Melioidosis: insights into the pathogenicity of *Burkholderia pseudomallei*

W. Joost Wiersinga, Tom van der Poll*, Nicholas J. White‡§, Nicholas P. Day‡§ and Sharon J. Peacock‡§*

Abstract | *Burkholderia pseudomallei* is a potential bioterror agent and the causative agent of melioidosis, a severe disease that is endemic in areas of Southeast Asia and Northern Australia. Infection is often associated with bacterial dissemination to distant sites, and there are many possible disease manifestations, with melioidosis septic shock being the most severe. Eradication of the organism following infection is difficult, with a slow fever-clearance time, the need for prolonged antibiotic therapy and a high rate of relapse if therapy is not completed. Mortality from melioidosis septic shock remains high despite appropriate antimicrobial therapy. Prevention of disease and a reduction in mortality and the rate of relapse are priority areas for future research efforts. Studying how the disease is acquired and the host–pathogen interactions involved will underpin these efforts; this review presents an overview of current knowledge in these areas, highlighting key topics for evaluation.

**Academic Medical Centre, Centre for Infection and Immunity Amsterdam (CINIMA), Amsterdam, The Netherlands.*
‡Wellcome Trust, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand.
§Centre for Clinical Vaccinology and Tropical Medicine, Nuffield Department of Clinical Medicine, University of Oxford, Churchill Hospital, Oxford OX3 7LJ, UK.
Correspondence to W.J.W.
e-mail:
W.J.Wiersinga@amc.uva.nl
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Melioidosis is a serious disease caused by the aerobic, Gram-negative soil-dwelling bacillus *Burkholderia pseudomallei* and is most common in Southeast Asia and Northern Australia. Melioidosis is responsible for 20% of all community-acquired septicaemias and 40% of sepsis-related mortality in northeast Thailand. Reported cases are likely to represent 'the tip of the iceberg'[1,2], as confirmation of disease depends on bacterial isolation, a technique that is not available in many of the affected areas. Melioidosis often affects individuals with one or more pre-existing conditions associated with an altered immune response, the most common being diabetes mellitus (50% of cases). The most severe clinical manifestation is melioidosis septic shock, which is often associated with pneumonia and bacterial dissemination to distant sites (FIG. 1).

Melioidosis can present with an array of clinical signs and symptoms and *B. pseudomallei* has been called 'the great mimicker'. There can be a prolonged period between exposure to the causative agent and the clinical manifestations of infection (the longest recorded incubation period documented is 62 years[3]). Furthermore, recurrence of infection is common despite adequate antimicrobial therapy[2]. *B. pseudomallei* is intrinsically resistant to many antibiotics (including penicillin, first- and second-generation cephalosporins, macrolides, rifamycins, colistin and aminoglycosides), but is usually susceptible to amoxicillin-clavulanate, chloramphenicol, doxycycline, trimethoprim-sulphamethoxazole, ureidopenicillins, ceftazidime and carbapenems[2,4]. Treatment is required for 20 weeks and is divided into intravenous and oral phases[2,4]. Initial intravenous therapy is given for 10–14 days; ceftazidime or a carbapenem are the drugs of choice. The overall mortality for primary disease is 50% in northeast Thailand (35% in children) and ~20% overall in the higher-technology setting of Northern Australia[2,5]. Interest in the pathogenesis of *B. pseudomallei* and the related bacterium *Burkholderia mallei* has increased following their classification as category B agents by the US Centers for Disease Control and Prevention. This review presents an overview of our current knowledge of disease acquisition and the host–pathogen interactions that follow.

Taxonomy and genomics

B. pseudomallei is an aerobic, motile, non-spore-forming bacillus (FIG. 2). The genus *Burkholderia* contains >30 species, the most pathogenic members of which are *B. pseudomallei*, *B. mallei* and, in certain clinical conditions such as cystic fibrosis, *Burkholderia cepacia* (FIG. 3). The genus also includes *Burkholderia thailandensis*, which coexists with

Genomic islands
Clusters of genes that have been imported from unrelated bacterial taxa through horizontal gene transfer, and which might help the bacterium to acquire a new (possibly pathogenic) lifestyle.

Shotgun sequencing
A genomic sequencing strategy that involves random fragmentation of large DNA segments. The fragments are sequenced, and programs with highly refined algorithms are used to reassemble the original DNA sequence.

Multilocus sequence typing
(MLST). A method for the genotypic characterization of prokaryotes at the infraspecific level, using the allelic mismatches of a small number of housekeeping genes. Designed as a tool in molecular epidemiology and used for recognizing distinct strains within named species.

B. pseudomallei in the soil in Thailand but rarely causes disease and is >10^5-fold less virulent than *B. pseudomallei* in Syrian hamsters or mice[6]. *B. mallei* causes glanders in horses and is potentially highly virulent in humans, but natural disease in any host is now extremely rare.

The genome of *B. pseudomallei* (strain K96243 from Thailand) has been sequenced and comprises two chromosomes of 4.07 Mb and 3.17 Mb[7]. The large chromosome carries many genes associated with core functions such as cell growth and metabolism, and the smaller chromosome carries more genes encoding accessory functions that could be associated with adaptation and survival in different environments. Approximately 6% of the genome is made up of putative genomic islands that have probably been acquired through horizontal gene transfer. These are mostly absent from the *B. thailandensis* genome (and are absent from the *B. mallei* genome[8]); it is unclear whether these regions have a role in disease pathogenesis. The Institute for Genomic Research (TIGR, Rockville, Maryland, USA) is currently sequencing nine further *B. pseudomallei* isolates and 25 *B. pseudomallei* bacteriophage genomes from various sources. In addition, shotgun sequencing of the *B. thailandensis* strain E264 genome is now complete. The molecular epidemiology of *B. pseudomallei* has been investigated using multilocus sequence typing (MLST), and the findings suggest a high rate of genetic recombination[9].

