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Interactions of *Burkholderia pseudomallei* and *Acanthamoeba castellanii* and Their Effects on Virulence in Human Monocytes

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Interactions of *Burkholderia pseudomallei* and *Acanthamoeba castellanii*
and their Effects on Virulence in

Human Monocytes

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A thesis submitted to the faculty of
Brigham Young University
in partial fulfillment of the requirements for the degree of

Master of Science

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ABSTRACT

Interactions of *Burkholderia pseudomallei* and *Acanthamoeba castellanii*
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in Human Monocytes

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Burkholderia pseudomallei, the etiological agent of melioidosis, is a saprophytic bacterium existing endemically in the water and soil of SE Asia and Northern Australia. This organism has shown the ability to remain dormant in its host for decades. *B. thailandensis* is a closely related non-pathogenic near neighbor that is also found in these soils. It has been suggested that free-living amoeba could be natural reservoirs for these organisms. The interactions of *Burkholderia* species and *Acanthamoeba castellanii*, a species of free-living amoeba, were studied to better understand the natural ecology of these organisms and to determine the effects amoeba interactions might have on pathogenesis. In this study, the adherence and persistence of several *B. pseudomallei* clinical isolates were compared to that of *B. thailandensis* within both amoeba and a human monocyte cell line. Results showed that *B. pseudomallei* isolates can enter amoeba and survive therein at varying levels of efficiency. Some isolates were able to persist inside the amoeba for up to three weeks. Optimal entry time into an amoeba trophozoite was found to be about three hours for all ten *B. pseudomallei* isolates. Interestingly, it was found that after internalization by amoeba, *B. pseudomallei* have a significantly increased ability to both attach to, and grow within human monocytes, suggesting that such interactions increase the virulence capabilities of soil isolates.

Keywords: amoeba, virulence, *Burkholderia*, *Acanthamoeba castellanii*

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INTRODUCTION

Microbiology

In 1911, the pathologist Alfred Whitmore and his assistant C.S. Krishnaswami, described a glanders-like disease among morphia addicts in Rangoon, Burma that was later named melioidosis (8). The etiological agent of melioidosis, *Burkholderia pseudomallei*, is a gram-negative, aerobic, motile saprophytic bacillus with one or more terminal flagella (44). In Thailand, melioidosis is the third most common cause of death resulting from an infectious disease following HIV/AIDS and tuberculosis (27). Clinical symptoms are extremely varied making it difficult to diagnose.

These bacteria are particularly motile in the early stages of their growth cycle and measure approximately 1.2µm in length (22). When stained, it has a bipolar appearance (44). This disease was named from the Greek “melis,” meaning distemper of asses, and “eidos,” meaning resemblance, by Stanton and Fletcher in 1932 (8). Over the years, this organism has been variously known as *Bacillus pseudomallei*, *Bacillus whitmorii*, *Malleomyces pseudomallei*, and *Pseudomonas pseudomallei* until in 1992, when it was finally changed to *Burkholderia pseudomallei* (8). This organism is found in standing water, including 50% of rice paddies, and soil, located usually at the rhizosphere or root zone, in endemic regions (8, 11, 22, 44). This bacterium grows best in soil which has a water content of 15% (3). As this organism is commonly found in the water and soil, it has had to adapt to survive under very harsh conditions. *B. pseudomallei* has been recovered from water sources ranging from pH 2-9 and is also able to survive prolonged nutrient deficiencies, exposure to antiseptic and detergent solutions, a wide

temperature range and dehydration, but is susceptible to killing by UV light (8). It has also been isolated from the roots of plants and from distilled water ten years after inoculation (3).

When grown under laboratory conditions, the colonies vary morphologically. They are mostly smooth in the initial growth stages on agar media, but can gain a rough, wrinkled appearance as they are allowed to mature (8). Because this organism has significant bioweapon potential, it has been categorized as a Class B select agent by the Centers for Disease Control and Prevention (22, 36, 44). Other mammals susceptible to infection by this organism are: camels, horses, sheep, cattle, goats, pigs, kangaroos, koalas, alpacas, deer, cats, dogs and captive marine animals (8). Some animals are more susceptible to this type of infection than others.

Epidemiology

This organism is endemic in Southeast Asia, Northern Australia, South America and has been described mainly in latitudes between 20°N and 20°S (8, 11). It has been described as an emerging infectious disease in Brazil (4, 22). In Australia, the disease was first recognized in sheep in 1949 in northern Queensland and it has been suggested that *B. pseudomallei* may have colonized Australia from SE Asia (8, 11). In other endemic regions, this organism accounts for approximately twenty percent of community acquired septicemias, and in Australia it has been the most common cause of fatal community-acquired bacteremic pneumonia (8, 44). In these endemic regions, about twenty-five percent of children will seroconvert every year during their first four years of life (44). Recently reported disease incidence rates are 19.6 and 20.0 per 100,000 people per year in Northern Australia and Papua New Guinea, respectively (27).

Most cases that occur outside of these endemic areas are in travelers that have returned from these regions (8). However, there have been two reported cases of melioidosis in North

America. One was an infection in Oklahoma after a farming accident and another was an infection in Georgia after a motor vehicle accident. The Oklahoma isolate has been very well studied and belongs to a distinct clade which could possibly represent a distinct species (22).

B. pseudomallei's two closest near neighbors are *Burkholderia mallei* and *Burkholderia thailandensis*. *B. mallei*, unlike *B. pseudomallei*, is non-motile and cannot persist in the environment. It has a very restricted host range infecting mainly equine species and causing a disease called glanders. It has been known to infect humans but usually only after contact with infected equine species or pure cultures (22, 38). *B. thailandensis* is avirulent in animals and humans, but is very similar to *B. pseudomallei* genomically (~90% sequence similarity) and morphologically (36). One important phenotypic difference between the two species is the ability of *B. thailandensis* to assimilate L-arabinose (17). *B. thailandensis* is also found in the soil in *B. pseudomallei* endemic regions.

The genome of *B. pseudomallei* is quite large consisting of two circular chromosomes with a combined length of 7.2Mb and an estimated 5800 genes (30). The first chromosome, 4.07 Mb, has a high number of genes involved in core housekeeping functions such as: cell wall biosynthesis, metabolism, nucleotide and protein biosynthesis, chemotaxis and motility. The second chromosome, 3.17 Mb, codes more for accessory functions that could be related to adaptation to environmental conditions (13). Multi-locus typing suggests that genomically *B. pseudomallei* is more closely related to *B. mallei* than other near neighbors. This also indicates that *B. mallei* evolved more recently than its other near neighbors and is most likely representative of an adaptation to a more specialized habitat (22). There are sixteen different genomic islands throughout the genome of *B. pseudomallei*, indicative of significant horizontal gene transfer which occurred both within *B. pseudomallei* strains and between *B. pseudomallei*

and other environmental bacteria (11). In addition, a number of drug resistance genes have been identified (26).

Several risk factors for infection with *B. pseudomallei* have been identified: diabetes mellitus, alcohol abuse, chronic renal disease and chronic lung disease (11). Melioidosis is most prevalent in rice farmers, servicemen, miners, adventure travelers, and indigenous peoples in endemic areas (22). There are three main routes of infection: inhalation, ingestion and inoculation through breaks in the skin. Of these three routes, ingestion is the least common. Other rare forms of transmission include person to person and one documented case of sexual transmission (8). Higher numbers of cases are associated with heavy rainfall and flooding, indicating involvement of environmental factors. Near drowning victims in these endemic areas also frequently become infected with the organism, most likely because of the high inoculums inhaled from water (8, 11). The peak season of melioidosis in Australia occurs two weeks after the summer rainfall in the tropical northern regions (22). Two accounts of melioidosis outbreaks due to contaminated water supplies have also occurred in Australia (34). Because of the high levels of this bacterium in the rice paddies, farmers working with minor cuts or abrasions have been known to become infected. There was also an incident of an inoculation which occurred during a snake bite (8). The minimum infectious dose for humans has not been calculated, but the LD₅₀ for C57BL/BALB/c mice when inoculated intravenously was between 10³ and 10⁵ bacteria (22).

This organism exhibits a pattern of long periods of dormancy, the longest documented case being 62 years in a veteran from the second world war (13). *B. pseudomallei* can survive for months or even years in the environment, which parallels its ability to reside in a dormant or quiescent state in host cells and tissues such as human macrophages and lymph-reticular organs (22). This

disease has also been called the “Vietnam Time Bomb” because of the number of servicemen that manifested the disease years after returning from Vietnam (22). It is suspected that large numbers of organisms were aerosolized when helicopters flew close to the rice paddies (8).

