DISSERTATION

THE ANALYSIS OF *Burkholderia pseudomallei* VIRULENCE AND EFFICACY OF POTENTIAL THERAPEUTICS

Submitted by

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ABSTRACT

THE ANALYSIS OF *Burkholderia pseudomallei* VIRULENCE AND EFFICACY OF POTENTIAL THERAPUTICS

Burkholderia pseudomallei is the causative agent of the disease melioidosis and is classified as a category B Select Agent. There are currently many challenges associated with both the study of this pathogen and its treatment in the clinical setting. Prior to these studies, there was no attenuated *B. pseudomallei* strain available that was exempt of Select Agent regulations and approved for study outside of biosafety level 3 (BSL-3) containment, and consequently basic research on this pathogen was largely hindered. The first purpose of these studies was to extensively characterize the attenuation of two *B. pseudomallei* mutant strains using melioidosis animal models. The two mutants constructed were Bp82 and Bp190, Δ*purM* derivatives deficient in adenine and thiamine biosynthesis. These mutants were found to be fully attenuated in immune competent and immune deficient mouse and hamster melioidosis models. Bp82 is currently exempt of all Select Agent regulations and can be safely handled in the BSL-2 setting, greatly accelerating research on this priority pathogen.

Since basic research on *B. pseudomallei* was not common in the Western world until its Select Agent classification, much is still unknown regarding the bacterial factors contributing to its virulence. A second purpose of this research was to determine whether resistance-nodulation-cell division (RND) efflux systems and iron acquisition siderophores impact the virulence of *B. pseudomallei* in a pneumonic murine melioidosis

model. This was examined using a clinical isolate naturally devoid of a characterized efflux system and the gene cluster for malleobactin siderophore synthesis, and by the construction of isogenetic mutants. The two characterized *B. pseudomallei* efflux pumps, AmrAB-OprA and BpeAB-OprB, were both found to be completely dispensable during *in vivo* murine infection. The removal of one or both of these systems did not reduce lethality of the mutant strains. Unlike that observed with similar bacterial pathogens, the lethality of *B. pseudomallei* was also not reduced upon the removal of either the malleobactin or pyochelin siderophores. This finding indicates *B. pseudomallei* is likely capable of utilizing alternative systems for iron acquisition within the host.

In addition to the challenges associated with the study of this pathogen, there are also many clinical challenges associated with melioidosis, providing a basis for the final two purposes of this research. One particular challenge is the high frequency of patient relapse, even after appropriate prolonged antibiotic therapy. A third purpose of this research was to determine whether traditional antibiotic therapy could be augmented by the co-administration of immunotherapy. Cationic liposome-DNA complexes (CLDC), which are potent activators of the innate immune system, were found to synergistically reduce intracellular *B. pseudomallei* concentrations in macrophages *in vitro* when combined with the antibiotic ceftazidime. In addition, this combination therapy also significantly increased mouse survival during both acute and chronic melioidosis. A similar enhancement to ceftazidime therapy was observed with recombinant IFN-γ, illustrating the potential of immunotherapy to improve clinical outcome and decrease patient relapse.

The lack of an effective approved vaccine for human use is another substantial clinical challenge associated with melioidosis and its prevention. The final purpose of these studies was to develop an effective mucosal vaccine, offering both short-term protection from acute pneumonic disease and long-term protection from disseminated chronic melioidosis. CLDC was identified as a highly effective mucosal adjuvant within complexed to heat-killed *B. pseudomallei*, and this adjuvant offered moderate protection from acute disease when combined with *Burkholderia* protein subunits. The longest-term protection from lethal challenge in our murine model, lasting beyond 100 days, was elicited by the fully attenuated live Bp82 strain. Since this strain is both fully attenuated and exempt of Select Agent regulations, it has great potential clinically for high-risk persons as an effective live vaccine strain.

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CHAPTER 1

REVIEW OF LITERATURE

Burkholderia pseudomallei AND MELIOIDOSIS

1.1 Pathogen of focus - *Burkholderia pseudomallei*

The *Burkholderia* genus is comprised of more than 30 species that occupy a variety of ecological niches. The majority of species within this genus are non-pathogenic soil-dwelling bacteria, but a few species are highly pathogenic to humans and can result in severe disease [1]. One clinically relevant *Burkholderia* species is *B. cepacia*, an opportunistic pathogen that commonly causes respiratory tract infections in cystic fibrosis patients. Two of the most pathogenic species within the *Burkholderia* genus are *B. mallei* and *B. pseudomallei. B. mallei* causes the disease glanders in horses, mules, and donkeys [1]. Humans too can acquire glanders following exposure to *B. mallei*, and this pathogen was used by Germany in World War I as a biological weapon [2]. *B. mallei* is not able to persist in the environment outside of the equine host [3], whereas *B. pseudomallei* can survive a variety of harsh environmental conditions for extended periods of time [4]. Another closely related species is *B. thailandensis,* which is far less pathogenic than both *B. mallei* and *B. pseudomallei*. *B. thailandensis* contains a functional arabinose synthesis operon that is largely absent in *B. pseudomallei* [5-6].

B. pseudomallei is highly pathogenic to humans and causes the disease melioidosis [7]. It is an oxidase positive Gram-negative bacillus that produces varying colony morphologies ranging from smooth in appearance to dry and wrinkled when grown on agar media [8]. *Burkholderia pseudomallei* is an environmental bacterium that is found in rice paddies, stagnant waters, and moist soils within endemic regions of the world [3, 9]. This is a hardy pathogen that can persist in low-nutrient environments for prolonged periods of time, and has remained culturable in distilled water for 10 years after initial inoculation [10]. This bacterium persists best in soils containing a water content of 15% [11], and is believed to obtain its required nutrients from organic rotting matter. Within the environment, *B. pseudomallei* is capable of infecting the free-living protozoa *Acanthamoeba astronyxis* [12]. *B. pseudomallei* is endemic to southeast Asia, northern Australia [4], and other tropical regions of the world located between 20°N and 20°S lines of latitude [13]. This pathogen was first described in 1911 among morphine addicts in Rangoon, Burma by Alfred Whitmore and C.S. Krishnaswami [14]. Since its discovery, it has been described using variety of names, including *Bacillus pseudomallei, Bacillus whitmori, Pseudomonas pseudomallei,* and has been known as *Burkholderia pseudomallei* since 1992 [15].

1.1(1) *B. pseudomallei* Genomics

B. pseudomallei strain K96243 has been fully sequenced and its genome is over 7Mbp contained within two chromosomes, which is one of the largest bacterial genomes characterized to date [3, 16]. Chromosome 1 is composed of genes primarily involved with housekeeping functions including metabolism, motility, and cell wall and protein biosynthesis, whereas chromosome 2 specific genes are thought to be involved with

bacterial adaptation within the environment and host [16]. *B. pseudomallei* contains a variety of horizontally acquired genomic islands that are not present within the closely related pathogen *B. mallei.*

B. pseudomallei is an organism with a genome of great plasticity. It is thought that horizontal gene transfer, recombination, and mutation all take place within this organism and greatly influence strain to strain variation [16-17]. There have been recent reports of genetic divergence even among isolates within the same melioidosis patient, indicative of within-host adaptation by *B. pseudomallei* [18]. In addition, phenotypic changes in colony morphology within a single strain have also been reported and observed in *B. pseudomallei* isolated from various tissue sites in melioidosis patients [8, 19-20].

1.1(2) Select Agent Listing

B. pseudomallei has gained increased attention in the Western Hemisphere in recent years due to its potential for use as a biological weapon [21-22]. Because of this pathogen's biodefense implications, *B. pseudomallei* is currently classified as a category B Select Agent by the Centers for Disease Control and Prevention (CDC). Agents within this category are those that are moderately easy to disseminate, result in moderate morbidity and low mortality rates, and require enhanced disease surveillance (http://www.bt.cdc.gov/agent/agentlist-category.asp). The Select Agent listing of this pathogen subjects all research using *B. pseudomallei* to strict Federal guidelines that govern its acquisition, possession and use [23]. Due to such guidelines, research utilizing *B. pseudomallei* in the United States can only be conducted by cleared

personnel. In addition, all research with this pathogen is required to take place within CDC inspected biosafety level 3 (BSL-3) containment facilities, greatly hindering research on *B. pseudomallei* by those research institutions lacking such containment laboratories.

For many bacteria on the Select Agent list, including *Bacillus anthracis*, *Francisella tularensis* and *Yersinia pestis,* there are already attenuated strains available that are excluded from Select Agent regulations (www.selectagents.gov/exclusions.html). Excluded strains are those that do not pose a severe threat to public health and safety, animal health, or animal products, and are thus removed from the list of select biological agents. This exclusion allows the attenuated strains to be handled in BSL-2 laboratories and greatly facilitates studies on these pathogens by institutions lacking approved BSL-3 containment. Prior to the avirulent *B. pseudomallei* characterized as part of this research [24], there was no attenuated *B. pseudomallei* strain exempt of Select Agent regulations. A variety of *B. pseudomallei* attenuated mutant strains have been constructed and tested *in vivo* to date (outlined in **Table 1.1** below). Most have these strains have been investigated in terms of protective efficacy rather than intensive demonstration of attenuation with the purpose of Select Agent exemption [25]. In addition, many were created using select agent non-compliant methods, therefore diminishing the likelihood of Select Agent exemption.

Table 1.1. Live attenuated *B. pseudomallei* strains. This table was adapted from [25].

Disrupted gene	Pathway/Gene Function	Median Lethal Dose/Route	Reference
Mannosyltransferase	Capsule polysaccharide synthesis (acapsular mutant)	$>10^6$ CFU i.p.	[26]
wcbC, wcbN	Capsule polysaccharide export (acapsular mutants)	$>10^3$ CFU i.n.	[27]
ilvI	Branched chain amino acid auxotroph	10^6 CFU i.p.	[28]
aroB	Aromatic amino acid auxotroph	$>10^6$ CFU i.n.	[27]
aroC	Aromatic amino acid auxotroph	Unknown CFU; i.p.	[29]
serC	Serine auxotroph	10^6 CFU i.p.	[30]
purN	Purine auxotroph	$<10^7$ CFU i.p	[31]
purM	Purine auxotroph	$>10^7$ CFU i.p.	
lipB	Lipoate protein lipase B auxotroph	$<10^3$ CFU i.n.	[31]
pabB	P-aminobenzoate auxotroph	$>10^7$ CFU i.p.	[31]
bipD	Type III secretion mutant	$>10^4$ CFU i.n.	[32]

1.1(3) Epidemiology of Melioidosis and Risk Factors

The primary endemic foci for *B. pseudomallei* infection are southeast Asia and northern Australia, approximately between 20°N and 20°S lines of latitude [4, 13]. *B. pseudomallei* was first identified in northern Australia in 1949 during an outbreak occurring in sheep in Queensland [33], and the first human case of melioidosis was reported in a diabetic patient in Townsville in 1950 [34]. The first cases occurred in the Northern Territory of Australia in the early 1960s [35]. The annual incidence of melioidosis disease in the Top End of the Northern Territory between 1989 and 1999 was estimated at 16.5 cases per 100,000, but rates as high as 41.7 per 100,000 were reported in 1998 during severe weather events [36-37]. During 1995 to 2000, the annual incidence in the Torres Straight Islands that lie between northern Queensland and Papua New Guinea was reported as 42.7 cases per 100,000, one of the higher incidences reported to date [38]. This is thought to be related to the high prevalence of diabetes and high seasonal rainfall within this region [38]. *B. pseudomallei* has been isolated by environmental sampling from soil, mud, and pooled surface water in northern Australia [4], and two outbreaks have been linked to contaminated drinking water [39-41].

Compared to Australia, the annual incidence of melioidosis is lower in Asia [4]. In northeast Thailand, the incidence was estimated at 4.4 melioidosis cases per 100,000 between 1987 and 1991 [42]. However, it is likely that many *B. pseudomallei* infections are often undiagnosed in these areas due to the lack of culture facilities in many endemic tropical locations [4, 43]. There are higher rates of seropositivity in Thailand compared to those reported in Australia, as approximately 80% are seropositive in Thailand as measured by indirect hemagglutination compared to 5-13% seropositivity in

Australia [44-46]. One possible rationale for this observation is the presence of other less-virulent *Burkholderia* species found within the soil, such as *B. thailandensis* that is commonly found in Thailand but not Australia [4]. However, it was recently reported that antibodies from melioidosis patients are incapable of recognizing *B. thailandensis* antigens [47], causing this to be an unlikely explanation for the discrepancy in seroprevalence. Similar to Australia, *B. pseudomallei* has been isolated from the soil and pooled surface water in Asia, and is commonly found within rice paddy fields in Thailand [4]. There were melioidosis cases in Thailand and among visiting tourists, and a cluster of cases in Indonesia that were linked to the 2004 Asian tsunami [48-51]. Melioidosis cases have also been reported in Laos, Vietnam, Malaysia, Indonesia, China, and Taiwan [43, 52-56].

There have been cases of melioidosis in the Americas. In the United States, there are reports of the disease in servicemen returning from duty in southeast Asia [57-58], and two cases were thought to have been acquired from Honduras [13]. There have also been sporadic melioidosis cases occurring in the Caribbean, and Central and South America [4, 43]. A cluster of melioidosis cases was reported in northeast Brazil in 2003 [59], and there was also a report of *B. pseudomallei* infection in a diabetic patient in Puerto Rico, likely infected by flooding during the rainy season [60]. One of the most controversial cases occurred in 1973 in the United States involving the “Oklahoma isolate” that was obtained from a soil-contaminated wound after a farming accident [61]. At the time, this isolate was thought to be *B. pseudomallei*, but later confirmed as a new species, known as *B. oklahomensis* [62].

Certain lifestyles in endemic regions have been shown to be risk factors for melioidosis, including rice paddy farming in Thailand and Aboriginal ethnicity in Australia.

Additional risk factors include diabetes mellitus, thalassemia, male gender, excessive alcohol consumption, renal disease, and chronic pulmonary disease [4, 63]. One commonality among many of these comorbidities proposed to increase susceptibility to *B. pseudomallei* infection is impaired neutrophil function [4, 46, 64-66]. It was recently demonstrated that neutrophils from diabetic patients have decreased phagocytosis of *B. pseudomallei* and reduced migration compared to non-diabetic subjects [67].

1.1(4) Transmission of *B. pseudomallei*

Following exposure, *B. pseudomallei* causes the disease melioidosis, and there are several thousand natural cases occurring in endemic regions of the world per year [7, 11, 20, 68]. Patients with immunocompromising conditions have an increased incidence of melioidosis [4, 36]. *B. pseudomallei* can be acquired by inhalation, ingestion, or through breaks in the skin, and person to person transmission of this pathogen is rare [3, 68].

Inhalation was initially regarded as the primary route for *B. pseudomallei* acquisition based on the finding that helicopter crews within endemic regions had increased incidence of disease, likely due to aerosolization of the bacteria from the soil [4, 69]. Increases in not only melioidosis cases, but also a shift to the pneumonic presentation of disease during seasons of heavy rainfall in endemic regions have also indicated inhalation as a means of natural exposure [70]. Currently, the subcutaneous inoculation route is hypothesized to be the primary means of *B. pseudomallei* acquisition within endemic areas [4]. This is based on the high incidence of melioidosis in rice paddy workers and the common report of injury prior to symptom onset in patients [36].

Such injuries and minor breaks in the skin are especially common to the feet of workers that have direct contact with the soil.

Ingestion is also regarded as a means of *B. pseudomallei* transmission based on pathological findings of gastrohepatic node infection in both pigs and humans [36, 71]. In addition, potable water contamination by *B. pseudomallei* has been linked to two separate melioidosis outbreaks in Australia [39-40]. Whether these outbreaks were actually due to ingestion of the contaminated water, or perhaps inhalation or subcutaneous inoculation, remains controversial. Sexual transmission of *B. pseudomallei* has been suggested, but has yet to be confirmed in the literature [72]. There is one documented case of *B. pseudomallei* transmission through breast milk and another single case of vertical transmission [73-74], but these routes are considered uncommon.

1.2 Clinical Manifestations of Melioidosis

The clinical presentation of melioidosis is dependent upon the infecting strain type, the route of exposure, and the host immune response [4]. There is a vast array of clinical manifestations associated with melioidosis, including pneumonia, septicemia, osteomyelitis, hepatic and splenic abscesses, skin infections, and neurological disease [4, 68]. Symptoms of melioidosis onset are dependent most heavily on the route of exposure, and the most common symptom at time of presentation is septicemia, usually involving bacterial dissemination to distant organs [20]. The lung is the most commonly affected organ in adult cases, and pneumonia is present in approximately 50% of melioidosis patients [75]. The lung can be infected directly by direct inhalation or

septicemic spread. The time from exposure to onset of symptoms can range greatly, as symptoms can present within just a single day, or in some cases, evidence of disease may not present for decades [49, 57]. Without prompt, appropriate antibiotic therapy, infection with *B. pseudomallei* is often fatal, as illustrated by the high mortality rates among untreated cases [20]. The overall mortality rate in adults is approximately 50% in Thailand and 20% in Australia [46, 75]. The reduced mortality in Australia is likely due to the availability of intensive care treatment.

The type of clinical presentation can vary largely based on geographic location. There is a high incidence of genitourinary infection with prostatic abscesses in Australia, occurring in approximately 18% of male melioidosis patients, whereas this presentation is uncommon in Thailand [75]. Suppurative parotitis is a common clinical finding in Thai children with melioidosis, characterized by fever and swelling of the parotid gland, but this observation is uncommon in Australia [76]. Brainstem encephalitis accompanied by flaccid paralysis is evident in approximately 4% of cases in northern Australia [75, 77], and similar neurological manifestations have been described in only a small number of child melioidosis cases in southeast Asia [78].

1.2(1) Acute and Chronic Stages of Melioidosis

The various forms of melioidosis can be classified into three overall categories, including acute, sub-acute, and chronic [9, 69]. Acute melioidosis involves either an acute pulmonary or acute septicemic presentation of disease. The acute pulmonary form, often preceding inhalation of *B. pseudomallei*, is characterized by respiratory distress, fever, and death within a few days if left untreated [9]. The acute septicemic

form is characterized by septic shock, meningitis, cellulitis, or skin lesions, and this form is also highly fatal within a short time period [9, 68]. Sub-acute disease is less severe and is characterized by prolonged fevers, and death can occur within weeks to months without appropriate antibiotic therapy. The chronic stage of melioidosis is the most common form, and involves symptoms lasting longer than 2 months [57]. This form often involves bacterial spread to disseminated sites, such as the spleen, liver, or brain. Chronic melioidosis can also be sub-clinical (or latent) in nature and without apparent symptoms, and this form is often undiagnosed until disease resurgence. Treatment of melioidosis is complicated because *B. pseudomallei* displays high levels of intrinsic resistance to many antibiotics. Prolonged antibiotic therapy (months) is prescribed [23], and even with appropriate therapy, patient relapse is still common [7, 79-80].

The acute, sub-acute, and chronic melioidosis forms of disease do not always occur in a defined order. The sub-clinical, latent form may be the initial stage presented and symptoms may not develop for years [81]. This latent form can also follow acute disease resolution, leading to eventual relapse. Recurrence of disease is most likely following immunosuppression or trauma [81], and is common in patients with diabetes, chronic pulmonary or renal disease, and alcoholism [46].

1.3 Melioidosis in Animals

A variety of animals are susceptible to *B. pseudomallei* infection, including horses, cattle, deer, cats, dogs, goats, sheep, pigs, kangaroos, camels, koalas, and even marine animals [4, 71, 82-86], and there have been epizootic outbreaks among animals within endemic regions. In 1957 there was *B. pseudomallei* infection among

pigs, sheep, and goats in Aruba, and there have been later outbreaks in the 1970s in France in zoos that contributed to both animal and human deaths [43, 87].

To study this pathogen *in vivo*, a variety of melioidosis animal models have been established and they have typically utilized rodent species [88-91]. Currently, hamsters are a common model used to study acute stage melioidosis as this species is highly susceptible to *B. pseudomallei*, and the mouse model is often utilized for studies on both the acute and chronic stages of disease. Mice have been extremely useful for the *in vivo* study of *B. pseudomallei* because different stages of the disease can be modeled based on manipulation of the challenge dose and route of inoculation, along with the strain of mouse utilized [3].

1.3(1) Murine Melioidosis Model

A variety of inoculation routes have been used in mouse melioidosis models including intraperitoneal, intravenous, and intranasal routes of infection [88, 90, 92-95]. Since the inhalational route is considered one of the most lethal routes of exposure and the route most relevant in biodefense-related research [89, 96], many models have involved intranasal or aerosol challenge. The BALB/c mouse is often used for melioidosis modeling and is considered more susceptible to *B. pseudomallei* than the C57/Bl6 mouse strain [90, 93, 97]. The human clinical melioidosis isolate *B. pseudomallei* 1026b is commonly used in murine models because it has been well characterized in the laboratory and is virulent to mice [98]. The intranasal LD_{50} for *B. pseudomallei* 1026b in BALB/c mice is approximately 900 CFU [99].

Similar to their human counterparts, both acute pneumonic and chronic disseminated melioidosis are observed in murine models [100], and the lungs, liver, and spleen are the primary targets of pathological involvement [75]. Delayed emergence of infection is consistently observed in antibiotic treated mice, indicating the relevance of this melioidosis model in the study of disease relapse [101]. Following lethal intranasal challenge with *B. pseudomallei,* mice typically succumb to acute disease end-point marked by respiratory distress, hunched posture, and ruffling, within just three days post-infection [24, 99-100]. Mice challenged with a sub-lethal inoculum or treated with therapy that subsequently survive acute pneumonic disease may show no symptoms of disease for 1-2 months, and it is currently unknown where *B. pseudomallei* reside during this asymptomatic latent period. However, the gastrointestinal tract is considered a likely location for colonization (Goodyear et al., article in preparation). Mice in the sub-clinical phase of melioidosis often have bacterial counts in the blood, lungs, liver, spleen, lymph nodes, and brain below the limit of detection (20 CFU/organ) despite eventual bacterial resurgence and progression to chronic melioidosis symptoms (Propst and Goodyear, unpublished observations). It is currently unknown where *B. pseudomallei* reside during the asymptomatic latent period, and published studies using animal models have failed to identify such reservoirs. Chronic disseminated disease in murine melioidosis models is typically associated with splenic infection and the formation of visible lesions, wasting, or neurological involvement [99-100] which will eventually progress to end-point symptoms similar to that seen in acute pneumonic disease (Propst and Goodyear, unpublished observations).

1.4 *B. pseudomallei* Pathogenesis

Initial infection with *B. pseudomallei* occurs at the epithelial cell layer of abraded skin in cases of cutaneous inoculation or the mucosal surface during inhalation or ingestion of this pathogen [81]. *In vitro* studies have revealed *B. pseudomallei* is capable of adhering to and invading many different epithelial cell types, including alveolar, bronchial, laryngeal, oral, and conjunctival cells [68, 102]. This initial attachment to epithelial cells appears to be dependent on both the polysaccharide capsule and type IV pili [103-104]. Previous studies have shown that attachment is mediated by *B. pseudomallei* binding to the asialoganglioside GM1-GM2 receptor complex on human pharyngeal epithelial cells [105].

Following attachment, *B. pseudomallei* can invade and multiply within both non-phagocytic and phagocytic cells [81, 106]. This pathogen can replicate in neutrophils and macrophages following either phagocytosis or invasion [3, 68]. Invasion of host cells is made possible by the presence of a *Burkholderia* secretion apparatus (bsa) type III secretion system (T3SS) and its effector protein BopE [107-108]. Following host cell entry, *B. pseudomallei* first enters into a phagosome. The T3SS effectors cause degradation of vacuolar membranes, allowing for efficient phagosomal escape by this pathogen and entry into the host cell cytoplasm [3, 32, 68]. The T3SS protein BopA enables *B. pseudomallei* to evade killing by host cell autophagy [109], an important defense mechanism against intracellular pathogens involving sequestering of bacteria in vacuoles and degradation by lysozyme fusion [110].

The polarly located *B. pseudomallei* protein BimA enables polymerization of host cell actin, leading to the formation of actin-based membrane protrusions on the bacterial surface. The presence of these actin tails contributes to bacterial motility and the

spreading to neighboring host cells [3, 111-112]. Actin polymerization is also thought to lead to the fusion of host cells and multinucleated giant cell (MNGC) formation, a common observation among both phagocytic and non-phagocytic cells infected with *B. pseudomallei* [113-114]. MNGC formation is thought to be induced by the T3SS effector protein BipB [115], and it is hypothesized that host cell fusion and subsequent MNGC formation contributes to *B. pseudomallei* cell-to-cell spread, evasion of host defenses, and persistence *in vivo* [3] .

This pathogen can cause both localized and disseminated disease [81]. Examples of localized disease include pneumonia or abscess formation, and the mechanism by which *B. pseudomallei* spreads from these initial localized locations to secondary organ sites within the host (the liver, spleen, blood, or brain) has not been fully elucidated. However, travel through macrophages within the lymphatic system has been considered likely, as this pathogen can successfully invade and survive within these cells [81]. *B. pseudomallei* can disseminate throughout the body and is capable of causing infection within a variety of locations in the host including the skin, blood, lungs, liver, spleen, genitourinary tract, brain, and parotid gland [68].

There has been some controversy, especially with neurological melioidosis, as to whether the pathogenesis during *B. pseudomallei* infection is the direct result of bacterial spread or due to the production of a toxin. It was reported in 1992 that *B. pseudomallei* was cultured from only one out of 7 melioidosis patients with brainstem encephalitis, indicating a potential exotoxin-induced neurological syndrome without direct central nervous system (CNS) infection [116]. However, a more recent report indicated the direct presence of *B. pseudomallei* within the CNS during cases of neurological melioidosis, demonstrating that *B. pseudomallei* invasion of the CNS and subsequent

inflammation may also be responsible for the neurological symptoms observed in melioidosis patients [77].

1.4(1) Role of Host Immune Response: Innate Immunity

Based on the findings that patients with diabetes, thalassemia, renal impairment, and alcoholism are at increased risk for melioidosis, the innate immune system is thought to play a primary role in controlling *B. pseudomallei* infection [4, 46]. Early studies indicated that *B. pseudomallei* is largely resistant to both the bactericidal activity of serum [117] and lysis by the terminal complement membrane attack complex [118]. *B. pseudomallei* can also survive and multiply within both macrophage/monocyte and neutrophil cell lines following phagocytosis [106]. This pathogen is capable of destroying the phagosome membrane within only 15 minutes of intracellular infection, escape phagosome-lysosome fusion, and avoid subsequent intracellular killing [119].

Research has indicated that *B. pseudomallei* is largely resistant to intracellular killing by neutrophils [67], which may explain why granulocyte-colony stimulating factor (G-CSF) does not consistently improve clinical outcome during advanced disease [120]. However, recent studies by Easton et al. have demonstrated a critical role for neutrophils independent of phagocytosis during pulmonary melioidosis. When neutrophil recruitment to the lungs was prevented with an anti-Gr-1+ cell-depleting monoclonal antibody, pulmonary disease was severely exacerbated following intranasal *B. pseudomallei* challenge in mice, and pulmonary burdens were increased by 1000-fold [121]. Key pro-inflammatory cytokines, including interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) that are imperative for defense against *B. pseudomallei* (discussed in

next section) were reduced by 98% in neutrophil-depleted mice. Such observations indicate that neutrophils likely play an important indirect role in the generation of early cytokines within the lungs during melioidosis [121].

Much research emphasis has been placed on macrophages for the study of intracellular *B. pseudomallei* infection [81]. Macrophages are imperative for the production of pro-inflammatory cytokines, including interleukin-12 (IL-12), that are important for host defense against this pathogen [122] (discussed in next section). Macrophages are a common site for intracellular *B. pseudomallei* infection, and many processes within these cells, including the production of reactive oxygen and nitrogen species, antimicrobial peptides, and lysosomal enzymes are imperative for intracellular bacterial killing. However, despite these defense strategies, *B. pseudomallei* can efficiently invade and replicate within macrophages [123-124]. Cells of macrophage/monocyte lineage are also thought to play a potential role in harboring *B. pseudomallei* during latent infection, but the precise location during latency currently remains unclear [4].

One potential mechanism proposed for the avoidance of macrophage killing by *B. pseudomallei* is related to their unique response to this particular pathogen. Macrophages exposed to *B. pseudomallei* were shown to produce lower levels of inducible nitric oxide synthase (iNOS) and tumor necrosis factor alpha (TNF-α) compared to macrophages exposed to other bacterial pathogens [125-126]. These observations provide a possible mechanism for the evasion of macrophage killing by this bacterial pathogen.

1.4(2) Role of Host Immune Response: Proinflammatory Cytokines

Interferon-γ (IFN-γ) is a proinflammatory cytokine produced primarily by T cells and natural killer cells [81], and this cytokine has been shown to be vital for defense against *Burkholderia* species. Previous research has demonstrated that IFN-$\gamma^{-/-}$ mice succumb to *B. mallei* infection within just 2-3 days following intraperitoneal challenge and have uncontrolled bacterial replication, whereas wild type mice receiving the same challenge dose survive beyond 40 days [127]. Santanirand et al. demonstrated there was greater than a 4000-fold increase in organ bacterial burdens of *B. pseudomallei* and a 5-log reduction in the median lethal intraperitoneal challenge dose when IFN-γ was neutralized in mice [94]. It has also been demonstrated that IFN-γ is crucial for control of *B. pseudomallei* replication within the lungs as IFN-$\gamma^{-/-}$ mice were also highly susceptible the intranasal challenge route [121].

A study using a mouse melioidosis model has demonstrated that during the early stages of *B. pseudomallei* infection, the dominant source of IFN-γ production is natural killer (NK) cells, with additional production by T cells, NK T cells, and macrophages [128]. However, this same study demonstrated that depletion of both T and NK cells surprisingly did not reduce the control of *B. pseudomallei*, therefore demonstrating significant redundancy in the various cellular sources of this vital cytokine and minimum threshold of IFN-γ required for efficient bacterial clearance [128].

Studies have been conducted to determine the mechanism by which IFN-γ increases host defense against this pathogen. Intracellular *B. pseudomallei* killing was shown to be increased *in vitro* when macrophages were pre-treated with IFN-γ. This was due to the induction of the inducible nitric oxide synthase (iNOS) and production of

reactive oxygen and nitrogen intermediates (ROI, RNI) in response to IFN-γ activation [81, 129]. Additional studies using animal melioidosis models have revealed that ROI play the bigger role in *B. pseudomallei* intracellular killing than RNI [130]. To decrease killing by reactive intermediates in response to IFN-γ activation, *B. pseudomallei* has been shown to repress iNOS expression [131]. IFN-γ has also been shown to induce autophagy, an intracellular defense against pathogens involving sequestering of bacteria into a phagosome and subsequent degradation by fusion with lysozymes [110]. Lysozyme fusion with intracellular phagosomes containing *B. pseudomallei* is detectable within infected macrophages, however the bacteria are able to efficiently evade killing by this mechanism and their replication quickly overwhelms the macrophage [132].

Interleukin-12 (IL-12) is a proinflammatory cytokine released by macrophages during early infection and is essential for inducing the production of IFN-γ by natural killer (NK) cells and T cells [122]. IL-12 has been shown to be imperative for defense against a wide variety of intracellular pathogens and is also thought to be important for protection from *Burkholderia* [127, 133-134]. IL-12$^{-/-}$ mice were found in a study by Haque et al. to succumb to intraperitoneal challenge with *B. pseudomallei* 26 days before wild type mice, illustrating the necessity of this cytokine for controlling early infection [128]. This same study also illustrated the importance of interleukin-18 (IL-18) during early infection, as neutralization of this cytokine also rendered mice more susceptible to infection. In addition, *in vivo* production of IFN-γ within this study was found to be largely dependent on IL-12, and to lesser extent on IL-18 production [128].