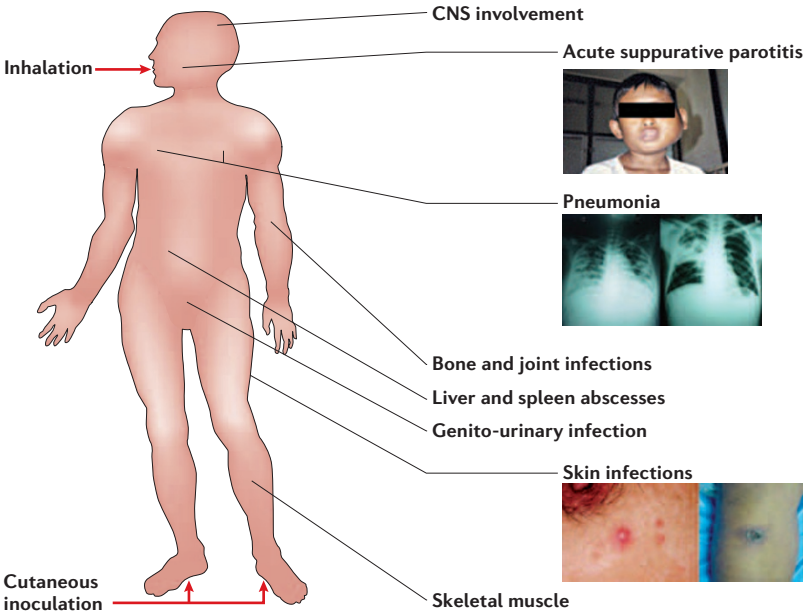

Figure 1 | **Selected clinical features of melioidosis, the 'great mimicker'.** The most severe clinical picture is melioidosis septic shock, which is often associated with bacterial dissemination to distant sites such as the lungs, liver and spleen. The lungs are the most commonly affected organ in adults, where there can be a localized or disseminated pulmonary infection, abscess formation or empyema. Chronic lung disease can also occur and can be difficult to distinguish from pulmonary tuberculosis. The clinical features of the disease in Thailand and Northern Australia (where most cases are reported) are largely shared, but there are some striking differences. Acute suppurative parotitis is the presenting feature in one-third of Thai paediatric cases but is uncommon in Australia; conversely, prostatic abscesses and brainstem encephalitis are more frequent in Australia[2,4]. Pictures courtesy of Dr Wirongrong Chierakul, Wellcome Trust, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand. CNS, central nervous system.

Whole-genome comparison between *B. pseudomallei* and *B. mallei* suggests that *B. mallei* has evolved through 'genomic downsizing' from a single clone of *B. pseudomallei* (this is consistent with a previous conclusion drawn from MLST[9]). A DNA microarray based on the whole-genome sequence of *B. pseudomallei* K96243 has been used to compare isolates of *B. pseudomallei*, *B. mallei* and *B. thailandensis*[10]. Deleted regions in *B. mallei* had significant genomic clustering compared with those in *B. thailandensis*, which were more uniformly dispersed. This indicates that the evolutionary processes that resulted in the divergence of these three species might have distinct mechanisms. Subtractive hybridization between *B. pseudomallei* and *B. thailandensis* has revealed several loci that are unique to the former[11,12]; publication of whole-genome comparisons between the two species is currently awaited.

Factors associated with disease acquisition

Melioidosis is found only in individuals who have been exposed to environments containing *B. pseudomallei*; infection is acquired through cutaneous inoculation, inhalation and aspiration. The factors associated with disease acquisition in endemic regions include environmental and host factors. There is no evidence that some isolates of *B. pseudomallei* are intrinsically more infectious than others.

Environmental exposure. There is a positive association between disease incidence and the extent of environmental contamination with *B. pseudomallei*[13–15]. However, few environmental-sampling studies have been published, and a more complete picture of the geographical distribution of *B. pseudomallei* can be derived from the reported cases of melioidosis. This topic has recently been reviewed by Cheng and Currie[4]. The disease is endemic in parts of Thailand, Northern Australia, Malaysia, Singapore, Vietnam and Burma. Possible endemic areas include Southern India, Southern China, Hong Kong, Taiwan, Brunei, Laos and Cambodia. Sporadic cases and occasional clusters have been reported in large areas of Asia, the Americas (notably Brazil), the Caribbean, the Pacific, Africa and the Middle East.

Weather conditions, route of acquisition and inoculum. Melioidosis is seasonal in the tropics, where most cases occur during the rainy season. This can be explained by increased contact with the organism. Rice farmers plant at the start of the monsoon and work in flooded rice paddies until harvest. Thai farmers rarely wear protective footwear and their feet often show signs of repeated trauma and injuries. Extreme weather can be associated with a shift in the mode of acquisition of infection. Aerosols are created during heavy rain, and this can result in repeated inhalation of the organism. Heavy rainfall and winds consistently cause a shift towards more pneumonia in patients presenting with melioidosis in Northern Australia[16]. Severe or penetrating injury and near-drowning are known risk factors for melioidosis, as highlighted by a study of a cluster of melioidosis cases in Southern Thailand following the 2004 tsunami[17].

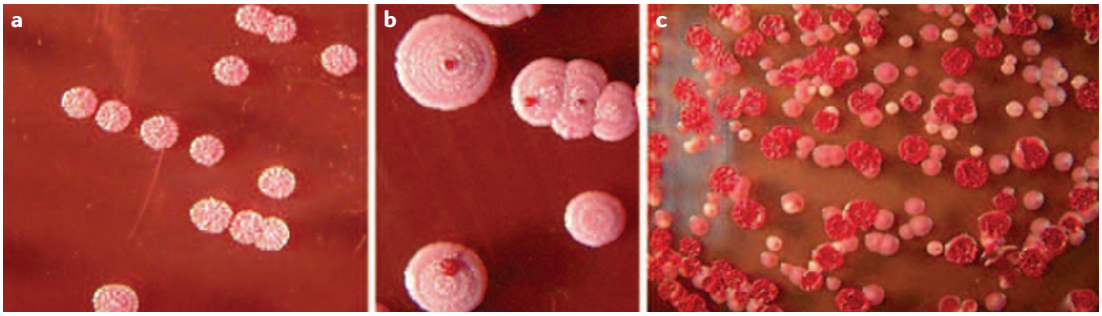

Figure 2 | **Clinical isolates of *Burkholderia pseudomallei*. a** | Typical colony morphology of *B. pseudomallei* on Ashdown's agar after incubation at 37°C in air for 3 days. **b**,**c** | Colony variation is commonly seen during culture of clinical isolates on Ashdown's agar. (**c**) shows the variable colony morphology that can be seen from a single sample; genotyping of these colonies showed that one clonal type was present. Colony variation can also be seen within a single colony, as shown in (**b**) in which the parental colony (pink) has given rise to a second morphotype (red). Pictures courtesy of Mrs Vanaporn Wuthiekanun and Mrs Narisara Chantratita, Wellcome Trust, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand.

Integrity of host immunity. In northeast Thailand, 80% of the population belongs to rice-farming families. Children have extensive contact with the organism, yet only one-fifth of all melioidosis cases here occur in children <14 years of age, with the disease incidence peaking later between the fourth and sixth decade of life. Most affected adults (>80%) have one or more underlying diseases (most commonly diabetes mellitus or renal failure). By contrast, children have an identifiable risk factor in <30% of cases (most commonly trauma). It is unclear whether affected children have a greater genetic susceptibility for disease. It is also possible that disease in childhood is caused by a subset of the bacterial population with increased pathogenic potential.

