The attenuated virulence of a *Burkholderia cenocepacia* K56-2 *paaABCDE* mutant is due to inhibition of quorum sensing by release of phenylacetic acid

By

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Abstract

Burkhoderia cenocepacia is one of 19 phenotypically similar but yet genetically distinct species collectively called as a *Burkholderia cepacia* complex. Bcc is known to possess large genomes, which encode for a variety of metabolic functions contributing to the high adaptability and successful survival in diverse environmental niches ranging from soil and water to human setting. *B. cenocepacia* is an opportunistic pathogen in patients with cystic fibrosis (CF), which is associated with high morbidity and mortality partially due to occurrence of cepacia syndrome, a rapid decline in respiratory functions and bacteremia. The pathogenesis of *B. cenocepacia* is multifactorial and attributed to various virulence factors encoded by its genome. Previous work in our laboratory has demonstrated that a functional phenylacetic acid (PAA) degradation pathway, particularly an intact ring hydroxylation complex, PaaABCDE is required for pathogenicity of *B. cenocepacia* in *Caenorhabditis elegans* host model; however, the reasons for such requirement were not understood. Based on preliminary data collected from metabolite filter diffusion assays, which have demonstrated that *B. cenocepacia* wild type filtrate (WT) was able to restore the attenuated pathogenicity phenotype of the insertional mutants of ring hydroxylation complex, *paaA* and *paaE*, we hypothesized that PAA derivatives released to the host environment might mediate the pathogenic phenotype. In addition to testing the bioactivity of potential metabolites present in the WT filtrate, the known intermediates such as 2-OHPAA and PAA were included; the latter has been detected in the supernatant of a newly generated mutant, in which a complete ring hydroxylation complex was deleted, a Δ*paaABCDE*. The mutant exhibited phenotypic characteristics similar to the insertional mutants. The exogenous addition of PAA further

decreased the pathogenicity of the Δ*paaABCDE* which cannot metabolize PAA, but did not affect the WT, due to active PAA consumption. In light of recent report showing the ability of PAA to interact with quorum sensing (QS) in *Pseudomonas aeruginsa*, we speculated whether the same phenomenon would be observed in *B. cenocepacia*. Using *Agrobacterium tumefaciens* biosensor strain we were able to detect reduced levels of acyl-homoserine lactones (AHL) in the supernatant of Δ*paaABCDE* when compared to the WT. In addition QS-regulated protease activity was abolished in Δ*paaABCDE* in the presence of PAA. These repressed phenotypes in Δ*paaABCDE,* which included protease activity and pathogenicity against *C. elegans,* increased with the addition of exogenous AHL demonstrating that the attenuated phenotype of *B. cenocepacia* Δ*paaABCDE* is due to QS inhibition by release of PAA, affecting AHL signaling. Taking these evidences together we were able to link the pathogenicity of *B. cenocepacia* with metabolism of PAA via QS. Since QS is a common feature of bacteria and the fact that CF infections are rather polymicrobial, interference of PAA with QS signaling of different bacteria is possible and can determine the outcomes of infection process. Nevertheless, the actual mechanism by which PAA inhibits QS signaling is to be determined. This work is an important dataset for further investigation the role of PAA in QS signaling inhibition and development of therapeutics that will target communication within polymicrobial communities.

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O'Grady E.P., Viteri D.F., Malott R.J., and Sokol P.A. (2009) Reciprocal regulation by the CepIR and CciIR quorum sensing systems in *Burkholderia cenocepacia*. *BMC Genomics* **10:** 441

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List of Abbreviations

1H-NMR	Proton Nuclear Magnetic Resonance
2-OHPAA	2-hydroxyphenylacetic acid
AHL	Acyl-homoserine-lactone
ACP	Acyl carrier protein
BCC	Burkholderia cepacia complex
BCESM	Burkholderia cepacia epidemic strain marker
BDSF	Burkholderia Diffusible Signal Factor
C4-HSL	*N*-butanoyl-homoserine lactone
C6-HSL	*N*-hexaoyl-homoserine lactone
C8-HSL	*N*-octanoyl-homoserine lactone
3O-C8-HSL	*N*-3oxo-octanoyl-homoserine lactone
3O-C12-HSL	*N*-3oxo-dodecanoyl-homoserine lactone
CF	Cystic Fibrosis
CFTR	Cystic Fibrosis Trnasmembrane Regulator
Cm	Chloramphenicol
CoA	Coenzyme A
DSF	Diffusible Signal Factor
ET-12	Electophoretic type 12
GC	Guanine Cytosine
GFP	Green Fluorescence Protein
Gm	Gentamycin
HPLC	High Pressure Liquid Chromatography

Km	Kanamycin
L4	Larva stage 4
LB	Luria Bertani
MLEE	Multilocus Enzyme Electrophoresis
Mpb	Mega base pair
MRSA	Methicillin-resistant Staphylococcus aureus
NGM	Nematode Growth Medium
OD_{600}	Optical Density measured at 600 nm
PAA	Phenylacetic acid
PCR	Polymerase Chain Reaction
QS	Quorum Sensing
SAM	*S*-adenosyl-L-methionine
SA	Salicylic acid
SD	Standard deviation
Tc	Tetracycline
TCA	Tricarboxylic acid
Tp	Trimetophrim
WT	Wild type

1. Introduction

1.1. *Burkholderia* genus.

The first *Burkholderia* species was discovered and identified as a causative agent of sour skin disease of onion in 1950 by phytopathologist Walter Burkholder (Burkholder, 1950). This species exhibited strong phenotypic similarities with phytopathogenic species of *Pseudomonas*; hence, was initially designated as a *Pseudomonas* species (Burkholder, 1950). Following the revision of *Pseudomonas* taxonomy this species was relocated into distinct taxonomic group creating a new genus *Burkholderia* (Yabuuchi *et al.*, 1992).To date the genus contains 62 distinctly described species that can be grouped into two main clades: pathogenic and non-pathogenic (Suarez-Moreno *et al.*, 2012). In the natural setting, such as in the rhizosphere, these bacteria can be found in symbiotic relationships with plants, participating in nitrogen fixation (Bontemps *et al.*, 2010) and plant protection (Parke *et al.*, 2001). In addition, some species are also known for their ability to degrade xenobiotic compound demonstrating their potential use as a tool in bioremediation (O'Sullivan *et al.*, 2005). Some species from *Burkholderia* genus have emerged as opportunistic pathogens in patients with cystic fibrosis (CF) (Isles *et al.*, 1984), chronic granulomatous disease (Speert *et al.*, 1994) and other medical conditions associated with a compromised immune system (Mahenthiralingam *et al.*, 1996; LiPuma,2010).Majority of those species belong to a distinct group that form the *Burkholderia cepacia* complex (Bcc).

1.1.1. *Burkholderia cepacia* complex

The species that are commonly identified as a *Burkholderia cepacia* complex (Bcc) (Vandamme *et al.*, 1997) is a group comprising at least seventeen species (Vanlaere *et al.*, 2009) with phenotypic similarities that are genetically distinct (Mahenthiralingam *et al.*, 2005). The Bcc are emerging opportunistic pathogens in CF patients and some immunocompromised individuals (Mahenthiralingam *et al.*, 1996; LiPuma, 2010). The significantly common isolates of Bcc recovered from patients are of *B. multivorans*, *B. dolosa* and *B. cenocepacia* (Reik R *et al.*, 2005). *B. cenocepacia* infections are also known for their patient to patient transmission nature (LiPuma *et al.*, 1990; Mahenthiralingam *et al.*, 1996) with the major epidemic strain, designated as an electrophoretic type 12 (ET-12), isolated and identified in Canadian and United Kingdom CF populations (Johnson *et al.*, 1994).

1.1.1.1. *Burkholderia cenocepacia* strains J2315 and K56-2

B. cenocepacia strains J2315 and K56-2 belong to ET-12, a highly transmissible lineage that was responsible for the transcontinental epidemic within CF populations in early nineties. Strains isolated from distantly located hospitals (United Kindom, Ireland, United States and Canada) were grouped based on their ET (Johnson *et al.*, 1994). An ET was determined using multilocus enzyme electrophoresis (MLEE), which assays mobility of several intracellular enzymes as the function of their amino acid composition and hence determining a total charge of the protein (Stanley *et al.*, 2003).

Majority of *B. cenocepacia* strains isolated from CF patients possess a conserved and unique DNA region, designated as the *Burkholderia cepacia* epidemic strain marker

(BCESM), which has been later applied as a clinical risk marker due to the transmissible nature of CF infection caused by *B. cenocepacia* (Mahenthiralingam *et al.*, 1996; Mahenthiralingam *et al.*, 1997). Although the presence of this marker does not correspond to the ability to colonize and establish infections, it indicates potency of the bacterium for patient to patient spread. The BCESM is located within a genomic island, designated *B. cenocepacia* island (*cci*) (Baldwin *et al.*, 2004), which contains genes important for metabolic functions such as fatty acid biosynthesis, amino acid metabolism and transport as well as genes encoding for pathogenic factors such as quorum sensing systems (Baldwin *et al.*, 2004).

The ability to colonize CF lungs demonstrates the highly adaptive nature, which is largely determined by the bacterial complex genome.

1.1.1.2. *B. cenocepacia* J2315 genome

Burkholderia species have evolved large multipartite genomes with high guanine-cytosine (GC) content and variety of genetic elements such as duplicated regions, prophage and genomic islands that accounts for the genomic plasticity (Holden *et al.*, 2009). The complete genome sequence of *B. cenocepacia* J2315 contains three circular replicons and a plasmid of a total size of 8.06 Mbps (Holden *et al.*, 2009). These replicons are referred to as chromosomes (c1-3) since they have been described to code for essential genes. Each of the replicons also encodes to ribosomal RNA genes, which represent a distinctive feature of chromosome and all three replicons are present in all sequenced Bcc strains. The majority of the housekeeping functions are encoded by the c1 while accessory functions of stress response and secondary metabolism are encoded on

c2 and c3 (Agnoli *et al.*, 2012; Agnoli *et al.*, 2014). Recently it has been proposed to reclassify c3 to mega plasmid pC3 since the loss of this replicon was proven to be not essential for culture growth in standard laboratory conditions (Bentley *et al.*, 2004; Agnoli *et al.*, 2012). The large and complex genome of *B. cenocepacia* encodes for enormous metabolic capabilities allowing *B. cenocepacia* to utilize a wide array of nutritional sources as well as to adapt to human host such as lungs of cystic fibrosis (CF) patients.

1.2. Cystic fibrosis

CF is an inherited genetic disorder caused by a mutation of the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) protein, which mainly functions as a chloride ion channel and is important in several mucosal surfaces including the lungs (Riordan *et al.*, 1989; Surette, 2014). There are number of hypotheses attempting to explain why abnormal function of CFTR results in a progressive pulmonary decline (Gibson *et al.*, 2003; O'Sullivan *et al.*, 2009). A portion of the explanation relies on the fact that dysfunctional CFTR alters the ionic composition, prevents a proper mucociliary clearance of airway surface fluid (Robinson *et al.*, 2002) or inactivating antimicrobial peptides (Hiemstra, 2007). As a consequence of impaired mucociliary clearance, the lung microbial community composition (Beck *et al.*, 2012) grows due to acquisition of new species from the air with some species becoming persistent and causing infectious process.

1.2.1. Poymicrobial infections in CF lungs

It is being rapidly acknowledged nowadays that CF infections are of polymicrobial nature meaning that there is a number of bacterial species that colonize lungs of CF patients simultaneously or interchangeably in an age dependent manner. Usually the first colonizers are *Staphylococcus aureus* and *Haemophilus influenza* followed by *Pseudomonas aeruginosa* (Lipuma, 2010), the most common opportunistic pathogen (Kato *et al.*, 2002). In a later course of the disease lungs are colonized by Bcc, *Stenotrophomonas maltophilia*, methicillin-resistant *S. aureus* (MRSA), non-tuberculous mycobacteria as well as by fungal species such as *Candida* spp. and *Aspergillus fumigates* (Surette, 2014). In addition to these conventional pathogens other species are present, which contribute to the pathogenesis directly or through synergy. Thus, the improvement of CF patient conditions after antibiotic treatment can be due to interruption or altering of the CF lung microbial community rather than primarily targeting the principal pathogen (Sibley *et al.*, 2011; Rabin *et al.*, 2012; Surette,2014).

Often the presence of *B. cenocepacia* strain from epidemic lineage ET12 is associated with occurrence of cepacia syndrome, a progressive decline in pulmonary function.

1.2.2 Cepacia syndrome

Initially, CF patients infected with *B. cenocepacia* were divided into to three groups based on the pattern of the disease symptoms: asymptomatic carriage, progressive decline in respiratory functions or fatal pneumonia (Isles *et al.*, 1984). The two latter patterns are mostly associated with cepacia syndrome, which is characterized by a high fever, rapid decline in pulmonary functions, leukocytosis, and abnormal rate of erythrocyte

sedimentation, bacteremia and sepsis (Isles *et al.*, 1984). It was believed that cepacia syndrome occurs relatively close to the initial colonization event (Isles *et al.*, 1984); however cases of cepacia syndrome occurring months or years later have also been reported (Dobbin *et al.*, 2000; Ledson *et al.*, 2002; Blackburn *et al.*, 2004). Although it was believed that cepacia syndrome is specific to *B. cenocepacia* infection, a case of cepacia syndrome observed in a CF patient infected with *B. multivorans* has been reported (Blackburn *et al.*, 2004). The cepacia syndrome caused by *B. cenocepacia* can be attributed to the arsenal of virulence factors produced by bacteria.

1.2.2.1. *B. cenocepacia* pathogenesis

B. cenocepacia pathogenesis is mediated via multiple virulence factors simultaneously rather than via a single element (Uehlinger *et al.*, 2009). The ability to establish infections inevitably correlates with the ability of bacteria to survive and persist in the host environment. Using signature-tagged mutagenesis approach, it has been shown that genes involved in metabolism, DNA repair and replication, regulatory functions, cell surface structures and transport functions are essential for the survival of *B. cenocepacia* in rat lung model (Hunt *et al.*, 2004).

Different sets of genes might be up or down regulated depending on the growth environment indicating an adaptation to certain niches. Comparative transcriptomics approach can analyze gene expression patterns as a function of nutrients available in the growth media. Therefore, genes that are upregulated under certain growth conditions that mimic CF sputum are believed to be involved in the virulence of *B. cenocepacia* during

lung infections (Drevinek *et al.*, 2008; Yoder-Himes *et al.*, 2009; Yoder-Himes *et al.*, 2010).

Among putative virulence factors of *B. cenocepacia* are cable pili, which facilitates bacterial colonization in CF lungs (Sun *et al.*, 1995; Tomich *et al.*, 2004); proteases, which target antibacterial peptides produced by human immune response (Corbett *et al.*, 2003; Kooi *et al.*, 2006); siderophores that acquire iron from the mammalian host environment (Sokol *et al.*, 1999; Sokol *et al.*, 2000; Visser *et al.*, 2004); and quorum sensing system, which regulates gene expression as function of the bacterial population density(Kothe *et al.*, 2003; Sokol *et al.*, 2003; Sokol *et al.*, 2007).

1.3. Quorum sensing in Gram- negative bacteria

Quorum sensing is a regulatory system that controls the expression of bacterial genes mostly related to virulence factors production in a cell density dependent manner. In Gram-negative bacteria, the system is composed of two proteins, autoinducer synthase (I) and transcriptional regulator (R). Originally quorum sensing (QS) phenomenon was reported in *Vibrio fischeri* where bioluminescence was viewed at high cell density and was regulated by two components: LuxI and LuxR proteins (Eberhard *et al.*, 1981). Therefore, QS identified later on were referred to as LuxI or LuxR type proteins or homologs. Autoinducer synthase catalyzes biosynthesis of autoinducer molecules, *N*-acyl-homoserine lactones (AHLs), from S-adenosyl-L-methionine (SAM), a precursor of a lactone ring group in AHL and acyl-acyl carrier protein (acyl-ACP), a precursor of an acyl chain (Hanzelka *et al.*, 1996), which is derived from metabolism of fatty acid. The acyl chain can vary in length from 4 to 18 carbons (Marketon *et al.*, 2002) and might

have a substituent such as oxo or hydroxyl groups on the third carbon of acyl chain (Churchill *et al.*, 2011). The same organism may produce more than one type of AHL molecule indicating the presence of more than just one synthase gene (Gotschlich *et al.*, 2001; Lutter *et al.*, 2001) resulting in the production of a mixture of AHL different in their acyl chain length or the presence of substituent. Synthesized AHL can passively or actively diffuse through the cell membrane (Pearson *et al.*, 1999). The AHL molecules are susceptible to chemical stresses such as alkaline pH (Byers *et al.*, 2002) or enzymatic degradation by lactonases and acylases (Huang *et al.*, 2003) terminating AHL signaling.

When a bacterial population increases in size, the amount of accumulating extracellular AHL reaches its threshold concentration allowing the recognition of AHL by its cognate protein regulator, which when bound to AHL becomes a DNA-binding transcriptional factor. Binding AHL at the C- terminus modulates DNA-binding abilities at N-terminus via a conformationally flexible linker (Churchill *et al.*, 2011) between these two domains in regulator resulting in either transcriptional activation or repression of targeted genes.

There are a small number of regulators that are found to be antagonized by the presence of AHL such as EsaR, a negative regulator from *Pantoea stewartii*, which represses gene transcription by binding to DNA in its AHL-free form (von Bodman *et al.*, 1998; Minogue *et al.*, 2002). Another way to mediate AHL response is via two-component system, which relies on phosphorylation cascade between membrane AHL sensor kinase and its cognate response regulator (Freeman *et al.*, 2000; Ryan *et al.*, 2008).

1.3.1. Quorum sensing in *B. cenocepacia*

B. cenocepacia possesses a number of QS systems, two of which are AHL-dependent: CepIR and CciIR. Opposite to it, a CepR2, an orphan LuxR- type transcriptional regulator is antagonized by AHL and mediate gene expression in pair with CepS, a regulatory protein belonging to the AraC family. Another, completely distinct QS system relies on production of diffusible signal factor (DFS) and a two component system, which influences gene expression via phosphorylation cascade triggered after recognition of DFS by sensor kinase . These QS systems are important for pathogenesis of *B. cenocepacia* during CF infections as the AHL (Chambers *et al.*, 2005; McKeon *et al.*, 2011) and DSF (Twomey *et al.*, 2012) production could be detected in the CF sputum.

1.3.1.1. CepIR QS system

The most conserved QS system in Bcc members consists of the *Vibrio fisheri* homologs LuxR and LuxI designated as CepR and CepI respectively (Lewenza *et al.*, 1999; Suppiger *et al.*, 2013). AHLs are synthesized by CepI, which is divergently transcribed from its cognate receptor protein, CepR. The major AHL molecule produced by CepI is *N*-octanoyl-homoserine lactone (C8-HSL) while *N*-hexanoyl-homoserine lactone (C6-HSL) is produced in a smaller amount. When found in complex, regulator-AHL, they activate or repress a number of virulence-related genes by binding to the consensus sequence in the promoter region of those genes (Lewenza *et al.*, 1999; Lewenza *et al.*, 2001). As in other AHL-regulated systems, AHL production in Bcc autoinduced; CepR negatively regulates its own transcription and positively regulates *cepI* expression (Lewenza *et al.*, 2001) (Figure 1).

In *B. cenocepacia* K56-2 protease production is positively regulates by CepIR (Lewenza *et al.*, 1999) while biosynthesis of ornibactin is under negative regulation of CepIR (Lewenza *et al.*, 2001).

1.3.1.2. CciIR QS system

The CciIR is a secondary signaling system in *B. cenocepacia* and is located on the genomic island *cci*. Since *cci* was found to be unique to epidemic CF isolates belonging to ET12 lineage, CciIR is found to be exclusive of *B. cenocepacia* strains belonging to that lineage. It is comprised of AHL synthase, CciI and its cognate receptor protein, CciR (Baldwin *et al.*, 2004). The major AHL molecule produced by CciI is C6-HSL while C8-HSL is produced in a smaller amount. Both genes are found in a single operon and co-transcribed from a putative promoter located upstream to *cciI* gene (Malott *et al.*, 2005). There is a regulatory interplay between CepIR and CciIR systems with the CciIR incorporating into previously established CepIR generating a tuned hierarchy between two systems (Figure 1). CepR is essential for the expression of CciIR gene cluster whereas CciR participates in negative autoregulation as well as negative regulation of *cepI* gene expression (Malott *et al.*, 2005). Using micorarrays studies CepR has been shown to exert mostly positive regulation on targeted genes while CciR regulates the same genes in an inverse manner (O'Grady *et al.*, 2009).

A CciIR system present K56-2 has been shown to regulate protease and swarming motility without influencing ornibactin production (Malott *et al.*, 2005).

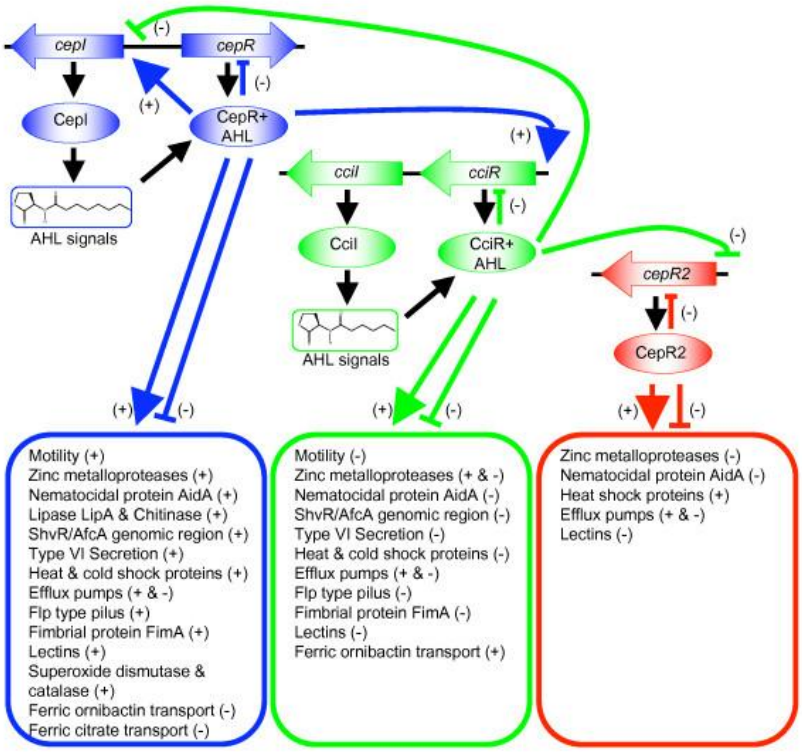


Figure 1. **Reciprocal regulation by the CepIR and CciIR quorum sensing systems in *B. cenocepacia*** (O'Grady *et al.*, 2009). CepIR and CciIR are both AHL- dependent quorum sensing systems while orphan CepR2 regulator is acting in the AHL independent manner. CepR-AHL complex is positively regulates *cepI* expression while employing a negative feedback in regulation of its own production similarly to transcriptional feedback employed by CciR-AHL comlex in CciIR regulatory recruit. In addition CciIR-AHL negatively regulates *cepR2* expression. In addition to the regulatory hierarchy between CepIR and CciIR systems, there is also a regulatory interplay. CciR-AHL complex negatively regulates *cepI* while CepR-AHL positively regulates *cciIR* expression creating a tuned response to a signaling.

1.3.1.3. CepR2 orphan LuxR-homolog

The number of LuxR homologs existing in bacteria is often greater than the number of its cognate LuxI homologs(Fuqua, 2006). Despite being unlinked to any specific LuxI homolog, these orphan LuxR proteins are still functional and predicted to respond to endogenous or exogenous AHLs. Although other *Burkholderia* species such as *B. pseudomallei*, *B. mallei* and *B. vietnamiensis* are known to posses LuxR homologs, CepR2 is only found in *B. cenocepacia* strains belonging to both epidemic (strain K56-2) and non epidemic (strain H111) lineages (Malott *et al.*, 2009); however, the regulation mechanism of CepR2 differs depending on which lineage the strain belongs to. Epidemic strains such as K56-2 contain additional QS, CciIR, which might alter regulatory hierarchy existing between different QS systems (Malott *et al.*, 2005).

