PHENYLALANINE CATABOLISM IN *BURKHOLDERIA CENOCEPACIA* K56-2

by

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ABSTRACT

Synthetic cystic fibrosis sputum medium (SCFM) is rich in amino acids and supports robust growth of *Burkholderia cenocepacia,* a member of the *Burkholderia cepacia* complex (Bcc). Previous work demonstrated that *B. cenocepacia* phenylacetic acid (PA) catabolic genes are up-regulated during growth in SCFM and are required for full virulence in a *Caenorhabditis elegans* host model. In this work, we investigated the role of phenylalanine, one of the aromatic amino acids present in SCFM, as an inducer of the PA catabolic pathway. Phenylalanine degradation intermediates were used as sole carbon sources for growth and gene reporter experiments. In addition to phenylalanine and PA, phenylethylamine, and phenylpyruvate could be used as sole carbon sources by wild type *B. cenocepacia* K56-2 but not by a PA catabolism defective mutant. These intermediates also induced a PA-inducible reporter system. Furthermore, proteomic analysis utilizing iTRAQ were used to study the protein expression of *B. cenocepacia* K56-2 grown in the amino acid-rich SCFM. Our results showed the over-expression of several proteins involved in amino acid and carbohydrate transport and metabolism. Interestingly, our results also showed the over-expression of flagellin and membrane efflux protein which are involved in the virulence of *B. cenocepacia*.

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LIST OF ABBREVIATIONS

°C	**= Degree Celsius**
2-DE	**= 2 Dimensional Protein Gel Electrophoresis**
Bcc	**= *Burkholderia cepacia* Complex**
CF	**= Cystic Fibrosis**
CFTR	**= Cystic Fibrosis Transmembrane Conductance Regulator**
CHAPS	**= 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate**
DTT	**= Dithiothreitol**
GFP	**= Green Fluorescence Protein**
HEPES	**= 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid**
IAA	**= Iodoacetamide**
iTRAQ	**= Isobaric Taq for Relative and Absolute Quantitation**
KEGG	**= Kyoto Encyclopedia of Genes and Genomes**
MOPS	**= 3-(N-morpholino)propanesulfonic acid**
PA	**= Phenylacetate**
PAGE	**= Polyacrylamide Gel Electrophoresis**
SCFM	**= Synthetic Cystic Fibrosis Sputum Medium**
SDS	**= Sodium Dodecyl Sulfate**
Tris	**= Tris(hydroxymethyl)aminomethane**

1-INTRODUCTION

1.0 *Burkholderia cepacia* Complex (Bcc)

Bcc is a group of closely related gram negative, rod-shaped bacteria which contains at least 17 different species (Mahenthiralingam and Vandamme 2005; Mahenthiralingam et al. 2000; Mahenthiralingam et al. 2005; Mahenthiralingam et al. 2008; Springman et al. 2009). *B. cepacia* was previously known as *Pseudomonas cepacia* and discovered as the cause of bacterial rot onion bulbs by W. H. Burkholder in 1950 (Burkholder 1950). However, based on 16S rRNA sequences, DNA-DNA homology values, cellular lipid and fatty acid composition, and phenotypic characteristics, *Burkholderia* was proposed as a separate genus and *B. cepacia* was designated as a type species in 1992 (Parke and Gurian-Sherman 2001; Yabuuchi et al. 1992).

1.1 Ecology

Bcc can be found throughout the environment (Mahenthiralingam et al. 2005). They have been isolated from soil, freshwater, seawater, and the rhizosphere of the plants (Coenye and Vandamme 2003) (Mahenthiralingam et al. 2005). Most of Bcc species are considered as beneficial in the natural environment due to their biopesticidal properties and the ability to degrade the toxic man-made compounds (Mahenthiralingam et al. 2005).

Most of *Bcc* strains do not usually infect healthy individuals but, they pose a threat as the opportunistic pathogen of immunocompromised individuals such as Cystic Fibrosis (CF) patients (Mahenthiralingam et al. 2005). The infection of CF patients with *Bcc* sometimes results in a condition called *Cepacia* syndrome (Isles et al. 1984; Mahenthiralingam et al. 2005). This is characterized by rapid decline of CF patients' condition due to necrotizing pneumonia and septicaemia which results in early death is associated to infection by Bcc (Isles et al. 1984). This syndrome however rarely occurs during infection by other CF pathogens (Banerjee and Stableforth 2000).

1.2 *Burkholderia cenocepacia* Strain K56-2

Most of Bcc species have been isolated from CF patients (Loutet and Valvano 2010). However, *B. cenocepacia* together with *B. multivorans* remains the most commonly isolated Bcc species from the CF patients in North America and Europe (Loutet and Valvano 2010; Reik R et al. ; Speert et al. 2002). The strain used in this study is *B. cenocepacia* K56-2, a clonal isolate of *B. cenocepacia* J2315. Both strains belong to the ET12 lineage, shown to be highly transmissible among CF patients in UK (Govan et al. 1993; Holden et al. 2009; Mahenthiralingam et al. 2000). Strain K56-2 is isogenic to strain J2315 except it lacks an insertion sequence (IS) element in O antigen biosynthesis gene cluster (Ortega et al. 2005). In addition, *B. cenocepacia* Strain K56-2 is proven to be useful for genetic analysis since it represents major epidemic CF clone and it is highly amenable to different molecular characterization techniques (Govan et al.

1993; Johnson et al. 1994; Lewenza et al. 1999; Mahenthiralingam et al. 1996; Mahenthiralingam et al. 2000).

All references to sequence analysis of *B. cenocepacia* strain K56-2 refer to the strain J2315 genome (Holden et al. 2009). The genome contains four replicons that consist of three circular chromosomes of 3.87 Mb, 3.217 Mb, and 0.87 Mb and a plasmid of 92 Kb (Holden et al. 2009). These replicons encode 3,537, 2,849, 776, and 99 predicted coding sequences (CDS) respectively (Holden et al. 2009).

Chromosome 1 mainly contains the CDS involved in the housekeeping functions. On the other hand, the majority of CDS found in chromosome 2 and 3 involved in the accessory functions such as horizontal gene transfer and protective responses (Holden et al. 2009). The example of the CDS involved in horizontal gene transfer includes the ones encoding many different pilli (Holden et. al. 2009). On the other hand, the CDS which encode the proteins involved in the efflux transport system, antimicrobial resistance and oxidative stress are responsible for the protective response in *B. cenocepacia* (Holden et al. 2009; Loutet and Valvano 2010). However, the presence of essential genes in chromosome 2 and 3 suggested that these two replicons are true chromosomes instead of megaplasmids (Holden et al. 2009).

1.3 Virulence Factors

B. cenocepacia is also known to have a number of recognized virulence factors that contribute to its pathogenicity such as the intrinsic resistance to multiple antibiotics, ability to form biofilm, the presence of haemolysin, and their quorum sensing ability (Loutet and Valvano 2010) (Bevivino et al. 2002; Chernish and Aaron 2003; Conway et al. 2002; Huber et al. 2002; Hutchison et al. 1998; Nzula et al. 2002; Venturi et al. 2004). Its inherent resistance to antibiotic such as chloramphenicol and ciprofloxaxin is due to the presence of active drug efflux pump (Burns et al. 1996). The decreased permeability of the outer membrane also inhibits the entrance of β-lactam antibiotic such as nitrofecin (Parr et al. 1987). *B. cenocepacia* is also resistant to trimethoprim due to the production of the trimethoprim-resistant enzyme, dihydrofolate reductase (DHFR) (Burns et al. 1989). In addition, *B. cenocepacia* lipopolysaccharide (LPS) structure contributes to the polymyxin resistance (Shimomura et al. 2003; Vinion-Dubiel and Goldberg 2003).

LysR-type transcriptional regulators have also been related to the virulence in *B. cenocepacia*. A mutation in this transcriptional regulator was shown affect change the colony morphology of *B. cenocepacia* to the shiny variant which is generally avirulent in the alfalfa seedling infection model (Bernier et al. 2007). This shiny variant of *B. cenocepacia* also showed a decrease in the biofilm formation (Bernier et al. 2007).

B. cenocepacia also possesses flagella, which is essential for the invasion of the host cell (Tomich et al. 2002). In addition, the flagella are also able to interact with Toll-

like receptor 5 (TLR5) which leads to the induction of the host immune responses (Urban et al. 2004). A pathogenicity island called *B. cenocepacia* island have also been identified in *B. cenocepacia*. This is the first pathogenicity island found in a Bcc species and was shown to be required for persistence and inflammation in rat lung infection model (Baldwin et al. 2004). Recently, phenylacetic acid (PA) catabolic pathway was also shown to be related to the virulence of *B. cenocepacia* (Hunt et al. 2004; Law et al. 2008; Loutet and Valvano 2010).

1.4 Cystic Fibrosis

Cystic fibrosis (CF) is an autosomal recessive disorder which affects approximately 1:3300 live caucasian births (Heijerman 2005). The disease is caused by a mutation in a gene encoding cystic fibrosis transmembrane conductance regulator (CFTR) which was first identified to be located on long arm of chromosome 7 in 1989 (Rommens et al. 1989). This mutation causes defective Cl^- transport across the affected epithelia, which is a distinctive feature of this disease (Welsh and Smith 1993).

CF manifests itself as exocrine pancreatic insufficiency, an increase in sweat Cl^- concentration, male infertility, and progressive loss of lung function caused by airway disease which is a major cause of mortality in CF patients (Heijerman 2005; Welsh and Smith 1993). CF patients are also susceptible to chronic infection by bacteria such as *Pseudomonas aeruginosa* and *Haemophilus influenzae*. The infection then leads to the induction of the patient's inflammatory response (Heijerman 2005).

There are two hypothesis to explain of the initial lung infection in CF patients. The first hypothesis is due to the elevated salt concentration in airway surface liquid (ASL) which is caused by defective CFTR, the function of innate antimicrobial peptides, defensins are inactivated which facilitates bacterial infection (Heijerman 2005). The second hypothesis is that abnormal sodium concentration and consequent failure of chloride secretion defective CFTR cause the depletion of water and volume of the ASL and periciliary fluid (Heijerman 2005). This leads to impaired mucocilliary clearance due to increased viscosity in both compartments and bacteria trapped in this mucus layer cause chronic infection (Heijerman 2005). Taken together, these two hypothesis suggest that the lungs of CF patients provide a favourable condition for bacterial infection due to the impared host immune system and the presence of mucus which support the adherence of the bacteria.

1.5 CF Sputum

CF sputum contains a complex mixture of mucus, bacteria, bacterial products, and inflammatory components such as antibodies, serum components, and dead host cells (Palmer et al. 2005). This serves not only as a physical substrate for bacterial growth, but also as a nutritional source for the infecting organisms (Ohman and Chakrabarty 1982; Palmer et al. 2005).

CF sputum contains different amino acids, small peptides, and carbohydrate which can serve as an energy source for bacterial growth (Ohman and Chakrabarty 1982; Palmer et al. 2005). It is able to support the growth of *P. aeruginosa* and induce the production of the quorum sensing molecule, *Pseudomonas* quinolone signal (PQS) which is related to virulence (Palmer et al. 2005). Recently, synthetic CF sputum medium (SCFM) which is a defined medium that nutritionally mimics CF sputum was developed (Palmer et al. 2007). *P.aeruginosa* grown in SCFM was shown to express the same genes as when it is grown in CF sputum (Palmer et al. 2007). In addition, the study showed that the aromatic amino acids in SCFM were responsible for the induction of the genes involved in the PQS production (Palmer et al. 2007). In addition, tryptophan which is also present in the CF sputum was shown to play a role as the precursor of this quorum sensing molecule (Chugani and Greenberg 2010). These studies suggest the relationship between aromatic amino acids which are present in SCFM and the virulence in the opportunistic pathogen of CF patients.

1.6 Aromatic Amino Acid Degradation

Phenylalanine, tyrosine, and tryptophan are the aromatic amino acids which are present in the SCFM (Palmer et al. 2007). In a fungus, *Aspergillus nidulans*, phenylalanine and phenylacetate is degraded through homogentisate pathway (Figure 1) (Fernandez-Canon et al. 1995). In bacteria such as *Escherichia coli* and *Rhodococcus* sp. strain RHA1, phenylalanine is degraded through phenylacetate (PA) catabolic pathway instead (Abe-Yoshizumi et al. 2004; Diaz et al. 2001; Navarro-Llorens et al. 2005). In *E.*

coli, phenylalanine is decarboxylated to 2-phenylethylamine (Diaz et al. 2001). An amine oxidase then converts 2-phenylethylamine to into phenylacetaldehyde (Diaz et al. 2001). Finally, a phenylacetaldehyde dehydrogenase oxidizes phenylacetaldehyde to PA (Figure 2). In addition, PA catabolic mutant of *Rhodococcus* sp. strain RHA1 was unable to grow on phenylalanine as the sole carbon source (Navarro-Llorens et al. 2005). This suggests that a functional PA catabolic pathway is required for the degradation of phenylalanine in *Rhodococcus* sp. strain RHA1.

Recently, *Pseudomonas putida* was shown to degrade phenylalanine through PA catabolic pathway and homogentisate pathway (Arias-Barrau et al. 2004; Herrera et al. 2010). The PA catabolic pathway involves the conversion of phenylalanine to phenylpyruvate (Figure 3) (Herrera et al. 2010). Phenylpyruvate is then converted to phenylacetyl-CoA. The later product is then further degraded to acetyl-CoA and succinyl CoA which enter tricarboxylic acid (TCA) cycle (Figure 3). In contrary to PA catabolic pathway, homogentisate pathway is involved in tyrosine degradation. Therefore, this degradation pathway involves the conversion of phenylalanine to tyrosine (Figure 3). Tyrosine is then further degraded to homogentisate (Herrera et al. 2010). The later is then further degraded to yield acetoacetate and fumarate which enters the TCA cycle.

On the other hand, tryptophan degradation proceeds through a completely different pathway in *E. coli*. This organism degrades tryptophan utilizing L-tryptophan indole-lyase (L-tryptophanase) and L-tryptophan permease (Diaz et al. 2001). Currently, little is known about the degradation of aromatic amino acids in *B. cenocepacia* except

for tryptophan catabolism. A previous study showed that in *B. cenocepacia* J2315, tryptophan which is converted to 2-amino-3-carboxymuconate semialdehyde is then further degraded to pyruvate and acetate via the intermediates 2-aminomuconate and 4-oxalocrotonate (Figure 4) (Colabroy and Begley 2005).

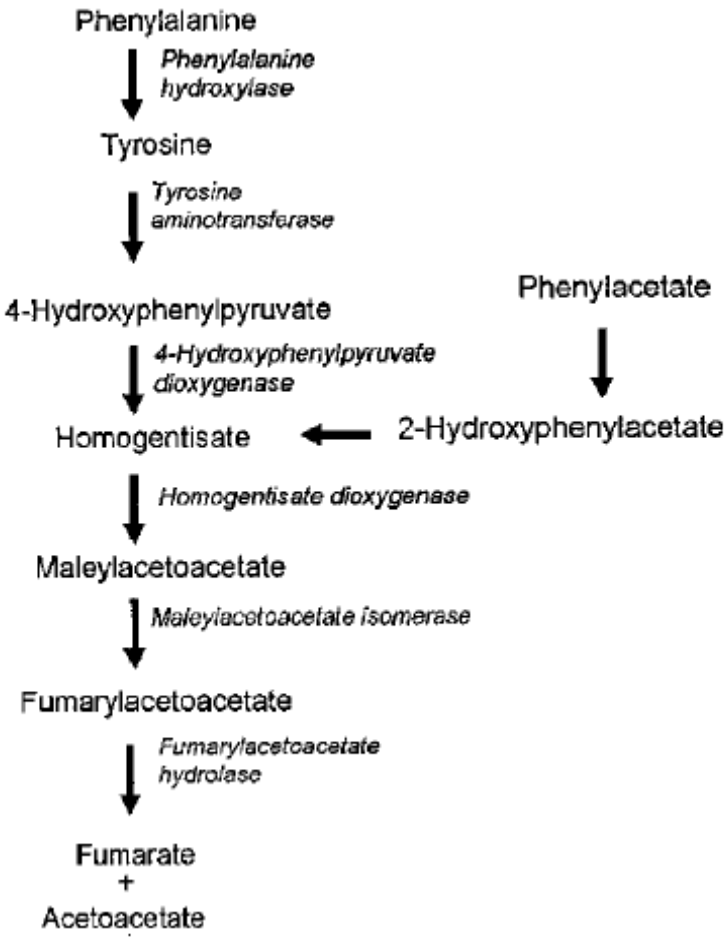


Figure 1. Phenylalanine and phenylacetate degradation in *A. nidulans* (Fernandez-Canon et al. 1995).

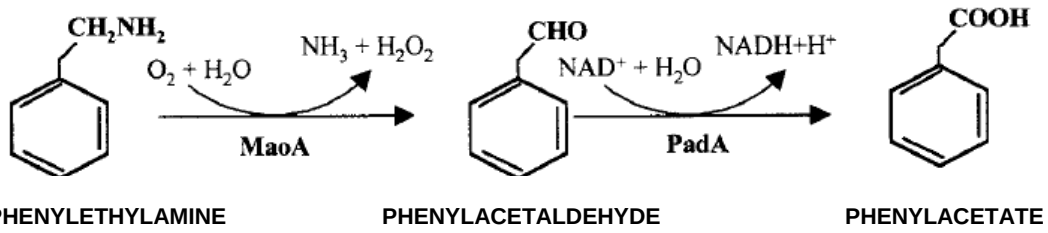


Figure 2. 2-Phenylethylamine is degraded to PA in *E. coli* (Diaz et al. 2001).

