PERSISTENCE OF *BURKHOLDERIA PSEUDOMALLEI*, *B. MULTIVORANS* AND *B. CENOCEPACIA*: DIFFERENTIAL ANALYSIS BY PROTEOMICS AND ACTIVATION OF PHOSPHOINOSITIDE-3 KINASE

by

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Abstract

Persistent infection in mammalian hosts is one intriguing aspect of the study of human diseases. In order to gain insight into the establishment of persistence, this thesis will evaluate the bacterial and host components associated with persistence of two groups within the *Burkholderia* genus: *Burkholderia pseudomallei* and *Burkholderia cepacia* complex. *Burkholderia pseudomallei* causes septicemic melioidosis with a high rate of relapse; however microbial determinants of relapse are unknown. Proteins were analyzed from sequential *B. pseudomallei* isolates from primary and relapsing melioidosis. Analysis by iTRAQ revealed that factors required for nitric oxide detoxification (HmpA) and necessary for anaerobic growth (ArcA, ArcC, and ArcB) were highly expressed in the relapse isolate. 2D-PAGE revealed up-regulation of a putative hcp-1 protein in the primary isolate, and flagellin and HSP20/alpha crystalline in the relapse isolate. These observations provide targets for further analysis of latency and virulence of *B. pseudomallei* in patients with relapsing melioidosis. The role of the host phosphoinositide3-kinase/Akt (PI3K/Akt) signaling pathway in the interaction of *Burkholderia cepacia* complex (Bcc) species with human immortalized macrophages was explored. *B. cenocepacia* and *B. multivorans* are the most clinically important and prevalent of the Bcc members and are serious opportunistic pathogens in cystic fibrosis (CF) patients. This study evaluated whether these pathogens can differentially induce PI3K/Akt signaling pathway, which is important in cell survival. *B. cenocepacia* activated PI3K/Akt in multiple cell types and this activation proceeded faster after exposure to *B. multivorans* than to *B. cenocepacia* in macrophages. Both species promoted cell survival by preventing caspase 9 cleavage, and bacteria internalization was necessary for this process. Unlike, *B. cenocepacia*, *B. multivorans* induced PI3K/Akt

mediated anti-apoptotic effect, faster NF-κB activation, and IκBα phosphorylation that was PIK3/Akt dependent. Macrophages exposed to *B. cenocepacia* induced greater pro-inflammatory cytokines release compared to *B. multivorans.* Internalization into macrophages of both species was PI3K regulated. These findings provide novel insights into the pathogenic mechanism underlying *Bcc* infection in CF. Together; this thesis provides data for the identification of mechanisms leading to the persistence of these bacteria in melioidosis and CF and may inform the design of therapeutic approaches for treatment in these diseases.

Preface

Ethics approval was obtained for collection of blood samples from UBC C&W Research Ethics Board (ethics certificate # H04-70193) and Biohazard Approval certificate UBC (protocol # B11-0013)

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A version of the study presented in Chapter 3 of this work will be submitted for publication. Billie Velapatino designed the study with assistance from David P. Speert. The environmental strain *B. cenocepacia* FC1666 used to produce Figure 3. 5.1 was a generous gift from Dr. John Lipuma (USA). LPS used in this study was kindly provided by Dr. Robert K. Ernst (University of Washington). Billie Velapatino designed and performed all experiments, analyzed data and wrote the chapter with assistance from David P. Speert.

Billie Velapatiño and David P. Speert. 2012. Apoptosis in macrophages is attenuated by *Burkholderia multivorans,* but not *Burkholderia cenocepacia,* via activation of the PI3K/Akt signaling pathway.

Preliminary data are presented in Chapter 4 and a version of this study will be submitted for publication. Billie Velapatino designed the study with assistance from David P. Speert. George Hall assisted with the preparation of buffers, culture media and plating of bacteria. Billie Velapatino performed all experiments, analyzed data and wrote the chapter with assistance from David P. Speert.

Billie Velapatiño, George Hall and David P. Speert. The role of the PI3K/Akt signaling pathway in uptake of *Burkholderia cenocepacia* and *Burkholderia multivorans* into macrophages.

Table of Contents

Abstract ..... **ii**

Preface ..... **iv**

Table of Contents ..... **vi**

List of Tables ..... **xi**

List of Figures ..... **xii**

List Abbreviations ..... **xiv**

Acknowledgements ..... **xvi**

Dedication ..... **xvii**

Chapter 1: Introduction **1**

1.1 The *Burholderia* genus 2

1.2 *Burholderia pseudomallei* and melioidosis 4

1.3 Putative virulence factor of *B. pseudomallei* 5

1.3.1 Quorum sensing 5

1.3.2 Capsular polysacharide and Lipopolysacharide (LPS) 6

1.3.3 Flagella and pilli 7

1.3.4 Type three secretion system (T3SS) 7

1.3.5 Type six secretion system (T6SS) 8

1.3.6 Bacterial morphotype switching 8

1.4 Intracellular life style of *B. pseudomallei* 9

1.5 Latency 11

1.6 *Burkholderia cepacia* complex and Cystic Fibrosis 12

1.7 Putative virulence factors of *B. cenocepacia* 14

1.7.1 Quorum sensing 15

1.7.2 Iron uptake 15

1.7.3 Surface polysaccharides 15

1.7.4 Motility and adherence 16

1.8 Intracellular life style of *Burkholderia cenocepacia* 16

1.9 *Burkholderia cepacia* complex and host signaling pathways 17

1.9.1 The Phosphoinositide 3-kinase (PI3K) 18

1.9.2 Family members of PI3K 19

1.9.3 PI3K/Akt signaling 20

1.10 Thesis objectives 23

Chapter 2: Analysis of potential mechanisms of latency in *Burkholderia pseudomallei:* the causative agent of melioidosis 25

2.1 Introduction 25

2.2 Hypothesis 27

2.3 Aim 27

2.4 Experimental procedures 28

2.4.1 Bacterial isolates 28

2.4.2 Pulse field electrophoresis gel 28

2.4.3 Total protein extractions 28

2.4.4 Two dimensional gel electrophoresis (2D-PAGE) analysis 29

2.4.5 Isotope tagging for relative and ansolute quantitation (iTRAQ) analysis 30

2.4.6 Western blotting 32

2.5 Results 33

2.5.1 Genetic profiles of the sequential *B. pseudomallei* isolates from primary and relapsing melioidosis from patient #1 33

2.5.2 Analysis of protein changes in *B. pseudomallei* proteome by 2D gel electrophoresis 34

2.5.3 Analysis of protein changes in *B. pseudomallei* proteome by iTRAQ 40

2.6 Discussion 45

Chapter 3: The activation of the PI3K/Akt signaling pathway by *Burkholderia cenocepacia* and *Burkholderia multivorans* in macrophages 51

3.1 Introduction 51

3.2 Hypothesis 54

3.3 Aim 54

3.4 Experimental procedures 55

3.4.1 Growth condition of bacteria and cells 55

3.4.2 Isolation and culture of condition of bacteria and cells 56

3.4.3 Cell stimulation, lysis and Western blotting 57

3.4.4 Apoptotic resistance analysis and flow cytometry 58

3.4.5 DNA fragmentation analysis by agarose electrophoresis 59

3.4.6 NF-κB/AP-1 activation–SEAP Reporter assay 59

3.4.7 Cytokine production 60

3.4.8 Statistical analysis 60

3.5 Results 61

3.5.1 Activation of PI3K/Akt signaling by *B. cenocepacia* and *B. multivorans* 61

3.5.2 Contribution of PI3K signaling to cell survival in *B. cenocepacia* and *B. multivorans*-challenged macrophages 65
3.5.3 *B. multivorans* suppresses apoptosis in macrophages via activation of PI3K/Aktsignaling pathway 67
3.5.4 PI3K/Akt signaling pathway promotes differential NF-κB/AP-1 activation in *B. cenocepacia* and *B. multivorans*–challenged macrophages 75
3.5.5 *B. cenocepacia* and *B. multivorans*–stimulated NF-κB/AP-1 activity is dependent on PI3K/Akt 80
3.5.6 PI3K/Akt promotes the release of cytokines in response to *B. cenocepacia* and *B. multivorans* in macrophages 82
3.6 Discussion 85
Chapter 4: The phosphoinositol-3-kinase–Protein Kinase B/Akt pathway is involved in *B. cenocepacia* and *B. multivorans* internalization into macrophages……..……92
4.1 Introduction 92
4.2 Hypothesis 95
4.3 Aim 95
4.4 Experimental procedures 96
4.4.1 Growth conditions of bacteria and cells 96
4.4.2 Growth curves analysis 96
4.4.3 Bcc internalization assay 97
4.4.4 Immunofluorescence staining and microscopy 97
4.4.5 Statistical analysis 98
4.5 Results 99

4.5.1 Growth curves for *B. cenocepacia* and *B. multivorans* 99

4.5.2 Effect of the inhibition of PI3K in the internalization of Bcc..... 100

4.5.3 *B. multivorans* internalization detected by confocal microscopy 102

4.6 Discussion 103

Chapter 5: Conclusions and future directions 106

5.1 Proteome analysis of persistent *B. pseudomallei* 106

5.2 The involvement of phosphoinositide 3-kinase/Akt signaling pathway in the interaction of Bcc with macrophages..... 108

5.3 Future directions 109

Bibliography 113

List of Tables

Table 2.1: Identification of differentially expressed *Burkholderia pseudomallei* proteins of the initial isolate (morphotype I) and relapse isolates (morphotypes I and III) patient #1 using 2D-PAGE, Mascot search engine and data from MALDI-TOF mass spectrometry……..…37

Table 2.2: Differentially expressed *Burkholderia pseudomallei* proteins with the highest fold change in expression in sequential isolates, Morphotype I (2644aI), Morphotype I (2644cI) and Morphotype III (2644cIII), identified using iTRAQ ….…….……...……...…..…..…41

List of Figures

Figure 1.1: Phylogenetic tree based on 16S RNA sequences of the Burkholderia genus 3

Figure 1.2: Activation and regulation of Akt 21

Figure 1.3: Cellular functions of Akt 22

Figure 2.1: Pulse Field Gel Electrophoresis profiles of *SpeI* restricted genomic DNA from the consecutive *B. pseudomallei* isolates from patient #1 33

Figure 2.2: 2D gel electrophoresis analysis of the consecutive *B. pseudomallei* isolates from patient #1 35

Figure 2.3: Magnification of areas of silver-stained 2D gels 36

Figure 2.4: Immunoblot of the detection of flagellin protein of the consecutive *B. pseudomallei* isolates from patient #1 38

Figure 2.5: 2D gel electrophoresis analysis of the consecutive *B. pseudomallei* isolates from patient #2 39

Figure 3.1: Akt phosphorylation in different cell types after challenge with *B. cenocepacia* or *B. multivorans* 63

Figure 3.2: Contribution of the PI3K/Akt signaling on THP-1 macrophage survival after challenge with *B. cenocepacia* or *B. multivorans* for 24h 66

Figure 3.3: Promotion of cell survival due to the activation of PI3/Akt signaling by *B. multivorans* challenge of macrophages 69

Figure 3.4: Effect of bacteria load on the *B. multivorans*-anti-apoptotic activity in THP-1 derived macrophages 71

Figure 3.5: Role of bacterial internalization on the *B. cenocepacia* and *B. multivorans*-induced anti-apoptotic activity in THP-1 derived macrophages 72

Figure 3.6: Caspase 9 inactivation in THP-1 derived macrophages in the presence of *B. cenocepacia* or *B. multivorans* 74

Figure 3.7: Differential NF-κB/AP-1 activation in *B. cenocepacia* and *B. multivorans*–challenged macrophages 77

Figure 3.8: NF-κB/AP-1 activation in macrophages exposed to three *B. cenocepacia* and three *B. multivorans* isolates 79

Figure 3.9: The NF-κB/AP-1 activation induced by *B. multivorans* is decreased by PI3K and Akt inhibitors 81

Figure 3.10: PI3K is required for cytokine production by macrophages challenged with *B. cenocepacia* or *B. multivorans* 83

Figure 4.1: Standard growth curves of *B. cenocepacia* and *B. multivorans* 99

Figure 4.2: Effect of the pharmacological inhibitor LY294002 of PI3K on Bcc internalization 101

Figure 4.3: Colocalization of *B. multivorans* with late lysosomal maker 102

Abbreviations

2D-PAGE	two-dimensional gel electrophoresis
Akt	serine/threonine kinase (also known as Protein Kinase B; PKB)
AP-1	activator protein
ATPase	adenosine triphosphate
Bcc	*Burkholderia cepacia* complex
Bsa	Burkholderia secretion apparatus
CF	cystic fibrosis
CFTR	cystic fibrosis transmembrane conductance regulator
CytD	cytochalasin D
ΔF508	deletion of phenylalanine at codon 508
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
ECL	enhanced chemiluminoscence
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme linked immunosorbent assay
GTPases	guanosine triphosphates enzimes hydrolase enzymes
IB3-1	CF cell line
Iκ-Bα	inhibitor κ-Bα
iTRAQ	isotope tagging for relative and absolute quantitation
IL-1β	interleukine-1 beta
IL-10	interleukine-10
JNK	janus kinase

LAMP-1	lysosomal-associated protein-1
LB	lurea bertani
LPS	lipopolysaccharide
LY	LY294002, inhibitor of PI3K
MAP kinase	mitogen activated protein kinase
M-CSF	macrophage colony stimulating factor
MOI	multiplicity of infection
MNGC	multinucleated giant cell
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NPFs	nucleation promotion factors
PFGE	pulsed field gel electrophoresis
PMA	phorbol-12-myristate 13-acetate
OD	optical density
SDS-PAGE	SDS-polyacrylamide gel
SEAP	secreted embryonic alkaline phosphatase
Stsp	staurosporine
THP-1	human monocytic THP-1 cell line
THP1X-Blue	reporter cell line for NF-κB
TLR	toll-like receptor
TNFα	tumor necrosis factor-alpha
T3SS	type three secretion system
T6SS	type six secretion system
WM	wortmannin

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Dedication

Para mi familia: Seferino, Juana,

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Chapter 1: Introduction

The establishment of a persistent infection in mammalian hosts is one intriguing aspect of the study of human diseases. In some diseases, the host might not clear the pathogen, and the initial acute infection is just a prelude to a long-lasting association with the host (Rhen et al., 2003). This association can be a balanced infection, where the host is almost unaffected, as is the case with some viruses (Rhen et al., 2003; Sinclair, 2008), and others might have the risk of reactivation into an active disease such as in the case of *Mycobacterium tuberculosis* (Gideon and Flynn, 2011). Numerous complications emerge from a persistent bacterial infection, such as an increase in transmission of the pathogen, spread of the disease and treatment failure because of the intrinsic or acquired resistance to antibiotics (Rhen et al., 2003). This is the case of some of the members of the *Burkholderia* genus; they cause infections, persist inside the host and are hard to eradicate. Therefore, gaining a clearer understanding of the basis for establishment of persistence of bacterial infection is vital to the treatment of certain chronic diseases. In order to gain insight into the establishment of persistence, this thesis will describe investigations of the bacterial and host components associated with persistence of two groups within the *Burkholderia* genus: *Burkholderia pseudomallei* and *Burkholderia cepacia* complex. Investigations of the microbial determinants of persistence and the effect of the bacteria on macrophages with emphasis on the role of a signaling pathway will be described. This initial chapter will summarize the knowledge of these bacteria and their role in human pathogenesis and finally the objectives will be presented.

1.1 The Burkholderia genus

Burkholderia was first proposed as a genus in 1992 by Yabuuchi et al., 1992. It was named after the American phytopathologist Walter Burkholder, who in 1950, described *Burkholderia* as the causative agent of sour skin in onion, it was also recognized as a plant pathogen and an opportunistic human pathogen occurring in soil and water as natural reservoirs (Mahenthiralingam and Vandamme, 2005). The genus *Burkholderia* comprises more than 62 species isolated from a wide range of niches, and it is divided in two main clusters as suggested by phylogenetical analysis of 16S RNA sequences, DNA-DNA homology values, cellular lipids, fatty acid composition and phenotypic characteristic (Drevinek and Mahenthiralingam, 2010; Suarez-Moreno et al., 2011; Yabuuchi et al., 1992). The first cluster includes human, animal and phytotrophic organisms, and most studies have focused on the pathogenic species of this group due to their clinical importance. The most pathogenic members are *B. pseudomallei* and *B. mallei* (which causes melioidosis and glanders respectively) and opportunistic pathogens (the 17 defined species of the *Burkholderia cepacia* complex), which cause disease in cystic fibrosis (CF) and chronic granulomatous disease (CGD) (Drevinek and Mahenthiralingam, 2010). The second cluster, with more than 30 non-pathogenic species, includes bacteria associated with plants or with the environment, and are considered potentially beneficial to the host (Suarez-Moreno et al., 2011), (Figure 1.1).

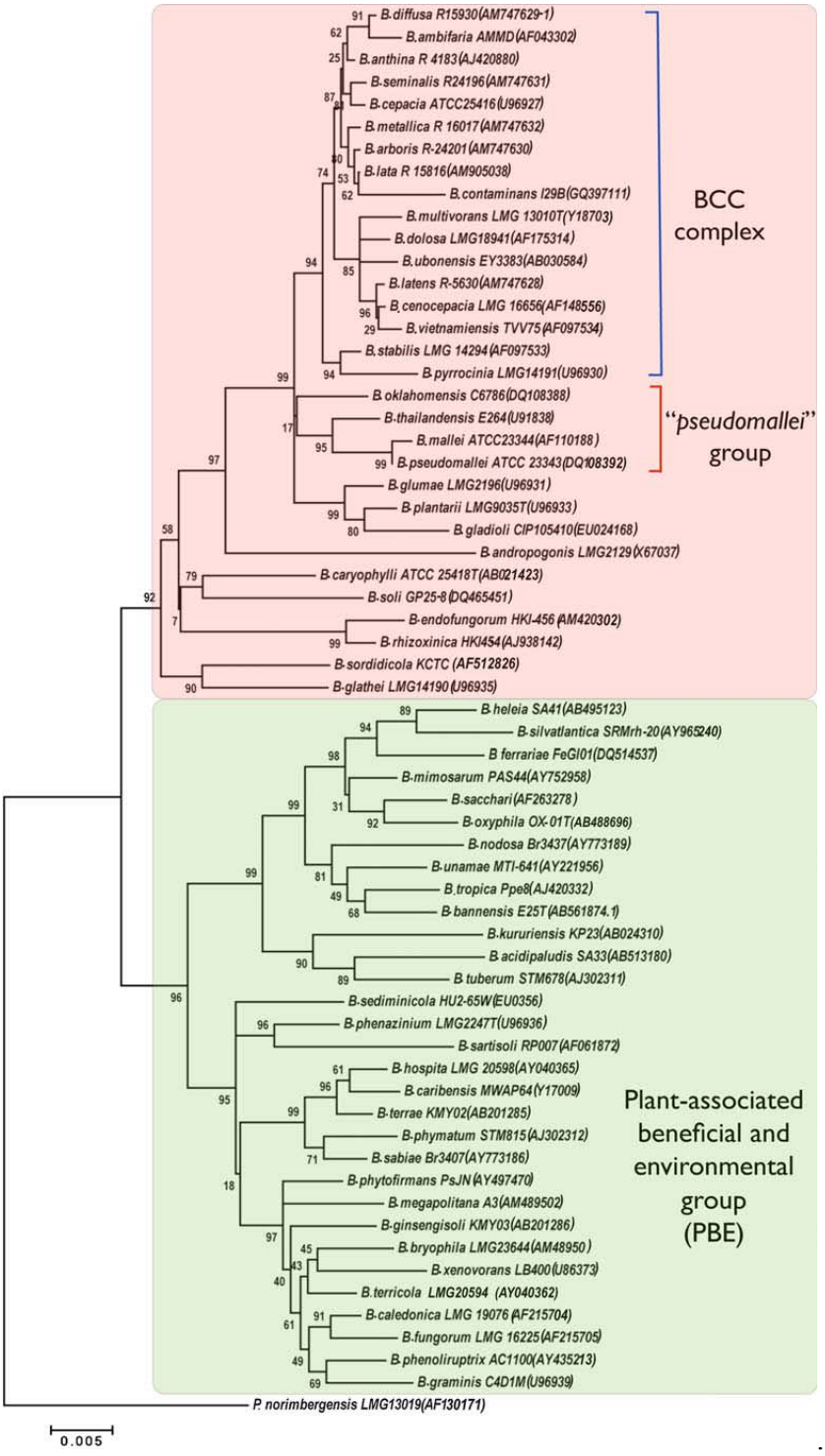


Figure 1.1: Phylogenetic tree based on 16S RNA sequences of the Burkholderia genus. In pink is the cluster of the pathogenic Burkholderia species and in green is the cluster of the plant-associated beneficial and environmental (PBE). From Suarez-Moreno et al., Microbial Ecology, 2011,by permission.

1.2 *Burkholderia pseudomallei* and melioidosis

Burkholderia pseudomallei is an aerobic, motile, non-spore forming, Gram-negative, facultative intracellular bacterium and is the causative agent of melioidosis (Cheng and Currie, 2005). Melioidosis; from the Greek *melis* meaning "a distemper of asses" with the suffixes -oid meaning "similar to" and -osis meaning "a condition", that is: a condition similar to glanders, is a serious disease common in Southeast Asia and Northern Australia and is responsible for 20% of the all community-acquired septicemias and 40% of sepsis-related mortality in northeast Thailand (White, 2003). Melioidosis can present with an array of clinical signs and symptoms; septic shock is the most severe clinical manifestation and is often associated with bacterial dissemination to distant sites as the lungs, liver and spleen. The lungs are the most commonly affected organ in adults, where infection can be a localized or disseminated with the formation of abscesses. Chronic lung disease can also occur and can be difficult to distinguish from pulmonary tuberculosis (Cheng and Currie, 2005; White, 2003). The incubation period is 9 days on average (Currie et al., 2000) and often affects individuals with one or more alterations in immune response such as occurs in diabetes mellitus or renal failure in adults. Melioidosis is seasonal in the tropics (such as in Thailand), where most of the cases occur during the rainy season and natural infection is thought to be transmitted primarily via percutaneous inoculation following exposure to muddy soils or surface water as in rice paddies (Currie, 2003). It is thought that under certain environmental conditions, such as tropical storms, cyclones, and typhoons, inhalation is the main mode of infection (Currie et al., 2008). Access to enhanced diagnostic laboratory facilities have helped to identify cases of melioidosis in places other than the endemic zones such as Indonesia, the Indian subcontinent, Southern China, Hong Kong, Brunei, Laos, Cambodia

and Taiwan, and cases have also occurred in the US (probably imported), the Caribbean and in Brazil (Currie et al., 2008).

Melioidosis is difficult to treat due to intrinsic resistance of *B. pseudomallei* to most antibiotics (Wiersinga et al., 2006). Despite long-term treatment of 20 weeks with intravenous and oral antimicrobials (White, 2003), recurrence is common and 75% of cases are due to bacteria persisting from the original infection occurring within a year; the remainder of relapse cases are due to infection with a different isolate (Maharjan et al., 2005). *B. pseudomallei* is considered as a bioterrorism threat and as a potential agent for biological weapons since it can cause disease following inhalation and it is difficult to treat with antibiotics; it is now on the list of category B agents of the US Centers for Disease Control and Prevention (Rotz et al., 2002; Wiersinga et al., 2006). At present there is no available human vaccine that protects against infection with *B. pseudomallei* (Patel et al., 2011), and with the current limitations of antibiotic treatment, the development of new therapeutic strategies for early diagnosis is crucial.

1.3 Putative virulence factor of *B. pseudomallei*

1.3.1 Quorum sensing

Quorum sensing is a form of bacterial communication that regulates the expression of multiple genes and the *B. pseudomallei* genome possess many signaling molecules in its communication system including *N*-decanoyl-homoserine-lactone and *N*-(3-oxotetra-decanoyl)-L-homoserinelactone (Ulrich et al., 2004). *B. pseudomallei* also expresses LuxI and LuxR cluster proteins that are responsible for synthesis and regulation respectively of quorum sensing molecules, and these clusters are involved in the pathogenesis in Syrian

hamsters after bacterial challenge (Ulrich et al., 2004). Expression of a multidrug efflux pump, BpeAB-OprB, can be regulated by *N*-decanoyl-homoserine-lactone and *N*-octanoyl-homoserine lactone, and this pump has been involved in cell invasion and cytotoxicity in non-phagocytic and phagocytic cells (Chan and Chua, 2005)

1.3.2 Capsular polysaccharide and Lipopolysaccharide (LPS)

B. pseudomallei produces an extracellular capsular polysaccharide: -3)-2-O-acetyl-6-deoxy-ß-D-manno-heptopyranose-(1- that is required for bacterial virulence in animal models (Reckseidler et al., 2001). The capsular polysaccharide is thought to act as a physical barrier that can block the access of opsonized bacteria to complement receptors thereby decreasing ligations and ingestion by phagocytes (Reckseidler et al., 2001; Steinmetz et al., 1995). *B. pseudomallei* LPS (formally termed type II O-antigen polysaccharide) exhibits less pyrogenic activity than other enterobacterial LPS in animal models and has a lower capacity to mediate activation of macrophages *in vitro* compared to *Escherichia coli* LPS (Matsuura et al., 1996; Utaisincharoen et al., 2000). It was also demonstrated that the LPS of *B. pseudomallei* signals through the pattern-recognition receptor Toll-like receptor (TLR) 2 and not through TLR4 (Wiersinga et al., 2007). Although both TLR2 and TLR4 contribute to cellular responsiveness to *B. pseudomallei in vitro* (West et al., 2008; Wiersinga et al., 2007), TLR2 detects *B. pseudomallei* LPS, and only TLR2 impacts on the host immune response *in vivo* (Wiersinga et al., 2007).