In *B. cenocepacia* K56-2 CepR2 does not participate in transcriptional regulation of *cepIR* or *cciIR*. However, CepR2 is negatively autoregulated and is also found to be under negative regulation by CciIR (Figure 1). Furthermore, CepR2 activity can be blocked by the presence of AHL (O'Grady *et al.*, 2009). CepR2 was been shown to regulate expression of genes located in all three chromosomes in AHL independent manner (Malott *et al.*, 2009; Ryan *et al.*, 2013). The *cepR2* gene is located on the c2 and is adjacent to a divergently transcribed gene BCAM0189, which is predicted to encode an AraC-family regulatory protein, CepS (Ryan *et al.*, 2013). The proposed model of co-regulation of a cluster of linked genes by CepR2/ CepS relies on CepR2 binding to the DNA inhibits CepS activity, which positively regulates gene expression. Accumulation of AHL at high cell density blocks CepR2 activity removing repression imposed by CepR2 from CepS (Ryan *et al.*, 2013).

CepR2 is negatively regulates proteolytic activity in K56-2. The QS- regulated activities such as biofilm formation, swarming and AHL production were observed to be not affected by the CepR2 while the genes related to virulence, chemotaxis, heat shock response and signal transduction are regulated mainly through repression by CepR2 (Malott *et al.*, 2009).

1.3.1.4. *Burkholderia cenocepacia* diffusible signal factor

Burkholderia cenocepacia diffusible signal factor (BDSF) is a structural analogue of a new family of extracellular fatty acid type signaling molecules called diffusible signal factors (DSFs), involved in bacterial as well as fungal quorum sensing (Wang *et al.*, 2004) that were originally identified in *Xhanthomonas campestris*, a Gram-negative pythopathogen (Barber *et al.*, 1997). In *X. campestris*, a RpfF protein is required for DSF biosynthesis while DSF recognition and signal transduction is mediated via two component system comprised of kinase encoded by *rpfC* gene and its response regulator encoded by *rpfG* (Slater *et al.*, 2000).

B. cenocepacia has a functional homologue of RpfF, an $RpfF_{Bc}$, encoded by BCAM0581 gene which belongs to crotonase superfamily with dehydratase and thioesterase activities and is required for biosynthesis of BDSF (Bi *et al.*, 2012). The BDSF perception is mediated via RpfR protein, a BDSF sensor receptor, which has enzymatic abilities to module cyclic-di-GMP levels via diguanylate cyclase and phosphodiesterase activities, possesses by the protein (Deng *et al.*, 2012). Upon binding of BDSF to RpfR, the phosphodiesterase activity is stimulated, which lowers cyclic-di-GMP levels and as a result affects a number of bacterial phenotypes such as motility, biofilm formation and expression of certain virulence factors (Hengge,2009; McCarthy *et*

al., 2010). The mentioned above phenotypes are also controlled by AHL-dependent systems indicating the overlapping regulation between distinct QS systems in *B. cenocepacia* (Schmid *et al.*, 2012; Suppiger *et al.*, 2013). Opposed to hierarchical circuit existing between CepIR and CciIR systems, the BDSF signaling system is believed to regulate the same set of gene in independent from CepIR manner (Schmid *et al.*, 2012).

The BDSF signaling systems is involved in positive regulation of several functions including protease production and biofilm formation and is found to be highly conserved among Bcc species (Deng *et al.*, 2012; Schmid *et al.*, 2012). In addition, the BDSF was able to inhibit the germ tube formation in *C. albicans* required for morphological transition of fungal pathogen to a virulent or a filamentous cell type demonstrating interkingdom signaling capacities of DSF-family molecules (Boon *et al.*, 2008).

1.3.2. Quorum sensing inhibition

The AHL dependent signaling inhibition can be achieved by targeting production or structure of AHL molecule or activity of AHL synthase and its cognate receptor protein. The mechanisms, which target AHL molecule fall in two main groups: an enzymatic degradation of AHL molecules, commonly referred as to quorum quenching, and blocking the regulator protein by compounds mimicking AHL structure, which prevents subsequent binding to targeted genes' promoter (Czajkowski *et al.*, 2009). AHL degrading enzymes, such as lactonases and decarboxylases, direct lactone ring opening while other enzymes, such as acylases or deaminases, break AHL to homoserine lactone and acyl side chain (Dong *et al.*, 2005).

1.3.3. Interspecies communication: *Pseudomonas-Burkholderia* interactions via AHL signaling

P. aeruginosa possesses two AHL- dependent QS systems, LsIR and RhlIR, which have been shown to regulate expression of many genes related to virulence (de Kievit *et al.*, 2000). LasI is responsible for production of *N*-3-oxododecanoyl-L-homoserine lactone (3O-C12-HSL) as a main molecule and a smaller amount of *N*-3-oxooctanoyl-L-homoserine lactone (3O-C8-HSL) and molecules (Pearson *et al.*, 1995). RhlI is responsible for production of *N*-butanoyl-L- homoserine lactone (C4-HSL) as a main signal molecule and C8-HSL in a smaller amount (Pearson *et al.*, 1995). In addition, LasR and RhlR proteins have been shown to be responsive to AHL molecules of appropriate range in length regardless to 3-oxo group substitution (Winson *et al.*, 1995; Passador *et al.*, 1996). In *B. cenocepacia* CepI directs synthesis of and a minor amount of C8-HSL. Although synthases direct production of specific AHL molecules, very often their cognate regulator are able to response to wider range of AHL molecules (Lutter *et al.*, 2001). Hence, the interspecies communication between *P. aeruginosa* and *B. cenocepacia* can be mediated via AHL molecules. Various factors such as bacterial strains, regulator sensitivity to AHL, and promoter affinity towards AHL-bound regulator protein are important in establishing the unidirectional communication between the species and determining the efficiency of the signaling (Riedel *et al.*, 2001; Lewenza *et al.*, 2002). To study AHL signaling as well as AHL production and secretion by the microorganisms is possible due to availability of biosensor strains that are genetically engineered to detect exogenous AHL molecules present in their growth environment.

1.3.4. Bacterial biosensor systems for AHL detection

The biosensor systems are bacterial strains that are genetically designed to phenotypically respond to detected AHL molecules present in the medium they are grown in (Steindler *et al.*, 2007). In Gram- negative bacteria biosensor systems rely on the LuxRI-type family of QS system (Steindler *et al.*, 2007). The main features of a typical biosensor or reporter strains are: (1) the lack of LuxI type synthase gene, which result in loss of AHL molecules production, (2) reporter gene under the positive regulation of the LuxI promoter, and (3) a functional LuxR-type regulator. The reporter genes are typically encode for a product with visually distinctive phenotype such as bioluminescence, green fluorescence or products that will cause a spectrophotometric changes in the medium during the assay. A choice of biosensor system is dependent on the length and substitution of an acyl chain of AHL molecules to be detected. *Chromobacterium violaceum* is mainly used to detect shorter acyl chains of C4 to C8 in length (McClean *et al.*, 1997) while *Agrobacterium tumefaciens* is able to detect a wider range of AHL including a long acyl chains of C10 and/or with substitution on the C3 position (Zhu *et al.*, 2003).

1.3.4.1. *Agrobacterium tumefaciens* as a reporter strain to detect AHL produced by *B. cenocepacia*

Agrobacterium tumefaciens is a Gram- negative bacterium, closely related to *Rhizobium*. It is a plant pathogen known to cause a crown gall disease mostly in dicotyledonous plant (broad leaves) such as rose family of trees (Escobar *et al.*, 2003). The disease is characterized by the development of tumor like swelling in the crown

section of the plant. The virulence factors of *A. tumefaciens* are encoded on oncogenic Ti plasmid, which contains and is regulated by TraIR QS. As classical AHL dependent QS, TraIR is composed of TraI, a 3-oxo-C8-HSL synthease and its cognate transcriptional regulator, TraR. TraR positively regulate *traI* expression.

A. tumefaciens strain A136 was developed to act as biosensor strain able to respond to AHLs with acyl chains of 4 to 12 carbons with a preference toward longer acyl chains and AHLs with 3-oxo group substitutions (Sokol *et al.,* 2003; Chambers *et al.*, 2005; Bernier *et al.*, 2008). The strain A136 is an avirulent derivative of *A. tumefaciens* that lost the ability to induce crown galls disease as a result of Ti plasmid loss (Watson *et al.*, 1975). Since *traIR* system is encoded by Ti (Fuqua *et al.,* 1994), elimination of the plasmid rendered A136 without QS. Next, a plasmid constitutively expressing TraR and a plasmid containing *luxCDABE* gene cluster under *traI* promoter were introduced into A136. Since *traI* is known to be positively regulated by TraR (Fuqua *et al.*, 1994); in the presence of AHL TraR will bind AHL and as a complex will bind at *traI* promoter sequence triggering *luxCDABE* expression and as a result production of bioluminescence.

1.4. Host models to study *B. cenocepacia* pathogenesis

The ability of *B. cenocepacia* to cause a disease is studied using variety of host model including insects, plant, nematodes and mammals. Depending on the host model, specific factors might be required to establish infections (Uehlinger *et al.*, 2009). *Caenorhabditis elegans* is a soil nematode, which is extensively used in the various areas of a research ranging from neural development in animals (Brenner,1974), aging process (Olsen *et al.*, 2006) and host pathogen interactions (Aballay *et al.*, 2002). The maintenance of *C.*

elegans in laboratory conditions is relatively simple due to short life span and rapid generation time. The sequenced genome of *C. elegans* has a large number of vertebrate orthologues as well as conserved elements of eukaryotic innate immune system important in studying human pathogens. The pathogenic phenotype of bacterial strain tested can be easily determined due to small and transparent bodies of *C. elegans* as well as time-defined development stages.

As a typical bacteriovore, *C elegans* is propagated on the slow growing non pathogenic *E. coli* OP50. To internalize the bacterial food such as OP50 into the intestine, nematodes contract the pharyngeal muscle. The bacteria are ground inside the pharynx, a neuromuscular pump that joints mouth to intestine. Hence, no intact OP50 cells can be found in the intestine. However, some human pathogens such as *P. aeruginosa* and *B. cenocepacia* are able to escape the grinder successfully reaching and proliferating inside the intestinal lumen. Colonizing bacteria causes infection and intact bacterial cells can be recovered from nematodes. In this study we used *C. elegans* DH26 strain, which is a temperature sensitive strain. Due to mutation causing defective spermatogenesis adult worms become sterile at the restrictive temperature of 25 ºC and cannot lay eggs, hence, allowing proper counting during the assay. *C. elegans* is a facile host model, to study chronic respiratory infections or human inherited diseases vertebrate models are used.

One of the common vertebrate or mammalian models are murine, which were developed to study chronic respiratory infection (Sokol *et al.,* 1999; Sokol *et al.,* 2000; Corbett *et al.*, 2003; Sokol *et al.*, 2003). Trachea of the murine are directly inoculated with agar beads covered with bacteria, allowed to establish infectious process in the lungs. The post infection outcomes are tested for any histopathological changes while

microbial count is done using lung tissue homogenate. The bacterial infection is considered invasive if bacterial cells can be recovered from the spleen tissue located in physical proximity to lungs (Cieri *et al.*, 2002). While chronic infections occurs via course of lung colonization when bacteria inoculated on agar beads are introduced into the animal, the acute infections are mimicked via aerosol inoculation of lungs to CFTR-deficient mice (Sokol *et al.*, 1999; Corbett *et al.*, 2003).

It is also possible to study Bcc infections in plant model, which include use of alfalfa and onion rot model (Aguilar *et al.*, 2003; Bernier *et al.*, 2003). Alfalfa might be not suitable to examine complex respiratory interactions but it serves as an alternative approach to preliminary analysis of the potential virulence of new isolated *B. cenocepacia* (Bernier *et al.*, 2003). Onion tissue maceration *in vitro* has been observed to be more severe and predominant in environmental isolates of Bcc (Wigley *et al.*, 1999).

1.4.1. Virulence factors required for efficient infection killing of *C. elegans*

The time that takes to kill nematodes when infected with pathogenic bacteria depends on the bacterial strain and type of media used for the assay. When grown on standard nematode growth medium (NGM), the killing occurs over the number of days and proceeds in an infection-like mode, hence this mode of killing is called slow (Tan *et al.*, 1999). On the other hand, the fast killing occurs when bacteria are grown on high osmolarity medium such as peptone-glucose-sorbitol (PGS) medium and the death of nematodes occurs within couple of hours (Tan *et al.*, 1999). This is shown to be a result of the presence of bacterial toxins, which are released during bacterial growth. Although the initial model was developed for *P. aeruginosa* (Tan *et al.*, 2000), the *C. elegans*

killing assays are also used to study the pathogenesis of *B. cenocepacia* (Kothe *et al.*, 2003; Law *et al.*, 2008).

One of the requirements for efficient slow killing of *C. elegans* by *B. cenocepacia* strain H11 is a functional CepIR QS system (Kothe *et al.*, 2003). It has been shown that CepIR system is required for bacteria to bypass the grinder barrier and reach the intestinal lumen. The *cepI* mutant, who has the attenuated virulence phenotype, was not able to colonize nematode intestinal; however, when the partially nonfunctional grinder nematodes were used, the intestinal colonization by GFP-*cep* mutant was observed. Although the *cepI* mutant could persist in intestinal lumen of grinder dysfunctional nematodes, it did not restore the virulence phenotype indicating the colonization per se is not sufficient enough to kill *C. elegans*, but rather the production of various QS-regulated factors are involved in the killing (Kothe *et al.*, 2003). The requirement of functional CepIR QS has not been yet experimentally demonstrated for K56-2 strain.

Another important factor required for efficient killing of nematode by bacterial is a nematocidal protein AidA (Huber *et al.*, 2004; Uehlinger *et al.*, 2009). Originally identified in *B. cenocepacia* strain H111, it is widely distributed among Bcc including *B. cenocepacia* strains J2315 and K56-2 (Huber *et al.*, 2004). AidA production is regulated by CepIR QS system. It is not required for common QS-regulated functions such as biofilm production and swarming nor proteases or siderophores production. Mutation in *aidA* gene causes an attenuation of pathogenicity phenotype in nematodes and inability to colonize the intestinal lumen even in the *C. elegans* dysfunctional grinder strain indicating that AidA protein is important in bacterial persistence in nematode (Huber *et al.*, 2004).

Functional enzymes of the purine biosynthesis pathway have been also found to be important for efficient *C. elegans* killing by *B. cenocepacia* strain H111. The auxotrophic mutants in purine production exhibited an attenuated pathogenicity phenotype that could be restored with the addition of the precursor intermediate, whose production step was impaired or the final product of the pathway (Schwager *et al.*, 2013). All of these enzymes are essential for the biosynthesis pathway since mutations in them cause a growth defective phenotype. However, the auxotrophy per se is not the reason for attenuation in virulence but rather it is likely to be the absence or lack of specific metabolites (Schwager *et al.*, 2013).

The intact phenylacetic acid (PAA) pathway is also required for the full pathogenicity in *C. elegans* model (Law *et al.*, 2008). *B. cenocepacia paaA* and *paaE* mutants, in which the *paaABCDE* gene cluster was interrupted, were defective in PAA degradation and were attenuated for virulence in the nematode host model *C. elegans* while mutants of the ring opening or beta oxidation steps were not (Law *et al.*, 2008).

1.5. Phenylacetic acid degradation pathway

Phenylacetic acid is a key intermediate in the degradation of phenylalanine, lignin-related aromatic compounds, and environmental pollutants (Schleissner *et al.*, 1994; Luengo *et al.*, 2001; Jimenez *et al.*, 2002; Ismail *et al.*, 2003), and is found in several bacteria (Martin *et al.*, 2009). So far it has been mostly investigated in a handful of microorganisms but recently it has emerged as a putative pathogenicity factor (Hunt *et al.*, 2004; Law *et al.*, 2008). The 14 genes responsible for PAA degradation form the *paa* gene cluster and are organized in one or three transcriptional units in *Escherichia coli*

(Ferrandez *et al.*, 1998; Ferrandez *et al.*, 2000) and *Pseudomonas putida* (Olivera *et al.*, 1998), respectively. The underlying biochemical mechanisms that convert PA into the TCA cycle intermediate succinyl-CoA have been elucidated in *E. coli* K12 and *Pseudomonas* sp. strain Y2 (Figure 2). PAA (Compound **1**, Figure 2) is first ligated to CoA by the phenylacetyl CoA ligase PaaK (Martinez-Blanco *et al.*, 1990; El-Said Mohamed, 2000). The Coenzyme A thioester of PAA (Compound **3**, Figure 2) is then oxidized to a 1,2-epoxyPAA-CoA (Compound **4**, Figure 2) by a complex of PaaABCDE enzymes (Teufel *et al.*, 2012). The epoxide is isomerized to the ring-expanded oxepin (Compound **5**, Figure 2) by PaaG, which is in turn transformed to 2,3-dehydroadipyl-CoA in a series of ring-opening and oxidation steps, where PaaZ cleaves the ring in the presence of PaaG, producing a highly reactive aldehyde that is oxidized to 3-oxo-5,6-dehydrosuberyl-CoA (Compound **6**, Figure 2) (Teufel *et al.*, 2011), PaaJ transforms the resulting ß-keto C_8 intermediate to 2,3-dehydroadipyl-CoA and PaaF catalyzes the subsequent conversion of the C_6 intermediate to 3-hydroxyadipyl-CoA (Teufel *et al.*, 2010). It is believed that in case of pathway overflow, accumulation of the toxic epoxide (Compound **4**, Figure 2) is avoided by rapid conversion to PAA by the reversible action of the PaaABCDE complex (Teufel *et al.*, 2012) and PaaI (Song *et al.*, 2006) or by the release of the dead-end product 2- hy hydroxyphenylacetic acid (2-OHPAA) (Compound **2**, Figure 2) (Grishin *et al.*, 2011) a shunt metabolite that does not feed back into the catabolism pathway.

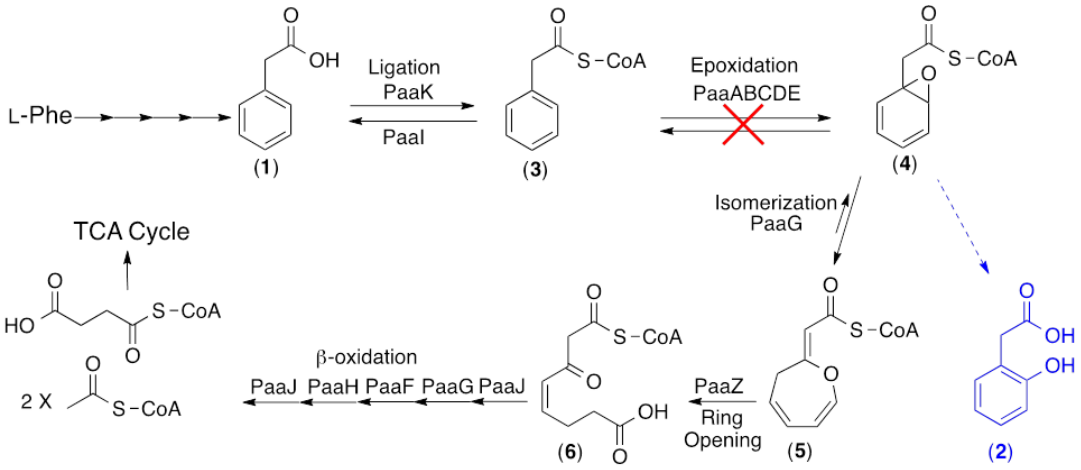


Figure 2. **Proposed PAA degradation pathway based on studies in *E. coli* and *Pseudomonas sp.*** The enzyme names according to Teufel *et al.* (Teufel *et al.*, 2012). Putative intermediates of the pathway: (**1**) PAA, (**2**) 2-hydroxyplenylacetic acid (2OHPAA), (**3**) PAA-CoA, (**4**) ring-1,2-epoxyphenylacetyl- CoA or epoxide, (**5**) ring-1,2-epoxyphenylacetyl- CoA or oxepin-CoA, (**6**) 3-oxo-5,6- dehydrosuberyl-CoA. The end products of the pathway are acetyl-CoA and succinyl-CoA. The enzymatic step, interrupted by the deletion of the *paaABCDE* gene cluster in *B. cenocepacia* K56-2 mutant TNP1, is denoted. The figure was created by Dr. Sorensen.

1.5.1. Phenylacetic acid pathway as a pathogenic factor

Phenylacetic acid degradation pathway first emerged as a putative pathogenic factor during the identification of genes important for bacterial survival in a rat lung infection model by signature-tagged transposon mutangenesis (Hunt *et al.*, 2004). The intact PAA pathway is also required for the full pathogenicity in *C. elegans* model (Law *et al.*, 2008). *B. cenocepacia paaA* and *paaE* mutants, in which the *paaABCDE* gene cluster was interrupted, were defective in PAA degradation and were attenuated for virulence in the nematode host model *C. elegans* while mutants of the ring opening or beta oxidation steps were not (Law *et al.*, 2008). The PAA degradation pathway is a source of potentially toxic compounds such as epoxide and aldehyde as well as intermediates that serves as a building materials and dead end product, all of those might potentially be involved in mediating the virulence of *B. cenocepacia* in *C. elegans* (Ismail *et al.*, 2003; Grishin *et al.*, 2011; Teufel *et al.*, 2012).

It has been also shown the PAA degradation pathway is highly active when *B. cenocepacia* is grown on LB medium and in conditions that mimic the amino acid-rich environment of the cystic fibrosis sputum (Hamlin *et al.*, 2009; Yoder-Himes *et al.*, 2009; Yudistira *et al.*, 2011) where Bcc thrives. Since PAA pathway is a central pathway where variety of aromatic compounds are converged and directed to TCA cycle, the importance of the active pathway may is in ability to efficiently utilize wide range of nutrients present in host environment as competitive advantage over other co-colonizing bacteria.

1.5.2. Phenylacetic acid interaction with quorum sensing system

Recent studies have shown the ability of PAA to interact with QS in *P. aeruginosa*, the most common cystic fibrosis pathogen (Kato *et al.*, 2002). The PAA was proposed to act as an antagonist of pathogenic responses in *P. aeruginosa* PAO1 strain (Musthafa *et al.*, 2012) where exogenous addition of PAA into the *C. elegans* slow assay resulted in an attenuation of pathogenic phenotype of PAO1 wild type strain as well as number of QS-regulated activities such as exoenzymes productions and motility were shown to be reduced in the presence of PAA providing a link between PAA and virulence via QS. Production of PAA by *P. aeruginosa* at high cell density levels was also demonstrated to reduce cytotoxicity (Wang *et al.*, 2013). Exogenous addition of synthetic PAA inhibited virulence traits (Musthafa *et al.*, 2012) known to be regulated by quorum sensing (QS) (Smith *et al.*, 2003; Schuster *et al.*, 2006). *P. aeruginosa* genomes do not clearly encode PAA metabolism enzymes (Wang *et al.*, 2013), so how PAA is produced or whether exogenous PAA is internalized and metabolized in *P. aeruginosa* is unknown. Although a direct involvement of QS systems was not demonstrated during these studies, a possible link between PAA metabolism and QS-regulated virulence emerged.

2. Rationale and Hypothesis

The previous work in our laboratory had demonstrated that mutations in ring hydroxylation complex, PaaABCDE cause attenuation in pathogenic phenotype of *B. cenocepacia* K56-2 indicating that this system is required for full infection in *C. elegans*. On the other hand mutations in enzymes involved in ring opening, PaaZ, and beta oxidation, PaaF, steps cause K56-2 to remain pathogenic (Law *et al.*, 2008). Phenolic content of supernatant from mutants of downstream step of the pathway was higher when compared to K56-2 WT while the interruption of ring hydroxylation caused a slight decrease (Law *et al.*, 2008). In addition preliminary experiments showed that wild type agar filtrate is able partially restore pathogenic phenotype of *paaA* insertional mutant (Law, 2009). These evidences led us to hypothesize that the pathogenicity of K56-2 in *C. elegans* might be mediated by the presence or absence of PAA or PAA derivatives, which are released from the pathway into the host environment. In light of recent evidence, which demonstrated that PAA has the ability to interact with QS of *P. aeruginosa* (Musthafa *et al.*, 2012), we also decided to investigate if this interaction exists in *B. cenocepacia* K56-2 strains. The specific objectives of this study were: to confirm the presence of released metabolites or intermediates from the pathway in bacterial filtrate, which are involved in pathogenicity, by using metabolite filter assay; to test the effect of isolated metabolite on pathogenic phenotype of K56-2 wild type and mutant strains, using nematode model; and if found bioactive to test the involvement of QS in pathogenicity towards *C. elegans*.