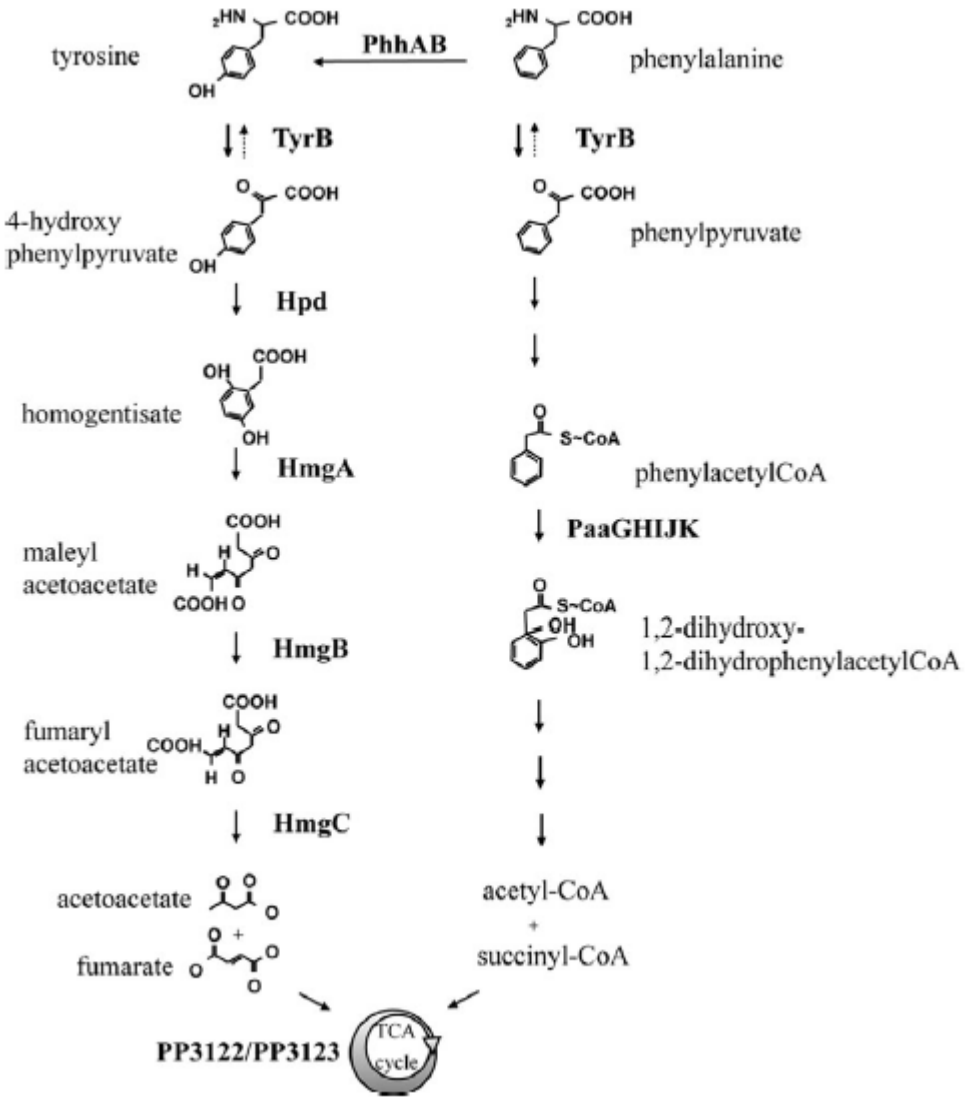


Figure 3. Phenylalanine catabolism in *P. putida*. Phenylalanine can be degraded through PA catabolic pathway and homogentisate pathway. The later involves the conversion of phenylalanine to tyrosine (Herrera et al. 2010).

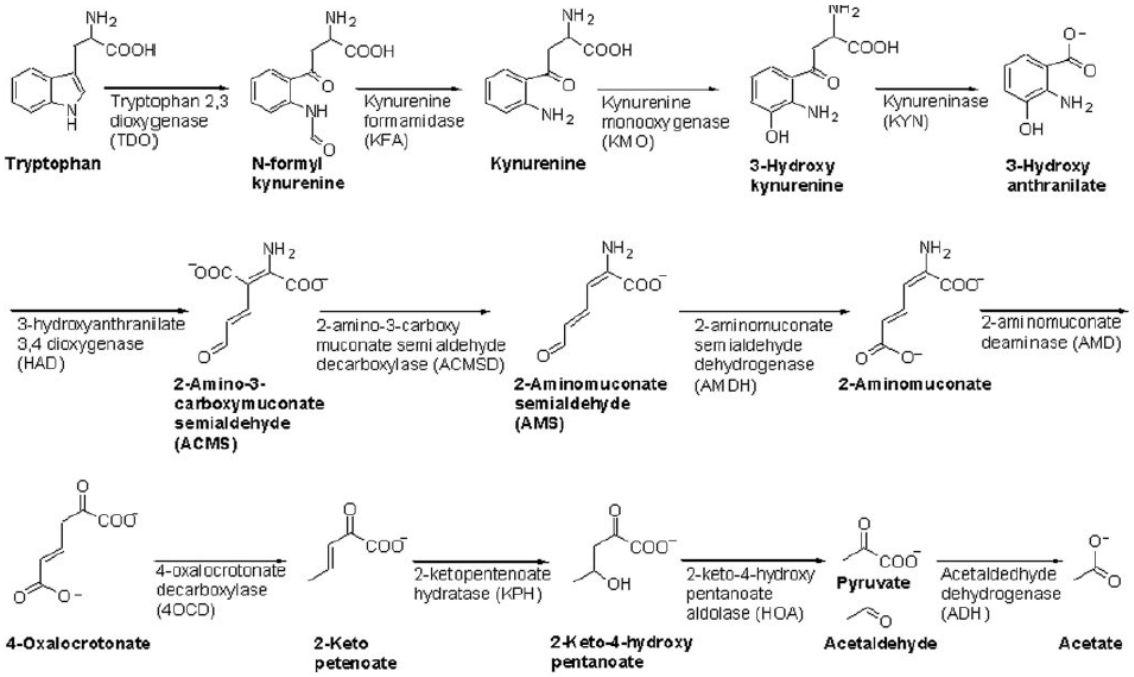


Figure 4. Tryptophan catabolism in *B. cenocepacia* J2315 (Colabroy and Begley 2005).

1.7 Phenylacetate (PA) Catabolism

PA catabolism is a central pathway for the degradation of many aromatic compounds such as phenylalanine, styrene, and 2-phenylethylamine (Luengo et al. 2001). Recently, PA catabolism has been characterized in *E. coli* K12 and *Pseudomonas* sp. strain Y2 (Teufel et al. 2010). The study showed that PA degradation yields succinyl-CoA and acetyl-CoA which enters TCA cycles (Figure 5).

In *B. cenocepacia*, PA catabolism was induced during growth in SCFM and minimal media containing phenylalanine as the sole carbon source (Hamlin et al. 2009). Furthermore, the same result was also shown in a transcriptomic analysis of *B. cenocepacia* grown in SCFM (Yoder-Himes et al. 2009). This suggests that phenylalanine may be degraded through PA catabolic pathway in *B. cenocepacia*.

PA catabolic pathway has also been related to the virulence in *B. cenocepacia* (Hunt et al. 2004; Law et al. 2008; Loutet and Valvano 2010). A disruption in a PA catabolic gene utilizing signature-tagged mutagenesis caused a defect in *B. cenocepacia* K56-2 *in vivo* survival. Additionaly, PA catabolism has also been shown to involved in the virulence of *B. cenocepia* K56-2 in the worm host model, *Caenorhabditis elegans*. These two studies strongly suggest the relationship between PA catabolism and virulence in *B. cenocepacia*. Since, PA catabolic pathway is also involved in the phenylalanine degradation, this also suggests the possible involvement of phenylalanine in the virulence of *B. cenocepacia*.

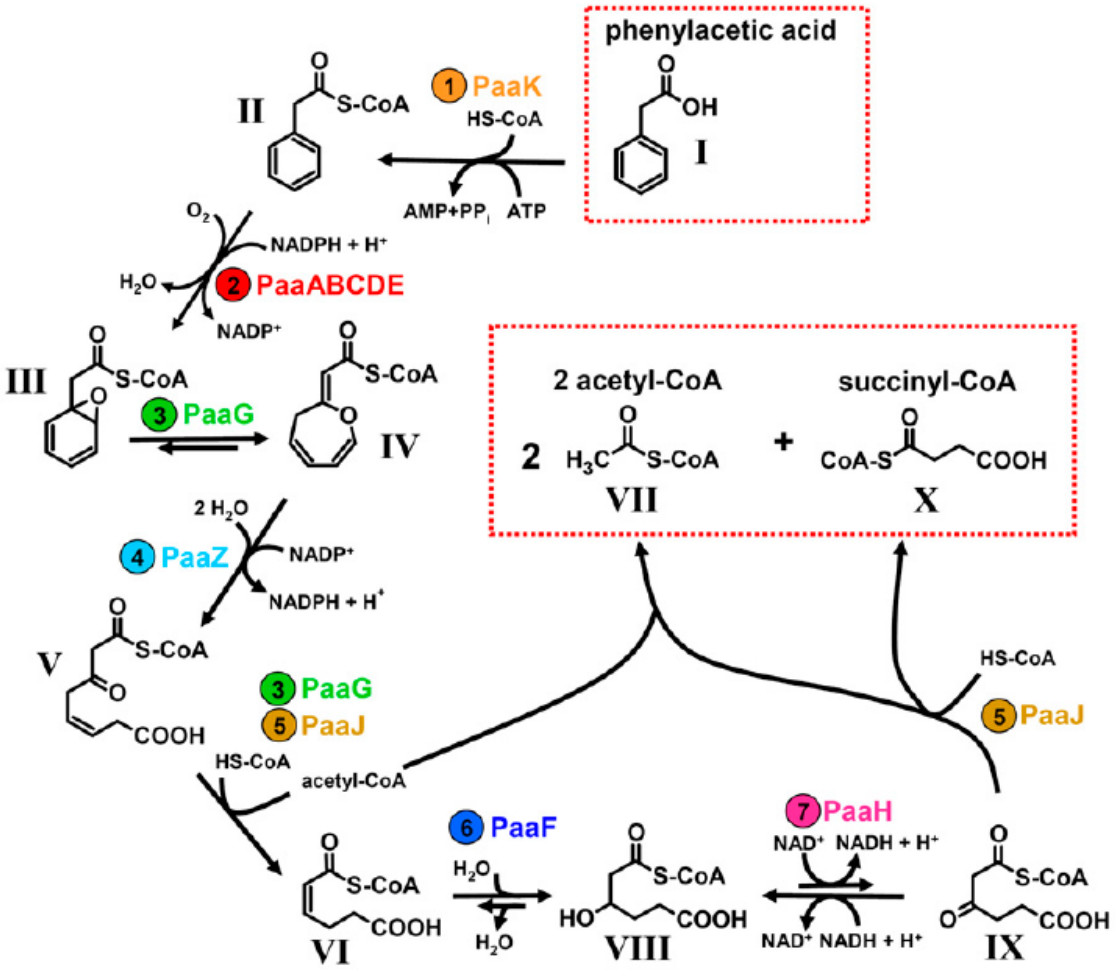


Figure 5. Phenylacetate catabolism in *E. coli* K12 and *Pseudomonas* sp. strain Y2. The figure shows the proposed enzymes and the degradation intermediates in the pathway. The enzymes are 1: phenylacetate-CoA ligase (AMP forming); 2: ring 1,2-phenylacetyl-CoA epoxidase (NAPDH); 3: ring 1,2-epoxyphenylacetyl-CoA isomerase (oxepin-CoA forming), postulated 3,4-dehydroadipyl-CoA isomerase; 4: oxepin-CoA hydrolase/ 3-oxo-5,6-dehydrosuberyl-CoA semialdehyde dehydrogenase ($NADP^+$); 5: 3-oxoadipyl-CoA/ 3-oxo-5,6-dehydrosuberyl-CoA thiolase; 6:2,3-dehydroadipyl-CoA hydratase; 7: 3-hydroxyadipyl-CoA dehydrogenase (NAD^+) (probably (*S*)-3-specific). The compounds are I: phenylacetate, II: phenylacetyl coA, III: ring 1,2-epoxyphenylacetyl-CoA, IV: 2-oxepin-2(3H)-ylideneacetyl-CoA, V: 3-oxo-5,6-dehydrosuberyl-CoA; VI: 2,3-dehydroadipyl-CoA, VII: acetyl-CoA, VIII: 3-hydroxyadipyl-CoA, IX: 3-oxoadipyl-CoA; X: succinyl CoA (Teufel et al. 2010).

1.8 Proteomic analysis

Proteomics can be defined as a large-scale study of proteins, usually by biochemical methods (Pandey et al. 2000). In the classical proteomics, two dimensional protein gel electrophoresis (2-DE) was used to separate the proteins obtained from a cell lysate (O' Farrell 1975; Pandey, et al. 2000). The proteins then were identified by utilizing the classical Edman degradation method (Edman 1949 ; Pandey et al. 2000). In this method, uncharged terminal amino group is reacted with phenylisothiocyanate (Edman 1949). The terminal amino acid is then cleaved as a thiazolinone derivative (Edman 1949). A further treatment of this compound yields phenylthiohydantoin-amino acid derivative which can be identified using chromatography or electrophoresis (Edman 1949). However, this method has been largely replaced by the more sensitive mass spectrometry (Pandey et al. 2000).

The mass-spectroscopy method relies on the tryptic digestion of the gel-separated proteins into peptides using (Pandey et al. 2000). This is because the peptides are easier to elute and to analyse with mass spectrometry compared to undigested protein (Pandey A et al.). There are two approaches in the identification of proteins utilizing mass-spectroscopy method (Pandey et al. 2000). The first approach involves the analysis of matrix assisted laser desorption/ionization (MALDI) coupled to time-of-flight (TOF) mass spectrometer (Berndt et al. 1999; Henzel et al. 1993; Pandey et al. 2000). The list of different peptide masses are used for protein identification. The mass spectra obtained are then searched against the database. The second approach utilizes ionization of

peptides directly from the liquid phase. This method is called electrospray ionization (ESI) (Fenn et al. 1989; Pandey et al. 2000). The peptide ions are then sprayed into a tandem mass spectroscopy to gain the sequence information. This method is more specific in protein identification. This is because the sequence information obtained from different peptides are used for the protein identification, as opposed to using the list of peptide masses (Pandey et al. 2000).

1.8.1 Two Dimensional Gel Electrophoresis (2-DE)

This approach is the most well-known method to separate and quantify proteins (Chevalier 2010; O'Farrell 1975). Native proteins extracted from the cell first have to be denatured, disaggregated, reduced and solubilized to disrupt molecular interactions between them to make sure that each protein can be resolved (Chevalier 2010; O'Farrell 1975). The samples are solubilized through the use of a buffer containing chaotropes (urea and/or thiourea), non-ionic detergent (Triton X-100), zwitterionic detergent (CHAPS), reducing agents (DTT), and carrier ampholytes. Most of the time, protease and phosphatase inhibitors are added to the buffer (Chevalier 2010).

Next, the proteins are separated in the first dimension by their isoelectric point (pI) which is a specific pH in which the net charge of a protein becomes zero. This is achieved through isoelectric focusing (IEF). Originally, this step was done by running the mixture of proteins in an IEF gel which contain a pH gradient (O'Farrell 1975). The pH gradient is created by using a mixture of carrier ampholytes with different pI (O'Farrell

1975). However, there are a number of problems with this type of gel such as pH gradient instability and the variability of carrier ampholytes between each batch (Gorg et al. 2004). Currently, this method has been largely replaced with the more stable immobilized pH gradients (IPG) strips (Gorg A et al. 2000; Gorg A et al. 2004).

To perform the IEF, a mixture of proteins, solubilization the buffer, and carrier ampholytes (which help the proteins to move) are loaded to an IPG strip (Chevalier 2010; Gorg et al. 2000; Gorg A et al. 2004). Electric field is then applied and negatively charged molecules (protein with ampholytes) move towards anode (positive electrode) while the positively charged molecules move towards (negative electrode) (Chevalier 2010). The proteins are unable to move further and focused when they are aligned according to their pI in which their global net charge is zero (Chevalier 2010). The strips are then equilibriated in the solution containing buffer with urea and glycerol in two steps. First, the equilibriation solution containing DTT is used to maintain a reducing condition. Then, the strips are equilibriated in the equilibration solution which contains iodoacetamide (IAA) to reduce the thiol groups and prevent their re-oxidation during electrophoresis (Chevalier 2010).

The proteins are then separated based solely on their molecular weight in the second dimension through SDS polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli 1970) and stained to visualize different protein spots (Chevalier 2010). The

spots of interest then can be further identified using mass spectroscopy (MS) analysis (Chevalier 2010).

1.8.2 Isobaric Tag for Relative and Absolute Quantification (iTRAQ)

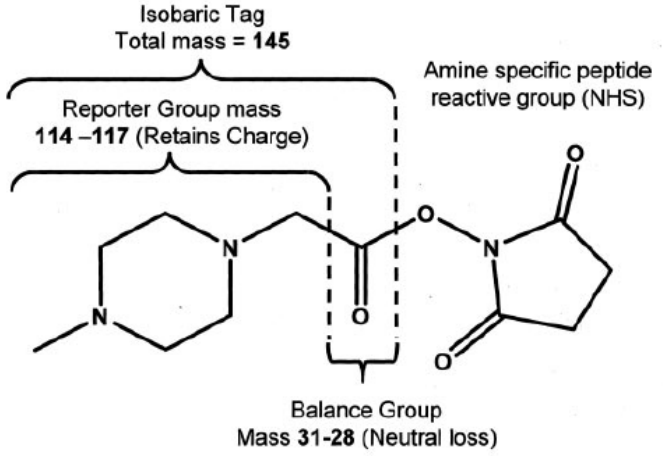
iTRAQ is a method of protein quantification using mass spectroscopy in which the relative protein levels in multiple sample can be analyzed simultaneously (Ross et al. 2004; Shadforth et al. 2005). This method uses a multiplexed set of isobaric reagents which form an amide linkage to any peptide amine (N-terminal and amino group of lysine side chain). These reagents contain different isobaric tags, each with a total mass of 145 Da. Each of these isobaric tags contains a distinct reporter group and a balance group, which maintains the same total mass between all different isobaric tags (Figure 6).

The derivatized peptides are indistinguishable in MS (Ross et al. 2004). However, the reporter groups are released upon the collision-induced dissociation (CID) in MS/MS, yielding signature ions (Ross et al. 2004). These signature ions then can be used to for quantitation of the proteins from different samples (Ross et al. 2004).

