Willcocks, Sam J; Denman, Carmen C; Atkins, Helen S; Wren, Brendan W; (2016) Intracellular replication of the well-armed pathogen Burkholderia pseudomallei. Current opinion in microbiology, 29. pp. 94-103. ISSN 1369-5274 DOI: https://doi.org/10.1016/j.mib.2015.11.007

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Intracellular replication of the well-armed pathogen
Burkholderia pseudomallei
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The Burkholderia genus contains a group of soil-dwelling Gram-negative organisms that are prevalent in warm and humid climates. Two species in particular are able to cause disease in animals, B. mallei primarily infects Equus spp. and B. pseudomallei (BPS), that is able to cause potentially life-threatening disease in humans. BPS is naturally resistant to many antibiotics and there is no vaccine available. Although not a specialised human pathogen, BPS possesses a large genome and many virulence traits that allow it to adapt and survive very successfully in the human host. Key to this survival is the ability of BPS to replicate intracellularly. In this review we highlight recent advances in our understanding of the intracellular survival of BPS, including how it overcomes host immune defenses and other challenges to establish its niche and then spread the infection. Knowledge of these mechanisms increases our capacity for therapeutic interventions against a well-armed foe.

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Current Opinion in Microbiology 2016, 29:94–103
This review comes from a themed issue on Host-microbe interactions: bacteria
Edited by Elizabeth Hartland and Anthony Richardson
For a complete overview see the Issue and the Editorial
Available online 22nd January 2016
http://dx.doi.org/10.1016/j.mib.2015.11.007
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Introduction
Burkholderia pseudomallei (BPS) is a motile, Gram-negative saprophyte that dwells in the soil and surface water of hot and humid countries, notably in South East Asia and Northern Australia. It is capable of infecting a wide variety of mammals and causes the emerging disease, melioidosis, in humans. Pathogenicity manifests as either a chronic pulmonary disease when particles of contaminated soil are inhaled, alternatively as life-threatening septicaemia if the bacteria are introduced into the blood stream through dermal puncture.

Humans are an accidental host and the lack of human-to-human transmission precludes host–pathogen evolution. Yet, BPS is very successful at survival in vivo, in part due to genomic islands acquired through horizontal gene transfer from other bacteria [1], and in part from adaptation to environmental stress factors that confer dual protection against host immune mechanisms. BPS possesses a large genome for a bacterium of around 7.25 million bases spread over two chromosomes. Accordingly, it possesses numerous virulence factors and demonstrates remarkable plasticity in adapting to microenvironmental conditions.

Several tools are available that help to identify virulence factors in the context of intracellular survival. A common approach is to compare the genome of BPS with a closely related avirulent species, B. thailandensis. A conceptually similar experiment is to compare virulent and attenuated clinical isolates derived from the same strain. Molecular based approaches such as PCR-based subtractive hybridisation have identified factors such as the membrane transporter, BPSL2033 that reduces the ability of BPS to replicate in macrophages [2]. More recently, the creation of initially small transposon-mediated signature-tagged mutant libraries in BPS [3] and now large scale libraries coupled with next generation sequencing [4] have opened new avenues to reveal the genes required by BPS to survive intracellularly.

In this review, we highlight recent reports relating to the mechanisms employed by BPS to survive and thrive inside host cells; summarised in Figure 1 and Table 1. Not only is this characteristic an essential requirement for pathogenicity, it also presents targets for the development of novel antimicrobial therapeutics against a hardy organism that naturally tolerates many existing antibiotics.

First contact with the host cell
BPS is not an obligate intracellular pathogen, but if cellular defense mechanisms are overcome it offers several advantages over an extracellular lifestyle, such as the evasion of humoral immunity, reduced exposure to antibiotics, and the potential establishment of latency. Intracellular living is also essential for localised spread from the site of infection, and eventual systemic dissemination.
Intracellular virulence of *Burkholderia pseudomallei*. *Burkholderia pseudomallei* (BPS) gains access to epithelial or phagocytic cells via flagella and adhesion-related virulence factors. BPS then goes on to express an arsenal of virulence factors to combat the host response. (i) **Uptake and entry**: either via phagocytic or epithelial cells. When entering non-phagocytic cells such as epithelial cells, BPS can attach and enter host cells. Flagella, pili, and other adhesins BoaA and BoaB are involved in this process. Passive uptake by tissue resident macrophages or neutrophils also occurs. (ii) **Survival and escape**: BPS once taken up by the host cell is enveloped in an endocytic vesicle or phagosome. Upon internalisation, *B. pseudomallei* is held within a phagosome, where the T3SS is required for BPS escape. Escape occurs before maturation and killing. Resistance to oxidative stress response from the host, and production of the serine protease ectoin are also crucial steps for phagosome survival and subsequent escape. (iii) **Dissemination and MNGC formation**: Once outside the phagosome, *B. pseudomallei* is able to replicate host actin is utilized for the polymerisation of elegantly structured Ena/VASP-like tails. These projections alongside the T6SS-1 result in successful dissemination to surrounding cells, resulting in the formation of multi-nucleated giant host cells (MNGC). Black text describes intracellular process of BPS, navy text lists genes important for intracellular survival are listed, references for which can be found in Table 1. Some of the host-combative responses are shown in red text, and the dashed-arrow indicates the connectivity between T3SS and T6SS. Actin tails are not structurally accurate.

The initial infection of a host cell depends upon invasion and adhesion factors that have been well reviewed [5]. In brief, BPS has the capability to invade cells either passively in the case of uptake by phagocytes such as resident tissue macrophages and recruited neutrophils; or actively in the case of epithelial cell infection. An advantageous trait of BPS in this regard are its flagella, which in wet soil or aquatic environments propel the bacilli against a concentration gradient toward nutrients that may otherwise be scarce; and in the mammalian host, enable penetration of protective mucous linings and surfactant to reach epithelial cells. Other factors such as the expression of Type IV pili are well-recognised adhesion factors, and BPS also possesses two further proteins, BoaA and BoaB that promote attachment to epithelial cells [6]. Host cell factors also play a role, such as protease-activated receptor-1, expressed by various cell types, that promotes cell ingress and is associated with BPS growth and dissemination [7].