3. Materials and methods

3.1. Bacterial Strains, Nematodes, and growth conditions

Bacterial strains and plasmids are listed in Table 1. *B. cenocepacia* K56-2 strains were grown at 37 °C in Luria Bertani (LB) medium, M9 medium (Sambrook *et al.*, 2001) or modified nematode growth medium (NGM) (Tan *et al.*, 1999) containing 3.5 g/ L peptone. Overnight cultures of the reporter strains *Agrobacterium tumefaciens* A136 (pCF218)(pMV26) were grown at 30°C in LB containing 4.5 μg/ mL of tetracycline (Tc) and 25 μg/ mL of kanamycin (Km). The growth of liquid culture was measured at optical density at 600nm (OD_{600}). Slow killing assays were performed using *Caenorhabditis elegans* temperature sensitive strain DH26, which is defective in fertilization at 25°C. *C. elegans* DH26 and *E. coli* OP50 were obtained from the *Caenorhabditis* Genetic Center (CGC, University of Minnesota, Minneapolis, USA). A general stock of *C. elegans* DH26 was maintained on NGM I containing 2.5 g/ L of peptone (Brenner, 1974) at 15 °C with OP50 as a food source. For slow killing and protease activity assays, synthetic *N*-octanoyl-DL-homoserine lactone (C8-HSL), phenylacetic acid (PAA) and 2-hydroxyphenylacetic acid (2-OHPAA) were purchased from Sigma Aldrich. Working stock of 100 mM PAA and 2-OHPAA was prepared by dissolving the compound in water and kept at the room temperature. To prepare 1 mM of C8-HSL working stock, the compound was dissolved in ethyl acetate and kept at -4 °C. PAA or 2-OHPAA was added directly to the cooled NGM prior to pouring the plates, to a final concentration of 1 or 5 mM. To prepare NGM supplemented with 1 μM C8-HSL, the concentrated working stock C8-HSL was spotted on the small Petri plates, allowed to dry and then the medium was poured following mixing throughout.

Table 1. **Bacterial strains and plasmids**

Strain or plasmid	Features	Reference or source
B. cenocepacia		
K56-2 (LMG18863)	WT strain, ET12 clone related to J2315, cystic fibrosis clinic isolate	(Mahenthiralingam *et al.*, 2000)
TNP1	K56-2 Δ*paaABCDE*	This study
E. coli		
DH5α	F- φ80 *lacZΔ*M15 *endA1 recA1 hsdR17*(r_K^- m_K^+) *supE44 thi-1 Δ gyrA96 (ΔlacZYAarg-F)U169 rel*A1	Invitrogen
OP50	Uracil auxotroph	*Caenorhabditis* Genetic Center (CGC, University of Minnesota, Minneapolis, USA)
SY327	*araD* Δ(*lac pro*) *argE* (Am) *recA*56 Rif^r *nalA* λ *pir*	(Miller *et al.*, 1988)
A. tumefaciens		
A136 (pCF218)(pMV26)	*traI-luxCDABE* biosensor strain	(Sokol *et al.*, 2003; Chambers *et al.*, 2005)
Plasmids		
pRK2013	Helper plasmid, Kan^r	(Figurski *et al.*, 1979)
pGPI-SceI	DHFR, I-SceI recognition sequence, Tp^r	(Flannagan *et al.*, 2008)
pDAI-SceI	DHFR promoter controlling e-ISce-I, Tc^r	(Flannagan *et al.*, 2008)
pAP20	ori_{PBBR1}, DHFR, Cm^R Cm duplicated region deleted	(Law *et al.*, 2008)
pTP1	pGPI-SceI, *paaABCDE*	This study
pTP2	pAP20, *paaABCDE*	This study

Kan, Kanamycin; Tp, Trimethoprim; Rif, Rifamycim; Cm, Chloramphenicol

3.2. Bacterial measurements and normalization of bacterial lawns

Inocula were prepared by taking bacterial strains from frozen glycerol stocks and growing them in LB for overnight. After washing the cultures with M9 medium, the cells were used to inoculate either NGM or M9 medium supplemented with glycerol or PAA as a sole carbon source. The growth kinetics were carried out on 96-well microtiter plates containing 150 μL of media with the bacterial inoculum adjusted to a starting optical density at 600 nm (OD_{600}) of 0.04. Microplates were incubated for 24 hours at 37 °C with shaking (200 rpm). The OD_{600} was measured using a Biotek Synergy 2 plate reader at interval of 1 hour, and values were converted to 1-cm-path-lenth OD_{600} by prior calibration with a GeneQuant^{TM} III 4283, version 4283V1.6.

To examine the growth on NGM plates supplemented with or without PAA, 50 μL of bacterial overnight cultures were adjusted to OD_{600} of 1.7, spread on small Petri plates and allowed to grow for 48 hours incubating the first 24 hours at 37 °C following an additional incubation for 24 hours at 25 °C (to mimic the initial steps of the killing assay standard procedure). After incubation, the plates were washed with 1 mL phosphate buffered saline (PBS) to remove the bacterial lawns and the cell suspensions were further diluted to estimate bacterial growth by measuring OD_{600}.

To ensure that slow killing assays on NGM with and without PAA were initiated with the same number of bacteria, the bacterial lawns were normalized to an OD_{600} equivalent to the growth of *B. cenocepacia* K56-2 WT on NGM by plating the normalized cell suspensions back to the same plates immediately before adding the worms.

3.3. Egg preparation and harvesting of synchronous *C. elegans* DH26 population

NGMI plates with *E. coli* OP50 lawn containing good amount of gravid worms and laid eggs were washed off with water into 16 mL snap cap tube to the final volume of 5 mL. The resulted worm suspension was treated with 1.5 mL of freshly made bleach mix (0.6 mL sodium hypochlorite, 0.5 mL sodium chloride, 0.4 mL water) and vortexed every minute for 3 minutes. The mixture was then aliquoted to four 1.5 mL centrifuge tubes and spun down (30 sec, 3500 rpm). The supernatant was aspirated to 100 μL and the pellet was resuspended with 1 mL water and spun down again. This step was repeated twice. After final aspiration the resulted pellet was spotted on bacteria free NGM plates till the next morning as an additional step for better synchronization, which allows all eggs to hatch and develop to L1 stage. Further development is arrested since no bacterial food is present. The L1 worms were then transferred to NGM plates with OP50 lawns to complete their development to L4 stage that they would reach next day afternoon.

3.4. Slow killing assay

C. elegans slow killing assays were performed as previously published (Law *et al.*, 2008). Small (60mm) Petri plates filled with NGM were seeded with 50 μL of stationary phase culture adjusted to an OD_{600} of 1.7. Plates were incubated overnight at 37 °C to allow formation of a bacterial lawn before 20 to 40 hypochlorite-synchronized L4 larvae of *C. elegans* DH26 were inoculated to each plate and then incubated at 25 °C for the duration of the assay. Plates were scored for live worms at the time of inoculation and every 24 hours subsequently for 5 to 7 days using a zoom stereomicroscope (Nikon SMZ 745). Worms were considered dead when unresponsive to touch with a sterile wire pick. Assays were performed in duplicate at least and analyzed using survival curves generated

by the Kaplan-Meier statistical method. The long rank test was used to compare survival differences for statistical significance using GraphPad Prism, version 5.02. A *p* values <0.05 were considered to be significant. In all assays OP50 was used as a control.

3.5. Chemical complementation assay

To assess the effect of PAA, 2-OHPAA or C8-HSL on bacterial pathogenicity phenotype in *C. elegans* these chemical were added to the slow killing assay standard NGM.

PAA or 2-OHPAA was added to the cooled medium prior to pouring. The assay on NGM supplemented with or without PAA was modified due to significant difference in the formed bacterial lawn between WT and TNP1. To ensure that slow killing assays were initiated with the same number of bacteria, the bacterial lawns were normalized to an OD_{600} equivalent to the growth of WT on NGM by plating the normalized cell suspensions back to the same plates immediately before adding the worms.

NGM supplemented with 1 μM C8-HSL was prepared by spotting the appropriate concentration from the concentrated stock solution on the bottom of empty small Petri plate, and allowed to ethyl acetate evaporate. Then, 10 mL medium per plate was poured, mixed well throughout and let to solidify.

3.6. Modified metabolite filter diffusion assay

Metabolite filter diffusion assay protocol was based on the similar method demonstrated by Gan *et al* (Gan *et al.*, 2002). Briefly, the bacteria were grown on nitrocellulose membrane placed upon agar medium for testing the production of diffusible metabolites. The previously described protocol in our laboratory (Law, 2009) was modified due to observed growth impairment on filter-conditioned plates in the

following manner: instead of allowing bacteria to grow on filter- conditioned plate, concentrated cell suspensions of OD_{600} equivalent to the growth of WT on NGM were plated following L4 larvae worms deposition. The concentrated cell suspensions were generated by harvesting the bacterial lawns formed during overnight incubation on NGM plates. The metabolite filter diffusion assay on plates with normalized bacterial lawns followed the standard routine of killing assay as described above.

3.7. Molecular biology techniques

Genetic manipulations with *B. cenocepacia* K56-2 strains were performed via triparental mating with *E. coli* pRK2013 as a helper strain. The pTP1 and pTP2 plasmids were maintained in *E. coli* SY2327 and DH5α Z-competent cells, respectively. Polymerase chain reactions were carried on Eppendorf Mastercycler ep gradient S thermal cycler with either Taq DNA polymerase (Qiagen) or HotStar HiFidelity Taq polymerase (Qiagen) with optimized conditions for each pair of primers. The DNA ligase and restriction enzymes (New England Biolabs) were used as recommended by manufacturers. QIAquick purification kit (Qiagen) and QIAprep Miniprep kit (Qiagen) was used to purify PCR products and plasmids, respectively.

3.8. Tri-parental mating with *B. cenocepacia*

Tri-parental mating with *B. cenocepacia* was carried out using *E. coli* pRK2013 helper strain and *E. coli* donor strain carrying the plasmid of interest. On the first day, donor strain was grown in 5mL LB for overnight in shaking incubator at 37 °C, whereas helper and donor strains were grown on LB agar with appropriate antibiotic (40 mg/ mL Kan for *E. coli* pRK2013). On the second day 100 μL of donor overnight culture were subinoculated into fresh LB medium and allowed to grow until reached OD_{600} ranging

between 0.3- 0.6 indicating an early log phase. Meanwhile cells of donor and helper strains were harvested from the plates, resuspended in PSB and OD_{600} was recorded. The volumes of each strain in final mix was adjusted to donor and helper as if OD_{600} of 0.3 in 1.5 mL suspension and recipient with OD_{600} of 1.0 in 0.5 mL. The resulted suspension was centrifuged for 1minute at 6000 rpm, supernatant was discarded and the pellet was resuspended in 100µl of SOC broth. Next, it was spotted in the middle of SOB plate and incubated lid side up for overnight at 37 °C. On the third day, the spot was collected with the sterile loop into 2 mL LB and the plate on LB plates with gentamycin to eliminate the helper strain growth and selective antibiotic specific to the donor strain. For homologous recombination 200 µL of undiluted suspension was plated and incubated for 48 hours at 37°C.

3.9. Colony PCR screening

Isolated colonies were picked form the plates using burn plastic tip and resuspended in the 100 µL of water. Two µl of resulted cloudy cell suspension were added to the 8 µL of master PCR mix prepared accordingly to the manufacturer's instructions in a small PCR tube with final volume of10 µL. Annealing temperature and extension time were adjusted to primers used in reaction and length of amplified fragment. The PCR product was analyzed by running 2 µL from each sample tube in 1 % agarose gel.

3.10. Construction of B. cenocepacia K56-2 *paaABCDE* unmarked deletion mutant

The unmarked deletion mutant was created using a system developed for genus *Burkholderia* by Flannagan *et al*. (Flannagan *et al.*, 2008) (Figure 3). Briefly, the fusion

product of two flanking regions, upstream and downstream fragments were amplified using appropriate primers (Table 2), two of which, STC213 and STC214, had an overlapping region that allowed a formation of a fusion product between two fragments (Dubarry *et al.*, 2010). The resulted fusion product was digested with *Eco*RV and *Xba*I and inserted into *Eco*RV-*Xba*I digested suicide plasmid, pGPI-SceI, carrying I-SceI recognition site for yeast homenuclease, and coding trimethoprim resistance. The resulted plasmid (Figure 4A), pTP1, was introduced into K56-2 by triparental mating, where it underwent its targeted insertion via homologous recombination. Conjugates were selected on LB medium supplemented with trimethoprim. The integration of the suicide plasmid with the insert of interest was confirmed by PCR using primers STC153 and STC154 that bind to external regions to MCS. Next, pDAI-SceI, expressing I-SceI nuclease and coding for tetracycline resistance genes, was introduced into K56-2: pTP1 via triparental mating. The double strand break caused by endonuclease triggered an intramolecular recombination event that could be resulted in reversion of the conjugate to a wild type allele or the deletion of the fragment between two flanking regions. Potential conjugates were trimethoprim sensitive and tetracycline resistance and the deletion event was identified by PCR analysis using primers STC215 and STC216. The positive clones were cured from the presence of pDAI-SceI plasmid by sequential subculturing in the antibiotic- free LB medium. The loss of plasmid was confirmed by the loss of tetracycline resistance using replica plate method. The mutation was confirmed by sequencing the flanking regions of *paaABCDE* cluster using primers SC213-216.

Figure 3. **A scheme for unmarked gene deletion using pGPI-SceI/pDAI-SceI system developed by Flannagan *et al*** (Flannagan *et al.*, 2008).

Step 1. Cloning upstream (H1') and downstream (H2') flanking regions of gene to be deleted (**B**) into suicide vector pGPI-SceI, which contains I-SceI recognition. The resulting plasmid is integrated into chromosome via homologous recombination between chromosomal (H1H2) and PCR amplified (H1'H2') flanking regions of the **B** conferring a trimethoprim resistance (Tp^R) to the bacteria.

Step 2. Introduction of pDAI-SceI plasmid, which constitutively expresses I-SceI endonuclease and encodes tetracycline resistance into bacteria with chromosomally integrated pGPI-SceI plasmid carrying flanking regions, H1'H2' of **B**. Recombination/ repair machinery is activated when double strand break in chromosome is generated by the I-SceI endonuclease cleavage at its recognition site.

Step 3. Homologous recombination between downstream regions (H2 and H2') will cause the exclusion the *paaABCDE* rendering a deletion mutant.

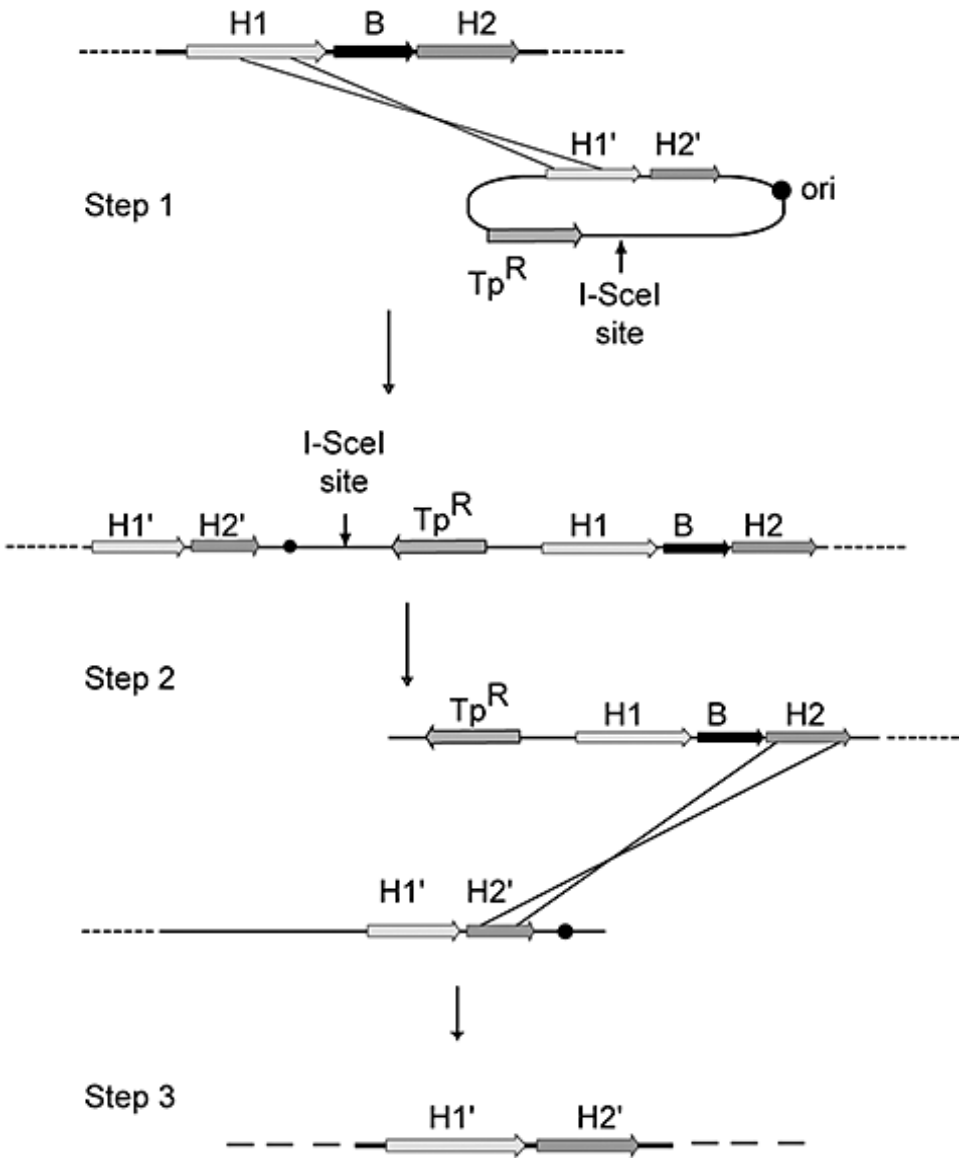

H1
B
H2
H1'
H2'
ori
Step 1
TpR
I-SceI
site
I-SceI
site
H1'
H2'
TpR
H1
B
H2
Step 2
TpR
H1
B
H2
H1'
H2'
Step 3
H1'
H2'

Table 2. **Primers used in this study**

Name	Oligonucleotide sequence, 5'-3'[ab]	Purpose or location
SC213	TTCGCC*ATGTACACGGAACGT TGA*GCGGCATCG	Amplification of *paaABCDE* downstream flanking region, 5'-3'
SC216	GCAGTGATATCCCATGATGC TGCCGACGAGGC	Amplification of *paaABCDE* downstream flanking region, 3'-5'
SC214	CGATGCCGC*TCAACGTTCCG TGTACAT*GGCGAA	Amplification of *paaABCDE* upstream flanking region, 3'-5'
SC215	AACTCTCTAGATCGACGGCC AGTTCGGTCACCC	Amplification of *paaABCDE* upstream flanking region, 5'-3'
SC230	AACTCTCTAGACGGCCCCAT CTGGAGGCTGATC	Amplification of *paaABCDE* downstream flanking region (internal to 215), 5'-3'
SC231	GCAGTGATATCTTCAGGTGC TTCGCCTCCTGCCA	Amplification of *paaABCDE* downstream flanking region (internal to 216), 3'-5'
SC153	GTGGATGACCTTTTGAATGA CCTTT	External to MCS in pGPI-SceI, 5'-3'
SC154	ACAGGAACACTTAACGGCT GACATG	External to MCS in pGPI-SceI, 3'-5'
SC003	GGCGTAGAGGATCTGCTCAT GTTTG	External to MCS in pAP20, 5'-3'
SC004	GCTACTGCCGCCAGGCAAA TTCTGT	External to MCS in pAP20, 3'-5'
SC255	AATTCTACATATGTACACGC AATCCCTCGACATC	Amplification of *paaABCDE*, forward 5'-3'
SC006	TAGCTCTAGATCAACGTTCG TCGAAGCTC	Amplification of *paaABCDE*, reverse 3'-5'

[a] Restriction sites are underlined.
[b] Overlapping region between *paaA* and *paaE* is italicized.

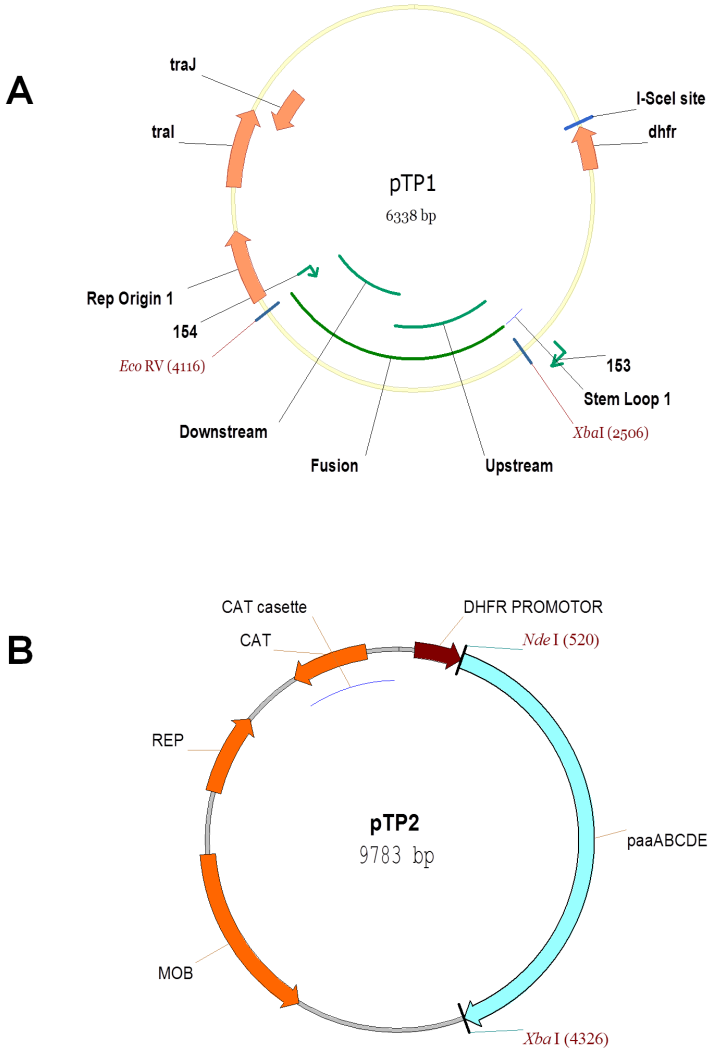


Figure 4. **Plasmid maps of pTP1 and pTP2 generated using VectorNTI.** (A) pTP1, a mutagenesis plasmid derived from the suicide vector pGPI-SceI with insert of fusion product between *paaABCDE* upstream and downstream flanking regions. (B) pTP2, a complementing plasmid derived from pAP20 vector with complete *paaABCDE* gene cluster under control of constitutive promoter, *dhfr*.

3.11. Complementation of TNP1

The constitutive expression vector pAP20 was constructed as previously described (Law *et al.*, 2008) and used to clone *paaABCDE* gene cluster. The complete operon was amplified from genomic DNA of K56-2 WT using primers STC255 and STC006. After XbaI/NdeI double digest vector pAP20 and *paaABCDE* amplificon were ligated. The resulted plasmid (Figure 4B), pTP2 was first transformed into *E. coli* DH5α following triparental mating into TNP1. The colony PCR with primers STC108 and STC109 was performed to confirm the presence of pTP2 in TNP1. Empty pAP20 vector was conjugated into K56-2 WT and TNP1 as a negative control for complementation experiments.