Another proinflammatory cytokine primarily produced primarily by macrophages, and to lesser extent by B and T cells, is tumor necrosis factor-α (TNF-α), and this has also been proposed to be important for defense against *B. pseudomallei* [4].

Neutralization of TNF-α increased susceptibility to infection in a murine melioidosis model, and TNF-$\alpha^{-/-}$ and TNF-α receptor $^{-/-}$ mice are both highly susceptible to *B. pseudomallei* infection [135-136]. However, using an *in vitro* model of *B. pseudomallei* and cytokine neutralization assays, our laboratory has demonstrated that this cytokine plays a far lesser role than IFN-γ in macrophage defense against this pathogen [100].

1.4(3) Role of Host Immune Response: Adaptive Immunity

Even though human immunodeficiency virus (HIV) infection is prevalent in Thailand, this does not appear to be a risk factor for melioidosis [137], indicating that cellular immunity may not play a significant role in *B. pseudomallei* infection. However, a study by Barnes et al. demonstrated that T cells may directly affect clinical outcome during melioidosis. T cells isolated from sub-clinical melioidosis patients were shown to have increased proliferation and IFN-γ production compared to T cells isolated from melioidosis patients with clinical disease, indicating that a strong cell mediated immune response is important for the control of infection [138]. Studies using a murine melioidosis model have demonstrated that while T cells appear to be dispensable during early stages of *B. pseudomallei* infection, $CD4^+$ T cells specifically play a vital role during later stages of infection. Mice depleted of $CD4^+$ T cells were found to have the shortest median survival time following intraperitoneal *B. pseudomallei* challenge compared to $CD8^+$ T cell-depleted or wild type mice [128].

Even though seropositivity is common within endemic regions, the antibody response resulting from natural environmental exposure to both *B. pseudomallei* and *B. thailandensis* does not appear to be sufficient for preventing either primary melioidosis or

disease relapse [4]. Melioidosis patients often have detectable antibody titers, with the dominating type being IgG, for years after infection [139]. In addition, *B. pseudomallei*-specific CD4+ and CD8+ T cells are detectable in the blood of melioidosis survivors compared to control subjects [140].

1.5 *B. pseudomallei* Virulence Factors

B. pseudomallei is capable of persisting in a variety of hostile environments in both nature and within the host. This is a highly resilient pathogen that can survive nutrient deficiency, extreme temperatures, acidic and alkali pH, many antiseptic solutions, and exposure to a wide variety of antibiotics [4]. In addition, studies using melioidosis animal models and *in vitro* cell culture have revealed a variety of bacterial factors thought to contribute to survival, pathogenicity, and long-term persistence within the host. Even though all virulence factors for this pathogen have yet to be completely elucidated, many bacterial factors have been identified to date. The major known virulence factors and their putative functions are outlined in **Table 1.2** below.

The presence of a capsule has been shown to increase *B. pseudomallei* survival within the blood by conferring resistance to complement deposition and decreasing phagocytosis [81, 141]. In addition, both the capsule and type IV pili are thought to mediate initial attachment of *B. pseudomallei* to host epithelial cells [103-104]. Following attachment, the presence of the *Burkholderia* secretion apparatus (bsa) type III secretion system (T3SS) allows for invasion, intracellular survival, and replication within both non-phagocytic and phagocytic cells [81, 106, 108, 142] (further discussed under section 1.4). Actin polymerization on the polar ends of *B. pseudomallei* induced by the BimA

protein facilitates efficient cell-to-cell spread within the host [112]. *B. pseudomallei* also contains a type VI secretion system (T6SS) which is thought to play a role in macrophage invasion and intracellular survival, but the complete role of this system is not yet fully understood [143].

B. pseudomallei produces a vast array of secreted enzymes, including protease, lipase, catalase, peroxidase, superoxide dismutase, hemolysins, and other virulence factors such as siderophores [4, 81]. The type II general secretory pathway (Gsp) is responsible for the secretion of protease, lipase, and phospholipase. However, studies using a variety of *gsp* and protease mutants have indicated these three enzymes do not play a major role in virulence [144-145]. This pathogen produces three different phospholipase C enzymes which are thought to play roles in nutrient acquisition and macrophage infection, and one of these enzymes (Plc-3) was required for full virulence in a hamster melioidosis model [81, 91, 146].

Table 1.2. Virulence factors identified for *B. pseudomallei* and their putative roles during infection.

Virulence Factor	Putative Role	Reference
Capsule (Type 1-O-PS) (*wab* operon)	Epithelial attachment; complement resistance	[103, 141]
Type III, IV O-PS	Required for full virulence in mice; precise roles unknown	[81, 147]
LPS O-antigen	Complement and defensins resistance	[148]
Quorum sensing *(pmlIR, bpsIR*)	Stationary phase gene regulation (metalloprotease, siderophore); required for virulence	[149-150]
Flagellin (*fli, flg*)	Motility and host cell invasion	[98, 151]
Type IV pili (*pilA*)	Epithelial cell attachment	[104]
Type III secretion system (*bsa* operon)	Translocators for delivery of effectors to host cell (bipB, bipC, bipD); Host cell invasion (*bipD, bopE*); Vacuolar escape (*bsaZ*); Evasion of autophagy (bopA); multinucleated giant cell formation/apoptosis (*bipB*); bacterial spread	[32, 107-109, 115, 142, 152-155]
Actin polymerization (bimA)	Intracellular spread to neighboring host cells	[112]
Type VI secretion system (*tss*)	Intracellular life cycle in macrophages, actin polymerization	[143, 156]
Type II secretion system	Secretion of protease, lipase, and phospholipase C	[144]
Phospholipase C (*plc-1, 2, 3*)	Phospholipid cleavage; nutrient acquisition; mutant strains are attenuated in hamsters	[146]
Siderophores (*mbaJ, mbaF, mbaJ, mbaI*, *fmtA; BPSS05087, fptA*)	Iron acquisition	[157]
RND efflux (*amrAB-oprA, bpeAB-oprB*)	Quorum sensing regulation (*bpeAB-oprB*); antibiotic efflux	[158-160]
Morphotype switching	Alteration of surface determinants *in vivo*	[8]

1.5(1) Iron Acquisition in *B. pseudomallei*

Many bacteria acquire iron by the secretion of siderophores. Siderophores are iron-scavenging molecules that have a high affinity for Fe^{3+} ions [161]. *B. pseudomallei* contains the hydroxamate siderophore, malleobactin (MbaA), that is expressed during iron-deficient conditions [81, 157]. Malleobactin is homologous to the pyoverdine siderophore produced by *Pseudomonas aeruginosa* [162]. The open reading frames *mbaA* and *mbaF* are involved with malleobactin biosynthesis, and the *fmtA* open reading frame encodes the FmtA receptor involved with its transport. The last two open reading frames involved in the same operon are *mbaJ* and *mbaI* [157]. Malleobactin is capable of releasing iron from transferrin, lactoferrin, and to a lesser extent from erythrocytes [163]. Malleobactin deficient *B. pseudomallei* mutants failed to grow under iron-limiting conditions *in vitro* [157], and homologous genes in the related species *P. aeruginosa* and *B. cepacia* are essential for full virulence in many experimental models [164-165]. However, the impact of malleobactin on *B. pseudomallei* virulence is largely unknown to date.

Based on homologous sequences to *P. aeruginosa, B. pseudomallei* is also thought to produce a second siderophore known as pyochelin [157, 166-167]. Pyochelin is encoded by the putative gene *BPSS0587,* and the putative pyochelin receptor protein, FptA, is encoded by the *fptA* open reading frame [157, 168]. In *P. aeruginosa,* a homologous receptor is used for iron delivery to the bacterial cell when complexed with the pyochelin siderophore [166]. An *fptA* deficient *B. pseudomallei* mutant (unable to utilize pyochelin) was shown to have no growth defects under iron-limiting conditions, suggesting that the malleobactin siderophore has an increased affinity for iron compared to pyochelin [157]. A mutant strain lacking both *mbaA* and *fptA* (deficient in both

malleobactin and pyochelin) showed the same level of *in vitro* growth in iron-deficient media as the single *mbaA* mutant [157].

1.5(2) Efflux Systems and Antibiotic Resistance

B. pseudomallei displays resistance to a diverse group of antibiotics including penicillins, third-generation cephalosporins, aminoglycosides, and rifamycins which greatly hinders therapeutic options clinically [4]. Antibiotic resistance is largely due to the presence of multi-drug efflux systems. Bacteria contain drug efflux transporters that are classified into five different families. These efflux systems are able to pump out a broad range of unrelated compounds including antibiotics [169]. The efflux pumps most prominent in Gram negative bacteria belong to the resistance-nodulation-cell division (RND) superfamily [169]. RND pumps are tripartite structures containing a transporter protein (located in the cytoplasmic membrane), a membrane fusion protein (spanning the cytoplasm), and an outer membrane protein (reaching the extracellular space). These systems are capable of effluxing a variety of compounds across the entire bacterial cell envelope. Genome sequencing of *B. pseudomallei* strains indicates the presence of at least 10 different RND efflux pumps that contribute to the intrinsic antibiotic resistance of this pathogen [16, 170]. To date, three of these efflux systems have been characterized in *B. pseudomallei*, including AmrAB-OprA, BpeAB-OprB, and BpeEF-OprC [158, 160, 171].

AmrAB-OprA was the first efflux system to be characterized and confers resistance to a variety of aminoglycosides, including tobramycin, kanamycin, and gentamicin. This system also confers resistance to the macrolides, erythromycin and

clarithromycin [160]. A second efflux system, BpeAB-OprB, was identified in 2004 [172] and is capable of effluxing the macrolides, fluoroquinolones, and tetracyclines [159]. BpeAB-OprB has also been reported to excrete quorum sensing molecules in *B. pseudomallei* strain KHW [158, 173]. It has been proposed that quorum sensing is dependent on this RND pump, and that BpeAB-OprB is required for the production of virulence factors controlled by quorum sensing, including biofilm formation, siderophore, and phospholipase C production [158]. However, it was most recently reported that BpeAB-OprB is not involved with quorum sensing or virulence factor production in *B. pseudomallei* strain 1026b [159], indicating potential variation between *B. pseudomallei* strains.

A third *B. pseudomallei* efflux system that has been identified is BpeEF-OprC and its substrates include trimethoprim and chloramphenicol [171]. The operon encoding this efflux pump also encodes the putative lipase, *BPSS0291* [171], indicating that efflux pumps could potentially play additional roles other than just antibiotic resistance during *in vivo* melioidosis infection. Since the true function for efflux pumps within bacteria is largely unknown [169], the impact these systems have on *B. pseudomallei* virulence during *in vivo* infection is also not understood.

B. pseudomallei contains various beta-lactamases which also contribute to its intrinsic antibiotic resistance. Genome sequencing has indicated the presence of Ambler class A, B, and D beta-lactamases [16]. The Bush-Jacoby-Medeiros class 2e beta-lactamase BPS-1 encoded by *blaA*, also known as *penA*, is known to confer resistance to the majority of cephalosporins [174]. Mutations in *blaA* result in resistance to beta-lactam/beta-lactamase inhibitor combinations and also to ceftazidime, a commonly used antibiotic to treat melioidosis [175]. Expression of the class D beta-

lactamases, OXA-42 and OXA-43, are also thought to contribute to resistance to ceftazidime [176].

1.6 Diagnosis of Melioidosis

Culture has remained the "gold standard" for definitive diagnosis of *B. pseudomallei* infection [20, 177-178]. It is recommended that samples of blood, urine, respiratory secretions, and throat swabs be cultured from patients with symptoms of melioidosis, and that wounds and pus be cultured during cases of cutaneous involvement [20]. The time to diagnosis with culture is typically 3 to 4 days and this constitutes one of its biggest downfalls. This is especially problematic in cases of *B. pseudomallei* septicemia, as patients often die within just 24 to 48 hours of hospital admission [177]. Culture also requires skilled laboratory personnel for interpretation of the results because normal flora can overgrow *B. pseudomallei* [177]. To help combat this problem with non-sterile specimens, Ashdown's agar is a selective media specific for *B. pseudomallei* containing trypticase soy agar, glycerol, crystal violet, neutral red, and gentamicin that is used for melioidosis diagnosis [179]. *B. pseudomallei* produces large purple-colored colonies when grown on this selective media. A modified Ashdown's agar containing colistin is also commonly used [180]. However, Ashdown's agar is not always readily available for use in all laboratories, especially within endemic regions of the world.

To decrease the time to diagnosis, various immunological-based diagnostic techniques have been explored, however there is currently no commercially available diagnostic test. Indirect hemagglutination antibody (IHA) tests for *B. pseudomallei*

specific antibodies have been developed, but have been considered poor for diagnostic purposes due to the common finding of seropositivity among healthy persons within endemic regions. It is estimated that 60-70% of Thai children are seropositive [181-182]. The detection of *B. pseudomallei* antigens is considered far more useful for diagnosing melioidosis because it indicates active disease [177]. Antigen tests have been developed for blood cultures and for directly testing patient specimens [4]. A test commonly used in Thailand is a latex agglutination test containing monoclonal antibodies specific for *B. pseudomallei* lipopolysaccharide and exopolysaccharide antigens. This test is used for blood culture fluid and was demonstrated to be both sensitive and specific [183-184].

Molecular tests have also been used for diagnosis of melioidosis. A polymerase chain reaction (PCR) test specific for the type three secretion system (T3SS) has been developed [185], but its effectiveness has had mixed results [186]. PCR-based diagnostic assays are most sensitive when conducted on direct specimens, and have had lower sensitivity when performed on blood cultures [185, 187]. The lower limit of detection for PCR identification typically falls below *B. pseudomallei* counts within the blood during melioidosis disease [20].

1.7 Melioidosis Therapies and Prevention

The current recommended intensive phase therapy for melioidosis is 50 mg/kg ceftazidime every 6-8 hours or 25 mg/kg meropenem every 8 hours for 10-14 days [23]. The duration of therapy is increased to 4-8 weeks for deep-seated infections. In cases of neurological, bone, joint, or prostatic melioidosis, 8/40 mg/kg trimethoprim-

sulfamethoxazole is also included in the intensive phase therapy. This initial treatment is then followed by oral eradication therapy consisting of trimethoprim-sulfamethoxazole for 3-6 months or longer, and doxycycline may also be included in the eradication therapy [23]. Whether or not doxycycline improves outcome is under current investigation in Australia [186]. In cases where trimethoprim-sulfamethoxazole cannot be used (pregnant women), amoxicillin-clavulanate is an alternative antibiotic, but has been shown to be associated with increased relapse rates [188].

In clinical trials conducted in the 1980s, the use of ceftazidime for intensive phase of therapy was shown to halve mortality over the previous conventional melioidosis therapy consisting of chloramphenicol/doxycycline/ trimethoprim-sulfamethoxazole [189-190]. No alternative antibiotics have definitively proven more effective than ceftazidime to date [186]. Since carbapenems are known to be highly active against *B. pseudomallei in vitro* [191], the efficacy of imipenem and meropenem have been compared to ceftazidime. In a clinical trial including more than 200 patients in Thailand, there was no difference in survival among patients treated with imipenem compared to ceftazidime. However, in patients surviving longer than 48 hours, imipenem had a lower rate of treatment failure, but these results remain controversial [186, 192]. In a trial comparing meropenem to ceftazidime therapy among melioidosis patients in Australia, outcomes were similar for patients in both groups. However, it was noted the more seriously ill patients were deliberately chosen to receive meropenem, suggesting that this antibiotic may be actually superior [193].

Even with appropriate antibiotics, the response to therapy is often poor, and fevers with melioidosis last approximately 9 days. Fevers lasting longer than 2 weeks or bacteremia persisting longer than one week despite antibiotic treatment constitutes

treatment failure [4]. Even with antimicrobial therapy, patient relapse is still common. Relapse occurs in approximately 13-23% of patients within a median time of 6-8 months [194-195]. Relapse is most often the result of reactivation of the original infecting strain of *B. pseudomallei* [195-196]. Relapse was shown to be increased in cases of severe disease, when doxycycline, quinolone, or ciprofloxacin-azithromycin were used during the oral eradication phase of treatment, or when eradication therapy was shorter than 8 weeks duration [4, 197-199]. This relapse is indicative of incomplete clearance of *B. pseudomallei* and stable colonization by this pathogen even after appropriate antimicrobial therapy and apparent recovery.

1.7(1) Immunotherapy for Treatment of Melioidosis

The use of immunomodulators for treatment of *B. pseudomallei* infection has not been thoroughly evaluated to date. However, since mortality from melioidosis is highest in cases of sepsis, treatments explored for improving the outcome of septic patients are considered relevant [186]. Recombinant human activated protein C (rhAPC) is known to reduce apoptosis in monocytes and decrease inflammation, and has been shown to improve clinical outcome from sepsis [200-201]. A melioidosis patient from a 2005 typhoon-related outbreak in Taiwan with acute pneumonic *B. pseudomallei* infection, septic shock, and multiple organ failure was treated with rhAPC and meropenem. This combination therapy consisting of an antibiotic and immunomodulator was shown to be successful, and was one of the first reports of rhAPC improving the outcome of melioidosis sepsis [202].

Hydrocortisone administration has not been shown to reduce mortality from septic shock in recent studies, even though this treatment hastens the reversal of shock itself [203]. Therefore, hydrocortisone therapy is not expected to improve survival in cases of melioidosis sepsis. Since diabetic patients have increased risk of melioidosis, it has been proposed that glycemic control could improve outcome from *B. pseudomallei* infection [4, 186]. However, insulin therapy was not found to improve outcome from sepsis in a clinical trial, and was actually shown to causes an increase in hypoglycemia-related adverse events [204].

Granulocyte-colony stimulating factor (G-CSF) is known to accelerate neutrophil recovery, and its impact on melioidosis outcome has been controversial. A 1998 study conducted in Australia analyzing survival from melioidosis sepsis following the administration of G-CSF indicated that G-CSF administration markedly reduced mortality [205]. However in a small trial conducted in Thailand, G-CSF increased survival time from sepsis caused by suspected melioidosis, but did not improve mortality [120]. These differing results could be largely dependent on the differing medical resources available in the trial locations [186].

Nonspecific activation of the innate immunity has been shown to improve outcome from *B. pseudomallei* infection in various melioidosis animal models. Unmethylated CpG oligodeoxynucleotides are potent stimulators of innate immunity and have been previously shown to elicit protection in mice from various intracellular bacteria [206]. It was reported by Wongratanacheewin et al. that intramuscular injection of CpG oligodeoxynucleotides administered prior to low-dose intraperitoneal *B. pseudomallei* challenge elicited survival in more than 90% of mice for 30 days [207].

1.7(2) CLDC Immunotherapy for *Burkholderia* Infection

Our laboratory has carried out studies similar to those conducted with CpG oligodeoxynucleotides (discussed in previous section). We have investigated the effectiveness of cationic liposome-DNA complexes (CLDC) to protect mice from pneumonic pathogens [99, 208]. CLDC is composed of cationic spherical lipid particles complexed with non-coding bacterial plasmid DNA, and have been previously shown to be potent activators of non-specific innate immunity [209]. These complexes can be delivered via a variety of routes including intravenously, intranasally, or intraperitoneally, and they have caused minimal adverse reactions in mice, dogs, and humans [100, 208, 210-211]. We recently reported that intranasal delivery of CLDC protected 100% of mice when administered prior to lethal *B. pseudomallei* or *B. mallei* challenge [99]. In this particular study, IFN-γ production induced by CLDC treatment was identified as the key cytokine mediating the protection elicited by the immunotherapy [99].

1.7(3) Prospects of a Melioidosis Vaccine

There is currently no approved vaccine for *B. pseudomallei.* A variety of vaccine formulations have been evaluated in animal melioidosis models to date, including killed whole-cell formulations, live-attenuated strains, a variety of *Burkholderia* protein subunits, capsular polysaccharide and lipopolysaccharide antigens, and DNA-based vaccines [25]. An overview of these approaches and their efficacy is outlined in **Table 1.3** below. Even though many of the vaccine formulations tested have offered short-term protection from acute melioidosis, a big challenge has been the lack of long-term, sterilizing protection from chronic disease following vaccination. Even some of the most

promising vaccine candidates fail to protect animals beyond 30-40 days post-infection [212], and *B. pseudomallei* is commonly isolated from the tissues of vaccinated survivors at the end of the observation periods [213].

Vaccine studies with killed whole-cell formulations have typically used non-adjuvanted irradiated or heat-killed *B. pseudomallei* [25, 213-214]. In a study by Barnes et al., mice were vaccinated subcutaneously with heat-killed *B. pseudomallei* and challenged intravenously. The majority of mice vaccinated with heat-killed bacteria alone failed to survive beyond 5 days post-intravenous challenge, illustrating the inability of killed, non-adjuvanted *B. pseudomallei* to protect from this challenge route [214]. Contrastingly, Sarkar-Tyson et al. demonstrated that intraperitoneal vaccination with killed *B. pseudomallei* alone offered protection beyond 40 days from aerosol challenge in 60% of mice [213]. However, it should be noted that a low aerosol challenge was used in this study, as not all unvaccinated mice succumbed to acute disease. Even greater protection was demonstrated by this same group following intraperitoneal challenge, as close to 100% of mice were protected for greater than 40 days [213]. However, it was noted by the authors that sterilizing immunity is not common in these studies, demonstrated by the finding that *B. pseudomallei* is routinely isolated from the tissues of vaccinated survivors [25].

Live attenuated strains have shown more promise than killed formulations in protecting mice from acute melioidosis [26, 28-29, 31-32, 215]. However long-term protection from chronic melioidosis is still lacking. In a study by Haque et al., 100% of mice vaccinated intraperitoneally with the live attenuated *B. pseudomallei* 2D2 strain were protected for 25 days following intraperitoneal challenge. Long-term sterilizing immunity was not produced however, as the majority of animals eventually succumbed

to chronic disease within 75 days [215]. Since the protection offered by this live attenuated strain was incomplete, the effectiveness of a boosting dose was analyzed. Boosted mice were found to have lower splenic bacterial burdens (compared to mice receiving a single vaccination) on day 1 after intraperitoneal *B. pseudomallei* challenge, but there was no significant difference in splenic burdens at any other time points analyzed (days 2, 6, 13 post-challenge) [215]. Additional studies in murine models have demonstrated the protective efficacy against lethal *B. pseudomallei* challenge increases when higher immunizing doses containing the live attenuated strain are used for vaccination [216]. In addition, the protective effect elicited by the live attenuated *B. pseudomallei* strain CL04 was shown to be greatly diminished when this strain was inactivated by gamma irradiation [216].

A variety of *Burkholderia* protein subunits have been tested for protective efficacy [212, 217-218]. The antigen showing the most promising to date has been LolC [25], an outer membrane protein that serves as an adenosine triphosphate-binding cassette transporter protein [212]. The majority of mice vaccinated subcutaneously or intraperitoneally with adjuvanted LolC were protected from melioidosis for over 30 days [212], similar to that seen with live attenuated vaccine formulations. However, long-term sterilizing protection was not offered by LolC or other outer membrane proteins tested to date [219]. Both lipopolysaccharide (LPS) and capsular polysaccharides have also been investigated as potential subunit vaccine candidates. Vaccination with either of these subunits intraperitoneally was shown to increase the time to death following intraperitoneal challenge in mice, but long-term protection was not observed [220].

Table 1.3. Summary of vaccine formulations investigated in murine melioidosis models. (Table continued onto next page).

Vaccine Formulation	Strain/Antigen	Route	Outcome	Source
Killed, whole-cell	killed Bp NCTC13178	s.c. vaccination i.v. challenge	no acute disease protection from killed Bp alone	[214]
	killed Bp K96243 killed Bp 576	i.p. vaccination i.p. challenge	> 80% protection for 20 days	[213]
	killed Bp K96243 killed Bp 576	i.p. vaccination low-dose aerosol challenge	~ 60% protection for 40 days	[213]
Live, attenuated strains	amino acid biosynthesis (*ilvI*) mutant, Bp 2D2	i.p. vaccination i.p. challenge Bp 576	100% protection for 25 days	[215]
	amino acid biosynthesis mutant (*aroB*), Bp 13B11	i.n. vaccination i.n. challenge Bp K96243	increased survival by ~ 2 days	[27]
	(*aroC*), Bp A2	i.p. vaccination i.p. challenge	BALB/c mice not protected	[29]
	serine biosynthesis mutant (*serC*)	i.p. vaccination i.p. challenge, Bp 576 and K92643	~80% survival for 25 days	[30]
	purine biosynthesis mutants (*purM*) (*purN*)	i.p. vaccination i.n. challenge i.p. vaccination i.n. challenge	Protection beyond 30 days Protection beyond 28 days (2/2 mice)	[130] [31]

Vaccine Formulation	Strain/Antigen	Route	Outcome	Source
Subunit vaccines	LolC (outermembrane protein) plus adjuvants	i.p or s.c. vaccination i.p. challenge Bp K96243	Protection for 30 days in > 80% mice; lack of chronic disease protection	[212]
	BipB, BipC, BipD (T3SS proteins) plus Freund's adjuvant	i.p. vaccination i.p. challenge Bp NCTC13178	No protection from antigens	[217]
	Omp85 (outermembrane protein) plus Freund's adjuvant	i.p. vaccination i.p. challenge Bp D286	~70% protection for 15 days; not sterilizing immunity	[218]
	Omp3 and Omp7 (outermembrane proteins) plus Freund's adjuvant	i.p. vaccination i.p. challenge Bp D286	~50% protection for 21 days; not sterilizing immunity	[219]
	Lipo-polycaccharide and capsular polysaccharide plus Ribi Adjuvant System (RAS)	i.p. vaccination i.p. challenge Bp NCTC4585	Increased time to death; Lack of chronic disease protection	[220]

1.7(4) Mechanism of Vaccine-Induced Protection

The live attenuated strain 2D2 was shown to protect 100% of mice from lethal *B. pseudomallei* challenge for 25 days [215], but failed to protect from long-term disseminated disease. The mechanism of acute disease protection following vaccination with this attenuated strain was examined by Haque et al. in 2006 to better understand the mechanism behind vaccine-mediated immunity to *B. pseudomallei* [215]. Splenocytes were obtained from immunized and control mice, and a significantly greater proportion of CD4+ and CD8+ T cells from vaccinated mice were found to produce IFN-γ in response to exposure to dead *B. pseudomallei* (compared to those cells isolated from unvaccinated controls). The proportion of IFN-γ producing T cells was greatest among the mice that received both a prime and booster vaccination, compared to those receiving just a single vaccination [215]. Additional experiments were conducted to determine whether the protection elicited by immunization is mediated by CD4+ or CD8+ T cells. Depletion of CD4+ T cells in immunized mice was found to completely abolish the protection offered by immunization, whereas CD8+ T cell depletion had no impact on protection. Such findings illustrate that vaccine-induced protection elicited by the live attenuated strain 2D2 is mediated by CD4+ T cells [215].

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CHAPTER 2

RATIONALE FOR RESEARCH AND SPECIFIC AIMS

2.1 Research Overview

The research project presented in this Dissertation is "The analysis of *Burkholderia pseudomallei* virulence and efficacy of potential therapeutics." The first two Specific Aims (Chapters 3 and 4) focus on the examination of virulence of a variety of *B. pseudomallei* mutant strains using a murine melioidosis model. Chapter 3 entails the extensive *in vivo* characterization of two fully attenuated mutant strains with the overall purpose of obtaining Select Agent exemption based on their complete attenuation. The identification of potential bacterial factors contributing to morbidity within the host is presented in Chapter 4. The latter two Specific Aims (Chapters 4 and 5) address melioidosis from more of a clinical perspective in terms of its treatment and prevention. The investigation of a potential enhancement to traditional antibiotic therapy by the co-administration of immunotherapy is discussed in Chapter 5, and the prevention of melioidosis with the development of an effective mucosal vaccine is addressed in Chapter 6.

2.1(1) Specific Aim 1 (Chapter 3 of Dissertation)

The basis for Specific Aim 1 is related to a major research challenge with *B. pseudomallei.* The overall long-term goal of this Aim was to greatly facilitate research on this pathogen by the research community. Basic research on *B. pseudomallei* is greatly hindered due to its Select Agent classification. All research must take place within biosafety level 3 (BSL-3) containment facilities, and studies using *B. pseudomallei* by those institutions lacking such containment laboratories are simply not possible in the United States. For many other bacterial strains on the Select Agent list, including *Bacillus anthracis*, *Francisella tularensis* and *Yersinia pestis,* there are already attenuated strains available that are excluded from Select Agent regulations (www.selectagents.gov/exclusions.html) and suitable for research within BSL-2 settings. Many of the attenuated *B. pseudomallei* mutants constructed to date were created using Select Agent non-compliant methods, and have been investigated in terms of protective efficacy rather than intensive demonstration of attenuation in immune competent and immune deficient animal models [1-4]. Prior to this research, there was no attenuated *B. pseudomallei* strain exempt from Select Agent regulations and approved for research outside of the BSL-3 setting.

The purpose of Specific Aim 1 was to create two *B. pseudomallei* attenuated mutant strains and fully characterize their attenuation *in vivo* using both immune competent and immune deficient animal models. Upon successful demonstration of attenuation, the strains can be filed for Select Agent exemption. The attenuated strains constructed as part of this research were Bp82, a 1026b ∆*purM* derivative, and Bp190, a K96243 ∆*purM* derivative. Both strains lack the capability for adenine and thiamine biosynthesis, and were constructed by Kyoung-Hee Choi and Takehiko Mima in the

Herbert Schweizer laboratory at Colorado State University. The attenuated mutant strains were tested in a variety of melioidosis models, including BALB/c mice, 129/SvEv mice, IFN-$\gamma^{-/-}$ mice, SCID mice, and Syrian hamsters. The hypothesis was that both strains would be fully avirulent *in vivo,* and incapable of replication and dissemination within a murine melioidosis model.

2.1(2) Specific Aim 2 (Chapter 4):

The focus of Specific Aim 2 is the identification and characterization of potential *B. pseudomallei* virulence factors. Since *B. pseudomallei* research within the Western world was largely lacking until its Select Agent classification [5], much is still unknown about this pathogen. Both iron acquisition and resistance-nodulation-cell division (RND) efflux systems have been proposed to play potential roles in virulence [6-7], but the impact of these systems during *in vivo* infection is largely unknown. *B. pseudomallei* is known to possess the hydroxamate siderophore malleobactin (MbaA) for iron acquisition from transferrin and lactoferrin [8-10], and is also thought to produce a second siderophore known as pyochelin [8, 11-12]. Homologous siderophores in related bacterial species have been shown to be required for full virulence [13-14]. *B. pseudomallei* is also believed to harbor at least 10 efflux pumps including the two characterized pumps AmrAB-OprA and BpeAB-OprB [15-16]. The BpeAB-OprB system has been proposed to be required for full virulence of *B. pseudomallei* [7], but this topic remains controversial.

