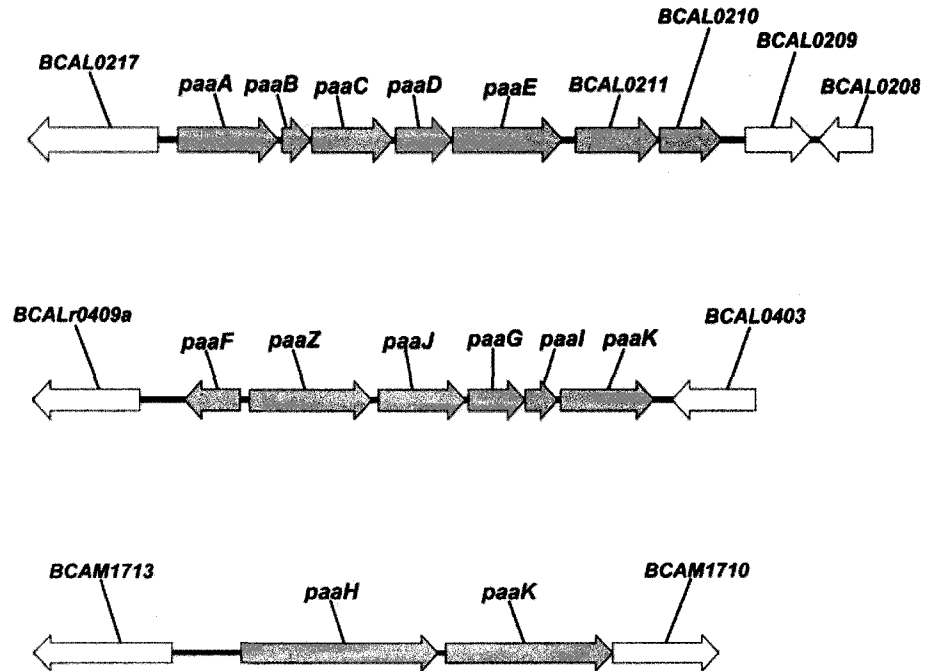
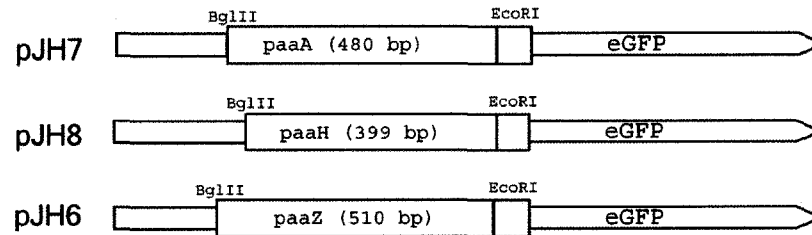


Figure 1. Proposed PA catabolic pathway of *B. cenocepacia* strain J2315. Genetic organization of the PA catabolic gene clusters in *B. cenocepacia* strain J2315.

A



B

AGATCTAGACGATCTGACGCACGGTTCGCCC6GCCACGTCTG6CGCGCGGTAGAGCGGGCGGGCGGTGCGG
 CGAGGTCGGGAATCGGCTGCAGCGCGGTTCGCCACGCAGCGACCCACCG6CCGGCGCGGGCCGACGCTG
 CAAGCGGGCGACACGAGAACGGCCGACCCAGCACAGCCGCGGCGAAACTGCTTCGAAATGACATCGGCA
 AAATCCTCATCGGAAAGCGGGCATTGTGCCGAGAAATGCGGGCCCCACGTGCGGAGCCGCACTTGCT
 CCGCTTGATCGTGCCCCGATGTCCTCGTTTACCCGAGTCCCGCCGCAAATCCACAACAAATTTATTA⁻³⁵
 CCGACCGGTCGGTTGGTTTATACTTCGCACACACTCAACCGGACGAGAGCCTTCGCCATGTACACGC⁻¹⁰
 TCCCTCGACATCCCCGGCAACGTCGCGCCGCTCGACGCCGCAACCGAGTCGCCCAGCAGACGCGGTTT
 GATGAATTC

AGATCTAGCGCACGTGACGACGCGTGGCCGGCGGCTCGATCCGACCCCGACAGGGCGGGCGACGATGGA
 CGGCGGTGACGGCGCGACACGCTTTCCCCCTGAAATCCGGCTATTTGTGCAATTTATCAGGCGTTCGG
 GGGTCACTATCTCACATTCGAACGCTTACGTTGACCTTTTCGGTTGATAAAAAGTTGCCCGATGTTTCGA
 TTTTTTGTGCTGCGTTGAGTTTCCGTAACGATGTCTTGCCCGACAAATTCGGGCTCGCCCTGACATAA⁻³⁵
 TACCGACCGGTCGGTTTATAATCGCTCGCACTTCCACTTCATGACGAGATCGCAAGCGATGGCTGTTTCC
 TCTGCACATTCGGTGAACTCGGGCGCACTCGTGCCGTGCGCCGTCGTCGGCGTGATCGGCGAATTC

AGATCTCGGCTTGCGGATCTCGCGGACCGTCTCCCAAGTTGCGCGTGATGTAGTCGCCCCGATAGACATC
 CATATAGGAGTAGGTCCGCATCATGCCGATGTCCGCACCGGCCGGAACGCCCTTTTCGCTCCCGGTCAC
 GACGATCGCGCCGATGTGCTCGTCGATCGAACGCCCTTACGCGCGGCGCCAGCTCATCCATCAGCGC
 ATCGTTTCAAGCGCATTCAGCGCCTTCGGACGGTTCAAGCGTCACCAAGACGACACGCCCCGGGTCTCCAC
 CAGGATGTTCTCGTAAGCCATCTATTTCTCTCGATCAATGAAATGAGAAACGCCCTCGCACATGCGAAT
 AGTTTGATGCTACCAATTCGCGACCAACCGGTCGGTTAATAATTCGATCCAATCCCTCTCAACGTTTCAT
 CAGGCGCGCCGATGACCCATGCCCTGTTACGAAGCACGAAGACACGCTGAAACACGCACTCGCCGCC
 ATCGAGAGCCGCGGGTACTGGAGCCCGTTCCGCCAATTC

Figure 3. A) Schematic diagram of P_{paaA} reporter plasmid (pJH7), P_{paaH} reporter plasmid (pJH8) and P_{paaZ} reporter plasmid (pJH6). B) Sequence of the inserted regions for P_{paaA} top, P_{paaH} middle and P_{paaZ} bottom. Predicted start codon is highlighted in bold. Putative ribosome binding site is boxed, predicted -10 and -35 regions are highlighted in grey and denoted above. The detected conserved inverted repeat is marked with arrows. Restriction sites are italicized.

that are structurally diverse (Grkovic et al. 1998). QacR binds its operator as a pair of dimers (four proteins in total) (Grkovic et al. 2001), and this is reflected in the dual overlapping repeat within the QacR operator (Ramos et al. 2005). The operator of QacR was aligned with the conserved IR from *paaA*, *paaZ*, and *paaH* promoters to highlight these findings (Fig. 4).

Interestingly, a 10 bp inverted repeat (GCCGG / CCGGC) was identified within the beginning of the coding sequence of the *BCAL0211* gene that is starkly similar to the above IR found in the core promoter of the *paaA*, *paaH*, and *paaZ* genes. Because TetR-type proteins are known to regulate their own transcription by self repression (Saenger et al. 2000), we aligned the sequence found in the *BCAL0211* gene with the *paaA*, and *paaZ* for comparison (TCOFFEE) (Fig 2 b).

4.1 Position Weight Matrix Construction of the Conserved IR and Search of the *B. cenocepacia* Genome Sequence

Because the PA pathway is a primary degradation pathway for many peripheral aromatic compounds in other bacterial species (Luengo et al. 2001), and the PaaX negative regulator represses some of these upstream pathways (del Peso-Santos et al. 2006; del Peso-Santos et al. 2008; Kim et al. 2004), we sought to determine if there may be similar IR sequences located in other regions of the *B. cenocepacia* chromosome besides the PA genes themselves. To test this hypothesis, a position weight matrix (PWM) (Wasserman and Sandelin 2004) of the conserved inverted repeat present in the promoter of the *paaA*,

<i>paaA</i>	A	C	C	G	A	C	C	G	G	T	C	G	G	T	T
<i>paaH</i>	A	C	C	G	A	C	C	G	G	T	C	G	G	T	T
<i>paaZ</i>	A	C	C	A	A	C	C	G	G	T	C	G	G	T	T
<i>qacR</i>	A	C	C	G	A	T	C	G	A	T	C	G	G	C	T

Figure 4. Alignment of the conserved inverted repeat detected in the *paaA*, *paaZ* and *paaH* promoters. QacR is shown for comparison, as another TetR-type transcriptional regulator. The *paaF* promoter sequence is the reverse complement of the *paaZ* promoter sequence and is shown for comparison.

paaH and *paaZ* plus the divergent *paaF* and *BCAL0211* was constructed (Fig 5). This was then used to search the entire genome sequence of *B. cenocepacia*. The coordinate positions of sequences detected and accompanying score are listed in Table 3.

PWM scores were calculated manually by a modified method originally described by Hertz and Stormo (Hertz and Stormo 1999) and Schnieder (Schneider and Stephens 1990) (Fig. 5). This PWM was then re-entered into the Target Explorer and used via the PATSER program to scan each of the *B. cenocepacia* chromosomes and megaplasmid. The default cutoff score in the program Target Explorer used to search the genome is recommended to be the lowest score of the training sequences. In this case, the lowest score of the training set was a sequence partially similar to the IR located in the upstream region of *BCAL0211* with a score of 12. The score was arbitrarily cut off at 17.0 because a multitude of scores matched below this value.

As expected, the top scores for the search were the *paaZ* (22.03), *paaA* and *paaH* (20.74 each), as these sequences were used to help formulate the matrix (Fig 5 a). The next highest score was BCAM0706 (18.41), and located within a putative TonB-dependent receptor (pseudogene), which contains a frameshift mutation according to the Sanger centre annotation (Holden et al. 2008). Upstream of this putative receptor is a putative nucleotide transporter gene. It is not known how these may function in relation to PA metabolism.