Immune response in the exposed but healthy population. Seroprevalence studies in northeast Thailand based on the indirect haemagglutination assay show that ~80% of people have antibodies against *B. pseudomallei* by the age of 4 years[18]. It is not clear whether healthy individuals with high antibody titres are infected and have a quiescent focus (analogous to a quiescent tuberculosis infection), or whether repeated environmental exposure in a primed individual maintains high antibody levels. The strong seasonality in disease presentation, combined with a reported mean incubation period of 9 days[19], suggests that primary disease occurs as a result of new infection rather than seasonal activation of a persistent focus. The possibility remains that the development of underlying risk factors causing immune dysfunction is seasonal, and this could be followed by seasonal disease breakthrough as bacteria escape from immune surveillance, but this is less biologically plausible. The lack of seasonality in documented relapse argues against this hypothesis. One episode of melioidosis does not protect susceptible individuals from further episodes of melioidosis due to re-infection[20].

Putative virulence factors

There is a paucity of knowledge in this area compared with the knowledge for other Gram-negative bacteria. The factors described here are included on the basis of a known role in virulence for other pathogens, or virulence in experimental models, and have been grouped according to the strength of existing evidence.

Strong putative candidates: quorum sensing. Quorum sensing is a cell-density-dependent communication system in Gram-negative bacteria that uses *N*-acyl-homoserine lactones (AHLs) for the coordination of gene expression[21,22]. LuxI proteins are responsible for AHL biosynthesis, and LuxR transcriptional regulators, following association with their cognate AHL(s), mediate gene repression or expression[21,22]. The *B. pseudomallei* genome is reported to contain three LuxI and five LuxR quorum-sensing homologues[22]. Mass-spectrometry analysis of *B. pseudomallei* culture supernatants has demonstrated the presence of many signalling molecules, including *N*-decanoyl-homoserine-lactone and *N*-(3-oxotetradecanoyl)-L-homoserinelactone[22]. Disruption of the eight genes encoding *luxIR* quorum-sensing homologues led to a significant increase in the LD_{50} (the infectious dose that is lethal to 50% of the animals infected) in Syrian hamsters after intraperitoneal challenge, and increased the time to death and reduced organ colonization in aerosolized BALB/c mice[22]. A LuxI-LuxR homologue termed PmlI-PmlR, which directs the synthesis of *N*-decanoyl-homoserine-lactone and is involved in regulation of a metalloprotease, is essential for full virulence in a mouse model[23]. A homologue termed BpsI-BpsR is also required for optimal virulence and the secretion of exoproducts[24].

Some quorum-sensing-controlled candidate virulence factors and processes such as siderophores, phospholipase C and biofilm formation are probably partially dependent on BpeAB-OprB, a multidrug efflux pump in *B. pseudomallei* that is also known to be responsible for conferring antimicrobial resistance to aminoglycosides and macrolides[25]. The *bpeAB-oprB* operon in turn might be regulated by quorum sensing, as *N*-decanoyl-homoserine-lactone and *N*-octanoyl-homoserine lactone can induce *bpeAB-oprB* expression[25]. BpeAB mutants are also associated with attenuated cell invasion and cytotoxicity of human lung epithelial (A549) and human macrophage (THP-1) cells[25].

Subtractive hybridization
A technique used to identify differentially expressed genes. The DNA species present in one sample are specifically enriched by hybridization with nucleic acids from another sample and by removing the associated double-stranded molecules.

Quorum sensing
A system by which bacteria communicate. Signalling molecules — chemicals similar to pheromones that are produced by an individual bacterium — can affect the behaviour of surrounding bacteria.

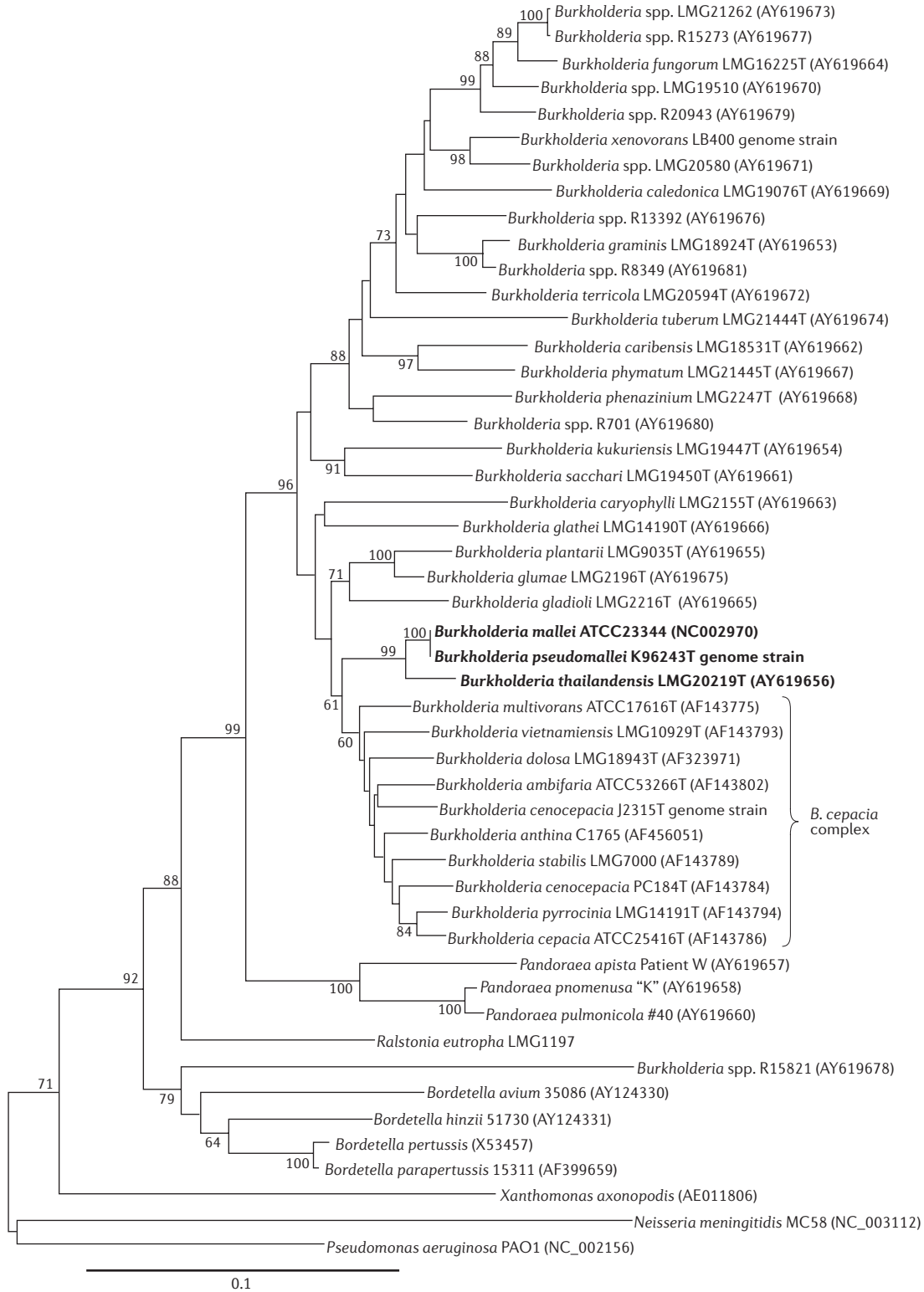

Figure 3 | **The phylogeny of the *Burkholderia* genus.** The phylogenetic tree is based on novel *recA* sequences. Most *Burkholderia* species are plant pathogens, but two groups can cause disease in humans. The *Burkholderia cepacia* complex is a group of important opportunistic pathogens for individuals with cystic fibrosis, with *Burkholderia cenocepacia* the cause of 70% of cases of *Burkholderia cepacia* complex infection[101]. *Burkholderia pseudomallei* and *Burkholderia mallei* have the potential to cause human disease, but the related *Burkholderia thailandensis* is rarely pathogenic. In this analysis, *recA* sequences from the related genera *Ralstonia*, *Bordetella*, *Xanthomonas* and *Neisseria* were included, and the tree was rooted with the *Pseudomonas aeruginosa* PAO1 *recA* gene. Reproduced with permission from REF. 102 © (2005) American Society for Microbiology.