The purpose of this Aim was to investigate the impact that *B. pseudomallei* siderophores and RND efflux systems have on virulence within a murine melioidosis

model. This was examined using both a clinical isolate and isogenetic mutants constructed within the laboratory. The two RND efflux systems evaluated within this research were the characterized AmrAB-OprA and BpeAB-OprB systems. The genomic region involved with iron transport that was analyzed within these studies was *mba,* containing a 13-gene malleobactin biosynthetic gene cluster [8]. A second iron transport factor analyzed within these studies was *fptA,* encoding the putative pyochelin receptor protein [8]. Strains deficient of *fptA* enabled us to examine the effect of the pyochelin siderophore as well as malleobactin on virulence of *B. pseudomallei*. The hypothesis was that virulence would be diminished in our murine model among the strains harboring deletions of efflux and iron acquisition genes, based on the report that homologous siderophores in related bacterial species are required for full virulence [13-14].

2.1(3) Specific Aim 3 (Chapter 5):

Specific Aims 3 and 4 focus on the treatment and prevention of melioidosis, with great potential relevance for the clinical setting within endemic regions and prophylaxis in the event of an intentional biological release. Even with antibiotic therapy, the overall mortality rate during melioidosis is still high, being approximately 50% in Thailand and 20% in Australia [17-18]. In addition, patient relapse is common after the discontinuation of therapy [19-21], illustrating the need for new approaches to improve the effectiveness of antimicrobial therapy for *B. pseudomallei* infection.

The purpose of Specific Aim 3 was to determine whether immunotherapy could augment the effectiveness of conventional antibiotic therapy for treatment of *B. pseudomallei.* This was examined *in vitro* using a macrophage infection model and also

in vivo using a murine melioidosis model. The antibiotic analyzed within these studies was ceftazidime, the current recommendation for intensive phase therapy for melioidosis treatment [22]. The immunotherapies investigated were cationic liposome-DNA complexes (CLDC) and recombinant IFN-γ. During the *in vivo* investigations, the impact of this combination therapy was assessed for both the short-term acute and long-term chronic stages of melioidosis. The hypothesis for Specific Aim 3 was that ceftazidime therapy would be enhanced with the addition of the immunotherapies. We predicted this combination therapy (immuno-antimicrobial therapy) would increase survival of the mice following lethal pneumonic *B. pseudomallei* challenge, and reduce bacterial loads both *in vivo* and *in vitro.* This hypothesis was largely based on our previous findings that CLDC is an effective immunotherapeutic against *Burkholderia* species [23].

2.1(4) Specific Aim 4 (Chapter 6):

The focus of Specific Aim 4 is the development of a mucosal vaccine for *B. pseudomallei.* There is currently no approved vaccine for human melioidosis, and an effective vaccine would likely be effective within both endemic regions of the world and among high-risk persons such as the military. A major current challenge among the various vaccine formulations investigated in murine models to date is the lack of long-term protection from chronic disease. Even some of the most promising vaccine candidates fail to protect animals into the chronic stages of disseminated disease [24]. In addition, *B. pseudomallei* is commonly isolated from the tissues of vaccinated survivors at the end of the observation periods [25]. These findings indicate a lack of sterilizing immunity elicited by the vaccines and illustrate the need for the identification of additional vaccine antigens, adjuvants, and effective delivery routes.

The purpose of Specific Aim 4 was to develop an effective mucosal vaccine for *B. pseudomallei* that would provide protection from both short-term acute and long-term chronic disease. Mucosal delivery of antigens (instead of systemic immunization) was chosen for these studies, as this route for delivery of antigens has been shown to most efficiently produce mucosal immunity [26-28]. The effectiveness of our vaccine formulations were tested via intranasal administration and one formulation was also tested orally. The challenge route utilized post-vaccination was pneumonic (intranasal) due to its biodefense implications.

The first goal of these studies was to determine whether the cationic liposome-DNA complexes (CLDC) would serve as an effective mucosal adjuvant. The vaccine formulations tested within these studies included killed bacteria, protein subunits conjugated to CLDC, and two live attenuated *B. pseudomallei* strains. The hypothesis for Specific Aim 4 was that the vaccine formulations delivered intranasally would provide both short and long-term protection from melioidosis, based largely on the effective mucosal immunity induced by this delivery route [26-28] and the vaccine formulations investigated. In addition, it was hypothesized that the live, attenuated formulations would produce the longest term protection, as live vaccines have shown some of the most effective protection to date in the literature [29-30].

2.2 References

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CHAPTER 3

A *Burkholderia pseudomallei* ∆*purM* MUTANT IS AVIRULENT IN IMMUNOCOMPETENT AND IMMUNODEFICIENT ANIMALS: CANDIDATE STRAIN FOR EXCLUSION FROM SELECT AGENT LISTS

The research presented in this chapter describes the construction and intensive demonstration of attenuation of two *B. pseudomallei* mutant strains. I acknowledge the contribution of Takehiko Mima and Kyoung-Hee Choi for the construction of all bacterial strains and genetic analyses described in this chapter.

3.1 Abstract

Burkholderia pseudomallei causes the disease melioidosis in humans and is classified as a category B Select Agent. Research utilizing this pathogen is highly regulated in the United States. and even basic studies must be conducted within biosafety level 3 (BSL-3) facilities. There is currently no attenuated *B. pseudomallei* strain available that is excluded from Select Agent regulations and can be safely handled

at BSL-2. To address this need, we created Bp82 and Bp190, Δ*purM* derivatives of *B. pseudomallei* strains 1026b and K96243 deficient in adenine and thiamine biosynthesis, but replication competent *in vitro* in rich medium. A series of animal challenge studies was conducted to assure that these strains were fully attenuated. Whereas the parental strains 1026b and K96243 and the complemented mutants Bp410 and Bp454 were virulent in BALB/c mice following intranasal inoculation, the Δ*purM* mutants Bp82 and Bp190 were avirulent even when administered at doses 4 logs higher than the parental strains. Animals challenged with high doses of the Δ*purM* mutants rapidly cleared the bacteria from tissues (lung, liver and spleen) and remained free of culturable bacteria for the duration of the experiments (up to 60 days post-infection). Moreover, highly susceptible 129/SvEv mice and immune incompetent mice (IFN-$\gamma^{-/-}$, SCID) were resistant to challenges with the Δ*purM* mutant Bp82. This strain was also avirulent in the Syrian hamster challenge model. We conclude that the Δ*purM* mutant Bp82 is fully attenuated and safe for use under BSL-2 laboratory conditions, and thus a candidate strain for exclusion from the Select Agent list.

3.2 Introduction

Humans develop melioidosis following cutaneous or inhalational infection with the Gram-negative bacterium *B. pseudomallei*. Melioidosis in humans is associated with a diverse spectrum of diseases, including acute pneumonia, osteomyelitis, hepatic and splenic abscesses, and neurologic disease [1-2]. Septic shock is the most severe clinical manifestation of *B. pseudomallei* infection and is typically associated with bacterial dissemination to the lungs, liver, and spleen [2]. Treatment of *B. pseudomallei*

infections is complicated because the organism is intrinsically resistant to many antibiotics [3]. Moreover, recurrence of infection is common in patients, even following appropriate antimicrobial treatment [4].

Melioidosis occurs primarily in Southeast Asia and Northern Australia but is increasingly found in other tropical and subtropical regions of the world [5], in visitors returning from endemic regions [6-7], or in tourists afflicted by natural disasters [8-11]. However, *B. pseudomallei* has gained attention in the Western Hemisphere in recent years due to its potential for use as a biological weapon [12]. Because of its biodefense implications, this pathogen is classified as a category B Select Agent by the U.S. Centers for Disease Control and Prevention. Select Agent listing subjects research using *B. pseudomallei* to strict Federal guidelines that govern its acquisition, possession and use [13]. Research with *B. pseudomallei* in the U.S. can only be conducted by cleared personnel in CDC inspected biosafety level 3 (BSL-3) containment laboratories. For some bacteria on the Select Agent list, including *Bacillus anthracis*, *Francisella tularensis* and *Yersinia pestis,* there are already *bona fide* attenuated strains available (www.selectagents.gov/exclusions.htm) that are excluded from Select Agent listings. These attenuated strains can be handled in BSL-2 laboratories which has facilitated studies of these bacteria. However, in the case of *B. pseudomallei* progress has been slow because no approved attenuated strains are available. We believe that availability of such strains would greatly facilitate and accelerate sorely needed basic research with this emerging Select Agent and priority pathogen. Additionally, attenuated strains have the potential to be used as live vaccine strains since in many cases pre-dosing of animals with the attenuated strains has been shown to afford protection against challenges with wild-type bacteria [14-18]. The ultimate goal of the proposed research was therefore to derive an attenuated *B. pseudomallei* strain that would be avirulent in

animal challenge studies, exempt from Select Agent registration, and thus could be widely distributed and used in BSL-2 laboratories.

Diverse *B. pseudomallei* mutants have been identified that exhibited various degrees of attenuation in animal models, including mutants deficient in branched chain amino acid biosynthesis [14], aromatic compound synthesis [17], mutants affecting capsule biosynthesis [19-21], mutants lacking a type IV pilin [22], and mutants lacking components of the type III secretion system [15]. Pilatz et al. [16, 23] conducted a transposon mutant screen aimed at identification of *B. pseudomallei* genes required for the intracellular life cycle and *in vivo* virulence. Amongst the most highly attenuated mutants was a *purM* mutant. This mutant, however, was created using a Select Agent non-compliant method (transposon mutagenesis with a tetracycline resistance marker), was not exhaustively studied in various animal models, and was generated in a strain that is not widely used by and available to the research community.

In the current study, we created Δ*purM* mutant derivatives of the readily available and well-studied *B. pseudomallei* strains 1026b [24] and K96243 [25]. These mutants were constructed by deleting the *purM* gene, which encodes phosphoribosyl formylglycinamide cycloligase. The product of the reaction catalyzed by this enzyme is aminoimidazole ribotide, a precursor for *de novo* adenine and thiamine biosynthesis. Here we report the results of studies conducted to evaluate the *in vivo* virulence of these Δ*purM* mutants of *B. pseudomallei*, with particular reference to virulence in immune deficient and hypersusceptible animal models.

3.3 Materials and Methods

3.3(1) Bacterial strains, media, and growth conditions

B. pseudomallei strain 1026b is a clinical isolate from a case of human septicemic melioidosis from Thailand. This strain is lethal to mice and has been extensively studied in the laboratory [24]. Bp82 is a Δ*purM* derivative of 1026b and adenine and thiamine auxotroph. K96243 is a clinical isolate from a case of fatal human melioidosis from Thailand and its sequence was the first for any *B. pseudomallei* strain to be published [25]. Strains Bp410 and Bp454 are Bp82 and Bp190 in which the Δ*purM* alleles have been replaced with wild-type *purM* sequences from 1026b. These strains are $purM^+$ and adenine and thiamine prototrophs. Strains were grown to saturation in Luria broth (LB) at 37°C with shaking, and then stored at -80°C in 15% glycerol until ready to use. For animal experiments, each strain was thawed just before use and the bacteria were diluted to the desired cell numbers using sterile phosphate buffered saline (PBS). For growth curves, each strain was grown overnight at 37°C in LB broth. The overnight culture was diluted 100-fold with either LB broth or M9 medium (22) with 10 mM glucose and 200 μl aliquots of the diluted cultures were transferred to a sterile 96 well black, clear bottom assay plate (Cat. No. 3603, Corning Incorporated, Corning, NY). Growth was recorded using a Synergy HT Multi-Mode Microplate Reader (BioTek Instruments, Winooski, VT) using the following settings: temperature 37°C; shake at slow speed; read plate every 30 min for up to 48 h at 600 nm. Where indicated, M9-glucose medium was supplemented with either 0.6 mM adenine or 0.0005% thiamine or both.

3.3(2) Attenuated mutant strain construction

A 1,545-bp fragment containing the *purM* gene and flanking DNA was PCR-amplified from strain 1026b genomic DNA using *Taq* DNA polymerase (New England Biolabs, Ipswich, MA) and primers 1487 (5'-CACACGTAGAACGTGCGATC) and 1585 (5'-CTTTCGAGAAGCTTTCGACGG; a newly introduced *Hin*dIII site is underlined) (purchased from Integrated DNA Technologies, Coralville, IA). The fragment was ligated into the TA cloning vector pCR2.1 (Invitrogen, Carlsbad, CA) resulting in pPS2277. Next, a 114-bp *Nru*I fragment was deleted from the *purM* coding sequence present on pPS2277 and replaced with a blunt-ended 776-bp *Sac*I fragment containing a zeocin resistance marker from pFZE1 [26]. This step resulted in pPS2336. A 2,253-bp *Hin*dIII fragment was purified from pPS2336 and used to transform 1026b utilizing a previously described procedure [26-27]. Zeocin resistant transformants were obtained and tested for purine auxotrophy by growing selected transformants on M9-glucose minimal medium plates [28] with and without 0.6 mM adenine. One representative colony, Bp80, growing only in the presence of adenine was retained for further study. A zeocin-susceptible derivative of Bp80 was derived by Flp recombinase-mediated excision of the zeocin resistance marker and curing of the Flp source plasmid pFLPe4 using previously described methods [26]. The presence of the Δ*purM* mutation in the resulting strain Bp82 was confirmed by PCR amplification of the fragment harboring Δ*purM* and the *FRT* scar using primers 1505 (5'-GATCTTCCATACCTGCTCGC) and 1508 (5'-GAATCCTCCGAAATCCGCTC), and sequencing of the resulting 975-bp PCR fragment. The K96243 Δ*purM* derivative Bp190 has previously been described [29]. Repair of the Δ*purM* lesions in Bp82 and Bp190 was achieved by allele replacement with an *Eco*RI fragment containing the 1026b *purM* gene and a previously described pEXKm5-based

sucrose counter-selection method [29]; the resulting adenine and thiamine prototrophs derived from Bp82 and Bp190 were named Bp410 and Bp454, respectively.

3.3(3) Animals

Specific pathogen-free female mice between 4 and 6 weeks of age were purchased from Jackson Laboratories (Bar Harbor, ME). Mice used in these studies were BALB/c, 129/SvEv, IFN-$\gamma^{-/-}$ (on the BALB/c background), and SCID (on the BALB/c background). Syrian hamsters 6 weeks of age were purchased from Charles River Laboratories (Wilmington, MA). Animals were housed in micro-isolator cages under pathogen-free conditions. All animal studies were approved by the Institutional Animal Care and Use Committee at Colorado State University.

3.3(4) Animal infections

All infections with *B. pseudomallei* were done using intranasal inoculation. Animals were anesthetized with 100 mg/kg ketamine (Fort Dodge Animal Health, Overland Park, KS) plus 10 mg/kg xylazine (Ben Venue Labs, Bedford, OH). The desired challenge dose of *B. pseudomallei* was suspended in PBS and 20 μl was delivered intranasally, alternating nostrils. Hamsters were infected in the same manner, but the inoculum was delivered in a total volume of 60 μl. For all survival studies, animals were monitored for disease symptoms twice daily and were euthanized according to pre-determined humane endpoints.

3.3(5) Enumeration of viable *B. pseudomallei* in organs

B. pseudomallei was quantified in lung, liver, and spleen tissues at 48 h post-infection in acute challenge studies and between days 30 and 60 post-infection in long-term survival studies. Lungs, spleens, and livers were removed aseptically and homogenized in sterile phosphate buffered saline using a stomacher (Teledyne Tekmar, Mason, OH). Viable bacterial counts were determined for each organ by plating serial 10-fold dilutions of organ homogenates on LB agar. Plates were incubated at 37°C for 48 h, then the numbers of colonies on the appropriate plate were scored visually and the organ burden of bacteria was expressed as colony forming units (CFU)/organ. Any plates containing organ homogenates that were sterile after 48 h incubation were incubated for an additional 2-3 days to ensure sterility.

3.3(6) Statistical analysis

Statistical analysis was performed using Prism 5.0 software (GraphPad, La Jolla, CA). Survival times were compared using Kaplan-Meier survival curves and the log-rank (Mantel-Cox) test. Organ bacterial burdens between 2 groups of mice were compared using a one sample *t*-test. Data were considered to be statistically significant for *p* values less than 0.05.

3.4 Results

3.4(1) Construction and properties of *B. pseudomallei* Δ*purM* mutants Bp82 and Bp190

A previously described recombinant DNA fragment transformation procedure, coupled to Flp-mediated excision of the zeocin resistance gene employed to initially mark and select the *purM* deletion, was used to generate a markerless chromosomal Δ*purM* mutant, Bp82 [26]. This mutant is missing codons 95-132 of *purM* and is expressing a truncated PurM protein of 99 amino acids whose translation terminates at a stop codon within the *FRT* scar (**Fig. 3.1A**). As a result, Bp82 does not produce a functional PurM enzyme and is an adenine auxotroph (**Fig. 3.1D** & **G**). Bp190 contains an in-frame *purM* deletion without the *FRT* scar which results in a mutant PurM protein that lacks the same 38 amino acids as the protein remnant in Bp82 but terminates with the native stop codon (**Fig. 3.1B**). However, while its growth is significantly attenuated in M9-minimal glucose medium lacking adenine (**Fig. 3.1F** & **H**) the K96243 derivative Bp190, in contrast to Bp82, is not a strict adenine auxotroph. The growth rates of 1026b and Bp82 as well as K96243 and Bp190 in LB medium are indistinguishable (not shown). Repair of the PurM defects in Bp82 and Bp190 resulted in prototrophs (Bp410 and Bp454) whose growth rates in M9-minimal glucose medium were indistinguishable from that of 1026b or K96243 (**Fig. 3.1G** & **H)**.

Because the product of PurM-catalyzed reaction, aminoimidazole ribotide (AIR), is a precursor of thiamine biosynthesis and *B. pseudomallei* possesses the genes for the biosynthesis of thiamine from AIR we assessed whether the Δ*purM* mutants were also thiamine auxotrophs. For both mutant strains, addition of thiamine alone did not affect growth in M9-glucose minimal medium (**Fig. 3.1D** & **F**). While growth of both mutants in

the same medium was significantly improved by adenine alone, only addition of adenine and thiamine restored growth to levels that were indistinguishable to those observed with 1026b and K96243 (compare panels **D** & **F** with **C** & **E**). The data confirm that both Δ*purM* mutants require both adenine and thiamine for normal growth in minimal medium.

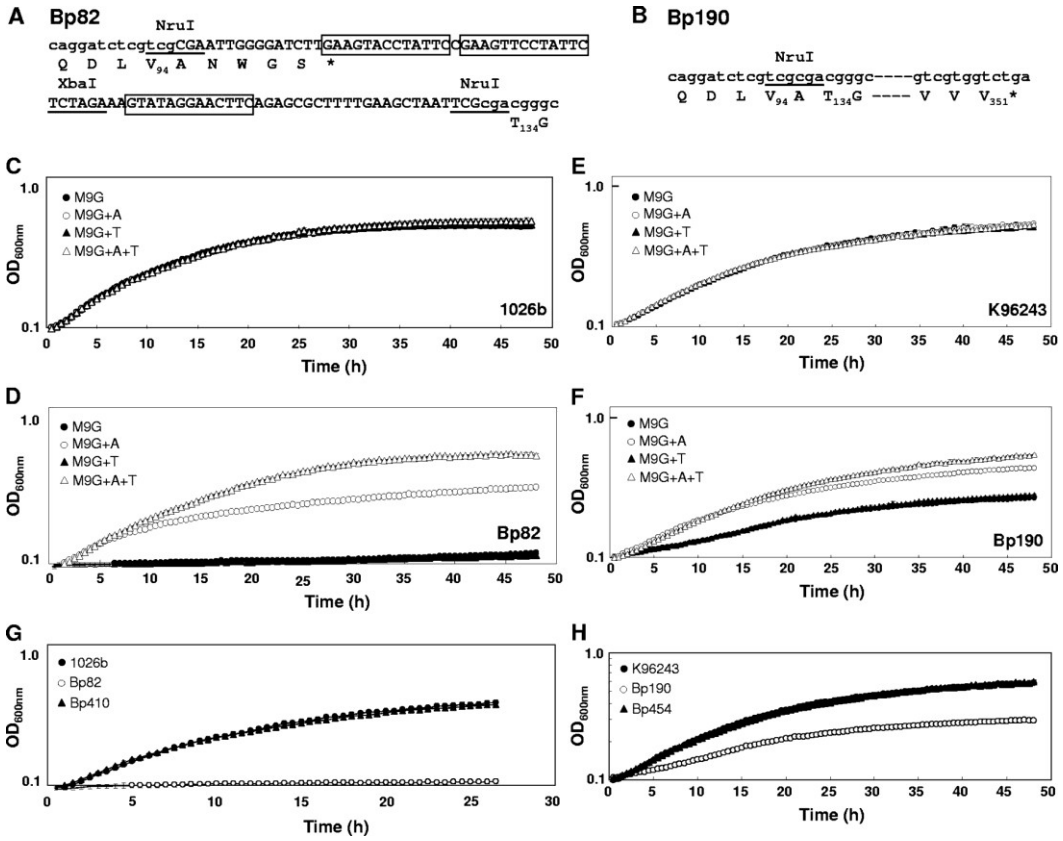

Figure 3.1. *purM* mutant alleles and *in vitro* growth of *B. pseudomallei* Δ*purM* mutants Bp82 and Bp190 and its *purM*$^+$ derivatives Bp410 and Bp454. (A) Sequence of the *FRT* scar region present in Bp82. Lower case letters indicate *purM* sequences. Capital letters indicate the 86-bp *FRT* scar sequence with the resident *Xba*I site and the Flp recombination sequences which are boxed. The residual PurM amino acid sequence is shown below the nucleotide sequence. *Nru*I sites mark the original junction sequences of *purM* and zeocin resistance-encoding cassette from pFZE1 and are the result from fusing a *Nru*I half site with a T4 DNA polymerase blunted *Sac*I site. (**B) Sequence of the deletion junction present in Bp190.** Deletion of an internal NruI fragment from *purM* resulted in deletion of 38 amino acids from PurM. The *purM* open reading frame terminates with the stop codon naturally found after valine 351. **(C)** to **(F) Growth of strains in minimal media.** The following strains were tested: 1026b and its Δ*purM* derivative Bp82; K96243 and its Δ*purM* derivative Bp190. The strains were inoculated into 200 μl of M9-glucose medium (M9G) with 0.6 mM adenine (M9G+A) or 0.0005% thiamine (M9G+T) or both adenine and thiamine (M9G+A+T) and growth at 37^{o}C was monitored by reading the optical density (600 nm) of the cultures at 30 min intervals. The data points are the mean of three independently monitored wells with standard deviations. **(G)** and **(H) Growth of prototype, mutant and complemented strains in minimal medium.** The following strains were tested: 1026b and its Δ*purM* derivative Bp82; K96243 and its Δ*purM* derivative Bp190. Bp410 and Bp454 which are Bp82 and Bp190, respectively, with *purM*$^+$ from 1026b replacing the Δ*purM* allele. The strains were grown in 200 μl of M9-glucose minimal medium without supplementation and growth at 37^{o}C was monitored as described above.

3.4(2) Deletion of *purM* renders *B. pseudomallei* non-virulent in BALB/c mice

B. pseudomallei 1026b is lethal to BALB/c mice following intranasal (i.n.) challenge, with an LD_{50} of approximately 900 CFU [30]. We therefore first determined whether the *purM* deletion would reduce the virulence of strain 1026b in BALB/c mice following i.n. challenge. BALB/c mice (*n* = 5 per group) were challenged with 5 x LD_{50} (approximately 5,000 CFU) of wild-type *B. pseudomallei* 1026b and survival was monitored. All mice reached end-point and were euthanized within 3 days post-infection (**Fig. 3.2A**). Next, BALB/c mice (*n* = 5 per group) were administered high-dose i.n. challenge with 1 x 10^6 and 1 x 10^8 CFU of Δ*purM* strain Bp82. None of the mice challenged with Bp82 died. The animals receiving 1 x 10^6 CFU did not develop signs of infection, while the mice challenged with 1 x 10^8 CFU had ruffling and mild respiratory symptoms lasting for 3-4 days following challenge. All mice were healthy at the time of sacrifice on day 30 post-infection, and the lung, liver, and spleen bacterial burdens for both groups challenged with the mutant strain were all below the limit of detection (LOD = 100 CFU per organ) (data not shown). These data indicated that the *purM* deletion resulted in a significant ($p < 0.01$) reduction in virulence for wild type BALB/c mice. The avirulent phenotype of Bp82 was attributable to deletion of the *purM* locus since repair of this locus with the 1026b *purM* gene by allelic exchange resulted in a strain (Bp410) which regained full virulence (**Fig. 3.2A**).

Although the K96243 derivative Bp190 was not a strict adenine and thiamine auxotroph, it was avirulent in BALB/c mice when inoculated intranasally at very high doses (**Fig. 3.2B**). As with Bp82, the avirulent phenotype of Bp190 was attributable to deletion of the *purM* locus since its repair with the 1026b *purM* gene by allelic exchange resulted in a strain (Bp454) which regained full virulence (**Fig. 3.2B**). The mice

challenged with 1 x 10^6 CFU Bp190 did not have clinical signs after challenge, and appeared healthy during the course of infection. At the time of sacrifice on day 60 post-infection, the lung, liver, and spleen bacterial burdens for both groups challenged with the mutant strain were all below the limit of detection (LOD = 50 CFU per organ) (data not shown). These data indicated that the *purM* deletion from K96243 also resulted in a significant ($p < 0.01$) reduction in virulence for wild type BALB/c mice.

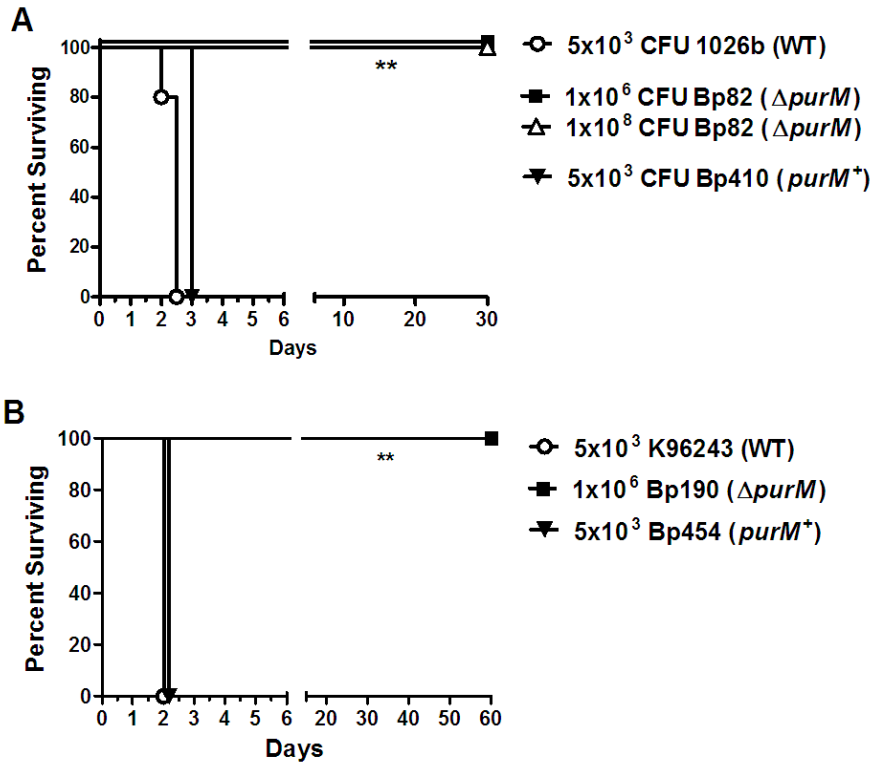

Figure 3.2. *B. pseudomallei* Δ*purM* mutants Bp82 (A) and Bp190 (B) are attenuated in BALB/c mice. (A) Mice (*n* = 5 animals per group) were challenged intranasally (i.n.) with either 5 x 10^3 CFU *B. pseudomallei* 1026b (wild type strain), 1 x 10^6 CFU or 1 x 10^8 CFU Δ*purM* strain Bp82, or 5 x 10^3 CFU Bp410 (Bp82 Δ*purM* lesion repaired with *purM* gene sequences from 1026b). Animal survival was assessed as described in Materials and Methods. The statistical differences in survival times were determined by Kaplan-Meier curves followed by log-rank test (**, $p < 0.01$ for Bp82 vs. 1026b, and Bp82 vs. Bp410). **(B)** Mice (*n* = 5 animals per group) were challenged i.n. with either 5 x 10^3 CFU *B. pseudomallei* K96243 (wild type strain), 1 x 10^6 CFU Δ*purM* strain Bp190, or 5 x 10^3 CFU Bp454 (Bp190 Δ*purM* lesion repaired with *purM* gene sequences from 1026b). (**, $p < 0.01$ for Bp190 vs. K92643, and Bp190 vs. Bp454). WT, wild type.

3.4(3) Genetic deletion of *purM* enables bacterial clearance of *B. pseudomallei* following intranasal challenge in immune competent mice

Experiments were conducted next to assess the degree to which Bp82 was able to replicate in BALB/c mice following intranasal (i.n.) challenge. BALB/c mice (*n* = 3 per group) were infected i.n. with 6 x LD_{50} (approximately 6,000 CFU) of *B. pseudomallei* 1026b or Bp82. At 48 h after infection, the animals were sacrificed and bacterial burdens were quantified in the lungs, liver, and spleen. Wild-type *B. pseudomallei* 1026b underwent significant replication within the lungs of infected mice during the 48 h post-challenge as evidenced by a greater than a 100-fold increase in bacterial burden. Moreover, *B. pseudomallei* 1026b also disseminated to the liver and spleen following i.n. inoculation (**Fig. 3.3A**). In contrast, at 48 h following challenge, Bp82 remained below the limit of detection for the assay within the lungs, liver, and spleen.

To further assess replication and dissemination of Bp82, BALB/c mice were subjected to higher i.n. challenge doses. Thus, one group of mice (*n* = 3 per group) was subjected to i.n. challenge with 1,000 x LD_{50} (1 x 10^6 CFU) and a second group of mice was challenged with 100,000 x LD_{50} (1 x 10^8 CFU). Forty-eight hours after infection, the mice were euthanized and bacterial burdens were quantified in the lungs, liver, and spleen. In a recently published study, we reported that 40% of the i.n. *Burkholderia* inoculum reaches the lungs within an hour of infection [31]. In mice i.n. challenged with 1 x 10^6 CFU Bp82 (assuming 4 x 10^5 CFU reached the lungs), there was a 3.5-log reduction in bacterial burden in the lungs 48 h after challenge, and bacterial counts within the liver and spleen were below the limit of detection for the assay (**Fig. 3.3B**). In mice receiving 1 x 10^8 CFU Bp82 (assuming 4 x 10^7 CFU reached the lungs), there was greater than a 2.5-log reduction in the bacterial burden within the lungs in the 48 h following challenge. The bacterial burden within the spleen was below the limit of

detection. A single colony grew on the liver plating from one mouse, whereas the burdens were below the limit of detection for the other mice (**Fig. 3.3C**). These results indicate neither efficient pulmonary replication nor dissemination to the liver or spleen in wild-type mice following high-dose i.n. challenge with Bp82.

The replication and dissemination of Bp190 (Δ*purM* mutant derived from K92643) was also assessed. BALB/c mice (*n* = 5 per group) were challenged i.n. with 1 x 10^6 CFU, and 48 h after infection, the mice were euthanized and bacterial burdens were quantified in the lung, liver, and spleen (data not shown). Assuming 40% of the inoculum reached the lungs following challenge (4 x 10^5 CFU), there was 2-log reduction in bacterial burden within the lungs at 48 h following challenge (mean $\log_{10}$ CFU/lung = 3.4). The bacterial burden within the spleen was below the limit of detection. Two of the five mice had *B. pseudomallei* detected within the liver at 48 h (mean $\log_{10}$ CFU/liver for all mice = 0.68) (data not shown). Compared to Bp82, strain Bp190 was not as efficiently cleared from the mice within the 48 h time period. This is consistent with *in vitro* growth data that indicated that Bp190 was not fully attenuated in M9 medium lacking adenine supplementation.