The next score found (18.41) is located at position 2306124 on chromosome 2 and positioned 189bp upstream from a putative 72 amino acid protein of unknown function. The putative protein also contains a domain of unknown function DUF1653. The next scores are located at

Table 3. Position Weight Matrix scores in a genomic scan of *B. cenocepacia*

	Genome coordinate	Upstream CDS	Score	Description	Location from predicted start
Chromosome 1	443094	PaaZ, PaaF	22.03	<i>paaZ, paaF</i>	-45, -70
	244306	PaaA	20.74	<i>paaA</i>	-44
	25578	BCAL0022	17.37	putative branched-chain amino acid ABC transporter ATP-binding protein	-235
	939333	BCAL0866	17.37	Amidohydrolase	+827
Chromosome 2	1910367	PaaH	20.74	<i>paaH</i>	-42
	781968	BCAM0706	18.41	Ton B dependant receptor (pseudogene)	-18
				Putative nicotinamide mononucleotide transporter (next in operon)	
	2306124	BCAM2071	18.41	Conserved hypothetical protein	-189
Chromosome 3	None				
Plasmid	None				

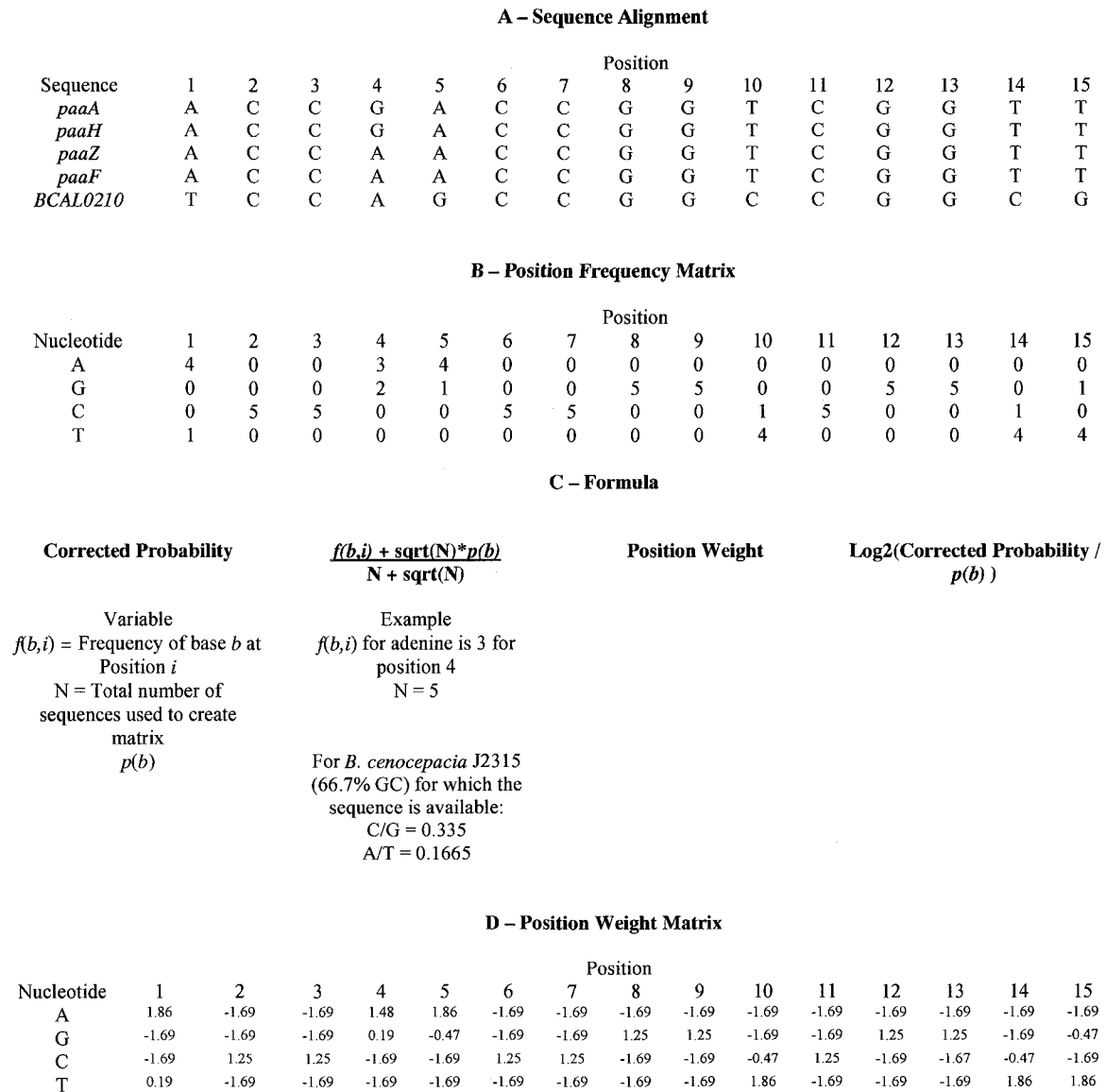


Figure 5. Construction of a Position Weight Matrix (PWM) of the conserved inverted repeat from the *paaA*, *paaH*, *paaZ*, *paaF* and *BCAL0211* genes. Panel A) Alignment of the sequences. Panel B) Position Frequency Matrix. Panel C) The formula used to calculate the Position Weight Matrix. Panel D) Calculated values for the PWM

positions 25578 on chromosome 1 with a score of 17.37. The location is 235 bp upstream from a putative branched-chain amino acid ABC transporter ATP-binding protein. Finally, at position 25578 on chromosome 1 is another location with a score of 17.37. Interestingly, this is located within the coding sequence (+827 nucleotides), of a putative amidohydrolase.

4.2 Function of the Enhanced Green Fluorescent Protein in a Constitutive Expression System – Characterization of *B. cenocepacia* Containing Plasmid pJH1

The fluorescent reporter used in this study is a modified version of the gfp originally isolated from the jellyfish *Aequorea victoria*. It contains the mutations S65T which adjusts the wild type excitation from 395 to 488. It also contains the mutation F64L which results in better folding at 37 °C (Cormack et al. 1996). Our results show that eGFP is functional in *B. cenocepacia*, and is effective as a reporter protein.

A plasmid was constructed containing the coding sequence for expressed eGFP under control of a constitutive dihydrofolate reductase promoter (DHFR), named pJH1. Briefly, eGFP was amplified from plasmid pSCrhaB4 (Cardona, unpublished data), and cloned into pAP20 (Law et al. 2008b), a broad host range plasmid that contained the DHFR promoter ahead of a multiple cloning site (Fig. 9 a). This plasmid was then conjugated into *B. cenocepacia* by triparental mating.

The functionality of plasmid pJH1 was assessed using both spectrofluorometry and fluorescence microscopy. For spectrofluorometry, *B. cenocepacia* cells were grown in 96 well format and measurements were taken using a Biotek Synergy 2 reader, cells were also assayed with a Shimadzu single cell spectrofluorometer for emission spectrum testing (Fig 6).

B. cenocepacia cells were grown in minimal media supplemented with various carbon sources. As expected, fluorescence increases linearly with optical density in various media types tested, indicating that plasmid copy number, and protein expression level, does not change significantly with time (Fig. 7). Importantly, this allows us to confirm subsequent results by correlating fluorescence and optical density of growth cultures and negate concerns over plasmid copy number. Levels for the pJH1 varied somewhat depending on different carbon sources used (unpublished data), likely as a result of a metabolic effect on the activity of the DHFR promoter.

Bacterial growth rates with *B. cenocepacia* containing pJH1 and *B. cenocepacia* wild type are similar, indicating no negative effect for plasmid maintenance (data not shown), which correlates with previous uses of the eGFP plasmid in *B. cenocepacia* (Lefebvre and Valvano 2002). For fluorescence microscopy measurements, *B. cenocepacia*/pJH1 exhibited bright fluorescence at 509nm when illuminated at 488nm (Fig. 8). No phenotypic difference in the cells containing the plasmid versus wild type cells was otherwise observed.

To test whether the constitutively expressing eGFP plasmid pJH1 is functional in other species, as it is based on the broad host range pAP20 which contains a pRB based origin of replication, the plasmid was conjugated into *P. aeruginosa* PA01 by triparental mating. *Pseudomonas* Isolation Agar (PIA) plates containing the conjugated strain exhibit fluorescence when examined under UV illuminating light, while the wild type bacteria do not exhibit fluorescence at these wavelengths. Thus, the eGFP protein is efficiently expressed from the DHFR promoter.

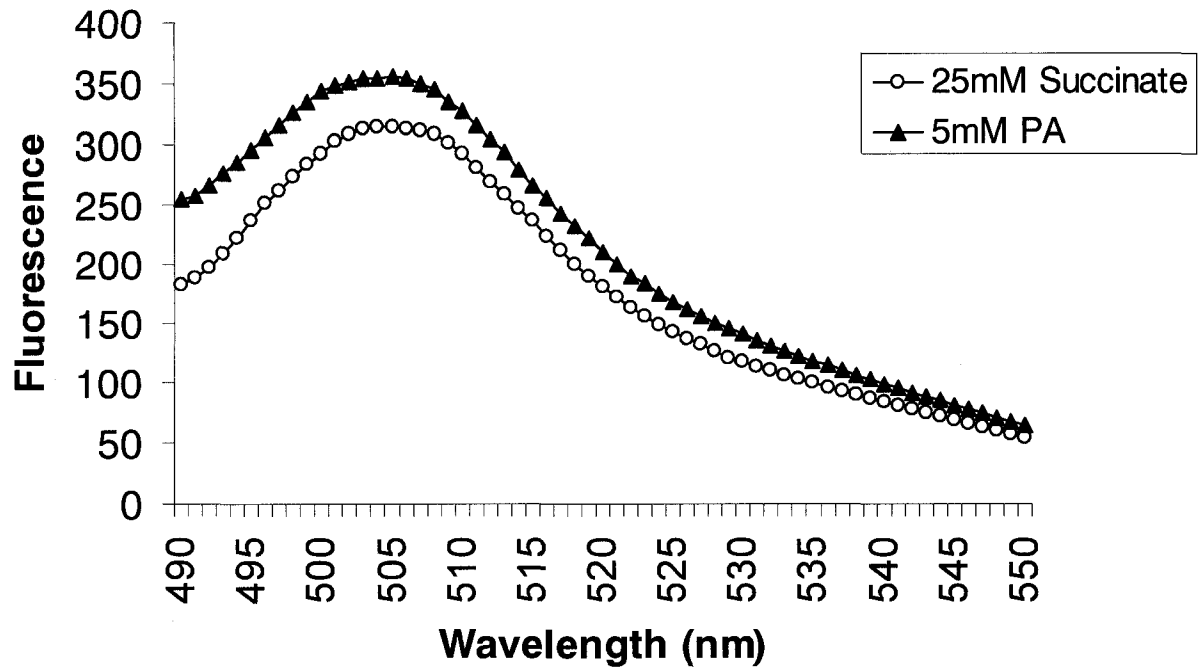


Figure 6. Emission spectrum testing of plasmid pJH1 (P_{DHFR}) constitutively expressing eGFP.

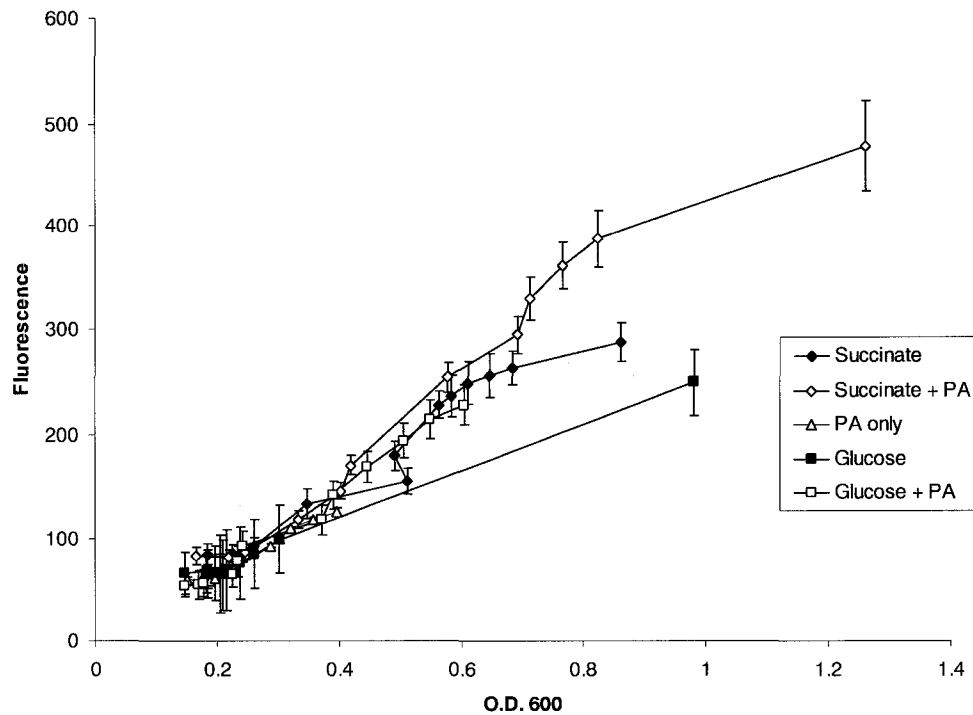


Figure 7. P_{DHFR} promoter strains grown for 13 hours in M9 minimal media supplemented with the following carbon source: 5 mM PA (open triangles), 5 mM PA and 25 mM glucose (open squares), 5 mM PA and 25 mM succinate (open diamonds), 25 mM glucose (filled squares), 25 mM succinate (filled diamonds). Error bars represent the standard deviation of three independent cultures.