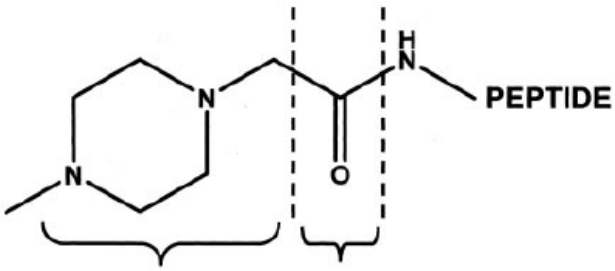
The work-flow of this method starts with the digestion of the protein samples with trypsin (Ross et al. 2004). The resulting peptides from each of different samples are then mixed with different multiplexed set of isobaric reagents (Ross et al. 2004). Next, the labelled peptides from different protein samples are mixed together and fractionated utilizing the strong cation exchange chromatography (SCX) (Ross et al. 2004). The

labelled peptides are then analysed utilizing liquid chromatography coupled to tandem mass spectroscopy (LC-MS/MS) (Ross et al. 2004). The signature ions which are produced upon CID in MS/MS are used for identifying and quantifying the proteins from different samples (Figure 7) (Ross et al. 2004).

a



b



m/z 114	(+1)	^{13}C	$^{13}C\ ^{18}O$	(+3)
m/z 115	(+2)	$^{13}C_2$	^{18}O	(+2)
m/z 116	(+3)	$^{13}C_2\ ^{15}N$	^{13}C	(+1)
m/z 117	(+4)	$^{13}C_3\ ^{15}N$		(+0)

Figure 6. iTRAQ reagent. The reagent contains an isobaric tag of mass 145 Da and an amine specific peptide reactive group (A). The isobaric tags contain reporter groups of mass 114, 115, 116, and 117 Da. The balance group of mass 31, 30, 29, and 28 brings the total mass of each isobaric tags to 145 Da. Different combinations of carbon, oxygen, and nitrogen isotopes are used to make the total mass of isobaric tags to 145 Da (B) (Ross et al. 2004)

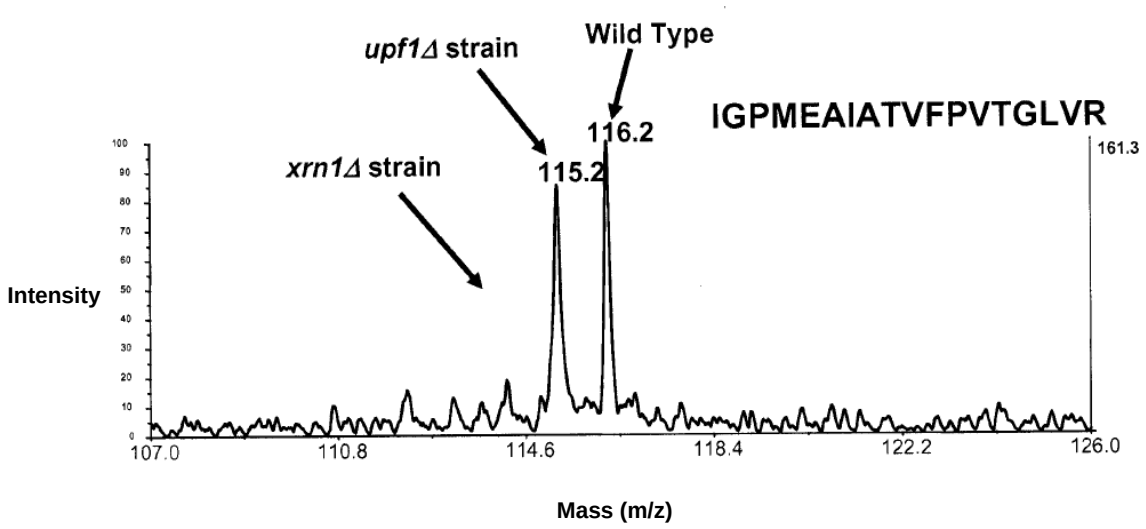


Figure 7. The quantitation of protein using iTRAQ. Xrn1 protein (containing the signature peptide IGPMEAIATVFPVTGLVR) from three different yeast strains were quantified. Isobaric tags with reporter group masses of 114, 115, and 116 Da were used to label the samples from *xrn1Δ* strain, *upf1Δ* strain, and wild type respectively. The figure shows the *xrn1Δ* strain did not produce Xrn1 protein Ross et al. 2004).

2-RATIONALE AND HYPOTHESIS

Synthetic CF sputum medium (SCFM) is a defined medium which was developed based on CF sputum. It is able to support the growth of *P. aeruginosa* and *B. cenocepacia* (Palmer et al. 2007; Yoder-Himes et al. 2009). When used as the growth media, SCFM and CF sputum, induces the expression of the same genes in *P. aeruginosa* including those responsible for the production of PQS (Palmer et al. 2007). In *B. cenocepacia*, SCFM was shown to also induce the expression of the virulence factors (Yoder-Himes et al. 2009). Furthermore, the phenylacetic acid (PA) catabolic pathway which was shown to be related to the virulence (Law et al. 2008) was also induced during the growth of *B. cenocepacia* in SCFM (Yoder-Himes et al. 2009).

SCFM like CF sputum contains aromatic amino acids such as phenylalanine, tyrosine, and tryptophan. Even though the level of tryptophan in CF sputum is low and often below the detection level (Palmer et al. 2005; Palmer et al. 2007). In bacteria, many of the aromatic compounds including aromatic amino acids like phenylalanine are degraded to PA before finally directed to tricarboxylic acid (TCA) cycle (Abe-Yoshizumi et al. 2004; Diaz et al. 2001; Navarro-Llorens et al. 2005). In addition, a previous study in *B. cenocepacia* showed that SCFM and phenylalanine were able to induce a PA-responsive reporter system (Hamlin et al. 2009). Therefore, we hypothesized that aromatic amino acids in SCFM are being used as carbon sources and nutritional cues by *B. cenocepacia*. In addition, these aromatic amino acids are being degraded to PA.

3-OBJECTIVES

3.0 To Determine the Aromatic Amino Acid which are Degraded to PA

Currently, little is known about the aromatic amino acids degradation pathway in *B. cenocepacia*. PA central catabolic was indicated to be a candidate for aromatic amino acids degradation pathway due to the fact that a previous study showed that PA sensitive reporter strain of *B. cenocepacia* K56-2/pJH7 was induced during growth in SCFM (Hamlin et al. 2009). To determine which aromatic amino acids enter the PA catabolic pathway in its degradation process, PA responsive reporter system were grown on MOPS-buffered minimal media containing different aromatic amino acids with the same concentration found in SCFM.

3.1 To Analyse Phenylalanine Degradation to PA

Since a previous study showed that a PA reporter system was also induced during growth in minimal media containing phenylalanine as the sole carbon source (Hamlin et al. 2009), we decided to further study the phenylalanine degradation in *B. cenocepacia*. First, *in silico* analysis utilizing Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa and Goto 2000; Kanehisa et al. 2006; Kanehisa et al. 2008) was done to determine the putative enzymatic steps of phenylalanine degradation to PA and all the putative degradation intermediate compounds. Then, a PA catabolic mutant strain was grown in minimal media containing these different phenylalanine degradation

intermediates to determine which steps that are used in phenylalanine degradation. In addition, PA reporter system was also grown in MOPS-glycerol with addition of the intermediates to determine whether any of these intermediate compounds was able to induce the system.

3.2 To Analyse the Protein Expression in *B. cenocepacia* Grown in CF-like Condition

A transcriptomic analysis in *B. cenocepacia* showed that the genes encoding potential virulence factors such as molecular chaperones, iron acquisition proteins, and proteins expressed in macrophages, are expressed in SCFM in contrast to soil-like condition (Yoder-Himes et al. 2009). Furthermore, several genes involved in PA catabolism, known to be related to virulence in *C. elegans* (Law et al. 2008), were also expressed during growth in SCFM (Yoder-Himes et al. 2009).

However, this study lacks proteomics confirmation. It is known that the changes in the gene expression do not always correlate directly to the changes in protein expression (Yoder-Himes et al. 2009; Zieske 2006). In order to study the protein expression in *B. cenocepacia* during growth in SCFM, particularly the proteins which are possibly involved in phenylalanine and PA catabolism, 2 different proteomic analysis approaches were used. The first preliminary analysis was done using the classical 2 dimensional gel electrophoresis (2-DE). Then, an approach utilizing the more sensitive isobaric tag for relative and absolute quantitation (iTRAQ) will be done to study the

difference in protein expression between the cells grown in amino acid rich SCFM and amino acid-lacking condition.

4-MATERIALS AND METHODS

4.0 Synthetic Cystic Fibrosis Sputum Medium (SCFM)

SCFM was prepared using the method previously described (Palmer et al. 2007). Amino acids were maintained as filter sterilized 100 mM stock in deionized water except tyrosine, aspartate, and tryptophan were dissolved in 1M, 0.5M, and 0.2M NaOH respectively. All the amino acids stocks were kept in the dark and stored in 4°C.

For making 500 mL of SCFM, buffered base first were made by mixing 3.25 mL 0.2 M NaH_2PO_4, 3.125 mL 0.2 M Na_2HPO_4, 0.174 mL 1 M KNO_3, 0.061 g NH_4Cl, 0.557 g KCl, 1.515 g NaCl, 1.0465 g MOPS (to final concentration of 10 mM) in 389.9 mL deionized water. Amino acids were added from 100 mM stock to buffered base (4.135 mL of L-aspartate, 5.36 mL of L-threonine, 7.23 mL of L-serine, 7.745 mL of L-glutamate·HCl, 8.305 mL of L-proline, 6.015 mL of L-glycine, 8.9 mL of L-alanine, 0.8 mL of L-cysteine·HCl, 5.585 mL of L-valine, 3.165 mL of L-methionine, 5.6 mL of L-isoleucine, 8.045 mL of L-leucine, 4.01 mL of L-tyrosine, 2.65 mL of L-phenylalanine, 3.38 mL of L-ornithine·HCl, 10.64 mL of L-lysine·HCl, 2.595 mL of L-histidine, 0.065 mL of L-tryptophan, 1.53 mL of L-Arginine·HCl). The pH of solution mixture then adjusted to 6.8 using HCl and filter sterilized through 0.2 μM-pore-size filter. After sterilization, these sterile components were added: 0.877 mL 1M CaCl2, 0.303 mL 1 M of $MgCl_2$, 0.5 mL of 3.6 mM $FeSO_4.7H_2O$, 1.5 mL of 1 M D-glucose, 4.65 mL 1 M L-

lactate. The SCFM was kept at 4°C. Table 1 shows the composition of SCFM with the concentration of each component (Palmer et al. 2007).

4.1 MOPS-buffered Minimal Media

MOPS-buffered minimal medium using glucose as the sole carbon source (MOPS-glucose) was prepared like previously described (Neidhardt et al. 1974). First, 10X MOPS mixture was prepared. To make 250 mL of 10X MOPS mixture, 20.93 g MOPS and 1.79 g tricine was added to approximately 75 mL deionized water. The pH of the mixture was adjusted to 6.8 using 10 M KOH and the total volume was brought to 110 mL. 2.5 mL of freshly made 0.01M $FeSO_4.7H_2O$ was added to the mixture. Finally, these solutions were added to mixture in this order: 12.5 mL of 1.9M NH_4Cl, 2.5mL of 0.276M K_2SO_4, 0.0625 mL of 0.02 M $CaCl_2.2H_2O$, 0.525 mL of 2.5 M $MgCl_2$, 25 mL of 5 M NaCl, and 96.75 mL of deionized water. This was filter sterilized through 0.2µm-pore-size filter. 10X MOPS mixture was stored at 4°C.

To make 500 mL of MOPS-glucose, 50 mL of 10X MOPS mixture, 5mL 0.132M K_2HPO_4, and 0.1mL 0.5mg/mL thiamine were added to 435 mL deionized water. The pH of the mixture was adjusted to 6.8 using 10 M NaOH and filter-sterilized through 0.2 µm-pore-size filter. Sterile 10 mL 1 M glucose was added to the mixture and MOPS-glucose was stored at 4°C. The exact concentration of MOPS-glucose components are listed in Table 2.

Table 1. Bacterial strains and plasmids

Strain or plasmid	Features[a]	References or sources
B. cenocepacia strains		
K56-2 (LMG18863)	ET12 clone related to J2315, CF clinical isolate	(Mahenthiralingam et al. 2000)
STC179-*paaA*	K56-2 *paaA*::SC175 Tp^r	(Law et al. 2008)
Plasmids		
pJH7	*paaA* promoter region (P_{paaA}) fused to *eGFP*	(Hamlin et al. 2009)

Table 2. Composition of SCFM

Components	Concentration (mM)
Ions	
Na^+	66.6
K^+	15.8
NH_4^+	2.30
Ca^{2+}	1.70
Mg^{2+}	0.60
Cl^-	79.1
NO^{3-}	0.35
PO_4	2.50
SO_4^{2-}	0.27
Amino acids	
Serine	1.40
Threonine	1.00
Alanine	1.80
Glycine	1.20
Proline	1.70
Isoleucine	1.10
Leucine	1.60
Valine	1.10
Aspartate	0.80
Glutamate	1.50
Phenylalanine	0.50
Tyrosine	0.80
Tryptophan	0.01
Lysine	2.10
Histidine	0.50
Arginine	0.30
Ornithine	0.70
Cysteine	0.20
Methionine	0.60
Other	
Glucose	3.20
Lactate	9.00
$FeSO_4$ (μM)	3.60

For growth experiments using phenylalanine degradation intermediates, 40 mL of MOPS-buffered minimal media containing 5 mM of phenylalanine, phenylpyruvate, phenylethylamine, phenylacetaldehyde, 2-hydroxy-phenylacetate or PA as the sole carbon source were used. To prepare this, 4 mL of 10X MOPS mixture, 0.4 mL of 0.132M K_2HPO_4, and 0.008 mL of 0.5 mg/mL thiamine were mixed together. Phenylalanine or its degradation intermediates was added to the concentration of 5 mM. The total volume was adjusted to 40 mL. Then the pH of the mixture was adjusted to pH 6.8 and filter-sterilized.

For the induction of PA catabolism by the presence of different PA precursors, MOPS-glycerol and MOPS-glycerol with addition of 0.5mM PA precursors were used as the growth medium. To prepare 100 mL of MOPS-glycerol, 10 mL of 10X MOPS mixture, 1 mL of 0.132M K_2HPO_4, and 0.02 mL of 0.5 mg/mL thiamine were mixed in 88.6 mL of MilliQ water. The pH of the mixture then was adjusted to 6.8. Finally, the mixture was filter-sterilized and 0.40 mL of 2.50 mM glycerol was added aseptically.

To prepare 40 mL of MOPS-glycerol containing 0.5mM phenylalanine, phenylpyruvate, or phenylethylamine, 4 mL of 10X MOPS mixture, 0.4 mL of 0.132M K_2HPO_4, and 0.008 mL of 0.5 mg/mL thiamine were mixed in 35.24 mL of MilliQ water. To this mixture, 0.2 mL of 100 mM phenylalanine, phenylpyruvate, or phenylethylamine were added. The pH of the mixture then was adjusted to 6.8. Finally, the mixture was filter-sterilized and 0.16 mL of 2.50 mM glycerol was added aseptically.

To prepare 40 mL of MOPS-glycerol containing 0.5mM phenylacetaldehyde, 4 mL of 10X MOPS mixture, 0.4 mL of 0.132M K_2HPO_4, and 0.008 mL of 0.5 mg/mL thiamine were mixed in 35.24 mL of MilliQ water. To this mixture, 0.2 mL of 100 mM phenylalanine, phenylpyruvate, or phenylethylamine were added. The pH of the mixture then was adjusted to 6.8. Finally, the mixture was filter sterilized and 0.16 mL of 2.50 mM glycerol was added aseptically.

4.2 Bacterial Strains and Growth Conditions

Bacterial strains and plasmids are listed in Table 3. All *B. cenocepacia* strains used were grown at 37°C in synthetic cystic fibrosis medium (SCFM) (Palmer et al. 2007) or MOPS-buffered minimal media (Neidhardt et al. 1974) containing different carbon sources and adjusted to pH 6.8.

4.3 Growth of *B. cenocepacia* in 96-well Format

To grow *B. cenocepacia* in 96 well microtitre plates, the strains were first grown overnight with shaking at 37°C in 5mL tubes containing SCFM. The cells were then collected by centrifugation at 7000 rpm for 5 minutes in the Centrifuge 5424 equipped with a FA-45-24-11 rotor (Eppendorf). The cells are then washed in phosphate-buffered saline (PBS) and diluted to optical density of 600 nanometers (OD_{600}) of 0.04 with SCFM or MOPS-buffered minimal medium containing the indicated carbon sources. Finally,

Table 3. MOPS-buffered minimal medium components

Components	Concentration (mM)
K_2HPO_4…….....	1.32
NH_4Cl…….....	9.52
$MgCl_2$…….....	0.523
K_2SO_4……	0.276
$FeSO_4$…….....	0.010
$CaCl_2$…….....	5x10-4
NaCl……...	50.0
MOPS…….....	40.0

150 µL-aliquots were deposited in 96-well plates. For continuous monitoring by automated reading of OD_{600}, plates were incubated in a Biotek Synergy 2 plate reader for 24 h at 37°C, with continuous fast shaking (1140 rpm). For endpoint readings, plates were incubated in an Excella 23 incubator shaker (New Brunswick Scientific) at 37°C and 210 rpm and only transferred to the Biotek Synergy 2 plate reader to monitor endpoint growth. OD_{600} values were converted to 1 cm-path length OD_{600} using a standard curve.

4.4 Reporter Activity Assays

B. cenocepacia K56-2 and *B. cenocepacia* K56-2/pJH7 (Hamlin et al. 2009) were grown overnight with shaking at 37°C in 5 mL of SCFM. The cells were then washed in PBS, diluted to an OD_{600} of 0.04 in 150 µL-aliquots of SCFM or MOPS-buffered minimal medium containing the indicated carbon sources, and arranged in 96-well format. Plates were incubated with continuous fast shaking (1140 rpm) at 37°C in a Biotek Synergy 2 plate reader for 24 h, until the stationary phase was reached. Fluorescence was quantified using excitation 485/20, emission 528/20 filter sets as previously described (Hamlin et al. 2009). The numbers 485nm and 528 nm refers to the wavelength of light which are allowed to pass the filter. The number 20 refers to the range between the upper and the lower limit of the light wavelength which are allowed to pass the filter. For example, 528/20 filter set means that the wavelengths of light which are allowed to pass the filter has to fall within the range of 518-538 nm. Relative Fluorescence was defined as the ratio between the arbitrary fluorescence and the OD_{600} of *B. cenocepacia* K56-

2/pJH7-containing wells minus the ratio between the arbitrary fluorescence and the OD_{600} of equivalent cultures of *B. cenocepacia* K56-2 wild type.