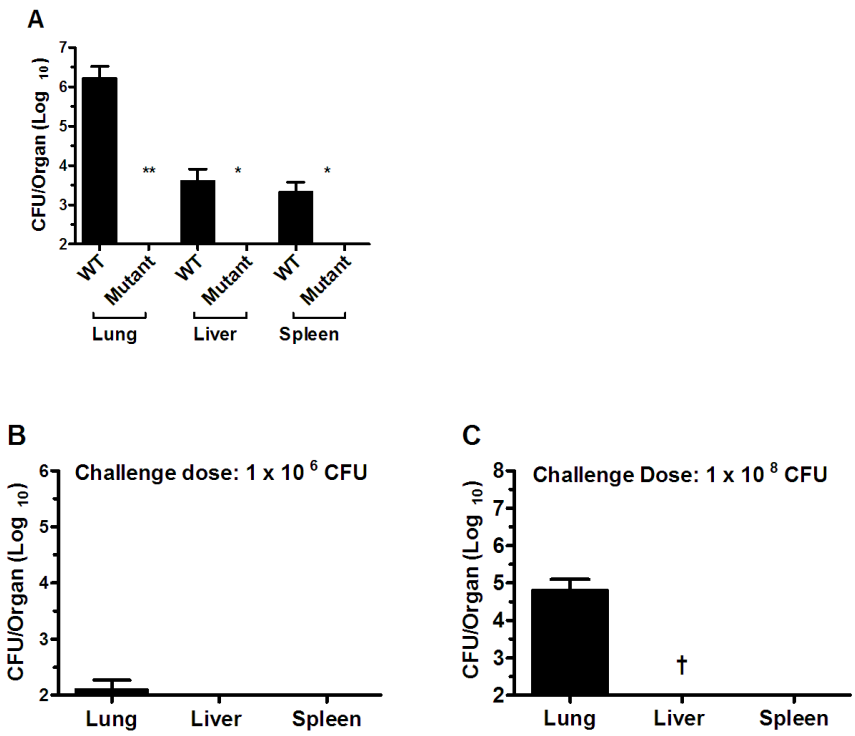

Figure 3.3. Bacterial burdens in the lungs, liver, and spleen 48 h after intranasal challenge with wild-type *B. pseudomallei* 1026b or Δ*purM* mutant Bp82.

(A) BALB/c mice (*n* = 3 per group) were challenged with 6 x 10^3 CFU of *B. pseudomallei* 1026b or Bp82. Bacterial burdens were quantified in each organ 48 h after challenge. Statistical differences were evaluated using a one sample *t*-test (**, $p < 0.01$, *, $p < 0.05$). **(B and C)** BALB/c mice (*n* = 3 per group) were challenged with 1 x 10^6 or 1 x 10^8 CFU Bp82 (challenge doses are indicated on the graphs) and bacterial burdens were quantified 48 h later. († indicates a single colony on the liver plating of one mouse).

3.4(4) *B. pseudomallei* Δ*purM* strains Bp82 and Bp190 are avirulent in hypersusceptible mice

We have previously observed that 129/SvEv mice are extremely susceptible to *Burkholderia* infection (A. Goodyear and S. Dow, unpublished observations). For example, the LD_{100} dose for both *B. pseudomallei* and *B. mallei* following intranasal (i.n.) challenge in 129/SvEv mice was found to be less than 100 CFU (data not shown). Therefore, we assessed whether Bp82 and Bp190 were lethal when inoculated in these hypersusceptible mice. 129/SvEv mice (*n* = 5 per group) were challenged i.n. with 100 CFU of wild-type *B. pseudomallei* 1026b or 1 x 10^6 CFU of Bp82. Mice (*n* = 5 per group) were also challenged with 200 CFU *B. pseudomallei* K92643 or 1 x 10^6 CFU Bp190. The high challenge dose used for Bp82 and Bp190 was more than 10,000 x LD_{100} for wild type *B. pseudomallei* strains 1026b and K92643 in 129/SvEv mice. In animals challenged i.n. with 100 CFU of *B. pseudomallei* strain 1026b, the euthanasia endpoint was reached by day 5 after infection. In contrast, challenge with 1 x 10^6 CFU Bp82 did not result in clinical signs or mortality in any of the animals (**Fig. 3.4**). Mice challenged with 200 CFU strain K92643 reached endpoint on day 2 after infection, whereas challenge with 1 x 10^6 CFU Bp190 did not cause clinical symptoms or mortality in any of the mice (**Fig. 3.4**).

All mice infected with Bp82 appeared healthy at the time of sacrifice on day 30 post-challenge. The bacterial burdens in lung, liver, and spleen for all mice infected with Bp82 remained below the limit of detection for the assay (data not shown). All mice infected with Bp190 were sacrificed on day 45 post-challenge, and the bacterial burdens in the lung, liver, and spleen were below the limit of detection at this time (data not shown). These data indicate that the *purM* deletion in both 1026b and K92643 eliminated virulence of *B. pseudomallei*, even in hypersusceptible mice.

Even though Bp190 was avirulent in both BALB/c and hypersusceptible 129/SvEv mice, this strain was less attenuated *in vitro* and not as dissemination deficient *in vivo* as Bp82. Thus further animal testing was only performed with Bp82.

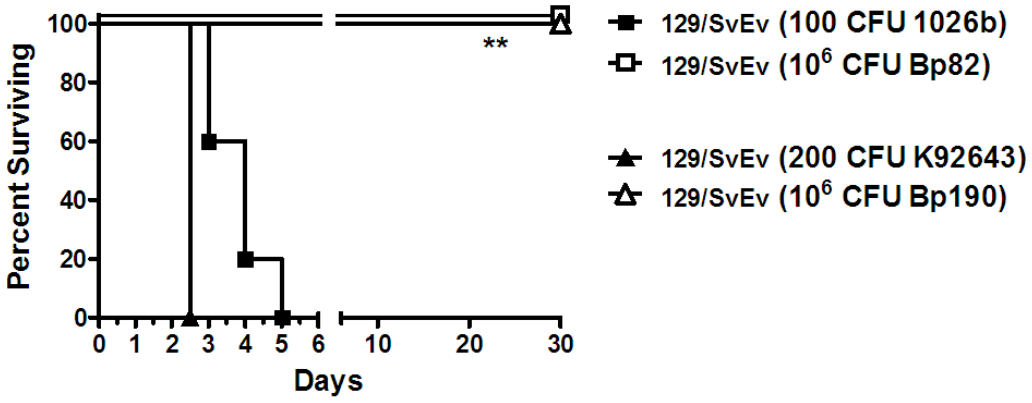

Figure 3.4. *B. pseudomallei* Δ*purM* strains Bp82 and Bp190 are avirulent in 129/SvEv mice. Mice (*n* = 5 animals per group) were challenged intranasally (i.n.) with either 100 CFU *B. pseudomallei* 1026b or 1 x 10^6 CFU Bp82. Mice (*n* = 5 animals per group) were also challenged with either 200 CFU *B. pseudomallei* K92643 or 1 x 10^6 CFU Bp190. Statistical differences in survival times were determined by Kaplan-Meier curves followed by log-rank test (**, $p < 0.01$ for Bp82 vs. 1026b, and Bp190 vs. K92643).

3.4(5) *B. pseudomallei* Δ*purM* strain Bp82 is avirulent in immune deficient mice

Previous studies have clearly demonstrated that interferon-gamma (IFN-γ) is vital for host defense against *B. pseudomallei* [32] and *B. mallei* infection [33]. To further investigate the degree to which Bp82 was attenuated, IFN-$\gamma^{-/-}$ mice (*n* = 3-4 mice per group) were challenged intranasally (i.n.) with a low dose (approximately 500 CFU) of wild-type *B. pseudomallei* 1026b or a high dose (1×10^6 CFU) of Bp82. While we did not experimentally determine the LD_{50} dose for *B. pseudomallei* in IFN-γ knockout mice, we estimate that the LD_{50} was in the range of 10 to 100 CFU. Therefore, the 10^6 CFU challenge dose with Δ*purM B. pseudomallei* Bp82 would represent approximately 100,000 times the estimated LD_{50} dose, and therefore similar to the difference in doses given the BALB/c mice. We observed that all mice challenged with *B. pseudomallei* 1026b developed severe disease and were euthanized on day 2 following challenge (**Fig. 3.5A**). In contrast, none of the IFN-$\gamma^{-/-}$ mice challenged with a high-dose of Bp82 developed clinical signs or succumbed to infection. The lung, liver, and spleen bacterial burdens for IFN-$\gamma^{-/-}$ mice infected with Bp82 were below the limit of detection at day 30 post-infection (data not shown).

The attenuation of *B. pseudomallei* Δ*purM* strain Bp82 was also evaluated in severe combined immune deficiency (SCID) mice. SCID mice are nearly devoid of B and T lymphocytes, thereby largely abolishing adaptive immune responses, while leaving innate immunity intact. SCID mice (*n* = 5 mice per group) were challenged with 1×10^4 CFU of *B. pseudomallei* 1026b or with 1×10^6 CFU of Bp82. The SCID mice challenged with wild type *B. pseudomallei* succumbed to infection within 5 days of challenge, which was a significantly increased time to death compared to the IFN-$\gamma^{-/-}$ mice (p=0.003) and wild type BALB/c mice (p=0.004) following challenge with 1026b. All

of the SCID mice challenged with Bp82 remained healthy throughout infection and survived (**Fig. 3.5B**). Bacterial burdens at day 30 post-infection were below the limit of detection for mice challenged with Bp82 (data not shown).

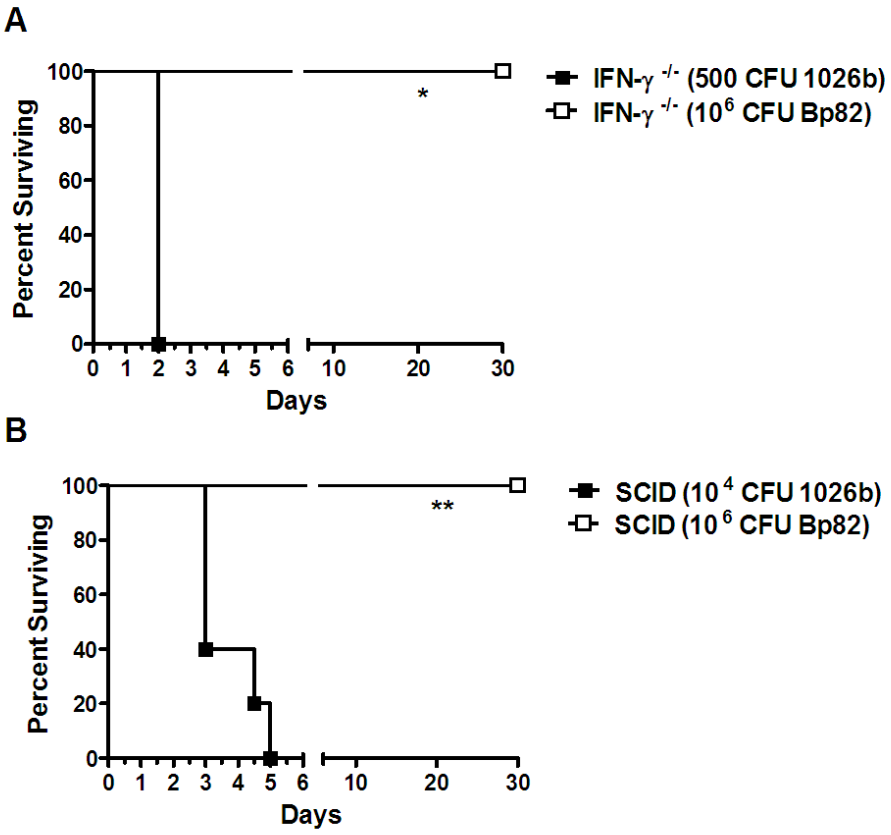

Figure 3.5. *B. pseudomallei* Δ*purM* mutant Bp82 is avirulent in immune deficient mice. (A) IFN-$\gamma^{-/-}$ mice (*n* = 3-4 mice per group) were challenged intranasally with either 500 CFU of *B. pseudomallei* 1026b or 1 x 10^6 CFU of Δ*purM* strain Bp82. **(B)** SCID mice (*n* = 5 mice per group) were challenged i.n. with either 1 x 10^4 CFU of *B. pseudomallei* 1026b or 1 x 10^6 CFU of Bp82. Statistical differences in survival times were determined by Kaplan-Meier curves followed by log-rank test (**, $p < 0.01$, *, $p < 0.05$ for Bp82 vs. 1026b).

3.4(6) *B. pseudomallei* Δ*purM* strain Bp82 is attenuated in Syrian hamsters

Syrian hamsters have previously been shown to be exquisitely susceptible to *B. pseudomallei* infection [34]. Therefore, we subjected Syrian hamsters to intranasal (i.n.) challenge with wild type and mutant *B. pseudomallei*. Syrian hamsters (*n* = 5 animals) were challenged i.n. with a low dose of wild type *B. pseudomallei* 1026b (approximately 400 CFU) or with a high dose (1×10^6 CFU) of Bp82 (**Fig. 3.6**). Animals challenged with *B. pseudomallei* 1026b developed acute illness and all were euthanized by day 4 post-challenge. In contrast, there were no signs of disease in the animals challenged with Bp82. All hamsters infected with Bp82 remained healthy for 30 days post-infection, and bacterial burdens within the lung, liver, and spleen were all below the limit of detection at the time of sacrifice.

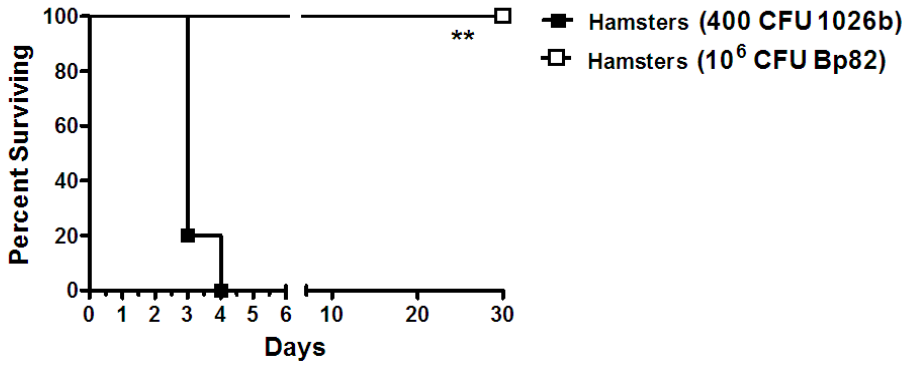

Figure 3.6. *B. pseudomallei* Δ*purM* strain Bp82 is avirulent in Syrian hamsters. Hamsters (*n* = 5 animals per group) were challenged intranasally with 400 CFU of *B. pseudomallei* 1026b or 1 x 10^6 CFU of Δ*purM* mutant Bp82. Statistical differences in survival times were determined by Kaplan-Meier curves followed by log-rank test (**, $p < 0.01$ for Bp82 vs. 1026b).

3.5 Discussion

Despite the medical importance of melioidosis, studying *B. pseudomallei* remains cumbersome, especially in Western countries where the awareness of melioidosis as a potential bioweapon has led to implementation of stringent security and containment requirements. Nowhere is this more evident than in the United States. The strict regulations governing the acquisition, possession and use of *B. pseudomallei* in the U.S. hinder even basic studies on the physiology, genetics, antimicrobial resistance, etc., of this important yet understudied pathogen and make mutant sharing an arduous endeavor because of the permit requirements. Therefore, there is a growing need for safe, attenuated mutants of *B. pseudomallei* that can be utilized under BSL-2 containment conditions.

As mentioned before, several *B. pseudomallei* mutants have been identified that exhibited various degrees of attenuation in cell culture or animal models. These mutants were created mostly for purposes of live vaccine studies. Attenuated mutants fall into roughly two categories, those that do not express virulence factors, e.g. capsule [19-21], mutants lacking a type IV pilin [22], and mutants lacking components of the type III secretion system [15], and those that exhibit metabolic defects, e.g. branched chain amino acid biosynthesis [14], aromatic compound synthesis [17], and purine biosynthesis [16, 23]. Most of these mutants, however, were either not created using Select Agent compliant methods, nor well characterized at the molecular level or thoroughly evaluated in various animal models to meet the criteria required for an organism to be considered for exclusion from the Select Agent list.

In this study we created attenuated mutants, characterized them at the molecular level, and thoroughly evaluated them in various animal models with the goal of defining a

strain that would meet the criteria for exclusion from the Select Agent list. We used Select Agent compliant methods to isolate and characterize defined Δ*purM* mutants. This target was chosen for mainly for two reasons. First, transposon-induced *B. pseudomallei purM* mutants were shown to be severely replication-deficient in cell culture and attenuated in a murine melioidosis model [16, 23]. Second, the purine biosynthetic pathway is a validated target for attenuated mutant construction in bacteria. It has been successfully targeted in attempts to isolate attenuated mutants of *Francisella tularensis* [18], *Brucella abortus* [35], *Shigella flexneri* [36], *Actinobacillus pleuropneumoniae* [37], and *Mycobacterium tuberculosis* [38]. In this study we constructed Bp82 and Bp190, Δ*purM* derivatives of strains 1026b and K96243, respectively. 1026b and K9623 were originally isolated from human melioidosis patients in Thailand. While 1026b is amenable to genetic manipulation and has been widely used for research in North America, K96243 is the sequenced prototype strain.

In vitro growth experiments demonstrated that Bp82 was an obligate auxotroph and required both adenine and thiamine for normal growth in M9-glucose minimal medium. In contrast, the growth of Bp190 was only partially attenuated in the same medium and normal growth again required addition of both adenine and thiamine. At present we do not understand the reason(s) for why Bp190 is not an obligate adenine auxotroph. A possible explanation may be that in contrast to the truncated 99 amino acid PurM protein present in Bp82, the Bp190 PurM contains an internal in-frame 38 amino acid deletion. This 313 amino acid PurM protein may retain some enzymatic activity sufficient to sustain partial growth in minimal media in the absence of adenine and thiamine supplementation. For both mutants we did not observe any suppressors of adenine and thiamine auxotrophy *in vitro*.

Both Δ*purM* mutants constructed in this study contain an internal deletion which is unlikely to be repaired by natural means. The growth rates of 1026b and K96243 and its Δ*purM* derivatives Bp82 and Bp190 in rich medium were indistinguishable indicating that their fitness under these conditions is very similar. These data indicate that Δ*purM* mutants a valid surrogates for many basic biological and applied studies, e.g. deciphering antibiotic resistance mechanisms or drug discovery research.

Using intranasal inoculation, the Δ*purM* mutants were fully avirulent in acute BALB/c infection models even at high (up to 10^6 CFU) challenge doses. For full demonstration of safety and lack of virulence *in vivo*, it is often necessary to conduct challenge studies in strains of animals that are extremely susceptible to bacterial infection and in immune deficient animals. The results of the present study show that the *B. pseudomallei* Δ*purM* mutants Bp82 and Bp190 were fully attenuated in hypersusceptible 129/SvEv mice, and Bp82 was also avirulent in the Syrian hamster model. In addition, the mutant strains failed to efficiently replicate *in vivo* or disseminate following intranasal challenge with high doses. It should also be noted that animals in these studies were infected via the inhalational challenge route, which is the most lethal route of infection and by which healthy laboratory workers would most likely be infected [39]. Moreover, the *B. pseudomallei* Δ*purM* strain Bp82 failed to cause mortality in immune-deficient mice, including IFN-$\gamma^{-/-}$ mice and SCID mice. Thus, by very stringent animal challenge criteria the *B. pseudomallei* the Δ*purM* strains created here are fully attenuated. This attenuation was solely due to the *purM* defect since repair of the Bp82 and Bp190 Δ*purM* allele with wild-type sequences resulted in adenine and thiamine prototrophy and restored virulence.

In summary, the extensive *in vitro* characterization and stringent animal challenge studies show that both *B. pseudomallei* ∆*purM* derivatives constructed and tested in this study are in principal viable candidates for exclusion from Select Agent lists. However, given the overall evidence we consider the 1026b *B. pseudomallei* ∆*purM* mutant Bp82 the superior attenuated strain candidate. It is fully attenuated *in vitro* when grown in adenine and thiamine deficient growth medium. In addition, it is avirulent *in vivo*, even following high-dose challenge in extremely susceptible wild-type and immune-deficient animals. Moreover, the mutant does not replicate *in vivo* and also does not establish chronic infections. Thus, we conclude that the *B. pseudomallei* ∆*purM* mutant Bp82 is to date the most viable candidate strain for exclusion from Select Agent lists and with good laboratory practice safe for use under BSL-2 conditions. Federal regulations permit such exclusions from the list of select biological agents in cases where it has been established that an attenuated strain of a select biological agent does not pose a severe threat to public health and safety, animal health, or animal products. Unlike *B. mallei* where variants exist that are severely attenuated for virulence in their natural host and thus likely candidates for exclusion from the select agent list [40], clinically attenuated *B. pseudomallei* strains that grow normally in laboratory media have yet to be discovered. Until such strains are discovered, genetically engineered and well-characterized strains such as the one described here are the only candidates for exclusion consideration and provide useful tools for an extended research community.

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CHAPTER 4

Burkholderia pseudomallei RND EFFLUX AND THE MALLEOBACTIN/PYOCHELIN SIDEROPHORES ARE DISPENSABLE FOR VIRULENCE

The studies described in this chapter focus on the impact that resistance-nodulation-cell division (RND) efflux systems and two major siderophores have on virulence and lethality of *B. pseudomallei.* I acknowledge the contributions of Lily Trunck, Takehiko Mima, and Brian Kvitko for the construction and genetic characterization of all the bacterial mutant strains described herein.

4.1 Abstract

Burkholderia pseudomallei is the causative agent of the fatal human disease melioidosis, and this pathogen is endemic to various tropical regions of the world. *B. pseudomallei* is currently classified as a category B Select Agent due to its potential use as a biological weapon. Even though this pathogen is associated with high morbidity and mortality, the specific bacterial factors contributing to virulence have not yet been completely elucidated. The purpose of these studies was to investigate the impact that

both siderophores and resistance-nodulation-cell division (RND) efflux systems have on the virulence of *B. pseudomallei in vivo.* This was examined using a *B. pseudomallei* clinical isolate (strain 708a) naturally devoid of both the AmrAB-OprA efflux system and gene cluster for malleobactin synthesis, and by the construction of various isogenetic derivatives. The *B. pseudomallei* efflux systems analyzed within these studies include the two characterized RND systems, AmrAB-OprA and BpeAB-OprB. The genetic deletion of one or both of these efflux pumps did not reduce lethality in our murine model, indicating these systems are dispensable for virulence and *in vivo* replication of *B. pseudomallei.* We also created mutant strains devoid of one or both of the major known *B. pseudomallei* siderophores including malleobactin and pyochelin. These iron transport systems were also found to be completely non-essential for virulence within our pneumonic melioidosis model. Contrary to what has been observed with other related bacterial species, these results indicate that both of the characterized RND systems and major known siderophores are not critical for virulence and lethality of this pathogen. *B. pseudomallei* appears to possess great redundancy of certain bacterial factors, and capable of utilizing iron transport systems other than malleobactin and pyochelin during iron-limiting conditions within the host.

4.2 Introduction

Burkholderia pseudomallei is the causative agent of the disease melioidosis. The genome of this pathogen is over 7Mbp contained within two chromosomes, and is one of the largest bacterial genomes characterized [1-2]. Since research on this pathogen was largely lacking in the Western world until its classification as a category B Select Agent, the precise bacterial factors contributing to virulence of *B. pseudomallei*

are not completely understood [3]. A number of *B. pseudomallei* virulence factors have been identified to date, which include the capsule, lipopolysaccharide, flagella, pili, quorum sensing, a type three secretion system, and morphotype switching [4-15]. However, additional bacterial factors have yet to be characterized. Our laboratory acquired a clinical *B. pseudomallei* isolate obtained from a human melioidosis patient with a large natural deletion of greater than 100kb of genetic material, including both resistance-nodulation-cell division (RND) efflux and iron acquisition genes [16]. Since siderophores and RND efflux systems have been proposed to have potential roles in virulence [17-18], the purpose of this research was to investigate the impact of these systems *in vivo* using a murine melioidosis model. This was examined using both the clinical isolate harboring the natural deletion, and also by the construction and characterization of various isogenetic mutants.

Siderophore production is imperative for iron acquisition by bacteria and has been considered a potential *B. pseudomallei* virulence factor [17]. *B. pseudomallei* contains a large biosynthetic cluster (*mbaA, mbaF, mbaJ, mbaI, fmtA*) encoding the hydroxamate siderophore, malleobactin (MbaA), that is known to be expressed during iron-deficient conditions [15, 19]. Malleobactin is homologous to the pyoverdine siderophore produced by the closely related bacterial species *Pseudomonas aeruginosa* [20], and is capable of releasing iron from transferrin, lactoferrin, and to a lesser extent from erythrocytes [21]. Malleobactin deficient *B. pseudomallei* mutants have been previously shown to be unable to grow under iron-limiting conditions *in vitro* [19]. In addition, homologous iron acquisition genes in both of the related species *P. aeruginosa* and *B. cepacia* are essential for full virulence in many experimental models [22-23]. However, the impact of malleobactin on *B. pseudomallei* virulence is largely unknown to date. Based on homologous sequences to *P. aeruginosa, B. pseudomallei* is also

thought to produce a second siderophore known as pyochelin [19, 24-25]. Pyochelin is encoded by the putative gene *BPSS0587,* and the putative pyochelin receptor protein FptA is encoded by the *fptA* open reading frame [19, 26]. In *P. aeruginosa,* a homologous receptor is used for iron delivery to the bacterial cell when complexed with the pyochelin siderophore [24]. The impact pyochelin has on *B. pseudomallei* virulence is also unknown to date.

Genome sequencing of *B. pseudomallei* strains indicates the presence of at least 10 RND efflux pumps [1, 27]. Two of these systems that have been characterized in *B. pseudomallei* are AmrAB-OprA and BpeAB-OprB [18, 28]. AmrAB-OprA confers resistance to both aminoglycosides and macrolides, and BpeAB-OprB contributes to macrolide and fluoroquinolone resistance [28-29]. It was reported by Chan et al. in 2005 that invasion of human lung epithelial cells and macrophages by *B. pseudomallei* was significantly reduced in the absence of the BpeAB-OprB efflux system [18]. This reduced invasion was proposed to be the result of impaired quorum sensing by the strain lacking BpeAB-OprB, as virulence was restored upon the addition of homoserine lactone molecules [18]. Within this same study, BpeAB-OprB was also required for siderophore and phospholipase C production, and for biofilm formation by *B. pseudomallei*, indicating a potential relation between this efflux pump and virulence of strain KHW [18]. Contrasting data was more recently reported by Mima et al. with the finding that BpeAB-OprB is not required for quorum sensing or siderophore production in *B. pseudomallei* strain 1026b [29]. The true purpose for efflux systems in bacteria is largely unknown [27, 30] and aside from antibiotic resistance, their role in the host during *in vivo* infection has yet to be elucidated, providing a foundation for the studies presented herein.

The impact that RND efflux and two different iron acquisition systems have on virulence was examined in these studies using a clinical isolate obtained from a human melioidosis patient encompassing a large natural deletion [16] and by the construction of isogenetic derivatives. The virulence of these strains was characterized *in vivo* using a murine melioidosis model. The two RND efflux systems evaluated within this research were the characterized *B. pseudomallei* AmrAB-OprA and BpeAB-OprB systems. The genomic region involved with iron transport that was analyzed was *mba,* a 13-gene malleobactin biosynthetic gene cluster and its extracytoplasmic sigma factor MbaS [19]. A second iron transport factor analyzed was FptA*,* encoding the putative pyochelin receptor protein [19]. Strains deficient of *fptA* enabled us to examine the effect of the pyochelin siderophore on virulence of *B. pseudomallei*. In summary, our results indicate that neither the efflux systems nor the iron acquisition genes analyzed within these studies are required for full virulence and lethality of *B. pseudomallei* in a pneumonic murine model of melioidosis.

4.3 Materials and Methods

4.3(1) Bacterial strains, media and growth conditions

All strains used in these studies are listed in **Table 4.1.** The wild type strain from which all efflux pump deletion mutants were derived is *B. pseudomallei* 1026b. The efflux pump mutants created from this strain include Bp50, Bp227, Bp340, and Bp400 (**Table 4.1**). Strain 1026b is a clinical isolate from a case of human septicemic melioidosis in Thailand. This strain is lethal to mice and has been extensively studied in the laboratory [8]. The strain from which all siderophore mutants were derived is *B.*

pseudomallei 1710b. Strain 1710b is also lethal to mice and is a clinical isolate from the blood of a melioidosis patient from Thailand [31]. The iron acquisition mutants derived from this strain include Bp327, Bp338, Bp416 (**Table 4.1**). *B. pseudomallei* 708a is a clinical isolate containing a natural deletion of genomic material of more than 100kb, including both *amrAB-oprA* and iron acquisition genes [16]. Refer to **Table 4.1** for a list of all deletions within this particular strain.

All strains were grown to saturation in Luria broth (LB) at 37°C with shaking, and then stored at -80°C in 15% glycerol until ready to use. For animal experiments, each strain was thawed just before use and the bacteria were diluted to the desired concentration using sterile phosphate buffered saline (PBS, Sigma-Aldrich, St. Louis, MO).

Table 4.1. Bacterial strains utilized in these studies.

B. pseudomallei	Relevant Properties/ Deleted Genes and Putative Gene Functions	Reference
Wild type strains:		
1026b	clinical isolate	[8]
1710b	clinical isolate	[31]
708a	clinical isolate; natural deletion of >100kb: 1. Δ(*amrAB-oprA*) (efflux pump) 2. Δ(BPSL1801-BPSL1800-BPSL1799 (putative fimbrial protein) 3. Δ*mba* (malleobactin siderophore synthesis gene cluster) 4. Δ*cob* (putative vitamin B_{12} biosynthetic pathway) 5. Δ*arcD* and Δ*arcABC* (arginine deiminase pathway) 6. Δ(BPSL1732-1731) (putative chemotaxis citrate transducer/chemotaxis protein)	[16]
Efflux mutants:		
Bp50	1026b with Δ(*amrAB-oprA*) (efflux pump)	[16]
Bp227	1026b with Δ(*bpeAB-oprB*) (efflux pump)	This study
Bp340	1026b with Δ(*amrAB-oprA*) (efflux pump)	This study
Bp400	1026b with Δ(*amrAB-oprA*)(*bpeAB-oprB*) (two characterized efflux pumps)	This study
Iron acquisition mutants:		
Bp327	1710b with Δ*mba* (malleobactin siderophore synthesis)	This study
Bp338	1710b with Δ(*mba*)(*amrAB-oprA*) (malleobactin siderophore synthesis, efflux pump)	This study
Bp416	1710b with Δ(*mba*)(*amrAB-oprA*)(*fptA*) (malleobactin siderophore synthesis, efflux pump, pyochelin receptor)	This study

4.3(2) Mutant construction and deletion determination of strain 708a

All of the deletion mutant constructs listed in **Table 4.1** were provided by the Herbert Schweizer laboratory (Colorado State University, Fort Collins, CO). Mutant strains constructed specifically for these studies were all created using Select Agent compliant methods. Next Gen sequencing, PCR and qRT-PCR were used to determine the deletion region of *B. pseudomallei* strain 708a.