Melioidosis manifests itself in a variety of symptoms, thus making it difficult to diagnose (5). It often presents as a febrile illness and usually takes one of three courses in infection: a rapidly progressing septicemia with or without pneumonia, a localized soft-tissue infection or a subclinical infection with delayed conversion to a clinically evident infection (22, 44). It frequently forms abscesses, especially in the lungs, liver, spleen, skeletal muscle and prostate. Four percent of patients will present with brain stem encephalitis (44). The lung is the most commonly affected organ, with either a cough and fever resulting from a primary lung abscess or pneumonia, or secondary to septicaemic spread. Sputum is purulent (44). A central nervous system infection is another rare clinical manifestation with a high mortality rate that occurs more commonly in Australia (22). The mortality rates are about 50% in Thailand and 20% in Australia, most likely due to better treatment facilities and programs in Australia (27, 44). The most common symptoms in endemic regions are also different, with acute suppurative parotitis being a unique syndrome found in children in SE Asia and prostatitis being more common in Australia (44). The major cause of death resulting from this infection is usually severe sepsis and organ failure (27).

Treatment

While melioidosis is a treatable disease, a very rigorous course of antibiotics is required. This is because *B. pseudomallei* is resistant to many common antibiotics and it also has the ability to lie dormant for many years making it difficult to completely eradicate (35). *B. pseudomallei* is

resistant to: third generation cephalosporins, penicillins, rifamycins, and most aminoglycosides. It also has relative resistance to quinolones and macrolides (8). On the other hand, *B. pseudomallei* is susceptible to kanamycin and also to ceftazidime which is usually the drug of choice (8, 44). Melioidosis is also commonly treated with a four drug regimen of chloramphenicol, doxycycline, and trimethoprim-sulfamethoxazole. In cases involving children or pregnant women, a treatment with amoxicillin-clavulanate is preferred. High dose parenteral treatment with antibiotics is administered for at least ten days, followed by a switch to oral antibiotics only after there is a clear sign of improvement of clinical symptoms. Usually, chloramphenicol is given for the first eight weeks of oral treatment, followed by the addition of doxycycline and cotrimoxazole for a full twenty week regime (44). Even after this intense treatment, ten to twelve percent of patients will relapse (44). Other treatment options such as antimicrobial peptides are currently being explored (23).

Virulence Factors

Secretion Systems

B. pseudomallei has many virulence factors in its large genome, which contribute to its pathogenesis. While not all of these virulence factors are well described or understood, some are much more proven. Many gram negative bacteria, such as *B. pseudomallei*, have a Type 3 Secretion System (T3SS) which allows them to inject virulence factors directly into the host cell. Often times, a T3SS is acquired through horizontal transfer (39). The proteins injected by a T3SS interfere with or block host immune responses, thereby helping the bacteria to invade host cells. There are many different organisms which possess T3SS, such as *Pseudomonas*, *Yersinia*, *Salmonella* and *Burkholderia*. Among these various organisms, the structure and function of the

T3SS is conserved. It consists of about 20 different proteins, most of which are involved in constructing the macromolecular complex which spans the inner bacterial membrane, the periplasmic space, the peptidoglycan layer, the outer bacterial membrane, the extracellular space and the host cellular membrane. This complex structure houses a needle complex also referred to as the injectisome, which is used to inject various effector proteins into the host (29).

B. pseudomallei has genes coding for three T3SS. Two of these are very similar to the plant pathogen-like T3SS of *Ralstonia solanacearum* and it is believed that they are involved in symbiotic or even pathogenic bacteria-plant interactions in rice paddies. The third T3SS is called *Burkholderia* secretion apparatus (Bsa) and it resembles the *Salmonella enteric* serovar Typhimurium pathogenicity island I (17). So far 9 *B. pseudomallei* effector proteins have been described, one of which is bobE which is a homolog of the *Salmonella* SopE (17). The full involvement of the T3SS in virulence of *B. pseudomallei* is not entirely understood, but it is clear that it is necessary.

B. pseudomallei also has six Type 6 Secretion Systems (T6SS) genes in its genome. Many other pathogenic organisms have a T6SS, such as *Vibrio cholera*, *Yersinia pestis*, *Francisella tularensis*, *Burkholderia mallei*, *Salmonella typhimurium*, pathogenic *Escherichia coli*, etc. The T6SS loci usually contain 15-25 genes (32). The majority of T6SS components that have been studied so far are not secreted, but they are necessary for the secretion of hemolysin coregulated protein and the valine-glycine repeat protein G (32). In one of the T6SS in *B. pseudomallei*, the expression of three genes is induced following macrophage invasion, but there is not a lot known about their roles in virulence (2).

Siderophore and Biofilms

B. pseudomallei possesses a siderophore to sequester iron for growth. This siderophore is called malleobactin, and it is very efficient at acquiring iron in an acidic pH. It is regulated by the *fur* gene which also regulates superoxide dismutase and peroxidase (8).

B. pseudomallei produces a biofilm which aids in the infection process, but does not appear to be essential for virulence. It is more likely that it plays a role in environmental survival (2). In the biofilm, there are slow growing bacteria within an extracellular polysaccharide matrix in a very complex three-dimensional structure. The production of biofilm is controlled by quorum sensing and biofilms have been observed in infected lung tissue from guinea pigs and humans (22). Quorum sensing is a means of cell-to-cell communication based upon cell density (2). RpoE is an alternate sigma factor that is involved in biofilm production and in RpoE mutants, there is a 50% reduction in biofilm production.

Adhesion

The polysaccharide capsule is believed to help *B. pseudomallei* in attachment to various host cells. There are three different varieties of capsules produced by *B. pseudoamallei*. One is a macrocapsule which is approximately 0.1-0.25 μ m in thickness, the second, a microcapsule of 0.086 μ m in thickness and lastly, there are some isolates that have no capsule (2). The capsule also aids in protecting the bacteria from opsonization, resulting in a decrease in phagocytosis and thus a hindered ability of the host to clear the infection. The capsule can also act as a barrier by blocking the access of the CR1 receptor on phagocytes (33). There is evidence that after the bacterium is internalized, it sheds its capsule.

Both flagella and pili have also been shown to aid in cell attachment (13). *B. pseudomallei* has flagella and type IV and Tad-like pili expressed on its surface (13). This organism has two to four polar flagella which provide motility independent of temperature. A gene required for flagella production is the *fliC* gene which encodes a 39.1-kDa protein. Antiserum against this gene product was able to block motility in almost all isolates examined (2).

Lipopolysaccharide (LPS)

Altering the LPS structure is a common evasion strategy of bacteria to avoid pattern recognition and other immune receptors of host cells (16). *B. pseudomallei* adapted this strategy with a unique, acid-stable structure between the inner core and lipid A linkage, along with longer amide-linked fatty acids. The longer fatty acid chains are predicted to decrease the level of recognition by CD14 on the surface of macrophages which in turn leads to a reduced inflammatory response. Other bacteria that are known to employ similar modifications are *Proteus*, *Salmonella*, *E. coli*, *Serratia* and *Pseudomonas* (16). In the absence of this unique LPS, there is an increase in IFN- β stimulation (2).

There have been three different *B. pseudomallei* LPS types described: two smooth serotypes A and B and one rare rough serotype. Serotype A accounts for 97% of strains, while the other two serotypes have been associated with strains that cause a disease relapse. No immunological cross reactivity among the varying serotypes has been described (2). High concentrations of LPS antibodies have been shown to improve survival in melioidosis patients. The LPS of both *B. pseudomallei* and *B. thailandensis* activates hTLR 4 (31).

Immune Response

B. pseudomallei is a facultative intracellular pathogen able to attach, invade and multiply within neutrophils, monocytes and macrophages without eliciting a strong immune response. Prior to invasion, *B. pseudomallei* activates the alternative pathway of complement and is phagocytosed; however, it is resistant to lysosomal defensins and cationic peptides. After phagocytosis, the T3SS delivers various effector proteins to the host cell, which can assist in vacuolar escape and intracellular motility. It is able to escape the phago-lysosome, as soon as fifteen minutes after ingestion, and it can replicate both intracellularly and extracellularly (2, 8).