4.5 Protein Sample Preparation for 2-DE

B. cenocepacia K56-2 was grown overnight at 37°C in SCFM. The cells were then washed in phosphate-buffered saline (PBS) and diluted to OD_{600} of 0.04 in the flask containing fresh 50 mL of SCFM or MOPS-glucose. The flasks were then incubated at 37°C room with continuous shaking. The cells were collected at mid-exponential phase when OD_{600} is ~1. This happens at 4 and 7 hours after the innoculation for the cells growing in SCFM and MOPS-glucose respectively through centrifugation at 5000 rpm at 4°C in Sorvall RC-5B Refrigerated Superspeed Centrifuge using equipped with a SS-34 rotor (DuPont Instruments). The cells were then washed with 50mM pH 7.5 Tris/HCl and resuspended in the same buffer supplied with protease inhibitor for sonication (Riedel et al. 2006). The washed cells were sonicated at power level 5 and 10% pulse for 7 rounds for 15 seconds with cooling in ice in between the sonication rounds (Invitrogen). The samples then centrifuged at 1000 xg at 4°C and the supernatant was kept in -70°C until ready to be used.

4.6 Protein Sample Preparation for iTRAQ

The cells were grown in a similar manner as the cells used for 2-DE protein sample preparation except, the overnight cells were sub-cultured into test tubes

containing fresh 5 mL of SCFM or MOPS-glucose and incubated in an Excella 23 incubator shaker (New Brunswick Scientific) at 37°C and 210 rpm. The cells were harvested at mid-exponential phase or when OD_{600} reached approximately 1 at 4 hours or 7 hours for the cells growing in SCFM and MOPS-glucose respectively, by centrifugation at 7000 rpm for 5 minutes in Centrifuge 5424 equipped with a FA-45-24-11 rotor. The cells were then washed by resuspending them in 500 µL of PBS and centrifuged at 7000 rpm for 5 minutes. The supernatant was discarded and the cells were resuspended in 300 µL of lysis buffer (10 mM pH 7.4 tris/HCl, 3 mM $CaCl_2$, 2 mM $MgCl_2$) supplied with protease inhibitor and 0.1% v/v NP40 for sonication. The cells were sonicated using the same protocol as protein samples preparation for 2-DE.

4.7 Protein Amount Estimation

Quantitation of total proteins was done by Coomassie plus (Bradford) protein assay kit (Pierce) for 2-DE and by microbichinchoninic acid (BCA) protein assay kit (Pierce) for the iTRAQ.

4.8 Isoelectric Focusing (IEF) and SDS-PAGE

pH 3-10 non-linear (NL) IPG strips (Invitrogen) were rehydrated overnight with 155 µL of sample rehydration buffer containing 20 mM Dithiothreitol (DTT) and 30 µg of protein sample. IEF was performed at 0.05 mA current limit, 0.1 watt, 500 V for 60 minutes, 1000 V for 60 minutes, and 3500 V for 180 minutes in ZOOM IPG runner using

ZOOM dual power supply (Invitrogen). Before applying them to SDS-PAGE, the strips were equilibrated with shaking 2 times for 15 minutes at room temperature first in the equilibration buffer, then in the alkylating solution. For the second dimension, the strips were transferred to NuPAGE 4-12% Bis-Tris gel with 1.0 mm IPG well (Invitrogen). Electrophoresis was performed at 200 V and 100 mA for 50 minutes.

4.9 Equilibration Buffer and Alkylating Solution

For preparation of equilibration buffer, first dilute 4X Lithium Dodecyl Sulphate (LDS) sample buffer (Invitrogen) to 1X with milliQ water. DTT then was added to a final concentration of 50mM. For the preparation of the alkylating buffer, Iodoacetamide (IAA) was added to a final concentration of 125mM into the 1X LDS sample buffer (Invitrogen) instead.

4.10 SDS-PAGE Gel Analysis of the Protein Samples for iTRAQ

The protein extracts were diluted to a concentration of 0.2 µg/µL by mixing with SDS loading buffer (10% glycerol, 50 mM pH 6.8 Tris-Cl, 20 g/L sodium dodecyl sulphate (SDS), and 1 g/L bromophenol blue) and adding DTT to a final concentration of 100mM. The mixture was then boiled in an analog block heater (VWR) at 100^0C for 3 minutes. 30 µL of the mixtures, each containing approximately 6µg of protein were loaded and separated on a 4-20% Precise Tris-HEPES PAGE gel (Thermo Scientific) in Tris-HEPES running buffer using XCell Surelock gel tank (Invitrogen).

4.11 Tris-HEPES Running Buffer

To make a stock solution of 10X Tris-HEPES running buffer, 121g of Tris buffer, 238 g of HEPES, and 10 g of SDS were mixed with milliQ H_2O to a total volume of 1 L. The stock solution was kept in 4°C and diluted to 1X with milliQ water before use.

4.12 Sample Processing for iTRAQ

Protein samples were processed as described previously (Dwivedi et al. 2009). Approximately 120 µg of protein samples from different growth conditions were adjusted to a volume of 200 µL with 100 mM ammonium bicarbonate buffer. The proteins were then reduced with 10 mM (final concentration) DTT for 60 minutes at 56°C followed by alkylation using 50 mM (final concentration) iodoacetamide (IAA) for 20 minutes at room temperature. The excess of IAA was neutralized by the addition of 17 mM DTT and by allowing the mixture to stand for 20 minutes at room temperature. Proteins were digested in trypsin sequencing grade (Promega) at 1:50 trypsin/protein w/w ratio for 16 hours at 37°C. Samples were frozen at -80°C and dried using speed vacuum and finally purified using Scalar C-18 (5 µm, 1x100 mm, 100 Å) column (Agilent Techonologies). Both eluents A (water) and B (90%) acetonitrile contained 20 mM ammonium formate buffer (pH 10). A Total of 60 fractions were collected using a gradient of 1% to 50% of eluent B in 67 minutes at a flow rate of 150 µL/min.

4.13 iTRAQ Labelling of Peptides

The peptides were labelled as described previously (Dwivedi et al. 2009). Peptides from approximately 100 µg of trypsin digested proteins were labelled with different iTRAQ reporter (Applied Biosystems) according to the manufacturer's procedure.

4.14 2D HPLC-MS Analysis

First Dimension

Mixed iTRAQ labelled peptides derived from ~120 µg of total protein were gradient fractionated on a C18 X-Terra column (5 µm, 1x100 mm, 100 Å, Waters). Both eluents A (water) and B (90% acetonitrile) contained 20mM ammonium formate buffer pH 10. A total of 30 fractions were collected using a gradient of 1-50% of solvent B in 30 minutes, at a flow rate of 150 µL/min. Fractions were dried and dissolved in 50 µL of eluent A (see second dimension) and 20 µL was injected for analysis in the second dimension.

Second dimension

A splitless nanoflow Tempo LC system (Eksigent) with sample injection via a PepMap100 trap column (0.3x5 mm, 5 µm, 100 Å, Dionex) and a 100 µm x 150 mm analytical column packed with 5 µm Luna C18(2) (Phenomenex) was used in the second dimension separation prior to tandem mass spectroscopy (MS/MS) analysis. Both eluents

A (2% acetonitrile in water) and B (98% acetonitrile) contained 0.1% formic acid as ion-pairing modifier. A 0.44% acetonitrile per minute linear gradient (0-35% B in 80 minutes, 500 nL/min) was used for peptide elution, followed by 5 minutes wash with 80% B.

A Qstar Elite QqTOF mass spectrometer (Applied Biosystems) was used in standard MS/MS data-dependent acquisition mode with a nano-ESI source. Survey MS spectra were collected (*m/z* 400-1500) for 1 second followed by 3 MS/MS measurements on the most intense parent ions (80 counts/sec threshold, +2 to +4 charge state, *m/z* 100-1500 mass range for MS/MS), using the manufacturer's "smart exit" and "iTRAQ" settings. "Smart exit" optionis a standard feature of Qstar Elite instrument, which runs under control of Analyst QS 2.0 software. It allows termination of MS/MS spectrum collection when preset values of peaks intensity (quality of the spectra) are reached. In this way instrument spends less time for MS/MS acquisition of abundant species and improves chances for detection of low-abundant ones. Spectral quality setting 5 (whole scale 1-20) was used throughout the experiments. Predefined "iTRAQ" settings adjust (increases) collisional energy to maximize intensity of reporter ions (114, 115, 116, 117 Da) in MS/MS spectra. Individual Wiff files generated after Qstar Elite analysis were converted to MGF (mascot generic file) format using Mascot.dll script in Analyst QS 2.0. Following this conversion MGF files of individual fractions were combined into one using a merging script.

4.15 Database Search and Identification

MS/MS data were analyzed using Global Proteome Machine (GPM-http://www.thegpm.org). *Burkholderia cenocepacia* J2315 protein index databases were used for the GPM search. The search parameters were as follows: fragment mass error 0.4 Da, complete modifications Cys alkylation with iodoacetamide, potential modifications Met-oxidation and deamidation of Asn and Gln residues. For iTRAQ analysis residue modifications were kept as 144.102063@N terminus (addition of 144.102063 Da to all N-terminal amino acids by the isobaric tag), 144.102063@K (addition of 144.102063 Da to all lysine residues by isobaric tag), 57.021464@C (addition of 57.021464 Da to all cysteine residues due to alkylation), and minimum fragment *m/z* as 1 and the GPM search was performed. The identified protein candidates with log(e) score ≤-1.3(95%) were used for further analysis.

4.16 Putative Phenylalanine Degradation Pathway

The map of phenylalanine catabolism pathway to PA and the putative genes involved in each step were derived from map00360 of the Kyoto Encyclopedia of Genes and Genomes (KEGG) database by using *B. cenocepacia* J2315 as the reference organisms (Kanehisa and Goto 2000). Alternatively, BCAL2986, BCAM0359, BCAL2359, and BCAM2591 were found through BLAST searches using the amino acid sequence of Bcen_0543 of *B. cenocepacia* AU1054, PP_2252 of *Pseudomonas putida*

KT2440, YDR380W of *Saccharomyces cerevisae*, and AN8078.2 of *A. nidulans* respectively as the query.

5-RESULTS PART 1 : ANALYSIS OF PHENYLALANINE CATABOLISM IN *B. cenocepacia* K56-2

To determine the growth phase in which PA degradation is induced during growth in SCFM, a PA responsive reporter strain *B. cenocepacia* K56-2/pJH7 (Table 1) (Hamlin et al. 2009), was grown on SCFM. In addition, the PA reporter system was also grown on MOPS-glycerol with addition of phenylalanine, tyrosine, and tryptophan to the similar concentration found in SCFM to determine the inducer of the PA catabolic pathway. We also investigated the putative steps of phenylalanine degradation to PA by growing a PA catabolic mutant strain (*B.cenocepacia* STC179-*paaA*) (Table 1) in MOPS-minimal medium containing phenylalanine or its degradation intermediates. Then, to study the induction of the PA catabolism, a PA reporter system was grown in MOPS-glycerol with addition of phenylalanine or its degradation intermediates as the inducer of the system.

5.0 Phenylalanine Induced PA-responsive Reporter System during Growth of *B. cenocepacia* in SCFM

PA catabolic pathway is the central route for the degradation of many aromatic compounds (Abe-Yoshizumi et al. 2004; Diaz et al. 2001; Navarro-Llorens et al. 2005). In addition, PA catabolic pathway was shown to be induced in *B. cenocepacia* grown in SCFM (Hamlin et al. 2009; Yoder-Himes et al. 2009). Here, we used a PA responsive reporter system, *B. cenocepacia* K56-2/pJH7 to monitor the activation of PA catabolic gene promoter during the growth in SCFM. The induction of the PA reporter system was

measured as the increase in the relative fluorescence. In addition, we also grow the PA-reporter system in MOPS-glycerol with addition of phenylalanine, tyrosine, or tryptophan to the same concentration found in SCFM (Palmer et al. 2007) to determine which of these aromatic amino acids can activate PA catabolic gene promoter.

We found that during growth in SCFM, the reporter system was induced when exponential phase of growth was reached and the induction diminished as the late stationary phase was reached (Figure 8a and b). The same result was obtained when the bacteria was grown with shaking at 37°C in 5mL SCFM (Figure 8c). In addition, our data also showed that only phenylalanine strongly induced the PA-reporter system but not tyrosine and tryptophan (Figure 9). This indicates that the PA-sensitive reporter system was induced during growth in SCFM due to the presence of phenylalanine.

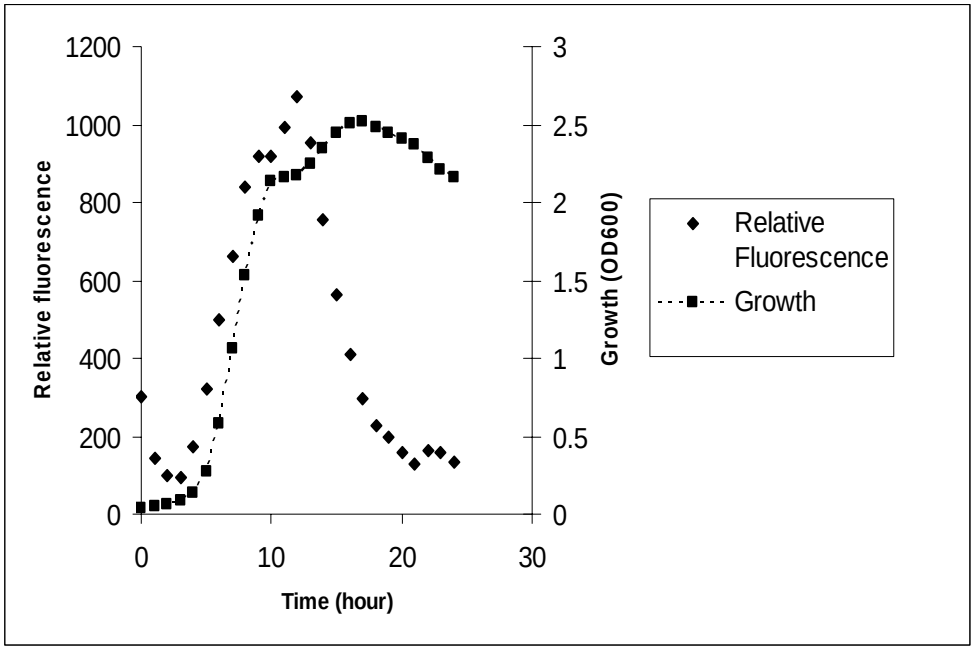
5.1 Phenylalanine is Degraded to PA

Although there is little confirmation, *in silico* analysis of microbial genomes suggests that phenylalanine undergoes degradation through the homogentisate pathway (Arias-Barrau et al. 2004). Recently, it was shown that *P. putida* is able to degrade phenylalanine through homogentisate and PA catabolic pathway (Herrera et al. 2010). Our *in silico* analysis utilizing the Kyoto Encyclopedia of Genes and Genomes (KEGG) database showed that phenylalanine can be degraded to PA in *B. cenocepacia* J2315 which is a clonal isolate of *B. cenocepacia* K56-2 (Figure 10, Table 4) (Kanehisa and Goto 2000; Kanehisa et al. 2006; Kanehisa et al. 2008; Mahenthiralingam et al. 2000). To

investigate which of the phenylalanine degradation pathways is active in *B. cenocepacia*, phenylalanine and its putative degradation intermediates were used as the sole carbon source for growth and as the inducer of the PA-reporter system.

Figure 11 shows that phenylalanine and its putative degradation intermediates were able to support the growth of *B. cenocepacia* K56-2 but not the *paaA* mutant strain, *B. cenocepacia* STC179. In addition, phenylalanine degradation intermediates were also able to induce the PA-responsive reporter system (Figure 12). Interestingly, phenylacetaldehyde was not able to support the growth of both wild type and *paaA* mutant strain but, it was able to induce the activation of PA catabolic gene promoter (Figure 11 and 12). Thus, the lack of growth was probably caused by the poor solubility of this compound when supplied exogenously. Finally, 2-hydroxyphenylacetate barely support the growth of both strains, in accordance to this compound not being a true intermediate but a dead-end product of PA degradation (Ferrandez et al. 1998).