4.3(3) Animals and pulmonary challenge model

Female BALB/c mice were used for these studies (Jackson Laboratories, Bar Harbor, ME). All mice were 6-12 weeks of age at the time of infection and were housed under pathogen free conditions. All animal studies were approved by the Institutional Animal Care and Use Committee at Colorado State University.

All animal infections with *B. pseudomallei* were performed using intranasal (i.n.) inoculation. Animals were anesthetized with 100 mg/kg ketamine (Fort Dodge Animal Health, Overland Park, KS) plus 10 mg/kg xylazine (Ben Venue Labs, Bedford, OH). The desired challenge dose of *B. pseudomallei* was suspended in PBS and 20 μl was delivered i.n. alternating nostrils. The challenge dose was confirmed by retrospective plating on LB agar. For all survival studies, animals were monitored for disease symptoms twice daily and were euthanized according to pre-determined humane endpoints. All procedures were performed in a biosafety level 3 (BSL-3) facility, in accordance with approved BSL-3 and Select Agent protocols.

4.3(4) Statistical analyses

Data were analyzed using Prism 5.0 software (Graph Pad, San Diego, CA). Survival times were analyzed by Kaplan-Meier analysis, followed by the log-rank test. For comparisons of survival times of more than one group in an experiment, the Bonferroni corrected threshold was applied to correct for multiple comparisons.

4.4 Results

4.4(1) *B. pseudomallei* 708a caused clinical disease in a human melioidosis patient despite the presence of a large deletion in chromosome 1

B. pseudomallei strain 708a is a virulent clinical isolate from a 32 year old male melioidosis patient in Thailand [32]. This patient had no risk factors for melioidosis, and presented with 21 days of fever and abdominal pain for 14 days. There was a splenic abscess present, and a splenectomy was required to control infection. Following ceftazidime and doxycycline treatment, the patient survived [16]. This clinical data indicates the ability of strain 708a to result in severe illness in the human melioidosis patient from whom it was isolated. Genotypic analyses later determined that strain 708a contains a large deletion of more than 100kb of genetic material within chromosome 1 [16]. The deletion region and notable genes involved are shown in **Fig. 4.1.** Despite the large deletion, this strain was virulent in this particular patient and capable of causing severe splenic disease**.**

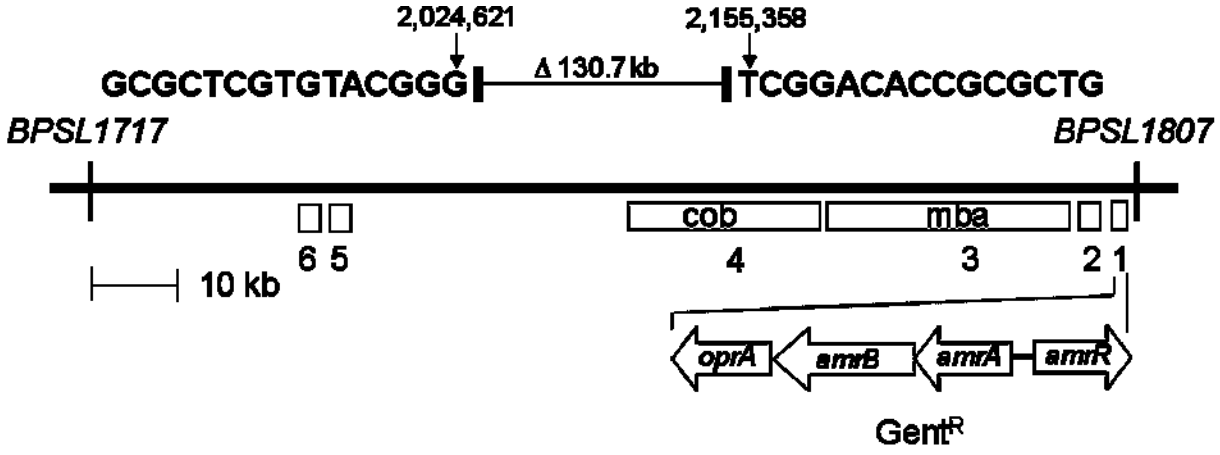

Figure courtesy of Trunck, Propst et al., 2009 [16].

Figure 4.1. Natural deletion in chromosome 1 of the *B. pseudomallei* strain 708a clinical isolate. The large deletion region in strain 708a is shown and the notable deleted genes are listed below:

1. Δ(*amrAB-oprA*) (efflux pump)
2. Δ(BPSL1801-BPSL1800-BPSL1799 (putative fimbrial protein)
3. Δ*mba* (malleobactin siderophore synthesis)
4. Δ*cob* (putative vitamin B_{12} biosynthetic pathway)
5. Δ*arcD* and *arcABC* (arginine deiminase pathway)
6. Δ(BPSL1732-1731) (putative chemotaxis citrate transducer/chemotaxis protein)

4.4(2) *B. pseudomallei* strain 708a is fully virulent in a murine inhalational challenge model

Our laboratory has previously shown that *B. pseudomallei* 1026b is lethal to BALB/c mice following intranasal (i.n.) challenge, with an LD_{50} of approximately 900 CFU [33]. For these studies, BALB/c mice (*n* = 5 per group) were challenged with 5 x LD_{50} (approximately 5,000 CFU) of wild type *B. pseudomallei* 1026b and survival was monitored. All mice reached end-point and were euthanized within 3 days post-infection (**Fig. 4.2**). We then wanted to determine whether the *B. pseudomallei* strain 708a containing the large genomic deletion had similar lethality in our pneumonic murine model. BALB/c mice (*n* = 4 per group) were challenged with 5 x 10^3 CFU strain 708a and survival was monitored. All mice reached end-point within 3 days post-infection, similar to that seen with *B. pseudomallei* 1026b (**Fig. 4.2**), indicating that strain 708a is fully virulent in our murine melioidosis model despite its large natural genomic deletion.

Mice (*n* = 5 per group) were also challenged with 5 x 10^3 CFU Bp50, an isogenetic Δ(*amrAB-oprA*) 1026b derivative of strain 708a. Unlike that seen with *B. pseudomallei* 708a, this challenge with Bp50 was not lethal to any of the mice, and all survived acute infection (**Fig. 4.2**). We found that a 10-fold higher intranasal challenge dose was required for the strain to be lethal in 100% of the mice and produce a similar time to death as that seen with *B. pseudomallei* strains 708a and 1026b. Mice (*n* = 5 per group) challenged with 5 x 10^4 CFU Bp50 reached acute disease end-point by day 4 post-infection (**Fig. 4.2**).

Our finding that the *amrAB-oprA* deletion did not compromise virulence of *B. pseudomallei* 708a, but did appear to reduce the virulence of the 1026b isogenetic derivative Bp50 was an unexpected result. For this reason, a second Δ*amrAB-oprA*

construct was created, which is strain Bp340. *In vivo* testing with this construct and additional efflux deletion mutants are presented in the next section.

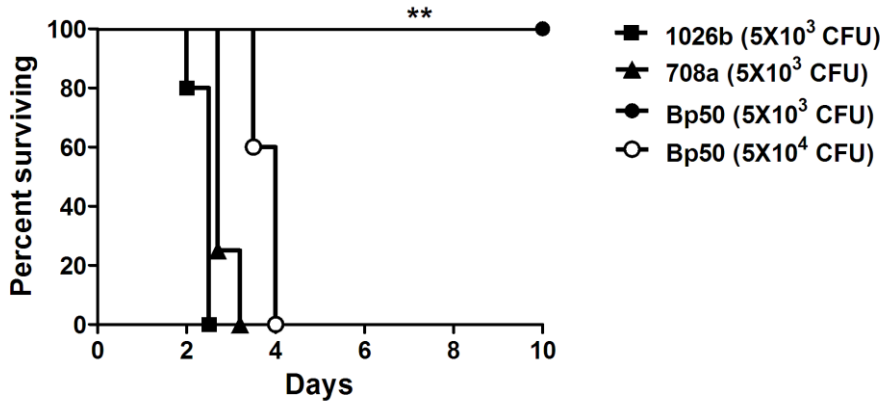

Figure 4.2. *B. pseudomallei* strain 708a is fully virulent in an acute murine melioidosis infection model. BALB/c mice (*n* = 4-5 mice per group) were infected intranasally with 5 x 10^3 CFU of 1026b, 5 x 10^3 CFU of strain 708a, and 5 x 10^3 or 5 x 10^4 CFU of isogenetic Δ(*amrAB-oprA*) strain 1026b derivative, Bp50. Statistical differences in survival times were determined by Kaplan-Meier curves followed by log-rank test. The Bonferroni corrected threshold was applied and comparisons with $p < 0.017$ were considered significant. **, $p < 0.01$ for strain 1026b vs. Bp50 (5 x 10^3 CFU challenge dose) and 708a vs. Bp 50 (5 x 10^3 CFU challenge dose). Data shown above are representative of 2 independent experiments.

4.4(3) The efflux systems AmrAB-OprA and BpeAB-OprB do not contribute to *B. pseudomallei* virulence *in vivo*

Despite the natural deletion of *amrAB-oprA* in *B. pseudomallei* strain 708a, this clinical isolate was fully virulent in our pneumonic melioidosis model (**Fig. 4.2**). Bp340, a second isogenetic Δ(*amrAB-oprA*) 1026b derivative was created and tested for lethality following inhalational challenge. When BALB/c mice were challenged with 1 x 10^3 CFU Bp340, all succumbed to end-point by day 2 post-infection (**Fig. 4.3**). Since the LD_{50} for wild type 1026b is approximately 900 CFU [33], Bp340 appears to be at least as virulent, and perhaps even more lethal than the parental 1026b strain with an intact *amrAB-oprA* operon. This finding indicates that AmrAB-OprA is dispensable for *B. pseudomallei* lethality in our murine model.

The impact of another characterized efflux system, BpeAB-OprB, was also tested in our studies. Even though this efflux pump is intact within strain 708a, we wanted to determine its impact on virulence *in vivo* since it has been proposed to impact both the invasive potential and cellular toxicity of *B. pseudomallei* [18]. When BALB/c mice were challenged with 2 x 10^3 CFU Bp227, a Δ(*bpeAB-oprB*) 1026b derivative, all mice reached end-point by day 2 post-challenge (**Fig. 4.3**), indicating this efflux system is also dispensable for strain 1026b virulence. A construct lacking both characterized efflux systems (Bp400) was also tested in our murine model. 1 x 10^4 CFU Bp400, a Δ(*amrAB-oprA*)(*bpeAB-oprB*) 1026b derivative, was lethal to 100% of mice challenged (**Fig. 4.3**). Combined, these results indicate that neither AmrAB-OprA nor BpeAB-OprB is required for full lethality of *B. pseudomallei* strains 708a and 1026b.

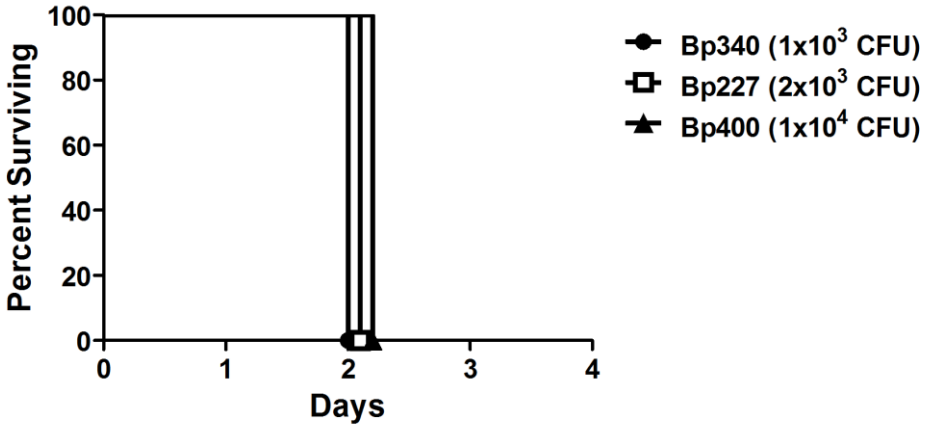

Figure 4.3. Neither AmrAB-OprA nor BpeAB-OprB is required for full virulence of *B. pseudomallei* 1026b in a pneumonic murine melioidosis model. BALB/c mice (*n* = 5 per group) were infected intranasally with $1x10^3$ CFU Δ(*amrAB-oprA*) Bp340, $2x10^3$ CFU Δ(*bpeAB-oprB*) Bp227, or $1x10^4$ CFU Δ(*amrAB-oprA*)(*bpeAB-oprB*) Bp400 and survival was monitored. All strains were lethal to 100% of the mice challenged.

4.4(4) Malleobactin and pyochelin siderophores do not serve as virulence determinants in *B. pseudomallei*

B. pseudomallei 708a was virulent in our animal studies despite the deletion of the 13-gene malleobactin biosynthetic gene cluster, *mba* (**Fig. 4.2**). We further characterized the impact of this siderophore by the construction of isogenetic deletion mutants lacking *mba.* These mutant strains were derived from the clinical isolate *B. pseudomallei* 1710b, as we believe this strain background is most similar to the clinical isolate 708a. Wild type *B. pseudomallei* 1710b was less lethal in our murine model than strain 1026b, as when BALB/c mice (*n* = 9 mice) were challenged intranasally with 2 x 10^3 CFU strain 1710b, there were acute disease survivors (**Fig. 4.4**). (Survivors would not be expected following a similar challenge dose with strain 1026b).

In order to determine the impact that iron acquisition genes had within this strain background, Bp327, Bp338, and Bp416 were all tested for virulence in our murine melioidosis model (*n* = 9-10 mice per group). Bp327 is a Δ(*mba*) 1710b derivative containing a 31 kb malleobactin biosynthetic gene cluster deletion of the same extent as *B. pseudomallei* 708a. BALB/c mice were challenged intranasally with 2 x 10^3 CFU Bp327 and all reached acute disease end-point by day 3 (**Fig. 4.4**). Since the natural deletion mutant, *B. pseudomallei* 708a, is also lacking the AmrAB-OprA efflux pump (in addition to the malleobactin biosynthetic gene cluster and other genes), the isogenetic Δ(*mba*)(*amrAB-oprA*) 1710 derivative Bp338 was also tested for lethality. Of the three 1710b derived mutants, Bp338 is most homologous in terms of genetic deletions to strain 708a. This strain was also lethal to 100% of the mice infected, as all animals reached acute disease end-point by day 3.5 post-challenge (**Fig. 4.4**).

The final deletion mutant tested *in vivo* was Bp416. This is a Δ(*amrAB-oprA*)(*mba*)(*fptA*) 1710b derivative that has a deletion of the putative pyochelin receptor protein, FptA, in addition to the efflux system and malleobactin biosynthetic gene cluster. Even though *fptA* is intact in *B. pseudomallei* strain 708a, Bp416 provided insight as to whether pyochelin, a second known *B. pseudomallei* siderophore, is imperative for iron acquisition within the host. All BALB/c mice challenged 1 x 10^3 CFU Bp416 reached end-point by day 2.5 post-infection. This strain is incapable of utilizing the two major siderophores (malleobactin and pyochelin) and is also lacking AmrAB-OprA, and still produced the shortest time to death among all four strains tested (**Fig. 4.4**). Also notable is that this particular group of animals received the lowest challenge dose of all four isolates. This challenge dose (used for Bp416) is near the LD_{50} intranasal challenge dose for wild type strain 1026b [34] and was 50% reduced compared to the sub-lethal challenge dose used for wild type 1710b, and still produced the shortest time death.

Combined, these results indicate that neither the malleobactin nor pyochelin siderophore is required for virulence following pneumonic challenge in a murine melioidosis model. If anything, deletion of these genes actually appeared to increase the virulence of the three deletion mutants, as they were all lethal to 100% of mice challenged, whereas wild type parental strain 1710b was not (**Fig. 4.4**). All three mutants had increased lethality and decreased time to death compared to wild type *B. pseudomallei* 1710b ($p < 0.01$ for all three mutant strains vs. 1710b).

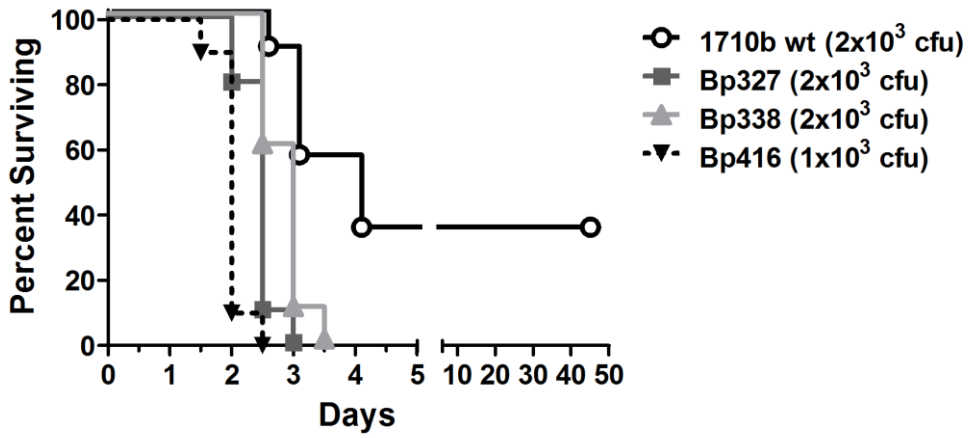

Figure 4.4. Deletion of *amrAB-oprA, mba,* and *fptA* does not reduce lethality of *B. pseudomallei* following inhalational challenge. BALB/c mice (*n* = 9-10 per group) were infected intranasally with 2 x 10^3 CFU wild type *B. pseudomallei* 1710b, Δ(*mba*) 1710b derivative Bp327, Δ(*mba*)(*amrAB-oprA*) 1710b derivative Bp338, or Δ(*mba*)(*amrAB-oprA*)(*fptA*) 1710b derivative Bp416. Statistical differences in survival times were determined by Kaplan-Meier curves followed by log-rank test. The Bonferroni corrected threshold was applied and comparisons with $p < 0.017$ were considered significant. ($p < 0.001$ for Bp327 vs. 1710b; $p < 0.01$ for Bp338 vs. 1710b; $p < 0.0001$ for Bp416 vs. 1710b).

4.5 Discussion

The bacterial factors that enable *B. pseudomallei* to cause disease have yet to be fully elucidated, and there is a gap in knowledge regarding its virulence determinants compared to other well-studied Gram-negative pathogens [17]. These studies were conducted to assess whether two characterized resistance-nodulation-cell division (RND) efflux pumps and known *B. pseudomallei* siderophores are imperative for *in vivo* infection within a pneumonic murine melioidosis model. We have demonstrated in these studies that both of the efflux systems analyzed, including AmrAB-OprA and BpeAB-OprB, are dispensable for *B. pseudomallei* lethality *in vivo.* In addition, neither malleobactin nor pyochelin is required for *B. pseudomallei* virulence during pneumonic infection, indicating the likely utilization of additional uncharacterized iron transport systems. These findings were based on the study of a clinical isolate naturally devoid of >100kb of genetic material (including both iron acquisition and efflux genes), and by the construction of a variety of isogenetic mutants.

The finding that neither AmrAB-OprA nor BpeAB-OprB is required for *B. pseudomallei* virulence in our murine melioidosis model is contrasting to previous research. It was reported by Chan et al. that *B. pseudomallei* invasion of both human lung epithelial cells and macrophages *in vitro* was significantly reduced in the absence of BpeAB-OprB [18]. However, when mice were challenged in our studies with the strains devoid of BpeAB-OprB and/or AmrAB-OprA, all were fully virulent. These isolates caused marked respiratory distress and were lethal to 100% of mice challenged, similar to that observed with wild type *B. pseudomallei* containing intact efflux systems. In fact, in the survival studies with the single efflux pump deletion mutants Bp340 and Bp227, the time to death (by day 2.0) appeared to be even slightly reduced compared to the

predicted time of death among mice infected with a similar challenge dose with the parental 1026b strain. The LD_{50} for strain 1026b is 900 CFU [34], and mice challenged with a 1-2 x 10^3 CFU (a relatively low lethal intranasal dose) would be expected to survive until approximately day 3 (data not shown). Our clinical observations of mice infected with the different efflux pump deficient strains (Bp340, Bp227, and Bp400) are likely indicative of no impaired invasion of murine pulmonary epithelial cells or reduction of virulence in the absence of BpeAB-OprB or AmrAB-OprA, contrasting to that reported by Chan et al. with strain KHW. These findings coincide with the previous reports of BpeAB-OprB being dispensable for virulence in *B. pseudomallei* strain 1026b [29]. Potential differences between strains 1026b and KHW merit further characterization *in vivo.* Challenge studies with efflux deficient mutants constructed from the KHW background are needed to determine differences in virulence determinants among different *B. pseudomallei* strains. In addition, whether the remaining uncharacterized RND efflux systems impact virulence of *B. pseudomallei* is also unknown at this time. Upon their future genetic characterization, this will need to be investigated *in vivo* using melioidosis models.

Siderophore production has been considered a likely *B. pseudomallei* virulence factor [17] and was also investigated within these studies. In the related species *Burkholderia cepacia,* the siderophore ornibactin is required for full virulence in a pulmonary rat agar bead infection model [22]. A mutant *B. cepacia* strain lacking ornibactin biosynthesis had a 4-log reduction within the lungs on days 7 and 28 post-infection, and produced reduced pulmonary pathology compared to a wild type strain [22]. In addition, the pyoverdine siderophore is required for infection and virulence of *Pseudomonas aeruginosa*, and is believed to contribute to iron acquisition by this pathogen *in vivo* [23]. Pyoverdine biosynthesis was shown to be upregulated when *P.*

aeruginosa is grown in sputum *in vitro* [35], and both pyoverdine and pyochelin were shown to contribute to *P. aeruginosa* persistence in the blood of a mouse infection model [36]. However, in the studies described herein, the deletion of either the malleobactin biosynthesis gene cluster or pyochelin receptor, subsequently preventing the usage of these major siderophores by this pathogen, failed to reduce the virulence of *B. pseudomallei*.

The clinical isolate 708a containing a natural deletion of the 13-gene malleobactin biosynthesis cluster (*mba*) was fully virulent in our murine model. This clinical isolate was known to cause severe splenic disease (resulting in a splenectomy) from the patient from whom it was isolated [16]. In addition, intranasal challenge with the isogenetic Bp416 mutant containing deletions of both *mba* and the pyochelin receptor *fptA,* which abolished both malleobactin and pyochelin usage by this strain, produced the shortest time to death of all the isogenetic mutants, even following a relatively low challenge dose. Mice infected with Bp416 received a challenge inoculum that was 50% reduced compared to that used for the wild type 1710b background strain, and yet still had the shortest time to death. In contrast to previous findings with the related bacterial species *B. cepacia* and *P. pseudomonas* [22-23], both malleobactin and pyochelin appear to be dispensable for *B. pseudomallei* replication *in vivo* and virulence. These results indicate that unlike what is observed with the related bacterial pathogens*, B. pseudomallei* is likely capable of using additional iron transport systems for iron acquisition *in* vivo, perhaps even alternating between pathways dependent on availability. One possibility is a heme-hemin receptor and transporter proteins, encoded by the genes *BPSS0244 and BPSS0243* [26]. As observed with other *B. pseudomallei* siderophores, these genes have been shown to be induced under iron-limiting conditions

and are hypothesized to play a potential role in iron acquisition [19, 26]. Additional studies are needed upon further characterization of these genes.

These studies have provided additional insight into virulence determinants of *B. pseudomallei.* The contrasting data observed between *B. pseudomallei* strains KHW and 1026b in regards to the efflux system BpeAB-OprB is indicative of potential genetic diversity among different *B. pseudomallei* isolates. This observation illustrates that potential virulence factors merit characterization using a variety of *B. pseudomallei* strain backgrounds. The finding that both pyochelin and malleobactin are dispensable for *B. pseudomallei* virulence in our murine melioidosis model further illustrates the plasticity and likely redundancy of certain bacterial factors like iron transport.

4.6 References

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CHAPTER 5

IMMUNOTHERAPY MARKEDLY INCREASES THE EFFECTIVENESS OF ANTIMICROBIAL THERAPY FOR TREATMENT OF *Burkholderia pseudomallei* INFECTION

The studies presented in this chapter focus on a significant enhancement to traditional antibiotic therapy by the co-administration of immunotherapies for treatment of *B. pseudomallei* infection. I acknowledge the tremendous contribution of Ryan Troyer for conducting the *in vitro* experiments described in this chapter.

5.1 Abstract

Burkholderia pseudomallei is a soil bacterium endemic to southeast Asia and northern Australia that can cause both acutely lethal pneumonia as well as chronic systemic infections in humans. Effective treatment of infection with *B. pseudomallei* requires rapid diagnosis and prolonged treatment with high doses of antimicrobials, and even with appropriate antibiotic therapy, patient relapse is common. Thus, new approaches to treat *B. pseudomallei* infection are needed. In the present study, we asked whether active immunotherapy with IFN-γ, a key cytokine regulating intracellular

replication of *B. pseudomallei*, could increase the effectiveness of conventional antimicrobial therapy for *B. pseudomallei* infection. Macrophage infection assays and *in vivo* pulmonary challenge models were used to assess the inhibitory effects of combined treatment with IFN-γ and ceftazidime on *B. pseudomallei* infection. We found that treatment with even very low doses of IFN-γ and ceftazidime elicited strong synergistic inhibition of *B. pseudomallei* growth within infected macrophages. *In vivo*, active immunotherapy markedly potentiated the effectiveness of low-dose ceftazidime therapy for treatment of infected mice in a pulmonary challenge model of *B. pseudomallei*. Combined treatment was associated with a significant reduction in bacterial burden and significant lessening of bacterial dissemination. We concluded therefore that immunotherapy with either endogenous or exogenous IFN-γ could significantly increase the effectiveness of conventional antimicrobial therapy for treatment of acute *B. pseudomallei* infection.

5.2 Introduction

Burkholderia pseudomallei is a soil bacterium endemic in southeast Asia and northern Australia [1], and other tropical and subtropical regions of the world [2]. This pathogen causes several thousand human cases of melioidosis every year [3-6]. Without prompt antimicrobial therapy, infection with *B. pseudomallei* is often fatal, as illustrated by high mortality rates for untreated cases [5]. Even after initial appropriate therapy, many patients are still susceptible to relapse or re-infection with *B. pseudomallei* [3, 7-8]. Because *B. pseudomallei* infection is difficult to eradicate, prolonged antimicrobial therapy (months) is often prescribed for infected persons [9]. *B. pseudomallei* also displays high levels of intrinsic resistance to many commonly used

antibiotics. Though *B. pseudomallei* is not endemic in North America, there is still considerable concern for this organism by the Centers for Disease Control and Prevention because of its high potential for use as a biological weapon. *B. pseudomallei* is currently classified as a category B Select Agent. There is also currently no vaccine available for preventing infection with *Burkholderia*.

For these reasons, new approaches to improve the effectiveness of antimicrobial therapy for *B. pseudomallei* infection are urgently needed. Previous studies have shown that non-specific activation of innate immunity by systemic (intraperitoneal) administration of CpG oligonucleotides prior to infection could provide protection against systemic challenge with *B. pseudomallei* [10]. We recently reported that mucosally delivered cationic liposome-DNA complex (CLDC) immunotherapy was particularly effective in protecting mice from inhalational challenge with both *B. mallei* and *B. pseudomallei* [11]. In that study, IFN-γ was identified as the key cytokine mediating the protection afforded by CLDC immunotherapy.

In the studies described herein we investigated whether active immunotherapy could be combined with conventional antimicrobial therapy to increase the effectiveness of *B. pseudomallei* treatment. Such an approach was evaluated previously, using the cytokine granulocyte-colony stimulating factor (G-CSF) in combination with ceftazidime, based on previous studies showing that neutrophils were key effector cells for controlling *B. pseudomallei* infection [12-14]. However, subsequent studies in mouse infection models revealed that G-CSF was not effective when combined with ceftazidime for treatment of *B. pseudomallei* infection [15].

In several other infection models, IFN-γ has been combined with antimicrobial agents to increase the effectiveness of treatment. For example, the combination of IFN-

γ with the antibiotics gentamicin and vancomycin enhanced the clearance of *Enterococcus faecalis* in an *in vitro* neutrophil infection model [16]. However, in a mouse model of *E. faecalis* infection, only low doses IFN-γ increased the effectiveness of antimicrobial therapy, while high IFN-γ doses were ineffective or deleterious [17]. In a *Francisella novicida* infection model, intranasal administration of recombinant IL-12 (rIL-12) (which induced IFN-γ production) increased the effectiveness of antimicrobial therapy [18]. Similarly, treatment of *Mycobacterium avium* infected mice with rIL-12 was found to increase the effectiveness of antimicrobial therapy in clearing bacterial infection [19].

Thus, there was reason to believe that immunotherapy, particularly with an immunotherapeutic capable of stimulating potent release of IFN-γ might be effective when combined with antimicrobial therapy for treatment of *B. pseudomallei* infection. Currently, first-line antimicrobials used for treatment of acute *B. pseudomallei* infection include ceftazidime, amoxicillin-clavulanic acid, carbapenems (meropenem or imipenem), and trimethoprim-sulfamethoxazole [5, 9]. Of these, ceftazidime is the current recommendation for intensive phase therapy, and has been evaluated the most extensively in mouse infection models and was therefore selected for the studies reported here [9, 15, 20-21]. For stimulation of IFN-γ release *in vivo*, cationic liposome-DNA complexes (CLDC) were used, as our prior studies have shown that CLDC are potent inducers of IFN-γ release [22-23].

Therefore, we designed experiments to investigate the interactions between IFN-γ immunotherapy and ceftazidime for control of intracellular infection with *B. pseudomallei*. Our results suggest that IFN-γ may be uniquely effective as an immunotherapeutic for increasing the susceptibility of intracellular *Burkholderia* to killing

by certain classes of antimicrobials. Thus, there is reason to believe that immuno-antimicrobial therapy is a promising new approach to improving the effectiveness of current antimicrobial drugs for treatment of *B. pseudomallei* infection.

5.3 Materials and Methods

5.3(1) Bacteria

B. pseudomallei strain 1026b was used for these studies [24]. This strain was inoculated in Luria broth (LB) and grown at 37°C with shaking for 16 h, and then stored at -80°C in 15% glycerol. Each vial was thawed just before use and the bacteria were diluted to the desired concentration using sterile phosphate buffered saline (PBS, Sigma-Aldrich, St. Louis, MO). The LD_{50} dose of *B. pseudomallei* in BALB/c mice by the intranasal route was determined by the Reed-Muench method to be 900 CFU [11]. The challenge dose used for the *in vivo* studies was 8 x LD_{50} (approximately 7,500 CFU).

5.3(2) Mice

Female BALB/c mice were used for these studies (Jackson Laboratories, Bar Harbor, ME). All mice were 6-12 weeks of age at the time of infection and were housed under pathogen free conditions. All animal studies were approved by the Institutional Animal Care and Use Committee at Colorado State University.