3.12. Quantification of PAA and 2-OHPAA in bacterial supernatant

To quantify the amount of PAA released by bacteria, 100 mL of NGM broth in 250 mL flask was inoculated with full inoculating loop from an overnight culture grown on LB agar. Bacteria were incubated for 24 hours at 37 ^{0}C with shaking at 200 rpm and harvested by centrifugation for 10 minutes at 7000 rpm. The supernatants were collected, acidified with concentrated hydrochloric acid (pH <2) and extracted twice with 50 mL ethyl acetate. The organic phase was then dried by Na_2SO_4, evaporated under reduced pressure to give a crude extract. The crude extract was then resuspended in methanol to concentration of 1 mg/ mL and analyzed by HPLC.

To quantify the amount of PAA consumed by bacteria, 5 mL of NGM broth containing 5mM PAA was inoculated with fresh overnight culture grown in LB, adjusted to an OD_{600} of 0.04. The bacterial inoculums were incubated for 24 hours at 37 ^{0}C with

shaking at 200 rpm and harvested by centrifugation for 3 minutes at 8000 rpm. One mL of supernatant was filter-sterilized using 0.2 μm filters (GE) and analyzed by HPLC.

3.13. HPLC analysis of bacterial supernatant

HPLC analysis was performed using Waters HPLC Separation Module 2695, combined with PDA Detector Model 2996. The column used was μBondapak™ Waters C_{18} (3.9 X 300mm) column particle diameter of 15-20μm, with 125 Å pores. The gradient was held at 3% methanol in 0.075% aqueous trifluoroacetic acid for 30 minutes, then linear gradient to 20% methanol was held for 10 minutes, following the linear gradient back to 3% methanol for 10 minutes and held there for 10 minutes. The total run time was 60 minutes. The flow rate was 1ml/minute. The eluent was monitored continuously at 210-600 nm, and HPLC traces were displayed at 210.3 nm. Under these conditions standard sample of PAA had a retention time of 8.9 minutes (σ=0.08, SEM=0.161, N=6) and phenylalanine retention time was 4.8 minutes (σ=0.09, SEM=0.183, N=7).

3.14. Chemical structure determination

Proton (^{1}H)-NMR spectra was applied to confirm the structure of detected metabolites in concentrated bacterial supernatant. ^{1}H-NMR spectra for standard PAA solution and PAA extracted from 4 L TNP1 supernatant were obtained on Burker Avance 300 at 300 MHz using deuterated chloroform ($CDCl_3$) as a solvent. Samples were prepared by dissolving 25-30mg of the compound in 0.7 mL $CDCl_3$. The chemical shifts values and integration of the signals observed in the ^{1}H-NMR of a standard sample of PAA were compared to the signal observed in ^{1}H-NMR spectra collected on the TNP1 supernatant extract.

3.15. Protease activity

Protease activity was tested qualitatively using 2% skim milk medium. To make 300 ml of 2 % skim milk medium (BD), 150 mL of 4% skim milk medium were combined with 150 mL of 2.5 % of agar medium and mixed well using magnetic stirrer. To examine the protease activity 3 μL of the bacterial overnight cultures were spotted on 2% skim milk medium and incubated at 37 ^{0}C. The zone of clearance including the zone of bacterial spotting was recorded and photographed after a period of 2 or 4 days. The 2% skim milk medium with 5mM PAA were prepared in the similar manner except 5 mM PAA and phosphate buffer at the volume equal as in NGM were added to the agar portion first and mixed well prior to combining the media. To examine the effect of C8-HSL on protease activity 5 μL of C8-HSL, dissolved in ethyl acetate to final concentrations of 1 or 10 μM were spotted on the solidified skim milk medium, allowed to dry and then bacterial inoculums were co-spotted.

3.16. Detection of AHLs in bacterial spent media (these experiments were performed by Dr. S. P. Bernier).

3.16.1. Production and extraction of spent media.

Briefly, *B. cenocepacia* cells from overnight cultures were spun down (2 min, 8000 rpm) and washed once in PBS. Eighty μL of washed cells were used to inoculate flasks (125 mL) containing 25 mL of NGM (1:300 dilution), which were incubated for 24 hrs at 37°C with shaking (175 rpm). Grown cells were pelleted by centrifugation (40 min, 4000 rpm, at 4°C) and culture supernatants were filtered with 0.22 μm filters (Millipore).

3.16.2. AHL detection assay.

Detection of AHLs in freshly extracted spent media from *B. cenocepacia* K56-2 strain derivatives was performed using the bioluminescent reporter strain *A. tumefaciens* A136 (pCF218)(pMV26) (Sokol *et al.*, 2003; Chambers *et al.*, 2005) as previously described (Bernier *et al.*, 2008). Briefly, overnight cultures from *A. tumefaciens* A136 (pCF218)(pMV26) were washed as described above and cells were resuspended in PBS and subsequently diluted 1:1500 (v/v) in LB. To determine whether AHLs were present in spent media from *B. cenocepacia* strains, various amounts of spent media were added to the diluted reporter strain for final concentrations varying from 0 to 10%. One hundred and fifty μL of diluted reporter-spent medium mixtures were added to each well of black, clear-bottom 96-well microtiter plates for which 60 μL of sterile mineral oil was added to prevent evaporation. Plates were placed into a Synergy H1 plate reader and incubated at 30°C with continuous shaking for up to 24 hours for which luminescence (counts per second, CPS) and growth readings (OD_{600}) were taken every 30 minutes.

4. Results

4.1. Complete deletion of ring hydroxylation gene cluster causes PAA-defective growth and diminishes the pathogenic phenotype of *B. cenocepacia* K56-2 in *C. elegans.*

We previously showed that an intact PAA degradation pathway is required for full pathogenicity of *B. cenocepacia* in the *C. elegans* host model (Law *et al.*, 2008). During these studies, a trimethoprim resistance marker interrupted the *paaA* or *paaE* genes of the ring-hydroxylation cluster. To ensure that the insertion element is maintained, however, trimethoprim must be added to the mutant cultures, which can cause metabolic effects. To avoid any antibiotic effect when growing mutant strains, we created a *B. cenocepacia* clean unmarked deletion mutant of the complete ring hydroxylation gene cluster. The complete exclusion of the *paaABCDE* locus generated a mutant strain, TNP1. We assumed that deletion of the complete cluster will cause similar phenotypic changes in bacteria to the same extent. To examine that, we tested TNP1 for PAA-dependency for intact growth in M9 with PAA as a sole carbon source and pathogenicity phenotype in *C. elegans*. As expected, TPN1 completely failed to grow on PAA (Table 3) indicating that deletion of the complete cluster causes PAA-defective growth phenotype similar to single gene insertional mutants, *paaA* and *paaE*. This phenotype was not due to a general growth defect as the mutant was able to grow to wild type levels in the undefined NGM and to 80% of wild type growth in M9 medium containing glycerol as the sole carbon source. Since glycerol is a poor-energy carbon source, the OD_{600} recorded after 24 hours of incubation of both strains appears low.

Table 3. **In vitro growth phenotypes of TNP1**[a]

Strain	Mean growth [OD_{600}]±SD[b](relative growth[c])		
	NGM	M9+50 mM Glycerol	M9+5 mM PAA
WT	1.09±0.03 (100)	0.17±0.04 (100)	0.56±0.03 (100)
TNP1	1.08±0.04(99)	0.14±0.05 (82)	0.05±0.01 (9)

[a] Growth was measured by determining OD_{600} after 24 hours (see Materials and Methods).
[b] Standard deviation (SD) of 4 independent experiments
[c] Percentage of growth relative to WT under the same conditions.

To examine the pathogenicity phenotype of TNP1, the killing ability of TNP1 was compared to the K56-2 WT in slow killing assays (Figure 5) using *C. elegnas* DH26 strain. As expected, TNP1 conferred the previously observed pathogenic phenotype of *paaA* and *paaE* in *C. elegans* (Law *et al.*, 2008). When worms were exposed to WT they exhibited restricted mobility and its survival rate fell to approximately 10-20% within the first three days of the assay. The worms were otherwise healthy as demonstrated in parallel with worms fed on the non-pathogenic *Escherichia coli* OP50. TNP1 exhibited the previously observed attenuated pathogenicity phenotype as the worms reached the adult stage, were motile for a longer period of time and their survival rate was higher.

4.2. Complementation analysis of TNP1

To ensure that observed phenotypic characteristic of TNP1 mutant is indeed due to complete deletion of *paaABCDE* gene cluster, complementation of TNP1 with *paaABCDE* gene cluster in trans was performed. Initially, the gene cluster was amplified from K56-2 WT genomic DNA and then cloned into the constitutive expression vector, pAP20. The resulting plasmid, pTP2 (Figure 4B) was then introduced into TNP1 via triparental mating. The positive conjugates were confirmed by colony PCR with primers forward and reverse to *paaABCDE* located in the non-integrative plasmid pTP2. The complementation of mutant's PAA growth defective phenotype was only observed after prolonged incubation time of nearly 70 hours (Figure 6); however, after following subculturing to the fresh medium the complementation was observed within 24 hours reaching 82% of the WT growth with similar growth kinetics.

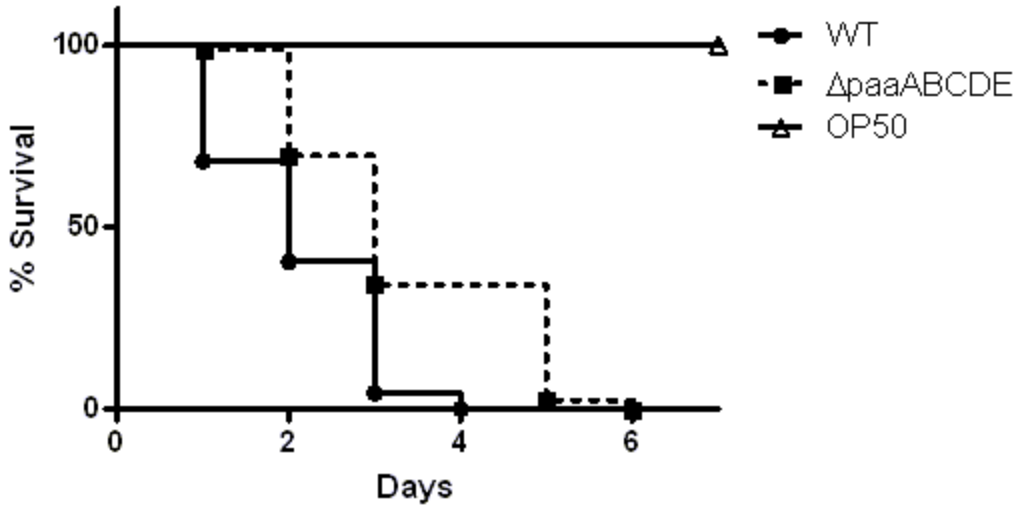


Figure 5. **Inactivation of PaaABCDE reduced the pathogenicity of *B. cenocepacia* TNP1 strain**. Killing ability of the K56-2 WT strain (*n*=69) was compared with that of TNP1 (*n*=79; *P* <0.0001) and *E. coli* OP50 (*n*=70, *P* <0.0001) as a negative control. The data is representative of 11 experiments.

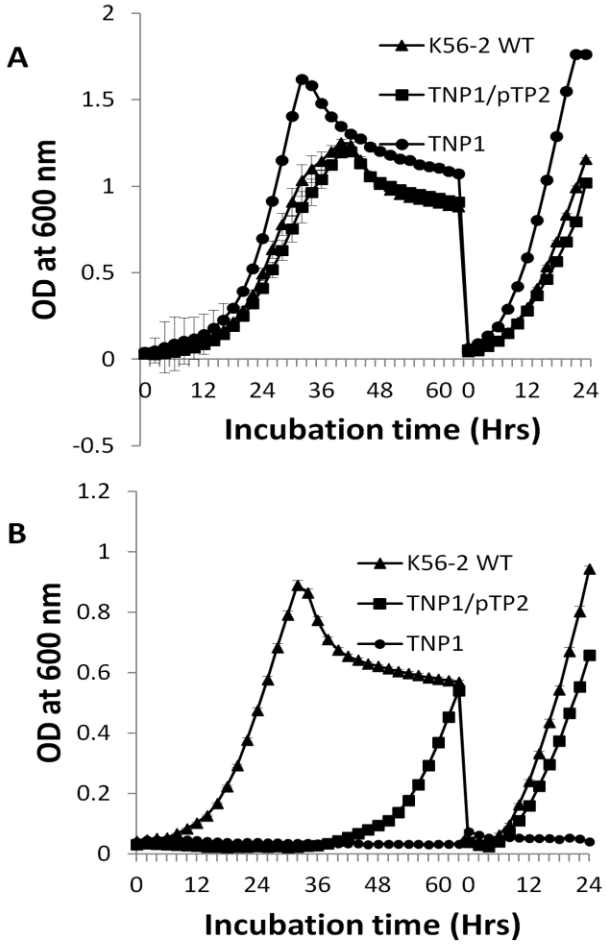


Figure 6. **Complementation analysis of TNP1 with *paaABCDE* in trans**. *B. cenocepacia* K56-2 WT, TNP1 and TNP1/pTP2 were grown in M9 medium with 10mM Glucose or 5mM PAA as a sole carbon source. After 64 hours of incubation at 37^0C, bacterial cells were subcultured into fresh medium at starting OD_{600} of 0.04 and incubated for another 24 hours. (A) Bacteria grown in M9 with 10 mM Glucose. (B) Bacteria grown in M9 with 5 mM PAA. The errors bas represent SD from two independent experiments.

4.3. Detection of PAA related metabolites/ intermediates in bacterial filtrate by using metabolite filter assay

Previous evidence using a *C. elegans* filter diffusion slow killing assay in our laboratory demonstrated that pathogenic phenotypes of *paaA* and *paaE* mutants grown on K56-2 filtrate-conditioned agar medium could be partially restored (Law, 2009). These observations lead us to hypothesize that metabolites released by K56-2 WT during the growth upon filter on NGM agar are involved in mediating pathogenicity in host model and since ring hydroxylation gene cluster mutants could be complemented with WT filtrate, it suggests that these metabolites are likely to be PAA catabolic pathway related. To ensure that WT filtrate would also restore pathogenic phenotype of TNP1, we performed filter diffusion assay using the new mutant.

4.3.1. Bacterial growth in filtrate-conditioned agar medium

When running metabolite filter assays with TNP1 we have observed that bacterial growth is affected by previous growth of filtrate producing bacteria (Figure 7); hence, during the assay the worms would to be exposed to different numbers of bacteria with confounding effects on pathogenicity. To further investigate this observation we performed a growth analysis on filter-conditioned plates. The filter-conditioned plates were generated by growing bacteria, which filtrate is to be examined, on nitrocellulose filter paper (0.2 μm, 48 mm) on NGM agar plates. After 24 h of incubation, filters with bacterial lawns were carefully removed. Next, these filter-conditioned agar plates were inoculated with 50 μL of fresh overnight cultures at OD of 1.7, to assess the pathogenicity restoration effect of filtrate. Following 24 hours, the developed bacterial lawns were collected with 1 mL PBS

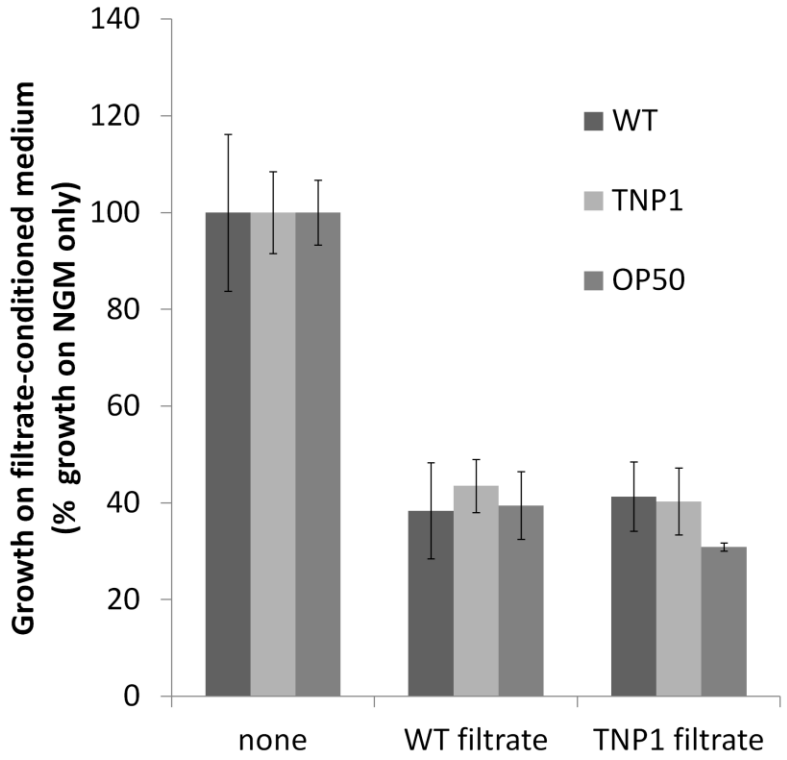


Figure 7. **Bacterial growth of *B. cenocepacia* K56-2 strains and *E. coli* OP50 is reduced in the presence of K56-2 WT and TNP1 filtrate**. First, filtrate producing bacteria were grown on filter on top of the NGM plate 24 hours and then removed with the filter. The resulting filtrate-conditioned plates were inoculated with fresh overnight culture of a test strains and incubated. The developed bacterial lawns were collected from the plates after 24 and bacterial loads were estimated by OD_{600}. The bars are mean values expressed as a percent of bacterial growth on NGM without filtrate. The error bars represent the SD of four independent experiments.

with help of loop after int. The cell suspensions then were diluted and OD was recorded. There is a general reduction of nearly 60 % in growth on filter-conditioned NGM for all bacterial strains tested when compared to the regular growth on NGM that was expressed as 100%.

4.3.2. Modification of metabolite filter diffusion assay

To ensure that metabolite filter diffusion assays were initiated with the same number of bacteria to allow proper comparison and eliminate any effect of reduced amount of bacteria consumed by the worms, the standard procedure was modified accordingly. The new protocol included additional step of normalizing the bacterial lawn to be spread on filter-conditioned plates to an OD_{600} equivalent to the growth of *B. cenocepacia* K56-2 WT on NGM. The normalized cell suspensions were plated back to the same plates immediately before adding the worms following standard procedure of slow killing assay. We then tested the modified assay protocol to see if it will confer the previous results received from using the old procedure. The assay included a number of control groups. Bacteria were grown on none-filtrate conditioned NGM so that any differences in pathogenicity when compared to growth on bacterial filtrate conditioned NGM could be attributed to the filtrate effect. To exclude the possibility that bacterial filtrate had a general enhancing or general attenuating effect on pathogenicity, the bacterial strains were grown on their own filtrate. In the test groups, the worms were infected with TNP1 and WT grown on WT and TNP1 filtrate conditioned NGM respectively. The number of live worms was recorded to generate the survival curves, which then were compared to the control groups to evaluate the change in pathogenic phenotype of bacteria in the presence of the filtrate (Table 4). From 4 independent assays, only in 2 assays WT filtrate

Table 4. **The change in pathogenicity of *B. cenocepacia* K56-2 strains in the presence of bacterial filtrate**[a]

Comparison		*C. elegans* survival [b]			
Phenotype		Change (*P* value)			
Trail	1-4	1	2	3	4
Strain	No filtrate	WT filtrate			
TNP1	Attenuated [c]	NS (0.0732)	NS (0.13)	↓ (0.008)	↓ (0.003)
WT	Pathogenic [d]	↑ (<0.0001)	NS (0.0896)	NS (0.672)	NS (0.1908)
OP50	None pathogenic	↓ (<0.0001)	NS (0.1032)	NS (0.06)	NS (1)
		TNP1 filtrate			
TNP1	Attenuated [c]	↑ (0.0001)	↑ (0.0277)	NS (0.4897)	NS (0.1861)
WT	Pathogenic [d]	↑ (<0.0001)	NS (0.672)	NS (0.3494)	↓ (0.0332)
OP50	None pathogenic	↓ (<0.0001)	NS (0.1714)	NS (0.06)	NS (1)

[a] The change in pathogenicity of *B. cenocepacia* strains was determined using *C. elegans* survival curves generated during metabolite filter diffusion assays.
[b] Change in survival of *C. elegans* infected with bacteria fed on filtrate conditioned NGM when compared to the no-filtrate control group
[c] The attenuated pathogenicity phenotype was determined by comparing the survival curves of *C. elegans* infected with TNP1 and *E. coli* OP50.
[d] The pathogenic phenotype was determined by comparing the survival curves of *C. elegans* infected with WT and *E. coli* OP50.
↑- Increase in worms' survival indicating attenuation effect in pathogenicity when compared to the no-filtrate group; ↓- Decrease in worms' survival indicating increase in pathogenicity when compared to the no-filtrate group; NS –not significant effect in worms' survival indicating that bacterial filtrate has no effect on bacteria consumed by worms.

could partially restore pathogenic phenotype of TNP1 as was evident by the significant decrease in survival of *C. elegans* in comparison to the control group (*P* value cut off is 0.05). However, in the two other assays WT filtrate had no significant affect on attenuated pathogenicity phenotype of TNP1. When TNP1 was grown on its own filtrate conditioned NGM, the pathogenic phenotype of TNP1 was decreased further and worms' survival was higher when compared to the control group in two out of 4 assays performed. The pathogenic phenotype of WT was mostly not affected by its own filtrate as was evident by no significant difference between *C. elegans* survival when compared to the control group; except one assay where WT filtrate diminished WT killing ability and the *C. elegans* survival was increased when compared to the control group. In 2 assays out 4, the pathogenic phenotype of WT was decreased or increased by the presence of TNP1 filtrate while in the rest of assays performed TNP1 filtrate did not affect pathogenicity of WT toward the worms.

4.4. The proposed dead end product of the pathway, 2-OHPAA is not affecting pathogenic traits of *B. cenocepacia*.

One of the known released metabolites is dead-end product of the pathway 2-OHPAA that is generated during spontaneous decomposition of the PAA-epoxide (Teufel *et al.*, 2010; Grishin *et al.*, 2011; Teufel *et al.*, 2012). 2-OHPAA is a potential candidate for a pathogenicity factor, which would be produced spontaneously in the WT. Although considered to be a dead end product of the pathway, 2-OHPAA can be utilized by both, WT and TNP1 as a sole (Yudistira *et al.*, 2011) or secondary carbon source (Figure 8). To test the possible role of 2OHPAA in attenuation of TNP1 we performed slow killing assays with exogenously added 2OHPAA (Figure 9). Our efforts at complementing the

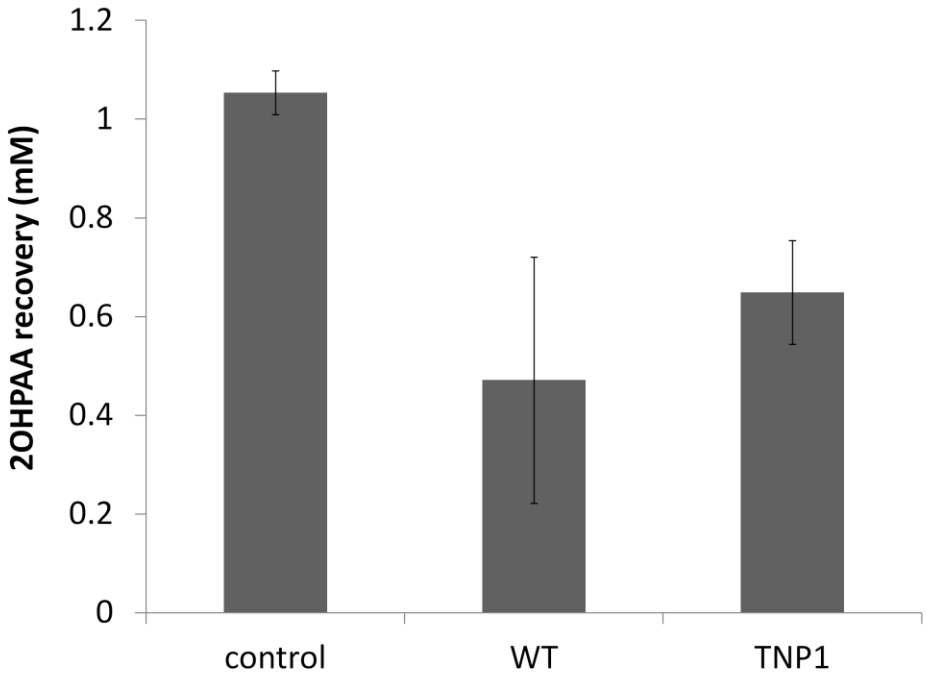


Figure 8. ***B. cenocepacia*** **K56-2 strains consume 2-OHPAA when supplemented as a secondary carbon source.** The ability to consume 2-OHPAA was measured as recovery of 2-OHPAA from NGM broth with 1 mM 2-OHPAA. The error bars represent the SD of four independent experiments.