A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600
400
200
0
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2
1.5
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Growth (OD600)
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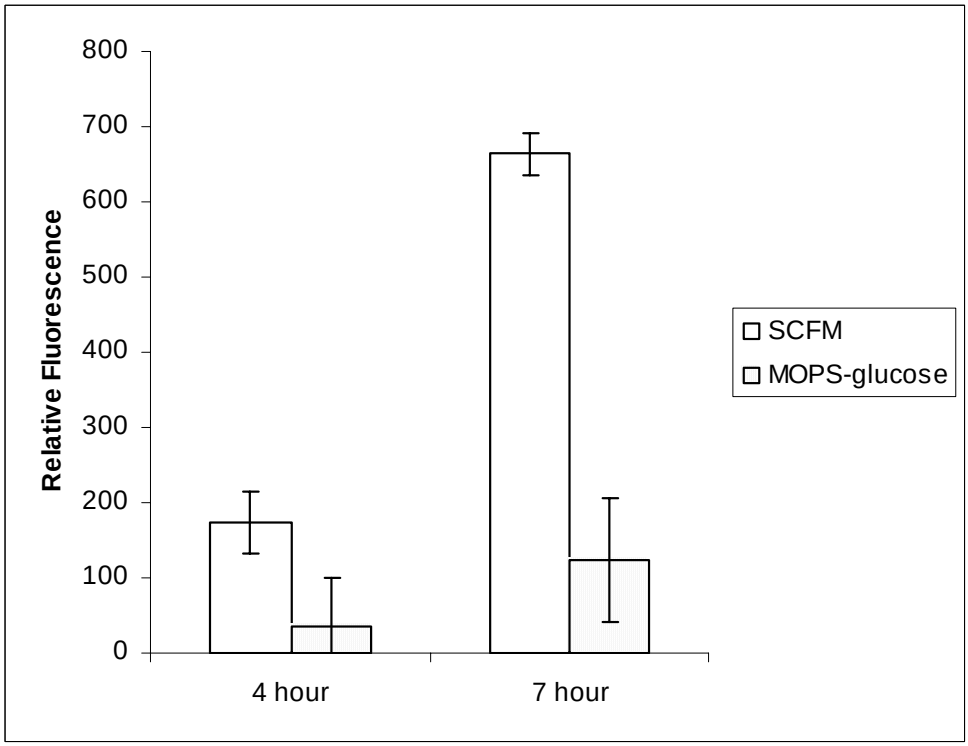
10
20
30
Time (hour)
Relative Fluorescence
Growth

B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200
100
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Relative Fluorescence
4 hour
7 hour
SCFM
MOPS-glucose

C

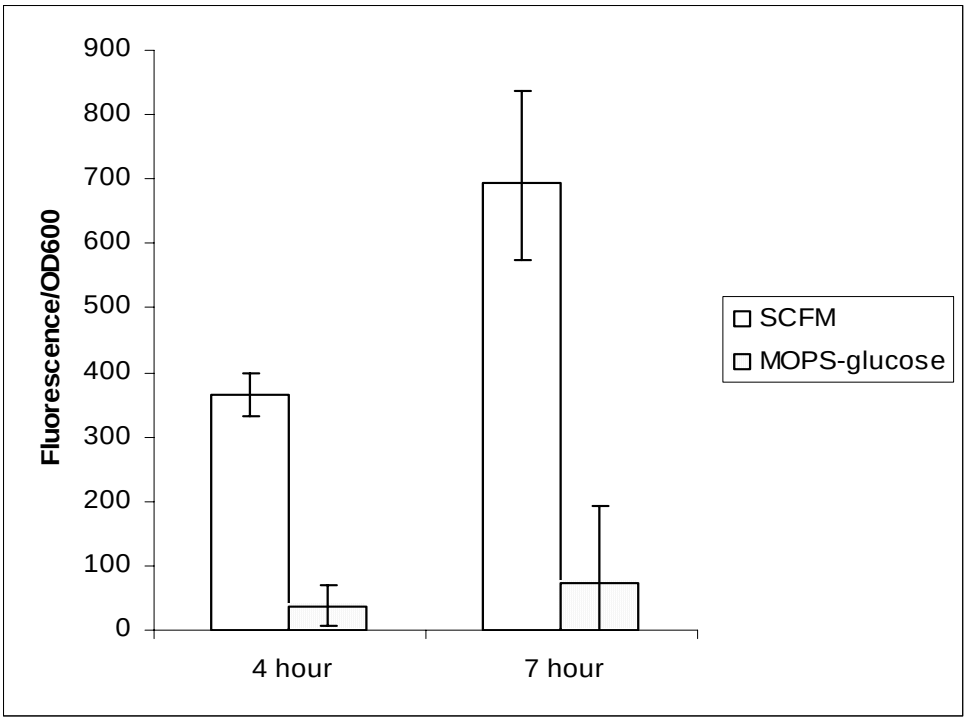

Figure 8. PA gene promoter is induced during growth in SCFM. A translational reporter plasmid containing the P_{paaA} gene promoter is responsive to SCFM during the exponential growth phase. *B. cenocepacia* K56-2/pJH7 cultures were inoculated in 96-well plates containing SCFM medium and incubated in a Biotek Synergy 2 plate reader at 37°C with shaking. OD_{600} and fluorescence were automatically recorded every hour (a). OD_{600} and fluorescence were also recorded at 4 and 7 hours of growth during incubation in 96-well plates (b) and test tubes containing 5mL of media (c). Relative fluorescence was calculated as described in Material and Methods. Error bars represent the standard deviation of three independent experiments.

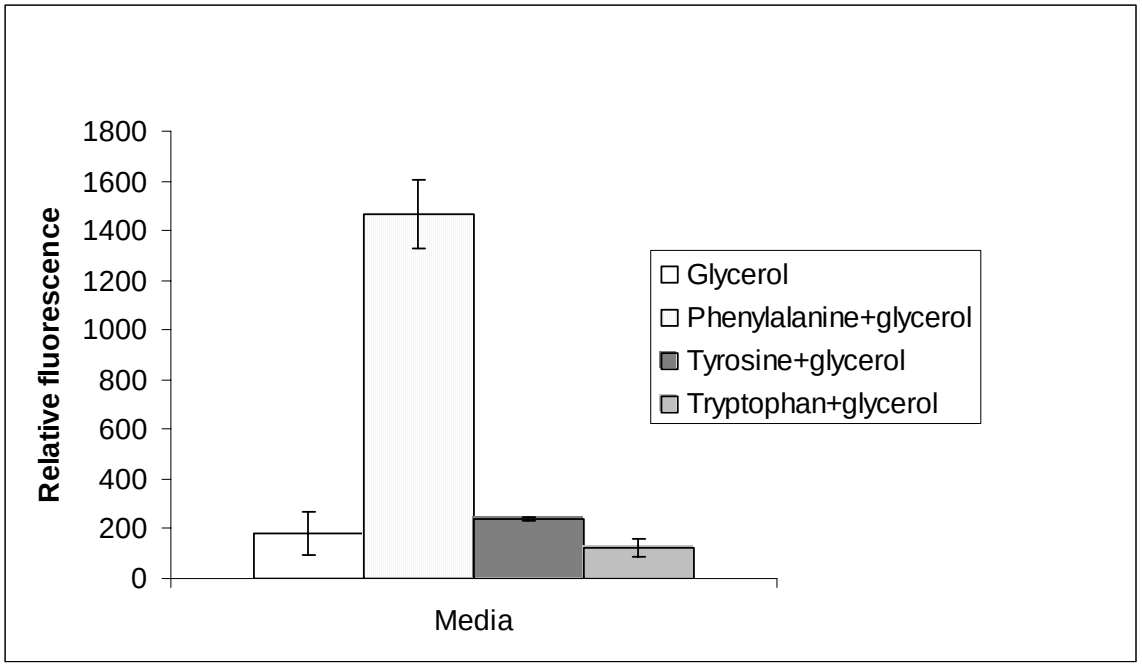

Figure 9. Phenylalanine was able to induce PA responsive reporter system but not tyrosine and tryptophan. *B. cenocepacia* K56-2/pJH7 was grown in MOPS-glycerol containing the same concentration of aromatic amino acid as found in SCFM (0.5 mM phenylalanine, 0.7 mM tyrosine, or 0.01 mM tryptophan). Error bars represents the standard deviation from 3 independent experiments.

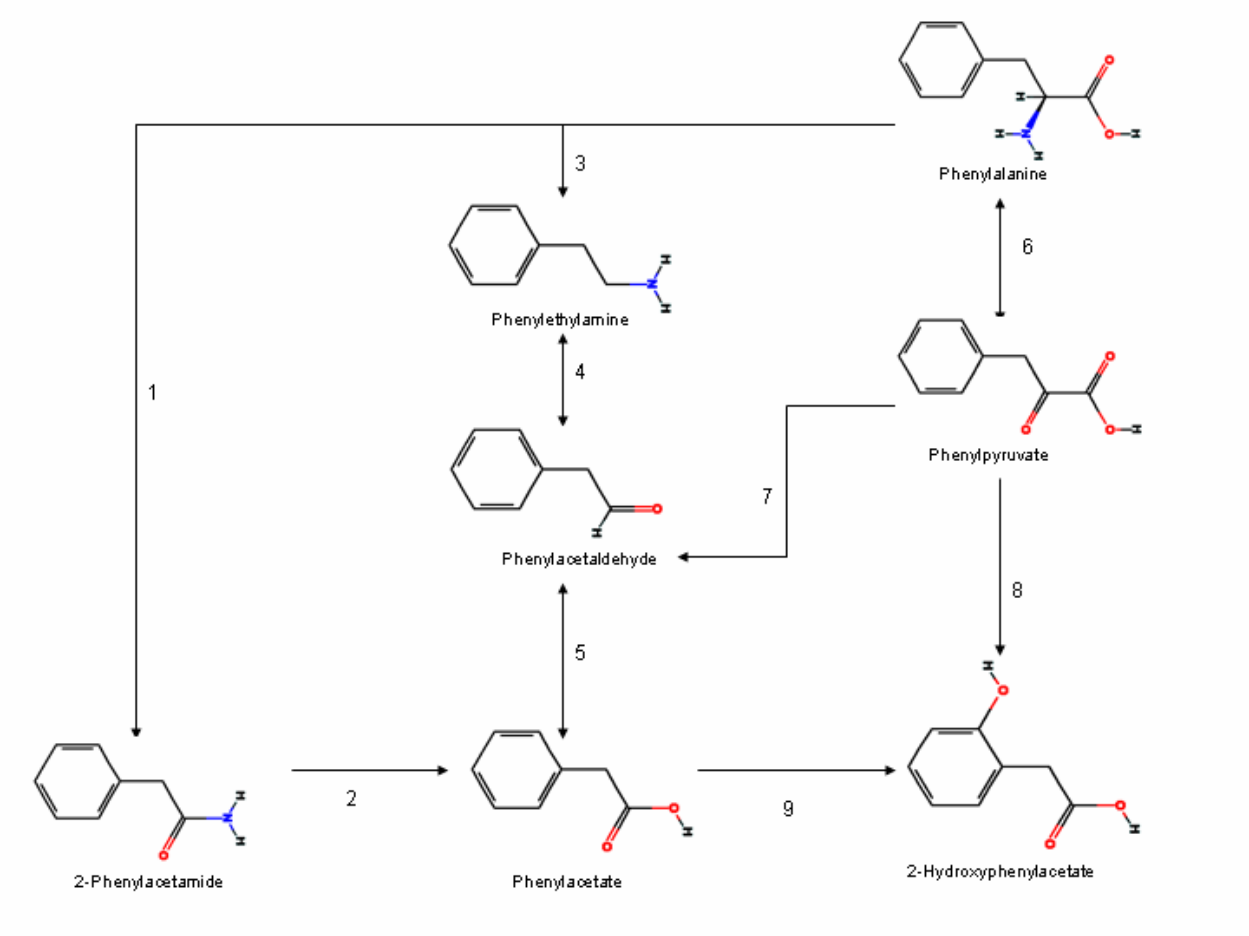

Figure 10. Putative phenylalanine degradation pathway to PA. The pathway was derived from map00360 obtained from Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa et al. 2000). The genes involved in each step are mentioned in Table 4.

Table 4. Enzymatic steps of phenylalanine degradation and their putative coding genes in *B. cenocepacia* J2315.

Step	ORFs [a]	Enzyme class [f]
1. Phenylalanine to 2-phenylacetamide	BCAL3299 (KatA) BCAM2107 (KatB)	oxidoreductase
2. 2-phenylacetamide to phenylacetate	BCAL2986[b,c] BCAL0148 BCAM0538 BCAM0641 BCAM1828 BCAM1315	amidase
3. Phenylalanine to phenylethylamine	BCAM0359[b,d]	Decarboxylase
4. Phenylethylamine to phenylacetaldehyde	BCAM0123 BCAM0112	monoamine oxidase
5. Phenylacetaldehyde to phenylacetate	BCAM1979	oxidoreductase
6. Phenylalanine to phenylpyruvate	BCAL0313 (HisC) BCAL0797 BCAL2303 (TyrB) BCAM1478	aminotransferase
7. Phenylpyruvate to phenylacetaldehyde	BCAL2359[b,e]	decarboxylase
8. Phenylpyruvate to 2-hydroxyphenylacetate	BCAL0207 BCAM1522 BCAM2503	Dioxygenase
9. Phenylacetate to 2-hydroxyphenylacetate	BCAM2591[f]	oxidoreductase

[a] unless noted, ORFS are described as putatively encoding the corresponding enzymes in *B. cenocepacia* J2313 according to KEGG database (Kanehisa et al. 2000).
[b] obtained as the hit with the lower expect value by BLAST search (Winsor et al. 2008) against *B. cenocepacia* J2315 using the indicated protein sequence queries, identified for the corresponding steps in the KEGG database.
[c] Bcen_0543 from *B. cenocepacia* AU1054.
[d] PP_2552 from *Pseudomonas putida* KT2440.
[e] YDR380W from *Saccharomyces cerevisae* (Hwang et al. 2009).
[f] AN8078.2 from *Aspergillus nidulans* amino acid sequence (Mingot et al. 1999).

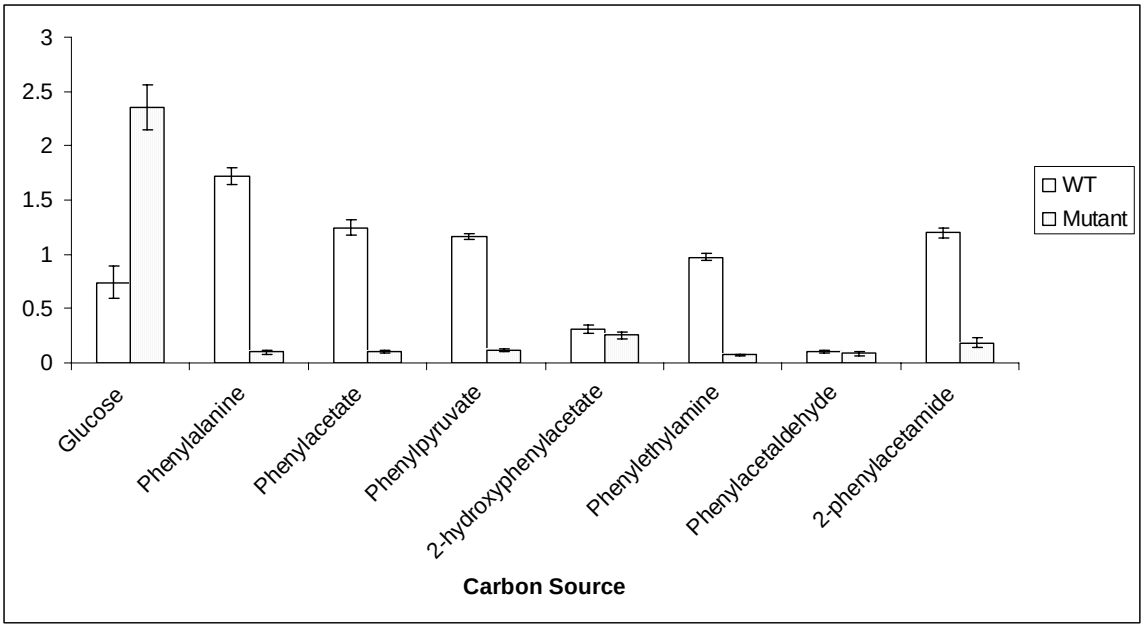

Figure 11. A PA degradation defective mutant is unable to grow in phenylalanine or putative phenylalanine degradation intermediates as sole carbon sources. *B. cenocepacia* K56-2 (wild type) or *B. cenocepacia* STC179-paaA (paaA) were inoculated in MOPS minimal medium containing 20mM glucose or 5mM of the indicated carbon sources and incubated for 24 h at 37^0C. Error bars represents the standard deviation from 3 independent experiments.

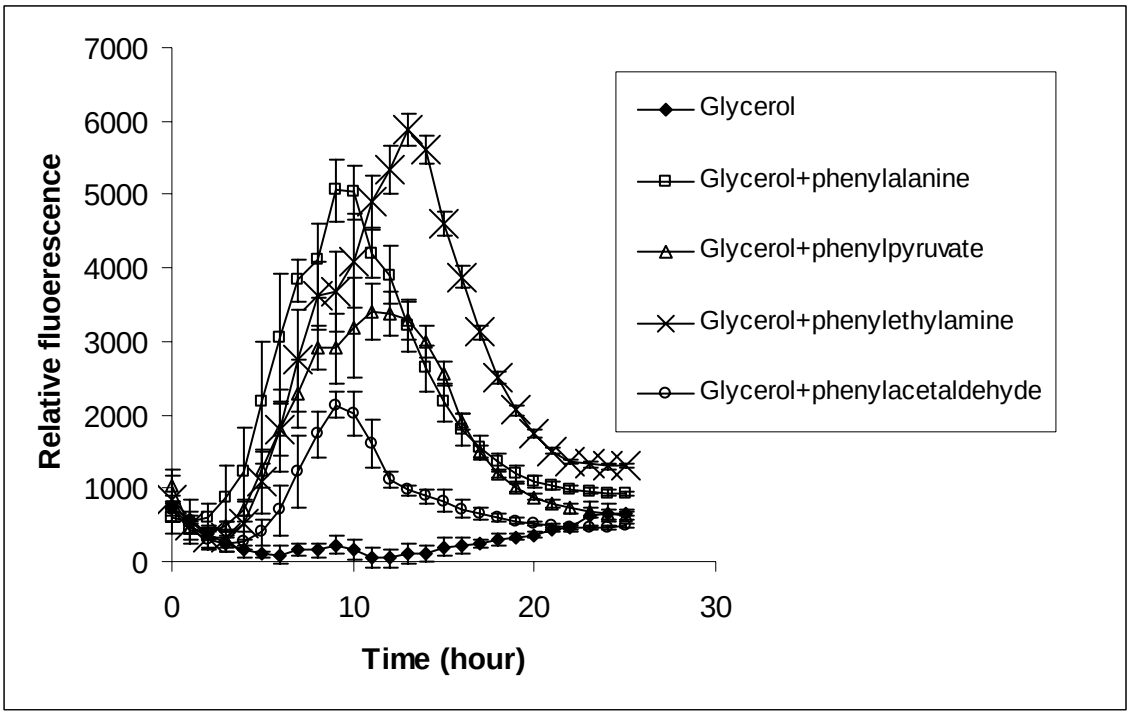

Figure 12. Phenylalanine and its degradation intermediates induced PA responsive reporter system. *B. cenocepacia* K56-2/pJH7 was grown in MOPS glycerol with addition of 0.5mM phenylalanine or its degradation intermediates. Error bars represent the standard deviation from 3 independent experiments.

6-RESULTS PART 2: PROTEOMIC ANALYSIS OF *B. cenocepacia* K56-2 GROWN IN CF-LIKE CONDITION

Proteomics based approaches were utilized to study the global protein expression of *B. cenocepacia* grown in SCFM, particularly the proteins which might be over-expressed due to the presence of phenylalanine. First, a growth curve experiment was performed in order to determine the time for the collection of the cells for the whole protein extraction. Then, two different proteomic approaches were used in this study. The first preliminary analysis was done utilizing the traditional 2-DE. The study then was followed in more detail through the analysis utilizing a more sensitive mass spectroscopy based approach, iTRAQ.