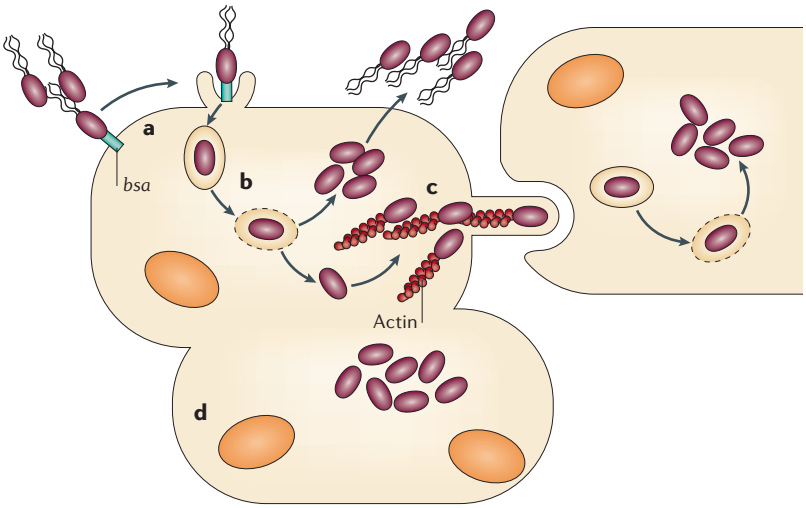

Figure 4 | **The intracellular lifestyle of *Burkholderia pseudomallei*. a** | Invasion of phagocytic and non-phagocytic cells. *B. pseudomallei* is capable of invading many cell types, including epithelial cells, and can survive and proliferate for prolonged periods within phagocytic cells. The Inv/Mxi/Spa-like type III secretion gene cluster, termed the *Burkholderia* secretion apparatus (*bsa*) system, encodes proteins that are required for invasion, escape from phagosomes and intercellular spread[29]. **b** | Endosome escape and intracellular proliferation. As early as 15 minutes after internalization, *B. pseudomallei* can escape from endocytic vacuoles into the cytoplasm of infected cells by lysing the endosome membrane. *B. pseudomallei* seems to be resistant to several host antimicrobial peptides (for example, protamine and certain defensins) and interferes with the synthesis of inducible nitric-oxide synthase (iNOS), which is known to have an important role in the killing of intracellular bacteria[73,79]. In addition, in certain circumstances *B. pseudomallei* can induce apoptosis in both phagocytic and non-phagocytic cells[75]. **c** | Cell-to-cell spread. Once inside the cytoplasm, *B. pseudomallei* induces the formation of actin-based membrane protrusions by continuous nucleation of actin at one pole of the bacterial cell[29,75,81]. The bacterial protein BimA is required for this process[82]. Cell-to-cell movement of *B. pseudomallei* occurs when a neighbouring cell phagocytoses this protrusion, thereby allowing the spread of *B. pseudomallei* without exposure to antibodies or immunoactive molecules. In the new cell, *B. pseudomallei* will subsequently escape from the secondary vacuoles and multiply intracellularly. **d** | Cell fusion. Uniquely among bacterial intracellular pathogens, *B. pseudomallei* can induce the formation of multinucleated giant cells by cell fusion[75].

Strong putative candidates: type III secretion system. *B. pseudomallei* contains three type III secretion system (TTSS) gene clusters, which contain between 16 and 18 reported genes[7,26–29]. One of these clusters (the TTSS3 cluster) shares homology with the *inv/spa/prg* TTSS of *Salmonella enterica* serovar Typhimurium (*S. typhimurium*) and the *ipa/mxi/spa* TTSS cluster of *Shigella flexneri*[26,28,29]. This cluster encodes a secretion apparatus that functions like a molecular syringe. A subset of type-III-secreted proteins ('translocators') interact with the eukaryotic cell membrane and inoculate other type-III-secreted 'effector' proteins into the target-cell cytosol, where they subvert host-cell processes (reviewed by Cornelis and Van Gijsegem[30]). Mutations that disrupt the *S. typhimurium* Inv/Spa/Prg apparatus inhibit bacterial invasion and enteropathogenesis. The gene cluster in *B. pseudomallei* (termed *bsa*, *Burkholderia* secretion apparatus) encodes proteins that are very similar to the *S. typhimurium* and *S. flexneri* type-III-secreted proteins required for invasion, escape from endocytic vacuoles, intercellular spread and pathogenesis[29].

Following internalization, *B. pseudomallei* escapes from endocytic vacuoles into the cytoplasm of infected cells (FIG. 4). Induction of actin polymerization at one pole leads to the formation of membrane protrusions and cell-to-cell spread. *B. pseudomallei* mutants lacking components of the Bsa secretion and translocation apparatus have reduced replication in murine macrophage-like cells, an inability to escape from endocytic vacuoles and cannot form membrane protrusions and actin tails[29]. Inactivation of BopE, a TTSS protein that is encoded adjacent to the *B. pseudomallei bsa* locus and is homologous to *Salmonella enterica* SopE/SopE2, a guanine nucleotide-exchange factor, leads to impaired bacterial entry into HeLa cells, indicating that BopE facilitates invasion[31]. In addition, the *B. pseudomallei bsa* locus encodes homologues of the *Salmonella* Sip translocator proteins (BipB, BipC and BipD)[29]. *Salmonella* SipB, SipC and SipD proteins are required for injection of effector proteins and invasion of epithelial cells *in vitro*[32]; mutation of the *B. pseudomallei bipD* gene impairs invasion of epithelial cells *in vitro*[31]. *B. pseudomallei* BipD mutants that lack a component of the translocation apparatus are attenuated following intraperitoneal or intranasal challenge of BALB/c mice, and have impaired bacterial replication in liver and spleen[32]. *B. pseudomallei* BipB has been shown to mediate the formation of multinucleated giant cells, cell-to-cell spreading of bacteria and apoptosis of infected host cells[33]. *bipB* mutants are also associated with attenuation following intranasal challenge of BALB/c mice[33].