The benefits of intracellular growth seem at odds with the characteristic of BPS to induce cell death of the infected cell. BPS is cytotoxic to dendritic cells (DC), monocytes and macrophages within hours of infection, with an absolute requirement for internalisation, caspase-1 activation, and the Type Three Secretion System (T3SS) in what has been described as a pyroptotic cell death mechanism [8]. The BPS flagellar hook-associated protein also induces cytotoxicity [9]. This rapid cell death is strongly linked to the multiplicity of infection and cell type. For example, epithelial cells seem somewhat resistant to BPS-mediated killing. Therefore, a low level infection that includes infection of epithelial cells may contribute to chronic persistence in the host, in contrast to highly inflammatory acute infection. Concurrently, BPS inhibits mechanisms of cell death that may control the infection though bacterial killing; infected neutrophils, for example, normally undergo apoptosis and are subsequently engulfed and degraded by macrophages, destroying their
| Table 1                                                                |
|-----------------------------------------------------------------------|
| Selection of major *B. pseudomallei* intracellular virulence traits and advancements in the literature, BPS locus ID from Burkholderia.com (Winsor et al., 2008) |

| Genetic feature | General role                                                                 | Reference(s)                        | BPS locus ID |
|-----------------|------------------------------------------------------------------------------|-------------------------------------|--------------|
| **Entry and uptake into host cells** |                                                                              |                                     |              |
| *Boa*           | Cell attachment; adhesin; autotransporter                                   | Balder et al. [6]                   | BPSL1705     |
| *bcaB*          |                                                                              | Lazar Adler et al. [74]             |              |
|                 |                                                                              | Lazar Adler et al. [70]             |              |
| *bpaC*          | Cell attachment; autotransporters; adhesin;                                 | Campos et al. [75]                  | BPSL1631     |
|                 | Type V secretion system; protects from complement killing                   | Lazar Adler et al. [70]             |              |
| **Type IV pilI, pilA** | Adhesion factor and intracellular mobility                            | Essex-Lopresti et al.;              | Fia1 and fia2 locus. |
|                 |                                                                              | Boddy et al.                        |              |
| **Metabolism and phagosome escape** |                                                                              |                                     |              |
| *fabI -1*       | Two enoyl-ACP reductases, bpmFabI-1 and bpmFabI-2, found in BPS one on each chromosome, involved in antibacterial activity | Liu et al. [53]                     | BPSL2204     |
| *fabI-2*        |                                                                              | Cumming et al. [52]                 | BPS30721 (unclear if active) |
| *sodC*          | Superoxide dismutase/oxidoreductase                                          | Vanaporn et al. [21]                | BPSL1001     |
| *ahpC*          | Alkyl hydroperoxide reductase; related to T-cell immunity                   | Loprasert et al. [76]               | BPS0492      |
| *CHBP*          | Delays host cell maturation and apoptosis;                                  | Reynolds et al. [77]                |              |
|                 | ATP/GTP binding protein unique to BPS                                       |                                     |              |
| *bopA*          | Type three secretion system; involved in epithelial cell invasion;          | Stevens et al. [78]                 | BPS1525      |
|                 | attenuated in murine model of infection                                      | Pumirat et al. [79]                 |              |
| *bopE*          |                                                                              | Vander Broek et al. [80]            |              |
| *bprD*          | Intracellular survival, type three secretion effector, virulence regulator  | Vander Broek et al. [80]            |              |
| *spR*           | Regulators of T3SS and T6SS LexA repressor                                  | Chirakul et al. [81]                |              |
| *eco*           | Serine protease activity releases BPS from vesicle, inhibits elastase       | Chen et al. [82]                    | BPSL1840     |
| *bsaN*          | Transcriptional regulator involved in modulation of T3SS and T6SS-1         | Chen et al. [83]                    |              |
| *BPSS1504*      | Affects Hcp1 secretion and integrity of the T6SS-1 apparatus                | Sun et al. [50]                     | BPS1546      |
|                 |                                                                              | Hopf et al. [64]                    | BPS1504      |
| *bssA*          | T3SS-3 ATPase/ATP synthase SpaL                                              | Gong et al. [84]                    | BPS1541      |
| *vgrG-5*        | T6SS spike protein; membrane fusion; intracellular spread                   | Toescsa et al. [66]                 | BPS1541      |
|                 |                                                                              | Schwarz et al. [67]                 | (unclear) |
| *virAG*         | Two component system, T6SS regulators, sensor and histidine kinase.         | Chen et al. (2013)                  | BURPS1710b_ |
|                 |                                                                              | AOS00-AOS32                         |              |
| *bsaU*          | Involved in early onset activation of caspase-1 pathway in macrophages;     | Sun et al. [50]                     | BPS1539      |
|                 | T3SS                                                                           | Bast et al. [40]                    |              |
| *purM*          | Phosphoribosylglycinamidase                                                  | Breitbach et al. [54]               | BPSL0908     |
| *purN*          | formyltransferase; phoshoribosylaminomimidazole synthetase                   |                                     | BPSL2018     |
| *fabI*          | Enoyl-ACP reductase; type II fatty acid biosynthesis pathway                 | Liu et al. [53]                     | BPSL2204     |
| *pabB/lipB*     | Lipote-protein ligase B; essential for intracellular life cycle and virulence in murine model | Breitbach et al. [54]               | BPSL0413     |
| *bipD, C, B*    | T3SS translocan proteins; phagolysosome survival; cell invasion protein     | Gong et al. [36]                    | BPS1529      |
|                 |                                                                              | Vander Broek et al. [80]            | BPS1531      |
|                 |                                                                              | BPS1532                              |              |
| *bpsC N (formerly scfC)* | TTSS1 ATPase; intracellular survival; murine pathogenesis                 | Warawa and Woods [85]               | BPS1390      |
| *sctN*          | Type III secretion system ATPase                                            | D’Cruze et al. [86]                 |              |
| *bsaQ*          | Type III secretion system; cell cycle inhibiting; invasion and escape from endocytic vesicles | Warawa and Woods [85]               | BPS1394      |
| *hcp1*          | Tail-spike T6ss1, MNGC cell formation, hemolysin-corelated protein substrate and or a secretion tube (also part of T6SS) | Burtnick et al. [87]                | BPS1498      |
|                 |                                                                              | Lim et al. [62]                     |              |
The host cell response to BPS infection