6.0 SCFM and MOPS-glucose Supports the Growth of *B. cenocepacia* K56-2

SCFM is a medium which nutritionally mimics CF sputum and is rich in amino acids (Palmer et al. 2007). To investigate what effects these amino acids have on the physiology of *B. cenocepacia*, particularly on phenylalanine and PA catabolism, proteomic analysis approaches were used. To collect the protein samples for this approach, the cells were grown under two different sets of conditions, using SCFM and MOPS-glucose (Neidhardt et al. 1974). The choice of MOPS-glucose was made based on the fact that this medium lacks amino acids but is able to support the growth of *B. cenocepacia* K56-2 to the same magnitude as SCFM, and SCFM is a also a MOPS-buffered medium.

Figure 13 shows that even though *B. cenocepacia* K56-2 grown in MOPS-glucose reached its exponential phase later, a similar OD_{600} as obtained when grown in SCFM was eventually reached. Since our data showed that PA catabolic gene promoter was activated during the exponential phase in the cells grown in SCFM (Figure 8), we decided to collect the cells for protein sample extraction when they reached mid-exponential phase at OD_{600} of ~1 (Figure 13 and 8c).

6.1 *B. cenocepacia* K56-2 Grown on Amino Acid-rich SCFM Showed a Difference in Protein Expression Compared to the One Grown on MOPS-glucose

For preliminary analysis, the whole protein extracts from the exponential phase cells of the two growth conditions were subjected to 2-DE. We found several protein spots which are more intense from the SCFM-grown cells protein samples (Figure 14). We were able to putatively identify two of these protein spots by referring to a proteomic study in *B. cenocepacia* H111 (Riedel et al. 2006). One spot with approximately molecular weight (MW) of 26 kDa and pI of 6 most likely belongs to acyl carrier protein (Figure 14). Another spot with approximate MW of 40 and pI of 4.5 is putatively belongs to flagellin (Figure 14). However, no further analysis was done on 2-DE. Instead, a more sensitive mass spectrometry based method, iTRAQ (Ross et al. 2004) was utilized in order to analyze the difference in the protein expression in more details.

We chose iTRAQ due to the limitation in the protein detection of the classical 2-DE, especially on the low abundance proteins (Ross et al. 2004; Zieske 2006). Protein

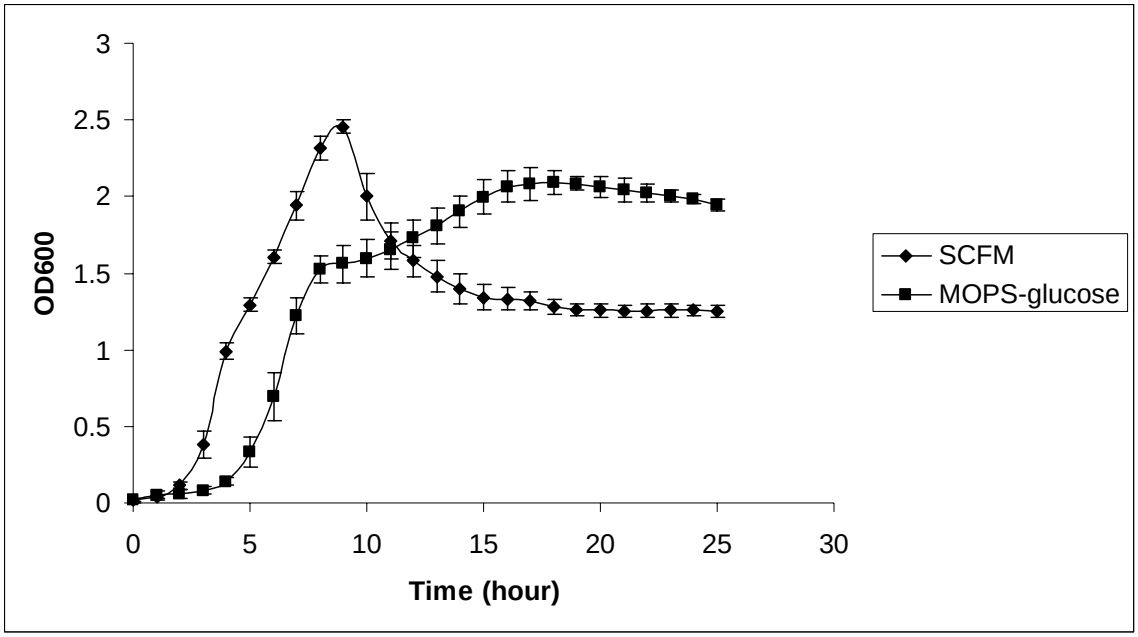

Figure 13. Growth of *B. cenocepacia* K56-2 in SCFM and MOPS-glucose. OD_{600} of bacteria were recorded every hour until the stationary phase is reached.

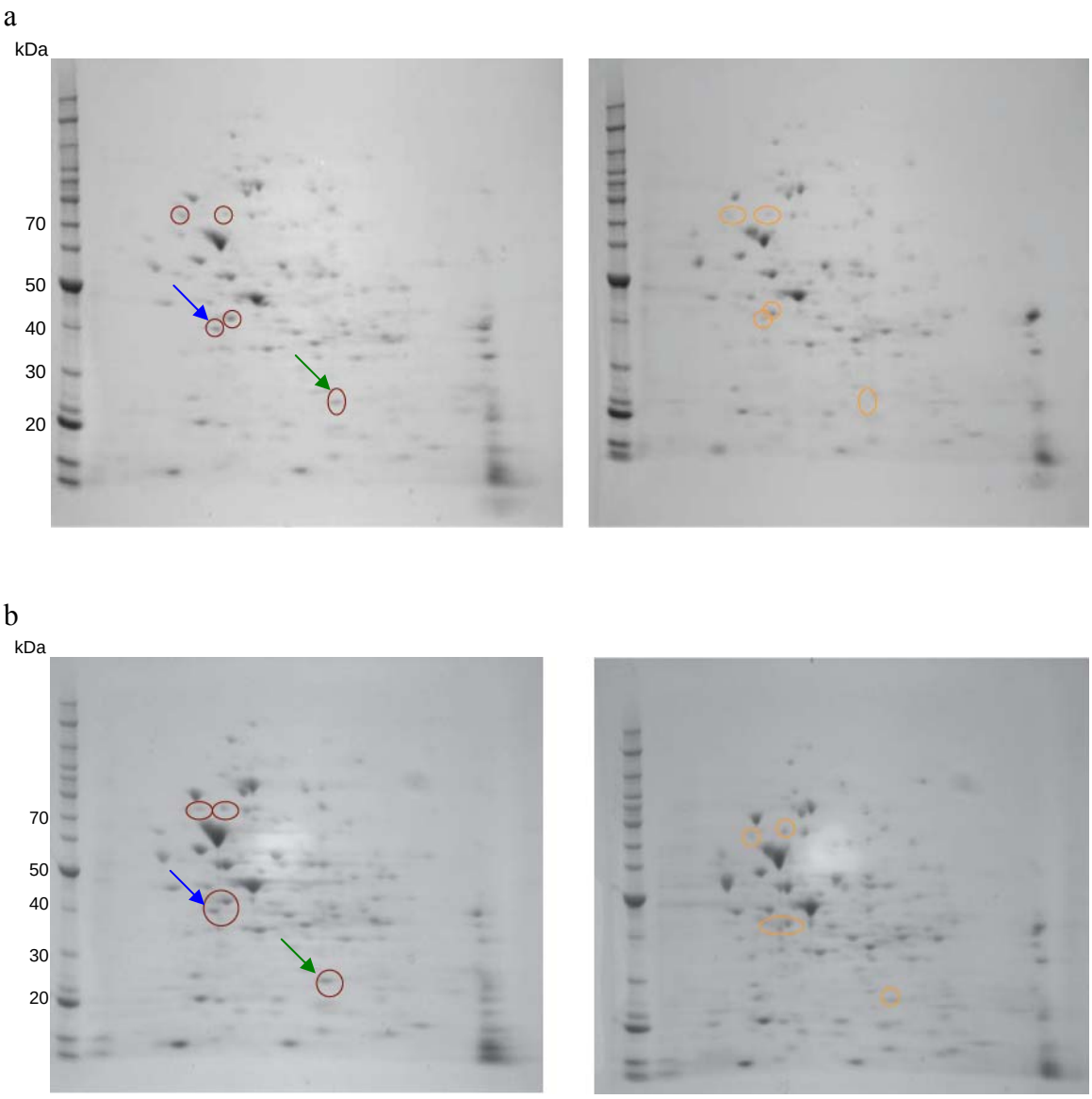

Figure 14. 2D gel analysis of the proteome of *B. cenocepacia* K56-2 grown on 2 different growth conditions. Whole protein extracts from SCFM-grown cells (left) and MOPS-glucose grown cells (right) using 30 ug of protein from first biological replicate (a) and 40 ug of protein from the second biological replicate (b) were used for 2-DE. The difference in the protein spot patterns was observed between protein samples obtained from the cells grown in SCFM and MOPS-glucose for both biological replicates. The blue and green arrows refer to the putative protein spots of acyl carrier protein and flagellin respectively.

samples from two biological replicates were used for preliminary analysis using SDS-PAGE to confirm the concentration of the samples. This preliminary analysis showed the presence of the bands with approximate size of 80 kDa and 120 kDa which are more intense from the samples obtained from SCFM-grown cells compared to the samples from MOPS-glucose grown cells (Figure 15).

Our iTRAQ results using two biological replicates showed that proteins that are involved in translation, ribosomal, structural, and biogenesis are over-expressed during the growth of *B. cenocepacia* K56-2 in amino-acid rich SCFM (Figure 16a, table 5a). In addition, we also found the over-expression of the proteins involved in amino acid, carbohydrate, and secondary metabolites metabolism (Figure 16a, Table 5a). Interestingly, flagellin and membrane efflux protein which are involved in the virulence of *B. cenocepacia* are also upregulated during growth in SCFM (Table 5a). We also found a protein which is annotated as a putative invasion protein which maybe related to the virulence of *B. cenocepacia*.

On the other hand, we also saw a different set of proteins involved in amino acid, carbohydrate, and secondary metabolites metabolisms were under-expressed during growth in SCFM. Interestingly, we found a significant amount of hypothetical proteins are being under-expressed in addition to transcriptional regulators (Figure 16b, Table 5b).

The analysis was also done on the proteins which are regulated differently only in one of the biological replicates (Table 6 and 7). Interestingly, a different set of ribosomal

proteins was shown to be over-expressed during growth in SCFM. The data also showed the presence of two more efflux proteins being regulated differently in the first biological replicates (Table 6). The data showed that BCAM2550, a multidrug efflux protein was under-expressed. This is probably due to the fact that some multidrug efflux systems are induced by the presence of antibiotics (Morita et al. 2006). Since there was no antibiotic added into SCFM, this may explain why BCAM2550 was under-expressed during growth in SCFM. We also found the presence of more hypothetical proteins which are regulated differently from analysing the individual biological replicates. The analysis also showed that during the growth in SCFM a protein involved in methionine biosynthesis is under-expressed in the first biological replicate (Table 6a). In addition, a valine biosynthesic protein is also found to be under-expressed in the second biological replicate during growth in SCFM (Table 7b).

We also found the proteins which are produced only during the growth of *B. cenocepacia* K56-2 (both biological replicates) in one condition but not the other (Table 8). The data showed that an ABC transporter ATP-binding protein (predicted to be involved in nickel ion transport) and a putative ketoreductase were only produced during the growth of *B. cenocepacia* K56-2 in SCFM (Table 8). In addition, a two component system, response regulator protein was not produced during growth in SCFM (Table 8).

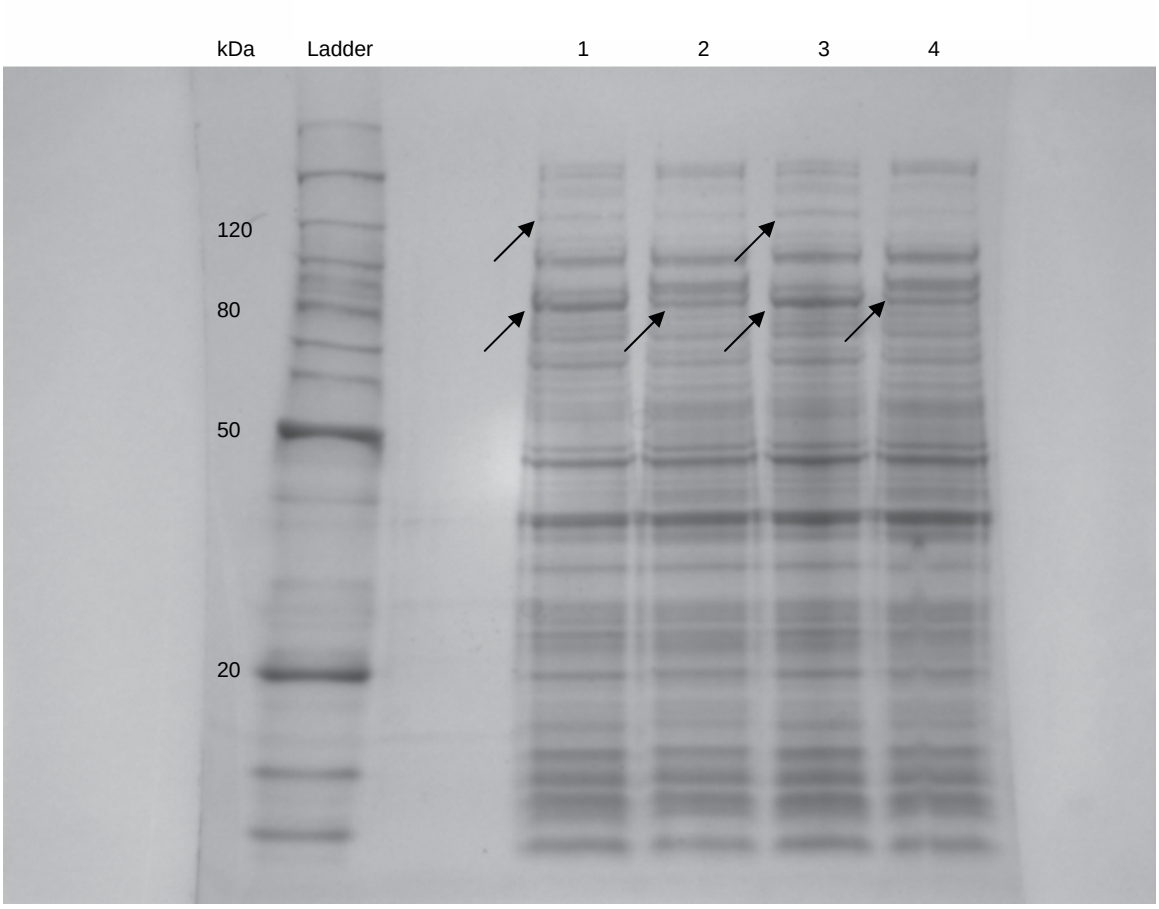

Figure 15. SDS-PAGE gel electrophoresis of the whole protein from *B. cenocepacia* K56-2 cells grown in two different sets of growth conditions. Lane 1, molecular weight marker; Lane 2 and 4, protein from SCFM-grown cells, first and second biological replicate respectively; Lane 3 and 5, protein from MOPS-glucose grown cells, first and second biological replicates respectively.

a.

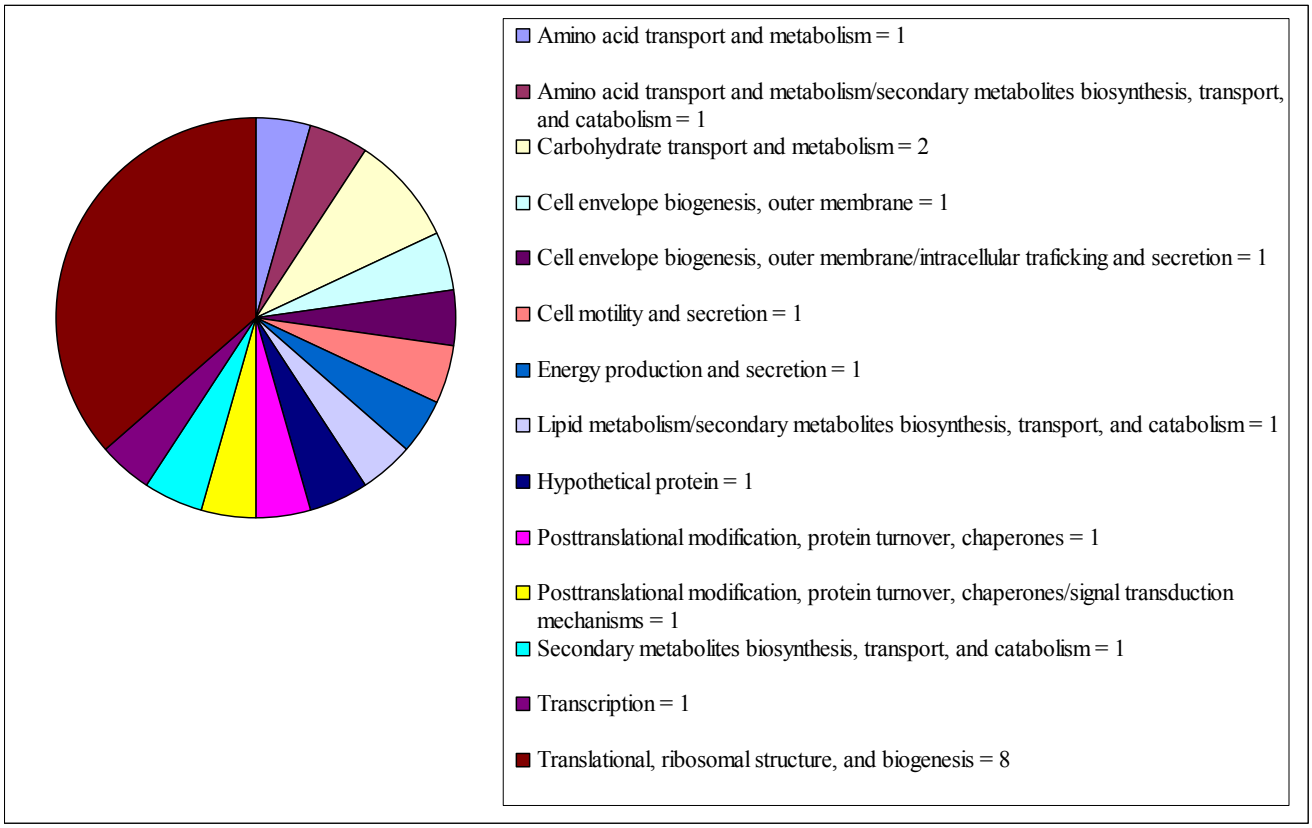

Figure 16. Functional category of proteins that are over-rexpressed (a) and under-expressed (b) during growth of *B. cenocepacia* K56-2 in SCFM compared to MOPS-glucose. There are a total number of 22 over-expressed proteins (a) and 21 under-expressed proteins. The numbers of proteins found for each category are shown in the figure.

b.