1.3.3 Flagella and pilli

B. pseudomallei is flagellated and motile, and flagella play a role in pathogenesis of a murine model, in which fewer bacteria invade lungs and spleen of BALB/c mice after infection with an aflagellate mutant (Chua et al., 2003; Essex-Lopresti et al., 2005). The type IV pilus is an important determinant for bacterial adherence, and *B. pseudomallei* contains a putative pilus structural protein (PilA), which has been shown to participate in virulence and in adherence to epithelial cells (Essex-Lopresti et al., 2005).

1.3.4 Type three secretion system (T3SS)

The type three secretion system (T3SS) is an apparatus that functions like a molecular syringe that spans the bacterial inner and outer membranes, enabling the bacteria to secret and translocate effector molecules into the cytosol when it is triggered by close contact with host cells. The *B. pseudomallei* K96243 genome contains three T3SS gene clusters (Holden et al., 2004), and T3SS-3 has been shown to be required for full virulence in a hamster model of infection whereas T3SS-1 or T3SS-2 appear not to be important for virulence (Warawa and Woods, 2005). The T3SS-3 locus in *B. pseudomallei*, designated *Bsa* (*Burkholderia* secretion apparatus), contains genes that encode proteins predicted to be required for the synthesis of the structural components (BsaQ, BsaU, and BsaZ), translocator (BipD), and effector proteins (BopA and BopE); it is suggested that a functional T3SS apparatus is required for invasion, escape from the endocytic vacuoles, intracellular spread and full virulence (Stevens et al., 2002). A recent study demonstrated the role of T3SS-1 ATPase, encoded by *bpscN*, in bacterial survival and replication in a respiratory melioidosis mouse

model perhaps by subverting normal phagosomal maturation and by slowing the recruitment of LC3, an autophagy marker (D'Cruze et al., 2011).

1.3.5 Type six secretion system (T6SS)

Type six secretion system (T6SS) plays an important role in virulence in different pathogens where they function to inject effector proteins into host cells (Pukatzki et al., 2007). *B. pseudomallei* possesses six copies of T6SSs and the T6SS-1 was first identified in infected macrophages *in vivo* expression technology study (Shalom et al., 2007). In this study, the expression of three genes within one of these T6SS clusters were induced following microbial invasion of RAW macrophages, and the encoded proteins were found to be important in cell invasion and intracellular survival (Shalom et al., 2007). The T6SS is also involved in bacterial actin-based motility and multinucleated giant cell (MNGC) formation; a recent study showed that *hcp1* (a secreted protein) deletion mutant from *B. pseudomallei* T6SS-1 demonstrated an attenuated virulence phenotype in hamsters, reduced intracellular growth and cytotoxicity, as well as lack of formation of MNGC *in vitro* in RAW 264.7 cells (Burtnick et al., 2011).

1.3.6 Bacterial morphotype switching

B. pseudomallei colonial morphology can vary within a culture as well as in culture from the same patient over time (Wiersinga et al., 2006). Seven colony morphotypes (I-VII) have been isolated from cultures in Ashdown's agar and their biological relevance was studied by Chantratita, et al., (2007) and Tandhavanant, et al., (2010). Morphotype I is the classical type described in the literature and it is present in 75% of the clinical isolates. This

morphotype gives rise to other morphotypes in a reversible manner, predominantly II and III, under *in vitro* conditions such as starvation, iron limitation and growth at 42 °C. Type II, III and VI differ significantly from type I in biofilm formation, elastase, protease, lipase activity, and swarming and swimming phenotypes. When examining isogenic types II and III, derived from type I *in vivo*, type II develops into more adapted fitness for persistence in a mouse model as well as a significant increase in intracellular replication advantage compared to the parental type I in epithelial cells *in vitro*. The isogenic type III established higher replication fitness in mouse macrophages *in vitro* than type I, which was associated with a switch to type II (Chantratita et al., 2007). In a different model using a human macrophages cell line, different strains from type I had significantly higher rates of replication and higher resistant to H_2O_2 than either type II or type III, but increased susceptibility to antimicrobial peptide LL-37 than type III (Tandhavanant et al., 2010).

1.4 Intracellular life style of *B. pseudomallei*

B. pseudomallei is able to invade many cell types including epithelial cells and can survive intracellularly and replicate for prolonged periods within phagocytic cells, although the precise mechanism of intracellular invasion is not known. The essential steps in its intracellular life style were recently reviewed by (Allwood et al., 2011): the first step includes the adhesion of the bacteria to the cell surface, and in non-phagocytic cells bacteria determinants such as PilA (type IV pili) and two novel adhesins (BoaA and BoaB) are crucial for the uptake in A549 and Hep2 cells. In the internalization step, the *Bsa* T3SS structural proteins BipD (translocator), BsaQ (structural component), and BopE (effector) are required for invasion of non-phagocytic cells. It was noted that BopE is translocated into the

cytoplasm and induces rearrangements in the actin cytoskeleton acting as a guanine nucleotide exchange factor for RhoGTPases that regulates an actin network. A two-component signal transduction system (IrIRS) is thought to regulate the expression of other genes important in the invasion of epithelial cell lines A549, HeLa, and CHO cells, but not macrophages. The *Bsa* T3SS putative effector BopE and BipD protein do not appear to play a role in the invasion of murine macrophages-like cells, J774.2 and RAW 264.7. After internalization, *B. pseudomallei* has the ability to escape from the endocytic vacuole (as early as after 15 min after uptake) into the cytoplasm of non-phagocytic and phagocytic cells by lysing endosomal membranes and therefore avoiding lysosomal degradation. The *Bsa* T3SS effector protein BopA is involved in the escape from phagosomes, in the formation of actin-tails and in the formation of multi-nucleated giant cells (MNGC) in RAW 264.7 cells. BsaZ, another *Bsa* T3SS structural component, participated in the escape from endocytic vesicles after 6 h of internalization in phagocytic cells. In the same way, other components (BsaQ and BsaU) showed a delayed escape phenotype in non-phagocytic cells. Following internalization and escape from endocytic vacuoles, *B. pseudomallei* is able to survive and spread intracellularly by means of using purine, histidine and para-aminobenzoate biosynthetic pathways and by using actin-based motility as a mechanism for cell-to-cell spread. The actin-based motility consists of the polymerization of the eukaryotic cytoskeletal protein actin at the surface of one pole of the bacterium enabling propulsion through the cytoplasm, formation of membrane protrusions into adjacent cells and simultaneous cell-to cell spread. Pathogens exploiting actin-based motility activate the Arp2/3 complex, which is involved in actin filament nucleation and organization. The complex is activated by nucleation promotion factors (NPFs) that initiate its conformational change to generate a new

filament from an existing one, forming a y- branched actin network. Pathogens are able to express NPFs like proteins that activate the Arp2/3 complex and initiate actin polymerization. *B. pseudomallei* uses actin-based motility in a unique way; BimA, an autosecreted protein, is involved in the formation of membrane protrusions, in cell to cell spread and can function as a NPF to induce actin-based motility in infected cells. However, BimA can stimulate actin polymerization in an Arp2/3 independent manner, suggesting that BimA alone might be sufficient for intracellular motility of *B. pseudomallei*. Additionally; hcp1, a secreted protein from the T6SS-1 cluster and a putative secreted protein bpsl1528 are involved in actin-based motility. Finally, cell fusion and the formation of MNGC might be due to different mechanism including participation of hcp-1, a RpoS regulon, effector protein BipB from the *Bsa* T3SS, and putative toxin-encoding genes *bpsl0590* and *bpsl0591*. Bacteria that escape from phagosomes can be subject to autophagy and ubiquitination by the host cell; however *B. pseudomallei* can avoid autophagic removal; the *Bsa* T3SS effector protein BopA has been involved in modulating this host response, but the exact mechanism is not known. A secreted product of the *bpss1512* gene from the T6SS cluster 5 was identified to interfere with the ubiquitination process, possibly by its deubiquitinase activity. The ability of *B. pseudomallei* to induce host cell changes through all these mechanisms might cooperate for optimal replication and cell-to-cell spread.

1.5 Latency

Healthy individuals in endemic zones and patients who recover from *B. pseudomallei* septicemia maintain high levels of antibodies for years, suggesting either continuous exposure to the organism (Vasu et al., 2003; Wiersinga et al., 2006) or sequestration of

bacteria in intracellular or cryptic sites (Gan, 2005). However the site (s) of latency and the mechanism by which *B. pseudomallei* persists remain unclear (Gan, 2005). Recurrent melioidosis is common, occurring in 75% of cases due to relapse rather than reinfection, especially when happening within a year of primary infection (Maharjan et al., 2005). The bacteria can remain latent for prolonged periods until activated by trauma or immunosuppression; there can be a prolonged period between exposure to the causative agent and the clinical manifestation: 62 years is the longest reported incubation period of latency that have been documented in an American Vietnam war veteran (Ngauy et al., 2005). This suggests that *B. pseudomallei* has the ability to enter a dormant state where it can avoid immune surveillance, most probably in an intracellular location (Gan, 2005).

1.6 *Burkholderia cepacia* complex and Cystic Fibrosis

Cystic fibrosis (CF) is the most common inherited disease of Caucasian populations with an incidence of approximately 1 in 2000 and results from a defective chloride channel called the CF transmembrane regulator (CFTR) (Doring and Gulbins, 2009; Mahenthiralingam et al., 2005; Riordan, 2008). The most common CFTR mutation that causes CF is deletion of the phenylalanine residue at position 508 (Δ508), and it is present in one or both alleles in approximately in 90% of CF patients (Riordan, 2008). Impaired CFTR function results in a high NaCl concentration and reduced volume of the airway fluid covering the apical surface of lung epithelia (Doring and Gulbins, 2009). The clinical features of CF include chronic lung infection with progressive deterioration of lung function, pancreatic insufficiency, male infertility, and meconium ileus in the newborn (Lukacs and Verkman, 2011). The median life expectancy for individuals with CF is currently 39 years in US and approximately 10 years longer in Canada, with lung disease the principal cause of

mortality and morbidity (Lukacs and Verkman, 2011). Susceptibility of the lungs of CF patients to respiratory infections appears to result from a combination of factors including: sticky mucus which impairs mucociliary clearance, high NaCl concentrations which reduce bactericidal activity of airway surface fluid, and an increase in the number of epithelial receptors for pathogenic bacteria (Doring and Gulbins, 2009; McKeon et al., 2010). Moreover, the CF lung environment is characterized by increased inflammation, which is exacerbated by bacterial lung infections acquired at an early age (Mahenthiralingam et al., 2008; Mahenthiralingam et al., 2005).

The *Burkholderia cepacia* complex (Bcc) is a group of closely related Gram-negative bacteria and an important virulent group of opportunistic pathogens in CF patients (Mahenthiralingam et al., 2008; Mahenthiralingam et al., 2002; Mahenthiralingam et al., 2005; McKeon et al., 2010). Bcc infections have been associated with three major outcomes in CF patients: asymptomatic carriage, chronic infection, and "cepacia syndrome", which is characterized by a rapid deterioration in lung function and, in some cases, bacteremia and septicemia, resulting in rapid death (Holden et al., 2009; McKeon et al., 2010). Among the multiple species of the Bcc, *Burkholderia cenocepacia* is the most prevalent in CF, causing more than 50% of infections in countries such as USA, Canada, France, Czech Republic, Portugal and Italy (Drevinek and Mahenthiralingam, 2010; Mahenthiralingam et al., 2002; Speert et al., 2002). Furthermore, in the *B. cenocepacia* species, the most transmissible and highly infectious strain is the ET12 (electrophoretic type 12) lineage; which is frequent among infected patients in Canada and the UK (Mahenthiralingam et al., 2002). Lately, *B. multivorans* has become a more common cause of CF infection; together with *B. cenocepacia*

it accounts for approximately 85-97% of all the Bcc infection in CF population (Drevinek and Mahenthiralingam, 2010) and it is the leading incident Bcc species infecting CF patients in the US (J. LiPuma, personal communication). *B. cenocepacia* appears to be more virulent and causes a much more serious infection than *B. multivorans*; however the mechanism behind this difference in virulence remains undetermined (Govan et al., 1996). Previous studies in our laboratory have demonstrated that *B. multivorans* can persist benignly in macrophages or epithelial cells for extended periods without causing inflammation, while *B. cenocepacia* is either readily cleared or kills the murine host inducing greater inflammation (Chu et al., 2004). Furthermore, *B. cenocepacia*, but not *B. multivorans*, interferes with the normal function of dendritic cells by inducing necrosis (MacDonald and Speert, 2008).

Of note, *B. pseudomallei* has also been recovered from individuals with CF; increasing numbers of reports have shown multiple cases of CF patients colonized with *B. pseudomallei* organism, suggesting that CF patients have the ability to acquire this organism outside the endemic areas or that they acquire and import them from such regions (Barth et al., 2007; O'Sullivan et al., 2011; Schulin and Steinmetz, 2001)

1.7 Putative virulence factors of *B. cenocepacia*

The mechanism of virulence of *B. cenocepacia* has been reviewed by Drevinek and Mahenthiralingam, 2010, and Loutet and Valvano, 2010. A summary of some of the virulence determinants involved in pathogenesis is presented here:

1.7.1 Quorum sensing

B. cenocepacia possess CepI synthase with a CepR regulator for cell-to-cell communication, and several genes have been identified to be regulated by this quorum sensing system such as siderophore synthesis, protease production, a T3SS, motility and biofilm formation (Leo, 2006).

1.7.2 Iron uptake

Members of the Bcc produce different siderophores (ornibactin, pyochelin, cepaciachelin and cepaciabactin) under conditions of iron depletion (Thomas, 2007). Ornibactin, the predominant siderophore produced by most of the strains of *B. cenocepacia,* is required for virulence in the rat agar bead model infection (Visser et al., 2004).

1.7.3 Surface polysacharides

In contrast to *B. pseudomallei*, *B. cenocepacia* LPS is capable of activating immune cells through the pattern-recognition receptor Toll-like receptor 4-mediated signaling (Bamford et al., 2007; Wiersinga et al., 2006; Wiersinga et al., 2007). Within the LPS molecular structure, the O- antigen portion is important for resistance to serum-mediated killing and also prevents bacterial binding to epithelial cells as well as phagocytosis by macrophages (Saldias et al., 2009). The lipid A and inner core oligosaccharide portion contains 4-amino-4deoxy-L-arabinose instead of a phosphate group, which confers resistance to cationic antimicrobial peptides (Loutet and Valvano, 2010). Some *B. cenocepacia* strains produce exopolysaccharide (EPS), which gives bacterial colonies a mucoid appearance, that contributes to persistence by inhibiting neutrophil chemotaxis and generation of scavenge

reactive oxygen species (Bylund et al., 2006). EPS from *B. cenocepacia* clinical isolates might also contribute to the chronicity of the Bcc infection in CF patients (Zlosnik et al., 2011; Zlosnik et al., 2008).

1.7.4 Motility and adherence

B. cenocepacia flagellum is an important virulence determinant that contributes to bacterial motility and adhesion and enables the organism to invade host cells (Tomich et al., 2002; Urban et al., 2004). Cable pili, a specific type of fimbriae that is expressed in *B. cenocepacia* strains from the ET-12 lineage, in association with the 22 kDa adhesin AdhA has been shown to play a role in the invasion of epithelial cells where it binds to cytokeratine 13 (Sajjan et al., 2000).

1.8 Intracellular life style of *Burkholderia cenocepacia.*

B. cenocepacia, exhibits the capacity to reside in macrophages, amoebae, dendritic cells and airway epithelial cells (Lamothe et al., 2004; MacDonald and Speert, 2008; Sajjan et al., 2006). The mechanism of survival and replication of *B. cenocepacia* in airway epithelial cells indicates that after internalization, *B. cenocepacia* interacts with early endosomes, but escape from late endosome and lysosome to enter autophagosomes and ultimately replicate in the endoplasmic reticulum (Sajjan et al., 2006). In phagocytic cells, *B. cenocepacia* can persist in bacterial-containing vacuoles (BcCVs) that for the initial 6 hours post-infection do not acidify and avoid fusion with lysosomes; this delay in phagolysosomal fusion is advantageous for the bacteria to activate other mechanisms that confer resistance to the hostile environment of the lysosome (Lamothe et al., 2004). A dysfunctional CFTR

(CFTR-defective macrophages or macrophages pretreated with a CFTR functional inhibitor) enhances the *B. cenocepacia*-mediated maturation defect of the BcCVs (Lamothe and Valvano, 2008). The involvement of virulence determinants, such as the type three secretion system (T3SS), might help bacteria to initiate infection as was demonstrated in a mouse infection model (Tomich et al., 2003). However, another study did not demonstrate the involvement of the T3SS in the survival of *B. cenocepacia* within phagosomes in macrophages (Lamothe et al., 2004). Furthermore, it is suggested that a second type four secretion system (T4SS) present in *B. cenocepacia*, Ptw, contributes to its survival and replication in both airway epithelial and macrophages cells early in the infection (Engledow et al., 2004; Sajjan et al., 2008b). Although, *B. cenocepacia* produces a wide variety of potential virulence factors (Mahenthiralingam et al., 2005; McKeon et al., 2010), the molecular mechanism underlying the process of persistence have not been elucidated.

1.9 *Burkholderia cepacia* complex and host signaling pathways

Host signal transduction pathways activated in response to bacterial contact or bacterial products that play a role in the pathogenesis and immune response of *B. cenocepacia* infection have been described by several studies (McKeon et al., 2010; Reddi et al., 2003; Sajjan et al., 2008b; Urban et al., 2004). Toll-like receptors (TLRs) play important roles in sensing microbial determinants and initiate a host immune response; for example it has been shown that TLR5, but neither TLR2 nor TLR4, significantly regulate *B. cenocepacia*-induced lung epithelial inflammatory response and that the adapter molecule MyD88 is required for activation (de et al., 2008). TLR5 is also expressed after bacteria challenge with wild type *B. cenocepacia* but not with a flagellum mutant in HEK293 cells,

resulting in both NF-κB activation and IL-8 secretion (Urban et al., 2004). *B. cepacia* LPS was also shown to induce secretion of IL-8 in A549 airway epithelial cells through CD14 (a specific LPS receptor) and by activation of the p38 mitogen-activated protein kinase (MAPK) pathway (Reddi et al., 2003). Another study showed that LPS from *B. multivorans,* unlike *B. cenocepacia*, can induce an inflammatory response via the MyD88-independent pathway and that this could be linked to clinical outcome in some patients (Bamford et al., 2007). *B. cenocepacia* and *B. multivorans* have the capacity to induce higher secretion of TNF-α and IL-1β cytokines compared to *Pseudomonas aeruginosa* (another CF pathogen) in the U937 macrophage cell line; moreover *B. multivorans* LPS was shown to signal predominantly through the c-Jun N-terminal kinase MAPK pathway (McKeon et al., 2010). However, to date there is only one recent study (Cremer et al., 2011) that had focused in the activation of the Phosphoinositide 3-kinase/Akt signaling pathway in response of *B. cenocepacia*; this pathway plays an important role in pathogenesis and inflammatory response to several pathogen infections and is the most important pathway for cell survival (Hazeki et al., 2007; Lee et al., 2011). The third chapter of this work will focus on the modulation of this pathway after Bcc exposure.

1.9.1 The Phosphoinositide 3-kinase/Akt signaling pathway

Phosphoinositide 3-kinase (PI3K) belongs to a family of lipids signaling kinases that phosphorylate phosphatidylinositol at the 3,4 or 5 position of the inositol ring after exposure of cells to various biological stimuli, resulting 3'phosphorylated inositol lipids. One of its products, phosphatidylinositol 3,4,5 triphosphate (PIP_3), has an important role as a second messenger by working as a docking platform for lipid-binding domains, such as the

pleckstrin homology (PH) domains of various cellular proteins targets such as serine/threonine kinase (Akt, also known as Protein Kinase B; PKB), Bruton's tyrosine kinase (Btk), 3-phosphoinositide-dependent kinase (PDK), protein kinase C, phospholipase C and others that trigger downstream kinase cascades (Engelman et al., 2006), (Figure 1.2). The PI3K pathway is commonly activated through surface receptors in response to hormones and growth factors. In cells such as monocytes and macrophages, PI3K is activated by immune complexes, TLR2, 3, 4 and 9 ligands, cytokines and chemotactic peptides (Hazeki et al., 2007).

1.9.2 Family members of PI3K

Mammalian PI3K can be subdivided into three classes (I, II, III), based on their primary and secondary structures, mechanism of regulation and substrate specificities (Wymann and Pirola, 1998). Of the class I PI3Ks, class IA and IB isoforms are the major sources of PIP_3 and have been the most extensively studied so far; they are heterodimeric proteins that consist of catalytic and regulatory adaptor subunits. Different genes encode class IA catalytic subunit: p110α, p110β and p110θ, and five genes encode the regulatory subunits: p85α, p85β, p55α, p50α, or p55γ. The class IB subtype includes a catalytic domain subunit p110γ and regulatory subunit p101 (Wymann and Pirola, 1998). Class IA PI3Ks are activated by growth factor receptor tyrosine kinases and class IB PI3Ks is activated by G-protein-coupled receptors (Cantley, 2002).

1.9.3 PI3K/Akt signaling

After PI3K activation and generation of PIP_3, which acts as a secondary messenger to recruit Akt to plasma membrane, Akt molecules are phosphorylated at two residues: threonine 308, which increases Akt activity by about 100-fold, and serine 473, which always parallels the activation of PI3K and is required for full activation of Akt (Figure 1.2). Activated Akt mediates phosphorylation of different substrates that leads to their activation or inhibition; in this way Akt contributes to diverse cellular roles, including cell survival, growth, proliferation, angiogenesis, metabolism, and migration. However, aberrant Akt activation underlies the pathophysiological properties of a variety of complex diseases, including type-2 diabetes and cancer (Manning and Cantley, 2007), (Figure 1.3). In humans there are three cellular homologues of Akt (Akt1, Akt2 and Akt3) and all of them are similar in structure and size; Akt1 is widely distributed and is implicated in cell growth and survival, whereas Akt2 is highly expressed in muscle and adipocytes and contributes to insulin-mediated regulation of glucose homeostasis. The distribution of Akt3 is more restricted with expression mainly in the testes and brain (Hers et al., 2011). The role of Akt2 isoform in phagocytosis has been shown in primary monocytes and PMA-differentiated THP-1 macrophages (Shiratsuchi and Basson, 2007).

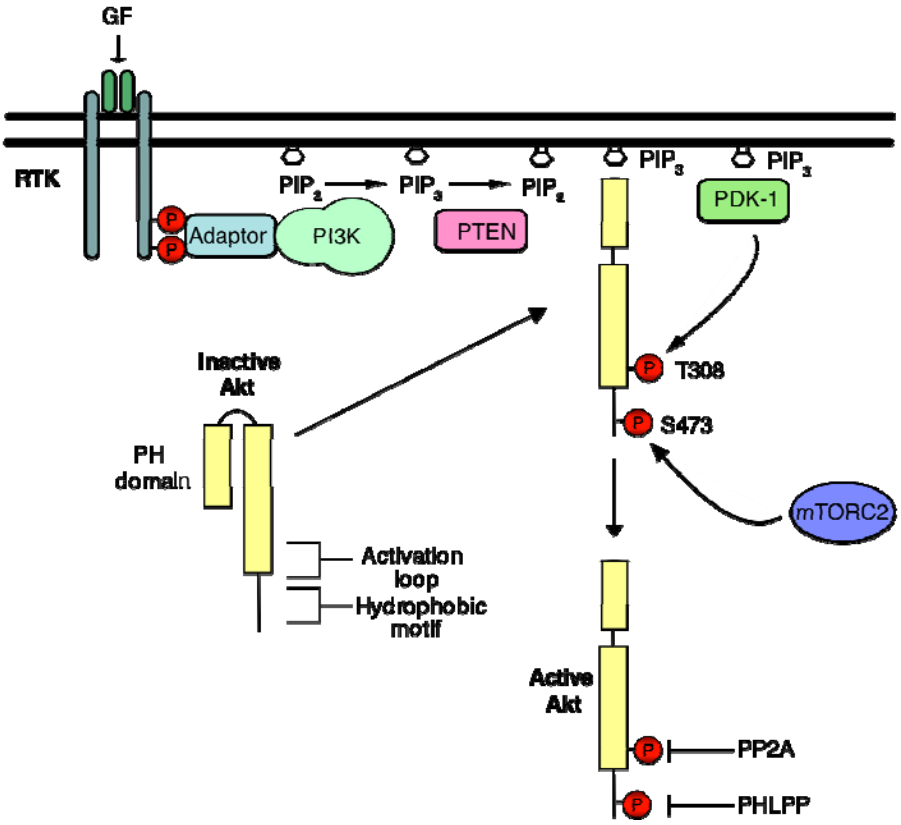


Figure 1.2: Activation and regulation of Akt. Receptor tyrosine kinases (RTKs) are activated by the binding of growth factor (GFs) to the extracellular domain. This results in receptor autophosphorylation and an increase in kinase activity. Class I phosphoatidylinositol 3-kinase (PI3K) binds either directly or through an adaptor protein to the activated receptor. PI3K phosphorylates phosphatidylinositol-4,5-biphosphate (PIP_2) to generate phosphatidylinositol-3,4,5-triphosphate (PIP_3). This reaction can be reversed by the action of PTEN (phosphate and tensin homology). Akt is normally maintained in an inactive state through an intramolecular interaction between the PH and kinase domains. However, the interaction between the PH domain of Akt and 3-phosphoinositide induces a conformational change in Akt, which enables co—recruited PDK1 to access the activation loop and phosphorylate Thr308. Dephosphorylation of this site is regulated by protein phosphatase 2A (PP2A). mTOR complex 2 (mTORC2) phosphorylates Akt in the hydrophobic motif on Ser 473 in a PI3K dependent manner. Dephosphorylation of Ser 473 is regulated by the phosphatase PHLPP. Akt dissociates from the membrane and phophorylates a wide range of substrates. From Hers et al., Cell Signalling 2011, by permission.