The TTSS1 gene cluster was first described in 1999 (REF. 34). This cluster is homologous to a TTSS found in the plant pathogen *Ralstonia solanacearum* but is absent from *B. mallei* and *B. thailandensis*[7,26,35]. TTSS2 is present in *R. solanacearum*, *B. pseudomallei*, *B. mallei* and *B. thailandensis*. Whereas TTSS3 has been shown to be required for full virulence in a hamster model of infection[27], the role of TTSS1 and TTSS2 in pathogenesis is not known.

Strong putative candidates: capsular polysaccharide. *B. pseudomallei* produces an extracellular capsular polysaccharide with the structure -3)-2-*O*-acetyl-6-deoxy-β-D-*manno*-heptopyranose-(1- (REFS 11,36). Previously characterized as a type I O-polysaccharide, it has more recently been considered to be a capsular polysaccharide based on its high molecular mass and genetic homology with group 3 capsular polysaccharides of other organisms. The capsular polysaccharide is required for *B. pseudomallei* virulence in experimental animal models[11,37]. Capsule expression is induced in the presence of serum, and the addition of purified *B. pseudomallei* capsule to serum bactericidal assays increases the survival of *B. pseudomallei* SLR5, a serum-sensitive strain, by 1,000-fold[38]. Phagocytosis is greater for a capsule-deficient mutant compared with the wild type in the presence of normal human serum[38]. Both observations can be explained by the finding that deposition of complement factor C3b on the bacterial cell surface is lower in the presence of capsule[38]. The capsule might act as a barrier, blocking access of the complement receptor-1 (CR1) on phagocytes to the C3b deposited on the bacterial surface[38].

Complement
A part of the innate immune system comprising serum proteins that can protect against infection.

Other putative candidates: lipopolysaccharide. *B. pseudomallei* lipopolysaccharide (LPS) (formally termed type II O-antigenic polysaccharide) seems to differ in several respects from the LPS of other Gram-negative organisms. *B. pseudomallei* LPS exhibits weaker pyrogenic activity in rodents compared with enterobacterial LPS, but stronger mitogenic activity in murine splenocytes[39]. LPS-mediated activation of a mouse-macrophage cell line *in vitro* is slower for LPS from *B. pseudomallei* compared with LPS from *Escherichia coli*[40]. Recognition of LPS by the host is of crucial importance for the initiation of a swift innate immune response to Gram-negative bacteria, primarily through activation of the pattern-recognition receptor Toll-like receptor (TLR)4 (see also further discussion)[41]. So, the fact that *B. pseudomallei* LPS is apparently less capable of activating immune cells might at least in part explain why TLR4 does not have a role in host defence against experimentally induced melioidosis in mice, whereas mice deficient for this receptor are highly susceptible to other Gram-negative infections[41].

B. pseudomallei LPS seems to be largely conserved across this species. LPS profiling of >700 *B. pseudomallei* isolates using proteinase-K digestion and SDS-PAGE silver-stained gels, a technique that examines the O-side chain, demonstrated that most isolates had a 'typical' ladder pattern of extracted LPS, 3% had an 'atypical' pattern, and 0.1% did not exhibit a ladder appearance at all[42]. The different LPS preparations have similar endotoxic activity in the Limulus amoebocyte lysate assay. However, there seems to be a difference in the host immune response to these molecules, as there is a lack of immunological crossreactivity on western blot between typical and atypical LPS using patient sera infected with typical and atypical LPS isolates[42].

The level of antibody to LPS on admission to hospital is higher in patients with melioidosis who survive compared with those who die, and in patients with non-septicaemic versus septicaemic melioidosis[43]. These antibodies might protect the host against death; alternatively, there could be an association between a raised anti-LPS antibody titre and a more efficient host immune response, including cell-mediated killing.

The LPS of *B. pseudomallei* (pathogenic) and *B. thailandensis* (non-pathogenic) have been compared. LPS profiling using proteinase-K digestion and SDS-PAGE silver-stained gel shows identical ladder patterns for most isolates of both species. The two species exhibit similar immunoblot profiles against pooled sera from patients with melioidosis, and with hyperimmune mouse sera[44]. LPS shedding profiles are also similar between the two species[45]. This has led to the suggestion that LPS is unlikely to be involved in the virulence and pathogenicity of *B. pseudomallei*[44]. Other possible explanations are that LPS from the two species is antigenically very similar but differs in biological activity or that LPS from both species has biological activity *in vivo*, but only *B. pseudomallei* has an additional complement of genes that promote successful invasion and bacterial dissemination within the host. Caution is required when interpreting the significance of LPS in *B. pseudomallei* virulence, as assays in which LPS is truncated could impair the insertion, stability and folding of other surface-anchored molecules.

Other putative candidates: flagella. *B. pseudomallei* is flagellated and motile. There is no difference in the ability of wild-type *B. pseudomallei* and an isogenic mutant defective in flagella expression to invade, and replicate in, human lung cells *in vitro*[46]. In one study, there was no difference between an isogenic mutant and wild-type *B. pseudomallei* in diabetic rat and Syrian hamster infection models[47]. In a second study, bacterial numbers were markedly reduced in the lung and spleen of BALB/c mice following intranasal infection of an aflagellate mutant compared with the wild type, and the mutant was less virulent following intraperitoneal infection of BALB/c mice, based on the LD_{50} (REF. 46).

Other putative candidates: type IV pili-mediated adherence. Adherence is an important virulence mechanism mediated by carbohydrate molecules, pilus and non-pilus adhesins. Type IV pili are important for virulence in many Gram-negative bacteria. The *B. pseudomallei* K96243 genome contains multiple type IV pilin--associated loci, including one encoding a putative pilus structural protein (PilA)[48]. A *pilA* deletion mutant has reduced adherence to human epithelial cells and is less virulent in the nematode model of virulence and the murine model of melioidosis, suggesting a role for type IV pili in *B. pseudomallei* virulence.

Other putative candidates. Other putative candidiates include a siderophore for iron acquisition[49] and secreted proteins such as haemolysin, lipases and proteases[50]. Bacterial colony morphology of clinical *B. pseudomallei* isolates can vary both within a given culture and in cultures from the same patient over time (FIG. 2). The relevance of this to human disease is the subject of intensive investigation in our laboratories.

Downregulation of virulence. An arabinose-assimilation operon that consists of nine genes is present in *B. thailandensis* and absent from the *B. pseudomallei* and *B. mallei* chromosomes. When this operon is cloned experimentally and re-introduced into a laboratory strain of *B. pseudomallei*, the mutant strain has a reduced ability to cause the death of Syrian hamsters compared with the parent strain[51]. On microarray analysis, several genes in the TTSS3 cluster are downregulated in the mutant when cells are grown in L-arabinose, suggesting a regulatory role for (a metabolite of) L-arabinose[51]. This could be one of many similar examples.