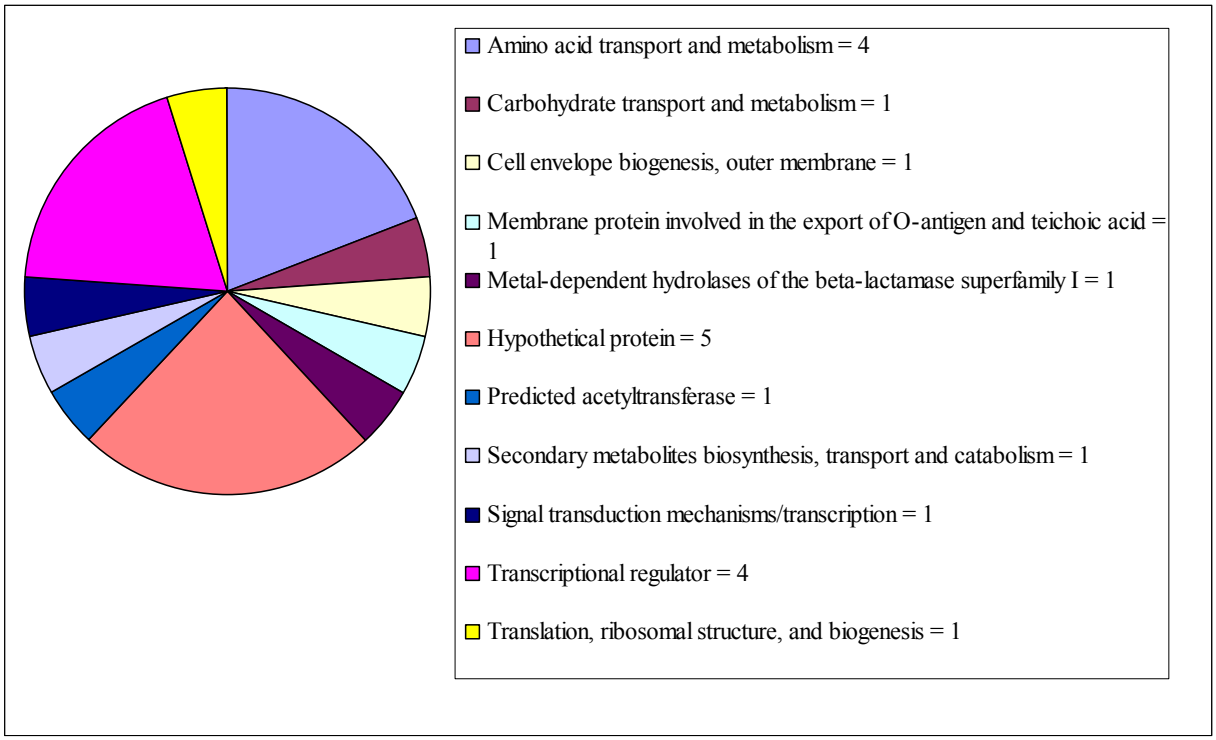
Amino acid transport and metabolism = 4
Carbohydrate transport and metabolism = 1
Cell envelope biogenesis, outer membrane = 1
Membrane protein involved in the export of O-antigen and teichoic acid = 1
Metal-dependent hydrolases of the beta-lactamase superfamily I = 1
Hypothetical protein = 5
Predicted acetyltransferase = 1
Secondary metabolites biosynthesis, transport and catabolism = 1
Signal transduction mechanisms/transcription = 1
Transcriptional regulator = 4
Translation, ribosomal structure, and biogenesis = 1

Table 5. List of the proteins from iTRAQ that are over-expressed (a) and under-expressed (b) during growth of *B. cenocepacia* K56-2 in SCFM compared to MOPS-glucose in both biological replicates

a.

Gene	Protein	GO Biological process prediction[a]	COG prediction category	1 iTRAQ[b]	2 iTRAQ[b]
BCAL1796	Putative saccharopine dehydrogenase	N/A	Amino acid transport and metabolism	3.59	2.97
BCAM1296	putative peptidase	N/A	Amino acid transport and metabolism / Secondary metabolites biosynthesis, transport, and catabolism	13.16	10.94
BCAM0767	Ribose import ATP-binding protein	D-xylose transport	Carbohydrate transport and metabolism	7.11	6.43
BCAL2748	Major Facilitator Superfamily protein	Multidrug transport	Carbohydrate transport and metabolism	7.81	4.93
BCAL1367	Putative invasion protein	N/A	Cell envelope biogenesis, outer membrane	5.44	5.82
BCAL1676	Multidrug efflux system outer membrane protein	Transport	Cell envelope biogenesis, outer membrane / Intracellular trafficking and secretion	5.02	4.35
BCAL0114	Flagellin	Ciliary or flagellar motility	Cell motility and secretion	5.98	4.16
BCAS0159	Putative dehydrogenase	N/A	Energy production and conversion	4.03	4.35
BCAL0995	Acyl carrier protein	Fatty acid biosynthetic	Lipid metabolism / Secondary metabolites biosynthesis, transport, and catabolism	3.88	3.25
BCAL0305	Hypothetical protein	N/A	N/A	3.70	3.56
BCAL3424	Thiol peroxidase	N/A	Posttranslational modification, protein turnover, chaperones	4.10	3.04
BCAL3278	Putative glutamate-ammonia-ligase adenylyltransferase	N/A	Posttranslational modification, protein turnover, chaperones / Signal transduction	6.48	3.27

			mechanisms		
BCAL2432	Putative benzoate membrane transport protein	N/A	Secondary metabolites biosynthesis, transport, and catabolism	11.67	4.12
BCAL0914	Putative DNA binding protein	N/A	Transcription	6.95	5.66
BCAL0558	Multifunctional tRNA nucleotidyl transferase/ 2'3'-cyclic phosphodiesterase/ 2'nucleotidase/phosphatase	N/A	Translation, ribosomal structure and biogenesis	6.65	3.55
BCAL1943	30S ribosomal protein S18	translation	Translation, ribosomal structure and biogenesis	3.63	2.86
BCAL2349	30S ribosomal protein S15	translation	Translation, ribosomal structure and biogenesis	3.67	3.32
BCAL1012	Elongation factor P	translational elongation	Translation, ribosomal structure and biogenesis	4.04	3.10
BCAL0225	50S ribosomal protein L7/L12	translation	Translation, ribosomal structure and biogenesis	4.65	3.87
BCAL0248	30S ribosomal protein S8	N/A	Translation, ribosomal structure and biogenesis	4.83	4.85
BCAL0236	50S ribosomal protein L23	translation	Translation, ribosomal structure and biogenesis	4.84	3.80
BCAL2765	30S ribosomal protein S20	translation	Translation, ribosomal structure and biogenesis	6.36	4.89

b.

Gene	Protein	GO biological process prediction[a]	COG prediction category	1 iTRAQ[b]	2 iTRAQ[b]
BCAM0983	Isopropylmalate isomerase large subunit	leucine biosynthetic process	Amino acid transport and metabolism	0.23	0.18
BCAL3004	Chorismate mutase	N/A	Amino acid transport and metabolism	0.36	0.44
BCAM2503	Putative amino acid dioxygenase	L-phenylalanine catabolic process, tyrosine catabolic process	Amino acid transport and metabolism	0.42	0.38
BCAL0547	Putative dipeptide ABC transporter ATP-binding protein	nickel ion transport	Amino acid transport and metabolism	0.47	0.42
BCAL3425	Putative sugar kinase	D-ribose catabolic process	Carbohydrate transport and metabolism	0.40	0.32
BCAS0533	Putative soluble lytic murein transglycosylase	N/A	Cell envelope biogenesis, outer membrane	0.50	0.41
BCAM1007	Putative polysaccharide biosynthesis protein	N/A	Membrane protein involved in the export of O-antigen and teichoic acid	0.27	0.18
BCAL2165	Metallo-beta-lactamase superfamily protein	N/A	Metal-dependent hydrolases of the beta-lactamase superfamily I	0.43	0.34
BCAL3350	Hypothetical protein	N/A	N/A	0.2	0.22
BCAM2005	Hypothetical protein	N/A	N/A	0.23	0.33
BCAM0148	Hypothetical protein	N/A	N/A	0.26	0.40
BCAL0938a	Hypothetical protein	N/A	N/A	0.3	0.3
BCAM1890	Hypothetical protein	N/A	N/A	0.48	0.45
BCAS0385	Putative acetyltransferase, GNAT family	N/A	Predicted acetyltransferase	0.20	0.17
BCAM1826	Putative N-hydroxyarylamine O-acetyltransferase	N/A	Secondary metabolites biosynthesis, transport, and catabolism	0.39	0.37
BCAM1545	LuxR superfamily regulatory protein	N/A	Signal transduction mechanisms / Transcription	0.45	0.38
BCAM2264	LysR family regulatory protein	N/A	Transcriptional regulator	0.34	0.46
BCAM0677	AraC family	N/A	Transcriptional	0.35	0.31

	regulatory protein		regulator		
BCAM1280	Putative GntR-family transcriptional regulator/aminotransferase	L-phenylalanine catabolic process	Transcriptional regulator	0.17	0.13
BCAS0354	AraC family regulatory protein	N/A	Transcriptional regulator	0.34	0.11
BCAL2721	NOL1/NOP2/Sun family protein	rRNA modification	Translation, ribosomal structure and biogenesis	0.48	0.39

[a]Biological process predictions were obtain from the Gene Ontology (Ashburner et al. 2000) through *Burkholderia* Genome Database (Winsor et al. 2008).
[b]iTRAQ reporter group ratio of the proteins obtained from SCFM-grown cells compared the ones obtained from MOPS-glucose grown cells.

Table 6. List of proteins which are over-expressed (A) and under-expressed (B) in the first biological replicate only

a

Gene	Protein	GO biological process prediction[a]	COG prediction category	iTRAQ[b]
BCAM0932	Outer membrane efflux protein	Transport	Cell envelope biogenesis, outer membrane / Intracellular trafficking and secretion	3.46
BCAL0234	50S ribosomal protein L3	N/A	Translation, ribosomal structure and biogenesis	3.49
BCAL0244	50S ribosomal protein L14	Translation	Translation, ribosomal structure and biogenesis	3.58
BCAM1633	Putative 2-nitropropane dioxygenase	N/A	Dioxygenases related to 2-nitropropane dioxygenase [General function prediction only]	3.58
BCAL0222	50S ribosomal protein L11	Translation	Translation, ribosomal structure and biogenesis	3.61
BCAL0261	50S ribosomal protein L17	Translation	Translation, ribosomal structure and biogenesis	3.66
BCAM0941	6-phosphogluconate dehydrogenase	Pentose-phosphate shunt	Carbohydrate transport and metabolism	3.69
BCAL2714	50S ribosomal protein L28	Translation	Translation, ribosomal structure and biogenesis	3.92
BCAM2354	Hybrid two-component system kinase-response regulator protein	Two-component signal transduction system (phosphorelay), phosphate metabolic process/ phosphate transport	Signal transduction mechanisms	3.93
BCAM0004	Putative partitioning protein ParB	Cytokinesis, chromosome segregation	Transcription	4.02
BCAM2594	Putative alcohol dehydrogenase	Metabolic process	Energy production and conversion / General function prediction only	4.12
BCAL0043	Putative extracellular ligand-binding protein	N/A	Amino acid transport and metabolism	4.17
BCAL1359	Hypothetical protein	N/A	hypothetical protein	5.65

b

Gene	Product	GO biological process prediction[a]	COG prediction category	iTRAQ[b]
BCAM0161	AraC family regulatory protein	N/A	Transcription	0.24
BCAM2169	putative outer membrane autotransporter	N/A	Function unknown	0.25
BCAM2550	Multidrug efflux system transporter protein CeoB	Transport	Defense mechanisms	0.41
BCAS0570	Putative alpha/beta hydrolase fold protein	Photosynthesis	Predicted hydrolases or acyltransferases (alpha/beta hydrolase superfamily) [General function prediction only]	0.42
BCAL3221	Putative capsular polysaccharide biosynthesis sulfatase membrane protein	N/A	Cell envelope biogenesis, outer membrane	0.42
BCAM0708	Phosphotransferase enzyme family protein	N/A	Putative homoserine kinase type II (protein kinase fold) [General function prediction only	0.43
BCAM1400	Putative decarboxylase	N/A	Function unknown	0.44
BCAM2194	Methylmalonate-semialdehyde dehydrogenase	Valine catabolic process	Energy production and conversion	0.44
BCAL2808	Putative periplasmic ABC transporter substrate-binding component	N/A	Carbohydrate transport and metabolism	0.47
BCAL1807	LysR family regulatory protein	N/A	Transcription	0.47

[a]Biological process predictions were obtain from the Gene Ontology (Ashburner et al. 2000) through *Burkholderia* Genome Database (Winsor et al. 2008).
[b]iTRAQ reporter group ratio of the proteins obtained from SCFM-grown cells compared the ones obtained from MOPS-glucose grown cells.

Table 7. List of proteins which are over-expressed (A) and under-expressed (B) in the second biological replicate only

a

Gene	Product	GO biological process prediction[a]	COG preediction category	iTRAQ[b]
BCAL0870	Putative oxidoreductase	Carbohydrate metabolic process	Energy production and conversion	2.95
BCAM1166	Hypothetical protein	N/A	hypothetical protein	2.95
BCAL0251	30S ribosomal protein S5	Translation	Translation, ribosomal structure and biogenesis	3.01
BCAL0372	Putative glutaredoxin	N/A	Posttranslational modification, protein turnover, chaperones	3.02
BCAL3222	Putative capsular polysaccharide biosynthesis dehydrogenase/reductase protein	Fatty acid biosynthetic process	Short-chain dehydrogenases of various substrate specificities [General function prediction only]	3.13
BCAL2962	Hypothetical protein	N/A	hypothetical protein	3.17
BCAL3286	Cobalamin adenosyltransferase protein	Cobalamin biosynthetic process	Function unknown	3.2
BCAM0854	Bifunctional exopolysaccharide biosynthesis protein (phosphomannose isomerase and GDP-D-mannose pyrophosphorylase)	Lipopolysaccharide biosynthetic process	Cell envelope biogenesis, outer membrane	3.33
BCAL0249	50S ribosomal protein L6	Translation	Translation, ribosomal structure and biogenesis	3.38
BCAL1561	Hypothetical protein	N/A	hypothetical protein	3.55
BCAL1506	Transcription elongation factor NusA	Transcription termination	Transcription	3.9
BCAL2693	LysR family regulatory protein	N/A	Transcription	4.54
BCAL0701	Hypothetical protein	N/A	Function unknown	5
BCAM2538	LysR family regulatory protein	Protocatechuate catabolic process, positive regulation of transcription, DNA-dependent	Transcription	7.16

b

Gene	Product	GO biological process prediction[a]	COG prediction category	iTRAQ[b]
BCAM0286	Putative alcohol dehydrogenase	Fermentation	Zn-dependent alcohol dehydrogenases [General function prediction only]	0.23
BCAS0098	Hypothetical protein	N/A	hypothetical protein	0.35
BCAM2652	TetR family regulatory protein	N/A	Transcription	0.36
BCAL1756	Putative metal dependent phosphohydrolase	N/A	Predicted kinase [General function prediction only]	0.37
BCAM0835	AraC family regulatory protein	N/A	Transcription	0.38
BCAM0630	Putative dehydrogenase	N/A	Amino acid transport and metabolism	0.39
BCAM0451	Putative extracellular endonuclease/exonuclease/phosphatase family protein	N/A	Predicted extracellular nuclease [General function prediction only]	0.41
BCAM0453	Hypothetical protein	N/A	hypothetical protein	0.41
BCAL2725	Lipoprotein signal peptidase	Proteolysis, protein secretion	Cell envelope biogenesis, outer membrane / Intracellular trafficking and secretion	0.42
BCAM2484	5-methyltetrahydropteroyltriglutamate- - homocysteine S-methyltransferase	Methionine biosynthetic process	Amino acid transport and metabolism	0.44
BCAL2006	Hypothetical protein	N/A	hypothetical protein	0.45
BCAM0469	Putative aldehyde dehydrogenase	Betaine biosynthetic process	Energy production and conversion	0.45

[a]Biological process predictions were obtain from the Gene Ontology (Ashburner et al. 2000) through *Burkholderia* Genome Database (Winsor et al. 2008).
[b]iTRAQ reporter group ratio of the proteins obtained from SCFM-grown cells compared the ones obtained from MOPS-glucose grown cells.

Table 8. Proteins which are produced by both biological replicates only during growth in one of the growth conditions

Gene	Product	GO biological process prediction[a]	COG prediction category	iTRAQ[b]
BCAM1773	ABC transporter ATP-binding protein	Nickel ion transport	Amino acid transport and metabolism / Inorganic ion transport and metabolism	Infinity
BCAM2754	Putative ketoreductase	N/A	Secondary metabolites biosynthesis, transport, and catabolism / General function prediction only	Infinity
BCAM0221	Two-component regulatory system, response regulator protein	N/A	Signal transduction mechanisms / Transcription	0

[a]Biological process predictions were obtain from the Gene Ontology (Ashburner et al. 2000) through *Burkholderia* Genome Database (Winsor et al. 2008).
[b]iTRAQ reporter group ratio of the proteins obtained from SCFM-grown cells compared the ones obtained from MOPS-glucose grown cells.

