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Functions of *Burkholderia* virulence factors: Input from proteomics and DNA microarray analyses

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Abstract

Burkholderia spp. consist of organisms that are extremely diverse and versatile living in the soil. Members of this genus, which include *B. pseudomallei, B. mallei, B. thailandensis* and *B. cepacia*, are capable of causing severe, life-threatening opportunistic infections in patients who are immunocompromised. The underlying virulence mechanisms of these bacteria, their interactions with the host including the host defense mechanisms may be reflected by changes in the expression of proteins of both the pathogen as well as the host. In this article, we review the current knowledge on these interactions of *Burkholderia spp*. pathogens and their host mainly from the perspective of data that was generated from recent proteomics and DNA microarray investigations.

Burkholderia species

The genus *Burkholderia* was first described in 1949, by Burkholder [1]. It consists of 43 obligately aerobic species of non-sporing, motile, bacillus, Gram-negative bacteria that are extremely diverse and versatile [2, 3]. These bacteria are commonly found in a variety of eco-systems worldwide, including the soil and groundwater. Although most species in the genus *Burkholderia* are harmless and not pathogenic for healthy individuals, a few are capable of causing severe, life threatening opportunistic infections in immunocompromised patients. Among the species, *B. pseudomallei* and *B.*

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mallei are the most pathogenic and have been exploited as potential biological weapons [Centres for Disease Control (CDC)] [4, 5]. In addition, their survival and persistence in the environment as well as in the host offer a notable example of bacterial adaptation [4].

Among the *Burkholderia spp*., *B. pseudomallei* and *B. cepacia* cause persistent and recrudescing infections in the human host. *B. pseudomallei*, the causative agent of the fatal disease melioidosis, is endemic predominantly in South East Asia and Northern Australia, while its sibling species, *B. mallei*, is the causative agent of glanders disease in horses, mules and donkeys. Humans can also acquire glanders from the infected animals by contact via ingestion or inhalation of the organisms. In contrast, *B. thailandensis*, a closely related species that shares 99% similarity of its genes with both the pathogenic species *B. pseudomallei* and *B. mallei*, is apparently non-pathogenic in humans.

Also recognised as a significant human pathogen, *B. cepacia* is naturally found in relatively high population in the moist soil and in particular the rhizosphere of plants and freshwater environments. It is usually found in hospitals as an opportunistic pathogen in wound and pulmonary infections and is associated with life-threatening pulmonary infections in patients with cystic fibrosis.

Pathogenesis of *Burkholderia spp.*

In order to establish an infection, bacteria have to colonise, invade, replicate, survive and persist in the host cells, as well as evade the host immune response [6]. Discovery of the bacterial virulence factors are important in understanding pathogenesis and interactions of the factors with the host. The virulence factors in bacteria may be associated with unique structural components of the cells (eg. capsules, fimbriae, LPS or other cell wall components) or actively secreted to invoke damage to the host tissues and/or protection of the bacteria against host defences.

Burkholderia Virulence Determinants

Virulence factors interact directly with host tissues or aid in concealing the bacterial surface from host defence mechanisms [7]. The virulence factors of *Burkholderia spp.* can be divided into several groups on the basis of the mechanisms of virulence and functions.

(1) Membrane surface proteins

Microbial adhesion to the host tissues can be mediated by individual proteins and carbohydrate molecules or by sophisticated organelles such as pilus and non-pilus adhesins [7, 8]. The importance of type IV pilus (TFP) for pathogenesis of *B. pseudomallei* has been reported [9]. Similarly, presence of type IV pilus has also been reported in *B. mallei* [10]. However, in *B. cepacia*, a 22 kDa cable pilus protein has been shown to facilitate bacterial binding to both mucin and CF respiratory epithelia, in mediating colonisation [11, 12, 13].