Figure 8. *B. cenocepacia* cells carrying plasmid pJH1 were grown in minimal media supplemented with glycerol for 24 hours. Cells were harvested and plated on 0.9% agarose slides, then examined microscopically under DIC and UV light (1000x).

4.3 Construction and Characterization of Plasmid pJH2, an eGFP reporter plasmid containing a multiple cloning site

As plasmid pJH1 contains the DHFR promoter with the eGFP gene, we sought to replace the DHFR promoter with a multiple cloning site suitable for the cloning of target insert DNA. To achieve this, plasmid pJH1 was subject to PCR, with primers annealing in opposite directions outside of the DHFR promoter. Each primer contained designed restriction enzyme recognition sequences on the 5' end, and the final restriction site was in common between the two primers (Table 2) such that the self ligated product of the reaction contains a multiple cloning site (Fig 9. b). This plasmid was conjugated into *B. cenocepacia* as previously described.

Growth rates for *B. cenocepacia* containing plasmid pJH2 and wild type are similar (data not shown), suggesting that plasmid maintenance does not affect growth rate. Importantly, the cells do not exhibit fluorescence when examined spectrophotomically (data not shown), microscopically or when colonies are examined under UV light and is thus suitable for the insertion of potential promoters to test for inducer/*in vivo* dependent activity.

4.4 Phenylacetic Acid Responsive P_{paaA}, P_{paaZ} and P_{paaH} Translational Reporter Plasmids

To evaluate whether the upstream regions of the PA genes contained promoters activated when cells are grown in phenylacetic acid, the promoter-*eGFP* translational reporter plasmids

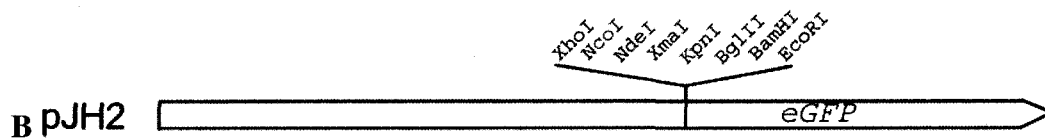
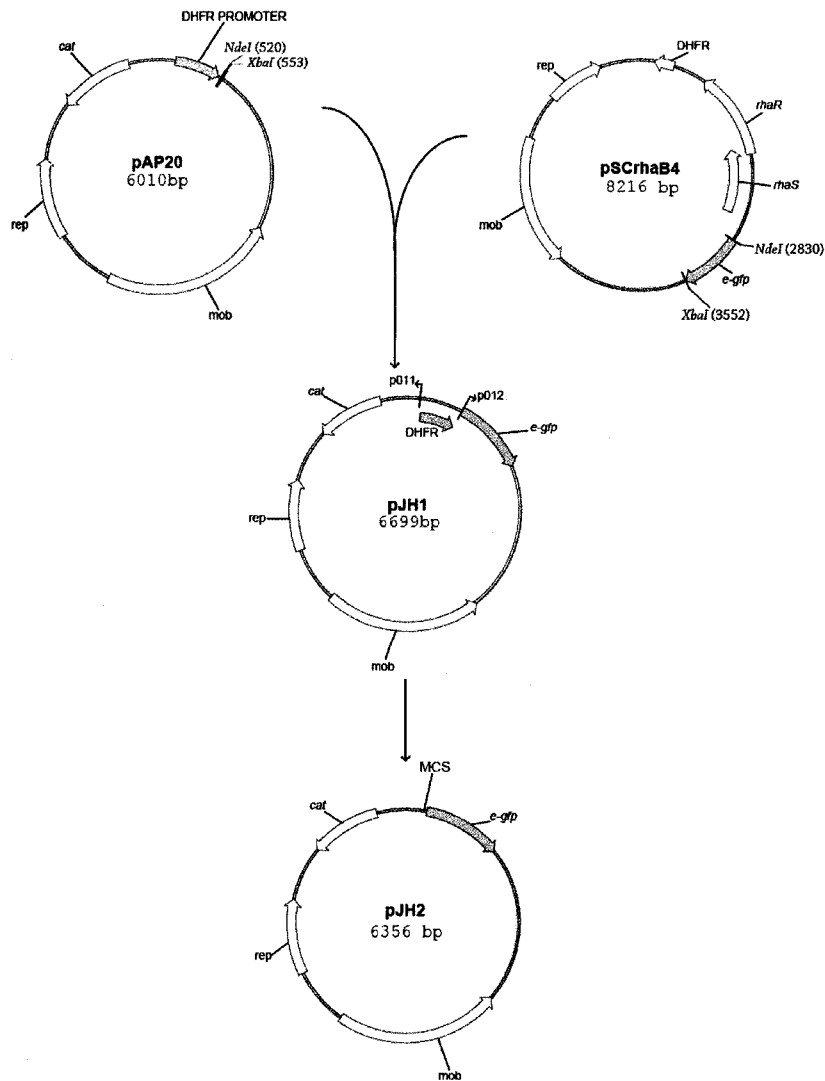
A

Figure 9. Construction of reporter plasmid pJH2 containing a multiple cloning site ahead of the eGFP gene (A). Plasmid pSCrhaB4 was digested with *XbaI* and *NdeI* to generate an eGFP gene fragment which was then ligated into the same sites in plasmid pAP20 to create pJH1. Plasmid pJH1 (P_{DHFR}) was then amplified using divergently oriented primers to replace the dihydrofolate reductase promoter (DHFR) with a multicloning site. Schemata of pJH2 multicloning site (B).

containing the upstream region from the first gene of each of the three PA clusters: *paaA*, *paaZ* and *paaH* fused to *eGFP* were constructed (Fig. 3 a). These constructs contained a C-terminal portion of 18-30 amino acids of the native protein (plus the upstream promoter) linked in frame to *eGFP* via a translated *EcoRI* site GAATTC (Glu – Phe) (Fig. 3 b). The eGFP protein has been reported to have been fused with whole functional proteins without affecting its activity (Tagge et al. 1997; Zimmer 2002) thus we chose to proceed with this method.

As expected, cells containing reporter plasmids with the *paaA* promoter (P_{paaA}), *paaH* (P_{paaH}) promoter or *paaZ* (P_{paaZ}) promoter exhibited fluorescence when measured by fluorometry when grown in minimal media containing PA (Fig. 10), but not in minimal media containing succinate, glucose or glycerol. The activity of P_{paaA} when grown in 25mM glycerol plus 5mM PA was 5.7 fold higher compared to growth in glycerol alone, and the activity of P_{paaH} and P_{paaZ} were each 2.9 fold higher with glycerol plus phenylacetic acid in the medium. This was also evidenced by colony streak on M9 minimal agar plates containing glycerol or glycerol plus PA when visualized under UV light (data not shown). These results agree with studies on the PA genes in other systems, where the PA gene promoter of *E. coli* and *Pseudomonas* have been shown to be induced several fold when cells are grown in the presence of PA.

Fluorescence was also increased linearly when plotted against optical density (Fig. 11), suggesting that plasmid copy number does not vary significantly during different phases of growth, similar to the observation for *B. cenocepacia* cells containing plasmid pJH1. To account for some media producing much greater cell density by late log phase, fluorescence signal, as measured by the 96 well microplate reader (materials and methods), was adjusted to an O.D. of 1 by dividing raw fluorescence by optical density.

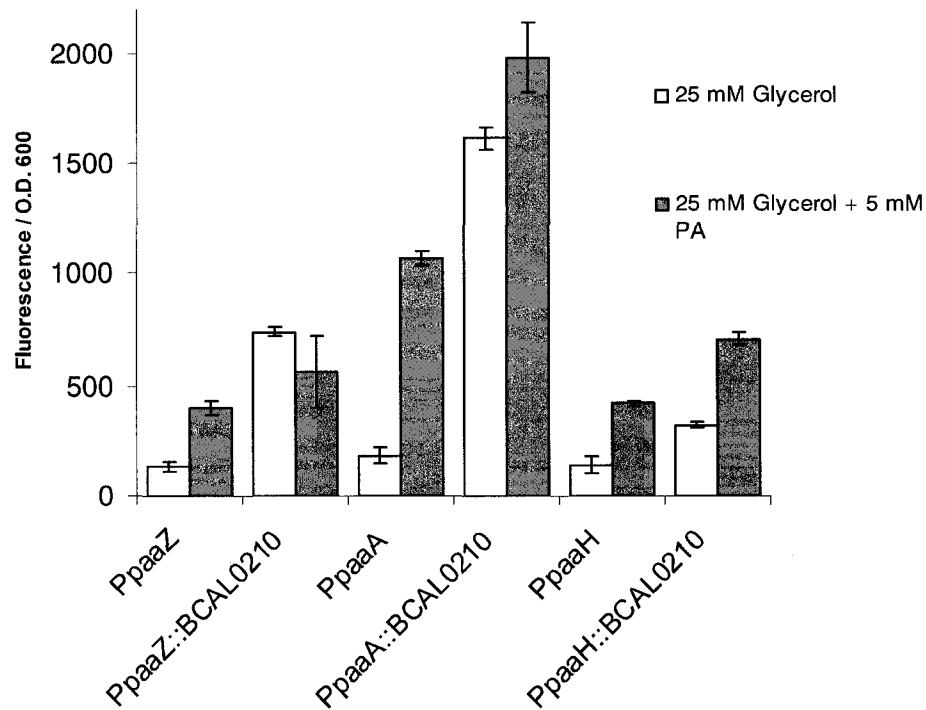
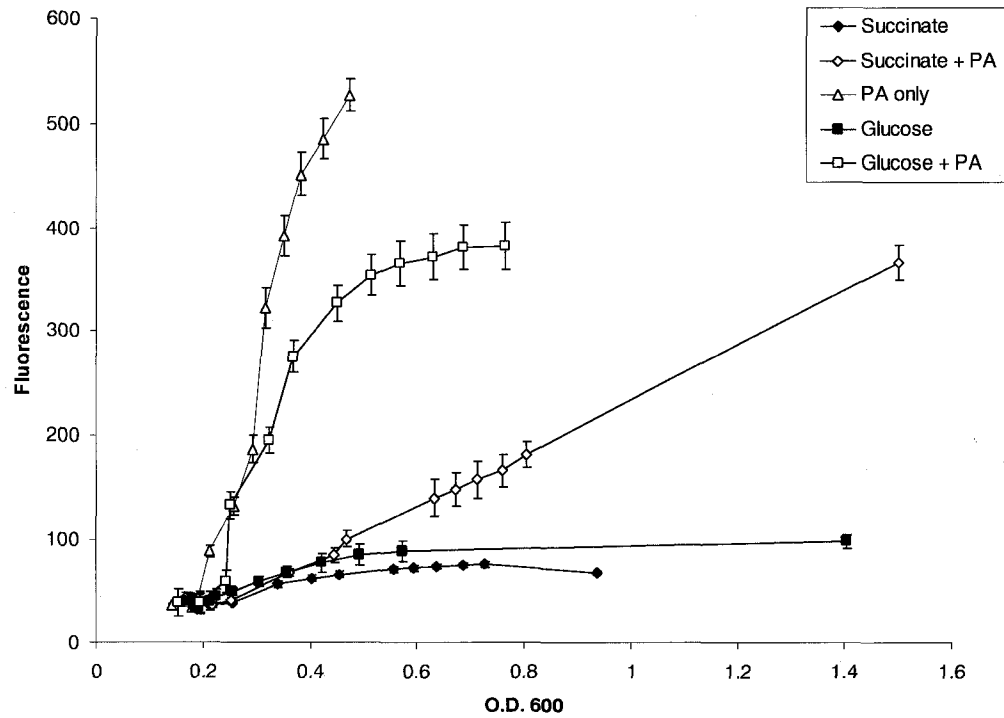
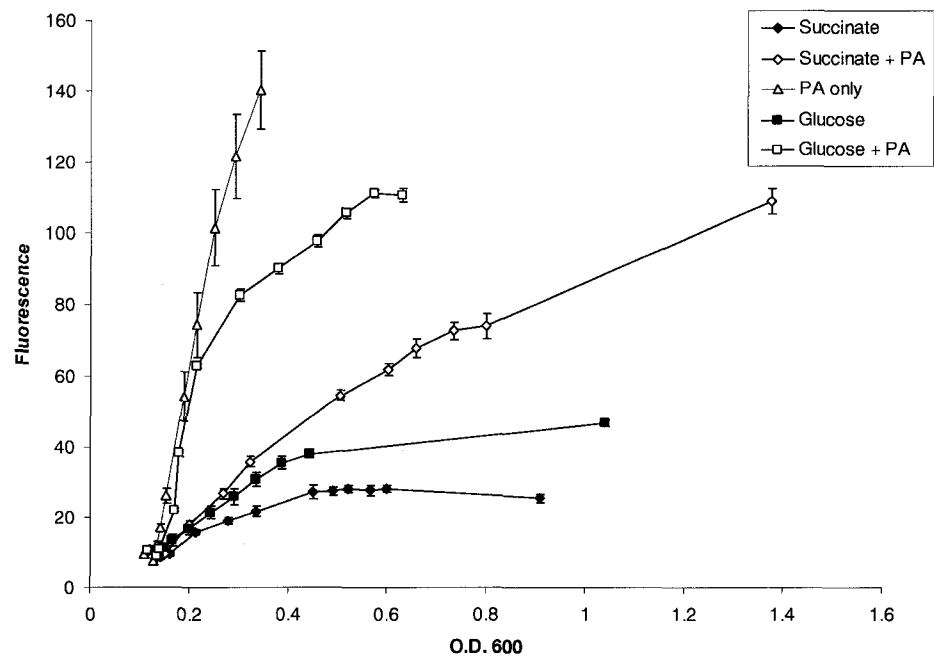