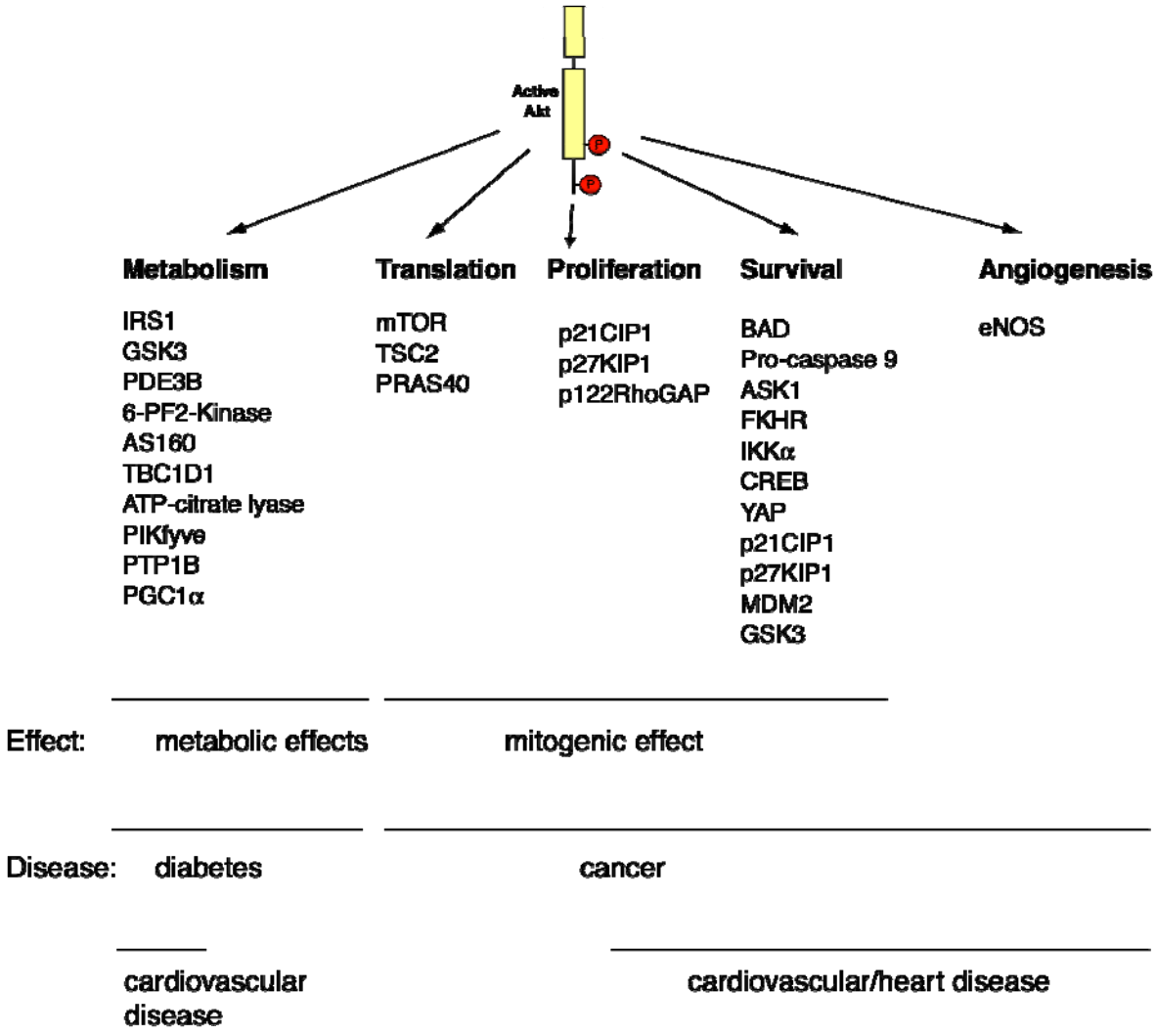


Figure 1.3: Cellular functions of Akt substrates. Phosphorylation by Akt leads to the activation or inhibition of many downstream effectors. Dysregulation of Akt substrates phosphorylation may lead to diseases From Hers et al., Cell Signalling 2011, by permission

1.10 Thesis objectives

In order to gain insights into the establishment of persistence, the **main goal** of the work described in this thesis **was to investigate the bacterial and host-pathogen interactions of two related bacteria within the *Burkholderia* genus: *Burkholderia pseudomallei* and *Burkholderia cepacia complex*.**

The **second chapter** of this thesis investigates the protein expression of the microbial determinants of *Burkholderia pseudomallei* which is the causative agent of melioidosis, a disease which carries a high mortality and morbidity rate in endemic areas of Southeast Asia and Northern Australia, by looking at the protein levels from sequential isolates from primary and relapsing melioidosis.

In the **third chapter**, the aim was to investigate the role of signaling pathways, specifically the phosphoinositide 3-kinase/Akt signaling pathway in macrophages, to measure cellular activation in response to two prevalent species from the *Burkholderia cepacia* complex that cause lung infections in CF patients: *Burkholderia cenocepacia* and *Burkholderia multivorans*. The differential activation of this pathway might reflect the different clinical outcome of the infection with these bacteria in CF patients.

Finally, while exploring the differential activation of the Phosphoinositide 3-kinase signaling pathway by these two bacteria, very preliminary data presented in the **fourth chapter** suggest the importance of this pathway for the internalization of these pathogens within macrophages. Ultimately, these studies contribute important knowledge about host-

pathogen interactions in persistent infections. Identifying the mechanisms leading to the persistence of these bacteria in melioidosis and CF, might contribute to the design of specific therapeutic approaches for the treatment of melioidosis as well as may increase the quality and length of life of CF patients.

Chapter 2: Analysis of potential mechanisms of latency in *Burkholderia pseudomallei:* the causative agent of melioidosis

2.1 Introduction

Burkholderia pseudomallei, the causative agent of melioidosis, is an aerobic, Gram-negative motile bacterium (White, 2003). Melioidosis is commonly characterized by acute pneumonia and septicemia in tropical regions of Southeast Asia and Northern Australia, and usually occurs in individuals with risk factors such as diabetes mellitus and renal impairment (White, 2003). There is no vaccine available for this disease, and antimicrobial therapy fails to eradicate the infecting organisms in around 10% of cases despite prolonged treatment lasting 12-20 weeks (Gan, 2005; Maharjan et al., 2005).

Seven different colony morphology types (morphotypes I-VII) have been described for *B. pseudomallei*, together with reversible switching between morphotypes under variable growth conditions (Chantratita et al., 2007). The ability of *B. pseudomallei* to remain dormant for prolonged periods of time after therapy of acute infection may be linked to colony variation (Gan, 2005). This characteristic may reflect bacterial phenotypic plasticity and adaptability, in response to environmental stimuli or stress, especially since the bacteria are soil saprophytes (Wiersinga et al., 2006).

The propensity of *B. pseudomallei* to become latent and to reactivate months or years later is similar to tuberculosis (Gan, 2005). Unlike tuberculosis, however, the re-emergent

isolate can be recovered from blood and from normally sterile body sites and may emerge as a new morphotype on the same genetic background (Chantratita et al., 2007). This relapsing type of invasive disease provides the opportunity to evaluate microbial determinants associated with phenotypic changes in the bacterium that may help explain the mechanism(s) of persistence in the human host.

The relevance of phenotypic plasticity in disease pathogenesis and relapse was explored by identifying a patient with melioidosis in whom disease relapsed after treatment with the recovery of different bacterial morphology. Here, we used two proteomic approaches: two-dimensional gel electrophoresis (2D-PAGE) and isotope tagging for relative and absolute quantitation (iTRAQ), to identify and compare protein profiles of *B. pseudomallei* isolates from the initial and relapsing infectious episodes.

2.2 Hypothesis

Burkholderia pseudomallei proteome changes over time *in vivo* with an altered metabolism in various forms during persistence melioidosis.

2.3 Aim

To identify proteins of *Burkholderia pseudomallei* that may be crucial during recurrent melioidosis disease; such proteins may play critical roles in maintaining survival of the bacteria in its human host.

2.4 Experimental procedures

2.4.1 Bacterial isolates

The isolates studied here were recovered from cultures from a patient with melioidosis whose disease relapsed within a year after therapy with oral amoxicillin-clavulanic acid. An isolate with morphotype I (the typical "cornflower head" colony (Chantratita et al., 2007) was recovered during the initial episode, and after six months isolates with morphotypes I and III (a large, smooth colony) (Chantratita et al., 2007) were recovered during the relapse episode; the three isolates were recovered from blood samples. A second patient with relapsing melioidosis within a year of primary therapy was studied; both the primary and relapse isolates were morphotype I. The primary infecting isolate was recovered from an abscess and the relapsing isolate was recovered from urine. Bacteria were grown aerobically on Luria-Bertani (LB) broth to stationary phase as previously described (Tandhavanant et al., 2010).

2.4.2 Pulse field electrophoresis gel (PFGE)

Genotyping of the sequential isolates were performed using pulsed field gel electrophoresis (PFGE) according to methods described in Maharjan et al., 2005. A single bacterial colony was streaked onto Columbia agar and incubated for 48 h at 37 °C in air. The colonies were harvested and suspended to an optical density at 540 nm of 0.7 in suspension buffer (75 mM sodium chloride, 25 mM EDTA, pH 7.5). This was mixed 1:1 with molten 2% low-melting-point agarose (Gibco), and pipetted into PFGE plug molds (Bio-Rad) and lysed overnight at 56 °C in lysis buffer (0.1% sodium dodecyl sulfate, 25 mM EDTA, pH

8.0) containing 500 μg/mL proteinase K (Invitrogen) and then rinsed three times with TE buffer (10 mM Tris, 10 mM EDTA). Prior to PFGE the plugs were digested overnight with 10 U SpeI (New England Biolabs) at 37 °C before being loading into a 1% agarose gel (Gibco) in 0.5 × TBE buffer (Tris-borate-EDTA). Each well was overlaid with 0.8% low-melting-point agarose. PFGE was performed on a CHEF-DRIII system (Bio-Rad) for 44 h at a temperature of 14 °C and at 6 V/cm using the following parameters: initial switch time, final switch time, and run time for block I, 10 to 60 V for 18 h; those for block II, 30 to 40 V for 18 h; and those for block III, 50 to 90 V for 8 h. Bacteriophage lambda concatemers were run as the standard (Promega). Gels were stained with ethidium bromide, washed in water, and photographed under UV light by Gel Doc 1000 system (Bio-Rad).

2.4.3 Total protein extractions

Extraction of whole cells proteins were performed as previously described (Chung and Speert, 2007) with some modifications. Briefly, *B. pseudomallei* cells grown in LB broth were resuspended in 1mL of cold lysis buffer composed of 5mM EDTA and 1mM PMSF in PBS for 2D-PAGE analysis or in 1mL of cold 5mM EDTA buffer for iTRAQ analysis. Protein concentration was determined by the BCA microassay kit (Pierce), and 200 μg or 1000 μg aliquots were frozen at –80 ºC.

2.4.4 Two-dimensional gel electrophoresis (2D-PAGE) analysis

Total proteins were purified from *B. pseudomallei* isolates grown to stationary phase in LB broth. Proteins were separated by 2D-PAGE in two replicates on separate days, following methods described previously (Chung and Speert, 2007) with some modifications.

Briefly, whole-cell protein extracts (200 μg) were treated with 2-D clean up kit (GE Healthcare) to remove interfering contaminants. Proteins were resuspended in rehydratation solution and applied to 24 cm long immobilized pH 4-7 gradient (IPG) strips as described (Chung and Speert, 2007). For the second dimension, the strips were placed on top of a 15 % SDS-polyacrylamide gel and electrophoresed as described (Chung and Speert, 2007). Protein spots were excised from silver stained 2D-PAGE gels and were sent for analysis using Mass Spectrometry at the Genome BC Proteomics Centre. The data obtained were identified by MASCOT LC-MSMS searches against the *B. pseudomallei* K96243 genome database (http://www.sanger.ac.uk) and Proteobacteria protein databases.

2.4.5 Isotope tagging for relative and absolute quantitation (iTRAQ) analysis

The same set of *B. pseudomallei* isolates from patient #1 were grown to stationary phase in LB broth and resuspended in EDTA buffer as described above. Whole-cell protein extracts (1000 μg) purified on two occasions on different days were sent for examination by one iTRAQ analysis to the Genome BC Proteomic Centre. The iTRAQ technique is a proteomic methodo for relative and absolute quantitation of proteins, and 4 or 8 analysis samples can be quantified simultaneously. In this technique, the introduction of stable isotopes using iTRAQ reagents occurs on the level of proteolytic peptides. The iTRAQ technology uses an NHS ester derivative to modify primary amino groups by linking a mass balance group (carbonyl group) and a reporter group (based on N-methylpiperazine) to proteolytic peptides via the formation of an amide bond. Due to the isobaric mass design of the iTRAQ reagents, differentially-labeled peptides appear as a single peak in MS scans, reducing the probability of peak overlapping. When iTRAQ-tagged peptides are subjected to

MS/MS analysis, the mass balancing carbonyl moiety is released as a neutral fragment, liberating the isotope-encoded reporter ions which provides relative quantitative information on proteins (Ernoult et al., 2008).

The iTRAQ procedure was carried out according to protocol the Genome BC Proteomic Centre. Briefly, protein samples were precipitated overnight in acetone at 4ºC followed by resolubilization in 0.5M TEAB, 0.2% SDS. Proteins were reduced with TCEP and alkylated with MMTS. Soluble proteins were then digested with trypsin (Promega) and labeled with the appropriate iTRAQ label. The iTRAQ labeled peptides were then combined and separated by strong cation exchange HPLC. SCX fractions containing peptides were then reduced in volume by speed-vac and analyzed by LC-MS/MS. The length of the reverse gradient used was 2 hours per HPLC strong cation exchange fraction. Samples were analyzed by reversed phase nanoflow (300nL/min) HPLC with nano-electrospray ionization using a quadrupole time-of-flight mass spectrometer (QStar pulsar i, Applied Biosystems) operated in positive ion mode. MS data were acquired automatically using Analyst QS 1.0 software Service Pack 8 (ABI MDS SCIEX, Concord, Canada). Database searches to identify peptides of the iTRAQ experiment were performed using ProteinPilot 3.0 software (Applied Biosystems) against the *B. pseudomallei* K96243 genome database. The following criteria were used to identify the differentially expressed proteins: (1) at least two unique high-scoring peptides; (2) error factor below 2 and *p*-values below 0.05 in each biological replicate; and (3) quantitative differences in proteins were observed across the two biological replicates (Kuzyk et al., 2009; Zlosnik and Speert, 2010).

2.4.6 Western blotting

Total proteins were separated by electrophoresis in a 12.5 % SDS-polyacrylamide gel (SDS-PAGE) and flagellin protein was detected by immunoblotting as previously described (Chung and Speert, 2007). Briefly, SDS-PAGE gels were transferred to polyvinylidene diflouride (PVDF) membrane (Millipore) and blocked with 5 % BSA or skim milk in Tris-buffered saline-0.1% Tween 20. Membranes were incubated with primary antibody at a dilution 1:10 000 for 24 h at 4 ºC with flagellin-specific rabbit polyclonal antiserum antibody kindly provided by Dr. Don Woods (Calgary, AB). A goat anti-rabbit IgG, 1:2000 (Cell Signaling) horseradish peroxidase-conjugated secondary antibodies was used before enhanced chemiluminoscence (ECL) detection.

2.5 Results

2.5.1 Genetic profiles of the sequential *B. pseudomallei* isolates from primary and relapsing melioidosis from patient #1

The PFGE banding patterns in the initial isolate; morphotype I, and the relapse isolates; morphotype I and morphotype III from patient #1, demonstrate identical genetic background. This suggests that the recurrent melioidosis in this patient represents a relapse due to recrudescence of the primary infecting isolate and that morphotype III might have been generated from morphotype I (Figure 2.1).

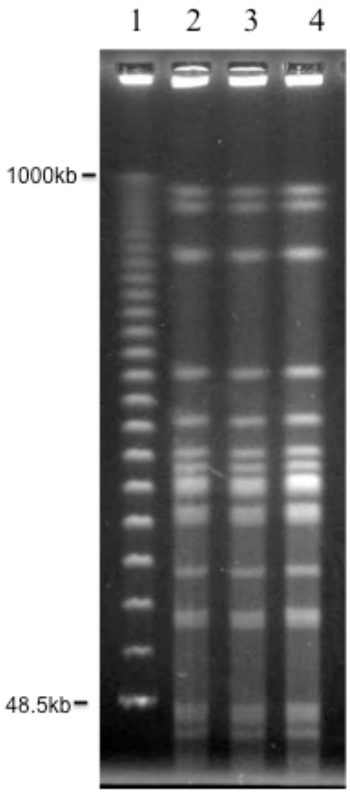


Figure 2.1: Pulse Field Gel Electrophoresis profiles of *SpeI* restricted genomic DNA from the consecutive *B. pseudomallei* isolates from patient #1. Lane 1: Lambda ladder (Bio-Rad), lane 2: initial isolate morphotype I (2644aI), lane 3: relapse isolate morphotype I (2644cI) and lane 4: relapse isolate morphotype III (2644cIII).

2.5.2 Analysis of protein changes in *B. pseudomallei* proteome by 2D gel electrophoresis

Differences in protein profiles between bacterial isolates as determined by using 2D-PAGE are shown in Table 2.1 and in Figure 2.2 for the first patient #1. A putative hemolysin-coregulated protein (Hcp) was present in the initial morphotype I and in the relapse morphotype I, but was absent in the relapse isolate morphotype III (Figure 2.3 spot 2). Two proteins of unknown function: BPSS0212 and BPSS1199 (Figure 2.3, spot 1 and 4, respectively) were also expressed at lower levels by the relapse isolate morphotype III alone (BPSS1199) or by both morphotypes I and III isolated at relapse (BPSS0212) as compared to the initial isolate (Table 2.1).

Analysis of bacteria isolated from this patient also demonstrated several up-regulated proteins in the relapse morphotype III compared with the primary infecting morphotype I isolate (Table 2.1). Of note, flagellin and HSP20/alpha crystalline family (Figure 2.3, spot 6 and spot 3 respectively) proteins showed substantially enhanced expression in morphotype III. Immunoblots confirmed an enhanced expression of flagellin in morphotype III compared to the initial isolates morphotype I (Figure 2.4). Protein up-regulation in the relapse isolate morphotype III was paralleled by down-regulation in the relapse isolate morphotype I for nearly all proteins, including HSP20/alpha crystalline and flagellin when compared to expression in the primary isolate.

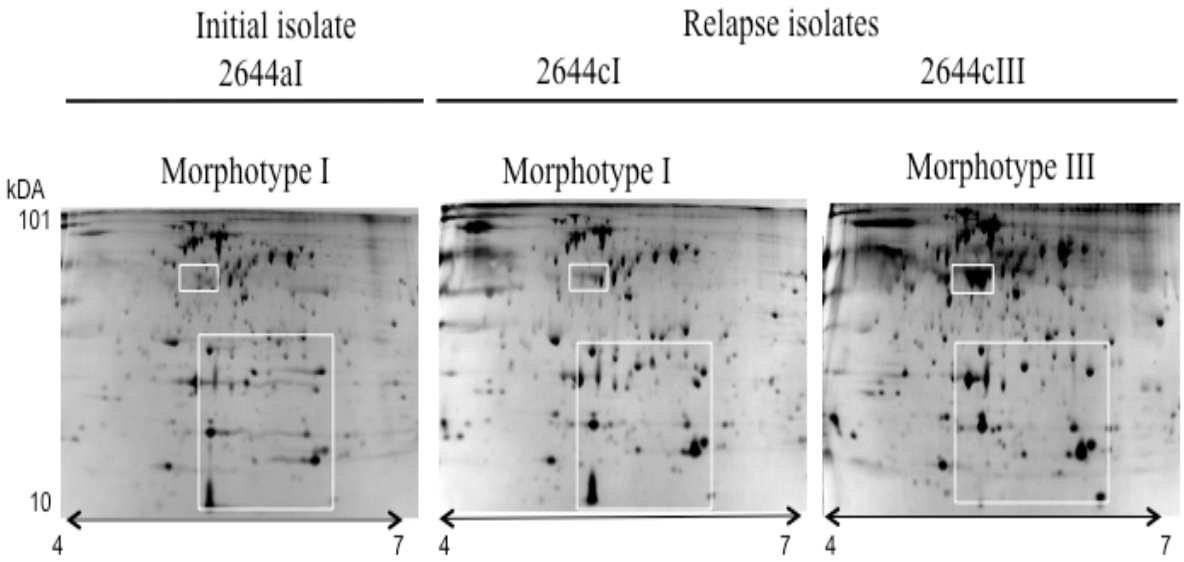


Figure 2.2: 2D gel electrophoresis analysis of the consecutive *B. pseudomallei* isolates from patient #1. Representative silver-stained 2D gels from initial isolate morphotype I (2644aI) and the relapse isolates morphotype I (2644cI) and morphotype III (2644cIII) show protein separation in the p*I* range 4 to 7 range strips. The majority of protein changes were observed between isolates 2644aI and 2644aIII (areas marked with white squares).

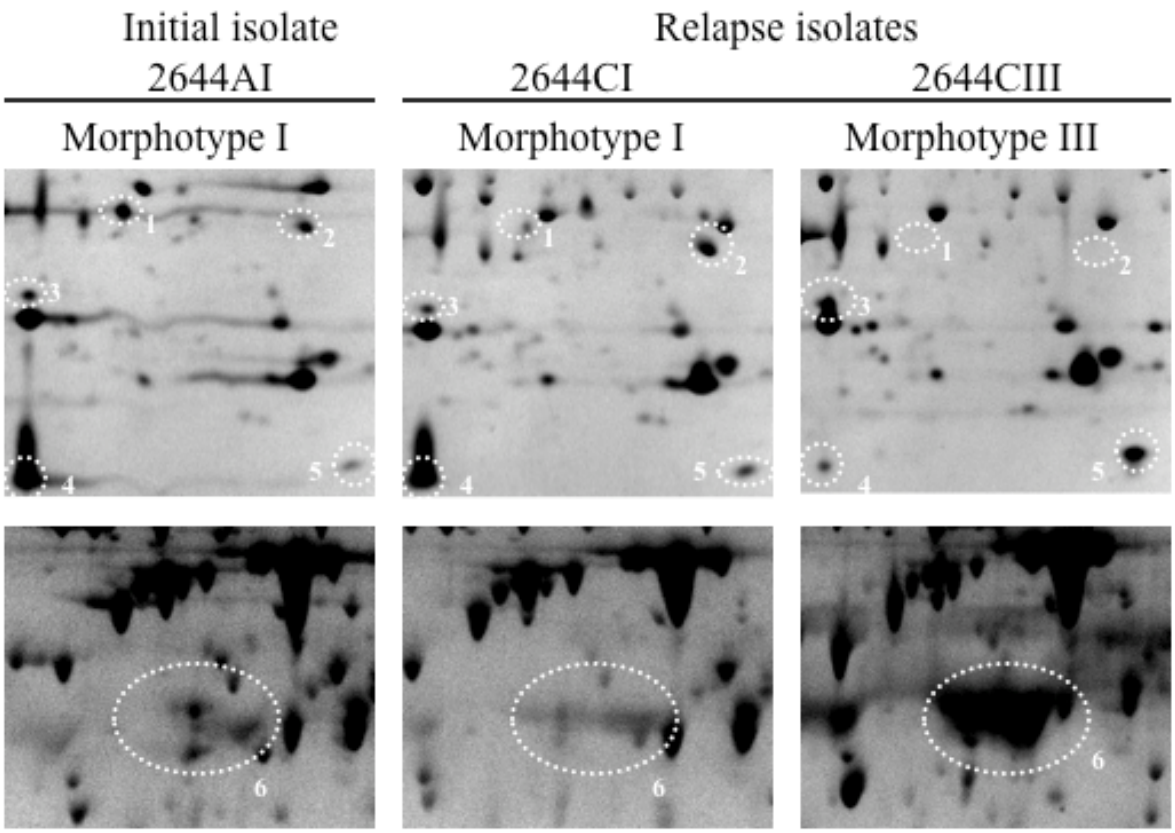


Figure 2.3: Magnification of areas of silver-stained 2D gels. Examples of protein spots that were differentially expressed by the initial infecting morphotype I isolate (2644aI) and relapse morphotype I (2644cI) and III (2644cIII) isolates. Spot numbers of proteins are shown in Table 2.1.

Table 2.1: Identification of differentially expressed *Burkholderia pseudomallei* proteins of the initial isolate (morphotype I) and relapse isolates (morphotypes I and III) patient #1 using 2D-PAGE, Mascot search engine and data from MALDI-TOF mass spectrometry.