Flagella proteins are also recognized to play a role in virulence as they allow motility of the bacterium to the cells at the target site of infection [14, 15, 16]. In *B. cepacia*, flagella also facilitate penetration of the host epithelial cell and contribute to the onset of systemic spread of the organism. Tomich *et al.* (2002) reported that the wild type motile *B. cepacia* is more invasive in lung epithelial cells compared to the non-motile mutant with a defective component of the motor-switch complex of the flagellar basal body [17]. In contrast, conflicting evidences have been reported for the involvement of flagella in the virulence of *B. pseudomallei*. While DeShazer *et al.* (1997) reported that no attenuation was observed in fliC *B. pseudomallei* 1026b transposon mutant in the diabetic rat or Syrian hamster melioidosis models [18], Chua *et al.* (2003) showed that a fliC mutant was indeed attenuated in BALB/c mice infected by either the intranasal or intraperitoneal routes [14]. In addition, Inglis *et al.* (2003) reported that the fliC mutant was unable to adhere to the cells of *A. astronyxis*, compared to the wild type [19]. In the case of *B. mallei*, frameshift or insertion mutations of the flagella biogenesis genes had also been reported. These mutations are most likely responsible for the lack of flagella and the non-motile phenotype [20].

(2) Secretory proteins - enzymes and toxins

Bacterial pathogens use distinct secretion systems to transport protein toxins from their cytoplasm into the host or extracellular matrix [21, 22]. The pathways have been classified based on the molecular nature of the transport machineries and their catalysed reactions as: (1) type I pili; (2) auto-transporters; (3) type I secretion; (4) type II secretion (general secretory

pathway and type IV pili; (5) type III secretion and flagella; (6) type IV secretion and DNA conjugation; (7) type V secretion, autotransporters proteins; and (8) type VI secretion [23]. In *Burkholderia spp.* the type II, III, and VI secretion systems have been described [24, 25, 26, 27, 28, 29].

Among the secretory proteins of *B. pseudomallei*, *B. mallei* and *B. cepacia* that are associated with virulence include endotoxin, exotoxin, and protease, as well as lecithinase, catalase, peroxidase, superoxide dismutase, cytotoxic exolipid, lipase, hemolysin, and water-soluble siderophore for iron acquisition from the host, which contributes to its survival and maintenance [30, 31, 32, 33, 34, 35, 36, 37].

(3) Cell wall and outer membrane components - lipopolysaccharide (LPS) or endotoxin

In Gram-negative bacteria, the outer membrane lipopolysaccharide (LPS) can protect against complement-mediated lysis. LPS has a dual role in pathogenesis; contribution to antimicrobial peptide resistance and promotion of a potent pro-inflammatory response [38]. It is also the most common bacterial component that is implicated in initiating sepsis [39]. Novem *et al.* (2009) reported that the LPS of *B. pseudomallei* stimulated lower levels of TNF-α, IL-6, and IL-10 in both human and murine macrophages, compared to the response to the LPS of *B. thailandensis in vitro* [40]. This may be attributed to differences of the lipid A structure of LPS between the two species. The Lipid A structures of *B. thailandensis*, *B. cepacia* and *B. mallei* were found to be similar and consisting of a mixture of tetra- and penta-acylated with substitutions of C14:0, C14:0(3-OH), and C16:0(3-OH), the C14:0(2-OH) structural substitution is apparently unique to *B. pseudomallei*.

(4) Other virulence factors - biofilm formation, siderophores and quorum sensing (QS)

Acquisition of iron from transferrin and lactoferrin is essential for microbial growth. *Burkholderia spp.* have evolved high-affinity iron uptake systems involving siderophores, which are low-molecular-weight iron chelators.

Burkholderia spp. are also known to form thick mature exopolysacharride (EPS) consisting of proteins and enzymes encoded by the *bce-I* gene cluster. In *Burkholderia*, the EPS is composed of branched acetylated heptasaccharide repeating units [41, 42]. These EPS contribute to the formation of mature biofilms in *B. cepacia, B. pseudomallei and B. mallei* strains, resulting in the persistence of their infections [43]. In addition, Dales *et al.* (2009) have reported that the *B. cepacia* biofilms were found to be more resistant to antibiotics compared to *P. aeruginosa* biofilms [44].