The engagement of host pattern recognition receptors during BPS infection have been well characterized, and it has been found that the toll-like receptor repertoire offer little protective benefit or worse, contribute to disease severity and mortality [12–17]. This may in part be due to the ability of BPS to resist various TLR-dependent innate immune killing mechanisms. One such mechanism, particularly in the murine model of infection, is the generation of reactive nitrogen species (RNS). In contrast to other Gram-negative bacteria, the LPS in the cell wall of BPS is a weak inducer of the enzyme, iNOS, partly due to its inability to stimulate IFN-β [18]. Accordingly, impairment of iNOS activity in a murine model of infection does not significantly alter killing capability [19]. A related mechanism is the respiratory burst, triggered by the engagement of pathogen associated molecular patterns (PAMP) by TLR. Reactive oxygen species (ROS) are able to denature enzymes as well as damage fatty acids and DNA, and direct exposure is lethal for BPS. While the effect of ROS-mediated killing of BPS in ex vivo isolated macrophages or macrophage-like cell lines is modest, in vivo studies have shown a significant role of ROS in host survival [19].

Unlike RNS, BPS is an inducer of ROS, perhaps unavoidably. A key protein in BPS invasion of host cells is BopE, which acts as a guanine nucleotide exchange factor for RhoGTPases, promoting actin cytoskeleton rearrangement (reviewed by Allwood et al. [5]). However, RhoGTPases also stimulate NAPH oxidase [20], promoting the respiratory burst. Therefore, the ability of BPS to neutralise ROS using proteins such as superoxide dismutase is a key aspect of their virulence in vivo [21]. Whole genome analysis has further revealed that during oxidative stress, BPS upregulates genes involved in cell wall repair and biosynthesis, as well as the peroxide scavenging enzymes, KatG and AhpC. Also discovered was an oxidative stress regulon under the control of the sigma factor, RpoE. RpoE is conserved among Gram-negative bacteria, and deletion in BPS resulted in reduced intracellular survival in macrophages and virulence in the murine model. RpoE seems to be important for maintaining expression of spermidine acetyltransferase, soprG, under oxidative stress. Spermidine is useful for the bacteria as it acts as a free radical scavenger but excessive levels are toxic, and so SpeG is required to prevent its accumulation to damaging levels [22,23].

Restricting the exposure of BPS to toxic levels of ROS in monocytes and macrophages is potentially realised through an additional mechanism. During the initial hours post-BPS infection, there is a reduction in CFU followed by growth recovery [24–26]; this temporarily correlates with activation of NADPH-oxidase that causes rapid, but only transient ROS production. Although direct modulation of NADPH-oxidase by BPS has not been experimentally demonstrated, its activity, as well as a large component of TLR-mediated signalling, depends on the transcription factor, NfκB, which BPS has been shown to actively inhibit. By undermining the transcriptional response to TLR-activation, BPS is able to disarm a key aspect of host immunity. The BPS virulence factor, TssM, inactivates NfκB through deubiquitinisation of its inhibitor, IkB, thus also impairing downstream interferon pathway activation [27]. TssM is located upstream of the T3SS, and downstream of the T6SS gene clusters. Deletion of the T3SS bsl cluster reduces its secretion, as does removal of the Type-Two Secretion-associated outer membrane channel, GspD [27,28].

It has been observed over many years that there is a clinical correlation between non-obese diabetes, predominantly Type 1 diabetes mellitus, and BPS infection; it is a recognized risk factor in endemic regions. Diabetes mellitus is an auto-immune disease with pleiotropic effects that generally impair the ability of the diabetic host to control bacterial infection. For example, a decreased capacity of macrophages to phagocytose and kill [29], LPS-induced generation of CD4 T-regulatory cells and impairment of TLR-mediated MyD88 inflammatory signalling [30].

Research into the molecular and immunological mechanisms that link diabetes with BPS in particular is beginning to reveal new insights into clinical observations. For example, a further mechanism by which BPS moderates
NfκB activity is by phosphorylation, and subsequent inactivation of, glycogen synthase kinase 3β (GSK3β), which usually supports NfκB activity. This is dysregulated in a rat model of diabetes, resulting in excessive TNF-α and IL12 cytokine production by peripheral blood mononuclear cells that may contribute to the higher risk of septic shock in diabetic patients with melioidosis [31]. Remarkably, BPS can alter the immune function of not only the infected host cell, but also uninfected bystander cells. BPS-infected neutrophils are able to locally inhibit CD4+ T-cell proliferation and IFN-γ production via increased surface expression of programmed death ligand-1 (PD-L1) [32]. This trait is exacerbated diabetes mellitus patients, and the resulting loss of effective CD4+ T-cell function may contribute to disease severity and the ability to prevent disease progression.

Phagosome escape and the type three secretion system

Following uptake by a professional phagocyte, BPS is contained in a membrane-bound vesicle that matures through endosomal contact to eventual fusion with the lysosome, an organelle that by virtue of acidic pH and degradative enzymes destroys the contents of the phagosome, allowing denatured proteins to be loaded onto MHCII molecules for surface expression and engagement of CD4 T-cell immunity.