Host–pathogen interactions during melioidosis

Innate immune response. On the first encounter with a pathogen, cells of the innate immune system recognize conserved surface motifs termed 'pathogen-associated molecular patterns' or PAMPs through host-cell pattern-recognition receptors. The family of TLRs are important members of this surveillance system which initiate the innate immune response, and form a key link between innate and adaptive immunity[52]. *B. pseudomallei* expresses

Limulus amoebocytelysate assay
A chromogenic assay used to monitor endotoxin production.

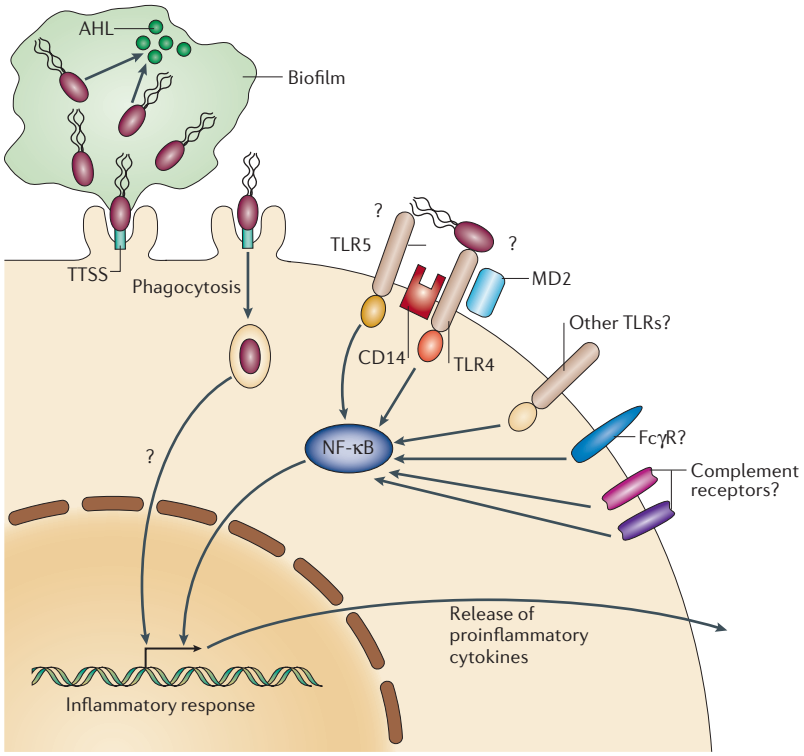

Figure 5 | **Host–pathogen interactions in *Burkholderia pseudomallei* infection: bacterial virulence meets innate immunity.** Proposed scheme of the first encounter between *B. pseudomallei* and the immune system. Putative virulence factors on the bacterial cell surface include lipopolysaccharide (LPS), capsular polysaccharides and flagella. There might also be a role for biofilm formation. Bacterial gene regulation through *N*-acyl-homoserine lactones (AHLs) could be crucial for bacterial survival *in vivo*. Type III secretion systems (TTSSs) might facilitate invasion of the bacterium into the host cell, and enable escape from endocytic host-cell vesicles. Monocytes are probably the most important immune cells in early infection. Based on evidence from other Gram-negative pathogens, there might be a role for the Toll-like receptors (TLRs). TLR4, with its co-receptors MD2 and CD14, might recognize *B. pseudomallei* LPS, whereas TLR5 might recognize flagella, although to date there are no published data to support this. The complement receptors CR1, CR3 and possibly FcγR mediate opsonin-dependent phagocytosis. Recognition of *B. pseudomallei* will cause activation of pro-inflammatory genes through nuclear factor (NF)-κB and lead to the activation of the immune response through the release of pro-inflammatory cytokines.

several PAMPs for which the corresponding TLR is known (FIG. 5). For example, in other Gram-negative species it has been shown that LPS activates the cells of the immune system through a receptor complex that consists of a ligand-binding molecule (CD14) and TLR4 as the signal transducer. Other candidate *B. pseudomallei* TLR ligands include peptidoglycan (TLR2), flagellin (TLR5) and bacterial DNA or CpG (TLR9). However, to date experimental data for a role for TLRs in melioidosis are lacking, although it is interesting to note that C3H/HeJ mice that carry a loss-of-function mutation in the *tlr4* gene are resistant to extremely high doses of *B. pseudomallei* LPS (up to 10,000 ng per mouse)[39].

The pro-inflammatory cytokine interferon (IFN)-γ has an important role in early resistance against *B. pseudomallei* infection. Inhibition of IFN-γ expression in mice lowered the LD_{50} from >5 × 10^5 to ~2 colony-forming units (CFUs) and was associated with an 8,500- and 4,400-fold increase in bacterial load in liver and spleen, respectively[53]. Inhibition of interleukin (IL)-12 or IL-18, the predominant endogenous inducers of IFN-γ production, resulted in increased mortality in the same model[53,54]. IFN-γ, IL-12 and IL-18 have a key role in the T_H1 cell-mediated immune response. Recent data indicate that activation of suppressor of cytokine signalling 3 (SOCS3) and cytokine-inducible Src homology 2-containing protein (CIS) in *B. pseudomallei*-infected macrophages correlates with a decreased IFN-γ signalling response and facilitates bacterial escape[55]. Further evidence for the role of a T_H1 response in protective immunity against melioidosis comes from studies with inbred mouse strains, in which T_H1-response-prone C57BL/6 mice are relatively resistant to *B. pseudomallei* when compared with the T_H2-response-prone BALB/c mice[56,57]. The pro-inflammatory cytokine tumour-necrosis factor (TNF)-α is also likely to be an important element of the early immune response, as passive immunization against this mainly macrophage-derived cytokine increased mortality in experimental murine melioidosis[53]. Serum IFN-γ, IL-12 and TNF-α concentrations are elevated in melioidosis patients[58,59]; the involvement of TNF-α in human disease is further suggested by a report that the –308 TNF-α promoter polymorphism, which is related to severity of disease for several other infectious diseases, was associated with both the occurrence and severity of melioidosis[60].

Plasma or serum concentrations of several other pro-inflammatory mediators are elevated in patients with melioidosis, including IL-6, IL-15, IFN-γ-inducible protein (IP)-10 and monokine induced by IFN-γ (Mig)[58,61], as well as the anti-inflammatory cytokine IL-10 (REF. 62). This indicates that multiple inflammatory pathways become activated, including those involving cellular activation. For example, IP-10 and Mig are chemokines primarily induced by IFN-γ which share a common receptor (CXC chemokine receptor 3, CXCR3), expressed by activated T cells and natural killer (NK) cells. Cytotoxic T cells and NK cells are further implicated in the immune response to *B. pseudomallei* through the observation that circulating levels of granzymes A and B are elevated. Granzymes are secreted by cytotoxic T and NK cells and are important for the initiation of apoptosis in the target cell, although their precise role in bacterial infection remains to be established[63]. NK cells and $CD8^+$ T cells harvested from the spleen of uninfected mice also release IFN-γ on stimulation with *B. pseudomallei in vitro*[64], and during experimental melioidosis NK and T cells have been identified as the predominant IFN-γ producers[54].