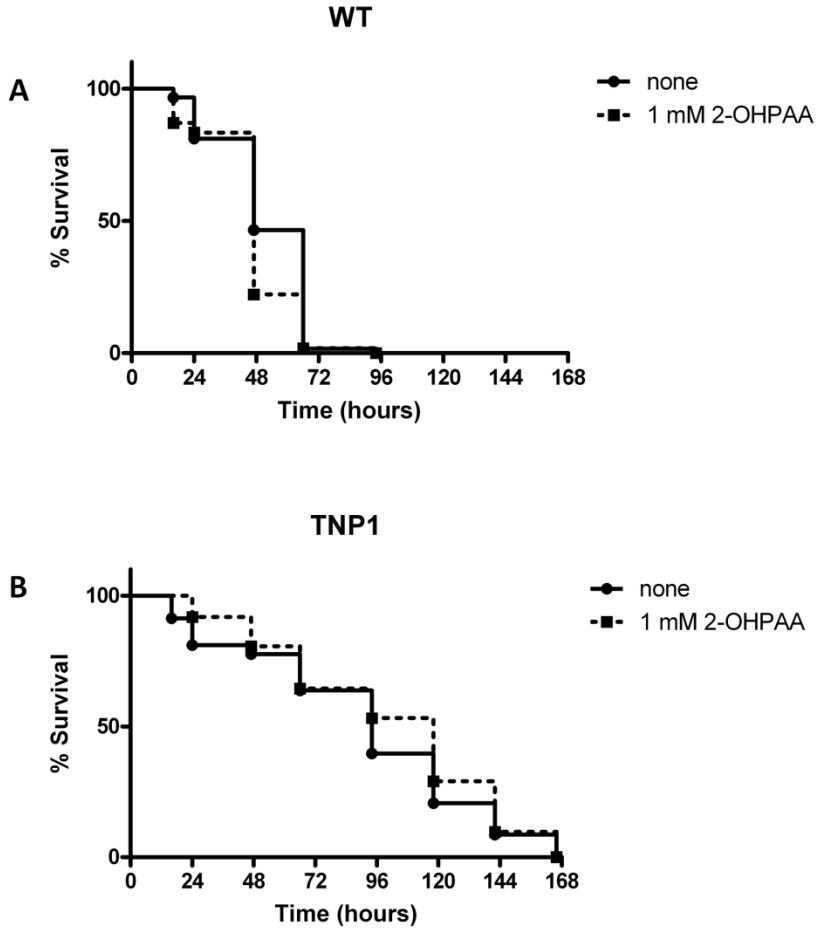


Figure 9. **The effect of exogenously added 2-OHPAA on pathogenic phenotype of *B. cenocepacia* K56-2 strains.** (A) Addition of 2-OHPAA does not affect pathogenic phenotype WT strain. The killing ability of WT grown on NGM (*n*=58) was compared to the one of WT grown on NGM with 1mM 2OHPAA (*n*=54, *P* =0.06). (B) The attenuated pathogenicity of *B. cenocepacia* TNP1 is not affected by the presence of 2-OHPAA. The killing ability of TNP1 grown on NGM (*n*=58) was compared to the one of TNP1 grown on NGM supplemented with 1mM (*n*=62, *P* =0.3137). The data is representative of 2 independent experiments.

defective phenotype of TNP1 with exogenously added 2-OHPAA were unsuccessful in proving the hypothesis. The attenuated pathogenicity phenotype of the mutant was not affected by the presence of 2OHPAA (Figure 9B). Furthermore, it did not cause any increase in pathogenicity of the WT (Figure 9A), which also did not produce any detectable amount of 2-OHPAA (Figure 10). Addition of 2OHPAA did not affect general well being of the worms as was evident by the healthy worms fed with *E. coli* OP50 grown on 2-OHPAA supplemented media.

4.5. TNP1 when grown on NGM releases PAA into the growth medium

To investigate if intermediates of PAA degradation pathway other than 2-OHPAA were involved in mediating the pathogenic phenotype of WT and TNP1 we compared the supernatants of WT with those of TNP1 by HPLC analysis rather than testing K56-2 filtrate restoration effect. To prepare bacterial supernatants, 100 mL stationary phase cultures were harvested by centrifugation, acidified with concentrated hydrochloric acid (pH <2) and extracted twice with 50 mL ethyl acetate. The organic phase was then dried by sodium sulfate (Na_2SO_4) and evaporated under reduced pressure to give a crude extract. The crude extract was then resuspended in methanol to concentration of 1 mg/ mL and analyzed by HPLC. In addition to bacterial supernatants, 100 mL of cell-free media control was included and prepared in the same manner to distinguish between metabolite released by bacteria and the growth media components. The HPLC traces of the organic extracts of control medium, WT and TNP1 supernatant were overlaid to compare released metabolites. While the overlaid HPLC traces of the organic extracts of media control and WT were indistinguishable, one predominant peak was observed in the

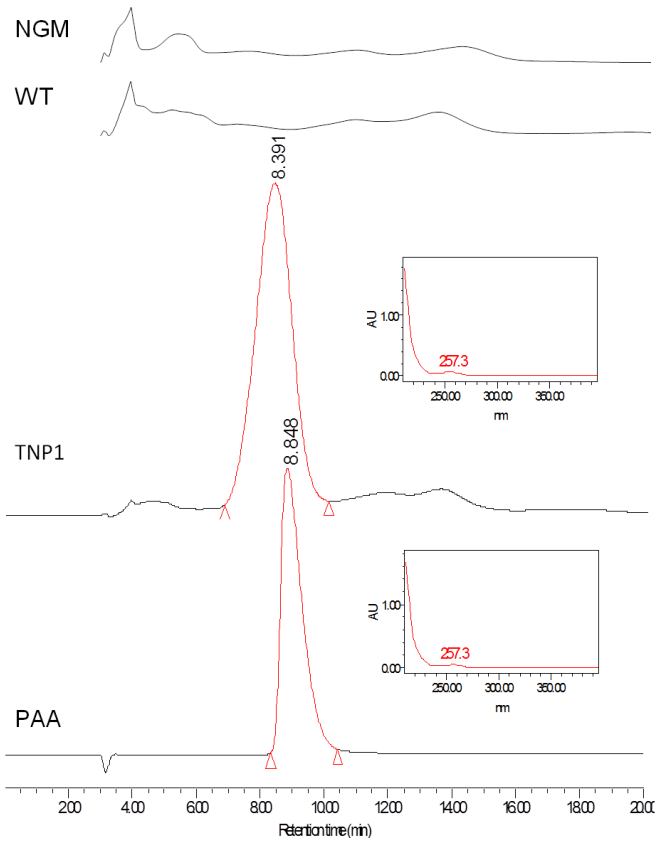


Figure 10. **HPLC analysis of bacterial culture extracts.** The figure shows the overlay of four HPLC traces from separate experiments using the gradient described in the method section. Each trace is displayed at 210.3 nm. The top trace (NGM) is the ethyl acetate extract from the media alone while the second trace (WT) is the extract from a culture of the K56-2 WT. The third trace (TNP1) is the extract from the deletion mutant where a peak with a retention time of 8.4 min was observed. The inset is the UV spectra (200-400 nm) recorded for this signal. The last trace is the extract of a 5 mM solution of a standard sample of PAA. The retention time and UV spectra appear identical to what was observed in the TNP1 mutant. This signal could not be detected in either the media (NGM) or wild type (WT) extracts.

TNP1 extract, which eluted at 8.4 minutes and corresponded to a standard PAA elution time and UV trace (Figure 10). Following the search in our standard chemicals library we found that it corresponded to a PAA elution time and UV trace. In order to calculate the PAA released by TNP1in the crude extract we created calibration curve (Figure 11) with known PAA concentration plotted against area under the PAA peak corresponding to its concentration. Under the harvesting conditions described above, a TNP1 culture of an OD_{600} of 1.4-3.4 contained 8.3 mM PAA. To confirm the peak at 8.4 minutes in TNP1 culture was PAA, a 4L TNP1 supernatant was extracted and the residues analyzed by proton (^{1}H)- NMR spectroscopy (Figure 12). The ^{1}H-NMR spectra revealed chemical shifts of protons on PAA molecule from bacterial extract and standard solution. Multiplet between 7.3-7.4 ppm correspond to aromatic protons on PAA. A broad singlet between 9.3- 10.7 ppm corresponds to the chemical shift of phenol group. The singlet observed at 3.7 ppm corresponds to methylene protons of PAA. Overall, the chemical shifts and integrations of standard PAA spectra were similar to those in TNP1 supernatant extract sample demonstrating the when grown in NGM the TNP 1 mutant but not the WT strain produces PAA, which is release to the supernatant.

4.6. PAA is actively degraded by *B. cenocepacia* K56-2

The observed release of PAA by the less virulent TNP1 but not by the more virulent K56-2 WT, suggested that the released PAA might play a role in attenuation of pathogenicity. During preliminary killing assays with exogenously added PAA into NGM plates, we noted that PAA caused WT to develop thicker lawns on agar media than these observed on TNP1 (Figure 13A). To investigate this observation, we inoculated NGM plates supplemented with 1 or 5mM PAA with 50 µl *B. cenocepacia* K56-2 WT, TNP1

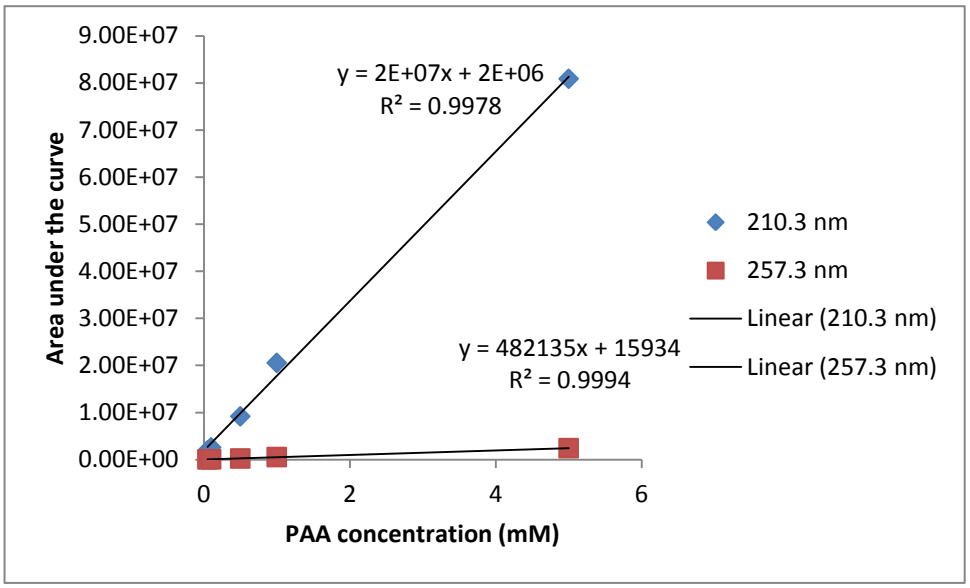


Figure 11. **PAA calibration curve was performed using standard PAA solutions with known concentrations.** Different concentrations ranging from 0.01 to 5 mM were analyzed by HPLC. The peaks from HPLC traces were integrated to get the area under the peak values that were plotted agains corresponding PAA concentration.

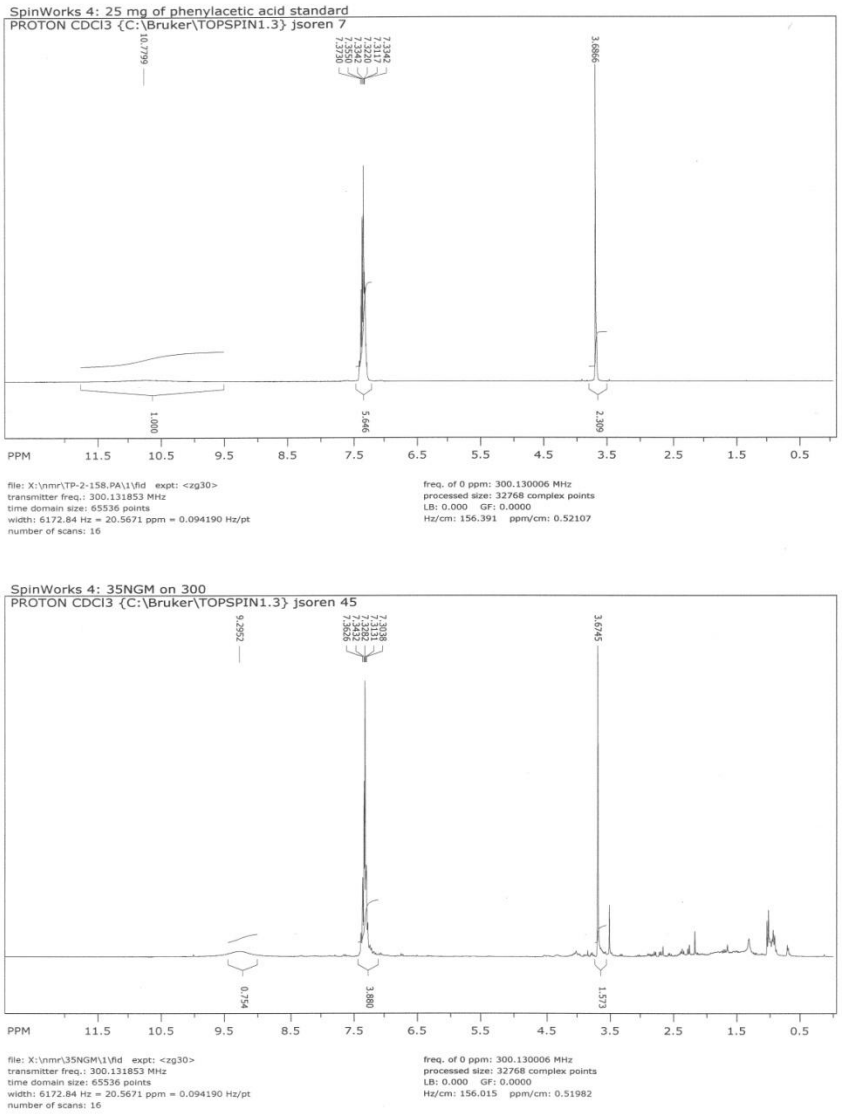


Figure 12. **The ^{1}H-NMR spectra revealed chemical shifts of protons on the PAA molecule.** Multiplet between 7.3-7.4 ppm correspond to the aromatic protons. A broad singlet between 9.3- 10.7 ppm corresponds to the chemical shift of proton of the phenol group. The singlet observed at 3.7 ppm corresponds to the methylene protons. The signals from standard PAA solution (upper spectra) were similar to signals from TNP1 extract (lower spectra).

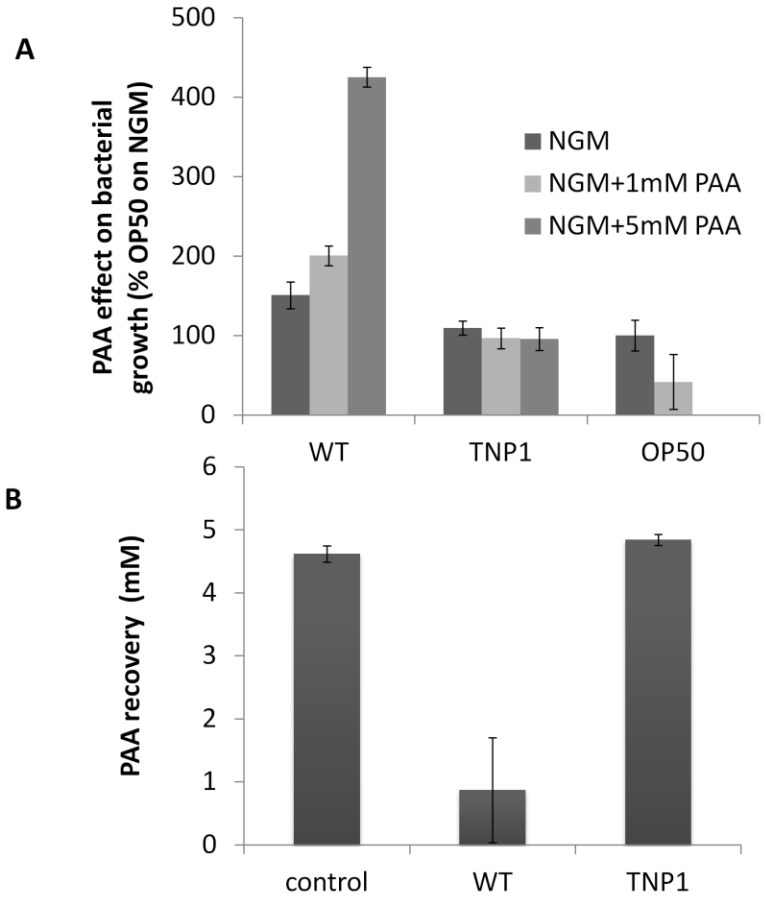


Figure 13. ***B. cenocepacia* K56-2 WT actively degrades PAA when grown on PAA supplemented NGM.** (A) Effect of PAA on bacterial growth on NGM plates. Bacterial lawns were grown for 48 hours, removed from the plates and resuspended on PBS to measure OD_{600}. The PAA effect on bacterial growth is expressed as percent of mean value of *E. coli* OP50 growth on NGM. The error bars represent the SD of two independent experiments. (B) PAA consumption by *B. cenocepacia* K56-2 WT and Δ*paaABCDE* . The ability to consume PAA was measured as recovery of PAA from NGM broth with 5 mM PAA. The error bars represent the SD of three independent experiments.

and *E. coli* OP50 overnight cultures adjusted to OD_{600} of 1.7. After 24 h incubation at 37°C, bacterial lawns were washed from the plates with 1mL PBS, generated cell suspensions were diluted and the bacterial content was estimated by OD_{600}. Based on this data, presence of PAA in the NGM greatly stimulated WT growth, while mutant grew on NGM independently of PAA concentration present. It is worth noting that *E. coli* OP50 exhibited a severe reduction of almost 60% on the NGM with 1mM PAA, whereas increasing PAA concentration to 5 mM resulted in complete absence of growth suggesting that this concentration could be toxic to *E. coli* OP50. These results suggest that PAA is consumed during the growth on the supplemented NGM. If PAA is being actively consumed, we will see the reduction of amount of PAA recovered from bacterial supernatants (Figure 13B). The bacterial supernatants were prepared by inoculating 5 mL of NGM with 1mM 2OHPAA with 50 µl of bacterial culture of OD_{600} and allowed to incubate for 24 h at 37°C. The next day 1 mL of bacterial culture or cell-free media were syringe filtered (0.2 µm) and analyzed by HPLC. While PAA was completely recovered after growth of TNP1, most PAA was consumed after growth of WT indicating that PAA is indeed actively degraded by WT. The ability to utilize PAA as an additional carbon source allows WT to have greater bacterial loads than these of TNP1.

4.7. Exogenous addition of PAA to the slow killing assays enhances the attenuated pathogenicity of TNP1.

The slow killing assays with exogenously added PAA were performed to assess the possible role of PAA in attenuation of TNP1 (Figure 14). Based on the observed differential growth on PAA supplemented media, we modified slow killing assay procedure by normalizing the bacterial lawn so to ensure that assays were initiated with

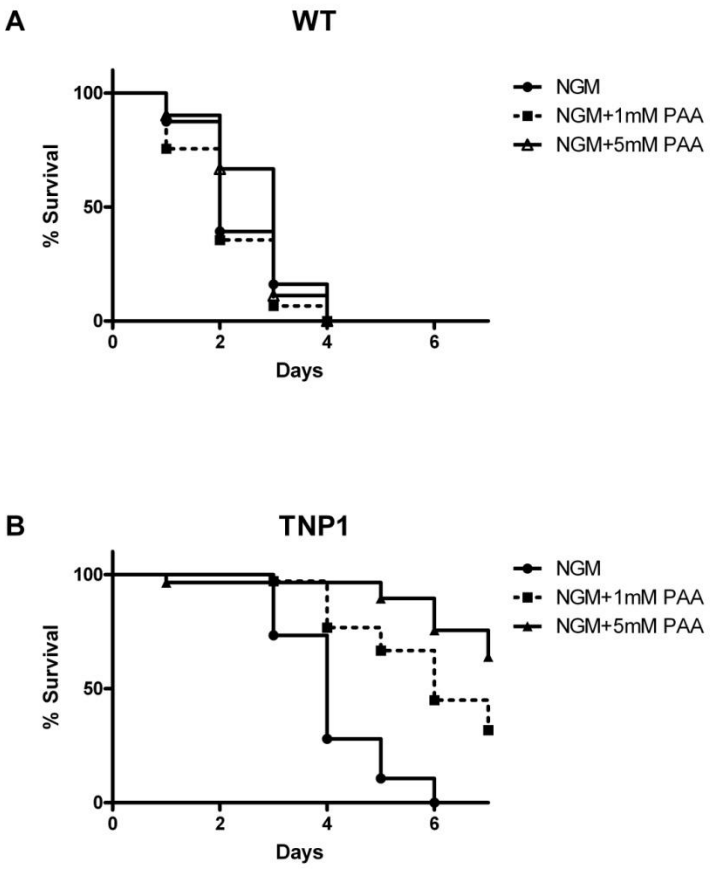


Figure 14. **The effect of exogenously added PAA on the pathogenic phenotype of *B. cenocepacia* K56-2 strains.** (A) Addition of PAA does not affect pathogenic phenotype of WT strain. The killing ability of WT grown on NGM (*n*=56) was compared to the one of WT grown on NGM supplemented with 1mM (*n*=72, *P* =0.1615) or 5mM PAA (*n*=45, *P* =0.1348) in bacterial growth-normalized slow killing assays. (B) The attenuated pathogenicity of TNP1 is enhanced in the presence of PAA as a function of its concentration. The killing ability of TNP1 grown on NGM (*n*=75) was compared to the one of TNP1 grown on NGM supplemented with 1mM (*n*=69, *P* < 0.0001) or 5mM PAA (*n*=86, *P* < 0.0001) in bacterial growth-normalized slow killing assays. The data is representative of 3 independent experiments.

the same number of bacteria to allow proper comparison between different types of media and eliminate any effect of reduced amount of bacteria consumed by the worms Addition of PAA did not affect the pathogenic phenotype of K56-2 (Figure 14A). However, a more severe attenuation of pathogenicity was observed for TNP1 grown on NGM supplemented with PAA (Figure 14B). The observed enhancement was a function of PAA concentration in the media. At the highest concentration used in the assay, worms exhibited a very prolonged high survival rate, whereas worms infected with TNP1 grown with 1mM PAA had an intermediate survival rate. Taken together these results suggests that exogenously added PAA has an pathogenicity attenuation effect but this effect could not be observed in *B. cenocepacia* K56-2 WT due to active degradation of PAA in PAA supplemented NGM. It is worth noting that the general effect of PAA on feeding behavior of *C. elegans* was not analyzed.