Methods to Elucidate Bacterial Virulence Factors or Host Response

Comparative or quantitative proteomics and mass spectrometry (MS) have become important techniques in the identification of low abundant virulence factors of pathogenic bacteria. This is essential in the understanding of pathogenesis and for identification of novel drug targets and vaccines. Comparative proteomics defines the differences in expression of proteins among different biological states (e.g., control vs. treatment, healthy vs. disease, specific genotype vs. wild type). Quantitative proteomics is a powerful approach used for both discovery and targeted proteomic analyses to understand the global proteomic dynamics in an organism. These techniques offer many advantages: I) fast resolution of the proteome of interest, II) relatively cheap, III) direct evaluation of p*I* and MW of the protein, IV) possibility to make replicates in the same run and V) allows multiple comparisons. Unfortunately it also suffers from several disadvantages: I) poor resolution of proteins with the extreme pH, II) very hydrophobic protein loss, III) absence of proteins of high and low molecular weights, IV) inability to detect proteins without lysine. In the past few years, several proteomics studies on different growth conditions or cellular fractions of *Burkholderia spp.* have been carried out. In this section, we review the comparative and quantitative analysis of *Burkholderia spp.* and the host factors.

Comparative proteomics

A total of 14 differentially expressed proteins (Table 1) were identified using stationary phase intracellular proteins of *B. pseudomallei* and *B. thailandensis* separated on two-dimensional gel electrophoresis (2D-GE) and analyzed using the matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF/MS) [45]. Of these 14 proteins, six were

highly expressed in *B. pseudomallei*. This included two hypothetical proteins, BPSL1549 and BPSL1958, which were suggested to have a possible role either as a specific protein or gene marker. This study therefore, illustrates the utility of comparative proteomics analysis to identify differences in the protein profiles between two closely related species that include pathogenic and non-pathogenic strains, and at the same time, enabled the identification of possible virulence markers of a bacterial pathogenic strain [45].

Table 1. Differentially expressed proteins of *B. pseudomallei* and *B. thailandensis.* Adapted and modified from (Wongtrakoongate *et al.* (2007) [45]).

Protein	*Burkholderia pseudomallei*	*Burkholderia thailandensis*
1. Phosphatidyl ethanolamine-binding protein	√	
2. Bacterial cell wall degradation protein, LysM	√	
3. Phosphoglucomutase	√	
4. Succinyl-CoA (3-ketoacid coenzyme A transferase subunit B	√	
5. Succinyl-CoA (3-ketoacid coenzyme A transferase subunit B		√
6. Chaperonin GroEL	√	
7. GroES	√	
8. Oxidoreductase (AhpC)	√	
9. Phasin-like protein (PhaZ)		√
10. Superoxide dismutase (SodA)	√	
11. Hypothetical protein BPSL 1549*	√	
12. Hypothetical protein BPSL 1958*	√	
13. Hypothetical protein BPSS 0212	√	
14. Hypothetical protein BPSS 0683	√	

The √ represents the present or the higher levels of protein expressions and asterisks represent the hypothetical proteins are not found in *Burkholderia thailandensis* genome database.

In order to map and identify *B. cenocepacia* H111 intra-, surface-associated and extracellular proteins, Riedel *et al.* (2006) performed a comparative proteomics study [46]. A total of 308 intracellular, 74 surface-associated, and 63 extracellular proteins spots were successfully identified. The majority of these proteins were found to be linked to housekeeping functions involved in energy production, amino acid metabolism, protein folding, post-translational modification and turnover, and translation. In addition, a significant number of truly secreted and membrane proteins were identified in the extracellular and surface-associated sub-proteomes [46]. This study demonstrated that the pre-fractionation protocol used was a highly valuable strategy for unraveling the cellular location of the identified proteins and provided a solid basis for the identification of strain-specific marker proteins that could serve as novel diagnostic tools.

Quantitative Proteomics

Recently, Wongtrakoongate and colleagues (2011) established an extended proteome reference map of *B. pseudomallei* constructed from early-stationary phase whole cell using 2D-GE coupled with MALDI-TOF/MS [47]. A total of 220 proteins were identified; many of them have been implicated in bacterial pathogenesis. Up to 20% of the identified proteins were found to be involved in post-translational modification and stress responses. Such a reference map is useful to support future functional analysis of the bacterial genes and environmental regulation and facilitate comparative proteomics with its sibling species.

Our study of the *B. pseudomallei* stationary phase culture secretory proteome by 2D-GE detected 113 protein spots [48]. Of these only 54 were in sufficient quantity for identification using MS. Further database search found these proteins to be metabolic enzymes, transcription/translation regulators, potential virulence factors, chaperones, transport regulators, and hypothetical proteins. Of these specifically, proteins such as succinyl-coA:3 ketoacid-coenzyme A transferase subunit A, putative heat shock protein, chaperonin GroEL, chitin binding protein, cell division protein FtsQ, NAD(P) transhydrogenase subunit alpha were also identified in other *Burkholderia spp*. [48].