5.3(3) Preparation and administration of cationic liposome-DNA complexes (CLDC)

CLDC were prepared as previously described [11, 22]. Briefly, sterile complexes of cationic liposomes were prepared using equimolar amounts of DOTIM (octadecanoyloxy {ethyl-2-heptadecenyl-3-hydroxyethyl} imidazolinium chloride) and cholesterol. Non-coding ultra-low endotoxin plasmid DNA was then added to liposomes at a final concentration of 0.1 mg/ml to form CLDC. Preformed CLDC were diluted in Tris-buffered 5% dextrose water (pH 7.4) and administered to mice intraperitoneally (i.p.) in a total volume of 200 µl. CLDC treatment was administered once i.p., 6 h after infection.

5.3(4) Recombinant IFN-γ

Recombinant murine interferon-γ (IFN-γ) was purchased from PeproTech (Rocky Hill, NJ) and reconstituted in PBS with 0.1% bovine serum albumin. Aliquots were frozen at -80°C until use. For *in vitro* studies, rIFN-γ was added at the indicated concentrations after bacterial infection and elimination of extracellular bacteria with kanamycin. For *in vivo* treatment, rIFN-γ was diluted in PBS with 0.1% bovine serum albumin and administered i.p. at 6 h and 18 h after infection in a total volume of 200 µl.

5.3(5) Ceftazidime

Ceftazidime was purchased from Sigma-Aldrich (St. Louis, MO) and diluted in PBS with 0.1% bovine serum albumin. Aliquots of the desired concentration were frozen at -20°C until use. The concentration of ceftazidime used for *in vivo* mouse treatments

was 25 mg/kg body weight, administered 6 h after infection and continued every 12 hours for a total of six treatments (administered at 6, 18, 30, 42, 54 and 66 h after infection).

5.3(6) *In vitro* macrophage infection assay to assess *in vitro* interaction between cytokines and ceftazidime

The mouse alveolar macrophage cell line AMJ.2 (American Type Tissue Collection, Manassas, VA) was used to investigate the ability of CLDC elicited cytokines to enhance the activity of antimicrobial drugs. AMJ.2 cells were cultured in complete medium, which consisted of MEM medium (Invitrogen, Carlsbad, CA) containing 10% FBS (Gemini Bio-Products, West Sacramento, CA), 2 mM L-glutamine (Invitrogen), 1x non-essential amino acids (Invitrogen), and 0.075% sodium bicarbonate (EMD Science, Gibbstown, NJ), without antibiotics added. *B. pseudomallei* was added to 2 x 10^5 AMJ cells per well of a 24-well plate at an MOI of 5 CFU per cell in 500 µl antibiotic-free medium. Plates were centrifuged at 2400xg for 2 min and then incubated for 1 h in at 37°C and 5% CO_2. Extracellular bacteria were then removed by washing the macrophages three times with 2 ml PBS, followed by treatment with medium plus 350 µg/ml kanamycin (Sigma-Aldrich) for 1 h. After incubation with kanamycin, the macrophages were washed three times with PBS.

Ceftazidime, CLDC supernatants or rIFN-γ, alone or in combination, were then added to the cultures and the macrophages were cultured for an additional 24 h. The treatment medium was then removed and cells were washed three times with 2 ml sterile PBS, then lysed with 1 ml sterile dH_2O in order to quantify intracellular bacteria. Serial dilutions of lysates were plated on LB agar plates and plates were incubated at

37°C for 48 h prior to counting colonies. In several experiments, bacteria present in the last PBS wash were plated and counted, then subtracted from the cell lysate counts to control for any extracellular bacteria not removed by thorough washing. In all treatment groups the amount of bacteria present in the last PBS wash was negligible compared to the amount of bacteria present in cell lysates. Thus, lysate counts reflected the number of intracellular bacteria.

The ability of cytokines elicited by CLDC immunotherapy to inhibit intracellular growth of *B. pseudomallei* was assessed by adding diluted supernatants prepared from overnight cultures of spleen cells from mice treated with CLDC, as described previously [11]. The concentration of IFN-γ in the CLDC supernatants used in these studies was found to be 1813 pg/ml by cytokine bead array (BD Biosciences, San Jose, CA), while the concentration of TNF-α was 160 pg/ml. Control supernatants were prepared from spleens of untreated mice. Neutralizing antibodies were used to determine whether IFN-γ or TNF-α was responsible for generating antibacterial activity in the *in vitro* macrophage infection assay. For this experiment, supernatants from CLDC-stimulated spleen cells were treated with 10 μg/ml anti-IFN-γ antibody (Clone R4.6A2; eBioscience, San Diego, CA) or with 10 μg/ml of anti-TNF-α antibody (Clone TN3-19.12; eBioscience) for 30 minutes prior to the addition of the supernatants to cells. Isotype antibodies for anti-IFN-γ (clone eBRG1) and anti-TNF-γ (clone eBio299Arm) were used as controls (eBioscience). Infected AMJ cells were incubated with supernatants for 24 h, and intracellular bacterial concentrations were determined as described above.

5.3(7) Pulmonary challenge model

All infections with *B. pseudomallei* were performed using intranasal (i.n.) inoculation. Animals were anesthetized with 100 mg/kg ketamine (Fort Dodge Animal Health, Overland Park, KS) plus 10 mg/kg xylazine (Ben Venue Labs, Bedford, OH). The desired challenge dose of *B. pseudomallei* was suspended in PBS and 20 μl was delivered i.n. alternating nostrils. The challenge dose was confirmed by retrospective plating on LB agar. For all survival studies, animals were monitored for disease symptoms twice daily and were euthanized according to pre-determined humane endpoints. All procedures were performed in a biosafety level 3 (BSL-3) facility, in accordance with approved BSL-3 and Select Agent protocols.

5.3(8) Determination of bacterial burden *in vivo*

Numbers of viable *B. pseudomallei* were quantified in lung, liver, and spleen tissues at 48 h after infection. Lungs, livers and spleens were removed aseptically and homogenized in 5 ml sterile phosphate buffered saline and homogenized using a Stomacher 80 Biomaster (Seward, Bohemia, NY). Viable bacterial counts were determined for each organ by plating serial 10-fold dilutions of organ homogenates on LB agar. Plates were incubated at 37°C for 48 h, and the numbers of colonies on the appropriate plate were scored visually. The organ burden of bacteria was expressed as colony forming units (CFU)/organ. The limit of detection for determination of bacterial burden in organ homogenates was 50 CFU/organ.

5.3(9) Statistical analyses

Data were analyzed using Prism 5.0 software (Graph Pad, San Diego, CA). Survival times were analyzed by Kaplan-Meier analysis, followed by the log-rank test. For comparisons of survival times of more than one group in an experiment, the Bonferroni corrected threshold was applied to correct for multiple comparisons. Other analyses were performed using Mann Whitney test (two group comparisons) or one-way ANOVA followed by a Tukey's multiple mean's comparison test (comparison of more than two groups). Possible synergistic interactions between antimicrobials and cytokines as detected using *in vitro* assays were assessed using two-way ANOVA, as described previously [25]. Differences were considered statistically significant for $p < 0.05$.

5.4 Results

5.4(1) CLDC-elicited cytokines synergize with ceftazidime to inhibit intracellular replication of *B. pseudomallei* in macrophages *in vitro*

An *in vitro* macrophage infection assay was used to determine whether immunotherapy could increase the effectiveness of ceftazidime therapy for inhibiting intracellular replication of *B. pseudomallei,* since macrophages represent a major target cell for *B. pseudomallei* infection *in vivo* [26-29]. Supernatants generated from spleens of mice treated *in vivo* with CLDC were used as a source of CLDC-stimulated cytokines.

Dose titration studies demonstrated that treatment with 10 μg/ml ceftazidime did not significantly inhibit *B. pseudomallei* replication in infected macrophages. This concentration of ceftazidime is well within the range of clinically achievable

concentrations of ceftazidime *in vivo* [30]. Similar titrations were performed for CLDC supernatants and we found that a 1:5 dilution was effective in partially inhibiting *B. pseudomallei* replication in infected macrophages.

Subtherapeutic concentrations of ceftazidime and CLDC supernatants were then combined to treat infected macrophages. Marked, synergistic inhibition of intracellular replication of *B. pseudomallei* was observed when cells were treated with the two agents in combination (**Fig. 5.1**). For example, combined treatment reduced intracellular *B. pseudomallei* concentrations by almost 2 $\log_{10}$, from 1.9×10^6 CFU to 2.0×10^4 CFU. This effect was not observed when supernatants from non-stimulated spleen cells were used with ceftazidime. Synergistic inhibition of *B. pseudomallei* was observed at CLDC supernatant dilutions up to 1:20 when combined with 10 μg/ml ceftazidime (**Fig. 5.1**). Thus, cytokines elicited by CLDC immunotherapy were capable of synergistically inhibiting intracellular *B. pseudomallei* replication when combined with a low dose of ceftazidime.

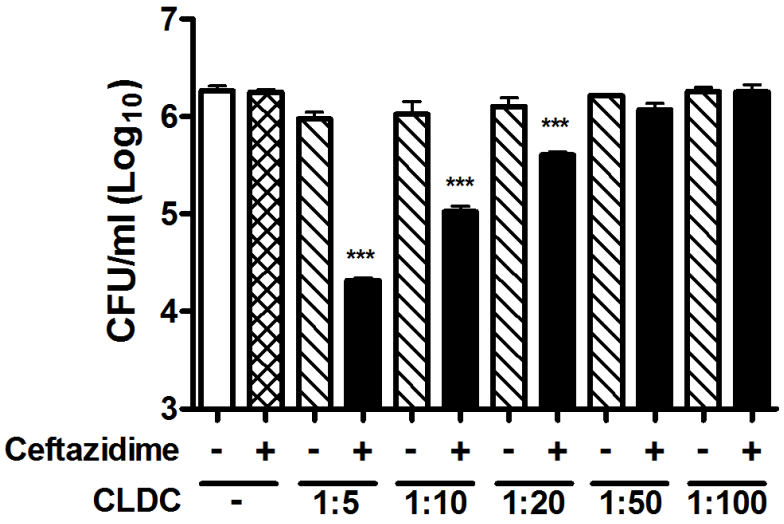

Figure 5.1. CLDC supernatants synergize with ceftazidime to inhibit intracellular replication of *B. pseudomallei*. AMJ.2 macrophages were infected in triplicate wells with *B. pseudomallei* strain 1026b for 1 h, and then treated with ceftazidime and/or CLDC spleen supernatants, alone or in combination, for 24 hours, as described in Materials and Methods. Concentrations of intracellular bacteria were quantified by serial dilution plating of macrophage lysates. The effects of CLDC supernatants at the dilutions indicated, alone or combined with 10 µg/ml of ceftazidime, on intracellular replication of *B. pseudomallei* were assessed. Mean bacterial concentrations in each treatment group were plotted and bars represent means ± SD. Synergistic interactions were assessed statistically by two-way ANOVA (***, $p < 0.001$). These data are representative of two independent experiments.

5.4(2) IFN-γ is responsible for synergistic inhibition of *B. pseudomallei* replication

Experiments were conducted next to identify cytokines present in CLDC supernatants that mediated the synergistic interaction with ceftazidime. Previous studies had identified interferon-γ (IFN-γ) as the most likely candidate cytokine [11, 22, 31], so the *in vitro* infection assay was repeated using CLDC supernatants that had been pre-treated with a neutralizing antibody to IFN-γ, as described in Materials and Methods. The effects of neutralizing tumor necrosis factor-α (TNF-α) activity were also evaluated. Neutralization of IFN-γ activity in CLDC supernatants eliminated nearly all of the synergistic antibacterial activity, thus identifying IFN-γ as the cytokine primarily responsible for the interaction with ceftazidime (**Fig. 5.2**). Neutralization of TNF-α activity had essentially no effect on the interaction of CLDC supernatants with ceftazidime (data not shown).

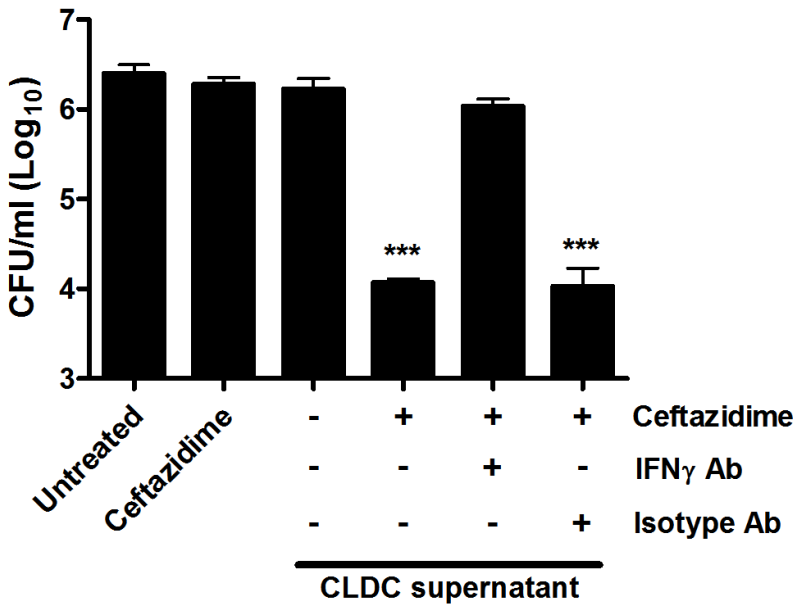

Figure 5.2. Synergistic inhibitory activity of CLDC stimulated supernatants is mediated by IFN-γ. Macrophages were infected in triplicate wells *in vitro* with *B. pseudomallei* for 1 h, then treated for 24 h with CLDC-stimulated spleen supernatants (1:5 dilution) alone or combined with ceftazidime at 10 µg/ml. CLDC supernatants were untreated, or treated with IFN-γ neutralizing antibody or isotype control antibody for 30 min before adding to infected macrophages. Intracellular bacterial numbers were determined 24 h after infection and mean bacterial numbers (± SD) were plotted. Synergistic interactions were assessed statistically by two-way ANOVA (***, $p < 0.001$). These data are representative of two independent experiments.

5.4(3) Synergistic inhibition of *B. pseudomallei* replication when IFN-γ is combined with ceftazidime

The preceding experiments identified IFN-γ as the primary cytokine mediating CLDC and ceftazidime synergistic activity. Therefore, we next determined whether recombinant murine IFN-γ (rIFN-γ) could reproduce the effects of CLDC supernatants in the *in vitro* infection assay. Infected macrophages were treated with ceftazidime (10 μg/ml) and a range of rIFN-γ concentrations from 1000 to 0.1 U/ml and the effects on intracellular *B. pseudomallei* infection were assessed (**Fig. 5.3**). We observed that the combination of rIFN-γ and ceftazidime elicited strong synergistic inhibition of *B. pseudomallei* replication, reducing bacterial counts from 1.9×10^6 CFU/ml in untreated cultures to 5.7×10^3 CFU/ml in cultures treated with 1000 U/ml rIFN-γ and ceftazidime. Concentrations of rIFN-γ as low as 1 U/ml elicited synergistic inhibition of *B. pseudomallei* intracellular growth (**Fig.5.3**). Titration of ceftazidime in combination with 100 U/ml rIFN-γ demonstrated that ceftazidime concentrations as low as 1 μg/ml could elicit synergistic inhibition of *B. pseudomallei* (**Fig. 5.3**).

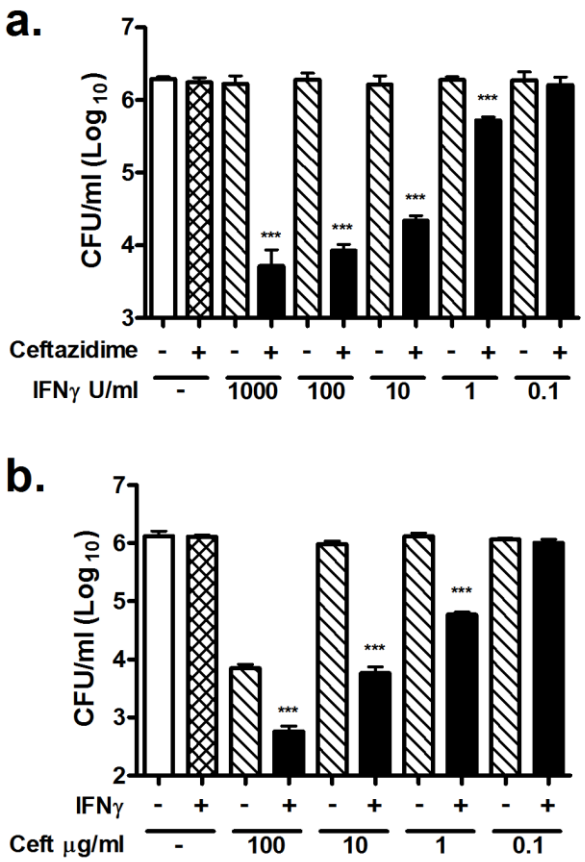

Figure 5.3. Recombinant IFN-γ and ceftazidime reciprocally increase inhibition of intracellular replication of *B. pseudomallei in vitro*. Macrophages were infected in triplicate wells *in vitro* with *B. pseudomallei* for 1 h, and then treated with rIFN-γ and ceftazidime for 24 h, and intracellular bacterial numbers were determined. **(A)** Decreasing concentrations of rIFN-γ were added to infected macrophages treated with a 10 μg/ml of ceftazidime and the effects on intracellular *B. pseudomallei* replication were assessed. For each treatment, the mean bacterial numbers were plotted and bars represent group means ± SD. **(B)** Decreasing concentrations of ceftazidime were added to macrophages treated with a fixed concentration of rIFN-γ (100 U/ml) and the effects on intracellular *B. pseudomallei* replication were assessed. Synergistic interactions were assessed statistically by two-way ANOVA (***, $p < 0.001$). These data are representative of two independent experiments.

5.4(4) Combined treatment with IFN-γ and ceftazidime rapidly suppresses intracellular replication and induces killing of *B. pseudomallei* in infected macrophages

Experiments were conducted next to characterize the *B. pseudomallei* inhibition kinetics following combined immuno-antibiotic treatment *in vitro*. Infected macrophages were treated with ceftazidime (10 μg/ml) and rIFN-γ (100 U/ml). The effects on intracellular *B. pseudomallei* infection were assessed at the time of treatment initiation and at 6, 12 and 24 h after treatment (**Fig. 5.4**). Following *B. pseudomallei* infection, bacterial counts were approximately 2 x 10^4 CFU/ml. In untreated cultures and cultures treated with ceftazidime alone or IFN-γ alone, intracellular *B. pseudomallei* counts rose continuously over time to >1 x 10^6 CFU/ml at 24 h post-infection. However, in cultures treated with both ceftazidime and IFN-γ, bacterial counts initially increased during the first 6 h of treatment, then subsequently decreased by 12 h and decreased further by 24 h to approximately 4 x 10^3 CFU/ml. Thus, at 12 h macrophages treated with ceftazidime and IFN-γ had significantly lower bacterial counts than macrophages treated with ceftazidime or IFN-γ alone. In addition, the progressive decrease in intracellular bacterial counts in ceftazidime plus IFN-γ treated cultures demonstrated that combined immuno-antibiotic therapy resulted in killing of *B. pseudomallei* in infected macrophages.

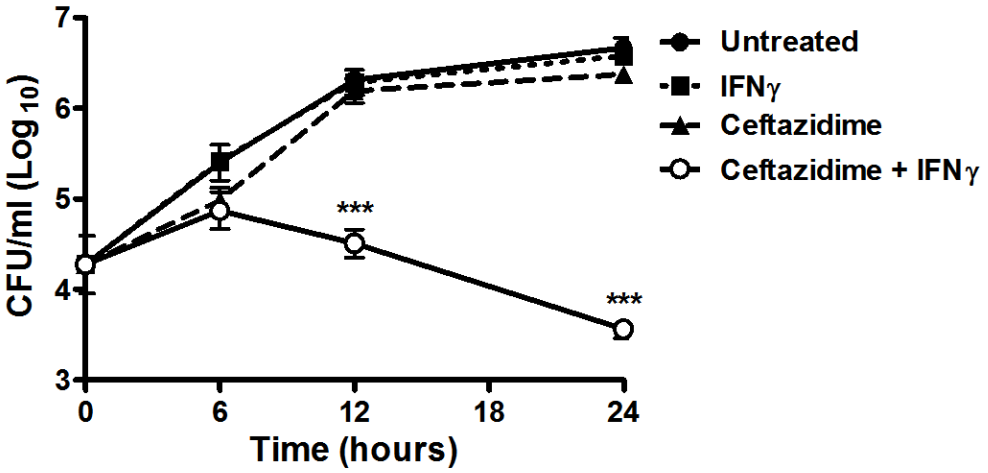

Figure 5.4. Time course of intracellular *B. pseudomallei* replication and killing following treatment with IFN-γ and ceftazidime. Macrophages were infected in triplicate wells *in vitro* with *B. pseudomallei* for 1 h, and then treated with rIFN-γ (100 U/ml) or ceftazidime (10 µg/ml) or both for the indicated time periods, and intracellular bacterial numbers were determined. Mean bacterial concentrations (± SD) were compared over time in the individual treatment groups using repeated measures ANOVA with the Bonferroni post-tests (***, $p < 0.001$, ceftazidime + IFN-γ combined group versus all other groups). Similar results were obtained in one additional experiment.

5.4(5) *In vivo* treatment with CLDC and ceftazidime generates significant protection from lethal pneumonic *B. pseudomallei* challenge

A murine model of acute pulmonary *B. pseudomallei* infection was used to determine whether the combination of immunotherapy plus ceftazidime treatment was also effective *in vivo*. Mice were infected intranasally (i.n.) with 8 x LD_{50} *B. pseudomallei* 1026b, and treated 6 h after challenge with ceftazidime administered intraperitoneally (i.p.). This treatment was repeated every 12 h for a total of 6 treatments. The dose of ceftazidime that consistently protected 20% or fewer acutely infected mice (when administered as single agent without immunotherapy) was 25 mg/kg. For the *in vivo* studies, a single dose of cationic liposome-DNA complexes (CLDC) was administered i.p. 6 hours after infection, as previous studies have found that the immune stimulatory effects of CLDC are prolonged [11, 23]. A dose of 20 μl CLDC administered i.p. was found to protect 20% or fewer *B. pseudomallei* infected mice (when injected as a single treatment without ceftazidime). Therefore, these sub-therapeutic doses of ceftazidime and CLDC were selected for the combination therapy studies.

For the combination therapy studies, mice were challenged i.n. with *B. pseudomallei*, and 6 h later treated with CLDC alone, ceftazidime alone, or both agents in combination. We observed that 90% of mice treated with the combination of CLDC and ceftazidime survived acute pulmonary challenge with *B. pseudomallei* (**Fig. 5.5A**). In contrast, only 10% of mice treated with ceftazidime or CLDC alone survived until day 20 post-challenge. Therefore, we concluded that combined treatment with a potent IFN-γ inducing immunotherapeutic (i.e., CLDC) significantly enhanced the efficacy of low-dose ceftazidime treatment for acute *B. pseudomallei* infection.

We also assessed the effects of the brief course of combined low-dose therapy on long-term survival following high-dose *B. pseudomallei* pulmonary challenge. Notably, five of the nine mice in the combination treated group that survived the initial 20 days after challenge (**Fig. 5.5A**) survived for an additional 40 days (**Fig. 5.5B**). When these animals were euthanized at the end of the observation period and their organs cultured, four of the five mice were found to be free of culturable *B. pseudomallei.* In contrast, the one surviving CLDC- treated mouse was euthanized on day 55 due to *B. pseudomallei* splenic infection, and the single ceftazidime-treated mouse euthanized at the end of the observation period had *B. pseudomallei* detected within the lungs. Thus, a brief 3-day course of treatment with combined low-dose immuno-antimicrobial therapy produced long-term cures in 40% of the treated animals. Therefore, it is reasonable to expect that longer treatment with full-dose therapy should be capable of generating even greater protection from chronic disseminated melioidosis.

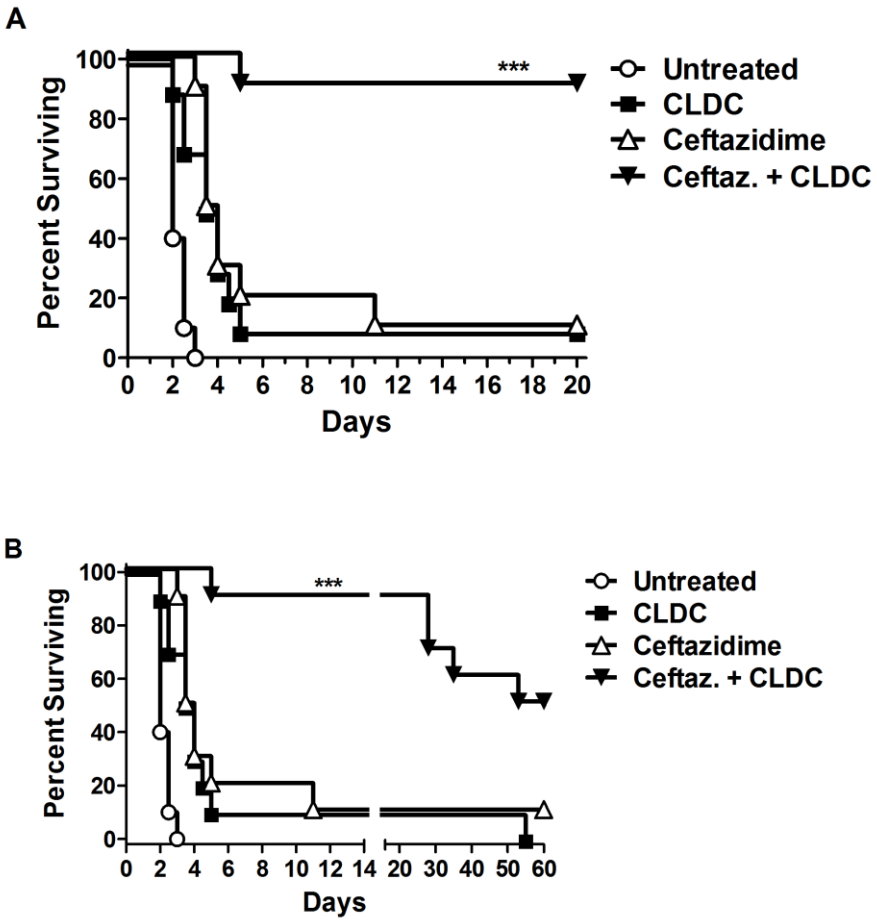

Figure 5.5. Low-dose ceftazidime (ceftaz.) plus CLDC immunotherapy effectively protects mice from acute and chronic infection with *B. pseudomallei*. (A) BALB/c mice (*n* = 10 mice per group) were challenged intranasally with 7.5 x 10^3 CFU *B. pseudomallei*. Six hours later mice were treated intraperitoneally with 25 mg/kg ceftazidime, 20 µl CLDC, or both agents in combination. The ceftazidime treatments were continued every 12 h for a total of six treatments, and the CLDC was administered once. Short-term (20-day) survival times were assessed. **(B)** Mice (*n* = 10 per group) that initially survived the 20-day short-term period were followed for an additional 40 days to assess the effects of treatment on long-term chronic infection. Statistical differences in survival times were determined by Kaplan-Meier curves, followed by log-rank test. The Bonferroni corrected threshold was applied for comparison of multiple survival curves, such that a *p* value of < 0.02 was considered significant for these analyses. (***, *p* < 0.001 for combination therapy vs. CLDC treatment, and for combination therapy vs. ceftazidime treatment). Data are representative of two combined experiments.

5.4(6) Combined immunotherapy and antimicrobial therapy suppresses *B. pseudomallei* replication and dissemination *in vivo*

The effects of combined CLDC and ceftazidime treatment on bacterial burdens in infected mice were assessed. Mice were sacrificed 48 h after pulmonary *B. pseudomallei* challenge, and bacterial burdens in lung, spleen, and liver tissues were quantified. Mice treated with ceftazidime and CLDC in combination had a significant decrease in bacterial burden in the lungs, spleen and liver compared to mice treated with either ceftazidime or CLDC alone, or compared to untreated mice (**Fig. 5.6**). These results indicated that combined therapy effectively suppressed bacterial replication in the lungs and also significantly inhibited bacterial dissemination to the spleen and liver.

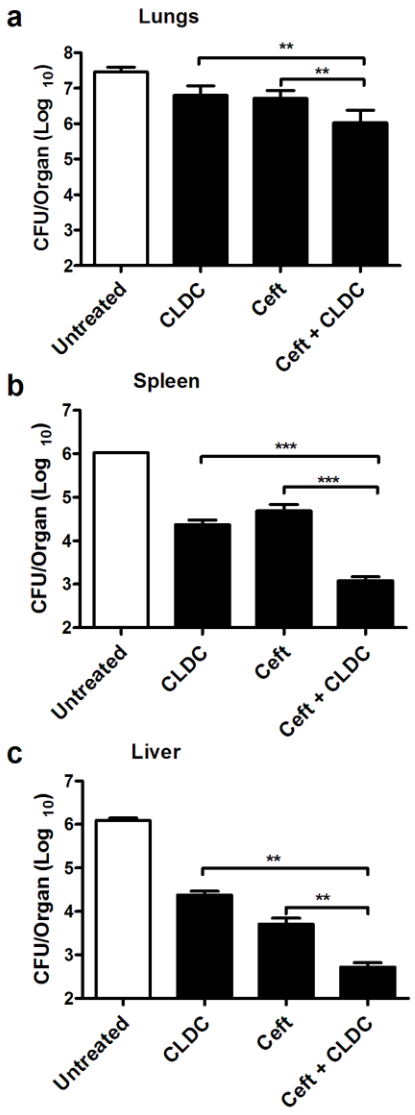

Figure 5.6. Combined treatment with CLDC and ceftazidime significantly decreases bacterial burden. BALB/c mice (n = 5 mice per group) were challenged intranasally with 8 x 10^3 CFU *B. pseudomallei*. Six hours later mice were treated intraperitoneally with 25 mg/kg ceftazidime, 20 µl CLDC, or both agents in combination. The ceftazidime treatments were continued every 12 h for a total of six treatments, and the CLDC was administered once. At 48 h after challenge, the mice were sacrificed and bacterial burdens were quantified in the lungs (**a**), spleen (**b**), and liver (**c**). Organ bacterial burdens were compared statistically, using one-way ANOVA and Tukey multiple means comparison test. (***, $p < 0.001$; **, $p < 0.01$). Significant reductions ($p < 0.01$) in bacterial counts of single-agent treated animals were also observed, but are not noted.

5.4(7) Treatment of mice with rIFN-γ significantly increases the effectiveness of ceftazidime therapy

Next, we investigated whether recombinant IFN-γ (rIFN-γ) could be substituted for CLDC and combined with low-dose ceftazidime treatment to generate increased *in vivo* protection from *B. pseudomallei* challenge. In dose titration studies *in vivo*, high doses of rIFN-γ (> 1 x 10^4 U rIFN-γ per mouse) administered intraperitoneally (i.p.) at 6 and 18 h after infection significantly protected mice from lethal inhalational challenge with *B. pseudomallei* (data not shown). However, lower doses of rIFN-γ (< 5 x 10^3 U) did not protect mice from lethal *B. pseudomallei* challenge (data not shown). Thus, a sub-therapeutic dose of 3 x 10^3 U IFN-γ per mouse, administered at 6 and 18 hours after infection, was selected for subsequent combination treatment studies. This dose consistently protected 20% or fewer mice when administered without ceftazidime (data not shown).