B. pseudomallei rapidly and efficiently activates complement, predominantly through the alternative activation pathway[65]. Complement activation results in the deposition of C3 on the bacterial surface and further opsonisation[65]. However, *B. pseudomallei* is resistant to the lytic action of complement[65], a feature shared with several other pathogens including *Borrelia* spp., *Salmonella* spp., *Neisseria* spp. and streptococci[66]. In one study, adherence and phagocytosis of *B. pseudomallei* by granulocytes and macrophages was dependent on the presence of opsonins and mediated by the complement receptors CR1 and

Natural killer (NK) cells
Lymphocytes that do not express the T-cell receptor or B-cell receptor and that mediate natural killing against prototypical NK-cell-sensitive targets.

Human leukocyte antigen (HLA). Also known as major histocompatibility complex (MHC), a glycoprotein that is found on the surface of antigen-presenting cells that presents antigen for recognition by T_H cells.

CR3 expressed on the macrophage membrane[65]. The capsular polysaccharide of *B. pseudomallei* contributes to resistance to phagocytosis by reducing the deposition of complement factor C3b (REF. 38).

Adaptive immune response. In patients with melioidosis, the levels of IgG, IgA and IgM correlate positively with disease severity, with higher levels in those with invasive compared with localized disease. Melioidosis is positively associated with specific human leukocyte antigen (HLA) class II molecules in Thai patients; the HLA class II DRB1*1602 allele was positively associated with septicaemic melioidosis, whereas DQA1*03 was negatively associated[67]. Patients who recovered from melioidosis showed evidence for an antigen-specific cell-mediated immune response, as reflected by enhanced lymphocyte proliferation and IFN-γ production in response to *B. pseudomallei* antigens[68]. In addition, asymptomatic seropositive individuals showed a stronger cell-mediated adaptive immune response as measured by *Burkholderia*-specific lymphocyte reactions compared with subjects with a history of clinical melioidosis[69], suggesting that a strong cell-mediated immune response might protect against disease progression. Conceivably, the production of IFN-γ by $CD4^+$ T cells activates macrophages to become more bactericidal, a notion that is supported by the finding that IFN-γ increases the intracellular killing activity of macrophages *in vitro*[70]. Of considerable interest, a very recent study has suggested that *B. pseudomallei*-specific $CD4^+$ T cells are important for late host resistance against murine melioidosis[54]. However, the importance of $CD4^+$ T cells in the control of infection is open to debate, as there does not seem to be an association between HIV infection and melioidosis[71].

***Intracellular survival of* B. pseudomallei.** *In vitro* models indicate that *B. pseudomallei* survives and replicates within neutrophils and monocytes[72–75], and uses multiple mechanisms to escape macrophage killing and evade host immunity. These include resistance to human defensins[72] and inhibition of DNA and protein synthesis within the host cell[76]. Ultrastructural studies suggest that *B. pseudomallei* might reside within membrane-bound compartments[73], particularly phagolysosomes[65], making use of its ability to survive and grow in acidic environments[77]. Furthermore, *B. pseudomallei* can invade mouse macrophages without activating inducible nitric-oxide synthase (iNOS), an enzyme that is required for the generation of reactive nitrogen intermediates and is important for intracellular bacterial killing[78]. *B. pseudomallei* can escape from endocytic vesicles into the cytoplasm (FIG. 4) by lysing the endosome membranes as early as 15 minutes after internalization by phagocytic cells[29,79,80]. Moreover, intracellular *B. pseudomallei* can be propelled by inducing continuous polymerization of actin at one pole of the bacterial cell[29,75,81], which results in the formation of membrane protrusions in host cells with the bacteria at the tip end. Such protrusions can project into an adjacent cell, facilitating the spread *B. pseudomallei* from one eukaryotic cell to another[75] (FIG. 4). A *B. pseudomallei*-specific protein termed BimA is required for the formation of actin tails. BimA is located at the pole of the bacterial cell at which actin polymerization occurs[82]. Mutation of the *bimA* gene in *B. pseudomallei* abolished actin-based motility of intracellular bacteria in a macrophage-like cell line. Actin-tail formation could be restored by inducible expression of the *bimA* gene on a plasmid, indicating the essential role for this gene in actin-tail formation. Of note, mutation of *bimA* does not influence the activity of the Bsa TTSS apparatus or bacterial escape from endosomes.

Multinucleated giant cells are occasionally seen in human tissue infected with *B. pseudomallei* and might represent giant macrophages[83]. It is possible that macrophages are a site of intracellular survival, given their relatively long half-life and reduced microbicidal capacity in comparison to neutrophils and monocytes[65]. A recent report indicated that *B. pseudomallei* might evade killing by macrophages through the induction of caspase-1-dependent host-cell death; this process requires a functional *bsa* TTSS[84]. Cell death was accompanied by the release of IL-1β and IL-18 (REF. 84). Survival within microcolonies encapsulated in a protective biofilm is an alternative explanation for prolonged quiescent survival within the host. *In vitro*, *B. pseudomallei* can survive for years in distilled water and can also enter and survive within free-living amoebae belonging to the genus *Acanthamoeba*; it is possible that this might enhance bacterial survival in soil and aquatic environments[85,86].

***Interactions with human epithelial cells* in vitro.** *B. pseudomallei* adheres to cultured human epithelial cell lines derived from alveolar, bronchial, laryngeal, oral, conjunctival and cervical tissues[87]. Adherence of *B. pseudomallei* (but not *B. thailandensis*) to cell lines *in vitro* was enhanced when incubated at a temperature of 30°C compared with 37°C (REF. 87). *B. pseudomallei* is more efficient in invasion, adherence and induction of cellular damage of respiratory epithelial cells compared with *B. thailandensis*[88]. A bacterial mutant defective in *pilA* (a putative type IV pilus gene) has reduced adherence to human epithelial cells[48]. The relevance of these observations for human disease pathogenesis is unknown.

New therapeutic and vaccine options

Potential new therapies targeting the immune response. Several conditions including diabetes mellitus, renal failure and alcohol abuse are important risk factors for the development of melioidosis. A common link is that these are associated with impairment of neutrophil function. This had led to interest in the therapeutic role of granulocyte colony-stimulating factor (G-CSF), a cytokine that increases the circulating neutrophil count and stimulates neutrophil function. A retrospective study conducted in Australia reported a reduction in mortality of melioidosis patients after the introduction of G-CSF as an adjunctive treatment for patients with septic shock[89]. A mouse model of melioidosis in which outcome was compared between mice given ceftazidime alone or in combination with G-CSF failed to show any benefit from G-CSF[90]. A randomized clinical trial of G-CSF is currently ongoing in Thailand[91].