Toll-like receptors (TLR) recognize various pathogen associated molecular patterns (PAMPs) from *B. pseudomallei* such as LPS and flagellin. MyD88 plays a role in the host immune response to melioidosis by activating TLRs. In patients suffering from septic shock, an increase in the activation of TLR1, TLR2, and TLR4 and its coreceptor CD14 has been described and macrophage-mediated killing of *B. pseudomallei* is due mainly to reactive nitrogen intermediates (RNI) (2). TLR2 has been associated with the growth and dissemination of *B. pseudomallei* throughout the body and it especially contributes to organ injury (45). The activation of TLR2 and TLR4 induces recruitment of various immune cells such as neutrophils, macrophages and NK cells (16). Proinflammatory cytokines are released and bacteria begin to replicate. *B. pseudomallei* can escape the cell by inducing apoptosis which can then lead to a secondary infection. *B. pseudoamllei* can cause apoptosis of macrophages and caspase-1-dependent cell lysis (2).

B. pseudomallei spreads from cell to cell through actin-mediated motility. It uses host actin-associated proteins to polymerize actin and move between cells. BimA, a protein secreted by *B.*

pseudomallei, interacts with actin and localizes it at the bacterial pole. Mutants of *bimA* are unable to polymerize actin. Because of its use of actin, *B. pseudomallei* can fuse host cells together creating multinuclear giant cells (MNGC) (19).

After initial infection, bacteria are able to spread to secondary sites, such as the spleen or liver. Inflammation allows increased access to the circulatory system, and although the exact mechanism of secondary spread is not known, it is possible that the bacteria use macrophages and the lymphatic system to migrate to other locations within the host (2). Relapses of melioidosis are common among those infected, although the exact mechanisms involved in latency and reactivation are not fully understood.

Free-living amoeba

Free-living amoeba are aerobic, mitochondriate, eukaryotic protists that can be found throughout the world naturally in various environments. Some examples are *Acanthamoeba* spp., *Balamuthia mandrillaris* and *Naegleria fowleri*, all of which can cause opportunistic disease in humans. The prevalence of human infections is not very high and thus, no efforts have been made to control free-living amoeba in the environment (46). These amoeba are ubiquitous and are found in many different environments around the world. They have been isolated from many different sources such as: soil, fresh and brackish waters, bottled mineral water, cooling towers of electric and nuclear power plants, heating, ventilating and air conditioning units, humidifiers, Jacuzzi tubs, hydrotherapy pools in hospitals, dental irrigation units, dialysis machines, dust in the air, bacterial, fungal and mammalian cell cultures, contact-lens paraphernalia, ear discharge, pulmonary secretions, stool samples, etc (14, 43). The ability to act as parasites within host tissues has resulted in these organisms being referred to as amphizoic amoebae (43).

Acanthamoeba spp. along with *Balamuthia* can cause a chronic and mostly fatal disease known as granulomatous amoebic encephalitis (GAE). The onset of this disease is slow and can develop as a chronic illness over weeks or even years. The most common symptoms of GAE are headache, stiff neck, and mental-state abnormalities. Other symptoms can also include nausea, vomiting, low-grade fever, lethargy, cerebellar ataxia, visual disturbances, hemiparesis, seizures and coma (43). This disease is most commonly found in those that are immunocompromised. Since the infections caused by these amoeba are rare, they are often hard to diagnose, and therefore many are confirmed during autopsy. The number of cases worldwide is not accurately known because of the lack of accurate diagnosis and reporting in many areas where infections occur.

Acanthamoeba can also cause keratitis, an inflammation of the cornea, which can lead to vision impairment (43). This disease is associated with trauma to the cornea or use of contaminated contact-lens products. Severe inflammation occurs along with photophobia, ocular pain and a 360° or paracentral stromal ring infiltrate. Generally speaking there is only one eye that is affected, but cases of bilateral keratitis have been documented. People who wear contact lenses are a risk group and it is estimated that as of August 2006 there were more than 5,000 reported cases in the United States alone (43). Unfortunately, there are not very effective treatments available for the various diseases caused by these species (14, 28).

Acanthamoeba castellanii was isolated in 1930 by Castellani after whom the species was named. It was isolated as a contaminant from a yeast culture plate and classified under the Super Group Amoebozoa. Within this class, there are more than 24 different species of *Acanthamoeba*. These organisms are classified into three main groups based on morphological criteria and cyst size (43). Group I includes species which have large amoebae with cysts that are approximately

16 to 30µm in size. The next group has the largest number of species with cysts measuring approximately 18µm or less. The third and final group contains species with the same cyst size as Group II, but with slight morphological differences (43). Through evolutionary studies and examination of rRNA genes, there have been fifteen different genotypes identified among these species (28). It has been shown that about 90% of the infectious *Acanthamoeba* isolates belong to the T4 genotype.

Acanthamoeba species have two main stages in their life cycle, a vegetative or trophozoite stage and a dormant cyst stage. During the trophozoite stage, the organisms are quite active and feed on bacteria and detritus that are present in the surrounding environment. Free-living amoeba in the trophozoite stage are phagocytic and can engulf entire bacterial cells (46). They reproduce through binary fission. Morphologically, *Acanthamoeba* spp. can be identified distinctly by their fine, tapering, thorn-like acanthopodia that arise from the surface of the body. In the trophozoite stage, the amoeba can range from 15 to 50µm in size, depending on the particular species (43). Amoeba form a cyst during periods of nutrient deficiencies, desiccation and other environmental stressors. While the amoebae are in the cyst stage, they are dormant. The cyst gives the amoeba added protection from various chemicals, and temperature and pH extremes, much like a spore does for a bacterium (24). These cysts are double-walled and can range in size from 10 to 25 µm. The ecocyst is the outer wall which contains lipids and proteins, and is wrinkled with folds and ripples. The endocyst or inner wall contains cellulose and can vary in shape (6, 43). The cysts are uninucleate and have a centrally placed very dense nucleolus. When favorable conditions return, the trophozoite stage is activated and they leave the cyst.

Amoeba-Bacteria interactions

Amoeba species exist in the soil and water where they interact with many types of bacteria. This provides a selective environment in which these bacteria evolve the ability to evade amoeba predation (1). Many bacteria have shown the ability to adhere to, enter, and survive inside of amoeba, and use the amoebal cysts to prolong their survival. Free-living amoeba have been described as interacting with: *Legionella pneumophila*, *Mycobacterium avium*, *Chlamydia*, *Francisella tularensis* and Rickettsiae (46). There have also been examples of amoeba-bacterial endosymbiotic relationships. In one instance, *Amoeba proteus* became dependent on its bacterial symbiont due to essential modifications in the gene expression of the symbiotic bacteria (46). It is also possible that these relationships could be used by the bacteria as a means of transmission. Bacteria have been observed in amoeba trophozoites and cysts. A study showed that when an *Acanthamoeba* trophozoite encysts, it produces a membrane-surrounded vesicle that can be filled with bacteria, and both the bacteria and the amoeba could be easily spread in an aerosol. The bacteria are usually found in the contractile vacuoles in the amoeba, but have also been observed in the cytosol (19).

There are clearly some advantages to the bacteria from bacteria-amoeba interactions. For example, *L. pneumophila* upon growing in *A. castellanii* has a shorter generation time, altered morphology, different surface properties and an increased invasiveness in macrophages and epithelial cells, along with increased virulence in experimental animals (9, 46). The bacteria also showed large vesicles in their cytoplasm and were able to express five new proteins after amoeba exposure. Another bacterium, *Vibrio mimicus*, increased in numbers tenfold in the presence of *A. castellanii*, and was able to survive longer than bacteria growing alone (1). Most of these organisms were found to be in the cytoplasm of the amoeba trophozoites after one and three days

of incubation, and were also observed in cysts at the same time points (1). As the amoeba were encysting, the bacteria were found in a space between the ecto- and mesocyst after three days of incubation in co-culture. This cyst stage allowed the bacteria to survive for more than two weeks in the amoeba. This same pattern was seen with several other bacteria including *B.*

pseudomallei. Because of the resistance of cysts to harsh environmental conditions, they give extra protection to any bacteria residing in the amoeba (1). *V. mimicus* was also able to survive inside encysted amoeba after a gentamicin treatment at a concentration of 1,000µg/ml.