Figure 10. Reporter strains P_{paaZ}, P_{paaA} and P_{paaH} in wild type background or *BCAL0210* mutant (strain JNRH1) were grown for 18 hours in M9 minimal media supplemented with 25 mM glycerol (white bars) or 5 mM PA and 25 mM glycerol (grey bars). Fluorescence was measured with a Biotek synergy plate reader with excitation wavelength 485/20 nm and emission wavelength 528/20 nm. Error bars represent plus or minus one standard deviation.

Fig 11. A**B**

C

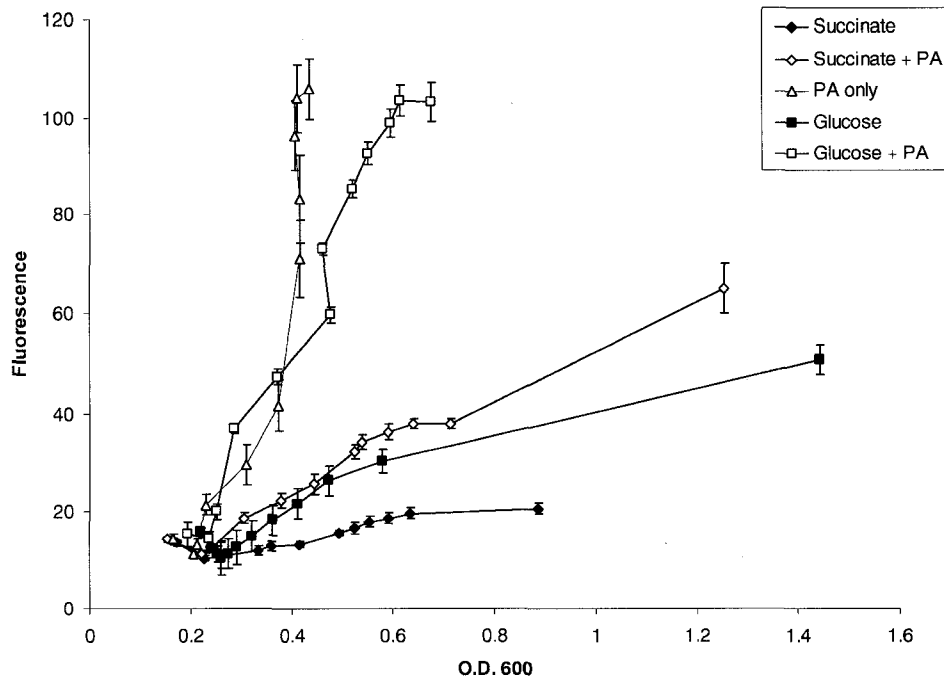


Figure 11. P_{paaA} promoter (A), P_{paaZ} promoter (B) P_{paaH} promoter (C) strains grown for 13 hours in M9 minimal media supplemented with the following carbon source: 5 mM phenylacetic acid (open triangles), 5 mM phenylacetic acid and 25 mM glucose (open squares), 5 mM phenylacetic acid and 25 mM succinate (open diamonds), 25 mM glucose (filled squares), 25 mM succinate (filled diamonds). Error bars represent the standard deviation of three independent cultures.

According to the KEGG database (Kanehisa and Goto 2000; Kanehisa et al. 2006; Kanehisa et al. 2008) phenylacetamide and phenylethelamine are expected to be degraded through the PA catabolic pathway in *B. cenocepacia* AU1054. To determine if these aromatic carbon sources funnel compounds downstream to the PA degradation pathway, and thus activate it, in *B. cenocepacia* K56-2, the PA reporter system P_{paaA} was grown in media containing these carbon sources. Indeed, this construct shows fluorescence expression similar to growth in PA when grown in phenylpyruvate or phenylacetamide (Table 4), but not in phenylacetaldehyde, which is a possible intermediate of the PA pathway in *B. cenocepacia* AU1054. These results suggest a possible alternative fate for phenylacetaldehyde, as normal growth was achieved on this carbon source (data not shown).

4.5 Phenylacetic Acid Responsive P_{paaA} Reporter is Activated During Growth in Synthetic Cystic Fibrosis Media and Phenylalanine

Aromatic amino acids have been shown to be present in cystic fibrosis lung sputum and their presence is capable of inducing virulence gene expression in the environmental pathogen *Pseudomonas* (Palmer et al. 2007). We therefore tested to see if phenylalanine proceeds through the PA pathway by inducing the P_{paaA} reporter. Indeed, our results show that when cells are grown in minimal media containing phenylalanine as the sole source of carbon, the P_{paaA} strain exhibits strong fluorescence when measured in late log phase (Table 4).

Additionally, we sought to determine whether the PA genes were activated in response to being grown in SCFM (Synthetic Cystic Fibrosis Media). SCFM is a chemically defined media based on the contents of CF sputum based media (Palmer et al. 2005). Our results show

Table 4. The activity of the P_{paal} reporter as a result of growth in M9 minimal salts media plus different carbon sources.

Media	Fluorescence / O.D. ₆₀₀
5 mM phenylacetic acid	1096 (107.4)
25 mM 2-hydroxy-phenylacetic acid	190.7 (19.67)
25 mM phenylalanine	1298 (276.9)
25 mM phenylpyruvate	1997 (88.67)
25 mM phenylacetamide	1331 (67.21)
SCFM	901.6 (259.7)

that the PA genes are indeed induced when grown in SCFM (Table 4), probably as a result of the presence of the aromatic amino acid phenylalanine. Notably, *B. cenocepacia* cultures grown in SCFM reached optical densities 30% higher than when grown in LB media alone (data not shown).

4.6 The Phenylacetic Acid Genes are Subject to Carbon Catabolite Repression

Aromatic compounds provide less available energy than their aliphatic counterparts due to their chemistry. It is known that aromatic compound degradation is subject to catabolic repression in the presence of more readily usable carbon sources in other bacteria (Aranda-Olmedo et al. 2005; Bruckner and Titgemeyer 2002; Martinez-Blanco et al. 1990). Since the P_{paaA} , P_{paaZ} and P_{paaH} reporter plasmids are differentially activated in the presence of PA, we sought to determine the effect of the growing the reporters in a mixture of PA plus various carbon sources.

Strains containing the P_{paaA} reporter were grown in 5mM PA only, or a mixture of 5 mM PA + 25 mM of succinate, lactate, glucose, arabinose, glycerol or sorbitol (Fig. 12). Cells were harvested after 18 hours of growth (late exponential phase), and analyzed for eGFP fluorescent activity. Results show that the TCA intermediates succinate and lactate repress the P_{paaA} reporter strongest (64.1% and 69.5% respectively). The saccharides glucose and arabinose cause a 50.1% and 42.8% repression respectively. Glycerol and sorbitol result in 2.9% repression (data not shown) and 16.9% activation respectively.

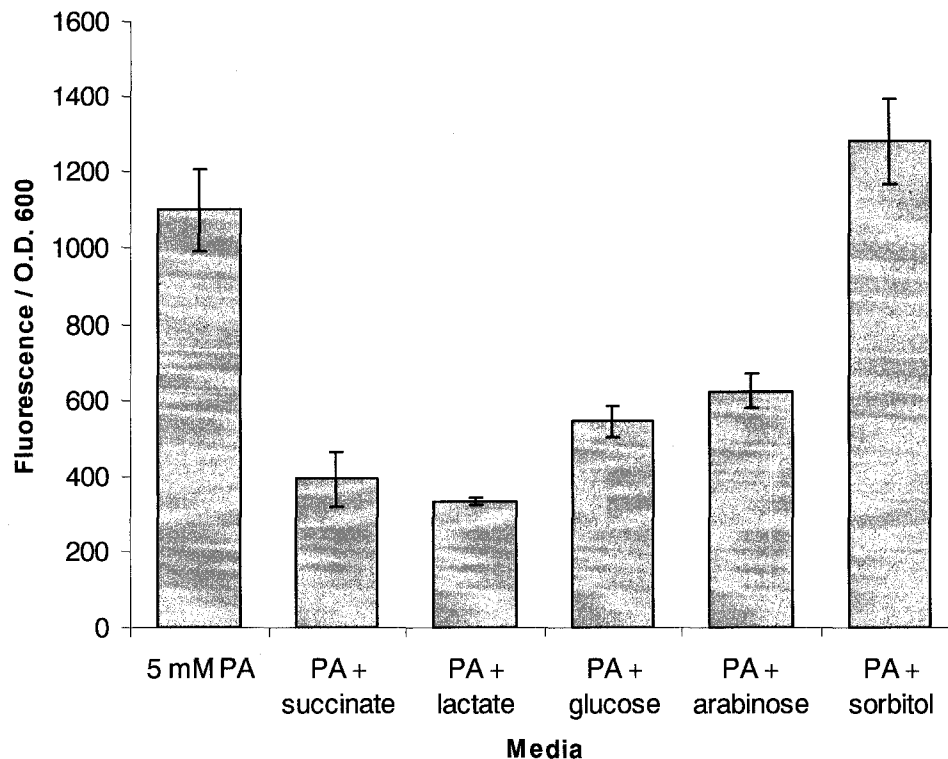


Figure 12. Reporter strain P_{paaA} in wild type background was grown for 18 hours in M9 minimal media supplemented with 5 mM phenylacetate or 5 mM phenylacetate and 25 mM succinate or 25 mM lactate or 25 mM glucose or 25 mM arabinose or 25 mM sorbitol. Fluorescence was measured with a Biotek synergy plate reader with excitation wavelength 485/20 nm and emission wavelength 528/20 nm.