As the phagosome matures, BPS encounters host proteases that are able to kill the compartmentalised invader. To counter this, BPS expresses a serine protease inhibitor called ectein that enables growth in macrophages and contributes to virulence in the murine model [33]. Ultimately, however, BPS is able to escape the phagosome before phagosomal fusion occurs, and for this, the T3SS is essential.

The role of the T3SS in BPS intracellular fitness, and therefore virulence cannot be overstated. To provide some context, the capsule of BPS is a well-studied virulence factor that confers protection against antibiotics and host immune effectors such as complement and antibody deposition. In a murine model of acute respiratory BPS infection, capsule mutants were shown to be attenuated 6.8-fold, while T3SS mutants were attenuated 290-fold [34]. Not only did T3SS mutants show reduced survival, but were also poorly able to disseminate beyond the site of infection. Mutation of genes encoding T3SS structural components, such as bsaO, impair BPS invasion of macrophages and multinucleated giant cell (MNGC) formation due to their inability to evade lysosomal degradation [35], as well as reducing BPS-induced cytotoxicity [8]. For this reason, T3SS-related proteins have become attractive therapeutic targets [36].

The secreted protein effectors that are exported via the T3SS have diverse effects. For example, a cell-cycle inhibitor, CHBP (BPSS1385), is able to arrest host cell division, delay apoptosis and modify the cytoskeleton [10]. BipC, located within the bsa locus, contributes to host cell adhesion and invasion, intracellular replication, cytotoxicity, phagosomal escape [37]. Adjacent to bipC on the chromosome is bipB, which has similarly been reported for its role in intracellular BPS infection, including induction of MNGC and cell-to-cell spread [38].

The T3SS-dependent liberation of BPS from the phagosome into the cytoplasm exposes the bacilli to intracellular, TLR-independent, pattern recognition receptors, namely the NOD-like receptors (NLR) and by extension to the machinery of the inflammasome [39]. Sensing of BPS PAMP, such as flagellin or the T3SS-associated proteins, BopE and BsaU by NLR4 leads to activation of caspase-1 in early infection [40], subsequently inducing rapid pyroptosis of infected cells, thus partly explaining the mechanism of BPS-induced cytotoxicity and the pre-requirement of T3SS-dependent phagosomal escape. While BopE is a secreted effector of the T3SS, BsaU is a component of the secretion apparatus itself, and its disruption compromises the secretion of other effectors, reducing NfκB-mediated inflammation in the host cell [41].

BPS infection additionally upregulates NOD2 expression [42], engagement of which leads to NfκB activation. While NOD2 is important for limiting early bacterial replication and partially contains BPS infection, it seems not to ultimately affect the outcome of survival in vivo [17]. BPS-mediated dysregulation of NfκB may play a role, as described above, as well an apparent NOD-2-dependent expression of suppressor of cytokine signalling 3 [42].

NOD2 activation has been reported to promote autophagy, a homeostatic process by which cellular contents are degraded and recycled. An autophagosome is formed around cytoplasmic material, meaning bacteria that have escaped the initial phagosome may once again become membrane entrapped. Autophagy has been shown to be induced by BPS infection in a T3SS-dependent manner, and is able to kill the bacteria [43,44]. However, the effectiveness of autophagy is undermined by expression of the T3SS effector protein, BopA. Although its mechanism of action remains to be fully elucidated, BopA is required for evasion of autophagy, and contributes to intracellular virulence [36*,44].

Metabolic challenges in the intracellular environment

Any intracellular organism finds itself in a conflict for resources with the host to maintain its metabolic activity. This is especially true for essential micronutrients such as iron, which is incorporated into molecules involved in multiple biological processes. Pathogens such as Mycobacterium tuberculosis achieve the sequestration of iron through the expression of siderophores that have a higher
affinity for the molecule than equivalent host molecules such as transferrin and lactoferrin. Ferric siderophores are then recognized by specific receptors on the bacterial surface. The BPS malleobactin siderophore family is crucial for its virulence and intracellular survival. They appear to be remarkably diverse in their peptide structure, suggesting characteristic adaptability of BPS to different microenvironmental conditions [45]. Genes that encode malleobactins as well as other BPS proteins relating to iron binding and uptake have been shown to be upregulated in experimentally induced models of iron deprivation, including oxidative stress [23,46,47]. Besides the dedicated iron-binding molecules, BPS expresses proteins with dual function such as BPSL2825 (PabB), a para aminobenzoate synthetase component important for metabolism and growth in vitro that takes on a critical role utilizing hemin as an iron source in vivo [47].

Metabolic flexibility is key to the ability of BPS to adapt as it moves from in terra to in vivo and it remains one of its defining features. While several Burkholderia spp. are similarly well equipped to thrive on multiple carbon sources, it has been postulated based on genomic interrogation that virulent species may regulate these pathways differently from avirulent species, with consequential effects on the expression of virulence factors [48]. An important requirement of pathogens, whether they are obligate or facultatively intracellular is their ability to metabolise fatty acids [49–51]. In bacteria, fatty acids are required for the formation of cell wall phospholipids and may be acquired either from existing host-derived fatty acids, through de novo synthesis or a combination of both. Since there is no evidence that BPS is able to acquire host-derived fatty acids, de novo fatty acid synthesis an attractive target for chemotherapeutic intervention. FabI is an enoyl-acyl carrier protein reductase, a key enzyme of the type II fatty acid synthesis system, and has been shown to be required for BPS virulence in the murine model of infection. It has since been successfully targeted by specific chemical inhibitors [52,53], representing a promising approach to drug design.

In addition to these processes, anabolic pathways, while perhaps not traditionally considered virulence factors are nonetheless essential for intracellular fitness. Anabolism requires ATP energy, for which the synthesis of purine, encoded by the pur gene cluster is required, purine also being a vital component of nucleic acids [54]. BPS differs from its host in that it is able to synthesise several amino acids and vitamins that humans cannot and instead acquire from dietary intake. This also makes them an attractive target for antimicrobial therapy. For example, histidine synthesis, encoded by the his gene cluster, is required for intracellular growth of BPS, and this area is also being explored for potential future vaccine design [54].