A. castellanii is found endemically in the soil and waters of SE Asia, an endemic region for *B. pseudomallei*, thus creating an environment conducive to frequent interactions between these two organisms (20, 37). *B. pseudomallei* has been shown to enter and survive inside of amoeba. When the bacteria adhere to amoeba trophozoites, a rapid rotation was observed, indicating a possible role of the flagella in adhesion. It uses a coiling phagocytosis mechanism to enter the host cell. This same mechanism of phagocytosis has been observed to occur between *L. pneumophila* and both *Acanthamoeba* spp and human monocytes (20). There are various species of free-living amoeba that *B. pseudomallei* can enter: *Acanthamoeba astronyxis*, *A. castellanii*, *A. palestinensis* and *A. polyphaga* (22). Once the bacteria are phagocytosed, they are able to escape from the vacuoles and enter the cytoplasm. After a 24 hour co-culture of *A. astronyxis* and *B. pseudomallei*, extracellular bacillary tufts and tangles were observed entangling the amoeba (20).

Inglis et al. also noticed varying responses among the different species of *Acanthamoeba* when co-cultured with *B. pseudomallei*. Vacuolation was less pronounced in *A. castellanii* and *A. polyphaga* when compared with *A. astronyxis*. Also, no external bacillary tufts were observed in

the first two species when incubated at 20°C, but the initial phagocytosis and bacillary tangles were more readily observed when co-cultures were incubated at 37°C (20).

Near neighbor *B. cepacia* has also been described as being able to survive, but not replicate within vacuoles of *A. polyphaga*, murine macrophages, and in a human monocyte cell line (25). It was observed that infected phagocytes, whether macrophages or amoeba, that contained intracellular bacteria eventually died. It was also found that the bacteria only replicated extracellularly and could be found within an acidified membrane-bound compartment which is separate from the phagolysosome. *B. cepacia* was, like many other bacterial species, able to survive for extended periods of time inside amoeba and macrophages: seventeen and six days, respectively (25).

The ability of *B. pseudomallei* to lay dormant for long periods of time can possibly be attributed to its ability to survive in human macrophages and avoid destruction (20). These mechanisms could be primed and developed in free-living amoeba. It is possible that similar methods of phagocytosis are employed by amoeba and human monocytes, although coiling phagocytosis of *B. pseudomallei* in human monocytes has not yet been observed (20). The relationship of *B. pseudomallei* and *Acanthamoeba* that has been described is very similar to that of *Legionella* and *Acanthamoeba*. *Legionella* has a symbiotic relationship with the free-living amoeba, and it is possible that *B. pseudomallei* is developing a similar relationship, and may use the amoeba as a reservoir in endemic areas (20).

Although it has been previously discovered that *B. pseudomallei* can enter free-living amoeba, it has not been quantifiably examined. It is important to gain a better understanding of this relationship and its effects on the virulence of this organism. The mechanisms these bacteria use

to adhere to, enter, and proliferate inside amoeba are the same mechanisms that are needed for entrance and survival inside mammalian cells (20, 46). *L. pneumophila* intra-amoebal interactions have been shown to enhance entry and virulence in human monocytes (20).

We hypothesize that there will be a diversity of virulence among various *B. pseudomallei* isolates relative to amoeba infections. Also, it is anticipated that *B. thailandensis* will have a lower response in all three indicators of virulence, especially optimal entry time, when compared to *B. pseudomallei*. In long-term survival, *B. pseudomallei* should be able to survive for weeks regardless of encystment of amoeba. In this study we show that *B. pseudomallei* clinical isolates can also adhere to, enter, and survive inside amoeba at varying efficiencies and that virulence in human monocytes is increased after being passaged inside amoeba.

MATERIALS AND METHODS

Strains and growth conditions. *Burkholderia pseudomallei* and *Burkholderia thailandensis* strains were obtained from various locations (Table 1). All isolates were grown on Columbia agar plates at 37°C or in Brain Heart Infusion broth with aeration at 37°C.

Cell lines and culture conditions. *Acanthamoeba castellanii* ATCC 30234 was grown in Peptone Yeast Glucose (PYG) broth (2% proteose peptone, 0.1% yeast extract, 0.1% sodium citrate, 1% 0.4M magnesium sulfate, 0.8% 0.05M calcium chloride, 1% 0.005M ferrous ammonium sulfate, 1% 0.25M dibasic sodium phosphate, and 1% 0.25M monobasic potassium phosphate) in the dark at 25°C until confluent in 75-cm² culture flasks (Corning). To harvest, the sides of the flask were rapped sharply to remove adherent amoeba which were then suspended in fresh media. Cell counts were determined using a hemacytometer.

Entry and adherence assay. In brief, 1ml of *A. castellanii* trophozoites were seeded into a 24-well plate (Corning) at a concentration of about 2×10^5 per well and allowed to incubate overnight in the dark at a temperature of about 25°C in PYG broth. The PYG broth was then removed and replaced with a High Salt buffer (HSB) (0.1% sodium citrate, 1% 0.4M magnesium sulfate, 0.8% 0.05M calcium chloride, 1% 0.005M ferrous ammonium sulfate, 1% 0.25M dibasic sodium phosphate, and 1% 0.25M monobasic potassium phosphate) and the plate was allowed to incubate for 1h at 37° C. Each well was then infected with 100µl (multiplicity of infection (MOI) ~100) of an overnight bacterial culture and returned to incubate for 30 min. For cell adherence, the cells were washed twice with HSB, lysed with 0.5% Saponin, and then plated using a Millipore Manifold filtration system. For entry assays, cells were washed once and then the media was replaced with HSB containing 250µg/ml Kanamycin (Sigma Aldrich). Cells were incubated for 2 hr, washed once with HSB, lysed with 0.5% Saponin, and plated in a similar manner as described above.

Intracellular survival assay. The assay was begun as previously described and the cells were washed once with HSB after the antibiotic incubation period. Fresh HSB was then added and the plate was returned to the incubator for approximately 24 hr under the aforementioned conditions. Amoeba were then lysed and the lysate was plated as previously described.

THP1 Infection with amoeba-passaged bacteria. Bacteria were passaged with amoeba as described above for a 24 hr infection. After incubation, 0.5% Saponin was added to each well, the lysate was removed to an eppendorf tube, bacteria were collected by centrifugation and washed twice with RPMI. The resulting suspension of bacteria was used to infect THP1 cells. Overnight cultures of the same isolates were used to infect THP1 cells as controls.

Eppendorf tubes with 1 ml of 10^6 THP1 cells were incubated at 37°C and 5% CO₂ for 10 min. Cells were infected with bacteria at an MOI of ~100, in triplicate. The tubes were then incubated in the same conditions for 30 min and washed twice with PBS. For cell association assays, the cells were lysed with a 0.5% Saponin solution followed by plating of serial dilutions in duplicate. For entry and 24 hr assays, cells were washed with PBS, and then re-suspended in 1 ml of media with 250µg/ml kanamycin. This was followed by a 2 hr incubation under the same conditions. The entry assay cells were washed once with PBS, and the cells were lysed and plated as previously described. After 24 hr, the remaining cells were lysed and plated.

Long-term amoeba survival assays. Amoeba were seeded into a 6-well plate (Corning) at a concentration of 2×10^5 amoeba/ml with 3ml/well and allowed to adhere for 24 hr. The media was replaced with HSB and the wells were incubated at 37 °C for 1 hr. The amoeba were infected at an MOI of ~100 and returned to the incubator for 30 min. The HSB was replaced with a 250µg/ml kanamycin solution in HSB for 2 hr. The cells were washed once with HSB and 3 ml of fresh HSB was added before the plates were incubated for various survival times. When the time point was reached, the HSB was removed and replaced with 3 ml of amoeba medium. The co-cultures were then allowed to incubate for 72 hr or until turbid, whichever happened first. The positive cultures were gram stained for confirmation of *B. pseudomallei* growth.

Long-term survival assays in the absence of amoeba encystment. The protocol described above was repeated except, 25 µg/ml cycloheximide was added in addition to the kanamycin treatment to prevent encystment of the amoeba. One hour before the HSB was changed to amoeba media, another cycloheximide treatment was performed. The co-cultures were then

allowed to incubate for 72 hr or until turbid, whichever happened first. The positive cultures were gram stained for confirmation of *B. pseudomallei* growth.

Statistical analyses. Bacterial count data for the 3 hour, amoeba, and THP1 studies were analyzed using generalized linear mixed models. Computations were done with the GLIMMIX procedure of SAS 9.2, using the maximum likelihood fitting method. Counts were assumed to follow the Poisson distribution, and a linear function of predictor variables was linked to the mean of the Poisson distribution via the logarithmic function. Dilution factor and titer were included as an offset in the model. The linear predictor included fixed effects for organisms, treatments, and hours as appropriate for the study. In all studies, the linear predictor also included random effects for replications and tubes within replications. Residual plots as well as the Pearson chi-square ratio were used to assess goodness-of-fit of the model. Pairwise differences between levels of fixed effects were tested using Wald tests.