Protein name (spot number)[a]	Category	Locus tag	Initial isolate	Change in relapse isolates		*pI*[b]	mass	SC (%)[c]	iTRAQ[d]
			I	I	III				
Conserved hypothetical protein (1)	Unknown function	BPSS0212	Present	Down	Absent	5.34	22451	41	67.20 (I)*
Hypothetical protein: Hemolysin-coregulated protein (Hcp) (2)	Type VI protein secretion system component	BPSS0171	Present	Present	Absent	5.56	17561	54	ND
Hypothetical protein (4)	Unknown function	BPSS1199	Present	Present	Down	5.83	11246	51	13.43 (I)
Cold shock transcription regulator protein (5)	Transcriptional regulation	BPSL3079	Present	Present	Up	5.7	7340	32	4.53 (III)
HSP20/alpha crystallin family protein (3)	Cellular processes, chaperone	BPSS2288	Present	Down	Up	5.14	16090	54	3.18 (III)
Thiol peroxidase	Stress response	BPSL2987	Present	Down	Up	5.12	17411	62	0.96 (III)
Flagellin (6)	Motility, host interaction	BPSL3319	Present	Down	Up	5.05	39243	30	1.79 (III) *
Peptidyl-prolyl cis-trans isomerase	Energy metabolism	BPSL0659	Present	Down	Up	4.84	11923	47	1.12 (III)
Probable phosphate-binding periplasmic abc transporter protein	Transport and binding proteins	BPSS0477	Present	Down	Up	4.8	36256	9	0.55 (IIII)
Acetyl-CoA carboxilase, biotin carboxyl carrier protein	Fatty acid biosynthetic process	BPSL2983	Present	Down	Up	4.66	16129	37	0.87 (III)

[a]Spot number numbers of proteins shown in Figure 2.3. [b]pI: Isoelectric point. [c]SC: Sequence coverage. [d]Average in fold induction of proteins by iTRAQ (morphotype from which protein was up-regulated). [*]Proteins statistically different detected by iTRAQ. ND: Non-detected.

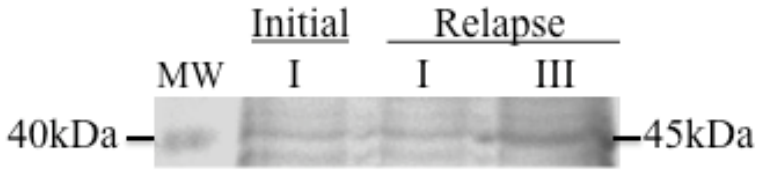


Figure 2.4: Immunoblot of the detection of flagellin protein of the consecutive *B. pseudomallei* isolates from patient #1. MW, molecular weight marker, Initial isolate morphotype I, Relapse isolates morphotypes I and III. Lanes contained 10 μg of total protein and separated on SDS-12.5% PAGE gel and immunoblotted with anti-flagellin polyclonal antibody.

Protein profiles for bacteria isolated from patient #2 were identical in both the primary infecting morphotype I and the relapse morphotype I isolates (Figure 2.5). Similarly to proteins profiles from morphotype I isolates from patient #1, flagellin protein showed the same level of expression between the two isolates (Area as marked in Figure 2.5, Figure 2.2 and Figure 2.3, spot 6 respectively), as well as nearly all proteins shown in Table 2.1.

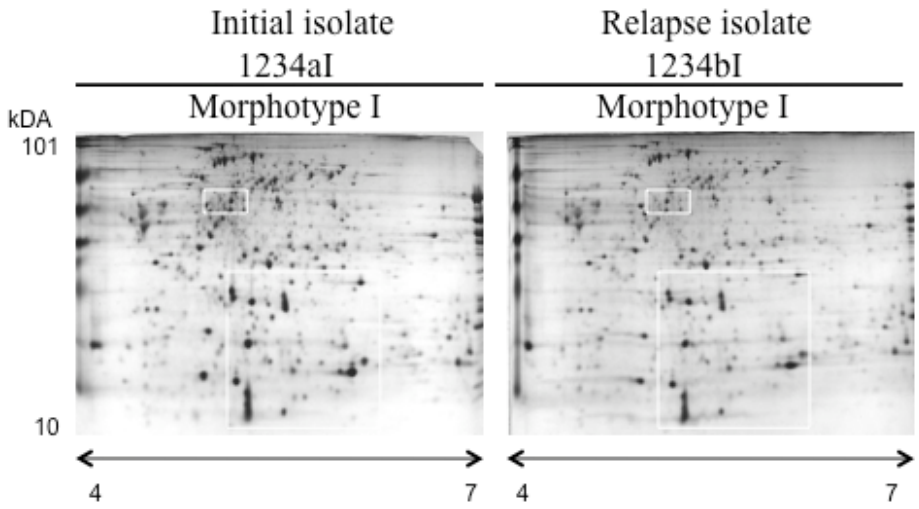


Figure 2.5: 2D gel electrophoresis analysis of the consecutive *B. pseudomallei* isolates from patient #2. Representative silver-stained 2D gels from initial isolate morphotype I (1234aI) and the relapse isolates morphotype I (1234cI) showing protein separation in the p*I* range 4 to 7-range strips. There were no major changes of protein expression between both isolates (areas marked with white squares) compared to relapse isolate morphotype III (2644cIII) from patient #1.

2.5.3 Analysis of protein changes in *B. pseudomallei* proteome by iTRAQ

Analysis of the sequential isolates using iTRAQ identified 974 proteins. The protein confidence threshold cut off applied was >1.3 (unused score: a measure of all the peptide evidence for the detected protein, calculated from the peptide confidence for peptides from spectra that have not already been completely "used" by higher scoring winning proteins) with at least two peptides with a 95% confidence. Thirty two proteins were differentially expressed in the initial (morphotype I) compared to the relapse (morphotype III) isolate. Of the differentially expressed proteins, seven were hypothetical with unknown function. Notable, in the same way as detected by 2D-PAGE, BPSS0212 was highly differentially expressed (67.20 fold change) compared to the relapse isolate morphotype III (Table 2.2). Other proteins, mainly involved in lipid metabolism and energy production, were also up-regulated in the initial isolate. A total of 18 proteins were differentially up-regulated in the relapse compared to the initial isolate. Of note, flavohemoprotein (HmpA) was highly expressed in morphotype III as well as enzymes that participate in the arginine deiminase pathway (arcA, arcC and arcB) (Table 2.2). We next compared the initial isolate to the second relapse isolate (also morphotype I); 2 proteins were up-regulated: Isocitrate lyase (AceA) and kumamolisin (Table 2.2).

Table 2.2 Differentially expressed *Burkholderia pseudomallei* proteins with the highest fold change in expression in sequential isolates, Morphotype I (2644aI), Morphotype I (2644cI) and Morphotype III (2644cIII), identified using iTRAQ.

Accession No.	Protein name	Gene name	Classification	Avg. (SD)[a]	Unused[b]	Cov (%)[c]	No Peptides (95%)
Morphotype I (2644aI) vs. Morphotype III (2644cIII)							
BPSS0213	hypothetical protein			99.08 (0)	28.89	86.2	62
BPSS1888	aromatic oxygenase		Inorganic ion transport and metabolism / General function prediction only	53.90 (9.77)	10	20.3	5
BPSS0212	hypothetical protein			67.20 (45.08)	26.52	35.9	24
BPSL0349	hypothetical protein			35.79 (4.64)	12	40.5	6
BPSL1549	hypothetical protein			30.76 (0.80)	21.58	85.8	19
BPSS1892	catechol 1,2-dioxygenase	catA	Secondary metabolites biosynthesis, transport, and catabolism	36.58 (9.42)	22.8	60	11
BPSS1356	hypothetical protein		Function unknown	64.09 (49.48)	111.11	66.2	87
BPSL0707	hypothetical protein		Energy production and conversion	27.29 (0.35)	7.71	18.8	4
BPSS0211	hypothetical protein			26.43 (3.77)	10	77.8	16
BPSL1955	succinyl-CoA:3-ketoacid-coenzyme A transferase subunit A	scoA	Lipid metabolism	19.71 (1.66)	14.36	51.3	14
BPSL1273	hypothetical protein		General function prediction only	16.05 (4.73)	17.38	70.7	12
BPSL1954	succinyl-CoA:3-ketoacid-coenzyme A transferase subunit B	scoB	Lipid metabolism	12.09 (0.86)	9.52	38.5	6
BPSL2733	LysR family transcriptional regulator		Transcription	22.10 (13.45)	28.22	68.7	18
BPSL1359	phosphate transport system, substrate-binding exported periplasmic protein	pstS	Inorganic ion transport and metabolism	14.70 (8.47)	44.42	78.6	39
BPSS1962	deoxyribose-phosphate aldolase	deoC	Nucleotide transport and metabolism	6.54 (2.29)	6	52.7	6
BPSS0882	hypothetical protein		Signal transduction mechanisms	7.72 (0.10)	16.4	77.3	10
BPSL3041	putative phenylacetic acid degradation oxidoreductase	paaZ	Energy production and conversion	14.00 (9.74)	22.18	41.4	18

Accession No.	Protein name	Gene name	Classification	Avg. (SD)[a]	Unused[b]	Cov (%)[c]	No Peptides (95%)
Morphotype I (2644aI) vs. Morphotype III (2644cIII)							
BPSL0880	putative superoxide dismutase	sodB	Inorganic ion transport and metabolism	3.82 (2.58)	12.08	76	24
BPSL2557	cold shock-like protein	cspA	Transcription	6.44 (1.41)	9.33	89.6	6
BPSS0269	lysine-arginine-ornithine transport system, binding exported protein	argT	Amino acid transport and metabolism / Signal transduction mechanisms	4.91 (0.60)	15.81	50	11
BPSL2697	chaperonin GroEL	groEL	Posttranslational modification, protein turnover, chaperones	4.78 (0.59)	134.57	89.6	209
BPSS1916	acetoacetyl-CoA reductase	phbB	Secondary metabolites biosynthesis, transport, and catabolism / General function prediction only	4.65 (0.63)	32.9	71.8	24
BPSL1535	acetyl-CoA acetyltransferase	phbA	Lipid metabolism	3.32 (0.02)	39.3	62.9	31
BPSS1288	gamma-glutamyltransferase 1		Amino acid transport and metabolism	5.24 (2.73)	26.16	39.6	16
BPSL0599	hypothetical protein			14.28 (15.97)	14	65.8	10
BPSS0802	outative extracellular ligand binding protein		Amino acid transport and metabolism	4.79 (2.99)	38.46	73.7	38
BPSL3043	enoyl-CoA hydratase	paaG	Lipid metabolism	6.58 (5.63)	8.21	44.9	6
BPSL1536	acetyacetyl-CoA reductase	phbB	Secondary metabolites biosynthesis, transport, and catabolism / General function prediction only	5.77 (4.48)	22.19	63.8	15
BPSS0913	methionine gamma-lyase		Amino acid transport and metabolism	7.75 (7.50)	34.02	50.1	29
BPSL3020	cell division protein FtsZ	ftsZ	Cell division and chromosome partitioning	10.22 (11.03)	18.01	37.9	12
BPSS0473	aldehyde dehydrogenase		Energy production and conversion	2.07 (0.10)	6	12.5	3
BPSL1550	putative betaine aldehyde dehydrogenase		Energy production and conversion	2.86 (1.47)	22.03	36.4	14

Accession No.	Protein name	Gene name	Classification	Avg. (SD)[a]	Unused[b]	Cov (%)[c]	No Peptides (95%)
Morphotype III (2644cIII) vs. Morphotype I (2644aI)							
BPSL2840	flavohemoprotein	hmpA	Energy production and conversion	29.93 (1.16)	14.13	39.60	13
BPSL1743	arginine deiminase	arcA	Amino acid transport and metabolism	17.12 (4.51)	55.45	81.10	62
BPSL1745	carbamate kinase	arcC	Amino acid transport and metabolism	11.58 (0.30)	14.21	44.00	14
BPSL1744	ornithine carbamoyltransferase	arcB	Amino acid transport and metabolism	12.27 (3.85)	37.20	69.40	46
BPSL0467	hypothetical protein		General function prediction only	6.77 (1.65)	6.00	16.10	3
BPSL2623	glutamate-1-semialdehyde 2,1-aminomutase	hemL	Coenzyme metabolism	6.45 (1.90)	8.04	12.70	4
BPSS1878	dehydrogenase		Energy production and conversion	5.54 (1.32)	10.17	22.00	6
BPSL0854	pyridoxamine 5'-phosphate oxidase	pdxH	Coenzyme metabolism	4.87 (0.06)	12.01	54.10	9
BPSS0840	zinc-binding dehydrogenase		Amino acid transport and metabolism / General function prediction only	15.51 (15.59)	18.29	44.20	15
BPSS1755	RNA polymerase sigma factor RpoD		Transcription	1.75 (0.19)	16.07	23.20	8
BPSL2301	pyruvate dehydrogenase subunit E1	aceE	Energy production and conversion	2.7 (1.16)	35.48	29.00	22
BPSS1958	hypothetical protein		General function prediction only	2.91 (1.55)	10.92	45.60	5
BPSS0355	aromatic amino acid aminotransferase	tyrB	Amino acid transport and metabolism	1.9 (0.14)	20.80	69.40	14
BPSS1944	alcohol dehydrogenase	adhA	General function prediction only	1.98 (0.32)	16.00	32.60	10
BPSL3216	elongation factor G	fusA	Translation, ribosomal structure and biogenesis	2.35 (0.86)	70.56	70.90	69
BPSL3389	trifunctional transcriptional regulator/proline dehydrogenase/pyrroline-5-carboxylate dehydrogenase	putA	Energy production and conversion	1.65 (0.02)	26.07	37.40	15
BPSL3319	flagellin	fliC	Cell motility and secretion	1.79 (0.23)	6.00	12.10	6
BPSS0281	4-aminobutyrate aminotransferase	gabT	Amino acid transport and metabolism	1.63 (0.07)	6.00	16.20	4

Accession No.	Protein name	Gene name	Classification	Avg. (SD)[a]	Unused[b]	Cov (%)[c]	No Peptides (95%)
Morphotype I 2644aI vs Morphotype I 2644cI							
BPSL2188	isocitrate lyase	aceA	Energy production and conversion	10.67 (12.09)	21.53	39.1	24
BPSS1562	kumamolisin		Posttranslational modification, protein turnover, chaperones	3.05 (3.16)	8	13.2	4

[a]Average of fold change of proteins from two biological replicates by iTRAQ. SD, standard deviation. [b]Unused score, a measure of all the peptide evidence for the detected protein, calculated from the peptide confidence for peptides from spectra that have not already been completely "used" by higher scoring winning proteins. [c]Sequence coverage (%), percentage of information generated to cover the full protein sequence.

2.6 Discussion

The proteome of sequential isolates of *B. pseudomallei* recovered from two patients with relapsing melioidosis were investigated. Several proteins were identified by iTRAQ when comparing morphotype I vs. morphotype III in patient #1. Of interest, the up-regulation of a putative superoxide dismutase, SodB, might explain the increased resistance to reactive oxygen intermediates of morphotype I compared to morphotype II and III (Tandhavanant et al., 2010). In *B. cenocepacia*, SodC, a periplasmic superoxide dismutase, is required for resistance to extracellular superoxide and a sodC mutant is more rapidly killed in a NADPH oxidase-dependent fashion, by murine macrophages than the wild type strain (Keith and Valvano, 2007). When comparing morphotype III vs. morphotype I, flavohemoprotein (HmpA) was highly expressed in relapsing isolate morphotype III; this protein is involved in detoxification of nitric oxide (Gardner et al., 1998). In this respect, studies have shown that HmpA activity in *Salmonella*-infected macrophages affects bacterial viability (McCollister et al., 2007; Stevanin et al., 2002). In another study, *Escherichia coli* mutants lacking Hmp survived less than the wild-type bacteria in macrophages despite similar binding and internalization rates (Stevanin et al., 2007). These Hmp mutants lack swarming motility and are aflagellate, an association that we observed in *B. pseudomallei* morphotype I by looking at the proteins expression; whereas the morphotype III relapse isolate demonstrated enhanced expression of both Hmp and flagellin proteins. Another set of proteins that were up-regulated are within the arginine deiminase pathway that participate in amino acid transport and metabolism: arcA, arcC, and arcB. In *Pseudomonas aeruginosa*, this pathway provides ATP synthesis under anaerobic conditions in the absence of exogenous

electron acceptors, provided that arginine is present in the growth medium (Vander Wauven et al., 1984). This alternative energy pathway is logically used under anaerobic stress; *B. pseudomallei* is able to grow anaerobically only in the presence of arginine and nitrate (Yabuuchi and Arakawa, 1993), a situation which may occur during the latent period between acute and relapsing infection. When comparing both morphotypes I (initial and relapse isolates) two proteins were up-regulated: (1) (AceA) is involved in energy production and is up-regulated in *Mycobacterium tuberculosis* after uptake into human macrophages (Graham and Clark-Curtiss, 1999). AceA is also associated with persistence of *M. tuberculosis* (McKinney et al., 2000) and of *B. pseudomallei*, in which its inhibition resulted in a switch from a chronic to an acute infectious state in an animal model (van Schaik et al., 2009). (2) The other up-regulated protein, kumamolisin, is important in post-transcriptional modification and it has collagenase activity (Wlodawer et al., 2004).

In the proteomic analysis using a 2D-PAGE approach, Hcp1 was identified in the initial isolate, morphotype I. This protein is a product of the newly described type 6 secretion system (T6SS) virulence cluster genes (Mougous et al., 2006; Pukatzki et al., 2007; Pukatzki et al., 2006; Schell et al., 2007). T6SS is induced after uptake of *B. pseudomallei* by macrophages (Shalom et al., 2007). Although the function of Hcp is unknown, it is thought to be part of a structural scaffold for the secretion of other proteins (Mougous et al., 2006; Pukatzki et al., 2007). At relapse, several proteins were up regulated in the morphotype III isolate; it was remarkable that proteins involved in facilitating chronic infection HSP20/alpha crystalline and flagellin were both up-regulated at this stage, the latter was validated by immunoblot. HSP20/alpha crystalline is one of the major immunoreactive

proteins of latent *M. tuberculosis* and is up-regulated when *M. tuberculosis* is grown *in vitro* in oxygen-depleted cultures (Lee et al., 1992; Starck et al., 2004). As in tuberculosis, this protein may play a role in the establishment of *B. pseudomallei* dormancy. Interestingly, the HSP20/alpha crystalline gene is strongly down-regulated at all-time points during chronic lung infection with *B. pseudomallei* in rats (van Schaik et al., 2008). However, protein up-regulation in the morphotype III relapse isolate was paralleled by down-regulation in the morphotype I relapse isolate for a wide range of proteins, including HSP20/alpha crystalline and flagellin. In *B. cenocepacia*, flagellin is up-regulated at high temperature and low pH (Chung and Speert, 2007), and fliD, a flagellar hook-associated protein, is one of the most highly up-regulated proteins throughout chronic lung infection in rats (van Schaik et al., 2008). Flagella expression was associated with morphotype III as detected by both 2D-PAGE and iTRAQ confirming a previous report in which flagella were detected in morphotype III by transmission electron microscopy (Chantratita et al., 2007).

The presence of two different morphotypes at relapse suggests that phenotypic switching is a dynamic process during which the initially infecting morphotype may co-infect with, and then be replaced by, a relapse morphotype (such as type III), which may be more fit for chronic infection. In this respect, morphotype III is resistant to human antimicrobial peptide cathelicidin LL-37 compared to morphotype I or II (Tandhavanant et al., 2010). In addition, morphotype III can switch to morphotype I under anaerobic conditions (Tandhavanant et al., 2010), which might explain the recovery of two different morphotypes during relapse in this particular patient.

Our findings raise several critical points. First, whilst it is clear that relapse isolates can exist in several different morphotypes, the relative proportion of change of morphotype between the initial and the relapse infection from morphotype I to III *in vivo* is low (Maharjan et al., 2005). Although, we speculate that each morphotype has a specific survival advantage in a given milieu, such as within a walled-off abscess versus an intracellular location, there is currently no evidence to support this suggestion. Second, our findings are complicated by the presence of two morphotypes in the relapse culture; further distinctions should be sought between bacterial adaptive changes that are directly associated with persistence *per se*, versus those simply associated with a different morphotype but not responsible for persistence. Such distinctions will be addressed by knocking out specific genes and then assessing microbial virulence in suitable *in vitro* models. For example, investigations on the adherence, survival, and replication of these mutants in several cell types might help to understand their role in virulence. In addition, appreciation of the factors that affect latency and virulence/invasiveness in melioidosis will require clarification of the mechanisms by which *B. pseudomallei* shifts between a state of dormancy and active growth.

The complexity associated with the presence of two morphotypes in the relapse culture for this patient was absent for a second patient, from whom the same morphotype (I) was observed from both primary and relapse cultures and with the same genetic background (data not shown). As with the initial and the relapse morphotype I strains in patient #1; flagellin protein showed the same level of expression as did the uncharacterized proteins BPSS0212 and BPSS1199. Hcp was also present in both the initial and relapse morphotype I

isolates in patient #2. It is remarkable that Hcp, the essential component of a functional T6SS in many bacterial species, was only expressed in morphotype I strains from both patients and was absent from morphotype III. Future experiments are needed to understand the role of T6SS in *B. pseudomallei* morphotype I, since this cluster may favour bacterial persistence as is the case for *Salmonella* (Parsons and Heffron, 2005) and *P. aeruginosa* (Mougous et al., 2006)

Proteomic investigations of bacteria from relapsing melioidosis have not previously been undertaken. The use of two complementary proteomic analyses is superior to either one in isolation for most fully assessing changes occurring during chronic infection. Initially, we used 2D-PAGE (a protein-centric approach) and selected for analysis the spots with most obvious differences in concentration. However, because this technique is time consuming and subjective, we then used a more robust and objective proteomic method: iTRAQ (a peptide-centric approach). All proteins, except BPSS0171, that were detected as differentially expressed by 2D-PAGE were also detected by iTRAQ, though with lower levels of differential expression (Table 2.1). Since these two techniques use different strategies for protein detection, they should be considered complementary rather than mutually exclusive (Thon et al., 2008).

This study has demonstrated differential protein expression by *B. pseudomallei* isolated during two episodes of melioidosis and subsequent relapse. The analyses described in this study may provide novel insights into mechanisms of persistence of intracellular organisms, since bacteria can be recovered from the bloodstream in infected patients rather

than relying on *in vitro* manipulation, as is the case in evaluation of *M. tuberculosis*. Further studies are required to identify phenotypic changes that facilitate long-term survival in the human host. Finally, the specific role of individual proteins can be assessed by manipulation of a targeted set of genes encoding the proteins identified in this study. Our objective in this study was to identify proteins of potential importance in enhancing survival of this difficult to treat pathogen. Our immediate goals are now to genetically manipulate the bacteria to determine which of the identified proteins influence resistance to normal anti-bacterial host defenses, thereby enhancing persistence.

Chapter 3: The activation of the of PI3K/Akt signaling pathway by *Burkholderia cenocepacia* and *Burkholderia multivorans* in macrophages

3.1 Introduction

The *Burkholderia cepacia* complex (Bcc) is a group of closely related Gram-negative bacteria and an important virulent group of opportunistic pathogens in cystic fibrosis (CF) and immunocompromised individuals (Drevinek and Mahenthiralingam, 2010; Isles et al., 1984; Speert et al., 2002). Bcc infections have been associated with three major outcomes in CF patients: asymptomatic carriage, chronic infection, and "cepacia syndrome", the latter of which is characterized by a rapid deterioration in lung function and, in some cases, bacteremia and septicemia, resulting in early death (Govan et al., 1996). Among the Bcc species recovered from patients with CF, *Burkholderia cenocepacia* and *Burkholderia multivorans* predominate, accounting for 85-97% of the infections (Drevinek and Mahenthiralingam, 2010). *B. cenocepacia* appears to be more virulent and causes a much more serious infection than *B. multivorans*; however the mechanism behind this difference in virulence remains undetermined (Govan et al., 1996). Previous studies have demonstrated that *B. multivorans* can persist benignly in macrophages or epithelial cells for extended periods without causing inflammation, while *B. cenocepacia* is either readily cleared or kills the murine host inducing greater inflammation (Chu et al., 2004). Furthermore, *B. cenocepacia*, but not *B. multivorans*, interferes with the normal function of dendritic cells by inducing necrosis (MacDonald and Speert, 2008).

Early interactions between bacteria and their host cells can determine the outcome of the infection: for instance, whether it results in a chronic non-invasive disease or an acute septicaemic fatal infection. Moreover, signal transduction pathways activated in response to bacterial contact or bacterial products play an important role in the pathogenesis of *B. cenocepacia* infection (McKeon et al., 2010; Reddi et al., 2003; Sajjan et al., 2008b; Urban et al., 2004). Comparing the two Bcc species, it has been shown that LPS from *B. multivorans,* unlike *B. cenocepacia*, can induce an inflammatory response via the MyD88-independent pathway and that this could be linked to clinical outcome in some patients (Bamford et al., 2007). There is little literature concerning the molecular basis of differential pathogenesis of *B. cenocepacia* and *B. multivorans* and the role of regulatory pathways in host response and the associated differential cellular consequences.