Initially, it was unclear as to which carbon source (succinate or glucose) had a stronger repressing effect on the P_{paaA} promoter, since at 18 hours, both show a strong repressing effect. A review of the literature shows that conflicting data exists on whether organic acids such as succinate (Ohtsubo et al. 2006), or saccharides (Martinez-Blanco et al. 1990) are inhibitors of aromatic catabolic gene expression. It is known that in *Pseudomonas*, glucose transport is not involved in catabolic repression, and glucose utilization genes are repressed in the presence of succinate (Collier et al. 1996), unlike the *E. coli* model.

We hypothesized that an end point reading is not sufficient to describe this process. Therefore, to clarify this for PA gene regulation in *B. cenocepacia*, each of the reporter strains was grown in minimal media supplemented with 5 mM PA, 5 mM PA + 25 mM succinate, 5 mM PA + 25 mM glucose, 25 mM succinate or 25 mM glucose. Fluorescence was measured hourly and scatter plotted against optical density to account for variations of growth in different media (Fig 11). Remarkably, cells grown in PA plus glucose showed no decreased fluorescence over early stages of growth (Fig 11 a,b,c). At around O.D 0.3, there is a shift in the slope towards lower overall fluorescence. This is observed in each P_{paaA} , P_{paaZ} and P_{paaH} promoters. This is contrasted with growth in succinate, which exhibits reduced fluorescence over all periods of growth. This suggests that in *B. cenocepacia* glucose transport does not affect catabolic repression, but rather subsequent downstream metabolites or internal energy signals such as the presence of organic acids.

4.7 Identification of *BCAL0210* as a TetR-type Transcriptional Regulator

After determining the PA responsiveness of the P_{paaA} , P_{paaZ} and P_{paaH} reporter strains, we next sought to determine a potential regulator for these promoters. Located immediately downstream of the *paaABCDE* gene cluster and oriented in the same direction are genes *BCAL0211* and *BCAL0210* (Fig. 1). *BCAL0211* encodes for a putative 273 amino acid protein that contains a conserved domain of unknown function (DUF1835 superfamily).

The putative 201 amino acid *BCAL0210* contains an N terminus TetR-domain between residues 16 and 62 encoding the helix-turn-helix motif common to 95% of bacterial DNA binding transcriptional regulators (Ramos et al. 2005). Given the vicinity to the *paaABCDE* gene cluster, and its similarity to other bacterial transcriptional regulators, we proceeded with a bioinformatic and genetic analysis of *BCAL0210*.

Similar to the model TetR regulator (Klock et al. 1985), it is expected that the C-terminus encodes a ligand binding domain, which upon binding an effector molecule, affects a conformational change, resulting in disassociation from target DNA and alleviation of repression. Results of a BLAST search indicated the entire putative *BCAL0210* protein shows similarity to AcrR (Expect value = $5e-7$) which is the regulator of a multi-drug efflux pump of *E. coli* (Ma et al. 1996; Su et al. 2007) (Fig. 13).



Figure 13. BLAST result of the predicted amino acid sequence of BCAL0210, located downstream of the *paaABCDE* gene cluster in the genome sequence of *B. cenocepacia*. Results show a conserved TetR-like DNA binding motif on the N-terminus and the entire sequence blasting as similar to AcrR (Expect 5 e-7), a multidrug efflux regulator from *E. coli*.

4.8 BCAL0210 Insertional Mutant Exhibits Phenylacetic Acid Independent Expression

The above analysis prompted us to create an insertional mutant in *BCAL0210* to determine the effect of the loss of *BCAL0210* on the regulation on the PA genes. A λ Pir dependent plasmid containing an internal fragment from *BCAL0210* was conjugated into λ Pir negative *B. cenocepacia*. The resultant trimethoprim resistant colonies were thus expected to contain the plasmid integrated into the chromosome within the *BCAL0210* gene. This was confirmed by colony PCR using primers annealing to both the plasmid and the chromosome (data not shown). The resultant strain was named JNRH1. Reporter plasmids containing the P_{paaA} , P_{paaH} and P_{paaZ} promoters were then conjugated into strain JNRH1 by triparental mating.

Results show that these strains exhibit fluorescence regardless of the presence of PA in the culture (Fig. 10). This PA independent activity suggests that *BCAL0210* encodes for a negative regulator, whose transcriptional regulatory ability is abolished in the JNRH1 mutant. Interestingly, fluorescence of the cultures was generally higher than reporter strains grown with PA. Gene *BCAL0209*, located several hundred base pairs downstream of *BCAL0210* encodes a putative GNAT family acetyl transferase. The proximity of *BCAL0210* and the presence of an AT rich region that may contain a promoter makes the possibility of polar effects unlikely.

4.9 A Conserved Inverted Repeat is Necessary for Negative Control of P_{paaA}

In an attempt to understand the nature of the conserved IR located upstream each of the *paaA*, *paaZ* and *paaH* genes, translational fusion plasmids containing mutations in the *paaA* IR were created. We hypothesized that the sequence is a motif recognized by a TetR-like

transcriptional regulator, due to it being a dual overlapping inverted repeat, similar to the QacR operator (Ramos et al. 2005) however much shorter (15bp vs 28bp) in length. This would then suggest that a potential DNA binding protein would bind as a pair of dimers, on each face of the DNA, and sterically hinder RNAP open complex formation, the most simple and commonly described method of DNA binding protein negative transcriptional regulation.

Therefore, we used plasmid mutagenesis with primers containing mismatched mutations on the 5' ends that annealed to plasmid pJH7 containing the P_{paaA} reporter to generate plasmids pJH10, pJH11 and pJH12. The plasmid pJH10 contains 14 mismatch mutations replacing nearly the entire IR within the *paaA* promoter. Plasmids pJH11 and pJH12 contain the mutations in the left and right half of the IR (as read 5' to 3') respectively. These plasmids were then transferred to *B. cenocepacia* wild type by triparental mating.

Reporter strains were grown in minimal media supplemented with 5 mM PA, 25 mM glycerol or both. Cells exhibited fluorescence at high levels, similar to wild type P_{paaA} grown in PA, or JNRH1/ P_{paaA} regardless of the carbon source used (Table 5), demonstrating that the sequence is indeed required for negative control of *paaA* promoter activity. We further postulate that the sequence is also necessary for negative control of the *paaZ* and *paaH* genes, due to the similar placement of the sequence in the core promoter, between the putative -35 and -10 elements.

Table 5. Mutations in the conserved IR of the *paaA* promoter result in PA independent expression. Parenthetical numbers represent the standard deviation of three separate cultures.

Plasmid	Fluorescence / O.D. ₆₀₀
pJH10	1579 (9.76)
pJH11	1345 (111)
pJH12	2159 (111)

4.10 Transcript Organization of the *paaABCDE*, *BCAL0211* - *BCAL0210* Gene Clusters

In order to determine if the PA responsive P_{paaA} translational reporter is reflective of *paaA* controlling the downstream genes of the *paaABCDE* cluster, a transcriptional analysis was performed. Whole RNA was isolated from *B. cenocepacia* cells grown in LB + 1 mM phenylacetate during early log phase (O.D. 600 = 0.3). Whole RNA isolated showed strong and distinct bands corresponding to 23s and 18s ribosomal RNA (data not shown). Copy DNA was then created using RT first strand synthesis system from stratagene. Copy DNA was then subjected to PCR using gene specific primers.

Results show that the *paaA*, *paaB*, *paaC*, *paaD* and *paaE* genes are contained on a single transcript and are thus co-regulated at the transcriptional level (Fig. 14). This is demonstrated by primers which anneal to the copy DNA, are able to make PCR amplicons between each of the genes. Since the genes *BCAL0211* and *BCAL0210* are located downstream from the *paaE* gene, we sought to determine the transcriptional organization of these genes as well. Primers were unable to generate an amplicon between *paaE* and *BCAL0211*. An amplicon was generated between *BCAL0211* and *BCAL0210*, suggesting that they are located on the same transcript. An amplicon was unable to be created between *BCAL0210* and the downstream gene *BCAL0209*.

4.11 *C. elegans* Infection Reporter Assay demonstrates P_{paaA} is Activated During Infection

Since previous findings in our laboratory have shown that a functional PaaABCDE ring hydroxylation complex is necessary for full virulence of *B. cenocepacia* in a *C. elegans* model of infection (Law et al. 2008b), we sought to determine whether the PA genes are

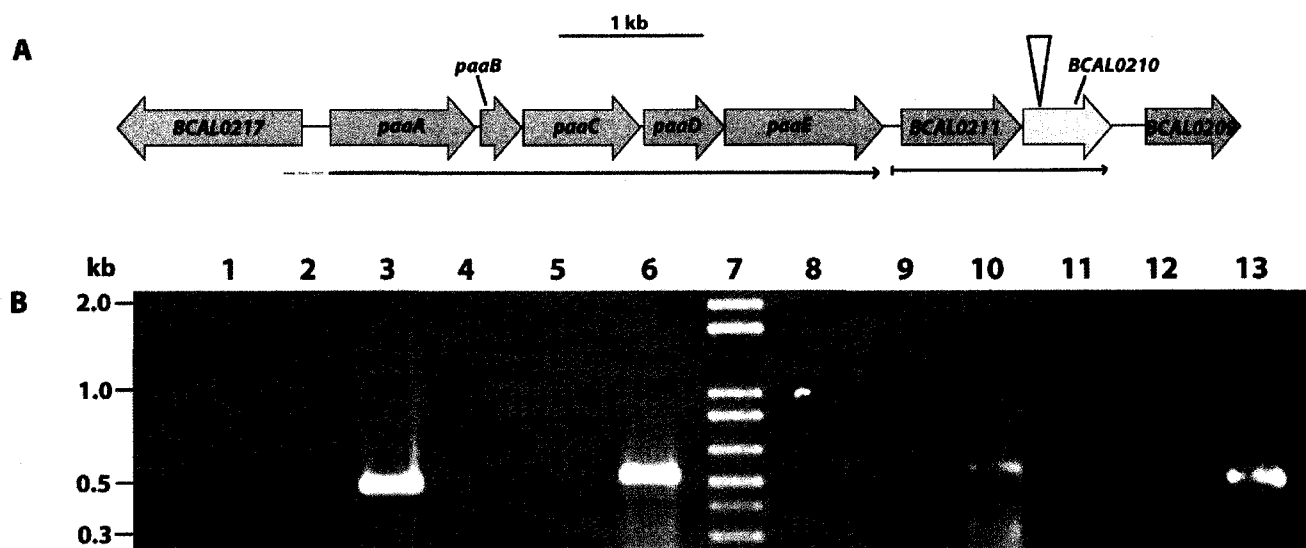


Figure 14. A) Reverse transcriptase (RT) organization of the *paaABCDE* genes and region. Solid line represents a single transcript. Vertical bar represents separate transcript. Red dash represents undetermined. B) RT-transcript profile of the *paaABCDE* ring hydroxylation complex genes. Lanes 1 (H₂O/RT), 2 (RNA/noRT), 3 (RNA/RT) *paaA* – *paaB*, lanes 4, 5, 6 *paaB* – *paaC*, lane 7 1kb ladder, lanes 8, 9, 10 *paaC* – *paaD*, lanes 10, 11, 12 *paaD* – *paaE*.

differentially activated during infection of the *C. elegans* lumen as compared to growth on minimal media. To accomplish this, an infection reporter assay was designed. Two day old synchronized worms were fed *B. cenocepacia* carrying the P_{paaA} fluorescent reporter. The worms were harvested after 48 hours of infection, mounted on agarose slides and visualized by fluorescent microscopy. The Zeiss Apotome was used to help remove out of focus fluorescence. Cells were limited to the intestinal lumen when comparing differential interference contrast Nomarski (DIC) and fluorescent images (Fig. 15).