Cell to cell spread and the Type Six Secretion System

BPS is able to spread beyond the site of initial infection, disseminating through the host and potentially infecting any organ or tissue. One strategy is to use the infected host cell itself as a vehicle. BPS has been shown to remain viable within, and induce maturation and migration of, immature DCs in vitro, a phenomenon seen in other infections as a route for transport to secondary lymphoid organs in vivo; although seemingly only a minority of BPS-infected DCs translocate to the lymph in vivo [55].

More localised spread occurs via a distinctive feature of BPS infection, namely its ability to create MNGC. Arising from the fusion of cell membranes between an infected cell and a neighbouring, uninfected cell, it is a phenomenon that enables continued intracellular replication, and cell to cell spread without the requirement of an extracellular step and the consequences that entails regarding host immunity. BPS is able to form MNGC in both phagocytic and non-phagocytic cells within hours of infection [56], and the eventual death of the MNGC results in plaque formation [57], that is damaging to the host, and may serve as a niche for further BPS replication.

It is well established that cell–cell spread and induction of MNGC requires bacterial motility. In addition to the two flagellin encoding genes, fla2 on chromosome 2 and fla1 on chromosome 1, BPS achieves intracellular motility through the action of the autotransporter, BimA. Localising to one pole of the bacillus, it binds host actin, polymerisation of which pushes the bacteria through the cytoplasm, leaving in its wake characteristic ‘comet tails’. BimA localisation is not itself directed to the poles, rather it is transported equally across the bacterial inner membrane [58]. BimA localisation depends on the polarity of its partner protein, BimC, which functions through an iron-dependent mechanism that remains to be fully described.

Interestingly, BimA is also expressed by B. thailandensis which is avirulent in humans. Recent published work has revealed the finer distinction responsible for virulence: whereas B. thailandensis BimA mimics the host Arp3/3 actin polymerase complex, BimA from BPS and B. mallei, another pathogenic member of the genus that causes the disease, glanders, in horses, instead mimic the far more efficient host Ena/VASP complex to initiate and elongate actin filaments [59,60]. The result is that while B. thailandensis BimA is able to generate branched actin networks, BPS BimA generates multiple bundles of filaments; moreover, BPS BimA is able to generate more actin tails and larger MNGC compared with B. mallei BimA, highlighting the key importance of actin-based motility for cell–cell spread in the host.

In addition to bacterial motility, a key requirement for cell fusion and MNGC formation is the Type Six
Secretion System (T6SS), disruption of which attenuates virulence in vivo. BPS possesses a predicted six T6SS gene clusters, which are thought to have evolved as a weapon for inter-bacterial competition [61] within the rhizosphere, the native microbiome of BPS.

While the T3SS is not directly required for MNGC formation, it is a prerequisite that the bacteria are able to escape the phagocytic vacuole [39,57]. Furthermore, there is cross-talk between the T6SS and T3SS: for example, T6SS1 expression is regulated by BprC, which is located in the T3SS gene cluster. The T6SS is upregulated by several orders of magnitude upon infection of host cells, and this is largely regulated by the two-component histidine kinase sensor, VirAG [62], which is itself regulated by a T3SS regulator, BsaN [63]. Mutagenesis studies have revealed that abrogation of both virAG and bprC function attenuates virulence in the murine model of BPS infection [63].

VirAG activates the promoter of a key T6SS effector, hemolysin-coregulated protein (Hcp), located in gene cluster five out of the six BPS T6SS gene clusters [63]. Solving the crystal structure of BPS Hcp has revealed unique variations in its tertiary structure that are not present in orthologues from avirulent species, hinting at its key role in virulence.

Hcp polymerases the hexameric ring structures that comprise the T6SS, forming an overall structure that is akin to the bacteriophage tailspike. Hcp is also secreted by the T6SS, dependant on the protein, BPSS1504 [64], and functions as a chaperone for other secreted effectors, localising to the infected cell plasma membrane. Exogenous recombinant Hcp also localises to the host cell surface, preferentially targeting antigen presenting cells. Accordingly, it has been found to be highly immunogenic and reacts strongly with sera from melioidosis patients [65]. The consequence of this is not clear, but it has been reported that binding of Hcp induces the anti-inflammatory cytokines, IL-10 and TGF-β, potentially interfering with the mounting of a robust immune response to other epitopes besides Hcp.

Activation of T6SS is a dynamic, multi-step process that begins with assembly of the inner tube, comprised of Hcp, its outer sheath and baseplate at the bacterial cell wall-host cell membrane interface. The tip of the T6SS spike is comprised of a trimer of the protein, Valine-glycine repeat protein G (VgrG) [66]. VgrG traverses the inner membrane, periplasmic space and peptidoglycan layer of the bacterial cell wall, while the elongation of the tail spike extends into the bacterial cytoplasm.

Upon contact with the host cell membrane, the T6SS outer sheath contracts and provides the kinetic energy for the inner tube to propel the VgrG tip, penetrating the host cell and potentially into the neighbouring cell membrane [67]. At this stage, T6SS effector proteins are also delivered. The recruitment of ATPase then disassembles the outer sheath proteins, which are recycled [68]. The contraction of the T6SS may serve to pull the two membranes close enough whereupon, according to the hemifusion paradigm, membrane fusion occurs when proximity is <1 nM [69], resulting in a fused, multinucleated cell. Although VgrG has been shown to be functionally conserved across Burkholderia spp., some species such as B. oklahomensis lack the ability of cell–cell spread, suggesting other requisites than VgrG alone [66].