The same model was used for bacterial counts in the entry study except that the bacterial counts were modeled as following a Negative Binomial distribution due to the presence of some extremely high counts.

RESULTS

***B. pseudomallei* can adhere to, enter, and survive inside amoeba at various efficiencies.** To determine the ability of *B. pseudomallei* to enter and survive inside of amoeba, we infected amoeba with ten clinical isolates selected from various geographical regions and times (see Table 1). The avirulent near-neighbor, *Burkholderia thailandensis*, was included for comparison. Results showed that *B. pseudomallei* was able to associate with, enter and survive inside *A. castellanii* with varying efficiencies, depending on the isolate ($p < 0.01$) (Fig 1). In

comparison, *B. thailandensis* was significantly less efficient ($p < 0.01$) at associating with, entering and surviving in amoeba.

B. pseudomallei PHLS 35, was the most efficient at attaching to the amoeba. It displayed a 60% association rate which was more than double the next highest isolate. PHLS 9 and 13178 were the least efficient *B. pseudomallei* isolates at attaching to the amoeba (Fig 1A). However, they were significantly better than the avirulent *B. thailandensis* control. Interestingly, isolate 13178 was one of the least efficient at associating with the amoeba, but it was one of the most efficient at entering the cells. The other two most efficient isolates in cell entry were PHLS 6 and PHLS 35 (Fig 1B). Again, the avirulent *B. thailandensis* had the lowest level of cell entry. In survival inside amoeba, there was one isolate that performed significantly better than the others. *B. pseudomallei* PHLS 6 was by far the most efficient survivor, with almost 80% of the cells surviving inside the amoeba at 24 hr. The other isolates averaged only about a 10% survival. Again, *B. thailandensis* had one of the lowest survival percentages (Fig. 1C).

***B. pseudomallei* enters *A. castellanii* optimally at 3 hours.** In order to examine the entry kinetics of *B. pseudomallei* into the amoeba, *A. castellanii* cells were infected with *B. pseudomallei* 13178 and *B. thailandensis* ATCC 700388 as a control. The co-cultures were allowed to incubate for 1, 2, 3, and 4 hours, to ascertain the optimal time for the largest entry percentage (Fig. 2). While there was a slow steady increase of *B. pseudomallei* entering the amoeba from 1 to 2 hr, there was a 20 fold entry increase from 2 to 3 hr. The rate of increase from 3 to 4 hours slowed to just slightly more than that of the 1 to 2 hour period. In comparison, the avirulent *B. thailandensis* showed a very low ability to enter this species of amoeba, with no entry seen until 3 hr of co-culture. The rate of entry from 3 to 4 hours was slightly lower than

the rate of *B. pseudomallei* from 1 to 2 hr, achieving only about a 0.2% cell entry after 4 hr of co-culture.

***B. pseudomallei* isolates differ widely with respect to 3 hr entry percentages.** Since the entry kinetics data for *B. pseudomallei* 13178 showed that three hours was the optimal amoeba entry time, experiments were undertaken to obtain similar data for the other nine *B. pseudomallei* isolates. After repeating the entry assay with all ten isolates using a three hour contact time, results showed significant variability in the entry abilities of the 10 isolates (Fig. 3). *B. pseudomallei* 13178 entered amoeba significantly better than PHLS 6, which entered significantly better than the remaining isolates. It is important to note that even the *B. pseudomallei* isolates with the lowest entry percentages (PHLS 35 and Darwin 2) were significantly better than the avirulent *B. thailandensis* isolate. The fact that PHLS 35 had one of the lowest entry percentages at 3 hr was surprising, especially since, as previously shown, PHLS 35 was one of the more efficient isolates at associating with amoeba and entering during a shorter incubation time.

***B. pseudomallei* is able to survive in *A. castellanii* for up to three weeks without encystment.**

To better understand *B. pseudomallei*'s ability to survive long-term within amoeba, *A. castellanii* cells were incubated for up to three weeks while being suspended in HSB. Amoeba were infected with an overnight culture (MOI ~100) and treated with kanamycin after a 30 min incubation period. Cells were then washed and replaced with fresh HSB. The HSB allows survival of the amoeba, but does not promote bacterial growth.

Five representative isolates (*B. pseudomallei* PHLS 6, PHLS 9, 13178, 80800117 and *B. thailandensis* 700388) were selected to infect the amoeba. Results showed that all 5 isolates were able to survive for up to three weeks in *A. castellanii* (Table 2).

***B. pseudomallei* does not require encystment to survive long-term in amoeba.** To determine whether encystment was required for survival of the bacteria, the previous co-culture experiment was repeated with the addition of cycloheximide in addition to the kanamycin, and an additional 1 hr treatment of cycloheximide to prevent encystment before adding PYG media. Results showed that encystment was not required for the *B. pseudomallei* to survive (Table 2). All five isolates, including *B. thailandensis*, were able to survive for up to three weeks in HSB, even without the protection of amoebal cysts.

***B. pseudomallei* is more virulent after being passaged in amoeba.** Since data from previous studies using different pathogens indicated that virulence was increased by passage through amoeba, experiments were performed to assess these effects using *B. pseudomallei*. Cell association, entry, and survival of *B. pseudomallei* 13178 and *B. thailandensis* ATCC 700388 were assessed for the human monocyte line THP1s. These bacteria were used to infect the monocytes both before and after passage through *A. castellanii* amoeba.

There was a striking difference in all 3 assays between cultured bacteria and amoeba-passaged bacteria for both *B. pseudomallei* and *B. thailandensis* (Fig. 4 and 5). The non-passaged bacteria were only about 8% efficient at associating with the amoeba, whereas the amoeba-passaged bacteria were close to 100% efficient at attaching. This same trend continued in the other two aspects, entry and survival. Interestingly, amoeba-passaged *B. thailandensis* had higher values (almost double) in all three assays when compared to amoeba-passaged *B. pseudomallei* (Fig. 5).

DISCUSSION

B. pseudomallei has a large genome and demonstrates a high degree of variance among clinical isolates, which most likely factors into the observed variability in virulence. It has been suggested that *B. pseudomallei* is an ‘accidental pathogen’ that most likely gained virulence in its natural soil environment, possibly through several horizontal transfer events, during interactions with free-living amoeba and other soil protozoa (20, 30, 37, 41). Amoeba share many characteristics with macrophages, the cells that first encounter many bacterial invaders (20, 46). By studying the interactions between *B. pseudomallei* and the common soil amoeba *A. castellanii*, insights into both the natural ecology of this bacterium and how it interacts with the innate immune system can be obtained.

Due to the variability in virulence among *B. pseudomallei* isolates, ten different isolates were selected to cover this variability (30, 41). The isolates for this study were selected based on location and date isolated, to maximize variation. All isolates are from human cases to ensure that they are indeed virulent and can cause disease. Many of these isolates have been used in previous studies and are believed to represent the virulence characteristic of this species (15, 40, 42).

Using the indicators of cell association, entry, and survival to measure virulence, it was found that the ten virulent *B. pseudomallei* isolates studied produced widely varied indices. Variation within a species is expected, especially for one with such a large genome like *B. pseudomallei*. The avirulent, near neighbor, *B. thailandensis*, not surprisingly, was not as adept at attaching, entering and surviving inside the amoeba. This is most likely because of its loss of certain necessary virulence factors that enable bacteria to infect and replicate in both protozoa or

mammalian cells. El-Etr et al. found, when comparing various *Francisella tularensis* isolates, that this species also had varying abilities to attach, enter, and survive in amoeba (12). The avirulent control in this study, the live vaccine strain (LVS), was statistically lower in these measures of virulence, similar to the responses of *B. thailandensis* in this study. Both *Burkholderia* and *Francisella* isolates are commonly found in the soil and water where amoeba are naturally present. Therefore, one would expect that these bacteria have evolved and adapted ways to avoid predation by amoeba, and maybe also have simultaneously acquired mechanisms to avoid destruction by mammalian immune cells. Other studies, using various amoeba species and *Burkholderia* species, have shown interaction among the two organisms (20-21, 25). These studies also showed that *Burkholderia* spp. interact with amoeba and are capable of surviving inside free-living amoeba.