Unmethylated CpG motifs in synthetic oligodeoxynucleotide enhance the uptake of bacteria by mouse macrophages in a concentration-dependent manner and induce nitric-oxide production by mouse macrophages[92]. CpG treatment one hour before bacterial inoculation offered protection in a murine model of *B. pseudomallei* infection, associated with the rapid induction of pro-inflammatory cytokines[93].

Vaccine development. There is no effective vaccine available that protects against *B. pseudomallei* infection. Current approaches under evaluation include conjugate, DNA, attenuated and heterologous vaccines[94]. Attenuated mutants that are invasive but have a reduced ability to multiply in phagocytes have been identified using transposon mutagenesis, and these induce a high degree of protective immunity in mice (K. Breitbach, personal communication). A mutant of *B. pseudomallei* that is auxotrophic for branched-chain amino acids induced a protective response against a subsequent challenge with an otherwise lethal dose of wild-type *B. pseudomallei* in an animal model[95]. Mice inoculated with a *B. pseudomallei bipD* mutant were partially protected against subsequent challenge with wild-type *B. pseudomallei*, although immunization with purified BipD protein was not protective[32]. However, it seems unlikely that live attenuated vaccination will be feasible for use in humans. Antibodies raised against *B. pseudomallei* flagellin markedly reduced bacterial motility and provided passive protection against experimentally induced *B. pseudomallei* infection[96]. Evaluation of LPS and capsular polysaccharide as subunit vaccines against experimental melioidosis demonstrated partial protection in a mouse model[97]. Inoculation of guinea pigs with *B. thailandensis* provided partial protection from a subsequent challenge with virulent *B. pseudomallei*[98].

In addition, it was recently postulated that the T-cell response to primary *B. pseudomallei* infection is biphasic, comprising an early cytokine — most notably IFN-γ induced — phase in which T cells seem to be functionally redundant for initial bacterial clearance, followed by a later antigen-induced phase in which *B. pseudomallei*-specific T cells (in particular $CD4^+$ T cells) are important for host resistance[55]. As a consequence, because *B. pseudomallei* infection generates a rapid and potent IFN-γ response from natural killer T cells and conventional T cells, it is has been suggested that an effective subunit vaccine against *B. pseudomallei* should target the generation of IFN-γ-secreting T cells[54]. This was further underscored by a recent vaccine-driven report suggesting that any potential vaccine would need to stimulate both cell-mediated and humoral immunity[99]. In this study, dendritic cells were used as a vaccine-delivery vector to induce cell-mediated immune responses to *B. pseudomallei*. Purified dendritic cells were pulsed with heat-killed whole-cell *B. pseudomallei* and used to immunize syngeneic mice. Strong cellular immune responses were elicited, although antibody responses were low. Subsequently, booster immunizations of either a second dose of dendritic cells or heat-killed *B. pseudomallei* were administered to increase the immune response. Immunized animals were challenged with fully virulent *B. pseudomallei*, and protection was shown in those with

Box 1 | **Questions for future research**

Epidemiology

- To what extent will new global environmental sampling studies and improvements in diagnostic microbiology give a more complete picture of the geographical distribution of *Burkholderia pseudomallei*? What is the real burden of disease from melioidosis worldwide?
- Are there any geographical or environmental differences that can account for the varying states of endemicity in regions with a climate that resembles highly endemic northeast Thailand, such as Vietnam and Laos?

Virulence and pathogenesis

- What is the precise role of putative genomic islands in virulence?
- Will the comparison of the genetic structure of isolates from the environment with those associated with invasive disease define whether some clones are more adept at causing melioidosis than others?
- To what extent is lipopolysaccharide an important factor in the virulence and pathogenicity of *B. pseudomallei*?
- Why is *B. pseudomallei* acute suppurative parotitis almost exclusively seen in Thai paediatric cases?
- Why is person-to-person transmission so rare despite the high number of bacteria detected in the sputum of patients?
- What is the mechanism that enables *B. pseudomallei* to hide from host defences for years or decades?
- What mechanism renders diabetes such a disproportionately important risk factor for disease acquisition?
- How is *B. pseudomallei* recognized by the innate immune system on first encounter?
- What is the role of $CD4^+$ lymphocytes in the control of infection by *B. pseudomallei*?

Treatment and prevention

- Which treatment option is most effective at reducing the high disease-relapse rates? What combination and duration of antibiotics is optimal?
- To what extent will better clinical care (including preventative measures, earlier clinical identification and better management of severe sepsis) improve the outcome of melioidosis in endemic areas?
- What will be the optimal vaccine strategy? And to what extent will a vaccine prevent severe disease due to *B. pseudomallei*?

strong humoral and cell-mediated immunity[99]. The possible utility of these observations for vaccine development awaits further investigation.

Conclusions and perspectives

The recent advent of molecular genetics and *in vitro* and *in vivo* infection models, together with availability of the complete genome sequence of *B. pseudomallei*, *B. mallei* and (in the near future) *B. thailandensis*, has advanced our understanding of the virulence mechanisms that govern the complex interaction between *B. pseudomallei* and host cells. Preserved mechanisms underlying the intracellular lifestyle of *B. pseudomallei* have been discovered by comparing the *B. pseudomallei* genome with the genomes of other intracellular microorganisms (for example, *Salmonella* and *Shigella* species)[86]. New techniques that allow integrative analyses at genomic, transcriptional and proteomic levels promise to provide further insights into the complex interaction between this pathogen and its environment[100]. The genetic diversity within the natural population will be characterized further using techniques such as MLST and microarray analysis, along with the identification of the *B. pseudomallei* secretome (all secreted proteins secreted) and immunome (identification of immunogenic proteins). Some virulence factors have now been characterized, and the study of host–pathogen interactions has shed some light on pathogenic mechanisms, but many key questions await investigation, and BOX 1 summarizes some important questions for future research on *B. pseudomallei* pathogenesis.

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Competing interests statement
The authors declare no competing financial interests.

DATABASES
The following terms in this article are linked online to:
Entrez Genome Project: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=genomeprj
Burkholderia mallei | *Burkholderia pseudomallei* | *Burkholderia thailandensis* | *Escherichia coli* | *Ralstonia solanacearum* | *Salmonella enterica* serovar Typhimurium | *Shigella flexneri*
UniProtKB: http://ca.expasy.org/sprot
BipB | BipC | BipD | BopE | BpsI | BpsR | CD14 | CIS | CR1 | CXCR3 | G-CSF | IFN-γ | IL-1β | IL-6 | IL-10 | IL-15 | IL-18 | iNOS | IP10 | Mig | OprB | PilA | PmlI | PmlR | SOCS3 | SopE | TLR2 | TLR4 | TLR5 | TLR9 | TNF-α

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http://www.jr2.ox.ac.uk/ndm/Tropical_Medicine
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http://www.sanger.ac.uk/projects/B_pseudomallei
The Institute for Genomic Research (TIGR):
htttp://www.tigr.org/pathema/b_pseudomallei
CDC list of bioterrorism agents:
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