Conclusions and future perspectives

Much has been learned in recent years about how BPS is able to infect human cells, evade immune killing mechanisms and replicate intracellularly. This review is not an exhaustive list of BPS virulence factors. Several are the subject of ongoing research to fully explain their function, such as boaB, bpaC and bpaE, which all contribute to virulence in the murine infection model [70]. Still more targets remain to be identified and understood, and newly emerging tools will help to expedite these efforts. For example, transposon-directed insertion-site sequencing (TraDIS) also known as TnSeq, has facilitated the discovery of the essential gene set of BPS [4] and B. thailandensis [71] and has very recently been applied to elucidate virulence genes in the acute model of in vivo infection [72]. A particular remaining challenge is to understand the establishment of chronic infection with BPS, which may demand novel treatment therapies in the clinical setting [73]. We have gained important insights into the pathogenicity of BPS, for example the significance of the T3SS and T6SS, and some of the basic cell biology of infection. Further work is needed to understand the subtleties of host-pathogen interactions that result in, or fail to result in, protective host immunity. Together these efforts will contribute to our ability to counteract the emerging threat of this well-armed pathogen.

References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- of special interest

1. Tumapa S et al.: Burkholderia pseudomallei genome plasticity associated with genomic island variation. BMC Genom 2008, 9:190.
2. Push SM et al.: Molecular characterization of putative virulence determinants in Burkholderia pseudomallei. Scientific World J 2014, 2014:590803.
3. Cuccui J et al.: Development of signature-tagged mutagenesis in Burkholderia pseudomallei to identify genes important in survival and pathogenesis. Infect Immun 2007, 75:1186-1195.
4. Moule MG et al.: Genome-wide saturation mutagenesis of Burkholderia pseudomallei K96243 predicts essential genes and novel targets for antimicrobial development. MBio 2014, 5:e00926-13.
Intracellular virulence of Burkholderia pseudomallei

Allwood EM et al.: Strategies for intracellular survival of Burkholderia pseudomallei. Front Microbiol 2011, 2:170.

Balder R et al.: Identification of Burkholderia mallei and Burkholderia pseudomallei adhesins for human respiratory epithelial cells. BMC Microbiol 2010, 10:250.

Kager LM et al.: Deficiency of protease-activated receptor-1 limits bacterial dissemination during severe Gram-negative sepsis (melioidosis). Microbes Infect 2014, 16:171-174.

Sun GW et al.: Caspase-1-dependent macrophage death induced by Burkholderia pseudomallei. Cell Microbiol 2005, 7:1447-1458.

Gourlay LJ et al.: From crystal structure to silico epitope discovery in the Burkholderia pseudomallei flagellar hook-associated protein FlgK. FEBS J 2015, 282:1319-1333.

Pumirat P et al.: Analysis of the prevalence, secretion and function of a cell cycle-inhibiting factor in the melioidosis pathogen Burkholderia pseudomallei. PLoS One 2014, 9:e86298.

Chanchamroon S et al.: Human polymorphonuclear neutrophil responses to Burkholderia pseudomallei in healthy and diabetic subjects. Infect Immun 2009, 77:456-463.

West TE et al.: Activation of Toll-like receptors by Burkholderia pseudomallei. BMC Infect 2008, 9:46.

West TE et al.: Toll-like receptor 4 gene expression and TLR4 variants are associated with susceptibility to melioidosis. Genes Immun 2012, 13:38-46.

Wiersinga WJ et al.: Toll-like receptor 2 impairment of host defense in gram-negative sepsis caused by Burkholderia pseudomallei (Melioidosis). PLoS Med 2007, 4:e248.

West TE et al.: Impaired TLR5 functionality is associated with survival in melioidosis. J Immunol 2013, 190:3373-3379.

West TE, Hawn TR, Skerrett Sj: Toll-like receptor signaling in airborne Burkholderia thailandensis infection. Infect Immun 2009, 77:5612-5622.

Myers ND et al.: The role of NOD2 in murine and human melioidosis. J Immunol 2014, 192:300-307.

Utasinchareon P et al.: Involvement of beta interferon in enhancing inducible nitric oxide synthase production and antimicrobial activity of Burkholderia pseudomallei-infected macrophages. Infect Immun 2003, 71:3053-3057.

Breitbart K et al.: Role of inducible nitric oxide synthase and NADPH oxidase in early control of Burkholderia pseudomallei infection in mice. Infect Immun 2006, 74:6300-6309.

Bokoh GM, Diebold BA: Current molecular models for NADPH oxidase regulation by Rac GTPase. Blood 2002, 100:2692-2696.

Vanaporn M et al.: Superoxide dismutase C is required for intracellular survival and virulence of Burkholderia pseudomallei. Microbiology 2011, 157(Pt 8):2392-2400.

Limsvuun K, Jones PG: Spermide acetyltransferase is required to prevent spermidine toxicity at low temperatures in Escherichia coli. J Bacteriol 2000, 182:5373-5380.

Jitprasutwit S et al.: Transcriptional profiles of Burkholderia pseudomallei reveal the direct and indirect roles of Sigma E under oxidative stress conditions. BMC Genom 2014, 15:787.

Wikraiphit C et al.: Activation of NADPH oxidase is essential, but not sufficient, in controlling intracellular multiplication of Burkholderia pseudomallei in primary human monocytes. Pathog Dis 2014, 71:69-72.

Archaroen S et al.: Fate of a Burkholderia pseudomallei lipopolysaccharide mutant in the mouse macrophage cell line RAW 264.7: possible role for the O-antigenic polysaccharide moiety of lipopolysaccharide in internalization and intracellular survival. Infect Immun 2007, 75:4298-4304.

Charoenlap J et al.: Differential intracellular fate of Burkholderia pseudomallei 844 and Burkholderia thailandensis UE5 in human monocyte-derived dendritic cells and macrophages. BMC Microbiol 2009, 9:20.

Tan KS et al.: Suppression of host innate immune response by Burkholderia pseudomallei through the virulence factor TssM. J Immunol 2010, 184:5160-5171.