The amount of time it takes bacteria to invade an amoeba is a probable indicator of virulence. Results from these studies indicated that three hours was the optimal time to measure entry of *B. pseudomallei* into the amoeba. Other species, such as *S. aureus*, show an optimal entry time of about 5 hr with *A. polyphaga*, while *M. avium* peaks at about 5 days of co-incubation with *A. castellanii* (10, 19). The *B. thailandensis* control was significantly lower than all ten *B. pseudomallei* isolates studied, indicating its lack of key virulence factors. Even though all of the isolates were not as effective as the standard *B. pseudomallei* 13178, even the least virulent *B. pseudomallei* was significantly higher than *B. thailandensis* at all four time points. Therefore, these assays seem to be a simple and effective way to quantitatively test for virulence in these species.

Many bacteria, after growing in the presence of amoeba, are more virulent in mammalian cells (9, 20, 46). It is possible that invasion of amoeba prime the expression of traits that allow

increased virulence in mammalian cells. *B. pseudomallei* and *Acanthamoeba* species, co-exist in the soil and therefore have had extensive opportunities to co-evolve. Free-living amoeba exhibit similar behavior to certain innate immune cells which operate in mammals (i.e. macrophages) (7). This may have provided *B. pseudomallei* a head start in adapting to the immune system of a more advanced host.

In a recent study, it was shown that *B. pseudomallei* can more efficiently adhere to and enter a human epithelial cell line compared to *B. thailandensis* (7). It was also shown that *B. pseudomallei* was able to survive and replicate inside of primary human monocyte-derived dendritic cells and macrophages, and that *B. pseudomallei* and *B. thailandensis* demonstrated a similar ability to survive, but only *B. pseudomallei* was able to replicate (7). The results of our experiments were similar in that both were able to survive, but no replication was observed.

Howard et al. also showed that *B. pseudomallei* virulence was enhanced after passaging through *A. astronyxis*, a different species of *Acanthamoeba* (18).

While increased virulence response was an expected result, the intensity of the response was unexpected. Cell association and entry assays, especially, showed more replication when compared to those using non-passaged bacteria. A possible explanation for this response, could be due to MOI. Amoeba have a finite number of cell surface receptors. Thus, according to the Poisson distribution, the higher MOI (used with the non-passaged bacteria) would result in a smaller number of bacteria that could invade a cell, while the lower MOI (used with the amoeba-passaged bacteria) would result in almost every bacterium being able to attach. Interestingly, *B. thailandensis*, after being passaged through amoeba, was able to associate with, enter, and replicate within a human monocyte line to a higher degree than *B. pseudomallei*. This is unexpected and opposite of what has been previously described (12). These two species are

found together in the same environments with amoeba, so it is possible that *B. thailandensis*, while non-pathogenic, has evolved mechanisms to survive within amoeba that are of increased benefit in this monocyte line. The percent survival results in THP1 cells were not particularly informative, probably due to the fact that the bacteria released from dead monocytes were able to grow unrestricted during the 24 hour incubation. This experiment needs to be repeated in an environment which does not support the growth of bacteria.

Encystment of amoeba trophozoites is rapidly induced by stress. Since the cyst provides notable protection from harsh environmental conditions, some bacteria have exploited this defense for their own protection and survival (12). *B. pseudomallei*, however, does not require a cyst to survive, which is not true for other more fastidious species, such as *Francisella* (12). Both organisms are able to survive up to three weeks in amoeba cysts, but when encystment is blocked, only *Burkholderia* spp. are able to survive for the same amount of time (Table 2). Another difference between these two systems is that *B. thailandensis* was able to survive without encystment, whereas *F. tularensis* LVS was unable to do so. Because *B. thailandensis* is a natural saprophytic organism found in *B. pseudomallei* endemic regions, it is not surprising that it was able to survive for an extended period of time inside the amoeba, where the laboratory created avirulent LVS could not. *B. pseudomallei* is capable of entering amoeba and it has been observed within both the cyst and trophozoite stage (18). Encystment of amoeba was observed during co-culture, especially after 24 hours. The exact role encystment plays in the natural survival of *Burkholderia* species in the environment remains to be determined.

Using free-living amoeba as an infection model for *B. pseudomallei* seems simpler and easier than some of the models that have been recently described. Some current models include BABL/c mice, which are highly susceptible to *B. pseudomallei*, Syrian hamsters, and various

human cell lines (5). More studies relating this model to *in vivo* infection models need to be performed. Facultative intracellular bacteria are commonly able to invade, survive and replicate within amoeba. For pathogens that co-exist naturally with amoeba like *B. pseudomallei*, it is worthwhile to further study the effects of these interactions on virulence and persistence in the environment. The fact that several bacteria, such as *L. pneumophila*, *M. avium* and *F. tularensis* have been shown to have increased virulence after being passaged with amoeba indicate that life inside the amoeba activates mechanisms that allow better survival in certain mammalian cells (9-10, 12). Further work is needed to specifically identify these mechanisms.

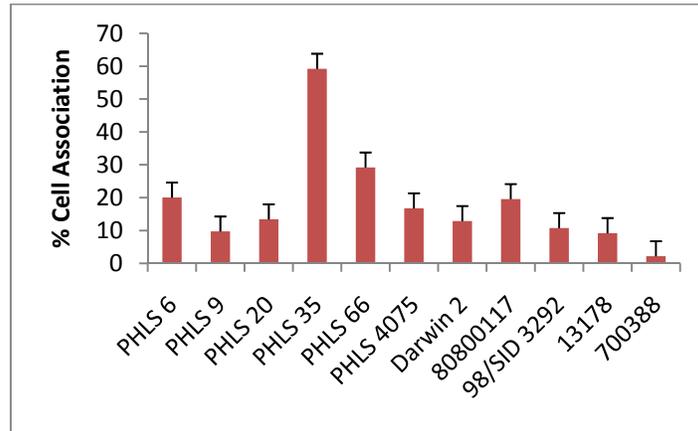
Gaining a better understanding of bacterial-amoeba interactions will provide a more complete picture of the pathogenesis of *B. pseudomallei* and the host immune response. *B. pseudomallei* is able to attach to, enter, and survive within the cell for up to three weeks with or without encystment, showing the stability of this organism. More importantly, it can use the free-living amoeba to enhance its pathogenic abilities in mammalian cells, indicating an important role of free-living amoeba in pathogenesis.

In conclusion, it was found that different *B. pseudomallei* isolates have varying levels of virulence, however, the optimal entry time into amoeba is ~3 hr for all ten virulent isolates assayed. Unlike *F. tularensis*, *B. pseudomallei* does not require amoeba encystment to survive. We showed survival for up to three weeks when amoeba encystment was blocked. Finally, the virulence of *B. pseudomallei* in a human monocyte cell-line was increased following passage through amoeba. The mechanisms involved in this phenomenon are presently unknown and will be the subject of future studies.

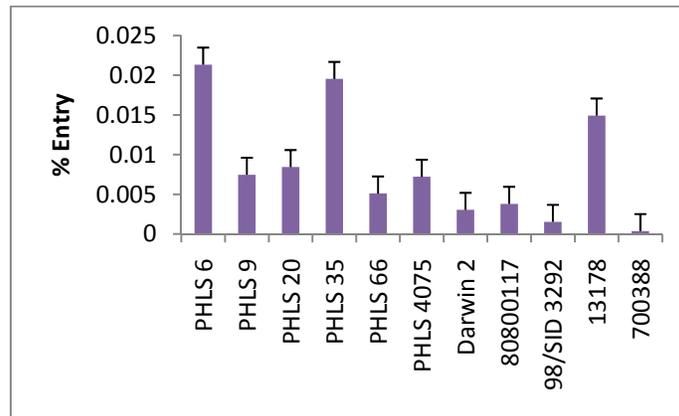
Table 1 *Burkholderia* strains used in this study.

Number	Organism	Isolate	Country of Origin	Source	Origin Date
1	<i>Burkholderia pseudomallei</i>	PHLS 6	Bangladesh	Human	1960
2	<i>Burkholderia pseudomallei</i>	PHLS 9	Pakistan	Human	1988
3	<i>Burkholderia pseudomallei</i>	PHLS 20	Thailand	Human-blood	1990
4	<i>Burkholderia pseudomallei</i>	PHLS 35	Vietnam	Human	1963
5	<i>Burkholderia pseudomallei</i>	PHLS 66	Kenya	Human-blood	1980
6	<i>Burkholderia pseudomallei</i>	PHLS 4075	Holland	Human-sputum	1999
7	<i>Burkholderia pseudomallei</i>	Darwin 2	Australia	Human-blood	2000
8	<i>Burkholderia pseudomallei</i>	80800117	Utah, USA	Human-blood	2008
9	<i>Burkholderia pseudomallei</i>	98/SID 3292	United Kingdom	Human	1998
10	<i>Burkholderia pseudomallei</i>	13178	Australia	Human	~2002
11	<i>Burkholderia thailandensis</i>	700388	Thailand	Rice field soil	1994

A.