Bacterial invasion of phagocytic cells results in the activation of several host proteins including the phosphoinositide3-kinase (PI3K) (Hazeki et al., 2007; Lee et al., 2011). PI3K is an integral component of a signal transduction pathway that regulates host cellular processes (Hazeki et al., 2007) and its activation results in the stimulation of phosphatidylinositol-dependent kinase, which phosphorylates and activates Akt (a serine threonine protein kinase B) (Franke et al., 1997). Akt mediates the downstream effects of PI3K by phosphorylating multiple targets in a variety of cells that regulates a variety of biological processes such as proliferation, autophagy, phagocytosis and cell survival (LÃ³pez-Neblina and Toledo-Pereyra, 2006; Mullonkal and Toledo-Pereyra, 2007). An anti-apoptotic action of Akt operates via the transcription factor nuclear factor-κB (NF-κB). When bound to its cytosolic inhibitor, IκBα, NF-κB is sequestered in the cytoplasm. Upon

its phosphorylation by IκB kinase (IKKs), IκBα is degraded. This allows NF-κB to move to the nucleous and activate the transcription of anti-apoptotic proteins. Akt associate with, and activates, IKKs (Duronio, 2008; Lopez-Neblina and Toledo-Pereyra, 2006). It has been shown that PI3K and Akt influences in *Helicobacter pylori*-mediated NF-κB activity in human gastric epithelial MKN45 cells (Takeshima et al., 2009) and in *B. cenocepacia* infected phagocytes RAW 264.7 cells (Cremer et al., 2011). The PI3K/Akt pathway also plays a role in host immune responses to bacteria or bacterial products (Hazeki et al., 2007; Lee et al., 2011) and pathogens such as *Salmonella*, *Helicobacter pylori* and *Francisella novicida* among others, have been shown to exploit this pathway to establish an infection, prevent apoptosis and modulate phagocytosis and the inflammatory response (Huang et al., 2005; Nagy et al., 2009; Rajaram et al., 2006). Recently, it has been shown that PI3K/Akt pathway is required to induce pro-inflammatory cytokines during *B. cenocepacia* infection in phagocytes (Cremer et al., 2011). However there is no information of the role of PI3K/Akt signaling pathway in cells exposed to *B. cenocepacia* and *B. multivorans.*

This study explored the signaling events in the PI3K/Akt signaling pathway after bacterial-host interaction, to determine if measures of cellular activation (phosphorylation, cell death processes, activation of downstream targets and cytokine production) are associated with the differential virulence and persistence of *B. cenocepacia* and *B. multivorans* in macrophages. We found that PI3K/Akt and NF-κB are differentially activated in a time-dependent manner and that *B. multivorans*, but not *B. cenocepacia*, induced PI3K-dependent cell survival. These findings provide novel insights into the pathogenic mechanism underlying *Bcc* infection.

3.2 Hypothesis

Burkholderia cenocepacia and *Burkholderia multivorans* differentially modulate the establishment of infection by regulating signaling pathways in the host.

3.3 Aim

To investigate the phosphoinositide3-kinase/Akt signaling pathway after bacterial-host interaction to determine if measures of cellular activation are associated with the differential virulence and persistence of *Burkholderia cenocepacia* and *Burkholderia multivorans* bacteria in macrophages.

3.4 Experimental procedures

3.4.1 Growth conditions of bacteria and cells

The *B. cenocepacia* K56-2 and *B. multivorans* C5568 isolates were recovered from patients with CF and were obtained from the Canadian *Burkholderia cepacia* complex Research and Referral Repository (CBCCRR) (MacDonald and Speert, 2008). Additionally, two clinical and one environmental isolates from each group of the two species were selected from the Bcc strain panel: *B. cenocepacia*: J2315, ATCC 17765 from the Bcc strain panel (Mahenthiralingam et al., 2000) and FC1666 from the natural environmental IIIa (FC1666), (kindly provided by Dr. John Lipuma, Ann Arbor, Michigan USA); and *B. multivorans*: MAC4, LMG 13010t and ATCC 17616. Bacteria were grown in 5mL Luria-Bertani (LB) broth overnight with shaking at 37 ºC to stationary phase. The human monocytic THP-1 cell line was obtained from the American type Culture Collection (ATCC) and was maintained in RPMI 1640 containing 10% Fetal Bovine Serum (Invitrogen), 2mM L-glutamine (Invitrogen), 1mM sodium pyruvate (Invitrogen) and 1% antibiotic-antimycotic (Invitrogen) at 37 ºC in 5% CO_2. The CF bronchial epithelial IB3-1 cell line kindly provided by Dr. Stuart Turvey (Vancouver, BC) and was cultured under standard protocols (Blohmke et al., 2008). The THP1-XBlue cells NF-κB/AP-1-Reporter Monocytes (InvivoGen) were maintained in accordance with the manufacturer's instructions. All cells were used within 45 days of establishing a new culture. Monocytic differentiation to the macrophage-like adherent phenotype was induced for 48 h with 0.1 μM of phorbol-12-myristate 13-acetate (PMA, Cell Signaling Technology), which is the most commonly used phorbol ester that binds and activates protein kinase C, causing a wide range of effects in cells and tissues.

PMA treatment compared to 1,25-dihydroxyvitamine D3 treatment, another stimulus that induces macrophage differentiation in monocytic cell lines, induces changes in cell morphology, increases the cytoplasmic to nuclear ratio, increases mitochondrial and lysosomal numbers, and alters differentiation-dependent cell surface markers similar to monocytes-derived macrophages (Daigneault et al., 2010). For all experiments, differentiated macrophages were cultured (starving conditions) in Minimum Essential Medium (MEM, Invitrogen) plus 10 mM Hepes (Invitrogen) or in LHC Basal medium (Invitrogen) for the IB3-1 cells for 16-24 h prior to bacterial challenge.

3.4.2 Isolation and Culture of Human Monocytes

Peripheral blood mononuclear cells (PBMCs) were isolated from the buffy coat of healthy blood donors according to the University of British Columbia Research Ethics Board protocol H04-70193 by density gradient centrifugation on Ficoll-Paque Plus (GE Healthcare) according to the manufacturer's instructions. Monocytes were isolated from the PBMCs by negative selection as previously described (MacDonald and Speert, 2008) and differentiated to macrophages using 0.1 μg/mL M-CSF (Biosource). For some experiments, monocytes were isolated by adhesion as previously described (Kiener et al., 1997). Briefly, 2×10^6 PBMC per well were distributed into 24-well plates and cultured in serum-free medium RPMI-1640 (Invitrogen) for 1 h. The non-adherent cells were removed with five PBS washes. The adherent cells (>70% monocytes) were then cultured for further bacterial challenge and measurement of cytokine release.

3.4.3 Cell stimulation, lysis and Western blotting

THP-1 derived macrophages (1×10^6 cell/well) were seeded in six-well tissue culture plates. Bacteria were diluted in starving medium (see above) to give a multiplicity of infection (MOI) of ~100, and co-culture with macrophages was maintained for graded durations at 37 ºC in 5% CO_2. Cells were collected and lysed with RIPA buffer (Sigma) including protease and phosphatase inhibitors (Sigma). Total proteins (30 μg) were separated on 12.5% acrylamide SDS-PAGE gel, transferred to polyvinylidene diflouride (PVDF) membrane (Millipore) and blocked with 5% BSA in Tris-buffered saline-0.1% Tween 20. Membranes were incubated with primary antibodies for 24 h at 4 ºC. Antibodies were purchased from Cell Signaling Technology and were used at the concentration of 1:1000: rabbit anti-phospho-AKT polyclonal (Ser473), rabbit anti-total AKT polyclonal, rabbit anti-phospho-IκBα monoclonal, rabbit anti-total IκBα polyclonal, rabbit anti-caspase 9 polyclonal, rabbit anti-α/β Tubulin polyclonal. Horseradish peroxidase-conjugated Goat anti-rabbit IgG (1:2000) or horse anti-mouse IgG (1:200) secondary antibodies were used before enhanced chemiluminoscence (ECL) detection. Bands on the blots were quantified by densitometry using Image J software 1.44e (Wayne Rasband, National Institutes of Health, USA). To compare protein levels of phosphorylation, bands were compared to control and normalized with the total level of AKT (Figure 1C). In some experiments, cells were incubated with 50 μM LY294002 (Sigma), 100 mM Wortmannin (Sigma), 10 μM PI-103 (Calbiochem), 50 μM Akt1/2 (Sigma), 10 μM AKTII (SH-5) (Sigma), 5 μM MG132 (Calbiochem), 10 μM Bay II-7082 (Invitrogen) for 30 min prior to bacterial challenge. Control cells were treated with equivalent volumes of dimethyl sulfoxide (DMSO) per mL of media. In some experiments, LPS (100 ng/mL) from *B. cenocpeacia* C6433 or *B.*

multivorans C5568, each kindly provided by Dr. Robert K. Ernst (University of Washington), was used as a positive control.

3.4.4 Apoptotic resistance analysis and flow cytometry

THP-1 derived macrophages challenged with bacteria at MOI of 10 for 4 h treated or untreated with LY294002 for 30 min were exposed to 2 μM of Staurosporine (Calbiochem), a broad-spectrum inhibitor of protein kinase, for 4 h. Cells were washed with PBS and harvested using 0.25% Trypsin/EDTA (Invitrogen) (Mimuro et al., 2007; Nagy et al., 2009). Cells were collected by centrifugation and resuspended in binding buffer (10 mM Hepes [pH 7.4], 140 mM NaCl, and 2.5 mM $CaCl_2$ at a concentration of 5×10^5 cell/mL. Cells were stained with FITC annexin V (BD Bioscience) and propidium iodine/RNAse (BD Bioscience) and calculated by quantitative flow cytometry. Ten thousand gated events were collected for each condition using FACSCalibour system and CellQuest, version 3.1, software (BD Bioscience). Data were analyzed using Flowjo 7.2.2 flow cytometry software (Tree Star, Ashland, OR) as described previously (MacDonald and Speert, 2008). Viable cells were measured by the percentage of annexin V− and propidium iodine/RNAse− macrophages. Early apoptosis of cells was measured by percentage of annexin V+ and propidium iodine/RNAse− macrophages, and late apoptosis of cells was measured by percentage of annexin V+ and propidium iodine/RNAse+ macrohages. For some experiments, cells were treated with 5 μg/mL cytochalasin D for 1 h and then the apoptotic resistance assay was performed.

3.4.5 DNA fragmentation analysis by agarose electrophoresis

DNA was extracted from floating and adherent THP-1derived macrophages that had been treated or not with LY294002 for 30 min, challenged with bacteria at MOI of 10 for 4h and then exposed to 2 μM staurosporine for 4 h to induce apoptosis using DNAzol Reagent (Invitrogen) according to manufacturer's instructions. Briefly, cells (1×10^6 cells/well) were seeded in six-well tissue culture plates and treated under different conditions. Cells were washed twice with PBS to remove unbound bacteria and lysed with 0.5 mL of DNAzol. Lysates were transfer into an assay tube, and DNA was precipitated with 1mL cold 100% ethanol and centrifugation at 10,000 × *g* at 4 °C. DNA pellets were washed twice with cold 70% ethanol and resuspended in 8mM NaOH. 10 μL was mixed with 0.32 μL of 1 M Hepes to a final pH of 7.0 and with loading buffer and subjected to agarose electrophoresis containing Sybr safe DNA gel stain (Invitrogen).

3.4.6 NF-κB/AP-1 activation–SEAP Reporter assay

The THP1-X Blue reporter cell line (Invivogen) was used to investigate NF-κB/AP-1 activation. THP1-X Blue cells derived from the human monocytic THP-1 cell line were transfected with a reporter plasmid expressing a "secreted embryonic alkaline phosphatase" (SEAP) gene under the control of a promoter inducible by transcription factors NF-kB and AP-1 (Invivogen). Differentiated THP-1 XBlue cells were seeded at 1×10^6 cell/mL into 96-wells plates and stimulated with bacteria with an MOI of 10. Infection was maintained for graded durations at 37 ºC in 5% CO_2 prior to quantification of SEAP. SEAP in the supernatant was detected by spectrophotometry using Quanti-Blue reagent (Invivogen)

according to the manufacturer's instruction and quantified at 620 nm OD. All measurements were performed using a microplate reader (Spectramax Plus).

3.4.7 Cytokine production

Cellular supernatants were collected after infection, stored at -80 ºC and assessed for cytokine concentration by enzyme-linked immunosorbent assays (ELISA). ELISA was performed using commercial kits for IL-10, TNF-α and IL-1β (BD Biosciences) (MacDonald and Speert, 2008). All measurements were performed using a microplate reader (Spectramax Plus).

3.4.8 Statistical analysis

All experiments were performed on at least 3 independent occasions. Statistical analysis was performed by Student's *t* test and repeated measure of ANOVA as appropriate, using GraphPad Prism (version 5.0). $P<0.05$ was defined as the threshold for statistical significant differences.

3.5 Results

3.5.1 Activation of PI3K/Akt signaling by *B. cenocepacia* and *B. multivorans*

PI3K catalyze the generation of phosphatydilinositol-3,4-biphosphate and phosphatydilinositol-3,4,5-triphosphate phospholipids, which results in Akt recruitment to the plasma membrane. Akt is phosphorylated at threonine 308 and at serine 473 residues leading to activation of its kinase activity (Coffer et al., 1998). To address whether the PI3K/Akt signaling pathway is activated in response to Bcc in different cell types, Akt phosphorylated on serine 473 residue was detected by immunobloting. Cells lysates at different time points after bacterial challenge were analyzed using specific phosphorylated Akt antibody, and all membranes were re-probed with Akt antibody to ensure equal loading of proteins in all lanes. As a positive control for Akt phosphorylation, cells were stimulated with *B. cenocepacia*–LPS (Figure 3.1). *B. cenocepacia* challenge of THP-1 derived macrophages induced strong phosphorylation of Akt (Figure 3.1A); this level of phosphorylation is observed in the IB3-1 cell line after bacterial, but not *B. cenocepacia* LPS, challenge (Figure 3.1B). In primary human monocyte-derived macrophages, Akt was phosphorylated with both *B. cenocepacia* LPS and bacteria alone after 4 and 5 days of cellular *in vitro* differentiation (Figure 3.1C). AKT phosphorylation experiments using heat-treated bacteria and 0.2 μm-filtered bacterial supernatant demonstrated lower level of phosphorylation as seen with live bacteria (Figure 3.1D). There was no significant difference in phosphorylation using different MOIs of *B. cenocepacia* (Figure 3.1E). The pharmacological PI3K inhibitors Wortmannin and LY294002 at concentration of 100 μM and 50 μM respectively abolished Akt phosphorylation (Figure 3.1F). To evaluate Akt

activation over time, THP-1 derived macrophages lysates were collected at intervals from 5 to 60 min after bacteria challenge (Figure 3.1G). Akt was activated in a time-dependent manner in *B. cenocepacia*–challenged macrophages, with phosphorylation reaching its peak at 60 min; in contrast, *B. multivorans*–challenged macrophages showed maximum Akt phosphorylation at 5 min. These data suggest that *B. cenocepacia* activated Akt in a PI3K-dependent manner in multiple cell types and that PI3K/Akt activation proceeded substantially faster after challenge by *B. multivorans* than by *B. cenocepacia* in macrophages.

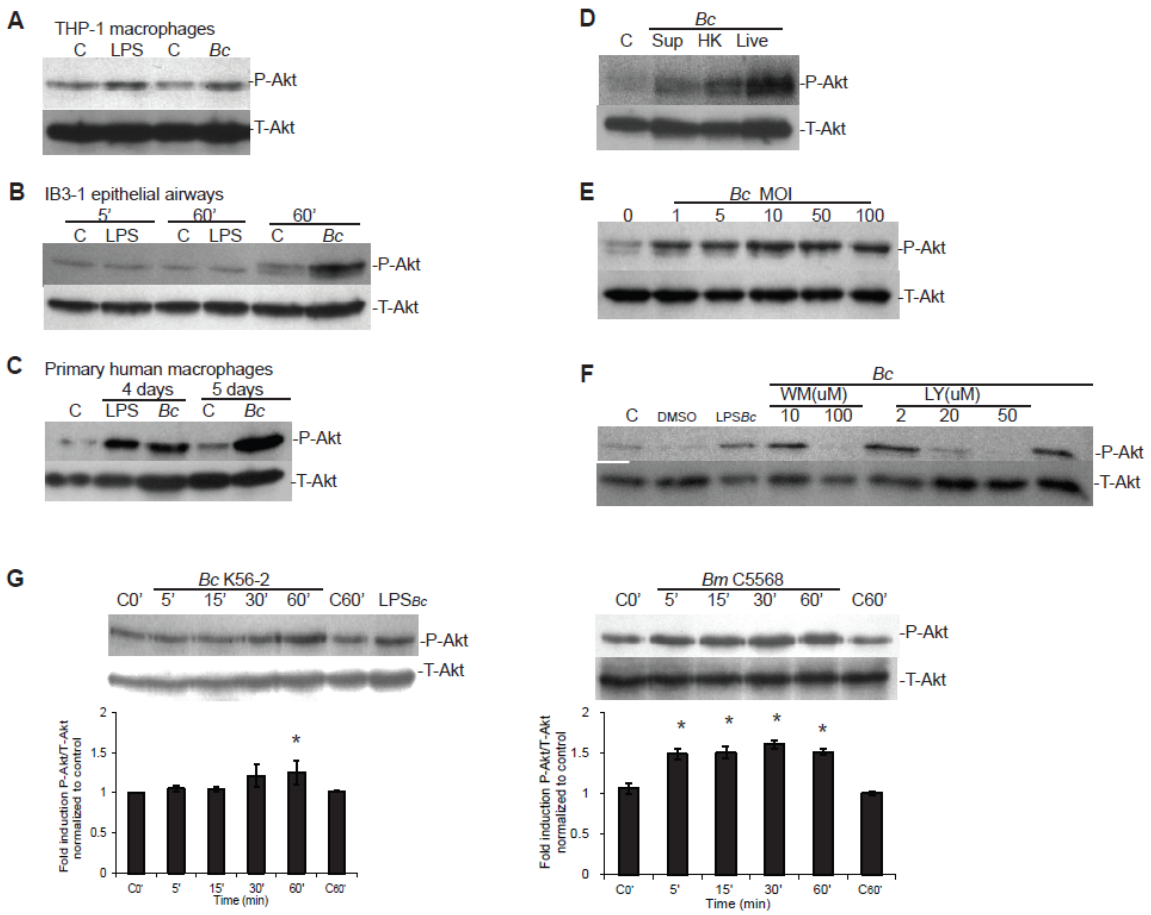


Figure 3.1: Akt phosphorylation in different cell types after challenge with *B. cenocepacia* or *B. multivorans*. Cell lysates were prepared and analyzed by immunobloting with antibody to either phosphorylated Akt (P-Akt) or total Akt (T-Akt). A representative blot is shown for each experiment with cells challenged at an MOI of 100 for 1 h. LPS (100 ng/μL) from *B. cenocepacia* was used as a positive control. **A**, PI3K-dependent activation of Akt by *B. cenocepacia* in THP-1 derived macrophages, **B**, in CF IB3-1 epithelial airways and **C,** in primary human monocyte-derived macrophages. **D**, THP-1 derived macrophages were exposed to either live *B. cenocepacia*, an equivalent number of heat-killed bacteria, or 5% (vol/vol) bacterial supernatant. **E**, THP-1 derived macrophages were incubated with different MOIs: 1-100. **F**, Effect of PI3K inhibition on *B. cenocepacia*–challenged cells. THP-1 derived macrophages were treated with Wortmannin (WM), LY294002 (LY) or equivalent volume of DMSO (negative control). The final lane shows the effect of live bacteria without inhibitor (positive control). **G**, Akt activation in a time–dependent manner by *B. cenocepacia* and *B. multivorans* in THP-1 derived macrophages. Data are from

immunoblots of bacterial–challenged cells for the times indicated and densitometric analysis from at least 3 independent blots performed on different days. Error bars indicates SE; * denotes $P < 0.05$ vs. unchallenged cells ($C_{0'}$) as measured by one-way analysis of variance with Dunnett's multiple comparisons test.

3.5.2 Contribution of PI3K signaling to cell survival in *B. cenocepacia* and *B. multivorans*-challenged macrophages

As it is known that the PI3K/Akt signaling pathway is involved in cell survival (Coffer et al., 1998); we next investigated the contribution of this pathway to *B. cenocepacia* and *B. multivorans*–mediated cell death via apoptosis. THP-1 derived macrophages were treated with LY294002, a PI3K inhibitor (Vlahos et al., 1994), and challenged with bacteria, after which the number of early and late apoptotic cells were quantified by flow cytometry. Cells exposed to *B. cenocepacia* or *B. multivorans* induced apoptosis, but the percentage of apoptotic cells increased dramatically in the presence of the PI3K inhibitor with each of the bacterial species (Figure 3.2). This phenotype was only observed after 24 h of incubation and not during early time points (data not shown). Cells treated with DMSO and LY294002 alone showed similar apoptosis percentages as cells treated with medium alone. These data demonstrated that activation of PI3K/Akt regulates macrophage cell survival in the presence of *B. cenocepacia* and *B. multivorans*.

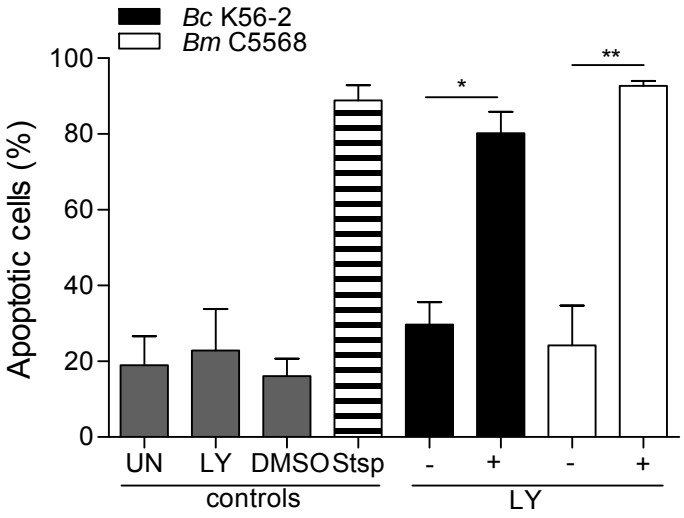


Figure 3.2: Contribution of the PI3K/Akt signaling on THP-1 macrophage survival after challenge with *B. cenocepacia* or *B. multivorans* for 24h. Combined percentages of early and late apoptotic cells for experiments performed on at least 3 occasions are shown. Cells were treated with 50 μM PI3K inhibitor LY294002 (LY) or control medium and incubated with *B. cenocepacia* or *B. multivorans*. Minus signs indicate cells incubated without LY. Cells treated with staurosporine (Stsp) served as an apoptosis positive control. Cells treated with DMSO and with medium alone (UN) served as apoptosis negative controls. Cells were stained with FITC annexin V and propidium iodine/RNAse, and apoptosis was quantified by flow cytometry. Error bars indicates SEs; * denotes $P < 0.05$ and ** denotes $P < 0.01$ as measured by one-way analysis of variance with Tukey's multiple comparisons test.

3.5.3 *B. multivorans* suppresses apoptosis in macrophages via activation of PI3K/Akt signaling pathway

Several reports have indicated that Bcc bacterial components (Cbl pili, hemolysin, ATP-using enzymes and azurin) have the capacity to induce cytotoxicity and cell death in macrophages, epithelial cells and neutrophils. (Cheung Jr et al., 2007; Hutchison et al., 1998; Melnikov et al., 2000; Punj et al., 2003). However, to demonstrate differential cell death induction it was necessary use long times of incubation, high MOIs or pure components. Therefore to expand previous data involving PI3K/Akt in cell survival, we investigated by performing the apoptotic resistant assay whether *B. cenocepacia* and *B. multivorans* had anti-apoptotic activity and whether the PI3K/Akt signaling pathway promoted this process. THP-1 derived macrophages were treated with LY294002 or control medium for 30 min and incubated with bacteria for 4 h. After incubation, cells were treated with staurosporine, a broad-spectrum inhibitor of protein kinases through the prevention of ATP binding to the kinase, to induce apoptosis, after which early and late apoptotic cells was quantified by flow cytometry. Both *B. cenocepacia* and *B. multivorans*-challenged macrophages were more resistant to staurosporine-induced apoptosis than cells without bacterial infection (staurosporine alone). However, in the presence of LY294002, *B. multivorans*-challenged cells did not inhibit apoptosis, and the percentage of apoptotic cells was higher than *B. multivorans*/staurosporine-treated cells and similar to cells treated with staurosporine alone. The percentage of apoptotic *B. cenocepacia*-challenged cells was similar to *B. cenocepacia*/staurosporine-treated cells (Figure 3.3A). A similar trend was observed using two other PI3K/Akt inhibitors: PI-103 (10 μM) and Akt1/2 (50 μM) (data not shown). Comparable results were obtained by evaluating DNA fragmentation, as a measure of

apoptosis, under the same conditions in which cells incubated with *B. cenocepacia*/staurosporine in the presence of LY294002 showed little or no fragmented DNA. On the other hand, cells incubated with *B. multivorans*/staurosporine in the presence of LY294002 showed obvious DNA fragmentation comparable to the staurosporine-treated cells (Figure 3.3B). These results suggested that *B. multivorans* plays an important role in modulating apoptosis by activating PI3K/Akt early in the infection of macrophages. Challenge of macrophages with staurosporine overrode the inhibition by *B. multivorans* plus LY294002, but not that of *B. cenocepacia* plus LY294002.