To quantify the visual measurements, NIH software Image J (Wayne Rasband, National Institute of Mental Health, Bethesda, Maryland, USA) was employed. Pixel intensity was averaged from 5 different micrographs from each condition, and the data displayed in bar graph format (Fig. 16). Because of some residual activity of the P_{paaA} promoter in the glycerol supplemented media, these micrographs and data are also shown. The difference between the two conditions (glycerol and *C. elegans* lumen) show that the promoter is indeed more active during infection of the *C. elegans* lumen. Average pixel intensity was similar (within standard deviation) for cells containing the P_{DHFR} promoter when grown on glycerol containing media or *C. elegans* lumen, demonstrating importantly that a similar amount of cells were measured in the two conditions. For cells containing the P_{paaA} promoter, measurements taken from the *C. elegans* lumen exhibited 67.1% stronger fluorescence than those grown on glycerol alone. Micrographs were optimized by using the Zeiss automatic exposure adjustment. Exposure times were averaged and compared: P_{paaA} glycerol = 943ms, P_{paaA} *C. elegans* = 988 ms.

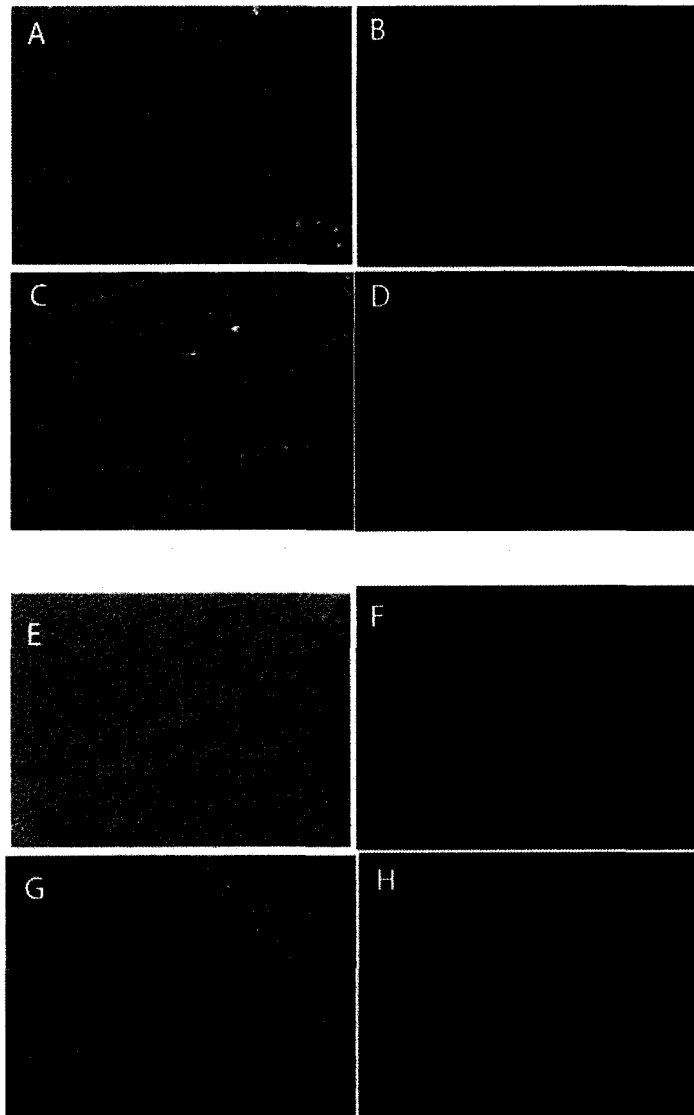


Figure 15. Example micrographs of *C. elegans* infection reporter assay. A) Differential Interference Contrast (DIC) (Nomarski) micrograph (1000X) magnification of *B. cenocepacia* / P_{DHFR} ; B) Fluorescent micrograph of the same position, displaying the constitutively expressing eGFP protein; C) DIC micrograph of *C. elegans* infected with *B. cenocepacia* / P_{DHFR} ; D) Fluorescence micrograph of panel C; E) Differential Interference Contrast (DIC) (Nomarski) micrograph (1000X) magnification of *B. cenocepacia* / P_{paaA} ; F) Fluorescent micrograph of the same position; G) DIC micrograph of *C. elegans* infected with *B. cenocepacia* / P_{paaA} H) Fluorescence micrograph panel G.

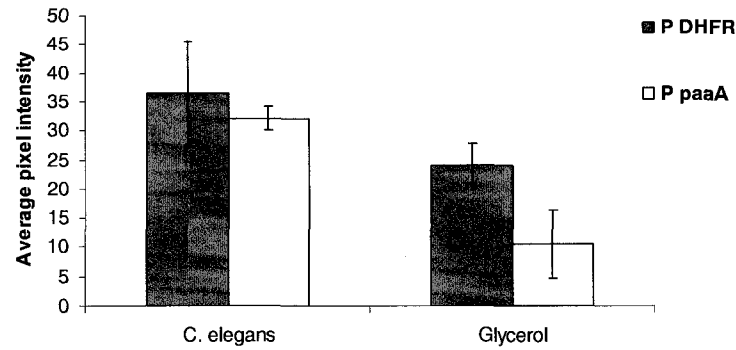


Figure 16. *B. cenocepacia* containing either the P_{DHFR} expressing eGFP constitutively (grey bars), or the P_{paaA} reporter (white bars) were grown for 48 hours at 37°C on M9 minimal agarose plates supplemented with 25 mM glycerol. At 48 hours, L4 age synchronized worms were added to half of the plates and infection allowed to proceed at 26 °C for 48 hours while the remainder were likewise incubated. Cells were visualized with a Zeiss fluorescence Axiovision microscope. Average pixel intensity was measured from separate samples using ImageJ software.

4.12 Determination of the Minimum Concentration of Phenylacetic Acid that Induces the P_{paaA} ,

P_{paaZ} and P_{paaH} reporter strains

Since we show that the PA genes are activated by the presence of PA, are subject to catabolic repression, and are induced during *B. cenocepacia* infection of the *C. elegans* intestine, we sought to quantify the minimum amount of PA required to activate PA gene expression. Our hypothesis was that the PA genes should be induced at low μM levels, since this may represent the physiological concentrations *B. cenocepacia* would be exposed to naturally. To determine this, cells were grown for 24 hours in 10 mM glycerol, a media previously determined not to exhibit significant catabolic repression upon P_{paaA} , P_{paaZ} or P_{paaH} expression.

At 24 hours, PA was added to the medium to a final concentration of 25 μM , 50 μM , 100 μM or 500 μM . Growth was resumed at 37 °C, and fluorescence measurements were recorded every 3 minutes. Results show that after 50 minutes, fluorescence increases for P_{paaA} and P_{paaZ} in the 25 μM condition (Fig. 17 a,b,c). The P_{paaH} reporter required 500 μM to be activated at the same time point. Additionally, fluorescence levels off at successive time points after induction in P_{paaA} and P_{paaZ} reporter constructs. We hypothesize that this is due to PA being exhausted from the media and subsequent repression of the promoters. These findings demonstrate highly efficient control of PA transcription within *B. cenocepacia*.

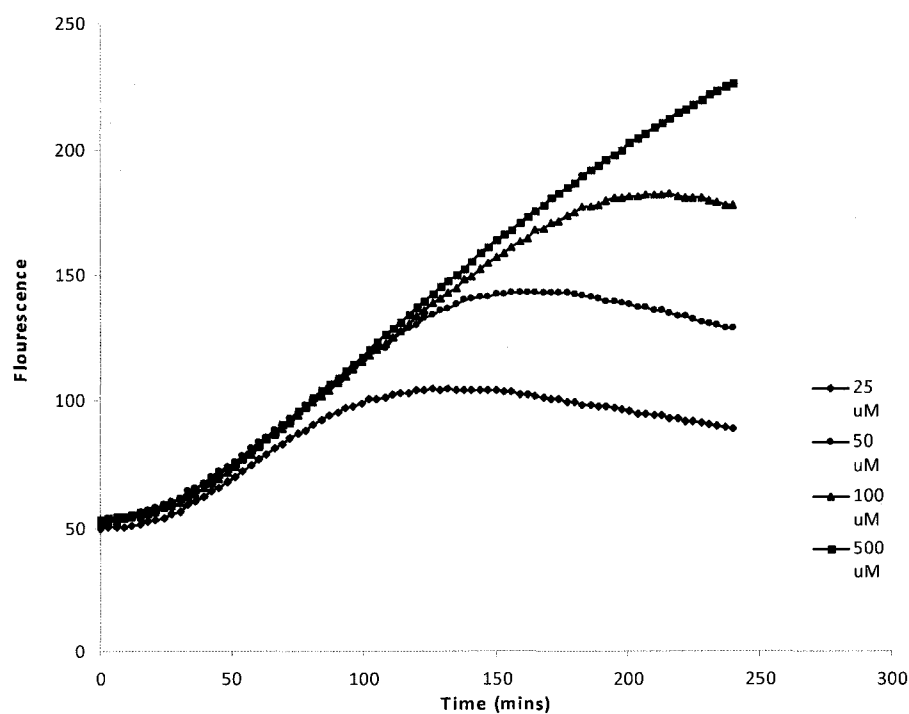
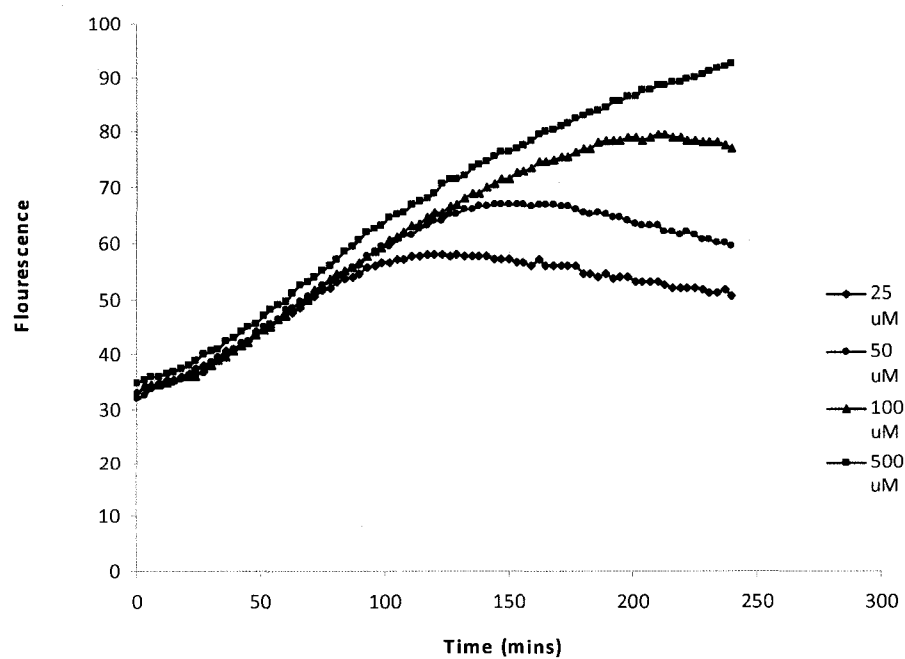
Fig. 17-A**Fig. 17-B**