B.



C.

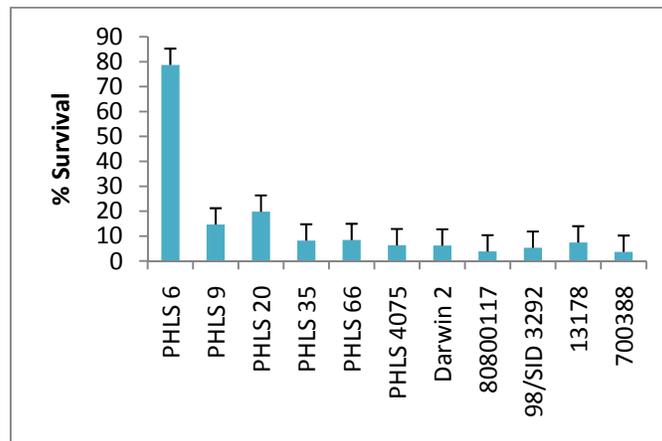


Figure 1. Cell association (A), entry (B) and intracellular survival (C) of *B. pseudomallei* in *A. castellanii* cell cultures. Isolate *B. thailandensis* 700388 was used as a control. Numbers shown are the percentage of bacteria at the time of sampling relative to the time zero inoculum. Isolate numbers are displayed on the x-axis. All assays were performed in triplicate.

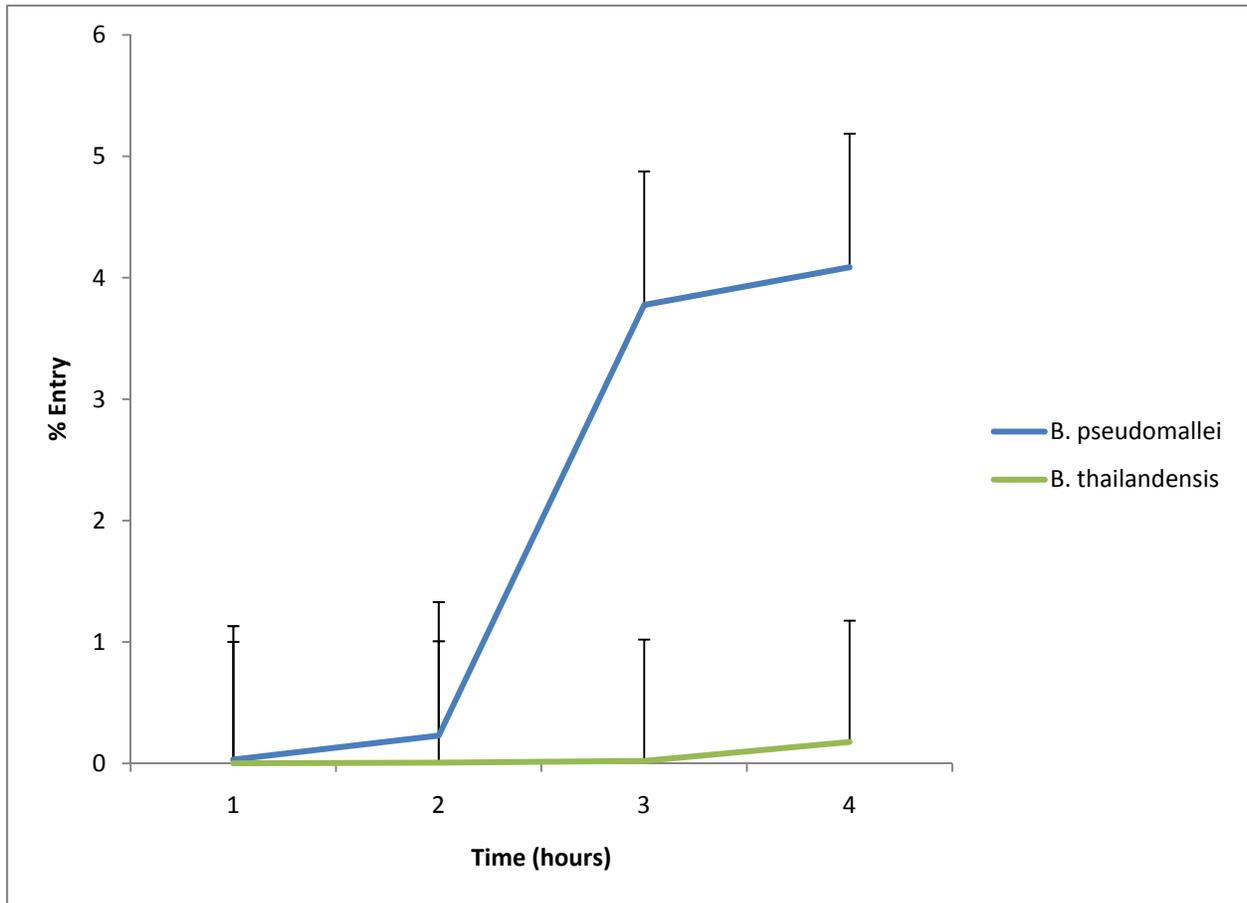


Fig 2. Entry time kinetics of *B. pseudomallei* and *B. thailandensis* in *A. castellanii* cells. Numbers shown are the percentage of bacteria at the time of sampling relative to the time zero inoculum. Time points in hours are displayed on the x-axis. All assays were performed in triplicate.