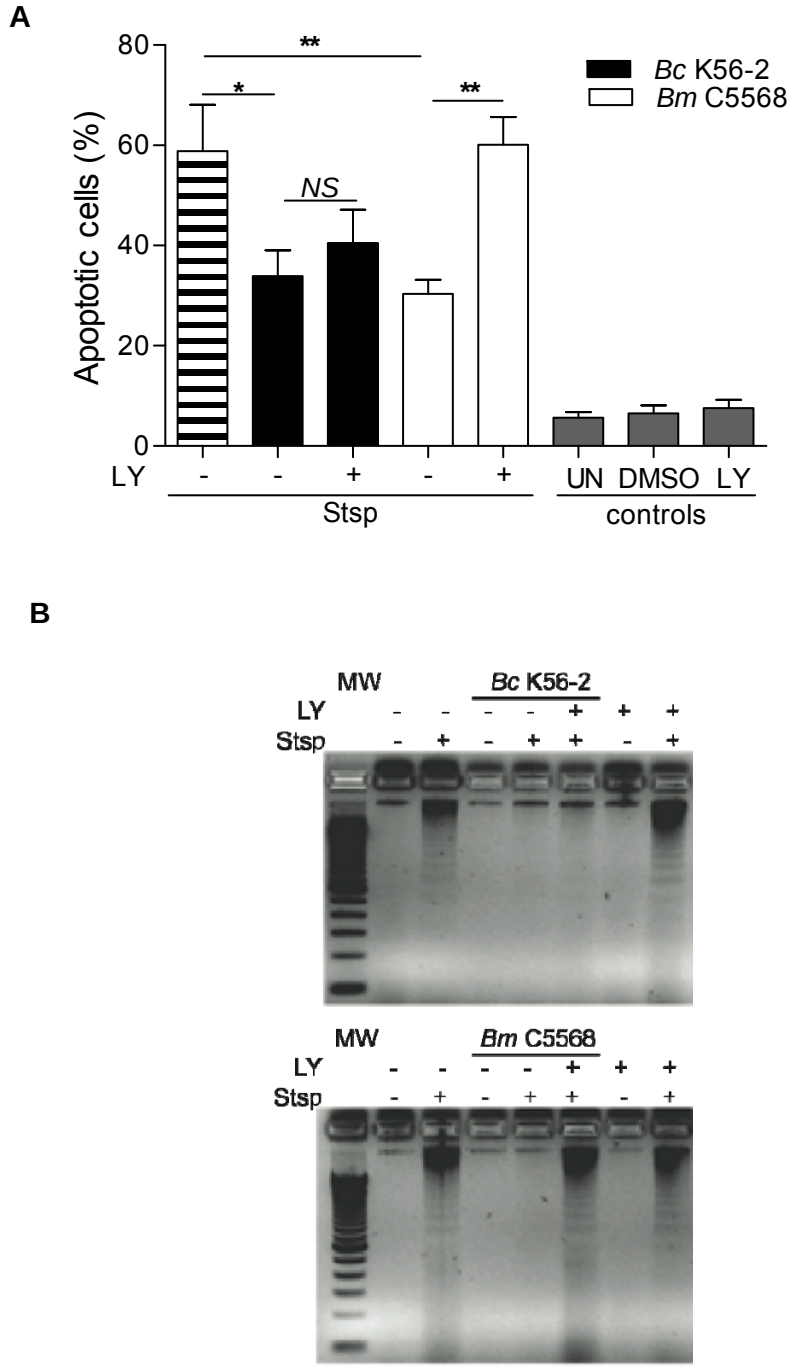


Figure 3.3: Promotion of cell survival due to the activation of PI3/Akt signaling by *B. multivorans* challenge of macrophages. **A**, Combined percentage of early and late apoptotic cells for experiments performed on 5 occasions. THP-1 derived macrophages were treated with 50 μM LY294002 (LY) or control media for 30 min, incubated with or without *B. cenocepacia* or *B. multivorans* at the MOI of 10 for 4 h and exposed to 2 μM

staurosporine (Stsp) for 4 h. Cells were stained with FITC annexin V and propidium iodine/RNAse, and apoptosis was quantified by flow cytometry. Error bars indicates SEs; * denotes $P < 0.05$ and ** denotes $P < 0.001$ as measured by one-way analysis of variance with Tukey's multiple comparisons test. **B**, Total DNA was extracted from detached and adherent cells and apoptotic DNA fragments were resolved in 1.8% agarose gel, stained with Sybr safe DNA gel stain, and visualized using UV transillumination. Molecular weight markers (MW) were run in the left hand lane.

To determine if the anti-apoptotic effect of *B. multivorans*–challenged was dependent on bacterial loads, experiments were performed with MOIs of 0 to 100. There was a dose dependent inhibition of apoptosis. Additionally, *B. multivorans*-challenged cells treated with DMSO slightly induced apoptosis; however, it was comparable to unchallenged cells (Figure 3.4).

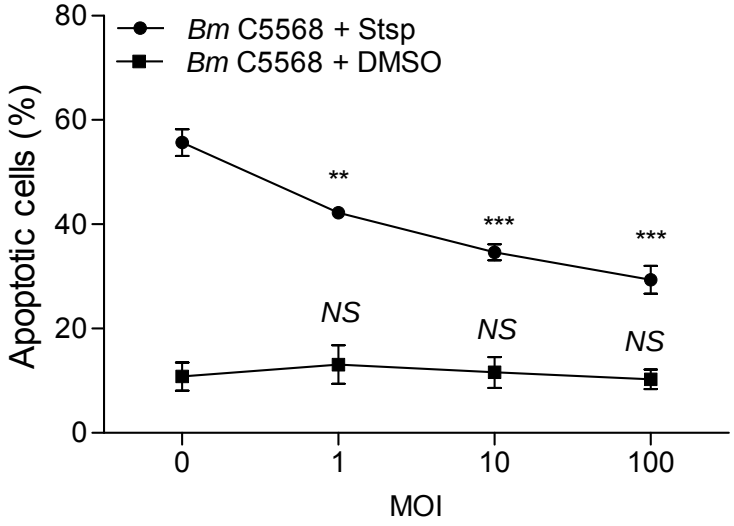


Figure 3.4: Effect of bacteria load on the *B. multivorans*-anti-apoptotic activity in THP-1 derived macrophages. Combined percentage of early and late apoptotosis was determined for cells that were exposed to *B. multivorans* at different MOIs: 0-100 for 4 h and then exposed to 2 μM staurosporine (Stsp) or DMSO for 4h. Data are average of 3 independent experiments. Cells were stained with FITC annexin V and propidium iodine/RNAse, and apoptosis was quantified by flow cytometry. Error bars indicates SEs; ** denotes $P < 0.01$, *** denotes $P < 0.001$ difference from cells treated with Stsp alone and *NS* denotes not significant difference from cells treated with DMSO alone as measured by one-way analysis of variance with Dunnett's multiple comparisons test.

To address whether bacterial internalization was necessary to induce anti-apoptotic activity in macrophages, a similar experiment was conducted in the presence of the actin-disrupting agent and uptake inhibitor, cytochalasin D. There was a greater apoptosis in cells treated with staurosporine than in its absence ($P = 0.011$ vs. *B. cenocepacia*-Stsp-treated cells and $P = 0.054$ vs. *B. multivorans*-Stsp-treated cells) (Figure 3.5) suggesting that bacterial internalization is necessary for these two bacterial species to induce their anti-apoptotic effects. Cytochalasin D treated cells did not induce significant apoptosis and percentage of apoptotic cells were comparable to untreated (UN) cells. Staurosporine plus cytochalasin D treated cells showed comparable DNA fragmentation patterns similar to staurosporine treated cells alone (data not shown).

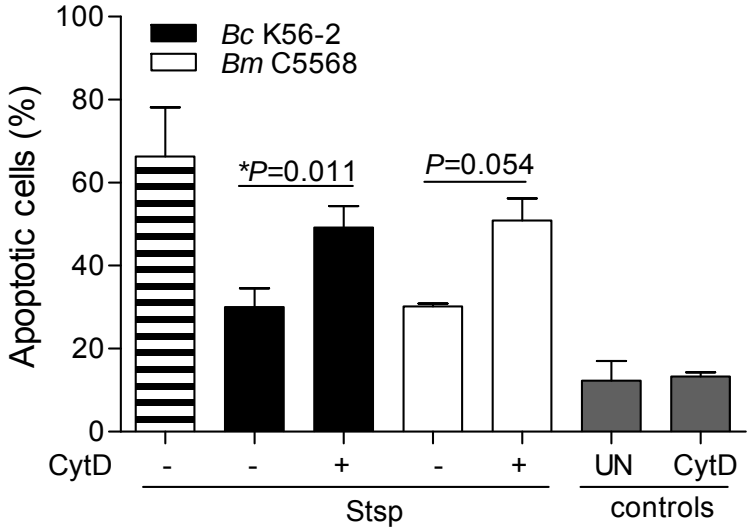


Figure 3.5: Role of bacterial internalization on the *B. cenocepacia* and *B. multivorans*-induced anti-apoptotic activity in THP-1 derived macrophages. Combined percentage of early and late apoptotic cells in experiments performed on 3 occasions. Cells were pre-treated with 5 μg/mL cytochalasin D (Cyt D) for 1 h and were exposed with or without *B. cenocepacia* or *B. multivorans* at an MOI of 10. Cells were then exposed to 2 μM

Staurosporine (Stsp) for 4 h. Cells were stained with FITC annexin V and propidium iodine/RNAse, and apoptosis was quantified by flow cytometry. Error bars indicates SEs; * denotes $P < 0.05$ in the presence or absence of Stsp as measured by Student's *t* test.

As it is known that activated Akt phosphorylates caspase 9 preventing its cleavage and therefore preventing apoptosis (Cardone et al., 1998), we next evaluated whether the *B. cenocepacia* and *B. multivorans*-mediated anti-apoptotic activity in macrophages involves caspase 9 inactivation. The method was similar to the anti-apoptotic resistant assay in which cells were left unchallenged or challenged with *B. cenocepacia* or *B. multivorans* at an MOI of 10 and then treated with staurosporine. Cells lysates were analyzed by immunoblotting using antibody specific for uncleaved (47 kDa) and cleaved caspase 9 (17 kDa) and membranes were re-probed with α/β Tubulin (55 kDa) antibody to ensure equal loading of proteins in all lanes. There was no caspase 9 cleavage in cells challenged with either *B. cenocepacia* or *B. multivorans* nor in cells challenged with *B. cenocepacia* and *B. multivorans* and Stsp–treated (Figure 3.6). These results suggest that both *B. cenocepacia* and *B. multivorans* promote cell survival by preventing caspase 9 cleavage.

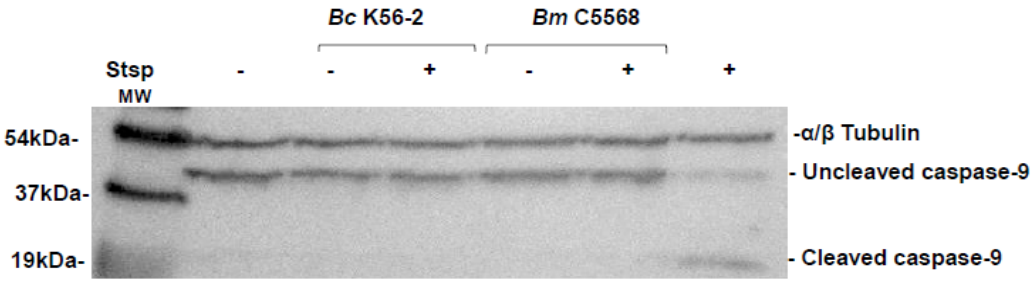


Figure 3.6: Caspase 9 inactivation in THP-1 derived macrophages in the presence of *B. cenocepacia* or *B. multivorans*. A representative blot is shown of 3 independent experiments. Cells were challenged at an MOI of 10 with or without *B. cenocepacia* or *B. multivorans* for 4 h and 2 μM Staurosporine (Stsp) for 4 h. Cell lysates were prepared and analyzed by immunoblotting with antibody for uncleaved and cleaved caspase 9 and with antibody for α/β Tubulin. Staurosporine (Stsp) treated cell was used as a positive control.

3.5.4 PI3K/Akt signaling pathway promotes differential NF-κB/AP-1 activation in *B. cenocepacia* and *B. multivorans*–challenged macrophages

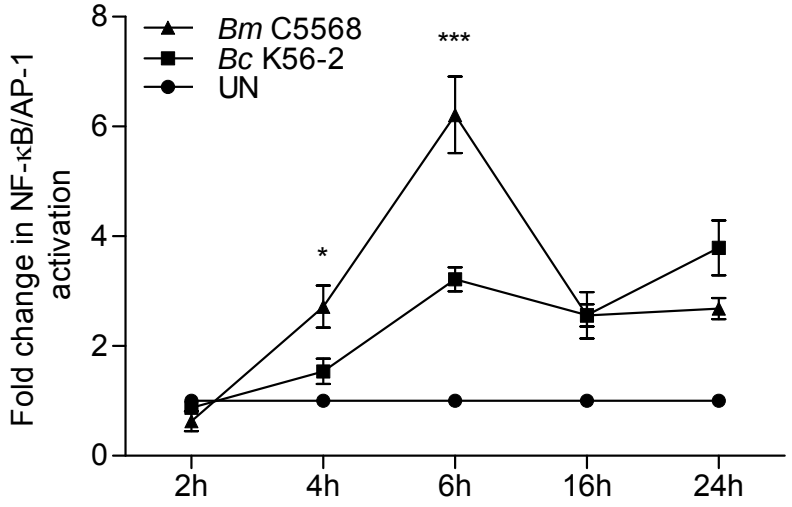
Akt phosphorylates and activates IKKα, which is a kinase that leads to the degradation of the NF-κB inhibitor IκB. When IκB is degraded, NF-κB is no longer bound to it, so it is released from the cytoplasm. NF-κB then goes to the nucleus and induces the transcription of anti-apoptotic genes (Duronio, 2008; Lopez-Neblina and Toledo-Pereyra, 2006). Therefore, to examine further downstream effects of PI3K/Akt pathway, we investigated the activation of NF-κB/AP-1in differentiated THP-1 X-Blue macrophages. Cells were exposed to bacteria at an MOI of 10 for up to 24 h (Figure 3.7A). Starting at 4 to 6 h, there were greater NF-κB/AP-1 activation in *B. multivorans*-challenged cells compared to those exposed to *B. cenocepacia* (2.7 vs. 1.53 fold change ($P < 0.05$) and 6.21 vs. 3.21 fold change ($P < 0.001$) respectively). There was no significant difference in NF-κB/AP-1 activation at later time points.

Since Akt is an upstream kinase implicated in Iκ-Bα phosphorylation (Lopez-Neblina and Toledo-Pereyra, 2006), we next extended the previous results by looking at phosphorylation of Iκ-Bα by immnunoblotting (Figure 3.7B). The kinetic analysis of *B. multivorans*-induced degradation and re-synthesis of Iκ-Bα revealed a faster increase of phosphorylated Iκ-Bα in 1 h and its gradual degradation over time. In contrast, *B. cenocepacia*-induced phosphorylation of Iκ-Bα increased after 2 h and continued up to 6 h. These results indicated that the faster *B. multivorans*–induced phosphorylation of Iκ-Bα leads to proteasome-mediated degradation of Iκ-Bα, therefore releasing NF-κB from the complex followed by its translocation to the nucleus to activate genes under its regulatory control.

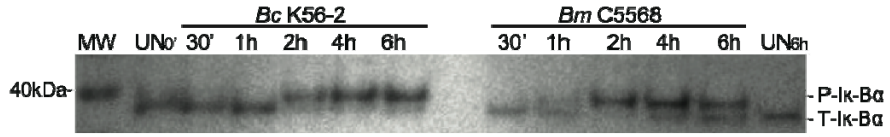
This observation correlated with the earlier NF-κB activation induced by *B. multivorans* as compared with *B. cenocepacia* (Figure 3.7A). NF- κB activation is terminated through cytoplasmatic resequestration of NF-κB, a process that depends on Iκ-Bα synthesis; this requires NF-κB transcriptional activity, which correlated with the reduction of NF-κB activation after 6h (Figure 3.7A).

We next determined whether NF-κB activation was dependent on bacterial load, as was shown with THP-1 cell apoptosis (Figure 3.4) with different MOIs: 10 to 0.01 at 6h. There was dose dependent NF-κB activation in *B. multivorans*-challenged cells compared to the unchallenged cells at MOIs of 10 and 1 ($P < 0.05$ and $P < 0.01$ respectively). NF-κB activation in *B. cenocepacia*-challenged cells was significantly enhanced over unchallenged cells only at an MOI of 10 ($P < 0.05$) (Figure 3.7C).

A


Bm C5568
Bc K56-2
UN
Fold change in NF-κB/AP-1 activation
0
2
4
6
8
2h
4h
6h
16h
24h
*
***

B


Bc K56-2
Bm C5568
MW
UN0'
30'
1h
2h
4h
6h
30'
1h
2h
4h
6h
UN6h
40kDa-
- P-Iκ-Bα
- T-Iκ-Bα

C

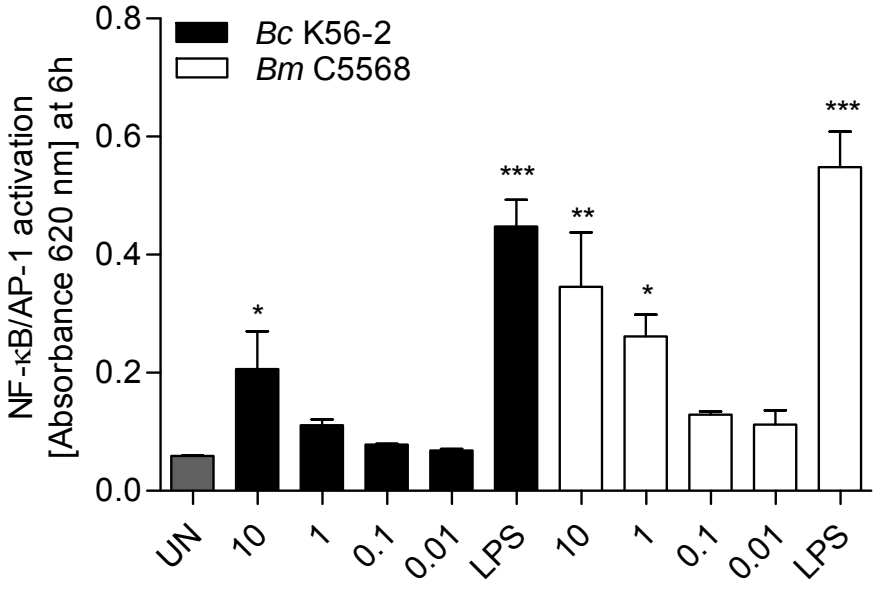

Bc K56-2
Bm C5568
NF-κB/AP-1 activation [Absorbance 620 nm] at 6h
0.0
0.2
0.4
0.6
0.8
UN
10
1
0.1
0.01
LPS
10
1
0.1
0.01
LPS
*
***
***
**
*
***

Figure 3.7: Differential NF-κB/AP-1 activation in *B. cenocepacia* and *B. multivorans*–challenged macrophages. **A**, Fold change of NF-κB/AP-1 activation in THP-1 X-Blue derived macrophages exposed with bacteria at an MOI of 10 incubated for the times indicated. Data are average of 4 independent experiments. Error bars indicates SEs; * denotes $P < 0.05$, ** denotes $P < 0.01$ and *** denotes $P < 0.001$ difference between the mean fold change of bacteria-challenged cells and unchallenged controls at indicated times points as measured by repeated measure of analysis of variance with Bonferroni post-test. **B**, A representative immunoblot of 3 independent experiments of cells lysates analyzed with antibodies to phosphorylated Iκ-Bα (P-Iκ-Bα) or total Iκ-Bα (T- Iκ-Bα). **C**, Dose-dependent bacteria effect on NF-κB/AP-1 activation. Cells were exposed with bacteria with MOIs: 10 to 0.01 for 6 h. *B. cenocepacia* LPS and *B. multivorans* LPS (100 ng/μL) were used as positive controls. Data are average of 3 independent experiments. Error bars indicates SEs; * denotes $P < 0.05$, ** denotes $P<0.01$ and *** denotes $P<0.001$ significance between challenged and unchallenged cells (UN) as measured by one-way analysis of variance with Dunnett's multiple comparisons test.

NF-κB/AP-1 activity was also measured for THP-1 X-Blue derived macrophages exposed to two other clinical isolates and one environmental isolate each of *B. cenocepacia* and *B. multivorans* together with the original pair of *B. cenocepacia* K56-2 and *B. multivorans* C5568. All of the three isolates of *B. cenocepacia* induced comparable NF-κB/AP-1 activation as *B. cenocepacia* K56-2 isolate, while two out three *B. multivorans* isolates induced greater NF-κB/AP-1 activation as *B. multivorans* C5568 (Figure 3.8).

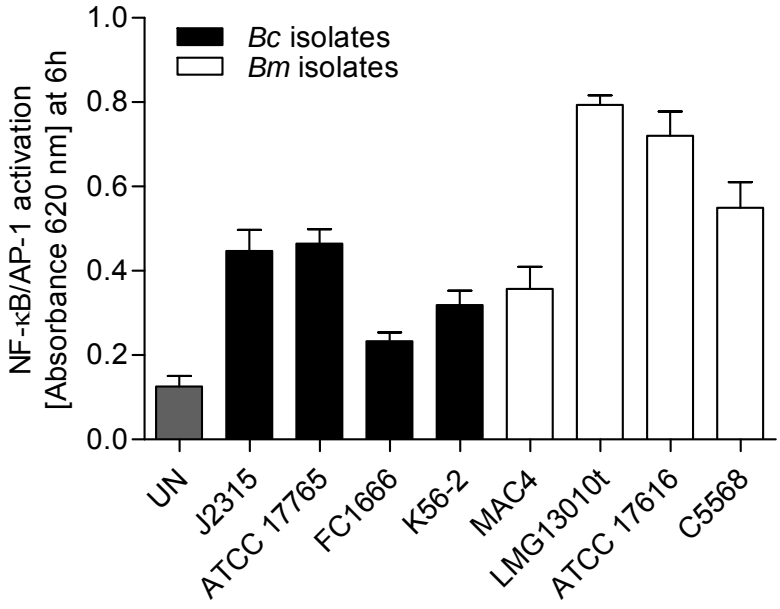


Figure 3.8: NF-κB/AP-1 activation in macrophages exposed to three *B. cenocepacia* and three *B. multivorans* isolates. Cells were challenged with bacteria with an MOI of 10 for 6 h. *B. cenocepacia* J2315 and ATCC 17765 are clinical isolates and FC1666 is a natural environmental isolate. *B. multivorans* MAC and LMG 13010t are clinical isolates and ATCC 17616 is a natural environmental isolate. Data are average of 3 independent experiments.

3.5.5 *B. cenocepacia* and *B. multivorans*–stimulated NF-κB/AP-1 activity is dependent on PI3K/Akt

Next, to evaluate whether *B. cenocepacia* and *B. multivorans*-induced PI3K activity affected NF-κB/AP-1 activation, THP-1 X-Blue derived macrophages were challenged with bacteria at an MOI of 10 and incubated for 6 and 24 hours in the presence of PI3K inhibitors (LY294002, Wortmaninn and PI-103) or Akt inhibitors (Akt1/2 and AktII). The Iκ-Bα and NF-κB inhibitors, MG-132 and Bay II-7082 respectively, were used as negative controls. The inhibitors caused a decrease in NF-κB/AP-1 activation in *B. multivorans*-challenged cells at 6 hours compared to cells incubated with bacteria alone (Figure 3.9). These findings suggest that NF-κB/AP-1 activation in bacterial challenged THP-1 cells is sensitive to inhibition of PI3K and Akt pathway and that there is more robust PI3K/Akt-dependent activation by *B. multivorans* than by *B. cenocepacia.*

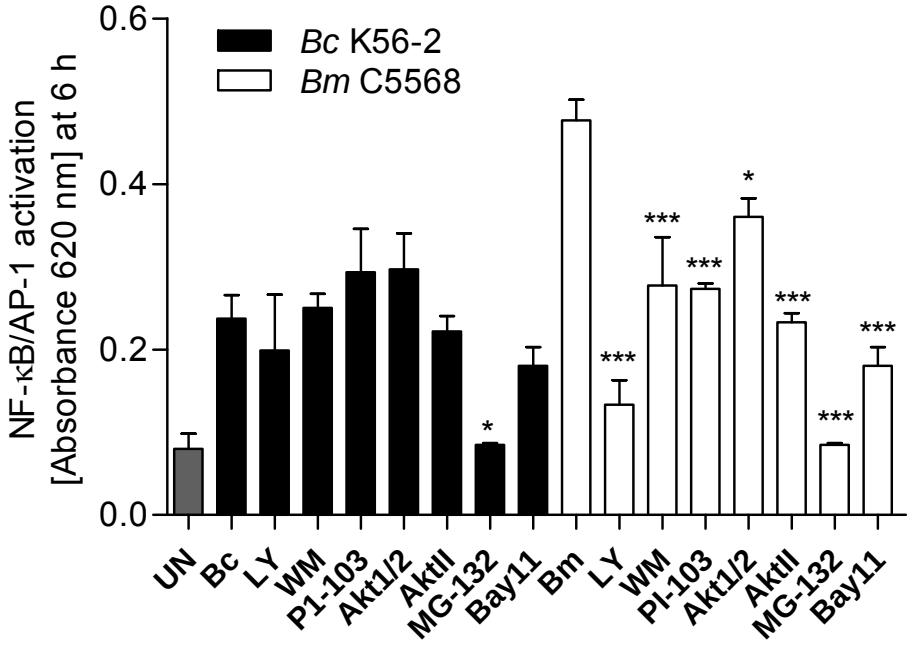


Figure 3.9: The NF-κB/AP-1 activation induced by *B. multivorans* is decreased by PI3K and Akt inhibitors. THP-1 X-Blue derived macrophages were challenged with bacteria at MOI of 10 and incubated for 6 h in the presence of PI3K (LY294002 (LY), Wortmaninn (WM) and PI-103) and Akt inhibitors (Akt 1/2 and Akt II). MG-132 and Bay II-7082 (Bay II) inhibitors were used as negative controls. Data are the average of 3 independent experiments. Error bars indicate SEs; * denotes $P < 0.05$, ** denotes $P < 0.01$ and *** denotes $P< 0.001$ significant difference from *B. cenocepacia* (*Bc*) or *B. multivorans* (*Bm*)-challenged cells alone as measured by one-way analysis of variance with Dunnett's multiple comparison test.