4.8. PAA interferes with QS-regulated protease activity in *B. cenocepacia*.

Exogenously added PAA into the growth medium of *P. aeruginosa* had previously shown to cause a reduction in QS- regulated virulence factors like pyocyanin, exopolysaccharide, protease and elastase production (Musthafa *et al.*, 2012). This observation and the fact that attenuation of *P. aeruginosa* cytotoxicity is associated with PAA accumulation (Wang *et al.*, 2013) suggested that non-metabolized PAA might also interact in a similar matter with QS in *B. cenocepacia* TNP1 strain. If this is the case, then the TNP1 mutant should exhibit attenuation of QS-regulated functions. To experimentally address this hypothesis we tested QS-regulated protease activity of *B. cenocepacia* strains (Lewenza *et al.*, 1999). The qualitative assays using a 2% skim milk supplemented with PAA at various concentrations were performed to examine the zone

of clearance around bacterial spotting as a direct indication of proteolytic activity (Figure 15 and 16). After 48 hours of incubation, the diameter of clearance zone including the colony was recorded, and expressed as a percent activity of WT on 2% skim milk medium. Both strains developed similar zones of clearance indicating an intact proteolytic activity in the absence of PAA. Although skim milk medium contains glucose and various amino acids including the phenylalanine, it was probably not sufficient for production and observed release of PAA by TNP1 to the medium. Subsequent addition of PAA at increasing concentration inhibited protease activity in a dose-dependent manner. The most severe effect was observed for TNP1 at 5mM PAA where proteolytic activity was completely abolished. The ability of PAA to decrease the proteolytic activity in a dose-dependent manner indicates the PAA affects at least one QS-regulated response in *B. cenocepacia* K56-2.

In addition to CepIR QS present in all Bcc, *B. cenocepacia* K56-2 has a second QS controlled by CciIR, that is coded in the part of genomic island, cci, which is involved in pathogenicity and metabolism. Both system are AHL-dependent and utilize C8-HSL or C6-HSL, however, at different proportion. CepIR system largely produces C8-HSL while CciIR system mainly relies on C6-HSL. *Agrobacterium* biosensor that was used in our study is 100 times more sensitive to C8-HSL than C6-HSL (Chambers *et al.*, 2005). It has been previously shown that the impact of CepI on *C. elegans* survival to be greater than the impact of CciIR (Uehlinger *et al.*, 2009). Furthermore, mutants of CepIR have a negative protease phenotype while mutations in CciIR have an opposite effect (Malott *et al.*, 2005). We, therefore, used synthetic C8-HSL molecules in our next experiments.

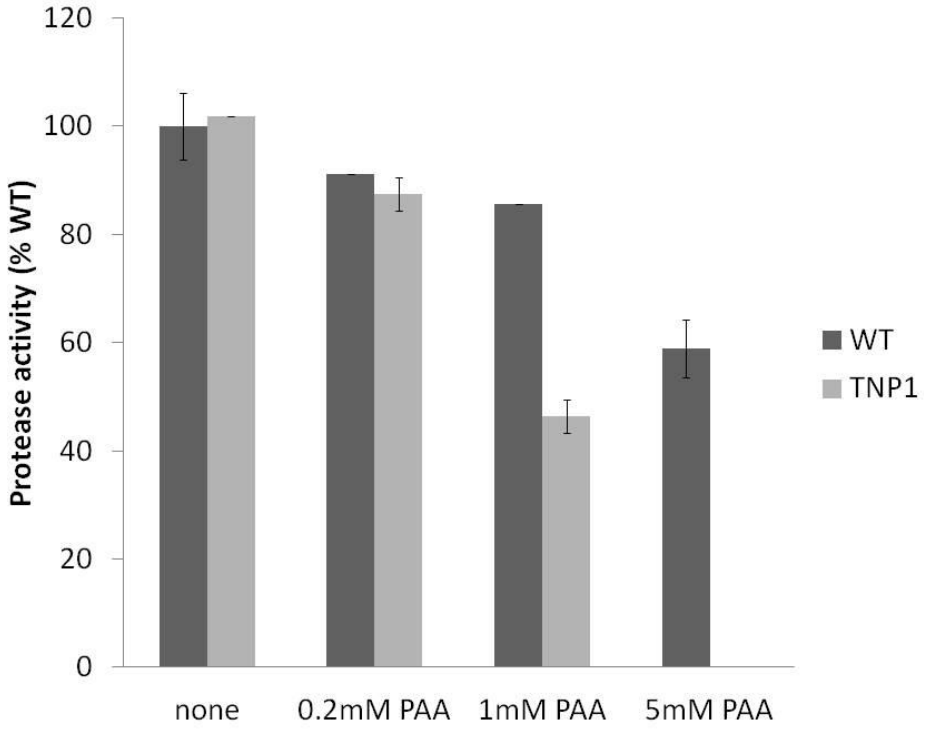


Figure 15. **PAA effect on protease activity of *B. cenocepacia* K56-2 strains.** (A) Protease activity was measured on agar containing 2% skim milk with or without the addition of PAA and expressed as the ratio between the diameter of the zones of clearance (including colonies) and the ones of WT on skim milk medium only. The error bars represent the SD of two independent experiments.

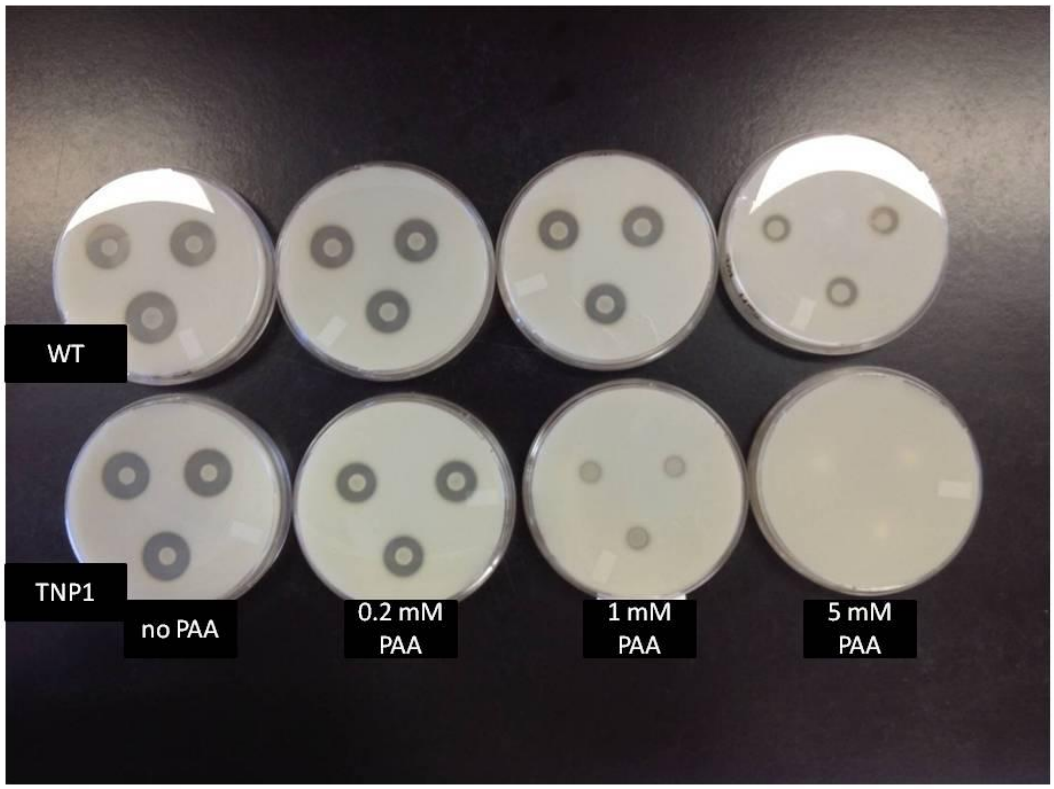


Figure 16. **Protease activity of *B. cenocepacia* K56-2 strains after 2 days of incubation on 2% skim milk with or without PAA.** The top row (from left to right) is WT grown on the skim milk plates with increasing PAA concentrations from 0 to 5 mM. The bottom row (from left to right) is TNP1 grown on the skim milk plates with increasing PAA concentrations from 0 (no PAA) to 5 mM.

We attempted to rescue the inhibition effect of PAA imposed on QS by addition of C8-HSL into assay media. We repeated protease activity assay on 2% skim milk with 5mM PAA, where the profound effect was found for the TNP1, in the presence of C8-HSL at concentration of 1 and 10 μM. After 4 days, the rescuing effect of C8-HSL molecule was observed for TNP1 (Fig. 17 and 18). The abolished proteolytic activity of TNP1 in the presence of PAA was partially restored when 1 and 10 μM C8-HSL were added, reaching 30 and 37% of K56-2 protease activity on skim milk medium, respectively. There is a slight increase in proteolytic activity of both strains when 1μM C8-HSL was added, however, increasing the concentration to 10 μM cause only slight increase in the activity.

4.9. Detection of AHLs is reduced in TNP1 supernatants

Endogenously produced or exogenously added PAA reduced the virulence of TNP1 against *C. elegans*. The effect of PAA on the pathogenicity of TNP1 was similar to the reduction of QS-regulated virulence factors and pathogenicity of *P. aeruginosa* against *C. elegans*, mediated by exogenous addition of PAA (Musthafa *et al.*, 2012). As both *P. aeruginosa* and Bcc bacteria use the QS signal molecules *N*-acylhomoserine lactones (AHLs) (Lewenza *et al.*, 2002) we hypothesized that PAA could decrease the pathogenicity of TNP1 through QS inhibition. In Bcc bacteria, AHLs, synthesized by the LuxI homolog CepI, bind the LuxR type transcriptional regulator CepR to activate a number of virulence-related genes (Lewenza *et al.*, 1999; Lewenza *et al.*, 2001). As in other AHL-regulatory systems, AHL production in Bcc is autoinduced. CepR negatively regulates its own transcription and positively regulates *cepI* expression (Lewenza *et al.*, 1999). The experiments testing the amount of AHL in K56-2 WT and TNP1 supernatant

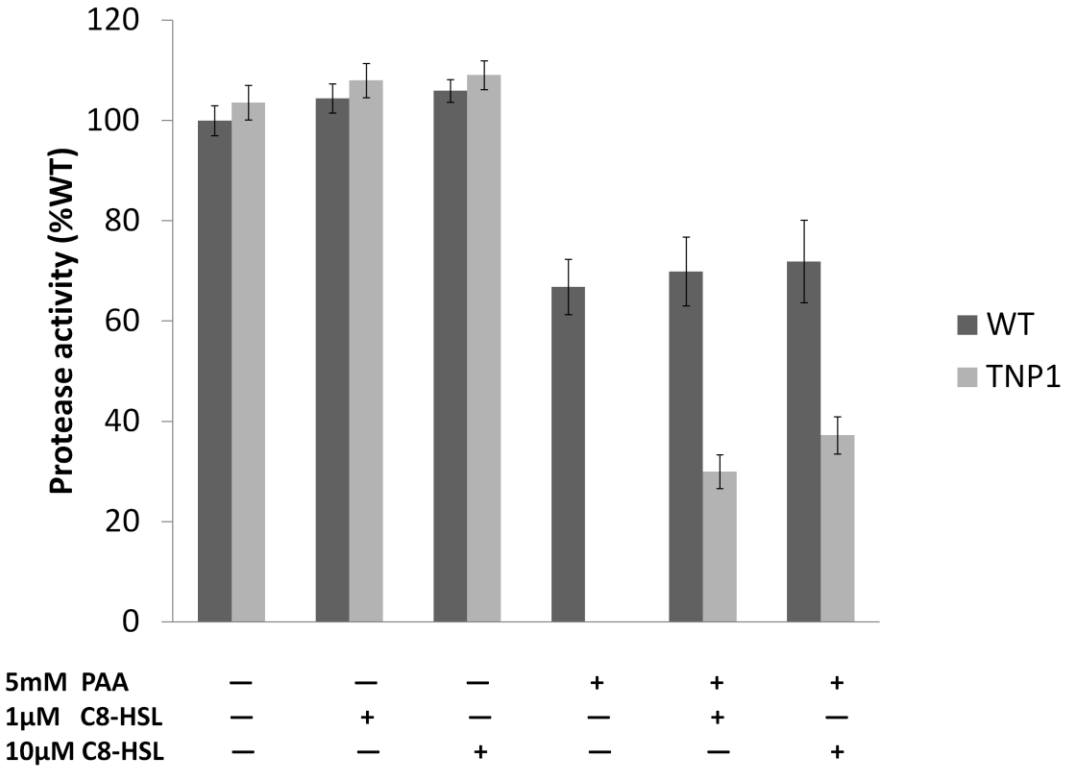


Figure 17. **C8-HSL counteracting effect on protease activity of *B. cenocepacia* K56-2 strains in the presence 5mM PAA.** Protease activity was measured on agar containing 2% skim milk with PAA and C8-HSL and expressed as the ratio between the diameter of the zones of clearance (including colonies) and the ones of WT on skim milk medium only. The error bars represent the SD of three independent experiments.

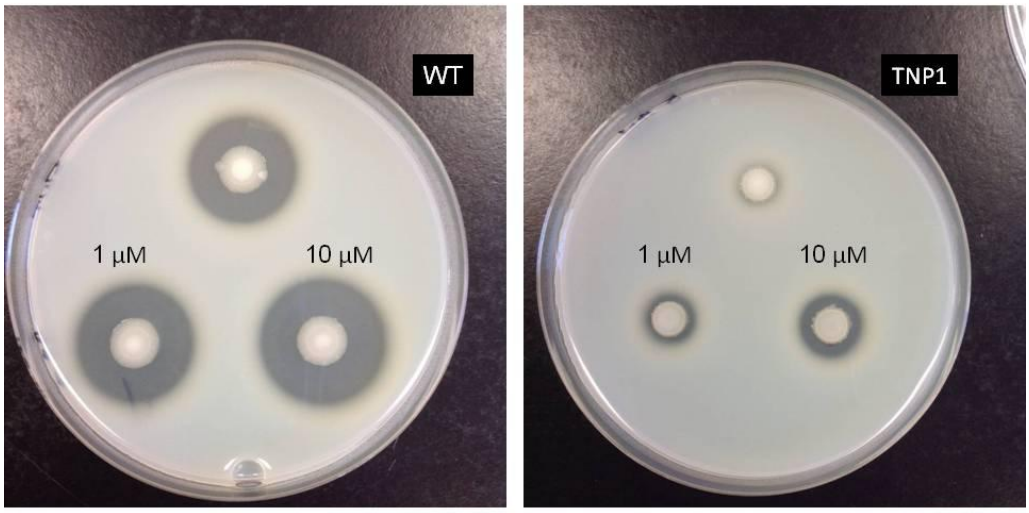


Figure 18. **Effect of C8-HSL on protease activity of *B. cenocepacia* K56-2 strains grown on 2% skim milk with 5mM PAA.** C8-HSL at a final concentration of 1 or 10 μM was spotted on agar first following the co-spotting with bacterial overnight cultures. The plates were allowed to dry and then incubated for 4 days. Zero μM is the ethyl acetate and served as a solvent control.

were carried out by our collaborator Dr. S. P. Bernier, a post doctoral fellow from Dr. Surette laboratory at McMster University To test our hypothesis, we used an AHL biosensor developed in *Agrobacterium tumefaciens* (Sokol *et al.*, 2003; Chambers *et al.*, 2005). The strain expresses the LuxR type QS regulator TraR from a constitutive promoter and the reporter *luxCDABE* from the AHL-responsive *traI* promoter. To determine whether the release of PAA by TNP1 strain impacted the production of AHLs by *B. cenocepacia* and/or its detection by the reporter system, low amounts of spent media obtained from 24 hours cultures of K56-2 WT and TNP1 strains were added to the reporter strain cultures and their respective expression profiles were compared directly (Figure 19A). For all tested concentrations of culture supernatant (i.e. 1, 2, and 5%), the signal emitted by the reporter strain was always higher when spent medium from the WT was used compared to TNP1. Higher concentrations of spent medium were not tested since it inhibited growth of *A. tumefaciens*. These data suggested that the excess of PAA released by TNP1 either led to a reduced production of AHLs or prevented AHLs from binding to TraR and the *traI* promoter of the reporter system. To assess the latter hypothesis, we added increasing concentrations of PAA to reporter cultures containing 1, 2, and 5% of spent medium from *B. cenocepacia* K56-2 WT to determine whether the reporter signal would get lower in the presence of PAA. Addition of 0.3 mM of PAA did not reduce the AHL-dependent response of the reporter strain compared to conditions without additional PAA (Figure 19B). Higher concentrations of PAA could not be evaluated since they inhibited growth of the reporter (data not shown). Altogether, a direct inhibition of AHL sensing by PAA does not seem to be sufficient to account for the reduced response to TNP1 supernatants. However, this inhibition of AHL sensing would

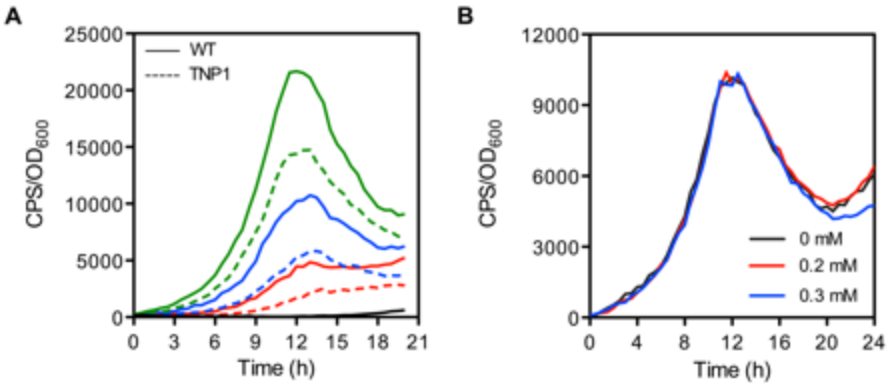


Figure 19. **PAA released by TNP1 leads to a reduced detection of AHLs by *A. tumefaciens* biosensor strain.** Relative luminescence due to AHL detection during growth of biosensor strain (A) with different amounts of spent medium (red 1%; blue 2%; green 5%) from WT (solid lines) and TNP1 (dashed lines) or (B) with 5% spent medium from WT with the addition of different concentration of PAA (black 0%; red 0.2%; blue 0.3%). The experiments were carried out by our collaborator Dr. S.P. Bernier.

lead to reduced AHL production in TNP1 by inhibiting the auto-induction feedback in AHL synthesis.

4.10. The attenuated pathogenicity phenotype of TNP1 in *C. elegans* is due to inhibition of a QS response.

The release of PAA by TNP1 mediated the reduction of AHL activity in *A. tumefaciens.* This observation together with the restoration of protease activity in TNP1 by addition of C8-HSL suggests that the inhibitory effect of PAA on pathogenicity can be counteracted by exogenous C8-HSL. To test this hypothesis, the effect of C8-HSL on the killing ability of B. cenocepacia K56-2 WT and TNP1 was measured during slow killing assays (Figure 20). In these conditions, the killing ability of K56-2 was unaffected as demonstrated by no significant difference between the survivals of worms on two types of media (Figure 20A). On the contrary, the attenuated pathogenicity of TNP1 was diminished; however, not to the wild type levels (Figure 20B). These results provide evidence that attenuation in pathogenicity of *B. cenocepacia* TNP1 in *C. elegans* is due to QS inhibition by endogenously produced PAA during growth in NGM. The effect of C8-HSL on bacterial growth was not analyzed.

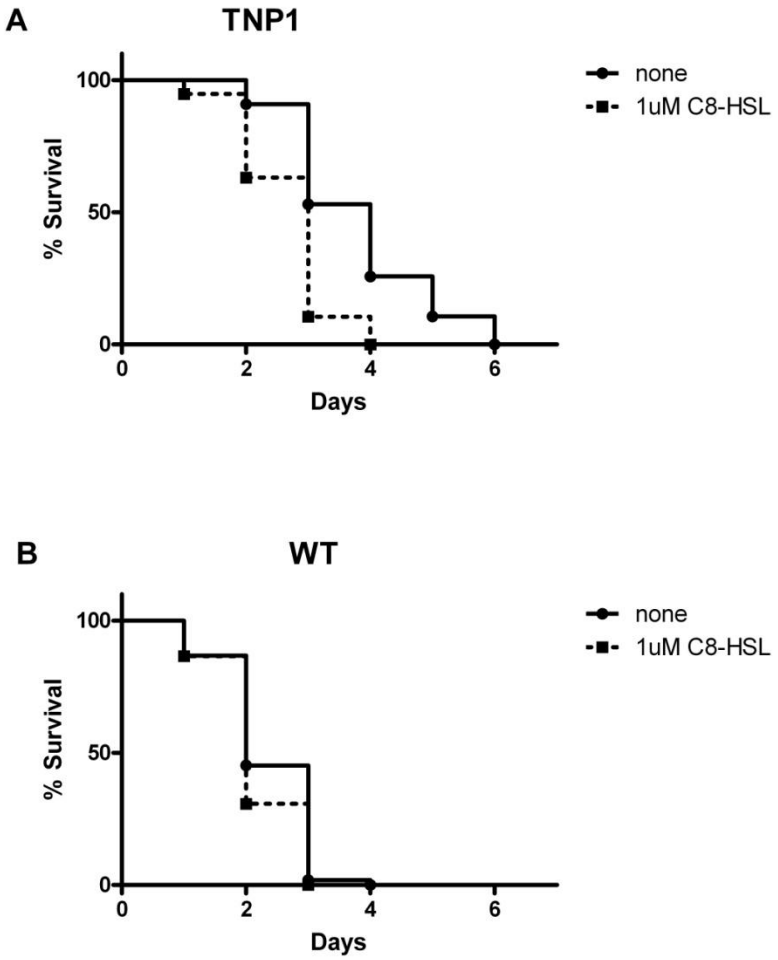


Figure 20. **C8-HSL effect on pathogenic phenotype of *B. cenocepacia* K56-2 strains.** (A) The attenuated pathogenicity of TNP1 is diminished in the presence of 1μM C8-HSL. The killing ability of TNP1 grown on NGM (n=66) was compared to TNP1 grown on NGM supplemented with 1μM C8-HSL (n=57, $P < 0.0001$). (B) Addition of 1μM C8-HSL does not affect pathogenic phenotype of WT strain. The killing ability of WT grown on NGM (n=61) was compared to WT grown on NGM supplemented with 1μM C8-HSL (n=45, P =0.7429). The data is representative of 3 independent experiments.

5. Discussion

5.1. Examinations of bacterial filtrate for the presence of PAA related bioactive metabolites

The goal of this work was to elucidate the reasons for the requirement of *paaABCDE* cluster of *B. cenocepacia* K56-2 for full pathogenicity of K56-2 in *C. elegans*. We initially hypothesized that *B. cenocepacia* K56-2 released PAA catabolic pathway related molecules were involved in pathogenesis in *C. elegans* (Law *et al.*, 2008). Hence, mutation arresting the pathway in one of the steps will cause accumulation of pathway intermediates. To test this hypothesis were performed the filter diffusion slow killing assays on normalized bacterial lawns using K56-2 WT and *paaABCDE* deletion mutant, TNP1. Overall, WT filtrate had a subtle effect on pathogenic phenotype of the TNP1 indicating the presence of metabolites or intermediates, which are absent in the TNP1 and might account the reduced pathogenicity. On the other hand, the decrease in pathogenicity of TNP1 in the presence of its own filtrate can be attributed to the release of PAA, which has been shown to enhance the attenuation (Figure 14). Although, the WT and TNP grown on no-filtrate conditioned medium exhibited their expected pathogenicity phenotype indicating that bacterial strains, media and temperature of incubation were proper, there are still other environmental factors that we were unable to deduce and to control, which caused a significant variability in the experiment.

The reduced growth on filtrate-conditioned medium can be attributed to the depletion of media nutrients by the previously grown bacteria, as well as potential excretion of bacterial waste products during 24 hours growth on filter. In addition, the diameter of filter paper did not completely cover the agar medium allowing worms to spend some

time in the filtrate free area, however, no general chemorepulsive nature of bacterial filtrate was observed and worms did not prefer certain area of the plate. These observations and the inconsistency of WT filtrate to restore pathogenicity inTNP1 are needed to be taken in the account when evaluating whether the filter diffusion assays is an efficient approach to examine the PAA related bioactivity of bacterial filtrate.