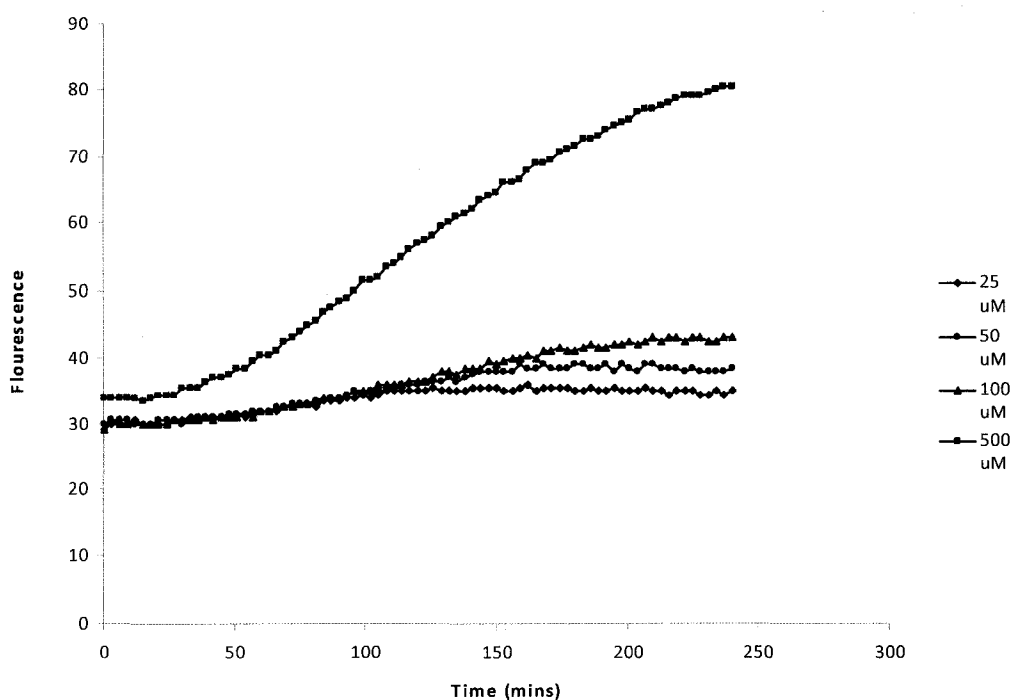
Fig. 17-C

Figure 17. P_{paaA} promoter (A), P_{paaZ} promoter (B) P_{paaH} promoter (C) were grown in M9 minimal media supplemented with 10 mM glycerol for 24 hours at 37°C with shaking at 200 RPM. Different amounts of phenylacetic acid (squares - 500 uM, triangles - 100 uM, circles 50 uM, diamonds - 25 uM) were then added to the medium, and growth resumed at 37°C. Fluorescence measurements were recorded at 3 minute intervals while growth proceeded for a total of four hours.

5 – DISCUSSION

5.0 Proposed Mechanism for Phenylacetic Acid Gene Regulation in *B. cenocepacia*

On the basis of our genetic analysis we propose that *BCAL0210* encodes for a transcriptional regulator that functions to negatively regulate the PA genes. Our results show that *BCAL0210* is necessary for PA dependent regulation of the *paaA*, *paaH* and *paaZ* genes (Fig. 10). It is likely that BCAL0210 binds a conserved inverted repeat DNA sequence (5' ACCNACCGGTNGGTT 3') located in the promoter of the *paaA*, *paaZ* and *paaH* genes, since our results show that this sequence is necessary for PA responsive control of *paaA* gene expression (Table 5). In the absence of phenylacetic acid, BCAL0210 would sterically hinder RNAP open complex formation by remaining positioned within the core promoter region.

We speculate that the conserved IR located in the upstream region of the *BCAL0211* gene may be a low affinity binding site for protein BCAL0210 and may thus be involved in self regulation. Our results show that *BCAL0211* and *BCAL0210* are located on the same transcript and thus co-regulated at the transcriptional level. Cloning of the *BCAL0211* promoter region was successful, but in every condition that we tested the promoter it would not show significant activity compared to the negative controls (data not shown). This may be due to a low basal level of transcription that is below the detection threshold of the eGFP reporter.

Open reading frame *BCAL0211* is located adjacent the *BCAL0210* regulator, and putatively encodes a 273 amino acid protein of unknown function. Our results (data not shown) show that *BCAL0211* and *BCAL0210* are located on the same transcript. Also, reporter P_{paaA} was conjugated into a previously available *BCAL0211* insertional mutant and a colony streak on

minimal media supplemented with 25 mM glycerol exhibited PA independent fluorescence at a similar level to the JNRH1 *BCAL0210* mutant (Fig. 18), possibly due to polar effects on downstream *BCAL0210*. A BLAST of *BCAL0211* reveals a domain of unknown function (DUF1835) located between residues 4 and 121. Since these genes are co-transcribed, it is possible that *BCAL0211* is involved in some fashion in the regulation of the PA genes. This remains an unexplored point of interest, due to the proximity to the PA genes, and the undefined character of the protein product.

5.1 Characterization of a New Fluorescent Reporter System Functional in *B. cenocepacia*

Our results were achieved by the development of a translational reporter plasmid, functional in *B. cenocepacia* containing a multicloning site, *eGFP* gene and a trimethoprim resistance cassette. This allowed for the rapid and efficient cloning of the upstream regions from each of the PA genes to generate the reporter plasmid.

In the absence of an inducer, there is a basal level of fluorescence from each of the P_{paaA} , P_{paaH} and P_{paaZ} reporter strains after 18 hours of growth (Fig XXX). The reasons for this are not completely clear. It could be possible that there is a titration effect of the potential regulator binding sites. This is because the plasmid of origin is mid copy number plasmid as it is originally based on pAP20 (Law et al. 2008b), a construct of pMLBAD (Lefebvre and Valvano 2002) a derivative of pBBR1 which is maintained at 20 – 30 copies per cell (Antoine and Loch 1992). Thus, binding sites for the putative negative regulator would be lowered. This would also suggest that there is a negative regulator rather than a positive regulator. The sequence of the PA operator suggests that 4 regulatory proteins bind each full sequence to restrict formation of

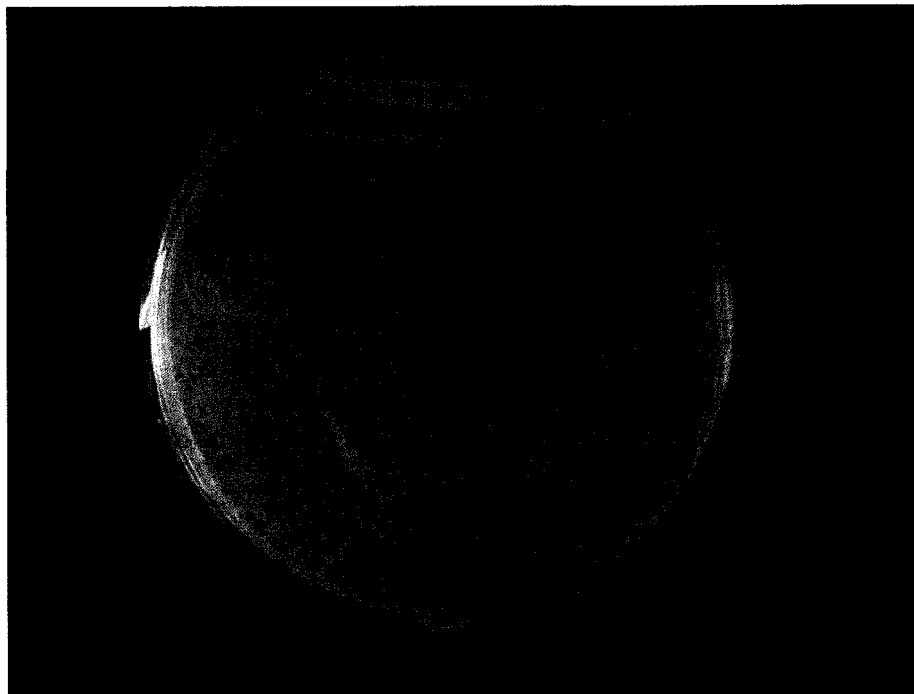


Figure 18. Reporter P_{paaA} was grown on M9 minimal media supplemented with 25 mM glycerol for 48 h at 37°C. Top *B. cenocepacia* wild type / P_{paaA} , left *B.cenocepacia* *BCAL0211::tp* / P_{paaA} , right *B. cenocepacia* JNRH1 / P_{paaA} . Cells were illuminated with UV light, viewed through a 500 nm filter and recorded with a Sony Cyber-shot DSC-P43 digital camera.

the RNAP open complex, thus there could be regulators binding, but not to the full extent necessary to completely inhibit RNAP. Additionally, this could be an actual observed physiological phenomenon, as PA may be expected to be present in after several rounds of bacterial replication and thus protein turnover, thus accounting for the observed fluorescence after 18 hours.

The eGFP protein is highly stable, with fluorescence of P_{DHFR} reporter colonies evident after 23 months of storage at 4°C in the absence of light (data not shown). Our results show that the eGFP chromophore is moderately sensitive to photobleaching during fluorescence microscopic examination of the specimen. Photobleaching properties of a chromophore may have useful applications such as Fluorescent Recover After Photobleaching (FRAP) (Jacobson et al. 1976), but a photobleaching resistant chromophore is desirable for accurate gene reporting studies. This was avoided by focusing the sample while in DIC mode, and using fluorescent light only during image acquisition. However, the replacement of eGFP with a more resistant protein would aid image analysis.

5.2 Other Uses for the pJH7 Plasmid

Recall mutations in P_{paaA} reporter were made to change base pairs within the IR and resulted in constitutive expression (Table 5). While disrupting the putative binding site confirmed the primary hypothesis that this conserved sequence is necessary for the control of P_{paaA} , the resultant expected levels of expression are not significantly (or severely) affected. The mutations were selected with the bias to transversions, no introduction of different an inverted repeat and different from the consensus IR. Thus, pJH10, pJH11 or pJH12, the mutant plasmids containing mutations in the left right and whole sequence presumably may be mutated using the

same technique to introduce specific base pairs of interest. For example, restriction sites maybe added, or another IR of interest may be inserted by plasmid based mutagenesis.

In conclusion, the promoter activity appears generally immune to changes within this region. This is expected because it is normally occupied by a transcriptional regulator and thus served no other purpose. Spacing should remain the same to retain the proper orientation for sigma factor binding.

5.3 Carbon Catabolite Control of the Phenylacetic Acid Genes

Our results show that the PA genes of *B. cenocepacia* are subject to CCR. We show that that the TCA intermediates succinate and lactate produce the strongest inhibitory effect on PA gene expression. Our data suggests that glucose itself does not cause catabolic repression but a downstream metabolite and subsequent higher intracellular energy signal is the cause of the observed repression. At roughly O.D. 0.5 the Glucose plus PA reporter is repressed when compared to the PA only condition in all 3 of the reporters observed (Fig. 11), suggesting an onset of repression.

Glucose and other sugars are used to make excess exopolysaccharide in an onion model (Bartholdson et al. 2008) and this may be a reason it is not used as an energy sensor. These findings agree with the examples from *Pseudomonas* that TCA intermediates also repress the synthesis of catabolic aromatic clusters. In that system, it has been shown that regulator CRC binds to aromatic transcripts to suppress translation (Moreno and Rojo 2008). Interestingly, a homolog of CRC protein is in the *B. cenocepacia* genome (BCAL0487). A simple experiment for

use with our system would be to create an insertional mutant in that gene and measure fluorescence of the promoters when PA is mixed with a TCA intermediate such as lactate.