3.5.6 PI3K promotes the release of cytokines in response to *B. cenocepacia* and *B. multivorans* in macrophages.

Finally, we investigated by ELISA the effect of the inhibition of *B. cenocepacia* and *B. multivorans*-induced PI3K activation on the release of cytokines. Pretreatment of THP-1 derived macrophages with LY294002 reduced production of cytokines by THP-1 cells after challenge by both (TNF-α and IL-10) or one (IL-1β) species of bacteria (Figure 3.10). When comparing cytokine-release capacity of each species, there was no difference in the amount of TNF-α produced by *B. cenocepacia* and *B. multivorans* ($P = 0.56$) (Figure 3.10A). However there was more IL-10 release (an anti-inflammatory cytokine) after challenge with *B. multivorans* ($P = 0.055$) (Figure 3.10B), and conversely, a higher of IL-1β release (a pro-inflammatory cytokine) by *B. cenocepacia* ($P < 0.001$) (Figure 3.10C and D).

We also evaluated the inhibitory effect of LY294002 on human monocytes from 3 healthy donors. Results demonstrated that there is a similar trend toward higher IL-1β release by *B. cenocepacia* challenged compared to *B. multivorans* ($P = 0.081$) and that IL-1β release was decreased in the presence of LY294002 (Figure 3.10E). By increasing the number of samples, this difference in induction might be statistically significant. Together these data suggest that the PI3K/Akt signaling pathway is involved in the inflammatory response to *B. cenocepacia* and *B. multivorans* in monocytes and macrophages.

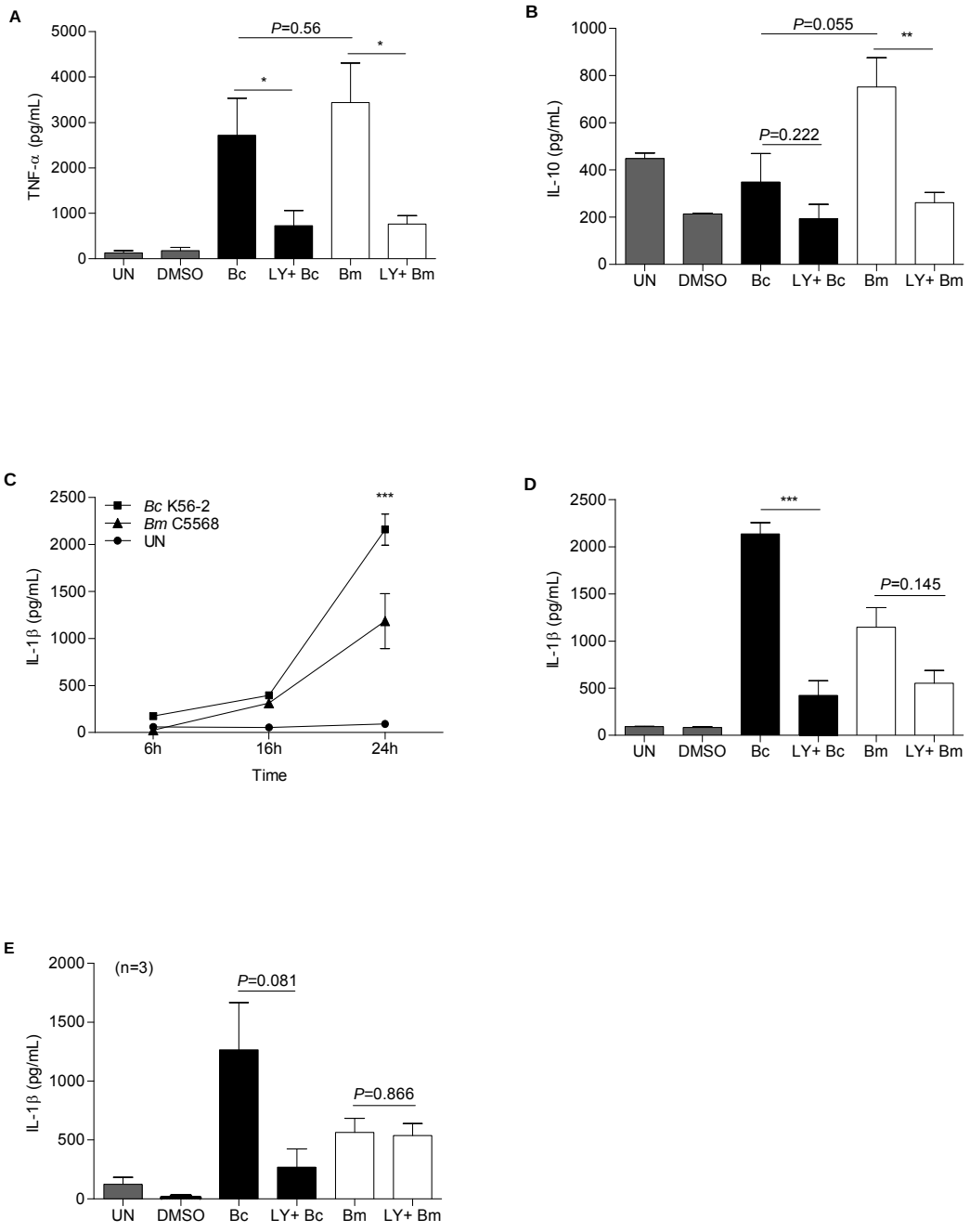


Figure 3.10: PI3K is required for cytokine production by macrophages challenged with *B. cenocepacia* or *B. multivorans*. Cytokine production was measured by ELISA from THP-1 macrophages exposed with bacteria at an MOI of 10 in the presence or absence of LY294002 (LY) after 24 h. Cytokine production by THP-1 cells: **A**, TNF-α, **B**, IL-10, **C** and **D,** IL-1β and **E**, IL-1β by human monocytes from 3 donors. Data are the average of 3 to 5 independent experiments. Error bars indicate SEs; * denotes $P < 0.05$, ** denotes $P < 0.01$

and *** denotes $P < 0.001$ significant difference between *B. cenocepacia* (*Bc*) or *B. multivorans* (*Bm*)–challenged cells alone or with inhibitors as measured by Student's *t* test. UN, unchallenged cells.

3.6 Discussion

Chronic lung infection with pathogens belonging to the Bcc progressively compromises lung function in patients with CF, ultimately leading to the death of the patient (Govan et al., 1996). Bcc isolates can survive intracellularly within epithelial cells and phagocytes, where their survival and persistence is believed to play a role in pulmonary infection and contributes to the persistent inflammation observed in CF patients (Sajjan et al., 2006; Saldias and Valvano, 2009). Macrophages represent a major component of the innate immune system and play several roles including anti-bacterial immune response (Linton and Fazio, 2003). Of the numerous signaling proteins that contribute to the large number of signals generated within phagocytic cells, the PI3K/Akt signaling pathway acts as a major player in the regulation of several processes including cell survival, and Akt has been shown to be highly expressed in immune cells such as macrophages (Lee et al., 2011).

Here, we explored the role of the PI3K/Akt signaling pathway in macrophages exposed to *B. cenocepacia* and *B. multivorans*, two prevalent species in CF lung infection that results in different clinical outcomes (Drevinek and Mahenthiralingam, 2010). Initially, using different cell types we found strong PI3K/Akt activation detected by Akt phosphorylation when exposed to *B. cenocepacia.* These results confirmed a recent study (Cremer et al., 2011) and previous reports that showed expression and phosphorylation of Akt in macrophages and epithelial cells in response to bacteria or bacterial components in other systems (Huang et al., 2005; Lee et al., 2011; Nagy et al., 2009; Rajaram et al., 2006). Of note, there was not Akt phosphorylation in the IB-3 CF epithelial airway cell line when

exposed to *B. cenocepacia* LPS alone, but only with live bacteria. Reports suggest that the TLR4-mediated response to LPS is minimal in airway cells due to a lack of the necessary co-receptors, including MD2 and CD14 (Jia et al., 2004; Schulz et al., 2002). Remarkably, *B. multivorans* initiated Akt phosphorylation significantly faster than *B. cenocepacia* in macrophages. Similarly some other pathogens showed rapid Akt phosphorylation (5 min; *Francisella tularensis* in macrophages (Rajaram et al., 2006) and others a more delayed phosphorylation (1 to 2 h; *Mycobacterium tuberculosis* in macrophages, *Salmonella, Pseudomonas aeruginosa* and *Helicobacter pylori*, in epithelial cells or other type of cells (Cho et al., 2010; Huang et al., 2005; Kierbel et al., 2005; Takeshima et al., 2009). A possibility that an autocrine mechanism could be activating Akt might explain Akt phosphorylation at 60 min in the case of *B. cenocepacia* exposed macrophages; the release of ligands or cytokines such as TNF-α containing vesicles (Ha et al., 2008) by cells after initial bacterial contact might then activate Akt at later time points. To address this possibility, filtered supernatants harvested from cells incubated with bacteria at different time points can be used to stimulate fresh cells and then look at levels of Akt phosphorylation by immunoblots. Additionally, the use of inhibitors to prevent cytokines release such as Brefeldin A, which inhibits transport of proteins containing vesicles, and the use of protein synthesis inhibitors (i.e cycloheximide) might help to understand the autocrine bacterial effect on macrophages.

We next evaluated the impact of differential Akt activation by *B. cenocepacia* and *B. multivorans* on macrophage survival was addressed by looking at the kinetics of PI3K/Akt mediated cell survival and activation of downstream effects.

Apoptosis was induced in THP-1 derived macrophages by adding staurosporine, a broad inducer of apoptosis, to evaluate how *Bcc* exposure affected apoptosis in these cells. We found that both *B. cenocepacia* and *B. multivorans* exposed cells were resistant to apoptosis, a phenotype previously observed in *Helicobacter pylori,* a chronic gastric pathogen (Mimuro et al., 2007). We also observed that the anti-apoptotic effect in *B. cenocepacia* and *B. multivorans* was by preventing the cleavage of caspase 9 and apparently through bacterial internalization. Remarkably, PI3K/Akt had been implicated in the anti-apoptotic effect only of *B. multivorans*, a phenotype observed in other infections (Chugh et al., 2008; Nagy et al., 2009). A possible explanation of this effect might involve the prevention of staurosporine/protein kinases complexes formation by *B. cenocepacia* and *B. multivorans* exposed macrophages, and although there is low-affinity of staurosporine/PI3K interactions complexes (Walker et al., 2000) the rapid PI3K activation in *B. multivorans* exposed macrophages might prevent staurosporine-induced apoptosis unless a more specific PI3K inhibitor is present.

Next, we evaluated the activation of NF-κB in macrophages exposed to *B. cenocepacia* and *B. multivorans*. Akt phosphorylates Iκ-Bα in the cytoplasm, which in turn activates NF-κB in the nucleus to induce anti-apoptotic gene expression, which prevents apoptosis and thus promotes survival (Duronio, 2008; Melnikov et al., 2000). We found that *B. multivorans*, in contrast to *B. cenocepacia*, rapidly modulates the NF-κB system in macrophages by inducing Iκ-Bα phosphorylation and degradation. As expected, inhibition of the PI3K/Akt pathway prevented NF-κB induction. It remains to be determined if this inhibition also abolishes Iκ-Bα phosphorylation. Further, the activation of IKK/NF-κB and

PI3K/Akt pathways are independent of each other in PMCs when exposed to *B. cenocepacia* and NF-κB p65 phosphorylation is GSK3β-dependent (Cremer et al., 2011). However, in other system, it has been shown that NF- κB transcriptional activity is PI3K-dependent and requires p65 phosphorylation (Takeshima et al., 2009). To aid in generalizing our observations on one strain from each of the two species, we determined the relevance of the differential NF-κB activation by examining other strains of *B. cenocepacia* and *B. multivorans*. Both clinical and natural environmental *B. cenocepacia* isolates induced similar low level NF- κB activation, whereas all but one clinical isolate of *B. multivorans* (MAC4) induced the characteristic enhanced NF- κB activation.

These results suggest that *B. multivorans* has evolved a strategy to adapt and survive within eukaryotic cells by rapidly activating PI3K/Akt to prevent cell death. Since humans are incidental hosts, this survival strategy may have first developed to allow co-existence in the environment in selected eukaryotes such as amoebae (Lamothe et al., 2004). Although, the pre-treatment with cytochalasin D prior to exposure to bacteria and to the apoptotic inducer suggested that uptake was important to prevent apoptosis, further experiments are necessary, as is the confirmation of the expression of anti-apoptotic genes because of the greater NF- κB activation. There are some examples in the literature in which pathogens have developed diverse strategies to interfere with PI3K/Akt signaling pathway thereby inhibiting apoptosis and establishing a durable infection (Mimuro et al., 2007; Nagy et al., 2009; Pendaries et al., 2006; Wang et al., 2007). However, there is no published information on the mechanism or bacterial factors of Bcc responsible for regulation of suppression of apoptosis of host cells.

Once the ability of *B. cenocepacia* and *B. multivorans* to differentially activate the PI3K/Akt signaling pathway and the downstream targets was established, it was important to examine whether the induction of cytokines was affected by the inhibition of PI3K. Macrophages produced similar amounts of TNF-α after challenge with either *B. cenocepacia* or *B. multivorans,* a phenomenon which was significantly decreased in the presence of PI3K inhibitors. These observations are consistent with published data, in which, LY294002 pre-treatment of PBMC inhibited TNF-α induced by *B. cenocepacia* (Cremer et al, 2011). Significant differences in IL-10 and IL-1β induction were observed; *B. multivorans* induced more IL-10 whereas *B. cenocepacia* induced more IL-1β. The latter observation was in accordance with previous data from broncholalveolar lavage fluid of infected BALB/c mice, in which the level of IL-1β in *B. cenocepacia* C6433-challenged mice was significantly higher than levels detected in *B multivorans* C5568-challenged mice (Chu et al., 2004), and with results showing that *B. cenocepacia* J2315 induced significantly more IL-1β than *B. multivorans* 13010 in macrophages (McKeon et al., 2010). Production of both cytokines was decreased in the presence of the PI3K inhibitor, LY294002. This observation might be related to the difference in persistence and induction of host response of these two species. It will be important to understand the relationship between PI3K/Akt and NF-kB activation with the production of cytokines in our system as has been demonstrated in a recent study (Cremer et al., 2011) and in other cell systems (Huang et al., 2005; Rajaram et al., 2006; Takeshima et al., 2009)

The use of cell lines might be a limitation of this study. However, we used primary cultured monocytes/macrophages in some of the experiments to confirm observed PI3K

activation in immortalized cells, and similar trends in the release of cytokines were observed. Another limitation of our studies is the use of the pharmacological inhibitor LY294002 to establish the role of PI3K in the anti-apoptotic effect. Such inhibitors lack sufficient specificity to prove a role of the proposed signaling pathway (Hazeki et al., 2007). However, in this study, we have used several other inhibitors and obtained consistent results. Further studies with cells lines that have mutations of PI3K and Akt or cells transiently transfected with AKT-specific small interfering RNA (siRNA) might be complementary approaches to analyze the specific role of the PI3K/Akt signaling pathway; but plasmids harboring these mutants are difficult to transfect into hematopoietic cells (macrophages in this case) (Hazeki et al., 2007). Several studies use HEK293 cells and mutants therefore; they obviate the technical problems of transfection, but the intracellular environment in this type of cells may be different from those of phagocytes (Hazeki et al., 2007) and extrapolation to the effects of Bcc on macrophages will be difficult to make. The use of mouse models might increase the understanding of the role of PI3K/Akt pathway, however, they also face the problem of the capacity of the Akt isoforms to compensate for each other and other kinases that can substitute for Akt function that regulate similar substrates (such as serum- and glucocorticoid-induced protein kinase (SGK) (Franke et al., 2003). Complementary experiments using these tools together with the use of PI3K and Akt inhibitors might help to understand the cell biology of the interaction of Bcc and macrophages.

In this study, we have explored the role of the PI3K/Akt signaling pathway in macrophages exposed to *B. cenocepacia* and *B. multivorans*. Collectively, our data indicate that although both Bcc species attenuate apoptosis, *B. multivorans*, unlike *B. cenocepacia*,

interfered with apoptosis by activating the PI3K/Akt pathway. It was remarkable that *B. multivorans* induced earlier Akt phosphorylation, greater NF-KB activation and released less pro-inflammatory cytokine. Data from this study may contribute to understanding the mechanism of persistence of these bacteria in the lung as well as to ultimately enhanced therapy for CF patients.

Chapter 4: The phosphoinositol-3-kinase–Protein Kinase B/Akt pathway is involved in *B. cenocepacia* and *B. multivorans* internalization into macrophages

4.1 Introduction

The *Burkholderia cepacia* complex (Bcc) is a group of closely related Gram-negative bacteria and an important virulent group of opportunistic pathogens in cystic fibrosis (CF) patients (Mahenthiralingam et al., 2008; Mahenthiralingam et al., 2005; McClean and Callaghan, 2009; Speert et al., 2002). *Bcc* infections in CF patients have been associated with the "cepacia syndrome" characterized by a rapid decline in pulmonary function that rarely occurs during infection with other CF pathogen. Among the multiple species of the Bcc, isolates of *B. cenocepacia* and *B. multivorans* are the most prevalent in CF, accounting for 85-97% of the infections (Drevinek and Mahenthiralingam, 2010). *B. cenocepacia* appears to be more virulent than *B. multivorans* and generally causes more serious infections; however the mechanism behind this difference in virulence remains unknown (Govan et al., 1996).

B. cenocepacia K56-2, exhibits the capacity to reside in macrophages, amoebae, dendritic cells and airway epithelial cells (Lamothe et al., 2007; Lamothe et al., 2004; Lamothe and Valvano, 2008; MacDonald and Speert, 2008; Sajjan et al., 2006). Survival and replication of *B. cenocepacia* in airway epithelial cells may proceed as follows: after internalization, *B. cenocepacia* interacts with early endosomes, but they escape from late

endosomes to enter autophagosomes and ultimately replicate in the endoplasmic reticulum (Sajjan et al., 2006). In phagocytic cells, *B. cenocepacia* can persist in bacterial-containing vacuoles (BcCVs) that for the initial hours post-infection do not acidify and avoid fusion with lysosomes; this delay in phagolysosomal fusion is advantageous for the bacteria to activate other mechanisms that confer resistance to the hostile environment of the lysosome (Lamothe et al., 2007; Lamothe and Valvano, 2008). The involvement of virulence determinants such as the type three secretion system (T3SS) might help bacteria to initiate infection as was demonstrated in a mouse infection model (Tomich et al., 2003). However, another study did not find the involvement of the T3SS in the survival of *B. cenocepacia* within macrophages phagosomes (Lamothe et al., 2007). Furthermore, it is suggested that a second type four secretion system (T4SS) present in *B. cenocepacia*, Ptw, contributes to its survival and replication in both airway epithelial and macrophages cells early in the infection (Engledow et al., 2004; Sajjan et al., 2008a). Recent publications suggested that the type six secretion system (T6SS) plays a role in the disruption of the actin cytoskeleton and delayed NADPH oxidase activation in macrophages infected with *B. cenocepacia* by deregulating the Rho family GTPases (Flannagan et al., 2011; Rosales-Reyes et al., 2011). Although, *B. cenocepacia* produces a wide variety of potential virulence factors (Mahenthiralingam et al., 2005; McClean and Callaghan, 2009), the molecular mechanism underlying the process of persistence has not been adequately studied. Moreover, the mechanism and role of *B. multivorans* in the invasion of macrophages have not been determined. Likewise, the involvement of host signal transduction pathways in Bcc internalization is poorly understood. It has been shown that invasion of several pathogens require phosphoproteins such as the phosphoinositide-3-kinase (PI3K). PI3K modulates many cytoskeleton-based cellular

processes, including adhesion, spreading, macropynocitosis and phagocytosis (Coffer et al., 1998). In this respect, PI3K has been shown to be necessary for the invasion of macrophages or epithelial cells by several bacteria, including *Listeria monocytogenes* (Ireton et al., 1996; Shen et al., 2010), *Helicobacter pylori* type I (Allen et al., 2005), *Escherichia coli* (Celli et al., 2001), *Streptococcus pneumoniae* (Agarwal and Hammerschmidt, 2009) and *Pseudomonas aeruginosa* (Kierbel et al., 2005). In the latter, not only was PI3K necessary, but also Akt phosphorylation was required for bacterial entry into cultured epithelial cells. Conversely, the uptake or entry of pathogens such as *Salmonella typhimurium* (Steele-Mortimer et al., 2000) and *Francisella novicida* (Parsa et al., 2006) was not affected by the inhibition of PI3K pathway, although the PI3K/Akt signaling pathway was activated in macrophages by these pathogens.

In this study I explored the effect of the PI3K signaling pathway in the internalization of *B. cenocepacia* and *B. multivorans* by macrophages, this pathway is likely to play a critical role in direct and transcriptional control of host cell survival that might benefit these two CF pathogens. My preliminary data suggest that PI3 kinase is important for the entry and internalization of *B. cenocepacia* but only early in the phagocytic process for *B. multivorans.* The colocalization of *B. multivorans* with a late phagosomal marker in macrophages was accomplished after 6 h post challenge.

4.2 Hypothesis

The Phosphoinositide-3-kinase/Akt signaling pathway plays a role in the internalization of members of the Bcc into macrophages.

4.3 Aim

To evaluate whether *Burkholderia cenocepacia* and *Burkholderia multivorans* requires the phosphoinositide-3-kinase/Akt signaling pathway to induce their own internalization into macrophages; such a strategy might be beneficial for survival within the hosts cells.

4.4 Experimental procedures

4.4.1 Growth conditions of bacteria and cells

Bacteria growth and cell maintenance were performed as previously described in Chapter 3 section 3.2.1 of this work. Briefly, the *B. cenocepacia* K56-2 and *B. multivorans* C5568 isolates were grown in 5mL Luria-Bertani (LB) broth overnight with shaking at 37 ºC to stationary phase (MacDonald and Speert, 2008). The human monocytic THP-1 cell line was obtained from the American type Culture Collection (ATCC) and was maintained in RPMI 1640 containing 10% Fetal Bovine Serum (Invitrogen), 2mM L-glutamine (Invitrogen), 1mM sodium pyruvate (Invitrogen) and 1% antibiotic-antimycotic (Invitrogen) at 37 ºC in 5% CO_2. Monocytic differentiation to the macrophage-like adherent phenotype was induced with 0.1 μM of phorbol-12-myristate 13-acetate (PMA, Cell Signaling Technology). For all experiments, differentiated macrophages were cultured (starving conditions) in Minimum Essential Medium (MEM, Invitrogen) plus 10 mM Hepes for 16-24 h prior to bacterial challenge.

4.4.2 Growth curves analysis

Standard growth curves were performed for *B cenocepacia* and *B. multivorans.* Bacteria were grown in 5 mL of LB broth at 37 °C with agitation for 16 h to stationary phase. Bacteria suspensions were adjusted to an optical density (OD) at 600nm of 0.1 in LB broth. Four hundred microliters of bacterial suspension was added in duplicate to 100-well microtiter plate. The growth rates were determined using Bioscreen C (Oy Growth Curves Ab Ltd) for 24h at 37 ºC under continuous shaking with readings every 15 min.

4.4.3 Bcc internalization assay

To determine the effect of PI3K pathway inhibition on the internalization of Bcc into macrophages, THP-1 derived macrophages, treated or not with 50 μM of LY294002 for 30 min, were exposed to *B. cenocepacia* and *B. multivorans* at an MOI of ~10 at 37 °C for 2 h. Bacterial dilutions were serially plated in LB agar to obtain the exact numbers of bacteria added in each experiment. Cells were washed 3 times with PBS and incubated with MEM plus 10 mM HEPES medium containing meropenem (8 ug/mL) to kill extracellular bacteria (MacDonald and Speert, 2008). After incubation up to 24 h in total, the cells were washed again 3 times with PBS and lysed in 0.25% Triton X-100 for 30 min. The released intracellular bacteria were enumerated by plating serial dilutions of cell lysates on LB agar plates (Kazmierczak et al., 2001; Sajjan et al., 2006). Experiments were performed in triplicate wells on 3 different occasions for each time point. The relative invasion (%) was calculated as follows: number of bacteria recovered / number of bacteria added × 100.