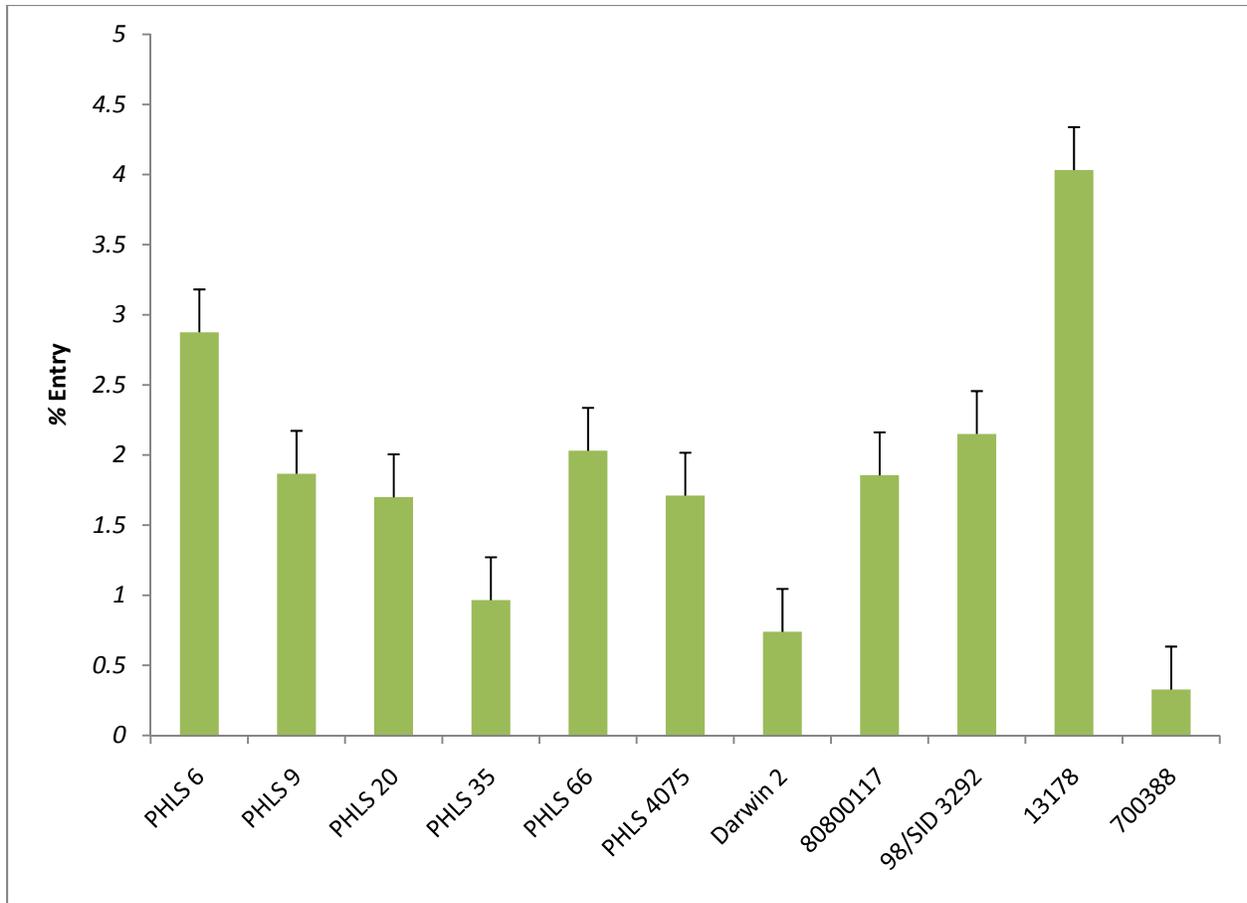
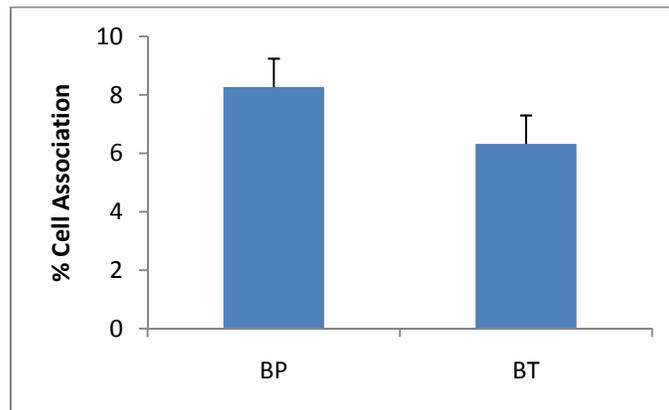
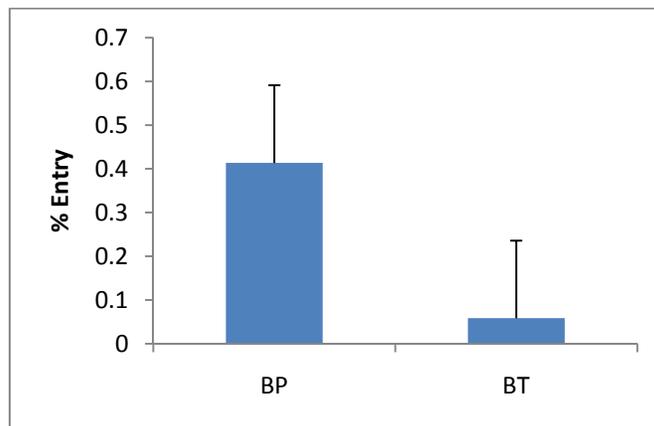


Figure 3. *A. castellanii* entry percentage of ten *B. pseudomallei* isolates and *B. thailandensis* 700388 after 3 hr of co-culture. Numbers shown are the percentage of bacteria after 3 hr relative to the time zero inoculum. Isolate numbers are listed on the x-axis. All assays were performed in triplicate.

A.



B.



C.

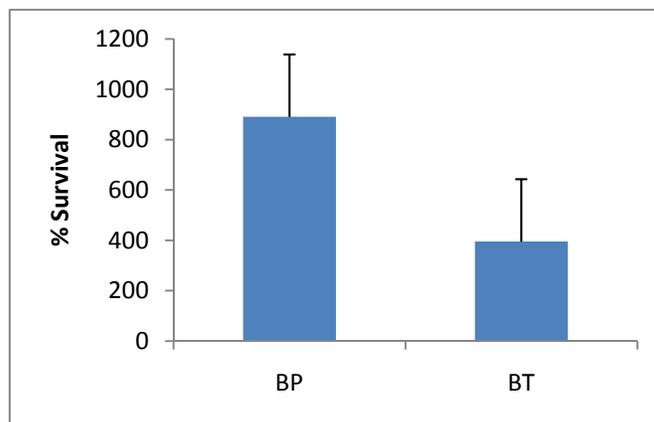
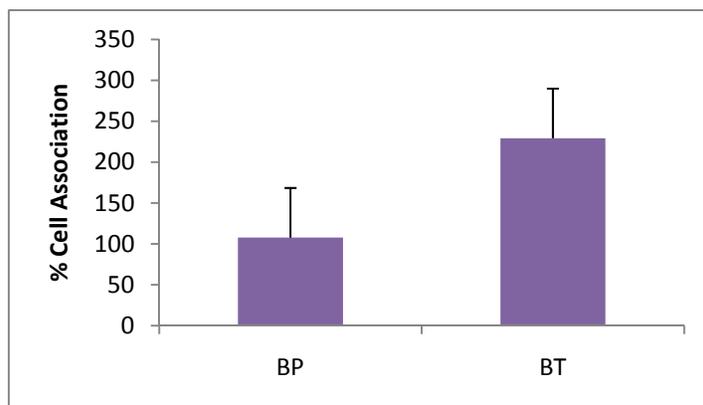
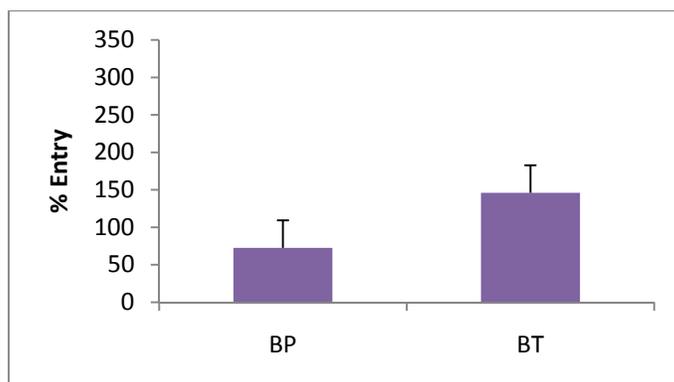


Figure 4. Cell association (A), entry (B) and survival (C) of non-passaged *B. pseudomallei* and *B. thailandensis* in the THP1 human monocyte line. BP is *B. pseudomallei* 13178 and BT is *B. thailandensis* 700388. Numbers shown are a percentage of bacteria at time of sampling relative to the time zero inoculum. All assays were performed in duplicate.

A.



B.



C.

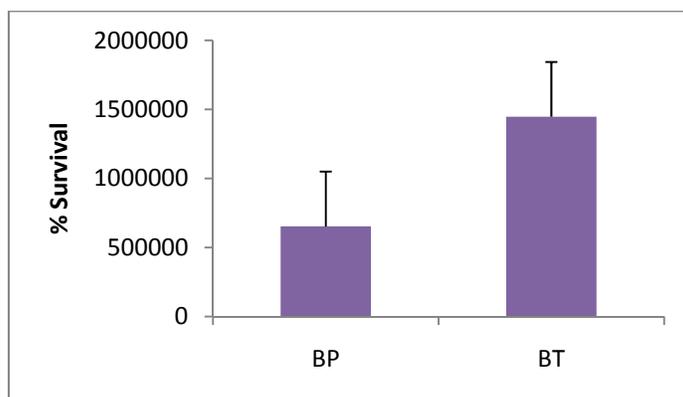


Figure 5. Cell association (A), entry (B) and survival (C) of amoeba-passaged *B. pseudomallei* and *B. thailandensis* in the THP1 human monocyte line. BP is *B. pseudomallei* 13178 and BT is *B. thailandensis* 700388. Numbers shown are a percentage of the bacteria at time of sampling relative to the time zero inoculum. All assays were performed in duplicate.

Table 2 Long-term survival of select isolates of *B. pseudomallei* and *B. thailandensis* in *A. castellanii*

Time (days)	<i>B. pseudomallei</i>								<i>B. thailandensis</i>		UI [†]	
	PHLS 6		PHLS 9		13178		80800117		700388		--	
	-C [*]	+C [‡]	-C	+C	-C	+C	-C	+C	-C	+C	-C	+C
3	+	+	+	+	+	+	+	+	+	+	-	-
7	+	+	+	+	+	+	+	+	+	+	-	-
14	+	+	+	+	+	+	+	+	+	+	-	-
21	+	+	+	+	+	+	+	+	+	+	-	-

* Amoeba cysts treated only with kanamycin.

‡ Amoeba cysts treated with cycloheximide and kanamycin.

† UI = Uninfected controls

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