Seventy percent of mice treated with the combination of low-dose rIFN-γ and low-dose ceftazidime survived for 20 days following inhalational challenge inhalational with *B. pseudomallei* (**Fig. 5.7A**). In contrast, only 10% of mice treated with rIFN-γ alone survived the challenge and none of the mice treated with low-dose ceftazidime alone survived until day 20 post-infection. Therefore, we concluded that treatment with rIFN-γ could also be used to significantly enhance the effectiveness of low-dose ceftazidime treatment.

We also examined the effects of rIFN-γ and ceftazidime combination treatment on long-term chronic infection with *B. pseudomallei.* When mice that survived the initial 20-day period following challenge were observed for 40 more days, six of the seven surviving animals treated with combination therapy eventually succumbed to chronic

infection (**Fig. 5.7B**). In addition, when the single surviving combination-treated mouse was euthanized at day 65 post-challenge, *B. pseudomallei* was present within the spleen. The long-term survival times of the combination-treated mice were significantly ($p < 0.001$) increased compared to the group of mice treated with only ceftazidime, but were not significantly different when compared to the rIFN-γ treated animals. However, the trend apparent in these results was that the combination of rIFN-γ immunotherapy combined with ceftazidime antimicrobial therapy was more potent than either therapy alone. It was also apparent from these results that combined short-term treatment with rIFN-γ and ceftazidime was not as potent as treated with CLDC plus ceftazidime, especially for generation of sustained protection from chronic *B. pseudomallei* infection.

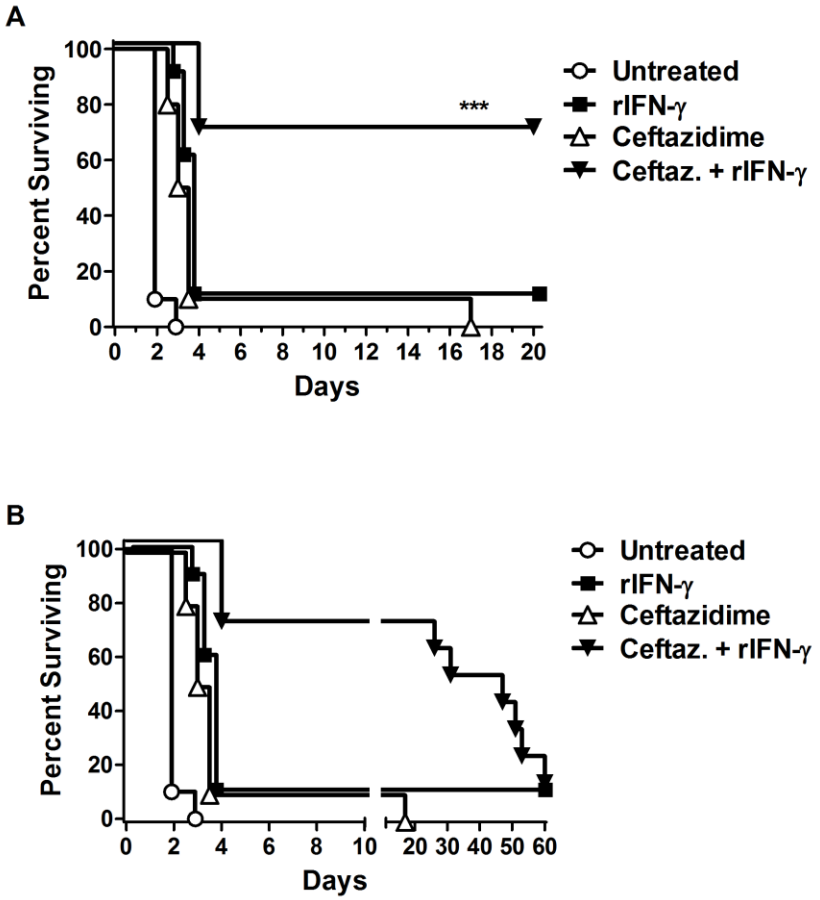

Figure 5.7. Treatment with low-dose ceftazidime plus rIFN-γ protects mice from acute but not chronic *B. pseudomallei* infection. (A) BALB/c mice (*n* = 10 per group) were challenged intranasally with 7.5 x 10^3 CFU *B. pseudomallei*. Six hours later mice were treated intraperitoneally with 25 mg/kg ceftazidime, 3 x 10^3 U rIFN-γ, or with both agents in combination. The ceftazidime treatments were continued every 12 h for a total of six treatments. Treatment with rIFN-γ was administered twice, at 6 and 18 h post-infection. Short-term (20-day) survival was assessed. **(B)** Mice (*n* = 10 per group) that initially survived the 20-day short-term period were followed for an additional 40 days to assess the effects of the combination treatment on chronic infection. Statistical differences in survival times were determined by Kaplan-Meier curves followed by log-rank test. The Bonferroni corrected threshold was applied for comparison of multiple survival curves and a *p* value of < 0.02 was considered significant for these analyses. (***, $p < 0.001$ for combination therapy vs. rIFN-γ treatment, and for combination therapy vs. ceftazidime treatment). Data are representative of two combined experiments.

5.5 Discussion

There is a clear need for new approaches to increase the effectiveness of antimicrobial therapy for *B. pseudomallei* infection, given the inherent antimicrobial resistance of *B. pseudomallei* and high probability of patient relapse even after prolonged, appropriate antibiotic therapy [3, 7-8] In the current study we demonstrated that treatment with recombinant IFN-γ, or a cationic liposome-DNA complex (CLDC) immunotherapeutic that efficiently induces IFN-γ production *in vivo*, significantly increased the effectiveness of conventional antimicrobial therapy for *B. pseudomallei* infection. These findings are important because they suggest a general strategy for improving the effectiveness of antimicrobial therapy for melioidosis. Immunotherapy has been shown previously to increase the effectiveness of antimicrobial therapy in mouse models of *Mycobacterium avium*, *Enterococcus faecalis*, and *Francisella novicida* infection [17-19]. The effectiveness of combined therapy has also been demonstrated in a *Cryptococcus* infection model [32-33]. However, to our knowledge this is the first report that demonstrates marked enhancement of antimicrobial therapy by immunotherapy for treatment of *B. pseudomallei* infection using an acute lethal challenge model.

Previous studies suggested that treatment with recombinant granulocyte-colony stimulating factor (rG-CSF) might be used to augment the effectiveness of antimicrobial therapy for treatment of *B. pseudomallei* infection [13]. These studies were based on the protective role of neutrophils in *B. pseudomallei* infection [14]. However, studies failed to confirm an *in vivo* role for treatment with rG-CSF for augmenting the effectiveness of ceftazidime for treatment of *B. pseudomallei* infection [15]. Our studies

indicated that immuno-antimicrobial therapy can be used effectively for treatment of *B. pseudomallei* infection, but that IFN-γ would be the preferred cytokine for this application.

These studies also suggest that combined immuno-antimicrobial therapy may be particularly effective for eliminating the chronic stage of *B. pseudomallei* infection. The brief 3-day course of treatment with combination therapy resulted in long-term chronic disease protection. This extended protection has the potential to be even augmented further with a longer duration of treatment. In addition, in these studies low-doses of ceftazidime and immunotherapy were used for the purpose of determining whether conventional antimicrobial therapy was markedly enhanced with immunotherapy. However, full-dose therapy should be capable of generating even greater protection from chronic *B. pseudomallei* infection.

The direct implications of these findings to human melioidosis patients have been addressed by clinicians within endemic melioidosis regions upon the publication of these results. Mortality from *B. pseudomallei* is especially high in septic patients even with appropriate antimicrobial therapy. In a 10-year prospective study conducted in northern Australia, 86% of melioidosis patients with septic shock died despite the administration of ceftazidime or carbapenems [34]. The septic melioidosis patient definitely constitutes one of the biggest clinical challenges with melioidosis. With the purpose of more accurately representing the human septic patient in our murine model and determining whether the combination therapy would improve clinical outcome, we conducted preliminary studies with slightly altered parameters to our model. Under the new parameters, mice were challenged intranasally with 5×10^4 CFU *B. pseudomallei* (approximately 1-log higher than used in our initial model) and treatment regimens were not initiated until 24 h post-challenge (18 h later than the previous model) to most

accurately mimic the clinical situation of advanced septic disease prior to therapeutic intervention. In addition, we tested a more clinically relevant, high-dose of ceftazidime in this revised model of 600 mg/kg (twice daily), and animals were treated with CLDC for an extended period of time (three total treatments instead of a single dose). Under these revised parameters to our *in vivo* model we saw no enhancement to ceftazidime therapy by the CLDC immunotherapy. The high *B. pseudomallei* challenge dose (greater than 50x LD_{50}) required to overcome the 600mg/kg dose of ceftazidime when administered as a single agent caused the mice to reach very advanced pneumonic disease by the time therapy was administered (24 h post-challenge). Even with the combination therapy, *B. pseudomallei* could not be effectively controlled under these parameters. We believe the enhancement to ceftazidime therapy with immunotherapy is most effectively illustrated using the initial model described in Materials and Methods, and believe immuno-antimicrobial therapy still has great potential for reducing patient relapse.

At present, the mechanism(s) by which IFN-γ interacts with ceftazidime to suppress intracellular replication of *B. pseudomallei* remains undetermined. Possible mechanisms include the induction of reactive nitrogen or reactive oxygen species by IFN-γ, which then subsequently increase the susceptibility of *B. pseudomallei* to killing by ceftazidime. However, preliminary experiments suggest that this is not the case. It is also possible that IFN-γ treatment could increase the permeability of macrophages to ceftazidime, but again preliminary experiments suggest that this is not the mechanism. Therefore, at present the mechanisms by which IFN-γ and certain antibiotics interact to generate synergistic killing of intracellular *Burkholderia* remain unknown. The CXC

chemokines (CXCL9 and CXCL10) induced by IFN-γ and known to possess antimicrobial activity are currently under investigation by our laboratory [35].

In summary, we report a strong synergistic interaction between IFN-γ and ceftazidime that suppresses *B. pseudomallei* in both acute and chronic infection models. The effect was demonstrated using an *in vitro* macrophage infection model and confirmed *in vivo* using a lethal bacterial challenge model. These experiments also suggest that immunotherapy capable of eliciting a more sustained release of IFN-γ may be more effective than short-term treatment with rIFN-γ. The combined immuno-antimicrobial treatment approach may be especially useful for reducing the duration of antimicrobial treatment and reducing the chance for patient relapse following antibiotic therapy.

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CHAPTER 6

THE DEVELOPMENT OF AN EFFECTIVE MUCOSAL VACCINE FOR *Burkholderia pseudomallei*

The studies described in this chapter investigate the protective efficacy of different mucosal vaccine formulations for melioidosis. I acknowledge Angela Duffy for assistance with the heat-killed/CLDC studies, Mark Estes for providing the *Burkholderia* protein antigens, the Herbert Schweizer laboratory for providing Bp82, the Tung Hoang laboratory for providing Bp422, and Andrew Goodyear for assisting with the oral vaccination studies and monitoring of the mice.

6.1 Abstract

Burkholderia pseudomallei causes the disease melioidosis and currently there is no approved vaccine for human use. A variety of vaccine candidates have been tested in animal melioidosis models, but those investigated to date have consistently failed to protect long-term from chronic melioidosis. The purpose of this research was to develop a mucosal vaccine for *B. pseudomallei* providing protection from both acute and chronic melioidosis, and to identify an effective mucosal adjuvant. A variety of vaccine

formulations were tested in these studies including killed bacteria, purified protein *Burkholderia* subunits, and two live attenuated strains. All vaccine candidates were administered intranasally and one of the attenuated mutants was also tested for efficacy following oral delivery. A cationic liposome-DNA complex (CLDC) adjuvant was found to significantly enhance protection from lethal pneumonic challenge. We found that adjuvanted killed bacteria and protein subunits significantly protected mice compared to non-adjuvanted antigens. Vaccination with either the adjuvanted killed bacteria or the two attenuated *B. pseudomallei* strains protected 100% of mice from acute pneumonic melioidosis, whereas the recombinant protein antigens, BimA, BopA, and LolC, offered less effective short-term protection. The best long-term protection from chronic melioidosis was elicited following intranasal vaccination with Bp82, a fully attenuated ∆*purM* 1026b mutant that is exempt from Select Agent regulations. Mice vaccinated with Bp82 were protected for 100 days following lethal pneumonic challenge. This attenuated strain was also administered orally to the animals, and significantly increased their time to death following pneumonic challenge. Bp82 has great potential as a live vaccine strain for high-risk persons.

6.2 Introduction

Burkholderia pseudomallei is a human pathogen endemic to Southeast Asia, Northern Australia, and other tropical regions of the world [1]. There is also concern for this pathogen in the Western Hemisphere due to its potential for use as a biological weapon [2]. This pathogen causes the disease melioidosis and there is currently no approved vaccine available. An approved vaccine would be advantageous in protecting those at risk within endemic regions and could also lessen the desire to weaponize this

pathogen for intentional release [3]. The overall purpose of these studies was to develop an effective mucosal vaccine for *B. pseudomallei* providing both short and long-term protection from melioidosis.

Vaccine research for melioidosis has been complicated by a variety of factors. Producing long-term protective immunity has proven to be one of the biggest challenges [4]. Since *B. pseudomallei* is a facultative intracellular pathogen, it is likely that both effective cell-mediated and humoral immune responses will be critical for long-term protection [5]. Humans have been shown to produce bacterial-specific antibodies following melioidosis [6-7]. However, recurrent *B. pseudomallei* infection within just one year of primary disease is commonly seen in melioidosis patients [8], illustrating the likelihood that antibody presence does not confer sterilizing immunity. Most vaccine research has been conducted in murine models and many vaccine candidates tested have produced short-term protection from acute melioidosis, but do not often confer long lasting protection from disseminated disease beyond 30-40 days [4, 9]. In addition, *B. pseudomallei* is commonly isolated from the tissues of vaccinated survivors at time of sacrifice, further indicative of a lack of sterilizing immunity [10].

A variety of *Burkholderia* vaccine approaches have been investigated in murine melioidosis models. Killed whole-cell formulations (composed of heat-killed or irradiated bacteria) have been tested for efficacy, but have not consistently produced long-term protection [10-12]. One of the more promising studies involved intraperitoneal vaccination with killed *B. pseudomallei* that offered protection beyond 40 days from aerosol challenge in the majority of mice [10]. It must be noted, however, that a low-dose aerosol challenge dose was used in this study as not all unvaccinated mice succumbed to acute disease. Live attenuated strains have shown more promise in

providing protection than killed formulations [13-18]. This was demonstrated by the finding that the protective effect elicited by the live attenuated *B. pseudomallei* strain CL04 was greatly diminished when this strain was inactivated by irradiation [19]. Attenuated mutant *B. pseudomallei* strains previously investigated have included auxotrophic strains deficient in amino acid synthesis [13, 15], purine biosynthesis [16], type III secretion [18], and strains defective in other biosynthetic pathways [20]. The attenuated strains tested have been most often administered intraperitoneally. Long-term protection is still an issue, as mice vaccinated intraperitoneally with a live attenuated *B. pseudomallei* strain 2D2 eventually succumbed to chronic disease within 75 days [17].

Outer membrane and type III secretion protein subunits have been examined as vaccine candidates [21-23]. The protein subunit that is considered one of the most promising is the lipoprotein-releasing system transmembrane protein, LolC [4]. LolC serves as an adenosine triphosphate-binding cassette transporter protein [22]. The greatest protection was observed when mice were vaccinated intraperitoneally with this subunit along with adjuvant immunostimulating complex (ISCOMS) and CpG oligodeoxynucleotide. Vaccinated mice did not begin succumbing to chronic disease infection until 30-50 days post intraperitoneal challenge with *B. pseudomallei* [22].

Previous *B. pseudomallei* vaccine research has most often used the intraperitoneal route for vaccination and subsequent challenge. Our purpose, however, was to develop a mucosal vaccine since mucosal delivery of antigens has been shown to most efficiently produce mucosal immunity [24-26]. The protective efficacy of all vaccine candidates within our research was tested using intranasal vaccination, and one of our attenuated strains was also delivered orally. Since the inhalational challenge

route is considered the most lethal route of *B. pseudomallei* infection [27] and also the route considered most likely during a potential biological attack, all *B. pseudomallei* challenges following vaccination were conducted intranasally. In this series of studies, we have explored a variety of vaccine candidates, including killed whole cell *B. pseudomallei*, live attenuated strains, and a series of protein subunits.

In addition to identifying vaccine candidates, we also explored the effectiveness of using a cationic liposome-DNA complex (CLDC)-based adjuvant in conjugation with *B. pseudomallei* antigens. We have previously demonstrated the effectiveness of CLDC when used as immunotherapy to enhance antibiotic therapy [28] and as a highly effective pre-exposure prophylaxis against lethal bacterial pathogens [29-30]. Similar lipid-based vaccine formulations have shown protection against other bacterial pathogens including *Mycobacterium tuberculosis* and *Vibrio cholerae* when administered intranasally to mice [31-33].

In summary, we have identified a promising CLDC mucosal vaccine adjuvant that greatly enhances mucosal protection when combined with heat-killed *B. pseudomallei* and purified *Burkholderia* protein subunits. The most effective vaccine candidate identified by our studies that offered protection from both acute and long-term chronic melioidosis for 100 days was the live attenuated strain Bp82, a fully attenuated 1026b Δ*purM* derivative. Because of its exemption from Select Agent regulations and previous demonstration of complete attenuation [34], this strain could serve as a potential vaccine candidate for high-risk persons in endemic regions or military personnel.

6.3 Materials and Methods

6.3(1) Bacterial strains, media and growth conditions

B. pseudomallei strain 1026b is a clinical isolate from a case of human septicemic melioidosis from Thailand. This strain is lethal to mice and has been extensively studied in the laboratory [35]. *B. pseudomallei* 1026b was the strain used for vaccination with heat-killed bacteria. Strain 1026b was also the virulent challenge strain used to test the protective efficacy of all vaccine formulations investigated within these studies.

Bp82 is a ∆*purM* derivative of 1026b, and adenine and thiamine auxotroph. This strain is completely attenuated in mice and hamsters [34], and was tested as a potential vaccine strain for protection against subsequent intranasal challenge with the fully virulent *B. pseudomallei* strain 1026b. A second attenuated strain that was investigated as a potential vaccine strain within these studies was Bp422 (kindly provided by Tung Hoang, University of Hawaii at Manoa, HI). Bp422 is a ∆*asd* derivative of 1026b, harboring a mutation within the aspartate-beta-semialdehyde dehydrogenase (*asd*) gene. This strain is a diaminopimelate (DAP) auxotroph in rich media, and a DAP, lysine, methionine, and threonine auxotroph in minimal media [36]. Due to the inability to synthesize DAP, Bp422 is unable to crosslink D-alanine in neighboring peptidoglycan strains during cell wall synthesis, resulting in cell death unless growth media is supplemented with DAP [37].

Strains 1026b and Bp82 were grown to saturation in Luria broth (LB) at 37°C with shaking, and then stored at -80°C in 15% glycerol until ready to use. For quantification, strains 1026b and Bp82 were plated on LB agar. Bp422 was grown to saturation in LB

supplemented with 200 μg/ml DAP, and then stored at -80°C in 15% glycerol until ready to use. For quantification, Bp422 was grown on LB agar plus 200 μg/ml DAP. For animal experiments, strains 1026b and Bp422 were thawed just before use and the bacteria were diluted to the desired cell numbers using sterile phosphate buffered saline (PBS). Bp82 was grown fresh prior to each intranasal vaccination. This strain was inoculated fresh into LB, grown to saturation, and diluted to the desired cell numbers using sterile phosphate buffered saline.

6.3(2) Mice

Female BALB/c mice were used for these studies (Jackson Laboratories, Bar Harbor, ME). Mice were between approximately 6-16 weeks of age at the time of experimentation and were housed under pathogen free conditions. All animal studies were approved by the Institutional Animal Care and Use Committee at Colorado State University.

6.3(3) Preparation of cationic liposome-DNA complex (CLDC) for adjuvant use within mucosal vaccines

Liposomes were prepared by combining cationic liposome DOTIM octadecenoyloxy (ethyl-2-heptadecenyl-3-hydroxyethyl) imidazolinium chloride and cholesterol in equimolar concentrations as described previously [38]. Non-coding plasmid DNA (0.2 mg/ml, Juvaris Biotherapeutics) was diluted in sterile Tris-buffered 5% dextrose water. The cationic liposomes were then added with gentle pipetting at a concentration of 100 μl of liposomes per 1 ml of solution, resulting in the spontaneous

formation of CLDC. To formulate the CLDC-adjuvanted vaccines, the protein subunits or heat killed *B. pseudomallei* were added to the CLDC. Vaccines were delivered to mice within 30 min of preparation.

6.3(4) Vaccination with heat-killed *B. pseudomallei* and CLDC

B. pseudomallei 1026b was heat-killed for intranasal vaccination of mice. The bacteria were washed, resuspended in PBS, and then heated to 80˚C for 1 h using a heat block. Complete bacterial killing was confirmed by plating on LB agar. To determine whether CLDC has potential as a mucosal vaccine adjuvant for protection against lethal *B. pseudomallei* challenge, BALB/c mice were vaccinated intranasally with CLDC adjuvant alone, 1 x 10^5 heat-killed *B. pseudomallei* organisms alone, heat-killed bacteria mixed with 10 µl CLDC, or left unvaccinated.

For all vaccinations, mice were anesthetized by intraperitoneal injection with 100 mg/kg ketamine (Fort Dodge Animal Health, Overland Park, KS) plus 10 mg/kg xylazine (Ben Venue Labs, Bedford, OH). All mice were vaccinated intranasally, alternating nostrils, with a total volume of 20 µl of the appropriate vaccine, and were boosted in the same manner 10 d later. Fourteen days after the boost, mice were subjected to lethal intranasal challenge with 7,500 CFU live *B. pseudomallei* 1026b (8 x LD_{50}). Mice were monitored for disease symptoms twice daily and were euthanized according to pre-determined humane endpoints.

6.3(5) Vaccination with protein subunits

The recombinant protein subunits evaluated as potential vaccine candidates within these studies included BimA, BopA, and LolC (all antigens kindly provided by Mark Estes, University of Texas Medical Branch, Galveston, TX). BimA is an autotransporter protein and BopA is a type III secretion system protein, both isolated from *B. mallei.* LolC is an ABC transporter protein from *B. pseudomallei.* These three protein subunits were tested as part of collaborative research with M. Estes to determine their cross-protection potential against both *B. pseudomallei* and *B. mallei.* The adjuvant tested within the protein subunit vaccines was CLDC.

BALB/c mice (*n* = 5-15 mice per group) were vaccinated intranasally with BopA protein alone (no adjuvant), BimA mixed with CLDC, BopA mixed with CLDC, LolC mixed with CLDC, or left unvaccinated. For all vaccinations, mice were anesthetized by intraperitoneal injection with 100 mg/kg ketamine (Fort Dodge Animal Health, Overland Park, KS) plus 10 mg/kg xylazine (Ben Venue Labs, Bedford, OH). All mice were vaccinated intranasally with 2 μg of purified recombinant protein in a total volume of 20 μl. Mice were boosted 1-2 times in the same manner 10-14 d later. Fourteen days after the final boost, mice were subjected to lethal intranasal challenge with approximately 3 x 10^3 CFU live *B. pseudomallei* 1026b (3 x LD_{50}). Mice were monitored for disease symptoms twice daily and were euthanized according to pre-determined humane endpoints.

6.3(6) Vaccination with live attenuated *B. pseudomallei* strains

Bp82, a Δ*purM* 1026b derivative, and Bp422, a Δ*asd* 1026b derivative, were the two attenuated strains tested for protective efficacy against lethal *B. pseudomallei*

challenge. Bp82 has been shown to be completely attenuated in mice [34], and mice were vaccinated with this strain using both intranasal and oral delivery. For intranasal vaccination, BALB/c mice (*n* = 5 mice per group) were vaccinated with 1 x 10^6 CFU Bp82 (freshly grown in LB at stationary phase). All mice were anesthetized for intranasal vaccination by intraperitoneal injection with 100 mg/kg ketamine (Fort Dodge Animal Health, Overland Park, KS) plus 10 mg/kg xylazine (Ben Venue Labs, Bedford, OH). All mice were vaccinated with Bp82 in a total volume of 20 μl and boosted in the same manner 14 d later. For oral vaccination with Bp82, BALB/c mice (*n* = 10 mice per group) were gavaged with inoculums ranging from 1 x 10^7 to 1 x 10^9 CFU Bp82 using a stainless steel gavage needle, and boosted 14 d later. Fourteen days after the intranasal or oral boosts, all mice were subjected to lethal intranasal challenge with approximately 5 x 10^3 CFU live *B. pseudomallei* 1026b (5 x LD_{50}). Mice were monitored for disease symptoms twice daily and were euthanized according to pre-determined humane endpoints.

Since Bp422 had not been previously tested *in vivo* for attenuation, BALB/c mice (*n* = 5 mice per group) were challenged intranasally with 1 x 10^7 CFU Bp422 and monitored for 80 d. And the end of this observation period, their lungs, livers, and spleens were plated for sterility to assess chronic colonization by this attenuated strain. Bp422 was also tested for vaccine efficacy against lethal *B. pseudomallei* challenge. BALB/c mice (*n* = 10 mice per group) were vaccinated intranasally with 1 x 10^7 CFU Bp82 and boosted in the same manner 3 weeks later. Two weeks following the boost mice were challenged intranasally with 4 x 10^3 CFU (4 x LD_{50}) *B. pseudomallei* 1026b. Mice were monitored for disease symptoms twice daily and were euthanized according to pre-determined humane endpoints.

6.3(7) Statistical analyses

Data were analyzed using Prism 5.0 software (Graph Pad, San Diego, CA). Survival times were analyzed by Kaplan-Meier analysis, followed by the log-rank test. For comparisons of survival times of more than one group in an experiment, the Bonferroni corrected threshold was applied to correct for multiple comparisons.

6.4 Results

6.4(1) Mucosal vaccination with heat killed bacteria plus CLDC adjuvant generates effective long-term protection against lethal pulmonary *B. pseudomallei* challenge

Experiments were conducted to identify an effective mucosal vaccine adjuvant for protection from *B. pseudomallei.* This adjuvant would be later tested for protective efficacy in conjugation with the *Burkholderia* protein subunits. The adjuvant examined by these studies was cationic liposome-DNA complexes (CLDC) that we have previously shown to offer prophylactic protection from lethal bacterial challenge and enhancement to antibiotic therapy [28, 30]. BALB/c mice were vaccinated and boosted intranasally with the CLDC adjuvant alone, heat-killed *Burkholderia pseudomallei*, or heat-killed bacteria mixed with CLDC. All mice were then subjected to lethal intranasal challenge and survival times were determined. All non-vaccinated control mice reached end-point prior to day 3 after challenge, and mice vaccinated with the CLDC adjuvant alone succumbed to end-point by day 4. In contrast, 4 of the 9 mice vaccinated with heat-killed bacteria alone survived for more than 40 d (**Fig. 6.1**). However, it is important to note that all of the surviving mice vaccinated with heat-killed *B. pseudomallei* only eventually succumbed to chronic disease by day 60 post-challenge (data not shown). In

contrast, 100% of mice vaccinated with heat-killed bacteria plus CLDC survived bacterial challenge for more than 40 d (**Fig. 6.1**), and 5 of these 9 mice survived beyond day 60 post-challenge (data not shown), indicating the ability of CLDC to enhance the protective effect elicited by the heat-killed bacteria. These results indicate the effectiveness of a CLDC-adjuvanted vaccine at offering protection from both acute and chronic melioidosis, and were imperative in the identification of a promising mucosal adjuvant for future vaccine studies.

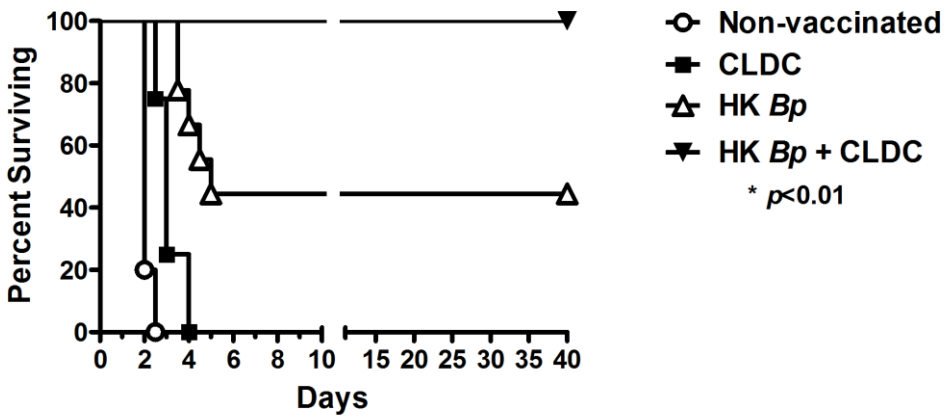

Figure 6.1. Mucosal immunization with heat-killed (HK) bacteria and CLDC adjuvant generates effective protection from both acute and chronic *B. pseudomallei* (*Bp*) infection. BALB/c mice (n = 4-5 mice per non-vaccinated control and CLDC alone groups, and 9 mice per heat-killed *B. pseudomallei* alone and heat-killed *B. pseudomallei* + CLDC groups) were primed intranasally with the adjuvant alone, 1 x 10^5 CFU heat-killed *B. pseudomallei* 1026b suspended in D5W buffer, or with heat-killed bacteria complexed to the CLDC adjuvant. Mice were boosted in the same manner 10 d later. All animals were then challenged intranasally with 7500 CFU live *B. pseudomallei* 1026b (8 x LD_{50}) 14 d following the boost and survival was monitored. Statistical differences in survival times were determined by Kaplan-Meier curves followed by log-rank test. The Bonferroni corrected threshold was applied and comparisons with $p < 0.013$ were considered significant. (*$p = 0.01$ for mice vaccinated with heat-killed bacteria alone vs. mice vaccinated with heat-killed bacteria adjuvanted with CLDC), indicating the significant effect elicited by the CLDC adjuvant on protection. Data shown are representative of 2 combined independent experiments.