4.4.4 Immunofluorescence staining and microscopy

Immunofluorescence assay was performed as previously described (Sajjan et al., 2006), with the following modifications: THP-1 derived macrophages (0.5×10^6) seeded in a 24 well plates containing glass coverslip (Fisher) were exposed to bacteria at an MOI of 50 and incubated up to 6 h. Cells were washed three times with PBS ++ (Invitrogen) to remove unbound bacteria and fixed with 2.5% paraformaldehyde (Electron microscopy science) at 37 °C for 10 min. Cell were permeabilized by incubation at room temperature with PBS ++ containing 10% goat serum (Sigma) and 0.2% saponin (Sigma) (SS-PBS) for 10 min. Cells were incubated with appropriately diluted primary antibodies for 60 min in SS-PBS. Bound

antibodies were detected by anti-mouse LAMP-1 CD107a (BD Bioscience) conjugated with either Alexa Fluor-568 or anti-rabbit JTC-Bcc conjugated with Alexa Fluor-488. Cells were counterstained and mounted with DAPI prolong gold (Invitrogen). Slides were viewed on a Fluoview Olympus confocal laser microscope at the UBC Bioimaging Facility using 60× objective, pseudocolored and assembled into Figures using Image J software 1.44e (Wayne Rasband, National Institutes of Health, USA).

4.4.5 Statistical analysis

All experiments were performed on at least 3 independent occasions. Statistical analysis was performed by repeated measure of ANOVA, using GraphPad Prism (version 5.0) or Student's *t* test as appropriate. $P<0.05$ was defined as the threshold for statistically significant differences

4.5 Results

4.5.1 Growth curves for *B. cenocepacia* and *B. multivorans*

Different growth rates may affect the number of intracellular bacteria following uptake by host cells. Consequently, prior to observation of internalization into macrophages, extracellular growth of *B. cenocepacia* and *B. multivorans* was compared in LB medium. Using a starting suspension of bacteria at OD of 0.1 at 600 nm there was a difference in growth rate starting at 1:15 h ($P < 0.01$); and the doubling time was 1:15 h and 1:00 h for *B. cenocepacia* and *B. multivorans* respectively (Figure 4.1).

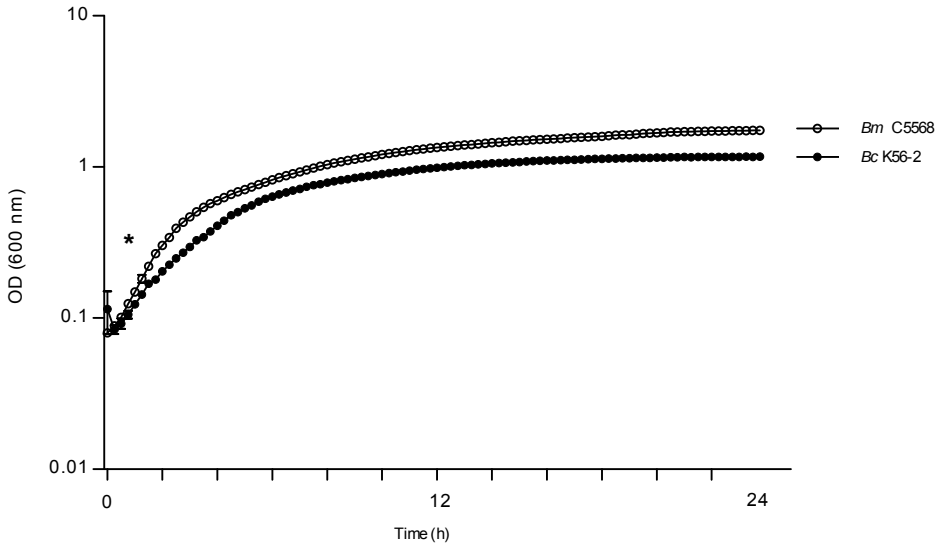


Figure 4.1: Standard growth curves of *B. cenocepacia* and *B. multivorans*. Growth curves were constructed in Luria Bertani broth using Bioscreen C for 24h at 37 ºC under continuous shaking with readings every 15 min. Data are averages of 2 independent experiments. Error bars indicates SEs; * denotes $P < 0.01$ difference between the mean of OD starting at 1:15 h until 24 h as measured by repeated measure of analysis of variance with Bonferroni post-test correction.

4.5.2 Effect of the inhibition of PI3K in the internalization of Bcc

To address the possibility that the PI3K signaling pathway is involved in the internalization of *B. cenocepacia* and *B. multivorans,* we assayed the effect of LY294002, a cell-permeable, low-molecular-weight inhibitor (Vlahos et al., 1994). THP-1 derived macrophages were exposed to *B. cenocepacia* and *B. multivorans* at an MOI of 10. After 2 h of incubation, cells were washed and incubated with medium containing the antibiotic meropenem to kill extracellular bacteria and incubated for up to 24 h in total. Figure 4.2 shows that LY294002 blocked *B. cenocepacia* internalization at all time points, whereas there were only differences in *B. multivorans* internalization at 4 and 24 h. In a separate experiment, the growth of bacteria in cell culture media in the presence or absence of 50 μM LY294002 was examined to determine the effect of LY294002 on bacteria growth. Results indicated that the reduced recovery of bacteria from LY294002-treated cells was due to inhibition of internalization rather the effect of LY294002 on bacteria growth (data not shown). Cells treated with Cytochalsin D, which inhibits actin rearrangement, were used as negative control for internalization. Internalization was reduced to less than 5% in both *B. cenocepacia* and *B. multivorans*-challenged cells at all-time points. Cell viability was assessed in all conditions; there were ~29 % apoptotic cells at 24 h but less than 10 % at early time points. Additionally, LY294002 and cytochalasin D alone do not induce significant apoptosis in macrophages (Chapter 3 of this work; Figure 3.2, 3.3 and 3.5). We also observed significantly higher percentages of internalization of *B. cenocepacia* within macrophages than *B. multivorans* at 8 and 16h ($P < 0.01$). This association was previously seen when comparing *B. cenocepacia* J2315 with *B. multivorans* 13010 in macrophages at the MOI of 10 in macrophages (McKeon et al.). Together, these data suggested that PI3K

activity is required for *B. cenocepacia* internalization into macrophages, whereas *B. multivorans* needs PI3K activity only at early and late time points.

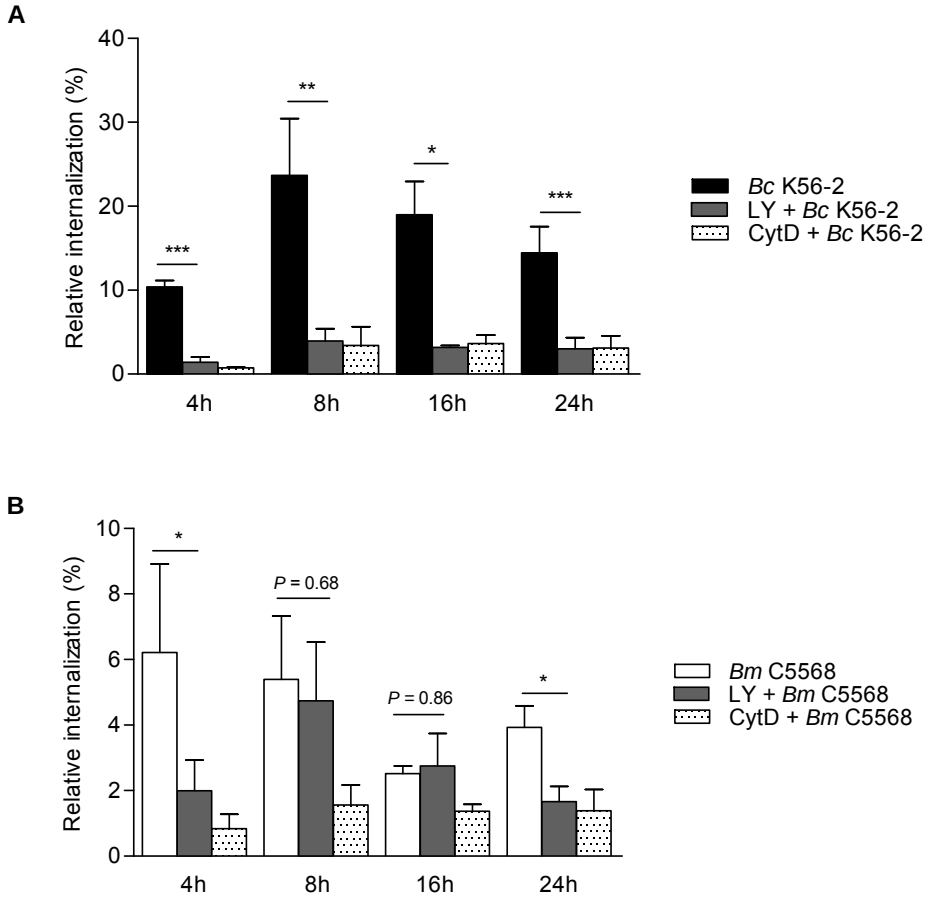


Figure 4.2: Effect of the pharmacological inhibitor LY294002 of PI3K on Bcc internalization. THP-1 derived macrophages were pre-treated or not with 50 μM of LY294002 (LY) for 30 min before exposure to **A.** *B. cenocepacia* K56-2 or **B**. *B. multivorans* C5568 for 2h. Cells were washed and medium containing meropenem antibiotic was added. Cells were incubated for 4, 8, 16 and 24 h in total after exposure to bacteria. Cells treated with cytochalisn D (CytD) (5 μg/mL) was used as a negative control for internalization. Data are average of 3 independent experiments done in triplicate each time. Error bars indicates SDs; * denotes $P < 0.05$, ** denotes $P < 0.01$ and *** denotes $P < 0.001$ as measured by Student's *t* test.

4.5.3 *B. multivorans* internalization detected by confocal microscopy.

To determine whether PI3K plays a role in the internalization of Bcc, we initially confirmed the internalization of bacteria in our system. THP-1 derived macrophages were exposed to bacteria at an MOI of 50 and incubated for 4 and 6 h. Cells were then fixed and the samples were analyzed by confocal and conventional fluorescence microscopy. Our preliminary data suggested that *B. cenocepacia* and *B. multivorans* are internalized by macrophages after 4 h (data not shown). By 6 h post-exposure there was an increasing percentage of internalized bacteria, and *B. multivorans* was partially co-localizing with LAMP-1, a late lysosomal maker (Figure 4.3). Experiments to confirm the uptake and internalization of *B. cenocepacia* at different time points are in progress.

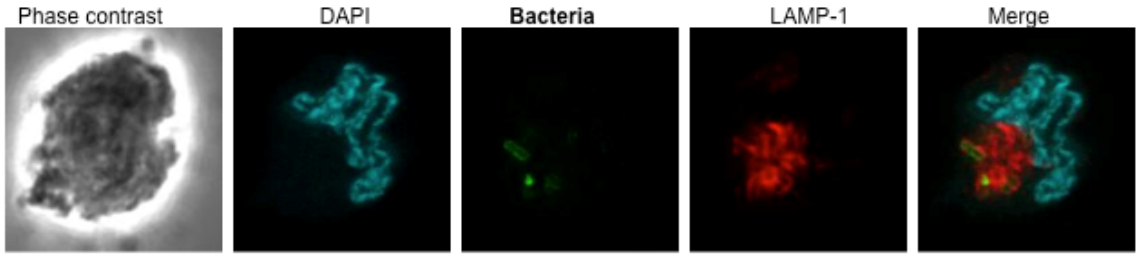


Figure 4.3: Colocalization of *B. multivorans* with late lysosomal maker. THP-1 derived macrophages were seeded in 24 well plates containing slides and exposed to *B. multivorans* C5568 at an MOI of 50 for 6h. Cells were washed to remove unbound bacteria, fixed and blocked with goat serum and incubated with antibody to LAMP-1 and *Bcc*. Cell were washed and bound antibody was detected by anti-mouse conjugated to Alexa Fluor-568 (red, for detection of LAMP-1) or anti-rabbit JTC-Bcc conjugated with Alexa Fluour-488 (green; for detection of bacteria).

4.6 Discussion

Phagocytosis is essential for the scavenging of dead cells and for eliminating invading microorganism and it is an essential component of the innate immune system (Kinchen and Ravichandran, 2008). The PI3K pathway is crucial player in the entry, phagosome formation and maturation in response to several pathogen infections (Coffer et al., 1998). *B. cenocepacia*, a member of the Bcc, has the capacity to alter phagosomal development and acidification (Saldias and Valvano, 2009)

In this study, we investigated whether the activation of PI3K signaling is necessary for *B. cenocepacia* and *B. multivorans* entry into cultured macrophages. Results showed that internalization of bacteria in the presence of a pharmacological inhibitor of PI3K was decreased. The effect of the inhibitor was observed at all time points in macrophages challenged with *B. cenocepacia*. In this respect, PI3K inhibition by wortmannin, another PI3K inhibitor, has also been shown to inhibit intracellular replication of *B. cenocepacia* K56-2 within epithelial cells (Sajjan et al., 2006). However, in a recent study, PI3K did not influence the intracellular replication of *B. cenocepacia* K56-2 within macrophages (Cremer et al., 2011). The difference between these findings and our results might be due to the use of mouse macrophages vs. human macrophages or in the different methodology used to challenge cells with bacteria (i.e. we did not perform a centrifugation step). In the case of internalization of *B. multivorans* into macrophages, PI3K pathway was important at early and late time points; one explanation could be that bacteria activate early PI3K pathway (Chapter 3 of this work) and once bacteria are inside cells, the signal turns off because of phagocytosis

progression. Bacterial killing by the host might be another explanation, since fewer bacteria were covered than *B. cenocepacia* at all-time points, in this way the inhibition of PI3K pathway might not have an effect on the internalization. Studies on the mechanism of intracellular killing as well as phagocytosis in *B. multivorans* challenged macrophages are needed.

In both *B. cenocepacia* and *B. multivorans* challenged cells; cytochalisn D prevented internalization as expected. These results will be confirmed by evaluating actin rearrangement after bacteria internalization by a different method such as staining F-actin with phalloidin; F-actin polymerization has been observed in CF airway epithelial cells, IB3, after the uptake of *B. cenocepacia* (Sajjan et al., 2006). Similarly, it was shown that *B. multivorans* promoted disruption of the active filament networking during epithelial infection (Schwab et al., 2003). In a murine cell line and human macrophages, it was demonstrated that *B. cenocepacia* alters the actin cytoskeleton of infected macrophages and delays NADPH oxidase complex assembly in a T6SS depend-manner (Flannagan et al., 2011; Rosales-Reyes et al., 2011). By the use of pharmacological inhibitors and by confocal microscopy, I will determine whether PI3K regulates *B. cenocepacia* and *B. multivorans*-induced actin polymerization in macrophages. Additionally, it will be important to evaluate the distribution of phosphatidylinositol-3,4,5-trisphosphate [$PI(3,4,5)P_3$], a short-lived phospholipid generated by PI3K, in Bcc interaction with macrophages. $PI(3,4,5)P_3$ is involved in the formation of phagosomes, in the modulation of the actin cytoskeleton and is essential for bacterial engulfment (Vieira et al., 2001). This phospholipid has been shown to accumulate at the site of entry of *Pseudomonas aeruginosa* and *Helicobacter pylori* early in

the interaction with epithelial cells and macrophages respectively, and its distribution was reduced in the presence of PI3K inhibitors (Allen et al., 2005; Kierbel et al., 2005). However, a recent study showed that the distribution of $PI(3,4,5)P_3$ and $PI(4,5)P_2$, another PI3K lipid product, were not altered and actin polymerization was not disrupted during *B. cenocepacia* infection (Flannagan et al., 2011).

By using confocal microscopy, very preliminary data demonstrated that *B. multivorans* internalized into macrophages after 6h partially colocalize with LAMP-1, a lysosomal marker. Unlike, *B. cenocepacia*, there is little information on the intracellular life style of *B. multivorans* in macrophages. In *B. multivorans*-challenged mice, bacteria were observed in membrane-bound vacuoles of macrophages in the alveolar space at day 4 of infection (Chu et al., 2004). However, more experiments using early phagosomal markers (EEA1, Rab5) are needed. Nevertheless, our goal in this study is to evaluate the influence of PI3K on Bcc entry. Our current experiments with confocal microscopy and inhibitors for these pathways are conducted to evaluate the activation of PI3K and Akt after bacteria challenge macrophages. Finally, results from these studies might provide knowledge of the activation of PI3K by *B. cenocepacia* and *B. multivorans* and the effects on macrophage function that could extend beyond modulation of bacterial engulfment.

Chapter 5: Conclusion and future directions

By examining the proteome of persistent *B. pseudomallei* from melioidosis patients and the interaction of two opportunistic CF pathogens, *B. cenocepacia* and *B. multivorans*, with macrophages, this thesis has explored the diverse topics of bacterial persistence in chronic disease. The aim of this section is to present the overall conclusions of the major findings of the thesis and finally future directions will be suggested.

5.1 Proteome analysis of persistent *B. pseudomallei*

The goal of this part of the thesis was to identify the bacterial determinants involved in relapsed melioidosis using proteomic approaches: two dimensional gel electrophoresis and isotope tagging for relative and absolute quantification. Whole proteins from morphotypes I and III, isolated from initial and relapsed infection respectively from a melioidosis patient, were investigated. Several proteins were up-regulated in morphotype III, which possesses a putative fitness advantage together with morphotype II for survival and persistence *in vivo* and *in vivo* models (Chantratita et al., 2007; Tandhavanant et al., 2010). Flavohemoprotein, arginine deiminase pathway, flagellin and HSP20/alpha crystalline family protein among other proteins were greatly up-regulated in morphotype III compared to I (Table 2.1 and 2.2), all of which could logically be involved in survival and persistent infections. Hcp-1, a member of the T6SS, was found to be up-regulated in morphotype I compared to the morphotype III isolate from this patient. It is important to mention that several hypothetical proteins with unknown functions were found in both morphotypes. A morphotype I isolated during the relapse infection of this patient was also investigated; the protein profile

resembled that of the initial isolate morphotype I. Analysis of proteins from a second patient displayed minor differences between isolates, both of which were morphotype I; protein profiles were similar to morphotype I in the first patient. This also served as our internal control for variations in the technique, which was highly reproducible.

The fact that we have tested isolates from only two patients and the low proportion of morphology switching between morphotype I and III *in vivo* (Maharjan et al., 2005) is one weakness of this study. Another critical point is that the expression of protein of these morphotypes on a rich bacteriological medium grown at the stationary phase might not be related to protein expression in the host. If variation exists between *B. pseudomallei* isolates that PFGE technique did not discriminated, a whole genome sequence might confirm that these isolates tested here are the same clone. Additionally, for more information, the use of a reference strain might be necessary to compare the proteomic profiles of these clinical isolates since there might be many proteins that are either missing or in addition. However, analysis of a reference strain should be performed together with the clinical isolates in order avoid changes in experimental conditions. Nevertheless, we found clear difference between morphotypes I and III that might have a biological significance and this thesis provides a list of the differentially up-regulated proteins for future studies.

Finally, two of the major strengths of this research are the use of clinical isolates from relapsed melioidosis patients as well as the use of two complementary proteomic approaches that resulted in a solid identification of proteins putatively involved in persistence. These data represent an important contribution to this emerging field by identifying proteins for

further investigation. We have already begun such a study with purified and crystallized alpha crystalline from collaborators at Seattle Biomedical Research Institute.

5.2 The involvement of phosphoinositide 3-kinase/Akt signaling pathway in the interaction of Bcc with macrophages

Through the investigation of the interaction of human macrophages and Bcc organisms, we found that *B. cenocepacia* and *B. multivorans* were able to differentially induce cellular processes *in vitro*. Although, both species were able to inhibit macrophage programmed cell death after the addition of a potent inducer of apoptosis, *B. multivorans*, unlike, *B. cenocepacia* utilized the host phosphoinositide 3-kinase/Akt signaling pathway to do so. This phenotypic difference might be the result of the capacity of *B. multivorans* to rapidly induce activation/phosphorylation of this pathway early in the interaction, as well as its rapid induction of NF-κB and phosphorylation of IκBα with concomitant induction of anti-apoptotic genes. Conversely, *B. cenocepacia* showed a more delayed activation of these cellular processes and the signaling pathway induced to prevent apoptosis remains to be determined. Both species were able to inactivate caspase 9 while preventing apoptosis, however, evidence that different apoptotic pathways (intrinsic vs. extrinsic) might be induced at different time points needs to be established.

Macrophages exposed to *B. cenocepacia*, unlike *B. multivorans*, induced predominantly pro-inflammatory cytokines, and it was mediated by the PI3K pathway. Furthermore, internalization into macrophages of *B. cenocepacia* and *B. multivorans* was differentially mediated by PI3K. The relevance of these different host cellular activations to

the overall pathogenesis in CF needs to be established in relevant systems. However, it is recognized that *B. cenocepacia* and *B. multivorans* interact in dramatically distinct ways in both *in vivo* with mouse macrophages (Chu et al., 2004) and *in vitro* with dendritic cells (MacDonald and Speert, 2008), which reflects their different clinical outcomes. *B. cenocepacia* is considered a more dangerous pathogen than *B. multivorans* for the CF patients whereas *B. multivorans* is more likely to persist without inducing inflammation (Govan et al., 1996). To treat persistent opportunistic pathogens in CF, it is necessary to understand first the complex and multifactorial processes of the interaction with the host and the signals they induce. The work performed in section 3 and 4 have made a contribution to this effort.

5.3 Future directions

In order to validate the differentially up-regulated proteins in *B. pseudomallei* from a patient with persistent melioidosis, several confirmations should be performed as well as mechanistic questions answered. First, with the use of molecular approaches and mRNA expression, relevant up-regulated proteins in the morphotype III relapse isolate need to be quantified not only in this particular isolate 2644cIII but also in other clinical isolates to determine if our data are generalizable. A possibility of coupling the proteomic data with transcriptome might increase the information of the differential unregulated proteins. Then, the construction of bacterial mutants together with their complemented and wild type variants will be necessary for the analysis of their role in pathogenesis of melioidosis. It will be important to examine the capacity of these mutants in the ingestion by phagocytes, macrophage survival, intracellular bacterial survival or replication, and susceptibility to host

antimicrobial peptides. These analyses could be performed using immortalized human cells, such as THP-1 cell line, or primary human macrophages. Although, Tandhavanant et al., 2010 tested morphotypes I, II, and III, these isolates were generated under *in vitro* conditions; it will be critical to look at the interaction of morphotype I and III isolates described in this study with macrophages to better understand their biological roles since they are natural mutants directly isolated from a melioidosis patient. One candidate protein to be tested will be the flavohemoprotein (HmpA) which is involved in detoxification of nitric oxide (Gardner et al., 1998), it is important for survival of *E. coli* in macrophages (Stevanin et al., 2007), and it is one of most highly up-regulated proteins in morphotype III (Table 2.2). Proteins from the arginine deiminase pathway, carbamate kinase and ornithine carbamoyltransfrerase were also up regulated in morphotype III and will be important to test. A recent study (Chantratita et al., 2012) also described the up-regulation of arginine deiminase and carbamate kinase in a morphotype III strain generated *in vitro* from morphotype I*;* although their role in survival in acid was demonstrated, their role in susceptibility to host defenses was not established and their involvement in persistence remains to be determined. HSP20/alpha crystalline family protein, thought to be important in latent *M. tuberculosis*, will be essential to analyze; macrophages can be exposed to pure protein and then the cell morphological changes, rates on cells survival (apoptosis/necrosis), release of cytokines among other cellular processes can be evaluated. These studies are now underway in the laboratory.

Immortalized cell lines are suitable in a model system, and they are a valuable tool since they provide a homogeneous population for the investigation of biochemical

mechanisms and signaling pathways; however primary cells are probably most closely correlated with the *in vivo* situation of CF and therefore a more relevant model system for understanding the pathogenesis of human disease. With an access to primary CF cells especially macrophages, the following experiments can be reproduced in order to validate the *in vitro* findings in chapters 3 and 4 of this thesis:

A. Validation of the anti-apoptotic activity of both *B. cenocepacia* and *B. multivorans* focusing on the PI3K signal pathway

   Parallel exposure of both *B. cenocepacia* and *B. multivorans* to CF macrophages and assessment of cell survival after pre-treatment with PI3K pathway inhibitors will indicate the bacterial effect on apoptosis.

B. Validation of differential PI3K phosphorylation and NF-κB activation

   Collection of proteins at different time points from CF cells after *B. cenocepacia* and *B. multivorans* exposure will determine the differential PI3K phosphorylation and NF-κB activation that will correspond to the cell survival phenotype in each case.

C. Validation of the internalization of bacteria in the presence of PI3K inhibitors

   Exposure of *B. cenocepacia* and *B. multivorans* to CF macrophages in the presence of PI3K inhibitor will demonstrate the role of a PI3K pathway in phagocytosis by CF mononuclear cells.

D. Cellular localization of *B. multivorans* in CF macrophages in the presence of PI3K/Akt signaling pathways inhibitors

B. multivorans does not appear to require activation of the PI3K pathway to be internalized by immortalized cells; therefore it will important to evaluate bacterial localization and the role of PI3K/Akt pathway in a CF cellular environment.

In summary, data presented in this thesis provide evidence that implicates modification of protein expression in *Burkholderia pseudomallei* during latency, resulting in an altered morphotype that may allow the bacteria to persist inside the host. Furthermore, the studies conducted with Bcc organisms, *B. cenocepacia* and *B. multivorans*; provide evidence that these organisms potentially differentially modulate the PI3K/Akt pathway in order to persist within macrophages. Thus, strategies to inhibit either expression of those proteins putatively responsible for latency or approaches to generate inhibitors to block the activation and modulation of signaling pathways, such as PI3K, may provide useful proof of principle for their role in chronic CF infections.

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