5.2. 2-OHPAA does not play role in pathogenicity of K56-5 towards *C. elegans*

One of the known metabolites to be released from the pathway as a dead end metabolite is 2-OHPAA (Teufel *et al.*, 2010; Grishin *et al.*, 2011; Teufel *et al.*, 2012). If 2-OHPAA indeed mediates pathogenicity in *B. cenocepacia*, interruption of the *paaABCDE* cluster will prevent formation of 2-OHPAA and as a result will cause an attenuation effect on pathogenicity. Our efforts at complementing the defective phenotype of the *paaABCDE* deletion mutant with exogenously added 2-OHPAA were, however, unsuccessful in proving this hypothesis. Although not involved in pathogenicity and being a dead end metabolite of PAA pathway, 2-OHPAA can serve as a carbon source for *B. cenocepacia* K56-2 WT and *paaABCDE* mutants (Yudistira *et al.*, 2011). Interestingly enough, 2-OHPAA cannot be used as a carbon source by *E. coli* strain (Ismail *et al.*, 2003), which is known to have PAA degradation pathway (Ferrandez *et al.*, 1998). The ability of *B. cenocepacia* to utilize 2OHPAA might serve as competitive advantage even in the population of bacteria degrading PAA furthermore demonstrating metabolic capacities of *B. cenocepacia*.

5.3. Delayed complementation of TNP1/pTP2

The complementation of PAA growth defective phenotype of TNP1 mutant was observed only after almost 3 days of incubation. Since pTP2 plasmid was sequenced and

did not have any mutation to affect *paaABCDE* expression and translation and it was able to complement *paaE* mutant, we thought that the cause might be in the TNP strain genotype. We reasoned that prolonged incubation might be required for the mutant strain to remove the repression imposed by the TetR- like regulator, encoded by the downstream to the *paaABCDE* gene cluster, BCAL0210. Removing the *paaABCDE* gene cluster results in the physical proximity of *paaA* promoter region located upstream to *paaABCDE* genes and intergenic region of BCAL0211. Due to physical proximity of the promoter the co-transcription of BCAL0211 and BCAL0210 genes, which had been shown previously to be co-transcribed might be initiated from the activated by the presence of PAA *paaA* promoter rather than BCAL0211 native promoter. In this case, the BCAL0210 gene product, TetR-like transcriptional regulator will be produced. Despite the repression imposed by the TetR-like regulator, there is a basal level of *paaK* expression that will be responsible PAA-CoA production. At certain time point the concentration of PAA-CoA produced will be sufficient to remove the repression of TetR-like negative regulator.

5.4. TNP1 unable to metabolize PAA releases it into growth medium while WT is actively degrades PAA.

Another approach to search for PAA related bioactive metabolites was to analyze the supernatant of WT for the presence of released metabolites and their absence in the supernatant of TNP1. The bacterial supernatants extracts were prepared from overnight cultures of bacteria grown in NGM broth since the host-pathogen interactions were studied on NGM and analyzed by HPLC. WT supernatants did not contain any released metabolites as was evident from WT trace that was nearly indistinguishable from the

trace of media control extract. However, trace of TNP1 showed presence of one predominant peak, which corresponded to PAA. Since TNP1 strain has a mutation in the ring hydroxylation complex, the pathway is arrested after ligation step. First enzyme of the pathway, PaaK, will activate PAA by adding CoA group to the molecule resulting in production of PAA-CoA molecules. Due to interruption of the second enzymatic complex of the pathway, PaaABCDE, the PAA-CoA is expected to be converted back to PAA by PaaI.

The PAA degradation pathway is a source of potentially toxic compounds and it is believed that a number of mechanisms are in place to avoid the accumulation of such toxic metabolites by rapid conversion to less toxic molecules, like the reversible action of the PaaABCDE complex to remove the potentially toxic epoxide (Teufel *et al.*, 2012). Theoretically, in case of overflow of downstream metabolites or arrested enzymatic steps, the epoxide will be reverted to PAA-CoA by PaaABCDE, and then converted to PAA by PaaI-mediated hydrolysis (Song *et al.*, 2006) leading to release of PAA. However, we could not find any growth condition where PAA was released by *B. cenocepacia* K56-2 WT. The reasons for this might be that the PAA degradation pathway is highly active when *B. cenocepacia* is grown on LB medium and in conditions that mimic the amino acid-rich environment of the cystic fibrosis sputum (Hamlin *et al.*, 2009; Yoder-Himes *et al.*, 2009; Yudistira *et al.*, 2011) where *B. cepacia* complex thrives. Hence, we arguably conclude that impeding overflow of a toxic epoxide, achieved through the release of a pathogenicity antagonist seems to be a costly mechanism to avoid intracellular accumulation of toxic compounds in an opportunistic pathogen.

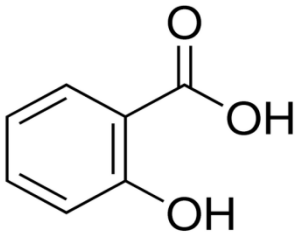
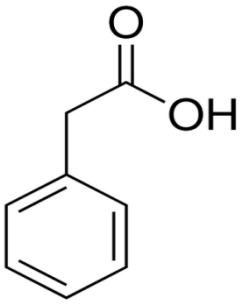
5.5. Endogenously produced PAA by TNP1 interferes with QS- regulated functions in *B. cenocepacia.*

In this study we reveal the involvement of QS in the K56-2 pathogenesis in *C. elegans*. AHL-mediated QS is known to be important in regulation of various virulence factors in Bcc such as proteases, siderophores, motility and biofilm production (Lewenza *et al.*, 1999; Huber *et al.*, 2002). Although proteases were shown not to be required for pathogenesis in nematode model (Kothe *et al.*, 2003; Uehlinger *et al.*, 2009), our results support that PAA interacts with at least one QS regulated activity in K56-2, similarly to results from *P. aeruginosa* PAO1 study (Musthafa *et al.*, 2012). Mutant reduced protease activity in the presence of 5 mM PAA could be compensated with the addition synthetic AHL demonstrating the counteracting interaction between PAA and AHL molecules. Furthermore, addition of synthetic AHL to the slow killing medium resulted in the partial restoration of mutant pathogenic phenotype in host model. Thus, we have demonstrated the cause of the attenuation and connected a microbial catabolic pathway with the QS response, providing further evidence to the link between metabolic capacities of bacterial pathogens and virulence (Anyanful *et al.*, 2005; Brown *et al.*, 2008; Boulette *et al.*, 2009; Keeney *et al.*, 2009; Eisenreich *et al.*, 2010).

5.5.1. Possible mechanisms of PAA-mediated QS signal inhibition

Although we have revealed the association between PAA release and QS signaling inhibition the actual mechanism still remains to be determined. PAA might interact with CepI and hence directly inhibiting AHL synthesis or it can competitively bind CepR regulator that indirectly will reduce AHL levels by inhibiting *cepI* synthesis. Salicylic acid (SA) is a plant hormone, which is structurally similar (Table 5) to PAA has also

Table 5. General comparison between salicylic and phenylacetic acids

General comparison between salicylic and phenylacetic acids	
Salicylic acid	**Phenylacetic acid**
$C_7H_6O_3$ 2-hydroxybenzoic acid Phytohormone 	$C_8H_8O_2$ Benzenacetic acid; α-toluic acid Phytohormone 

been shown to reduce protease activity and attenuate pathogenicity towards *C. elegans* in *P. aeruginosa* PA14 (Prithiviraj *et al.,* 2005). Using structure-based virtual screening SA was identified as a QS inhibitor in *P. aeruginosa* PA01 (Yang *et al.,* 2009). Although these evidences support the anti QS nature of PAA, the mechanism, by which SA inhibits QS, also remains unknown. Another speculation is that mutant strain in addition to PAA releases PAA-CoA as well, which would be produced if PaaI- mediated hydrolysis of PAA-CoA is lower than PaaK- mediated ligation. PAA-CoA is the inducer of the PAA degradation pathway since it has been previously shown to remove the repression imposed by the negative regulator suggesting the ability of at least of PAA-CoA to interact with transcriptional regulators (Ferrandez *et al.,* 2000; Garcia *et al.,* 2000).

5.6. The biological relevance of PAA degradation pathway in the polymicrobial infections

The biological relevance of the PAA degradation pathway may emerge in the context of microbial population dynamics, an area of great promise for further understanding microbial disease mechanisms (Peters *et al.,* 2012). In the lung of CF patients, successive colonization of *Staphylococcus aureus*, *Haemophilus influenza,* and *Pseudomonas aeruginosa* (LiPuma, 2010), always occur in the setting of the complex microbial community (Sibley *et al.*, 2008; Sibley *et al.*, 2009). Clinical data suggests that co-colonization by *P. aeruginosa* and Bcc is more deleterious to the pulmonary condition than colonization by each of the species alone (Jacques *et al.*, 1998). Due to physical proximity and the fact that *P. aeruginosa* and Bcc species are able to utilize AHL molecules, it is reasonable to assume that there is cross talk between bacteria via QS (Lewenza *et al.*, 2002; Eberl *et al.*, 2004). Interestingly, *P. aeruginosa* is capable of

releasing PAA when grown to high-density levels, which in turns attenuates cytotoxicity (Wang *et al.*, 2013), and exogenously added PAA inhibits QS related functions (Musthafa *et al.*, 2012). In contrast to *P. aeruginosa*, for which PAA is inhibitory, *B. cenocepacia* can grow with PAA as a sole carbon source (Law *et al.*, 2008) and PAA is even preferred over other nutrients during growth on NGM. Given that the release of PAA by a member of the cystic fibrosis microbial population could lead to attenuation of virulence, we propose that removal of PAA by Bcc might be important to CF lung disease progression. By actively degrading PAA, *B. cenocepacia* could be acting as a housekeeper removing the quorum-inhibition compound, restoring the pathogenicity of the population and promoting its own growth and fitness in the population. The addition of synthetic AHL to the killing assay did not increase pathogenicity of *B. cenocepacia* wild type. While the reasons are unknown we suggest that in the experimental conditions tested *B. cenocepacia* might have reached its maximum pathogenicity achievable by QS response.

5.7. New approach for therapeutics development

Our results highlight the importance of understanding microbial pathogens in the context not only nutritional sources available in the host but also their interacting microbial partners. Linking the catabolism to pathogenicity via interspecies communication within polymicrobial infections can serve as a new platform for development of new therapeutics that will be efficient against community as whole rather than single species. In polymicrobial infections, a given drug can have different effects on different species. While PAA attenuates cytotoxicity in *P. aeruginosa* (Wang *et al.*,

2013) it clearly induces growth in *B. cenocepacia* and only attenuates pathogenicity of *B. cenocepacia* in a defective PAA-degradation genetic background. Thus, an antivirulence molecule for *P. aeruginosa* may not have effect in the cystic fibrosis lung environment due to active degradation by Bcc.

6. Conclusions

The phenylacetic acid degradation pathway of *Burkholderia cenocepacia* is active during cystic fibrosis-like conditions and is necessary for full pathogenicity of *B. cenocepacia* in nematode and rat infection models (Hunt *et al.*, 2004; Law *et al.*, 2008); however, the reasons of such requirements are unknown. The goal of this work was to elucidate the reasons why the *paaABCDE* cluster of *B. cenocepacia* K56-2 was required for full pathogenicity in *C. elegans*. The initial hypothesis that *B. cenocepacia* K56-2 might release intermediates of the PAA catabolic pathway that were involved in the pathogenic mechanism against *C. elegans* was based on data obtained from metabolite filter diffusion assay performed previously in our laboratory (Law *et al.*, 2008). However, due to reduced growth on filtrate-conditioned medium, the original protocol was modified accordingly. The results obtained from the modified protocol could only moderately support the hypothesis since we were able to show subtly restoration effect of WT filtrate on attenuated pathogenicity TNP1 mutant. Nevertheless, inconsistency in the WT filtrate bioactivity and the general effect on growth were revealed to be the essential points in performing this type of assay. In parallel with metabolite filter assays we also tested a known metabolites of the pathway such as the by-product of PAA degradation, 2-OHPAA (Teufel *et al.*, 2010; Grishin *et al.*, 2011; Teufel *et al.*, 2012). We hypothesized that interruption of the ring hydroxylation complex would prevent formation of 2-OHPAA causing the attenuation effect. However, our efforts at complementing the defective phenotype of Δ*paaABCDE* with exogenously added 2-OHPAA were unsuccessful and 2-OHPAA was never detected in WT supernatants. In light of the recent report demonstrating the ability of PAA to interfere with QS signaling in *P. aeruginosa*

prompted us to investigate whether the same phenomenon would be observed in *B. cenocepacia* K56-2. In this work, we show that the attenuated pathogenicity of TNP1 is due to QS inhibition. Unlike *B. cenocepacia* WT, unmetabolized PAA is released by TNP1 extracellularly in conditions that allow infection in *C. elegans* such as NGM. In the same conditions, detection of AHL by *A. tumefaciens* biosensor strain in TNP1 was reduced. Furthermore, addition of PAA into skim milk medium inhibited protease activity of TNP1 and further decreased its pathogenicity when added to the NGM killing assay while no significant effect was observed for the WT due to active PAA degradation. Phenotypes repressed in TNP1, protease activity and pathogenicity against *C. elegans*, increased with the addition of exogenous AHL. Thus, we demonstrate that the attenuated phenotype of *B. cenocepacia* Δ*paaABCDE* is due to QS inhibition by release of PAA, affecting AHL signaling. Thus, we have revealed the cause of the attenuation and connected a microbial catabolic pathway with the QS response, providing further evidence to the link between the metabolic capabilities of bacterial pathogens and virulence.

Future work will focus on elucidating the actual mechanism of QS signaling inhibition by PAA that will shed a light on interspecies communication as well as biological relationships within complex polymicrobial communities.

Bibliography

Aballay A., and Ausubel F.M. (2002) *Caenorhabditis elegans* as a host for the study of host-pathogen interactions. *Curr Opin Microbiol* **5:** 97-101.

Agnoli K., Frauenknecht C., Freitag R., Schwager S., Jenul C., Vergunst A., *et al.* (2014) The third replicon of members of the *Burkholderia cepacia* Complex, plasmid pC3, plays a role in stress tolerance. *Appl Environ Microbiol* **80:** 1340-1348.

Agnoli K., Schwager S., Uehlinger S., Vergunst A., Viteri D.F., Nguyen D.T., *et al.* (2012) Exposing the third chromosome of *Burkholderia cepacia* complex strains as a virulence plasmid. *Mol Microbiol* **83:** 362-378.

Aguilar C., Bertani I., and Venturi V. (2003) Quorum-sensing system and stationary-phase sigma factor (rpoS) of the onion pathogen *Burkholderia cepacia* genomovar I type strain, ATCC 25416. *Appl Environ Microbiol* **69:** 1739-1747.

Anyanful A., Dolan-Livengood J.M., Lewis T., Sheth S., Dezalia M.N., Sherman M.A., *et al.* (2005) Paralysis and killing of *Caenorhabditis elegans* by enteropathogenic *Escherichia coli* requires the bacterial tryptophanase gene. *Mol Microbiol* **57:** 988-1007.

Baldwin A., Sokol P.A., Parkhill J., and Mahenthiralingam E. (2004) The *Burkholderia cepacia* epidemic strain marker is part of a novel genomic island encoding both virulence and metabolism-associated genes in *Burkholderia cenocepacia*. *Infect Immun* **72:** 1537-1547.

Barber C.E., Tang J.L., Feng J.X., Pan M.Q., Wilson T.J., Slater H., *et al.* (1997) A novel regulatory system required for pathogenicity of *Xanthomonas campestris* is mediated by a small diffusible signal molecule. *Mol Microbiol* **24:** 555-566.

Beck J.M., Young V.B., and Huffnagle G.B. (2012) The microbiome of the lung. *Transl Res* **160:** 258-266.

Bentley S.D., and Parkhill J. (2004) Comparative genomic structure of prokaryotes. *Annu Rev Genet* **38:** 771-792.

Bernier S.P., Beeston A.L., and Sokol P.A. (2008) Detection of *N*-acyl homoserine lactones using a *traI-luxCDABE*-based biosensor as a high-throughput screening tool. *BMC Biotechnol* **8:** 59-6750-8-59.

Bernier S.P., Silo-Suh L., Woods D.E., Ohman D.E., and Sokol P.A. (2003) Comparative analysis of plant and animal models for characterization of *Burkholderia cepacia* virulence. *Infect Immun* **71:** 5306-5313.

Bi H., Christensen Q.H., Feng Y., Wang H., and Cronan J.E. (2012) The *Burkholderia cenocepacia* BDSF quorum sensing fatty acid is synthesized by a bifunctional crotonase homologue having both dehydratase and thioesterase activities. *Mol Microbiol* **83:** 840-855.

Blackburn L., Brownlee K., Conway S., and Denton M. (2004) 'Cepacia syndrome' with *Burkholderia multivorans*, 9 years after initial colonization. *J Cyst Fibros* **3:** 133-134.

Bontemps C., Elliott G.N., Simon M.F., Dos Reis Junior F.B., Gross E., Lawton R.C., *et al.* (2010) *Burkholderia* species are ancient symbionts of legumes. *Mol Ecol* **19:** 44-52.

Boon C., Deng Y., Wang L.H., He Y., Xu J.L., Fan Y., *et al.* (2008) A novel DSF-like signal from *Burkholderia cenocepacia* interferes with *Candida albicans* morphological transition. *Isme j* **2:** 27-36.

Boulette M.L., Baynham P.J., Jorth P.A., Kukavica-Ibrulj I., Longoria A., Barrera K., *et al.* (2009) Characterization of alanine catabolism in *Pseudomonas aeruginosa* and its importance for proliferation in vivo. *J Bacteriol* **191:** 6329-6334.

Brenner S. (1974) The genetics of *Caenorhabditis elegans*. *Genetics* **77:** 71-94.

Brown S.A., Palmer K.L., and Whiteley M. (2008) Revisiting the host as a growth medium. *Nat Rev Microbiol* **6:** 657-666.

Burkholder W.H. (1950) Sour skin, a bacterial rot of onion bulbs. *Phytopathol* **40:** 115-117.

Byers J.T., Lucas C., Salmond G.P., and Welch M. (2002) Nonenzymatic turnover of an *Erwinia carotovora* quorum-sensing signaling molecule. *J Bacteriol* **184:** 1163-1171.

Chambers C.E., Visser M.B., Schwab U., and Sokol P.A. (2005) Identification of *N*-acylhomoserine lactones in mucopurulent respiratory secretions from cystic fibrosis patients. *FEMS Microbiol Lett* **244:** 297-304.

Churchill M.E., and Chen L. (2011) Structural basis of acyl-homoserine lactone-dependent signaling. *Chem Rev* **111:** 68-85.

Cieri M.V., Mayer-Hamblett N., Griffith A., and Burns J.L. (2002) Correlation between an in vitro invasion assay and a murine model of *Burkholderia cepacia* lung infection. *Infect Immun* **70:** 1081-1086.

Corbett C.R., Burtnick M.N., Kooi C., Woods D.E., and Sokol P.A. (2003) An extracellular zinc metalloprotease gene of *Burkholderia cepacia*. *Microbiology* **149:** 2263-2271.

Czajkowski R., and Jafra S. (2009) Quenching of acyl-homoserine lactone-dependent quorum sensing by enzymatic disruption of signal molecules. *Acta Biochim Pol* **56:** 1-16.

de Kievit T.R., and Iglewski B.H. (2000) Bacterial quorum sensing in pathogenic relationships. *Infect Immun* **68:** 4839-4849.

Deng Y., Schmid N., Wang C., Wang J., Pessi G., Wu D., *et al.* (2012) Cis-2-dodecenoic acid receptor RpfR links quorum-sensing signal perception with regulation of virulence through cyclic dimeric guanosine monophosphate turnover. *Proc Natl Acad Sci U S A* **109:** 15479-15484.

Dobbin C.J., Soni R., Jelihovsky T., and Bye P.T. (2000) Cepacia syndrome occurring following prolonged colonisation with *Burkholderia cepacia*. *Aust N Z J Med* **30:** 288-289.

Dong Y.H., and Zhang L.H. (2005) Quorum sensing and quorum-quenching enzymes. *J Microbiol* **43 Spec No:** 101-109.

Drevinek P., Holden M.T., Ge Z., Jones A.M., Ketchell I., Gill R.T., and Mahenthiralingam E. (2008) Gene expression changes linked to antimicrobial resistance, oxidative stress, iron depletion and retained motility are observed when *Burkholderia cenocepacia* grows in cystic fibrosis sputum. *BMC Infect Dis* **8:** 121.

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

Eberhard A., Burlingame A.L., Eberhard C., Kenyon G.L., Nealson K.H., and Oppenheimer N.J. (1981) Structural identification of autoinducer of *Photobacterium fischeri* luciferase. *Biochemistry* **20:** 2444-2449.

Eberl L., and Tummler B. (2004) *Pseudomonas aeruginosa* and *Burkholderia cepacia* in cystic fibrosis: genome evolution, interactions and adaptation. *Int J Med Microbiol* **294:** 123-131.

Eisenreich W., Dandekar T., Heesemann J., and Goebel W. (2010) Carbon metabolism of intracellular bacterial pathogens and possible links to virulence. *Nat Rev Microbiol* **8:** 401-412.

El-Said Mohamed M. (2000) Biochemical and molecular characterization of phenylacetate-coenzyme A ligase, an enzyme catalyzing the first step in aerobic metabolism of phenylacetic acid in *Azoarcus evansii*. *J Bacteriol* **182:** 286-294.

Escobar M.A., and Dandekar A.M. (2003) *Agrobacterium tumefaciens* as an agent of disease. *Trends Plant Sci* **8:** 380-386.

Ferrandez A., Garcia J.L., and Diaz E. (2000) Transcriptional regulation of the divergent *paa* catabolic operons for phenylacetic acid degradation in *Escherichia coli*. *J Biol Chem* **275:** 12214-12222.

Ferrandez A., Minambres B., Garcia B., Olivera E.R., Luengo J.M., Garcia J.L., and Diaz E. (1998) Catabolism of phenylacetic acid in *Escherichia coli*. Characterization of a new aerobic hybrid pathway. *J Biol Chem* **273:** 25974-25986.

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

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

Freeman J.A., Lilley B.N., and Bassler B.L. (2000) A genetic analysis of the functions of LuxN: a two-component hybrid sensor kinase that regulates quorum sensing in *Vibrio harveyi*. *Mol Microbiol* **35:** 139-149.

Fuqua C. (2006) The QscR quorum-sensing regulon of *Pseudomonas aeruginosa*: an orphan claims its identity. *J Bacteriol* **188:** 3169-3171.

Fuqua W.C., and Winans S.C. (1994) A LuxR-LuxI type regulatory system activates *Agrobacterium* Ti plasmid conjugal transfer in the presence of a plant tumor metabolite. *J Bacteriol* **176:** 2796-2806.

Gan Y.H., Chua K.L., Chua H.H., Liu B., Hii C.S., Chong H.L., and Tan P. (2002) Characterization of *Burkholderia pseudomallei* infection and identification of novel virulence factors using a *Caenorhabditis elegans* host system. *Mol Microbiol* **44:** 1185-1197.

Garcia B., Olivera E.R., Minambres B., Carnicero D., Muniz C., Naharro G., and Luengo J.M. (2000) Phenylacetyl-coenzyme A is the true inducer of the phenylacetic acid catabolism pathway in *Pseudomonas putida* U. *Appl Environ Microbiol* **66:** 4575-4578.

Gibson R.L., Burns J.L., and Ramsey B.W. (2003) Pathophysiology and management of pulmonary infections in cystic fibrosis. *Am J Respir Crit Care Med* **168:** 918-951.

Gotschlich A., Huber B., Geisenberger O., Togl A., Steidle A., Riedel K., *et al.* (2001) Synthesis of multiple *N*-acylhomoserine lactones is wide-spread among the members of the *Burkholderia cepacia* complex. *Syst Appl Microbiol* **24:** 1-14.