Given our finding that the PA genes are each subject to catabolic repression, a lower energy state may preferentially create ppGppp to prevent RNAP transcription of ribosomal genes and thus free up available polymerase for other sigma 70 promoters such as the PA genes.

5.4 Induction of the PA genes in Synthetic Cystic Fibrosis Media (SCFM)

Our findings show that the P_{paaA} promoter is induced during growth in a Synthetic Cystic Fibrosis Medium (SCFM) (Table 4). The fluorescence levels observed are not as strong as those grown in PA alone or in 25 mM phenylalanine. We hypothesized that the presence of lactate in the media maybe a cause of this repressing effect. Thus, we tested to see if lactate is a repressor of the P_{paaA} promoter (Fig 12). Indeed our results show that lactate is a strong repressor of the P_{paaA} promoter activity.

The SCFM media was originally formulated based upon an analysis of compounds present in actual cystic fibrosis clinical sputum samples. Compounds include amino acids, lactate, ferric iron and other compounds. As an imitation media, it showed general microarray features similar to actual cf sputum in *P. aeruginosa* microarray induction experiments. In general, *B. cenocepacia* grows to a much denser final optical density than LB media (data not shown), suggesting that the formulation is nutrient rich.

5.5 Induction of the P_{paaA} in a Host Model of Infection

Our findings show that the P_{paaA} promoter is induced 67% in the *C. elegans* lumen (Fig. 16). These data support the report that functional *paaABCDE* ring hydroxylation complex genes are necessary for full virulence when infecting *C. elegans* because we demonstrate that *paaA* promoter, controlling the *paaABCDE* transcript, is activated during infection.

C. elegans placed on plates containing fresh lawns of wild type *B. cenocepacia* display a sick phenotype after 48 hours infection. Upon microscopic examination (DIC) the intestine appears distended, with bacterial infection limited to the intestinal lumen. Worms fed on P_{DHFR} constitutive expressing eGFP exhibit bright fluorescence in the distended lumen.

The apotome feature on the Zeiss microscope was used to obtain optical sections of the sample. This results in fluorescence from above and below the plane of focus to be effectively removed from the final image in a method called structured illumination (Neil et al. 1997; Schaefer et al. 2004). The “high” grid was used for all 1000x images.

The ImageJ software from NIH was used for a new method of image analysis. Axiovision Z1 files were saved as Tagged Image Files (Tif) for import into Image J. Quantification of area by measuring pixel intensity has previously been used in *C. elegans* for the reporting of gene induction (Chavez et al. 2007). Our method is unique in that it compares the difference in fluorescence in a reporter, while fluorescent levels of the control remain constant (within error). This is necessary primarily because of two factors: The *C. elegans* lumen is of course three dimensional and fluorescence from above and below the plane of focus could potentially add to the measured overall pixel intensity and thus produce a false positive. The second is that *C.*

C. elegans contains autofluorescent vacuoles of lipofuscin that accumulate with age (Yin 1996), and are located adjacent, but not inside the intestinal lumen. Unfortunately, these vacuoles are excited by nearly the same wavelengths as eGFP protein. These issues are addressed by use of the apotome, counting fluorescence in a defined area within the lumen, and measuring fluorescence intensity in the positive control infection containing pJH1, which shows no increase in fluorescence levels within error.

5.6 Metabolism and the Presence of Inducing Compounds

It has been observed that mutants in the ring hydroxylation complex *paaABCDE* cause decreased pathogenicity in the *C. elegans* model (Law et al. 2008a). The simplest explanation for these data is that PA, likely via phenylalanine, provides an additional energy source for the bacteria. *B. cenocepacia* is known to contain several proteases which could liberate phenylalanine from host proteins as an energy source during host infection. However, due to the same intestinal load during infection with the *paaABCDE* mutant and wild type bacteria when infecting *C. elegans*, this may not be the case.

5.7 Metabolic Coupling of the Phenylacetic Acid Gene Operons

The observation that the de-repressed P_{paaA} is roughly 3-fold stronger than either the P_{paaH} or P_{paaZ} promoters piqued our attention (Fig. 10). This may be due to unaccounted for deficiencies in the vector construct or plasmid reporter i.e.) pJH6 or pJH8 N-terminal fusions may be negatively affecting translation. We consider this unlikely based on the responsiveness of the system, and also the consistency of the P_{paaZ} and P_{paaH} response. Of primary concern however, is this of biological significance? The presence of a poly A tract upstream the *paaA* -35

element (Fig. 3 b) suggests that an UP element (Ross et al. 2001) is present, and likely accounts for the increased activity. The fact that the PA genes are organized into three gene clusters may partly be due to a difference in the requirement for their encoded protein products (enzymatic complexes), and thus by differing transcript levels this could be accomplished.

This hypothesis predicts that the product of the *paaABCDE* gene cluster, a ring hydroxylation complex that converts PA-CoA into 2OH-PA-CoA is beneficial to have in higher amounts than the other PA degradation complexes (PaaZ ring hydroxylation). This could be simply due to the optimal kinetic coupling of the product of the 2OH-PA-CoA to the PaaZ ring opening complex. In *E. coli* and *Pseudomonas spp.* the PA genes are organized as a single operon, however. In *Pseudomonas sp.* Strain Y2, a second functional gene cluster for the catabolism of PA has been described (Bartolome-Martin et al. 2004), where a conserved operator is located upstream of two gene clusters located adjacently on the chromosome.

Another possibility is that the ring hydroxylation complex has an additional cellular function in the induced state, and that PA-CoA, the presumed inducer of the system, is a signal to this additional function. Interestingly The P_{paaH} reporter requires 10 fold more PA to induce activity than the P_{paaA} or P_{paaZ} reporter. This may reflect it's location on chromosome 2 and may have been acquired after the PA genes on chromosome 1 and thus produce less efficient of a response.

5.8 Defense Mechanisms

The high activity of the PaaABCDE cluster could function as a defense mechanism. *C. elegans* may exude compounds in reaction to host infection and this hydroxylating them may be a method of deactivation.

Additionally, the ring hydroxylation complex and or paaK or paaK2 may ligate other aromatic compounds “X”, for example host antibiotics, to X-CoA and lead them to hydroxylation and subsequent disarmament. On a local level, consider that over the entire domain, BCAL0210 blasts as AcrR (a TetR type protein), which encodes for a negative regulator of a drug efflux system (Fig. 13). AcrR is known to recognize a wide range of structurally dissimilar compounds via the inducer binding domain that can bind and release AcrR from its operator (Su et al. 2007). Thus, other aromatic substrates may operate on BCAL0210 and allow the PaaABCDE to hydroxylate them. The resultant products could then proceed to another CoA degradation pathway or be exuded via one of the multiple drug efflux systems.

5.9 Host as a Growth Environment

Previously it has been shown that nutritional factors serve as signal molecules to induce virulence factors in *Pseudomonas* spp. (Palmer et al. 2007). In that study, a synthetic cystic fibrosis medium (SCFM) was developed and demonstrated that the presence of aromatic amino acids is necessary to observe this effect. In that study, it was shown phenylalanine degradation genes were up-regulated 32 fold and interestingly our results show phenylalanine strongly induces the P_{paaA} genes (Table 4).

It is becoming increasingly apparent that bacterial regulation of catabolism plays a role in virulence gene expression. For example, in *Neisseria meningitidis* preferentially catabolizes lactose over glucose, and mutants deficient in lactose transport are unable to colonize some tissues. An explanation has been proposed that suggests because intermediates of lactate consumption feed directly into the sialylation pathway, this leads to more effective surface sialylation and thus immune evasion.

Recently, it has been shown that the presence of aromatic amino acids triggers the production of the intercellular communication signal (Pseudomonas Quinolone Signal) PQS produced by *P. aeruginosa*. It is hypothesized that since PQS and aromatic amino acids share biosynthetic precursors, it is metabolically advantageous for aromatic amino acids to serve as a signal for increasing PQS production.

5.10 Alternative Fates for PA-CoA

Based on these previous findings, we can speculate that compounds that feed into the PA pathway may provide a metabolic shortcut to the synthesis of compounds necessary for full virulence. Since it has been shown that a *paaABCDE* encoded ring hydroxylation complex is necessary for full virulence of *B. cenocepacia* in the *C. elegans* host model, and the end product of the ring hydroxylation complex is 2-hydroxy-phenylacetate-CoA, this compound may serve as a metabolic precursor for a virulence compound or mechanism. The fact that *B. cenocepacia* mutants in subsequent steps that further degrade PA do not result in decreased virulence in the *C. elegans* model, strongly suggests something to do with the upstream product PA-CoA.

5.11 Position Weight Matrix Analysis

We constructed a PWM using the IR from sequences *paaA*, *paaZ*, *paaH*, *BCAL0211* and *paaF*. The IR appears to be exclusive to the *paaA*, *paaZ* and *paaH* gene promoters. *BCAL0022* encodes for a putative “branched chain” amino acid transporter. It might be possible that this gene is involved in the transport of phenylalanine and thus co-regulation by a downstream metabolite of phenylalanine degradation, PA, may be beneficial in controlling expression

5.12 Formulation of Aerosolized CF Therapeutics

Some CF antibiotics are delivered by aerosol to provide a more targeted dose with less toxic side effects. Some of the formulations of these aerosols include aromatic compounds such as parabens (Kuhn 2001). These are additives which are used to adjust the properties of the aerosolized product. Likely, consideration has not been given to whether these aromatic compounds might have a secondary antagonistic effect. It is currently unclear whether aromatics used in the formulation of aerosolized CF therapeutics proceed via the PA pathway. This could be determined by using minimal media supplemented with glycerol and physiological doses of the therapeutic to be tested. Because of the finding that the presence of aromatic amino acids induce gene changes in *Pseudomonas*, and our findings that the PA pathway is induced in a host model of infection, further studies should be performed to determine if these additives are safe when used against *P. aeruginosa* or BCC infection. This may have been a previously unconsidered factor and may help in more effective clinical decisions. This would help avoid triggering some of the potential defensive or offensive mechanisms of *B. cenocepacia* as a result of exposure to small amounts of aromatics.

6 - CONCLUSIONS

The observation that a functional phenylacetic acid (PA) PaaABCDE ring hydroxylation is necessary for full virulence of *B. cenocepacia* in a *C. elegans* host model prompted us to explore the regulatory aspects of the PA genes in *B. cenocepacia*.

We show that the PA gene promoters are responsive to PA and other compounds expected to proceed via the PA pathway. We also show the PA gene promoters are subject to carbon catabolite repression in the presence different carbon sources. Interestingly, over early phases of growth, succinate exhibits stronger repression than glucose. The *paaA* promoter induced during growth in synthetic cystic fibrosis media (SCFM) and during infection of *C. elegans*.

Through genetic analysis we determined that interruption of gene *BCAL0210* results in de-repression of the PA gene promoters, and mutations in a conserved sequence in the core promoter of *paaA* results in a loss of PA dependent activity. The *paaABCDE* genes form an operon located on a single 3.9kb transcript. *BCAL0210* and another protein of unknown function form a separate operon immediately downstream of these genes.

A model for regulation of the PA genes is proposed, which is subject to biochemical confirmation. We speculate as to why the *paaABCDE* gene cluster is induced in a host model of infection. Likely, phenylalanine may play a role as its presence explains induction in SCFM and *C. elegans*.

It is hoped this work leads to a further understanding of aromatic catabolism and virulence of the opportunistic environmental pathogen *B. cenocepacia*.

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