Burtnick MN, Brett PJ, DeShazer D: Proteomic analysis of the Burkholderia pseudomallei type II secretary host cell factor. Analyst 2014, 139:3214-3226.

Rich J, Lee JC: The pathogenesis of Staphylococcus aureus infection in the diabetic rodent model. Diabetes 2005, 54:2904-2910.

Wang J et al.: Multiple mechanisms involved in diabetes protection by lipopolysaccharide in non-obese diabetic mice. Toxicon Appl Pharmacol 2015, 295:149-158.

Maniam P et al.: Regulatory role of GSK3beta in the activation of NF-kappaB and modulation of cytokine levels in Burkholderia pseudomallei-infected PBMC isolated from streptozotocin-induced diabetic animals. Trop Biomed 2015, 32:36-48.

Buddhia S et al.: Programmed death ligand 1 on Burkholderia pseudomallei-infected human polymorphonuclear neutrophils impairs T cell functions. J Immunol 2015, 194:4413-4421.

This article is notable for describing a mechanism by which BPS may suppress host signalling to dampen adaptive immunity, particularly in type 2 diabetes, a major risk factor for melioidosis.

Ireland PM et al.: The serine protease inhibitor Ecolitin is required for full virulence of Burkholderia pseudomallei. Microb Pathog 2014, 67-70:55-58.

Gutierrez MG, Pfeffer TL, Warawa JM: Type 3 secretion system cluster 3 is a critical virulence determinant for lung-specific melioidosis. PLoS Negl Trop Dis 2015, 9:e3441.

Muangsombut V et al.: Inactivation of Burkholderia pseudomallei bsaQ results in decreased invasion efficiency and delayed escape of bacteria from endocytic vesicles. Arch Microbiol 2008, 190:623-631.

Gong L et al.: The Burkholderia pseudomallei type III secretion system and BopA are required for evasion of LC3-associated phagocytosis. PLoS One 2011, 6:17852.

This article highlights the importance of the type three secretion systems in the evasion of host innate immunity through the transport of effector proteins such as BopA and BipD.

Kang WT et al.: Functional characterization of effector protein BipP, a type III secretion system protein, in Burkholderia pseudomallei pathogenesis. J Infect Dis 2015, 211:827-834.

Suparak S et al.: Multinucleated giant cell formation and apoptosis in infected host cells is mediated by Burkholderia pseudomallei type III secretion protein BipP. J Bacteriol 2005, 187:6556-6560.

This article describes a notable feature of BPS infection, the formation of multinucleated giant cells, and the role of the protein, BipP.

Teh BE et al.: Type three secretion system-mediated escape of Burkholderia pseudomallei into the host cytosol is critical for the activation of NFkappB. BMC Microbiol 2014, 14:115.

Bast A et al.: Caspase-1-dependent and -independent cell death pathways in Burkholderia pseudomallei infection of macrophages. PLoS Pathog 2013, 10:e1003986.

Hil CS et al.: Interleukin-8 induction by Burkholderia pseudomallei can occur without Toll-like receptor signaling but requires a functionaltype III secretion system. J Infect Dis 2008, 197:1537-1547.

Pudla M et al.: Nucleotide-binding oligomerization domain-containing protein 2 regulates suppressor of cytokine signaling 3 expression in Burkholderia pseudomallei-infected mouse macrophage cell line RAW 264.7. Innate Immun 2011, 17:532-540.

Rincha D et al.: Macrophagy is essential for killing of intracellular Burkholderia pseudomallei in human neutrophils. Autophagy 2015, 11:748-755.
44. Cullinane M et al.: Stimulation of autophagy suppresses the intracellular survival of Burkholderia pseudomallei in mammalian cell lines. Autophagy 2008, 4:744-753.

45. Franke J, Ishida K, Hertweck C: Plasticity of the malleobactin pathway and its impact on siderophore action in human pathogenic bacteria. Chemistry 2015, 21:8010-8014.

46. Alice AF et al.: Genetic and transcriptional analysis of the siderophore malleobactin biosynthesis and transport genes in the human pathogen Burkholderia pseudomallei K96243. J Bacteriol 2006, 188:1551-1566.

47. Pilat S et al.: Identification of Burkholderia pseudomallei genes required for the intracellular life cycle and in vivo virulence. Infect Immun 2006, 74:3576-3586.

48. Challacombe JF et al.: Interrogation of the Burkholderia pseudomallei genome to address differential virulence among isolates. PLoS One 2014, 9:e119591.

49. Lee W et al.: Intracellular Mycobacterium tuberculosis exploits host-derived fatty acids to limit metabolic stress. J Biol Chem 2013, 288:6788-6800.

50. Sun Y, O'Riordan MX: Branched-chain fatty acids promote Listeria monocytogenes intracellular infection and virulence. Infect Immun 2010, 78:4667-4673.

51. Vaughan AM et al.: Type II fatty acid synthesis is essential only for malaria parasite late liver stage development. Cell Microbiol 2009, 11:506-520.

52. Cummings JE et al.: The Burkholderia pseudomallei enoyl-acyl carrier protein reductase FabI is essential for in vivo growth and is the target of a novel chemotherapeutic with efficacy. Antimicrob Agents Chemother 2014, 58:931-935.

53. Liu N et al.: Mechanism and inhibition of the Fabl enoyl-ACP reductase from Burkholderia pseudomallei. J Antimicrob Chemother 2011, 66:564-573.

54. Breitbach K, Kohler J, Steinmetz I: Induction of protective immunity against Burkholderia pseudomallei using attenuated mutants with defects in the intracellular life cycle. Trans R Soc Trop Med Hyg 2008, 102(Suppl. 1):S89-S94.

55. Williams NL et al.: Migration of dendritic cells facilitates systemic dissemination of Burkholderia pseudomallei. Infect Immun 2014, 82:4233-4240.