Identification of these proteins would greatly facilitate future studies aimed at mapping proteins that are differentially expressed in response to certain stimuli or during colonisation of a particular environmental niche as well as unraveling global

regulatory networks operating in these organisms.

In order to identify the mechanisms underlying the adaptive strategies employed by the bacterial isolates, protein expression of clonal isolates of *B. cenocepacia* (cytoplasmic and membrane-associated proteins) were compared using two-dimensional difference-gel electrophoresis (2D-DIGE) [49]. The first isolate was recovered from a chronically infected CF patient, while the second isolate from the same patient after 3 years following persistent infection and intravenous therapy with ceftazidime/gentamicin, was found to be multidrug resistant. The second mutlidrug resistant isolate demonstrated higher expression of proteins involved in energy metabolism, translation, nucleotide synthesis, protein folding and stabilisation, peptidoglycan synthesis, membrane lipids synthesis, lipopolysaccharide synthesis and iron binding transport compared to the first isolate (Table 2). The expression profiles suggested that protein synthesis and DNA repair processes were more active in the second isolate, thus conferring a persistence advantage for the bacteria to survive in the CF airways and lungs.

Table 2. Comparison of protein expression the two different clonal isolates of *B. cenocepacia*

Proteins	First isolate	Second isolate
Energy metabolism	↓	↑
Translation	↓	↑
Nucleotide synthesis	↓	↑
Protein folding and stabilization	↓	↑
Peptidoglycan synthesis	↓	↑
Membrane lipids synthesis	↓	↑
Lipopolysaccharide synthesis	↓	↑
Iron binding transport	↓	↑

In another study, 83 proteins were obtained when culture supernatants of *B. cepacia* were analysed using 2D-GE. Among these proteins, flagellar hook-associated domain protein (FliD), flagellar hook-associated protein (FlgK), tonB-dependent siderophore (Fiu), elongation factor G (FusA), phosphoglycerate kinase (Pgk) and sulfatase (AslA) were reported to be involved in virulence [50]. These identified secretory proteins may be used as targets for the development of new strategies to control the infection using agents that can block their release.

As a summary, the proteomics studies described have enabled scientists to identify, compare, quantitate and differentiate proteins expressed by *Burkholderia spp*. In addition, these studies can enable identification of definitive proteins that may play a role in disease causation or virulence. Proteomics also aids in identification of variations within inter- or intra species *ie* within the *Burkholderia* genus or as demonstrated within a species *ie* different strains of *B. cenocepacia* under varying defined conditions or during infection [49]. This therefore provides a sound basis for the identification of strain-specific protein markers that could serve as novel diagnostic tools, differentiate strains within a species based on presence or absence of proteins or expression of proteins during different biological states. In addition, the cellular location of the identified proteins may also provide valuable clues for the role of a particular protein within the cell.

Host Transcriptional Responses to *Burkholderia spp.*

DNA microarray technology allows the understanding of complex cross-talk between the host and the pathogen. In order to improve our understanding of pathogenesis of disease, it is necessary to identify virulence-associated microbial genes as well as host-defense strategies and characterise the cues to which they respond and mechanisms by which they are regulated [51, 52, 53]. In recent years, reports on the use of microarray analyses in infection assays have provided first insights into the complexity of acute host–bacterial interactions which can occur due to many host cells changes that include modulation of RNA expression, target receptor induction, actin cytoskeletal rearrangements, signal transduction pathway activation, and vacuolar trafficking [54].

Most studies carried out on the host transcriptional response upon exposure to *Burkholderia spp.* have been performed to monitor changes in gene expression that can occur in different host cells on contact with *Burkholderia spp.* either *in vitro* using the epithelial or macrophage cells or *in vivo* using the mice models.