7-DISCUSSION

Research on catabolic pathways of pathogenic bacteria during growth in the host is emerging as a relevant field in bacterial pathogenesis (Brown et al. 2008). Up to now, aromatic amino acid catabolic pathways as virulence determinants are largely overlooked in bacterial pathogens. However, these carbon utilization routes may be especially relevant to CF infection given the presence of aromatic amino acids in the CF sputum. During growth in CF conditions, the PA catabolic pathway was activated but the reasons underlying this induction were unknown (Hamlin et al. 2009; Yoder-Himes et al. 2009).

7.0 Phenylanine is Degraded only through PA Catabolic Pathway in *B. cenocepacia* K56-2

In this work, we showed that SCFM supports robust growth of *B. cenocepacia* K56-2/pJH7 and induced the PA-sensitive reporter system during exponential phase of growth (Figure 8). This indicates that PA catabolic genes are activated during growth in SCFM and is consistent with PA catabolism being the part of the central metabolism *B. cenocepacia*. Our data also showed that the presence of phenylalanine at the similar level found in SCFM was able to induce PA reporter system (Figure 9). This indicates that PA catabolism maybe active during growth in SCFM due to presence of phenylalanine. In addition, the fact that PA reporter system was induced when the cells entered exponential phase may indicate that phenylalanine is *B. cenocepacia* favorite carbon source (Figure

8). However, the induction of the PA reporter system was diminished in the stationary phase of growth (Figure 8). This may indicate the depletion of phenylalanine in SCFM.

Our result also showed that tyrosine and tryptophan did not induce the PA responsive reporter system during the exponential phase of growth in *B. cenocepacia* K56-2 (Figure 9). This indicates that tyrosine and tryptophan are not responsible for the induction of *paaA* reporter system during the growth of *B. cenocepacia* K56-2 in SCFM. Therefore, this suggests that both tyrosine and tryptophan are not degraded through the PA catabolic pathway.

The concept that phenylalanine degradation products enter the PA catabolism is further supported by the fact that the putative phenylalanine degradation intermediates were also able to induce the PA responsive reporter system (Figure 12). In addition, our result showed that when used as the sole carbon source, phenylalanine and its degradation intermediates were able to support growth of wild type but not the *paaA* mutant strain (Figure 11). These results confirm the presence of several possible steps for the degradation of phenylalanine to PA as predicted by the KEGG database (Figure 10). This also suggests that despite of the presence of homogentisate catabolic genes in *B.cenocepacia* J2315 genome, phenylalanine is degraded through PA catabolic pathway in *B. cenocepacia* K56-2. In addition, the fact that the *paaA* mutant strain did not grow on phenylalanine as the sole carbon source indicates phenylalanine is degraded only through PA catabolic pathway in *B. cenocepacia* K56-2. If *B. cenocepacia* could degrade phenylalanine through homogentisate pathway (which involves the conversion of

phenylalanine to tyrosine), then the *paaA* mutant strain should grow on phenylalanine as the sole carbon source.

Interestingly, the *paaA* mutant strain showed an increased final OD_{600} when growing on glucose as the sole carbon source. A previous study in *B. cenocepacia* K56-2 showed all the genes in *paaABCDE* operon are co-transcribed (Hamlin et al. 2009). In addition, *paaA* was interrupted by the insertion of a suicide plasmid containing a downstream transcriptional terminator (Flannagan et al. 2007; Law et al. 2008). This interruption might have introduced a polar mutation in *paaABCDE* operon and blocked the expression of all the genes in the operon. This may lead to *paaA* mutant strain conserving the energy for the expression of these genes for growth instead. Therefore, this may explain why the *paaA* mutant strain showed an increased growth in MOPS-glucose compared to the wild type strain.

7.1 *B. cenocepacia* K56-2 Grown in the CF-like Condition Showed a Difference in the Global Protein Expression Compared to the One Grown in MOPS-glucose

We also investigated the phenylalanine and PA catabolism in *B. cenocepacia* through proteomics. A transcriptomics study in *B. cenocepacia* showed that PA catabolic genes were upregulated during growth in SCFM. However, the changes in the gene expression do not always correlate directly to the changes in protein expression (Yoder-Himes et al. 2009; Zieske 2006). Therefore, we decided to confirm this result through the proteomic analysis. A preliminary analysis utilizing 2-DE showed a difference in protein

spots pattern between the whole protein extracts from *B. cenocepacia* K56-2 grown in SCFM and MOPS-glucose (Figure 14). In addition, a SDS-PAGE analysis also showed the presence of the bands that are more intense from the protein samples obtained from SCFM-grown cells compared to the samples obtained from MOPS-glucose grown cell (Figure 15). These data suggests a difference in the global protein expression between *B. cenocepacia* grown in the 2 different sets of growth condition. In addition, this also shows that the study merits a further investigation by utilizing a more sensitive proteomic analysis method, iTRAQ.

7.2 Proteomic Analysis utilizing iTRAQ Showed a Preference in Carbon Source utilization in *B. cenocepacia*

Our proteomic analysis data utilizing iTRAQ found that a number of proteins predicted to be involved in the carbohydrate and amino acid metabolism were regulated differently during growth in SCFM compared to MOPS-glucose (Table 5). This indicates that there is a preference of carbon source utilization in *B. cenocepacia*. This concept is also further supported by the under-expression of some proteins predicted to be involved in the transcriptional regulation. However, some of the metabolic proteins which are under-expressed were predicted to be involved in amino acid biosynthesis. For example BCAM0983 and BCAM2484 which are involved in leucine and valine biosynthetic process respectively. This indicates that instead of synthesizing leucine and valine, *B. cenocepacia* grown in SCFM prefers to metabolize these amino acids from the growth

medium. In addition, this may suggest that it is more efficient for *B. cenocepacia* to transport these amino acids from the medium compared to synthesizing it.

7.3 The Putative Proteins which are involved in Phenylalanine Degradation to PA were not Over-expressed during Growth of *B. cenocepacia* in SCFM

The proteomic analysis failed to identify the putative phenylalanine catabolic proteins (as predicted by the KEGG database, Table 4) as being over-expressed during growth in SCFM compared to MOPS-glucose. In addition, we also did not find any of the PA catabolic protein being over-expressed. This is in contrary to our experimental data utilizing PA reporter system and *paaA* mutant strain (Figure 8, 9, 11, 12) which shows that phenylalanine is utilized and being degraded to PA during growth of *B. cenocepacia* in SCFM.

One of the possible explanations is the phenylalanine and PA catabolic proteins are low abundance proteins. This may result in the inaccurate analysis of the phenylalanine and PA catabolic protein level during growth of *B. cenocepacia* in SCFM. In addition, phenylalanine concentration is low compared to the other carbon sources such as glucose, lactate, lysine, and many other amino acids present in SCFM. This may cause *B. cenocepacia* to produce phenylalanine degradations proteins in lower level compared to the other catabolic proteins. Furthermore, there are other differences in the composition of SCFM compared to MOPS-glucose. This includes the difference in the

concentration of nitrogen, sodium, potassium, calcium, magnesium, chloride, phosphate, sulphur, and iron. These differences may have a role in the expression of these proteins.

Interestingly, we found that BCAM2503, predicted to be involved in phenylalanine and tyrosine catabolic process, as being repressed on SCFM (Table 5b). In addition, this protein is predicted by the KEGG database to be involved in the conversion of phenylpyruvate to 2-hydroxyphenylacetate (Table 4, Figure 10). One of the possible explanation for this finding is BCAM2503 is actually involved in the tyrosine catabolism. Therefore, a possible reason that BCAM2503 was under-expressed may be because tyrosine is not a preferred carbon source for the growth of *B. cenocepacia* in SCFM.

Alternatively, the degradation of phenylpyruvate to 2-hydroxyphenylacetate is not preferred by *B. cenocepacia*. This concept is supported by our result which showed that 2-hydroxyphenylacetate barely support the growth of *B. cenocepacia* (Figure 11). In addition, our result showed that PA supported the growth of *B. cenocepacia* better than 2-hydroxyphenylacetate (Figure 11). Since phenylpyruvate is a degradation intermediate of phenylalanine catabolism, this result suggests that *B. cenocepacia* prefers to degrade this aromatic amino acid to PA, which supports the growth better than 2-hydroxyphenylacetate.

We also find BCAM1280 was under-expressed (Table 5b). This protein is predicted to be a GntR-family transcriptional regulator (Table 5b). In addition, BCAM1280 is also predicted to be involved in phenylalanine catabolism (Table 5b)

(Vuralhan et al. 2003). In *E. coli*, GntR is involved in the repression of the operon which is involved in the gluconate uptake and catabolism (Izu H et al. 1997). If BCAM1280 is a transcriptional repressor, then the result supports the fact that phenylalanine is degraded to PA during growth in SCFM (Figure 8 and 9).

7.4 The Virulence Factors are Expressed by *B. cenocepacia* during growth in SCFM

The proteomic analysis also showed that virulence factors were over-expressed by *B. cenocepacia* grown in SCFM compared to the one grown in MOPS-glucose. The examples are the efflux transporter proteins (BCAL1676 and BCAM0932) and a flaggelin (BCAL0114) (Table 5 and 6). We also found a protein which is annotated as a putative invasion protein (BCAL1367) (Table 5). A BLAST search using amino acid sequence indicated that this protein is annotated as lytic transglycosylase in other *Burkholderia* species. This protein is known to be involved in the modification of bacterial peptidoglycan, including the creation of flagella insertion sites and pores for the secretion system (Scheurwater et al. 2008).

A possible explanation for the over-expression of the virulence factors during the growth of *B. cenocepacia* in SCFM compared to MOPS-glucose maybe due to the difference in nutritional composition. For example SCFM contain a variety of amino acids. The presence of amino acids, especially aromatic amino acids has been related to virulence in *P. aeruginosa* through an increase in the PQS production (Palmer et al.

2007). In addition, high concentration of iron in the CF sputum was shown to stimulate the biofilm formation in *B. cenocepacia* (Berlutti et al. 2005).

In addition, the proteomic analysis also showed a number of LysR-type transcriptional regulators being expressed differently during growth in SCFM compared to MOPS-glucose. LysR-type transcriptional regulators have been shown to be related to the virulence in *B. cenocepacia*. A mutation in a LysR-type regulator was shown to transform *B. cenocepacia* into the shiny variant (Bernier et al. 2007). In addition, this shiny variant is avirulent in the alfalfa seedling infection model and has reduced biofilm formation (Bernier et al. 2007). However, the relationship between virulence and the LysR-type regulators found in our proteomic analysis is currently unknown.

Interestingly, we found a multidrug efflux transporter protein (BCAM2550) was under-expressed during growth of *B. cenocepacia* in SCFM. This may indicate that this protein is only induced by the presence of a specific antimicrobial. Since we did not add any antimicrobials to our media, this explains why BCAM2550 is under-expressed during growth in SCFM.

7.5 The Hypothethical Proteins are Expressed by *B. cenocepacia*

Our data from proteomic analysis also found a number of hypothetical proteins being expressed differently during growth of *B. cenocepacia* in the 2 set of different conditions (SCFM and MOPS-glucose) (Table 5, 6, and 7). A BLAST search was done

using the amino acid sequence of these hypothetical proteins. The BLAST using BCAL3350, BCAM2005, BCAL0938a, BCAM1166, and BCAS0098 amino acid sequences showed that the homologues of these proteins were also annotated as the protein with an unknown function. BCAL0305 was annotated to be involved in the toluene tolerance in other species of *Burkholderia* which indicates the possible involvement of this protein in the defense mechanism of *B. cenocepacia*. *Burkholderia ambifaria* MC 40-6 homologues of BCAM0148 and BCAL1359 were annotated to be the type VI secretion system proteins. BCAM1890 homologues are annotated as aldehyde dehydrogenase and aspartate racemase in *B. cenocepacia* AU1054 and *B. ambifaria* MC40-6 respectively. This indicates that BCAM1890 maybe involved in the nutrient metabolism. On the other hand, BCAL1561 homologues in *B. ambifaria* MC40-6 was annotated as a phage tail collar protein. Currently, there is no experimental characterization of these hypothethical proteins. Therefore, role of these proteins in *B. cenocepacia* is currently unknown.

7.6 The Proteins which are Expressed only during Growth in One of the Growth Conditions

The proteomic analysis also showed the presence of two proteins which are produced only during growth in SCFM (Table 8). These proteins are BCAM1773 and BCAM2754. In addition, we also found one protein, BCAM0221 which was only produced during *B. cenocepacia* growth in MOPS-glucose. This indicates that the regulation for the production of these proteins maybe related to the nutritional

components of the growth medium. For example, BCAM1773 and BCAM2754 were produced only during growth of *B. cenocepacia* in SCFM. This is maybe due to the presence of a SCFM component which is not available in the MOPS-glucose. On the contratry, BCAM0221 production in the minimal medium, MOPS-glucose maybe due to a response mechanism in *B. cenocepacia* to cope with the limitation of some nutrients.

7.7 Ribosomal Proteins are Over-expressed during Growth of *B. cenocepacia* in SCFM compared to MOPS-glucose

Our result showed the over-expression of ribosomal proteins during growth of *B. cenocepacia* in SCFM compared to MOPS-glucose. This maybe caused by the fact that SCFM contains more variety of nutrition. For example, in addition to glucose and lactate, SCFM also contain nineteen different amino acids (Table 2). Therefore, the over-expression of ribosomal proteins might be due to production of different metabolic enzymes by *B. cenocepacia* during growth in SCFM.

8-CONCLUSIONS

In this study, we have used a PA-sensitive reporter system strain of *B. cenocepacia* to investigate whether the aromatic amino acids presence in SCFM are degraded to PA. We found that only phenylalanine but not tyrosine nor tryptophan was able to induce the reporter strain. To investigate this further, a wild type *B. cenocepacia* PA catabolism mutant strain was grown in minimal media containing phenylalanine and its degradation intermediates as the sole carbon source. Phenylalanine and its intermediate molecules, except for phenylacetaldehyde were able to support the growth of the wild type but not the mutant strain. This indicates that in *B. cenocepacia*, phenylalanine is degraded only through PA catabolic pathway. The reason why phenylacetaldehyde was not able to support the growth of *B. cenocepacia* was probably because either it is a toxic intermediate of phenylalanine catabolism or due to inability of the bacteria to uptake the compound when it is supplied extracellularly. Furthermore, both wild type and mutant strains were able to grow to a lesser magnitude when supplied with 2-hydroxyphenylacetate as the sole carbon source. This might be because 2-hydroxyphenylacetate is not degraded to PA but using an alternative catabolic pathway instead. In order to study the phenylalanine degradation to PA further, PA-sensitive reporter strain was grown in minimal media containing glycerol as the carbon source containing different phenylalanine degradation intermediate as the inducer for the reporter system. The result showed that all of the phenylalanine degradation intermediates used in the experiment was able to induce the reporter system. This

indicates phenylalanine is degraded to PA through several different steps in *B. cenocepacia*.

We also used different proteomic approaches to study the global protein expression in *B. cenocepacia* K56-2 grown in amino acid-rich SCFM compared to amino acid-lacking MOPS-glucose. The result of a preliminary analysis utilizing the traditional 2-DE showed the difference in the global protein expression between the cells grown in the two different conditions. In order to study this in more detail, we utilized a more sensitive mass spectrometry based approach, iTRAQ. iTRAQ analysis showed a difference in the global protein expression between *B. cenocepacia* grown in the two different conditions. The proteins involved in amino acid and carbohydrates metabolism were differentially regulated in the two different conditions indicating a preference in the carbon source used when the cells are grown in SCFM. In addition, we also see the ribosomal proteins are being differentially regulated. We also found two proteins which are produced only during growth of *B. cenocepacia* in SCFM and one protein which is only produced during growth in MOPS-glucose. These results may be triggered by the difference in the nutritional composition of SCFM compared to MOPS-glucose. We also found a number of hypothethical proteins being expressed by *B. cenocepacia*, Interestingly, we found flagellin, a putative invasion protein, and a membrane efflux protein which are involved in the virulence of *B. cenocepacia* are over-expressed during growth in SCFM. This suggests the presence of the SCFM components which can trigger the virulence factors production in *B. cenocepacia*. Surprisingly, despite finding differences in PA-sensitive transcriptional reporter system, we did not find the proteins

involved in phenylalanine nor phenylacetate degradation in this study. This might be because these proteins are low abundance proteins.

9-FUTURE DIRECTIONS

Our results showed that *B. cenocepacia* degrades phenylalanine only through PA catabolic pathway. In addition, we also showed the presence of several putative enzymatic steps for phenylalanine degradation to PA. We also able to obtained the putative genes which maybe involved in each of these enzymatic steps from the KEGG database. It will be interesting to see whether these putative genes are induced by the presence of phenylalanine. One possible approach to confirm this is with real time reverse transcriptase-PCR (real time RT-PCR). In order to do this, RNA can be extracted from *B. cenocepacia* grown in MOPS-glucose and MOPS-phenylalanine for analysis utilizing rela time RT-PCR. In addition, to see whether these genes are upregulated during growth in SCFM, a real-time RT-PCR experiment using RNA extracts from *B. cenocepacia* grown in SCFM and MOPS-glucose can be done. Alternatively, proteomic analysis of the cells grown in MOPS-glucose and MOPS-phenylalanine utilizing iTRAQ can be performed. Since, we suspect that these proteins might be the low abundance proteins, an iTRAQ analysis specific for these proteins can be done using the cells grown in SCFM compared to MOPS-glucose.

We also find a number of virulence factors being over-expressed dutring growth of *B. cenocepacia* compared to MOPS-glucose. It will be interesting to see what components present in SCFM which induces the expression of these virulence factors. This can be done by proteomic analysis using protein samples of the cells grown MOPS-glucose and MOPS-minimal medium containing different amino acids as the sole carbon

source. Alternatively, proteomics comparison can be done between the protein samples obtained from the cells grown in traditional MOPS-glucose and MOPS-glucose containing higher concentration of different inorganic compounts. For example, the comparison can be done between MOPS-glucose and MOPS-glucose with iron concentration similar to what is found in SCFM.

We also found the hypothetical proteins being expressed in *B. cenocepacia*. The characterization of these proteins will help to understand *B. cenocepacia* better. In addition, a new antimicrobial target may be found by characterizing these hypothetical proteins. The characterization can be done through the protein purification and X-ray crystallography in order to obtain the structural information on proteins. The structural information then can be compared with the other existing protein structures in order to find similarities. This can be used to build a hypothesis on what role that the protein has in the cell function. Then, this hypothesis can be followed by creating a mutant strain and analyzing the phenotype produced.

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