Grishin A.M., Ajamian E., Tao L., Zhang L., Menard R., and Cygler M. (2011) Structural and functional studies of the *Escherichia coli* phenylacetyl-CoA monooxygenase complex. *J Biol Chem* **286:** 10735-10743.

Hamlin J.N., Bloodworth R.A., and Cardona S.T. (2009) Regulation of phenylacetic acid degradation genes of *Burkholderia cenocepacia* K56-2. *BMC Microbiol* **9:** 222.

Hanzelka B.L., and Greenberg E.P. (1996) Quorum sensing in *Vibrio fischeri*: evidence that S-adenosylmethionine is the amino acid substrate for autoinducer synthesis. *J Bacteriol* **178:** 5291-5294.

Hengge R. (2009) Principles of c-di-GMP signalling in bacteria. *Nat Rev Microbiol* **7:** 263-273.

Hiemstra P.S. (2007) The role of epithelial beta-defensins and cathelicidins in host defense of the lung. *Exp Lung Res* **33:** 537-542.

Holden M.T., Seth-Smith H.M., Crossman L.C., Sebaihia M., Bentley S.D., Cerdeno-Tarraga A.M., *et al.* (2009) The genome of *Burkholderia cenocepacia* J2315, an epidemic pathogen of cystic fibrosis patients. *J Bacteriol* **191:** 261-277.

Huang J.J., Han J.I., Zhang L.H., and Leadbetter J.R. (2003) Utilization of acyl-homoserine lactone quorum signals for growth by a soil pseudomonad and *Pseudomonas aeruginosa* PAO1. *Appl Environ Microbiol* **69:** 5941-5949.

Huber B., Riedel K., Kothe M., Givskov M., Molin S., and Eberl L. (2002) Genetic analysis of functions involved in the late stages of biofilm development in *Burkholderia cepacia* H111. *Mol Microbiol* **46:** 411-426.

Huber B., Feldmann F., Kothe M., Vandamme P., Wopperer J., Riedel K., and Eberl L. (2004) Identification of a novel virulence factor in *Burkholderia cenocepacia* H111 required for efficient slow killing of *Caenorhabditis elegans*. *Infect Immun* **72:** 7220-7230.

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

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

Ismail W., El-Said Mohamed M., Wanner B.L., Datsenko K.A., Eisenreich W., Rohdich F., *et al.* (2003) Functional genomics by NMR spectroscopy. Phenylacetate catabolism in *Escherichia coli*. *Eur J Biochem* **270:** 3047-3054.

Jacques I., Derelle J., Weber M., and Vidailhet M. (1998) Pulmonary evolution of cystic fibrosis patients colonized by *Pseudomonas aeruginosa* and/or *Burkholderia cepacia*. *Eur J Pediatr* **157:** 427-431.

Jimenez J.I., Minambres B., Garcia J.L., and Diaz E. (2002) Genomic analysis of the aromatic catabolic pathways from *Pseudomonas putida* KT2440. *Environ Microbiol* **4:** 824-841.

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

Kato K., and Takayama T. (2002) *Pseudomonas aeruginosa* infection and cystic fibrosis. *Lancet* **359:** 262.

Keeney K., Colosi L., Weber W., and O'Riordan M. (2009) Generation of branched-chain fatty acids through lipoate-dependent metabolism facilitates intracellular growth of *Listeria monocytogenes*. *J Bacteriol* **191:** 2187-2196.

Kooi C., Subsin B., Chen R., Pohorelic B., and Sokol P.A. (2006) *Burkholderia cenocepacia* ZmpB is a broad-specificity zinc metalloprotease involved in virulence. *Infect Immun* **74:** 4083-4093.

Kothe M., Antl M., Huber B., Stoecker K., Ebrecht D., Steinmetz I., and Eberl L. (2003) Killing of *Caenorhabditis elegans* by *Burkholderia cepacia* is controlled by the *cep* quorum-sensing system. *Cell Microbiol* **5:** 343-351.

Law R.J. (2009) The Relationship between Phenylacetic acid Catabolism and Pathogenicity of *Burkholderia cenocepacia* K56-2 in the *Caenorhabditis elegans* Host Model. The Relationship between Phenylacetic acid Catabolism and Pathogenicity of *Burkholderia cenocepacia* K56-2 in the *Caenorhabditis elegans* Host Model.

Law R.J., Hamlin J.N.R., Sivro A., McCorrister S.J., Cardama G.A., and Cardona S.T. (2008) A functional phenylacetic acid catabolic pathway is required for full pathogenicity of *Burkholderia cenocepacia* in the *Caenorhabditis elegans* host model. *J Bacteriol* **190:** 7209-7218.

Ledson M.J., Gallagher M.J., Jackson M., Hart C.A., and Walshaw M.J. (2002) Outcome of *Burkholderia cepacia* colonization in an adult cystic fibrosis centre. *Thorax* **57:** 142-145.

Lewenza S., and Sokol P.A. (2001) Regulation of ornibactin biosynthesis and *N*-acyl-L-homoserine lactone production by CepR in *Burkholderia cepacia*. *J Bacteriol* **183:** 2212-2218.

Lewenza S., Visser M.B., and Sokol P.A. (2002) Interspecies communication between *Burkholderia cepacia* and *Pseudomonas aeruginosa*. *Can J Microbiol* **48:** 707-716.

Lewenza S., Conway B., Greenberg E.P., and Sokol P.A. (1999) Quorum sensing in *Burkholderia cepacia*: identification of the LuxRI homologs CepRI. *J Bacteriol* **181:** 748-756.

Lipuma J.J. (2010) The changing microbial epidemiology in cystic fibrosis. *Clin Microbiol Rev* **23:** 299-323.

LiPuma J.J. (2010) The changing microbial epidemiology in cystic fibrosis. *Clin Microbiol Rev* **23:** 299-323.

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

Luengo J.M., Garcia J.L., and Olivera E.R. (2001) The phenylacetyl-CoA catabolon: a complex catabolic unit with broad biotechnological applications. *Mol Microbiol* **39:** 1434-1442.

Lutter E., Lewenza S., Dennis J.J., Visser M.B., and Sokol P.A. (2001) Distribution of quorum-sensing genes in the *Burkholderia cepacia* complex. *Infect Immun* **69:** 4661-4666.

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

Mahenthiralingam E., Simpson D.A., and Speert D.P. (1997) Identification and characterization of a novel DNA marker associated with epidemic *Burkholderia cepacia* strains recovered from patients with cystic fibrosis. *J Clin Microbiol* **35:** 808-816.

Mahenthiralingam E., Campbell M.E., Henry D.A., and Speert D.P. (1996) Epidemiology of *Burkholderia cepacia* infection in patients with cystic fibrosis: analysis by randomly amplified polymorphic DNA fingerprinting. *J Clin Microbiol* **34:** 2914-2920.

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

Malott R.J., Baldwin A., Mahenthiralingam E., and Sokol P.A. (2005) Characterization of the cciIR quorum-sensing system in *Burkholderia cenocepacia*. *Infect Immun* **73:** 4982-4992.

Malott R.J., O'Grady E.P., Toller J., Inhulsen S., Eberl L., and Sokol P.A. (2009) A *Burkholderia cenocepacia* orphan LuxR homolog is involved in quorum-sensing regulation. *J Bacteriol* **191:** 2447-2460.

Marketon M.M., Gronquist M.R., Eberhard A., and Gonzalez J.E. (2002) Characterization of the *Sinorhizobium meliloti sinR/sinI* locus and the production of novel *N*-acyl homoserine lactones. *J Bacteriol* **184:** 5686-5695.

Martin F.J., and McInerney J.O. (2009) Recurring cluster and operon assembly for Phenylacetate degradation genes. *BMC Evol Biol* **9:** 36.

Martinez-Blanco H., Reglero A., Rodriguez-Aparicio L.B., and Luengo J.M. (1990) Purification and biochemical characterization of phenylacetyl-CoA ligase from *Pseudomonas putida*. A specific enzyme for the catabolism of phenylacetic acid. *J Biol Chem* **265:** 7084-7090.

McCarthy Y., Yang L., Twomey K.B., Sass A., Tolker-Nielsen T., Mahenthiralingam E., *et al.* (2010) A sensor kinase recognizing the cell-cell signal BDSF (*cis*-2-dodecenoic acid) regulates virulence in *Burkholderia cenocepacia*. *Mol Microbiol* **77:** 1220-1236.

McClean K.H., Winson M.K., Fish L., Taylor A., Chhabra S.R., Camara M., *et al.* (1997) Quorum sensing and *Chromobacterium violaceum*: exploitation of violacein production and inhibition for the detection of *N*-acylhomoserine lactones. *Microbiology* **143 (Pt 12):** 3703-3711.

McKeon S.A., Nguyen D.T., Viteri D.F., Zlosnik J.E., and Sokol P.A. (2011) Functional quorum sensing systems are maintained during chronic *Burkholderia cepacia* complex infections in patients with cystic fibrosis. *J Infect Dis* **203:** 383-392.

Miller V.L., and Mekalanos J.J. (1988) A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires toxR. *J Bacteriol* **170:** 2575-2583.

Minogue T.D., Wehland-von Trebra M., Bernhard F., and von Bodman S.B. (2002) The autoregulatory role of EsaR, a quorum-sensing regulator in *Pantoea stewartii* ssp. *stewartii*: evidence for a repressor function. *Mol Microbiol* **44:** 1625-1635.

Musthafa K.S., Sivamaruthi B.S., Pandian S.K., and Ravi A.V. (2012) Quorum sensing inhibition in *Pseudomonas aeruginosa* PAO1 by antagonistic compound phenylacetic acid. *Curr Microbiol* **65:** 475-480.

O'Grady E.P., Viteri D.F., Malott R.J., and Sokol P.A. (2009) Reciprocal regulation by the CepIR and CciIR quorum sensing systems in *Burkholderia cenocepacia*. *BMC Genomics* **10:** 441.

Olivera E.R., Minambres B., Garcia B., Muniz C., Moreno M.A., Ferrandez A., *et al.* (1998) Molecular characterization of the phenylacetic acid catabolic pathway in *Pseudomonas putida* U: the phenylacetyl-CoA catabolon. *Proc Natl Acad Sci U S A* **95:** 6419-6424.

Olsen A., Vantipalli M.C., and Lithgow G.J. (2006) Using *Caenorhabditis elegans* as a model for aging and age-related diseases. *Ann N Y Acad Sci* **1067:** 120-128.

O'Sullivan B.P., and Freedman S.D. (2009) Cystic Fibrosis. *Lancet* **Vol 373:** 1891-1904.

O'Sullivan L.A., and Mahenthiralingam E. (2005) Biotechnological potential within the genus *Burkholderia*. *Lett Appl Microbiol* **41:** 8-11.

Parke J.L., and Gurian-Sherman D. (2001) Diversity of the *Burkholderia cepacia* complex and implications for risk assessment of biological control strains. *Annu Rev Phytopathol* **39:** 225-258.

Passador L., Tucker K.D., Guertin K.R., Journet M.P., Kende A.S., and Iglewski B.H. (1996) Functional analysis of the *Pseudomonas aeruginosa* autoinducer PAI. *J Bacteriol* **178:** 5995-6000.

Pearson J.P., Van Delden C., and Iglewski B.H. (1999) Active efflux and diffusion are involved in transport of *Pseudomonas aeruginosa* cell-to-cell signals. *J Bacteriol* **181:** 1203-1210.

Pearson J.P., Passador L., Iglewski B.H., and Greenberg E.P. (1995) A second *N*-acylhomoserine lactone signal produced by *Pseudomonas aeruginosa*. *Proc Natl Acad Sci U S A* **92:** 1490-1494.

Peters B.M., Jabra-Rizk M.A., O'May G.A., Costerton J.W., and Shirtliff M.E. (2012) Polymicrobial interactions: impact on pathogenesis and human disease. *Clin Microbiol Rev* **25:** 193-213.

Prithiviraj B., Bais H.P., Weir T., Suresh B., Najarro E.H., Dayakar B.V., *et al.* (2005) Down regulation of virulence factors of *Pseudomonas aeruginosa* by salicylic acid attenuates its virulence on *Arabidopsis thaliana* and *Caenorhabditis elegans*. *Infect Immun* **73:** 5319-5328.

Rabin H.R., and Surette M.G. (2012) The cystic fibrosis airway microbiome. *Curr Opin Pulm Med* **18:** 622-627.

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

Riedel K., Hentzer M., Geisenberger O., Huber B., Steidle A., Wu H., *et al.* (2001) *N*-acylhomoserine-lactone-mediated communication between *Pseudomonas aeruginosa* and *Burkholderia cepacia* in mixed biofilms. *Microbiology* **147:** 3249-3262.

Riordan J.R., Rommens J.M., Kerem B., Alon N., Rozmahel R., Grzelczak Z., *et al.* (1989) Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. *Science* **245:** 1066-1073.

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

Ryan G.T., Wei Y., and Winans S.C. (2013) A LuxR-type repressor of *Burkholderia cenocepacia* inhibits transcription via antiactivation and is inactivated by its cognate acylhomoserine lactone. *Mol Microbiol* **87:** 94-111.

Ryan R.P., Fouhy Y., Garcia B.F., Watt S.A., Niehaus K., Yang L., *et al.* (2008) Interspecies signalling via the *Stenotrophomonas maltophilia* diffusible signal factor influences biofilm formation and polymyxin tolerance in *Pseudomonas aeruginosa*. *Mol Microbiol* **68:** 75-86.

Sambrook J., and Russell D.W. (2001) Molecular cloning: A laboratory manual, 3rd ed. Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory Press.

Schleissner C., Olivera E.R., Fernandez-Valverde M., and Luengo J.M. (1994) Aerobic catabolism of phenylacetic acid in *Pseudomonas putida* U: biochemical characterization of a specific phenylacetic acid transport system and formal demonstration that phenylacetyl-coenzyme A is a catabolic intermediate. *J Bacteriol* **176:** 7667-7676.

Schmid N., Pessi G., Deng Y., Aguilar C., Carlier A.L., Grunau A., *et al.* (2012) The AHL- and BDSF-dependent quorum sensing systems control specific and overlapping sets of genes in *Burkholderia cenocepacia* H111. *PLoS One* **7:** e49966.

Schuster M., and Greenberg E.P. (2006) A network of networks: quorum-sensing gene regulation in *Pseudomonas aeruginosa*. *Int J Med Microbiol* **296:** 73-81.

Schwager S., Agnoli K., Kothe M., Feldmann F., Givskov M., Carlier A., and Eberl L. (2013) Identification of *Burkholderia cenocepacia* strain H111 virulence factors using nonmammalian infection hosts. *Infect Immun* **81:** 143-153.

Sibley C.D., and Surette M.G. (2011) The polymicrobial nature of airway infections in cystic fibrosis: Cangene Gold Medal Lecture. *Can J Microbiol* **57:** 69-77.

Sibley C.D., Parkins M.D., Rabin H.R., and Surette M.G. (2009) The relevance of the polymicrobial nature of airway infection in the acute and chronic management of

patients with cystic fibrosis. *Curr Opin Investig Drugs* **10:** 787-794.

Sibley C.D., Parkins M.D., Rabin H.R., Duan K., Norgaard J.C., and Surette M.G. (2008) A polymicrobial perspective of pulmonary infections exposes an enigmatic pathogen in cystic fibrosis patients. *Proc Natl Acad Sci U S A* **105:** 15070-15075.

Slater H., Alvarez-Morales A., Barber C.E., Daniels M.J., and Dow J.M. (2000) A two-component system involving an HD-GYP domain protein links cell-cell signaling to pathogenicity gene expression in *Xanthomonas campestris*. *Mol Microbiol* **38:** 986-1003.

Smith R.S., and Iglewski B.H. (2003) *P. aeruginosa* quorum-sensing systems and virulence. *Curr Opin Microbiol* **6:** 56-60.

Sokol P.A., Malott R.J., Riedel K., and Eberl L. (2007) Communication systems in the genus *Burkholderia*: global regulators and targets for novel antipathogenic drugs. *Future Microbiol* **2:** 555-563.

Sokol P.A., Darling P., Lewenza S., Corbett C.R., and Kooi C.D. (2000) Identification of a siderophore receptor required for ferric ornibactin uptake in *Burkholderia cepacia*. *Infect Immun* **68:** 6554-6560.

Sokol P.A., Darling P., Woods D.E., Mahenthiralingam E., and Kooi C. (1999) Role of ornibactin biosynthesis in the virulence of *Burkholderia cepacia*: characterization of *pvdA*, the gene encoding L-ornithine N(5)-oxygenase. *Infect Immun* **67:** 4443-4455.

Sokol P.A., Sajjan U., Visser M.B., Gingues S., Forstner J., and Kooi C. (2003) The CepIR quorum-sensing system contributes to the virulence of *Burkholderia cenocepacia* respiratory infections. *Microbiology* **149:** 3649-3658.

Song F., Zhuang Z., Finci L., Dunaway-Mariano D., Kniewel R., Buglino J.A., *et al.* (2006) Structure, function, and mechanism of the phenylacetate pathway hot dog-fold thioesterase PaaI. *J Biol Chem* **281:** 11028-11038.

Speert D.P., Bond M., Woodman R.C., and Curnutte J.T. (1994) Infection with *Pseudomonas cepacia* in chronic granulomatous disease: role of nonoxidative killing by neutrophils in host defense. *J Infect Dis* **170:** 1524-1531.

Stanley T.G., and Wilson I. (2003) Multilocus enzyme electrophoresis: a practical guide. *Mol Biotechnol* **24:** 203-220.

Steindler L., and Venturi V. (2007) Detection of quorum-sensing *N*-acyl homoserine lactone signal molecules by bacterial biosensors. *FEMS Microbiol Lett* **266:** 1-9.

Suarez-Moreno Z.R., Caballero-Mellado J., Coutinho B.G., Mendonca-Previato L., James E.K., and Venturi V. (2012) Common features of environmental and potentially

beneficial plant-associated *Burkholderia*. *Microb Ecol* **63:** 249-266.

Sun L., Jiang R.Z., Steinbach S., Holmes A., Campanelli C., Forstner J., *et al.* (1995) The emergence of a highly transmissible lineage of *cbl+ Pseudomonas* (*Burkholderia*) *cepacia* causing CF centre epidemics in North America and Britain. *Nature Medicine* **1:** 661-6.

Suppiger A., Schmid N., Aguilar C., Pessi G., and Eberl L. (2013) Two quorum sensing systems control biofilm formation and virulence in members of the *Burkholderia cepacia* complex. *Virulence* **4:** 400-409.

Surette M.G. (2014) The cystic fibrosis lung microbiome. *Ann Am Thorac Soc* **11 Suppl 1:** S61-5.

Tan M.W., and Ausubel F.M. (2000) *Caenorhabditis elegans*: a model genetic host to study *Pseudomonas aeruginosa* pathogenesis. *Curr Opin Microbiol* **3:** 29-34.

Tan M.W., Mahajan-Miklos S., and Ausubel F.M. (1999) Killing of *Caenorhabditis elegans* by *Pseudomonas aeruginosa* used to model mammalian bacterial pathogenesis. *Proc Natl Acad Sci USA* **96:** 715-720.

Teufel R., Friedrich T., and Fuchs G. (2012) An oxygenase that forms and deoxygenates toxic epoxide. *Nature* **483:** 359-362.

Teufel R., Gantert C., Voss M., Eisenreich W., Haehnel W., and Fuchs G. (2011) Studies on the mechanism of ring hydrolysis in phenylacetate degradation: a metabolic branching point. *J Biol Chem* **286:** 11021-11034.

Teufel R., Mascaraque V., Ismail W., Voss M., Perera J., Eisenreich W., *et al.* (2010) Bacterial phenylalanine and phenylacetate catabolic pathway revealed. *Proc Natl Acad Sci U S A* **107:** 14390-14395.

Tomich M., and Mohr C.D. (2004) Genetic characterization of a multicomponent signal transduction system controlling the expression of cable pili in *Burkholderia cenocepacia*. *J Bacteriol* **186:** 3826-3836.

Twomey K.B., O'Connell O.J., McCarthy Y., Dow J.M., O'Toole G.A., Plant B.J., and Ryan R.P. (2012) Bacterial *cis*-2-unsaturated fatty acids found in the cystic fibrosis airway modulate virulence and persistence of *Pseudomonas aeruginosa*. *Isme j* **6:** 939-950.

Uehlinger S., Schwager S., Bernier S.P., Riedel K., Nguyen D.T., Sokol P.A., and Eberl L. (2009) Identification of specific and universal virulence factors in *Burkholderia cenocepacia* strains using multiple infection hosts. *Infect Immun* **9:** 4102-4110.

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

Vanlaere E., Baldwin A., Gevers D., Henry D., De Brandt E., LiPuma J.J., *et al.* (2009) Taxon K, a complex within the *Burkholderia cepacia* complex, comprises at least two novel species, *Burkholderia contaminans* sp. nov. and *Burkholderia lata* sp. nov. *Int J Syst Evol Microbiol* **59:** 102-111.

Visser M.B., Majumdar S., Hani E., and Sokol P.A. (2004) Importance of the ornibactin and pyochelin siderophore transport systems in *Burkholderia cenocepacia* lung infections. *Infect Immun* **72:** 2850-2857.

von Bodman S.B., Majerczak D.R., and Coplin D.L. (1998) A negative regulator mediates quorum-sensing control of exopolysaccharide production in *Pantoea stewartii* subsp. *stewartii*. *Proc Natl Acad Sci U S A* **95:** 7687-7692.

Wang J., Dong Y., Zhou T., Liu X., Deng Y., Wang C., *et al.* (2013) *Pseudomonas aeruginosa* cytotoxicity is attenuated at high cell density and associated with the accumulation of phenylacetic acid. *PLoS One* **8:** e60187.

Wang L.H., He Y., Gao Y., Wu J.E., Dong Y.H., He C., *et al.* (2004) A bacterial cell-cell communication signal with cross-kingdom structural analogues. *Mol Microbiol* **51:** 903-912.

Watson B., Currier T.C., Gordon M.P., Chilton M.D., and Nester E.W. (1975) Plasmid required for virulence of *Agrobacterium tumefaciens*. *J Bacteriol* **123:** 255-264.

Wigley P., and Burton N.F. (1999) Genotypic and phenotypic relationships in *Burkholderia cepacia* isolated from cystic fibrosis patients and the environment. *J Appl Microbiol* **86:** 460-468.

Winson M.K., Camara M., Latifi A., Foglino M., Chhabra S.R., Daykin M., *et al.* (1995) Multiple *N*-acyl-L-homoserine lactone signal molecules regulate production of virulence determinants and secondary metabolites in *Pseudomonas aeruginosa*. *Proc Natl Acad Sci U S A* **92:** 9427-9431.

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

Yang L., Rybtke M.T., Jakobsen T.H., Hentzer M., Bjarnsholt T., Givskov M., and Tolker-Nielsen T. (2009) Computer-aided identification of recognized drugs as *Pseudomonas aeruginosa* quorum-sensing inhibitors. *Antimicrob Agents Chemother*

53: 2432-2443.

Yoder-Himes D.R., Konstantinidis K.T., and Tiedje J.M. (2010) Identification of potential therapeutic targets for *Burkholderia cenocepacia* by comparative transcriptomics. *PLoS One* **5:** e8724.

Yoder-Himes D.R., Chain P.S., Zhu Y., Wurtzel O., Rubin E.M., Tiedje J.M., and Sorek R. (2009) Mapping the *Burkholderia cenocepacia* niche response via high-throughput sequencing. *Proc Natl Acad Sci U S A* **106:** 3976-3981.

Yudistira H., McClarty L., Bloodworth R.A., Hammond S.A., Butcher H., Mark B.L., and Cardona S.T. (2011) Phenylalanine induces *Burkholderia cenocepacia* phenylacetic acid catabolism through degradation to phenylacetyl-CoA in synthetic cystic fibrosis sputum medium. *Microb Pathog* **51:** 183-196.

Zhu J., Chai Y., Zhong Z., Li S., and Winans S.C. (2003) Agrobacterium bioassay strain for ultrasensitive detection of *N*-acylhomoserine lactone-type quorum-sensing molecules: detection of autoinducers in *Mesorhizobium huakuii*. *Appl Environ Microbiol* **69:** 6949-6953.