56. Pegoraro G et al.: A high-content imaging assay for the quantification of the Burkholderia pseudomallei induced multinucleated giant cell (MNGC) phenotype in murine macrophages. BMC Microbiol 2014, 14:98.

57. French CT et al.: Dissection of the Burkholderia intracellular life cycle using a photothermal nanoblade. Proc Natl Acad Sci USA 2011, 108:12095-12100.

58. Lu Q et al.: A polar-localized iron-binding protein determines the polar targeting of Burkholderia BimA autotransporter and actin tail formation. Cell Microbiol 2015, 17:408-424.

59. Gouin E, Torres JJ, Cossart P: Intracellular bacteria find the right cell. Cell 2015, 161:199-209.

60. Benanti EL, Nguyen CM, Welch MD: Virulent Burkholderia species mimic host actin polymerses to drive actin-based motility. Cell 2015, 161:348-360.

61. Ooi WF et al.: The condition-dependent transcriptional landscape of Burkholderia pseudomallei. PLoS Genet 2013, 9:e1003795.

62. Lim VT et al.: Extended loop region of Hcp1 is critical for the assembly and function of type VI secretion system in Burkholderia pseudomallei. Sci Rep 2015, 5:8235.

63. Chen Y et al.: Regulation of type VI secretion system during Burkholderia pseudomallei infection. Infect Immun 2011, 79:3064-3073.

64. Hopf V et al.: BPSS1504, a cluster 1 type VI secretion gene, is involved in intracellular survival and virulence of Burkholderia pseudomallei. Infect Immun 2014, 82:2006-2015.

65. Chieng S, Mohamed R, Nathan S: Transcriprome analysis of Burkholderia pseudomallei TESS identifies Hcp1 as a potential serodiagnostic marker. Microb Pathog 2015, 79:47-56.

66. Toesca IJ, French CT, Miller JF: The Type VI secretion system spike protein VgrG5 mediates membrane fusion during intercellular spread by pseudomallei group Burkholderia species. Infect Immun 2014, 82:1436-1444.

67. Schwarz S et al.: VgrG-5 is a Burkholderia type VI secretion system-exported protein required for multinucleated giant cell formation and virulence. Infect Immun 2014, 82:1445-1452.

68. Gallagher LA et al.: Sequence-defined transposon mutant library of Burkholderia thailandensis. MBio 2013, 4:e00604-13.

69. Gutierrez MG, Yoder-Himes DR, Warawa JM: Comprehensive identification of virulence factors required for respiratory melioidosis using Tn-seq mutagenesis. Front Cell Infect Microbiol 2015, 5:78.

70. Nandi T, Tan P: Less is more: Burkholderia pseudomallei and chronic melioidosis. MBio 2013, 4:e00709-13.

71. Lazar Adler NR et al.: Autotransporters and their role in the virulence of Burkholderia pseudomallei and Burkholderia mallei. Front Microbiol 2011, 2:151.

72. Campos CG, Byrd MS, Cotter PA: Functional characterization of Burkholderia pseudomallei trimeric autotransporters. Infect Immun 2013, 81:2788-2799.

73. Loprasert T et al.: Compensatory increase in hspC gene expression and its role in protecting Burkholderia pseudomallei against reactive nitrogen intermediates. Arch Microbiol 2003, 180:498-502.

74. Reynolds C et al.: T cell immunity to the alkyl hydroperoxide reductase of Burkholderia pseudomallei: a correlate of disease outcome in acute melioidosis. J Immunol 2015, 194:4814-4824.

75. Stevens MP et al.: A Burkholderia pseudomallei type III secreted protein, BopE, facilitates bacterial invasion of epithelial cells and exhibits guanine nucleotide exchange factor activity. J Bacteriol 2003, 185:4992-4996.

76. Pumirat P et al.: Global transcriptional profiling of Burkholderia pseudomallei under salt stress reveals differential effects on the Bsa type III secretion system. BMC Microbiol 2010, 10:171.

77. Vander Broek CW et al.: Quantitative proteomic analysis of Burkholderia pseudomallei Bsa type III secretion system effectors using hypersecreting mutants. Mol Cell Proteomics 2015, 14:905-916.
81. Chirakul S et al.: Characterization of BPSS1521 (bprO), a regulator of Burkholderia pseudomallei virulence gene expression in the mouse model. PLoS One 2014, 9:e104313.

82. Holden MT et al.: Genomic plasticity of the causative agent of melioidosis, Burkholderia pseudomallei. Proc Natl Acad Sci U S A 2004, 101:14240-14245.

83. Chen Y et al.: Characterization and analysis of the Burkholderia pseudomallei BsaN virulence regulon. BMC Microbiol 2014, 14:206.

84. Gong L et al.: Burkholderia pseudomallei type III secretion system cluster 3 ATPase BsaS, a chemotherapeutic target for small-molecule ATPase inhibitors. Infect Immun 2015, 83:1276-1285.

85. Warawa J, Woods DE: Type III secretion system cluster 3 is required for maximal virulence of Burkholderia pseudomallei in a hamster infection model. FEMS Microbiol Lett 2005, 242:101-108.

86. D’Cruze T et al.: Role for the Burkholderia pseudomallei type three secretion system cluster 1 bpscN gene in virulence. Infect Immun 2011, 79:3659-3664.

87. Burtnick MN et al.: The cluster 1 type VI secretion system is a major virulence determinant in Burkholderia pseudomallei. Infect Immun 2011, 79:1512-1525.

88. Stevens MP et al.: Identification of a bacterial factor required for actin-based motility of Burkholderia pseudomallei. Mol Microbiol 2005, 56:40-53.

89. Sitthidet C et al.: Actin-based motility of Burkholderia thailandensis requires a central acidic domain of BimA that recruits and activates the cellular Arp2/3 complex. J Bacteriol 2010, 192:5249-5252.

90. Sarovich DS et al.: Variable virulence factors in Burkholderia pseudomallei (melioidosis) associated with human disease. PLoS One 2014, 9:e91882.