***Burkholderia pseudomallei* (*in vivo* model)**

In the human host, relapse of melioidosis has been associated with the ability of *B. pseudomallei* to modulate macrophage bactericidal effect in favour of its intracellular survival and persistence [55, 56]. In a murine acute-phase melioidosis model that was developed, the microarray technology platform was used to compare the transcriptome of infected liver and spleen with un-infected tissues [57]. It was found that genes involved in immune response, stress response, cell cycle regulation, proteasomal degradation, cellular metabolism, and signal transduction pathways were differentially regulated. Although a broad range of innate immune mechanisms were activated in the host during early infection, it was then suppressed at the later stage, in addition to sub optimal activation of the downstream complement system. Therefore, this may explain the promotion of un-controlled spread of the bacteria resulting in a fulminant infection.

***Burkholderia pseudomallei* and *Burkholderia thailendensis* (*in vitro* model)**

Using an *in vitro* model, Wongprompitak and colleagues (2008) [58] compared the effect of exposure (for two hours) of *B. pseudomallei* and *B. thailandensis* (the non-pathogenic counterpart of *B. pseudomallei*) on the human lung A549 epithelial cells. The results demonstrated differential regulation of genes mainly involved in anti-apoptosis, inflammation, immune response, cell adhesion, apoptosis cytokine production, and immune cell recruitment. *B. pseudomallei* was also found to inhibit the production of pro-inflammatory cytokines including TNF-α, IL-11, IL-6, cytokine regulatory protein and adhesion molecules, which may have lead to the suppression of host inflammatory response and bacterial elimination through reduction in the recruitment of innate immune cells to the site of infection. In contrast, the expression of these genes in the A549 cells was found to be activated upon exposure of *B. thailandensis* [58].

Burkholderia pseudomallei* and *Burkholderia cepacia

We have similarly studied the transcriptional responses of A549 cells when exposed to *B. pseudomallei* and *B. cepacia* live bacteria as well as their concentrated secretory proteins (data unpublished). Our results showed that the levels of alteration of host transcriptional responses were apparently higher using secretory proteins rather than the live bacteria for both *B. pseudomallei* and *B. cepacia*. The host genes that were found to be differentially regulated during infection with *B. pseudomallei* and *B. cepacia* included those involved in the metabolic and cell cycle pathway, apoptosis and the immune response. The host metabolic processes and the cell cycle pathways were transcriptionally up-regulated and in combination, alteration of these pathways may provide clue for the need of the host cells to survive and proliferate in order to sustain cell injuries caused by the secretory proteins and/or to allow prolonged survival and replication of the *B. pseudomallei* and *B. cepacia* in the host cells. Inhibition of apoptosis of the A549 cells was also found and may indicate a role in easing replication of the bacteria and prolonging their intracellular survival, thus favoring bacterial persistence. In addition, suppression of the pro-inflammatory molecules could result in the reduced recruitment of the innate immune cells to the site of infection, which in turn could influence the degree of host inflammatory response and bacterial elimination. In general, pathogens have been shown to modulate the epithelial bactericidal response in favor of its intracellular survival and persistence in the human host, and this process may be associated with disease relapse.

Further Role of Microarray Analysis in Pathogenesis of Disease

In order to survive and propagate in the new habitat of the mammalian host, the *Burkholderia spp.* need to develop persistence as a mode of adaptation to the new environment. This may be achieved by bacterial molecules that are released to the environment in adaptation and as a result interact with the host. These bacterial molecules could therefore be attractive targets for therapeutic intervention, particularly in persistence, where antibiotic drugs are ineffective. Results of the microarray gene expression study can therefore clues on interaction of these molecules with the host and can then be exploited for therapeutic interventions, thus removing the *Burkholderia spp.* intracellular persistence in the infected host.

Conclusion

The proteomics and DNA microarray research in *Burkholderia spp.* has been useful in elucidating the functionality of proteins expressed by the bacteria as well as the host. The utilisation of these techniques has leapfrogged the understanding of the mechanism of virulence of these organisms as well as the host-pathogen interaction. Recent proteomics and DNA microarray studies have provided early insights into the underlying virulence mechanisms of the bacteria, their interactions with the host and the host defense mechanisms. However, there is also a need to analyse and interpret the complex biological processes and functionality of the proteins involved in the causation and persistence of these infections. At the same time, in order to obtain definitive maps on the pathogenicity of the disease, consideration of other factors like strain to strain variation of the bacteria and host genetic diversity will also need to be considered.

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