6.4(2) Mucosal vaccination with CLDC adjuvanted *Burkholderia* protein subunits elicits protection from acute pneumonic melioidosis

Once CLDC was identified as an effective mucosal adjuvant for protection from both acute and chronic melioidosis (**Fig. 6.1**), this adjuvant was tested in conjugation with a variety of *Burkholderia* protein subunits. BimA and BopA, both *B. mallei* proteins, and the *B. pseudomallei* protein, LolC, were all tested within these studies. BALB/c mice were vaccinated with BimA, BopA, or LolC, all in conjugation with the CLDC adjuvant. To assess the impact of the CLDC adjuvant in conjugation with the individual protein subunits, another group of mice were vaccinated with the BopA subunit alone. Control mice remained unvaccinated. Mice were primed and boosted 1-2 times, and challenged with live *B. pseudomallei* 1026b two weeks after the final boost. Our data indicated short-term protection elicited by the three adjuvanted protein antigens lasting for 20 d in 40-60% of the mice (**Fig. 6.2**). However, this protection waned over time, as the majority of the mice succumbed to disseminated chronic melioidosis by day 60 post-infection. When mice were vaccinated with the BopA subunit alone, there was no protective effect initiated and these mice succumbed to acute disease similar to that observed in non-vaccinated controls. These results indicate the necessity of the CLDC adjuvant within this mucosal vaccine for pneumonic protection from *B. pseudomallei.*

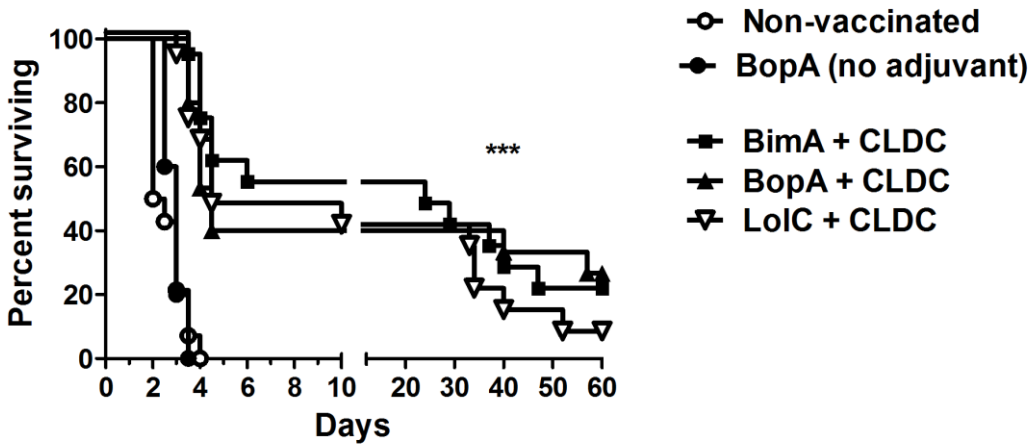

Figure 6.2. Mucosal vaccination with *Burkholderia* protein subunits in conjugation with CLDC elicits short-term protection from pneumonic *B. pseudomallei.* BALB/c mice (*n* = 5 mice) were vaccinated with the BopA subunit alone, and other groups (*n* = 15 mice per group) were vaccinated with BimA, BopA, or LolC, all in conjugation with the CLDC adjuvant. Control mice (*n* = 14 mice) were left unvaccinated. Vaccinated mice were primed and boosted 1-2 times, and challenged with 3 x 10^3 CFU live *B. pseudomallei* 1026b (3 x LD_{50}) two weeks after the final boost. Survival was monitored for 60 d post-challenge, and mice were euthanized according to pre-determined humane end-points. Approximately 40-60% of the mice were protected until day 20 from lethal *B. pseudomallei* challenge. All antigens tested conferred increased survival and time to death compared to non-vaccinated control animals (***, $p < 0.0001$ for BimA, BopA, and LolC vaccinated groups vs. non-vaccinated controls). Data shown are representative of 3 combined independent experiments.

6.4(3) Intranasal vaccination with Bp422, a *B. pseudomallei* 1026b Δ*asd* derivative, offers protection from acute *B. pseudomallei* infection

Two different attenuated mutant *B. pseudomallei* strains were tested in these studies as potential live vaccine strains. Bp422, a Δ*asd* 1026b derivative, was tested for both attenuation and as a live vaccine strain in our murine melioidosis model. This strain is a diaminopimelate (DAP) auxotroph and unable to crosslink D-alanine in neighboring peptidoglycan during cell wall synthesis [36-37]. To ensure *in vivo* attenuation of this strain, BALB/c mice were challenged intranasally with 1 x 10^7 CFU Bp422 (10,000x LD_{50} for wild type 1026b) and monitored for survival. Despite the observation of ruffling and mild respiratory symptoms in the initial days following challenge (most-likely due to the high burden of LPS), this Bp422 was completely non-lethal to the mice (**Fig. 6.3**). At 75 d post-infection, the lungs, livers, and spleens were all plated from infected animals to rule out long-term colonization by this mutant. All organs were sterile at this time, indicative of complete bacterial clearance and no persistent colonization by Bp422 (data not shown).

Bp422 was also tested for vaccine efficacy against lethal *B. pseudomallei* challenge. BALB/c mice were vaccinated intranasally with 1 x 10^7 CFU Bp82 and boosted 3 weeks later. Two weeks after the boost mice were challenged intranasally with 4 x 10^3 CFU (4 x LD_{50}) *B. pseudomallei* 1026b and survival was monitored. Non-vaccinated control mice succumbed to acute disease end-point by day 3 post-infection, whereas 100% of mice vaccinated with Bp422 were protected from the lethal challenge for 16 d (**Fig. 6.4**). However, Bp422 vaccination failed to protect from long-term chronic melioidosis, as all mice eventually succumbed to disseminated infection end-point by 60 d post-challenge with *B. pseudomallei* 1026b (**Fig. 6.4**).

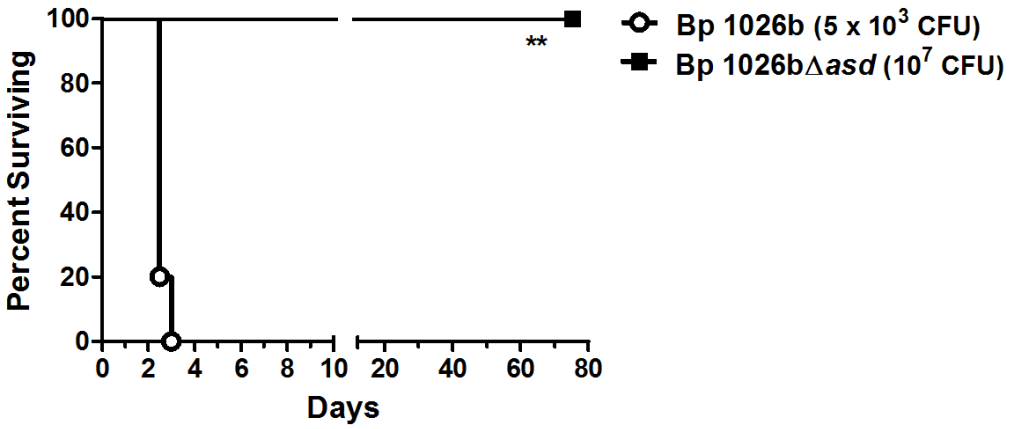

Figure 6.3. Bp422, a Δ*asd* 1026b derivative, is completely avirulent to mice. BALB/c mice (*n* = 5 mice per group) were challenged intranasally with 1 x 10^7 CFU Bp82 and survival was monitored. For purpose of comparison, survival from mice challenged with 5 x 10^3 CFU wild type 1026b is shown. (**, $p = 0.0016$ for mice challenged with Bp422 mutant vs. wild type 1026b strain). And the end of this observation period, their lungs, livers, and spleens were plated for sterility, and no *B. pseudomallei* bacteria were isolated (data not shown).

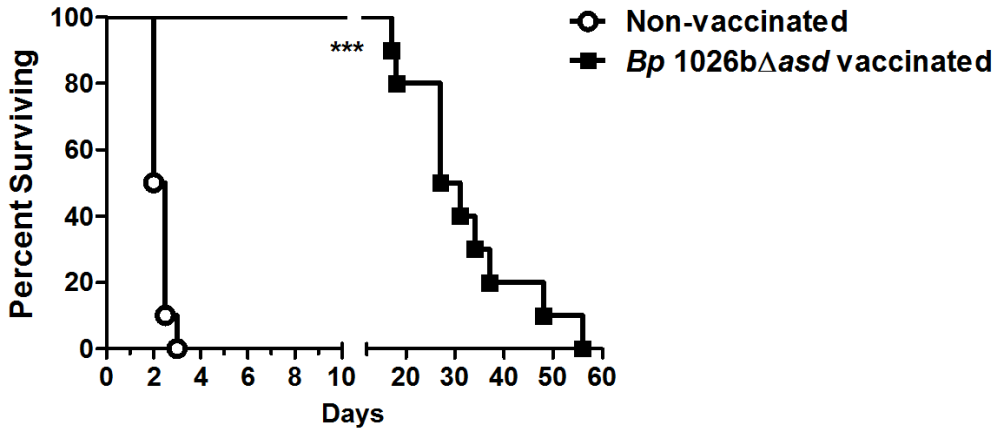

Figure 6.4. Vaccination Bp422, a Δ*asd* 1026b derivative, elicits protection from acute pneumonic melioidosis. BALB/c mice (*n* = 10 mice per group) were vaccinated intranasally with 1 x 10^7 CFU Bp422, an attenuated Δ*asd* 1026b derivative, and boosted in the same manner 3 weeks later. Control mice remained unvaccinated. Two weeks following the boost, mice were challenged intranasally with 4 x 10^3 CFU (4 x LD_{50}) *B. pseudomallei* 1026b. Mice were monitored for disease symptoms twice daily and were euthanized according to pre-determined humane endpoints. (***, $p < 0.0001$ for vaccinated mice vs. non-vaccinated controls). Data shown are representative of 2 combined independent experiments.

6.4(4) Intranasal vaccination with Bp82, a fully attenuated *B. pseudomallei* 1026b Δ*purM* derivative, offers protection from lethal *B. pseudomallei* disease for 100 days

A second attenuated mutant tested for protective efficacy against pneumonic *B. pseudomallei* challenge was Bp82, a Δ*purM* 1026b derivative. Complete attenuation in both immune competent and immune deficient has been previously demonstrated within our previous studies [34], and Bp82 has since been excluded from Select Agent regulations. Two different routes of vaccination were investigated, including both intranasal and oral delivery. For intranasal vaccination, BALB/c mice were vaccinated with 1 x 10^6 CFU freshly growing Bp82 and boosted two weeks later. Fourteen d following boost, mice were challenged intranasally with 5 x 10^3 CFU *B. pseudomallei* 1026b (5 x LD_{50}) and survival was monitored (**Fig. 6.5**). All of the mice were protected from acute pneumonic melioidosis, as 100% survived for 37 d following lethal pneumonic challenge. Three of the 5 animals went on to survive beyond 100 d, illustrating the ability of Bp82 to protect long-term from disseminated *B. pseudomallei* infection. (Two of these animals succumbed to chronic disease post-100 d).

It is also worthy to note that during this long-term observation period lasting greater than three months, the mice vaccinated with Bp82 had less chronic disease symptoms than all other vaccinated survivors within these studies. It was observed that mice vaccinated intranasally with this attenuated mutant had markedly less ruffling, squinty eyes, weight loss, and splenic lesion formation compared to the animals vaccinated with other formulations tested in these studies (heat-killed, protein subunit, and Bp422 vaccinated mice). Also worthy of mention is that the Bp82 vaccinated survivors unexpectedly experienced a cage flood within the first 20 d post-challenge in which this stress induced on the animals could have potentially negatively impacted long-term survival.

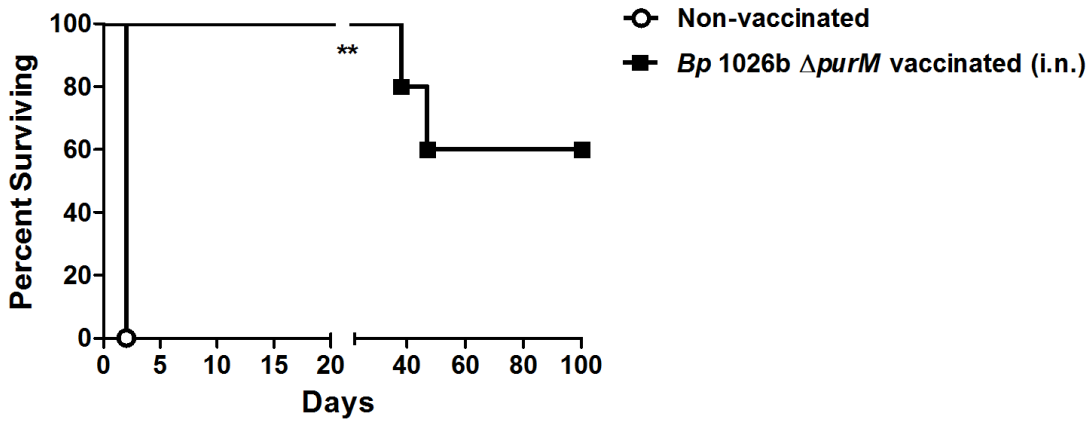

Figure 6.5. Intranasal vaccination with Bp82, a fully attenuated *B. pseudomallei* 1026b Δ*purM* derivative, offers protection from both acute and long-term chronic melioidosis. BALB/c mice (*n* = 5 mice per group) were vaccinated intranasally with 1 x 10^6 CFU Bp82 and boosted in the same manner 2 weeks later. Control mice remained unvaccinated. Two weeks following the boost, mice were challenged intranasally with 5 x 10^3 CFU (5 x LD_{50}) *B. pseudomallei* 1026b. Mice were monitored for disease symptoms twice daily and were euthanized according to pre-determined humane endpoints. (**, $p = 0.003$ for vaccinated mice vs. non-vaccinated controls).

6.4(5) Oral vaccination with an attenuated strain increases survival time following lethal intranasal *B. pseudomallei* challenge

The pneumonic delivery of attenuated bacterial strains to humans has not been widely approved. However, oral delivery of avirulent strains is often considered more acceptable, and live attenuated strains have been approved for human use to date, including the *Salmonella* vaccine strain Ty21a and other attenuated *Salmonella* strains [39-40]. In addition, an oral inactivated whole-cell *Pseudomonas aeruginosa* vaccine has been tested in a phase 1 clinical trial with the overall the purpose of reducing pulmonary *P. aeruginosa* colonization [41]. Based on the similarity between *P. aeruginosa* and *B. pseudomallei*, and the overall acceptance of the oral vaccine route, we wanted to determine whether oral vaccination with Bp82, a Δ*purM* fully attenuated strain, would protect mice from pneumonic *B. pseudomallei* challenge. Since Bp82 is exempt from all Select Agent regulations and is fully attenuated [34], it has potential as a possible human vaccine strain. For oral vaccination, BALB/c mice were gavaged with inoculums ranging from 1×10^7 CFU to 1×10^9 CFU, as we found that protection was not affected by the vaccine dose (data not shown). Mice were boosted orally two weeks later, and challenged intranasally with 5×10^3 CFU *B. pseudomallei* 1026b ($5 \times LD_{50}$) two weeks following the boost. For the majority of the mice, survival was increased by 1 d (**Fig. 6.6**). However, 20% of the vaccinated mice were completely protected from melioidosis for more than 30 d following pneumonic challenge. When comparing the orally vaccinated mice to non-vaccinated controls, there was a significant increase in survival time ($p = 0.003$).

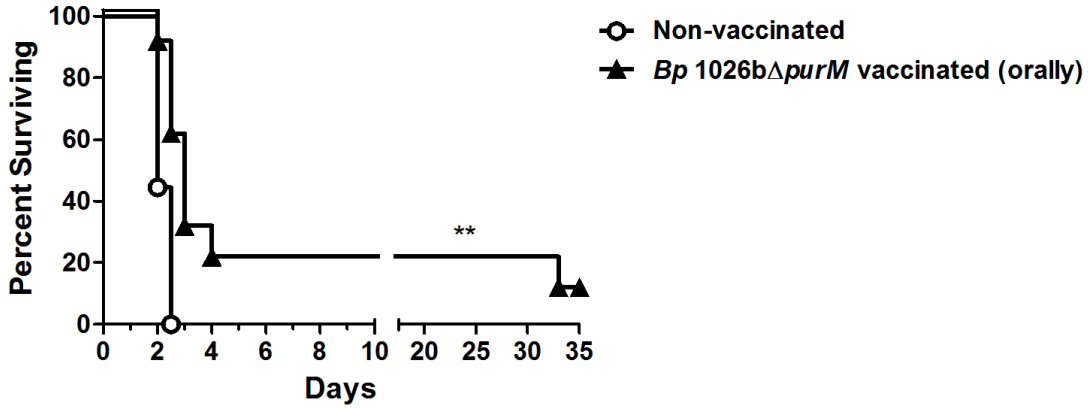

Figure 6.6. Oral vaccination with Bp82, a fully attenuated live *B. pseudomallei* strain, increases survival time following pneumonic challenge. BALB/c mice were orally vaccinated by intragastric gavage with 1 x 10^7 CFU to 1 x 10^9 CFU Bp82, a Δ*purM* 1026b derivative, and boosted orally two weeks later. Two weeks following boost, mice were challenged intranasally with 5 x 10^3 CFU *B. pseudomallei* 1026b (5 x LD_{50}) and survival was monitored. Mice were monitored for disease symptoms twice daily and were euthanized according to pre-determined humane endpoints. Control mice remained unvaccinated. (**, p = 0.003 for vaccinated mice vs. non-vaccinated controls). Data shown are representative of 2 combined independent experiments.

6.5 Discussion

Melioidosis is a highly fatal disease and there is currently no approved vaccine for human use [42-43]. The primary purpose of this research was to develop an effective mucosal vaccine for *B. pseudomallei.* We examined the protective efficacy of a variety of *B. pseudomallei* vaccine formulations, including killed whole bacteria, purified protein subunits, and two live attenuated strains. We examined the protection elicited by the different vaccine formulations from both acute pneumonic infection and also disseminated chronic melioidosis over several months. Previous melioidosis vaccine research has most often used the intraperitoneal vaccination route; however mucosal delivery of antigens has been shown to most efficiently produce mucosal immunity [24-26]. Therefore, the protective efficacy of all vaccine formulations tested within our studies was examined following intranasal vaccination and one live attenuated strain was delivered orally. All challenges following vaccination were conducted intranasally, as the inhalational challenge route is considered the most lethal route of *B. pseudomallei* infection [27] and the route considered most likely during an intentional biological release.

The first goal of this research was to identify an effective mucosal vaccine adjuvant for use with purified *Burkholderia* protein subunits. Antigens adjuvanted by lipid-based adjuvants have previously been shown to elicit protection when administered mucosally against bacterial pathogens [31-33], and we have previously investigated cationic-liposome-DNA complexes (CLDC) as a *Burkholderia* pre-exposure prophylaxis [29] and as an enhancement to antimicrobial therapy [28]. Based on its proinflammatory properties, we chose to investigate the adjuvant potential of CLDC when delivered mucosally. The CLDC adjuvant was first tested in conjugation with heat-killed *B. pseudomallei*, and this vaccine formulation protected 100% of mice from lethal

pneumonic challenge for 40 d. Less than half of mice vaccinated with heat-killed bacteria alone were protected, illustrating the ability of the CLDC adjuvant to elicit protective mucosal immunity from both acute and chronic melioidosis. In previous vaccine studies using killed *B. pseudomallei*, mice were often vaccinated with non-adjuvanted bacteria [10]. However, our findings indicate the ability of an effective adjuvant to enhance protection with whole-cell killed bacterial formulations, and provide a probable rationale why complete protection has not been commonly reported in the literature with killed bacteria alone [4, 10, 12]

CLDC was also tested in conjugation with BimA and BopA, two recombinant protein *B. mallei* antigens, and the *B. pseudomallei* protein subunit, LolC. Since subunit vaccines are more often approved for human use than killed or attenuated live strains [4], these experiments provide insight into potential clinically relevant vaccines. Intranasal vaccination with each subunit adjuvanted by CLDC moderately protected mice from acute pneumonic disease, but failed to offer long-term protection from chronic melioidosis. LolC has been considered a promising vaccine candidate within the literature to date [4], however intranasal vaccination of mice within our studies protected only half of the animals from acute disease and this protection waned over time. In a study by Harland et al., intraperitoneal vaccination with adjuvanted LolC protected 100% of mice for greater than 20 d following intraperitoneal *B. pseudomallei* challenge [22]. We believe the enhanced protection observed in that study is due to the intraperitoneal route being far less virulent in murine melioidosis models than the pneumonic challenge route used in our studies [44]. Of all the vaccine formulations tested within our studies, the recombinant protein subunit vaccines were the least effective against acute pneumonic melioidosis, indicating the likelihood that an effective mucosal vaccine may need to encompass multiple antigens.

Live attenuated *B. pseudomallei* vaccines are considered the most effective of the melioidosis vaccine formulations investigated in mouse melioidosis models to date, as they consistently protect the majority of vaccinated mice from acute disease [4, 13-18]. However, as seen in other vaccine formulations, sterilizing immunity has also been lacking in the studies and has only been reported in a C57BL/6 model [13], which some consider to be a mouse strain more resistant to *Burkholderia* infection [45]. Our vaccine studies using live attenuated strains were largely consistent with findings in the literature. Intranasal vaccination with either of the attenuated strains tested protected 100% of mice from acute disease for at least 20 d post-infection with lethal *B. pseudomallei* 1026b. However, it should be noted that vaccination with Bp422, a live attenuated 1026b ∆*asd* derivative, was far less effective at protecting mice from long-term chronic disease than vaccination with heat-killed *B. pseudomallei* complexed with CLDC. Mice vaccinated with CLDC adjuvanted heat-killed bacteria were protected beyond 40 days post-infection, whereas mice vaccinated with Bp422 began to succumb to chronic melioidosis end-point shortly after 20 d.

A second live attenuated mutant tested in our studies for protective efficacy was Bp82, a fully attenuated ∆*purM* 1026b derivative [34]. Even though both Bp82 and Bp422 were constructed from the same strain background (1026b), Bp82 was shown to produce superior protection, indicating protection is dependent on the type of attenuated strain used. Intranasal vaccination with this strain demonstrated protection from not only acute pneumonic melioidosis, but long-term protection in 60% of the animals lasting beyond 100 d. To our knowledge, this is approaching the longest survival time recorded in the literature to date, especially following the highly lethal intranasal challenge route in BALB/c mice. This study definitely needs to be repeated, and long-term protection likely could be enhanced even further by adjuvanting Bp82 with CLDC or by boosting animals

during the chronic disease stage. Based on the protective efficacy of this attenuated strain and its exemption from Select Agent regulations, we believe this strain could serve as a likely vaccine option for high-risk persons, similar to the *Francisella tularensis* live vaccine strains (LVS) approved for use in exposed laboratory workers and military personnel [46-47].

Since pneumonic delivery of an attenuated *B. pseudomallei* strain is unlikely for human approval, we also investigated the protective efficacy elicited by oral delivery of Bp82. Oral delivery of attenuated *Salmonella* strains and an inactivated whole-cell *Pseudomonas* strain has been previously approved for human use [39-41], indicating the acceptance of this delivery route. It is also worthy to note that oral vaccination has been shown to elicit protection from pneumonic challenge in a variety of infection models, including *P. aeruginosa* and *F. tularensis* [48-50]. Previous findings have shown that intestinal vaccination induces IgA antibody production within the lower airways and protects against pulmonary bacterial infection [48-49]. Therefore, we investigated whether oral vaccination with Bp82 would protect mice from lethal *B. pseudomallei* pneumonic challenge. Even though oral vaccination significantly increased the time to death following lethal intranasal challenge, only 20% of vaccinated mice were protected beyond 4 d post-infection. This is far less protection than that observed by KuoLee et al. following oral vaccination with LVS and pneumonic *F. tularensis* challenge [50]. However, the protection could potentially be enhanced by orally boosting the mice with Bp82 in the period after lethal *B. pseudomallei* challenge. Protection may also be enhanced by adjuvanting Bp82 with CLDC, as we have recently discovered that CLDC adjuvanted vaccines are effective when administered orally [51].

In summary, these studies have identified CLDC as effective mucosal vaccine adjuvant and based on efficacy and safety, this adjuvant has great potential for human

use [52-53]. Adjuvanting additional promising *Burkholderia* antigens would be beneficial for future investigations. Even though approval of live attenuated strains for human use is less likely than fully inactive vaccine formulations [4], vaccination with attenuated *B. pseudomallei* strains have shown the most promise in terms of protective efficacy in our studies. Intranasal vaccination with the fully attenuated Δ*purM* 1026b strain effectively protected mice from acute and chronic melioidosis for a more than three months, and has great promise as a potential melioidosis vaccine candidate.

6.6 References

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CHAPTER 7

CONCLUDING REMARKS

7.1 Significance of Work

The research projects described in this Dissertation has made contributions to both the *Burkholderia pseudomallei* research community and the clinical side of melioidosis in terms of its prevention and treatment. A fully attenuated *B. pseudomallei* strain was extensively characterized *in vivo* and granted exemption of Select Agent regulations, greatly facilitating *B. pseudomallei* studies for the first time at biosafety level 2 (BSL-2) instead of BSL-3 containment. Virulence determinants were also investigated *in vivo* providing additional insight into the bacterial factors required for successful persistence *in vivo.* Clinically relevant findings include the identification of immunotherapies that efficiently enhance traditional antimicrobial therapy for the treatment of melioidosis, and the discovery of novel vaccine formulations and antigen delivery routes that provide long-term disease protection.

7.2 Specific Aims 1 and 2

B. pseudomallei is endemic to Southeast Asia and Northern Australia, and causes the fatal disease melioidosis in humans [1]. There are currently many challenges associated with this pathogen. One particular challenge is the Federal regulations that greatly hinder research with *B. pseudomallei.* Select Agent listing of this pathogen subjects research to strict Federal guidelines that govern its acquisition, possession and use. Research with *B. pseudomallei* in the United States can only be conducted by cleared personnel in inspected BSL-3 containment laboratories [2], greatly preventing research on this pathogen by institutions not possessing such facilities. The purpose of **Specific Aim 1** (Chapter 3) was to develop a fully attenuated *B. pseudomallei* strain that could be granted exemption of Select Agent regulations and safely handled in the BSL-2 setting. This Specific Aim addressed the challenge of the strict regulations restricting research on this pathogen, as the availability of such attenuated strains would both facilitate and accelerate sorely needed basic research with this priority pathogen.

The two attenuated strains constructed were Bp82 and Bp190, Δ*purM* derivatives of *B. pseudomallei* strains 1026b and K96243. Both strains are deficient in adenine and thiamine biosynthesis, but replication competent *in vitro* in rich medium. Both mutants were completely avirulent in mice following inhalational challenge with doses ranging from 3 to 5-logs greater than the established LD_{50} for wild type *B. pseudomallei* [3]. Bp82 was the mutant characterized most extensively *in vivo*, and this strain failed to replicate within the lungs or disseminate to the liver and spleen following inhalational challenge in mice. Both Bp82 and Bp190 were completely non-lethal to a strain of mice known to be hypersusceptible to *B. pseudomallei*, and Bp82 was also shown to be

completely avirulent to immune deficient mice and even Syrian hamsters. Upon this demonstration of complete attenuation in both immune competent and immune deficient animal models, Bp82 has been granted exemption of Select Agent regulations and is now approved for research within the BSL-2 setting. This is the most characterized attenuated strain of *B. pseudomallei* created to date, and is also the first *B. pseudomallei* strain approved for research outside of BSL-3 containment. The other attenuated *B. pseudomallei* strains constructed previously have not been as extensively characterized *in vivo* using immune deficient and hypersusceptible species, nor granted Select Agent exemption [4-7]. The availability of Bp82 to the research community greatly facilitates basic research on this pathogen.

B. pseudomallei was a bacterial pathogen largely unheard of in the Western world until its classification as a category B Select Agent [8]. Basic research on this pathogen has largely been lacking until recent years in the United States, and as a result, much is still unknown about *B. pseudomallei*. Many bacterial factors potentially contributing to virulence within the host have yet to be fully elucidated, thus providing a basis for **Specific Aim 2** (Chapter 4). The purpose of this Aim was to determine whether siderophores and resistance-nodulation-cell division (RND) efflux systems impact virulence within a murine melioidosis model. This was analyzed using a clinical *B. pseudomallei* isolate (strain 708a) naturally devoid of both the malleobactin siderophore and AmrAB-OprA efflux pump, and by the construction of a variety of deletion mutants.

B. pseudomallei 708a is a clinical melioidosis isolate that contains a natural deletion of more than 130kb of genetic material, including genes for malleobactin siderophore synthesis (encoded by the *mba* gene cluster) and a characterized efflux

system (*amrAB-oprA*) [9]. Despite such deletions, this strain caused clinical disease in the patient from which it was isolated and was also lethal in our murine melioidosis model, similar to that observed with the wild type strains 1026b and K96243. Upon the characterization of *B. pseudomallei* mutants lacking RND efflux systems, it was further determined that neither the AmrAB-OprA nor BpeAB-OprB system is required for full virulence following pneumonic challenge in a murine melioidosis model. Removal of one or both of these characterized systems failed to reduce the lethality of the mutant strains. In addition, deletion of the malleobacin siderophore (*mba*) and pyochelin receptor (*fptA*) failed to reduce lethality within our murine model, indicating that both the malleobactin and pyochelin siderophores are dispensable during *B. pseudomallei in vivo* infection. In fact, simultaneous removal of the genes *mba, fptA, and* the efflux system, *amrAB-oprA,* actually appeared to increase lethality of strain Bp416 in our murine model. These findings are contrasting to previous finding in the literature proposing the BpeAB-OprB efflux systems and siderophores are required for the virulence of *B. pseudomallei* or related bacterial species [10-13]. Our results indicate that *B. pseudomallei* is capable of utilizing additional iron transport pathways other than pyochelin and malleobactin during melioidosis that need to be characterized by future research, and the potential for great genetic diversity between different strain backgrounds.

7.3 Specific Aims 3 and 4

There are many challenges in the clinical setting with the melioidosis, in terms of both treatment of the disease and its prevention in endemic regions or for high-risk persons. The current antibiotic regimen recommended for treatment of melioidosis is months in duration [2], and even with antibiotic therapy, relapse still occurs in

approximately 13-23% of patients [14-15]. This relapse is most often the result of reactivation of the original infecting strain of *B. pseudomallei* [15-16]. New approaches are needed to improve the effectiveness of antimicrobial therapy for *B. pseudomallei* infection, providing the rationale for **Specific Aim 3** (Chapter 5). Its purpose was to determine whether immunotherapy could enhance the effectiveness of ceftazidime treatment. This was analyzed using both an *in vitro* macrophage infection model and a murine melioidosis model.

The first immunotherapy investigated within these studies was cationic liposome-DNA complexes (CLDC) which we have previously shown to be protective against *Burkholderia* and *Francisella* species [3, 17]. When combined with ceftazidime, CLDC synergistically increased intracellular bacterial killing by alveolar macrophages *in vitro* and produced multi-log reductions in *B. pseudomallei* counts. This finding was found to translate effectively to our *in vivo* murine melioidosis model as well, as nearly 100% of mice were protected from acute melioidosis when CLDC was combined with a low, subtherapeutic dose of ceftazidime. In addition, the brief 3-day course of treatment with combination therapy resulted in effective long-term chronic disease protection in our animals. Since recombinant IFN-γ (rIFN-γ) has been used in the clinical setting [18] and we demonstrated the protective effect from the combination therapy is dependent on IFN-γ [3], we also examined its effectiveness in combination with ceftazidime for treating melioidosis. The majority of mice were still protected from acute disease, but the rIFN-γ was not as effective as the CLDC in the prevention of disseminated, chronic melioidosis. Combined, these results indicate that ceftazidime effectiveness may be augmented in the clinical setting and disease relapse reduced when combined with an immunotherapy such as CLDC. Based on the efficacy of CLDC to produce a sustained IFN-γ release

over time and its safety in a variety of models, this immunotherapy has potential for use in the human melioidosis setting [19-21].

In addition to the challenges associated with melioidosis treatment, another obstacle lies in the prevention of this disease. Due to the high mortality rates with melioidosis, prevention strategies are vital and could be extremely effective, especially within endemic regions of the world [22]. There is currently no approved vaccine for human use and the diphasic nature of melioidosis, often characterized by an acute pneumonic/septic stage followed by chronic disseminated disease, has proven to be a challenge in many of the vaccine candidates investigated in animal models to date. There has commonly been a lack of long-term, sterilizing protection following vaccination in many of the murine vaccination studies conducted [23-24]. The intraperitoneal route has been most community utilized in previous studies for antigen delivery. However, since mucosal delivery of antigens has been shown to most efficiently produce mucosal immunity [25-27], the purpose of **Specific Aim 4** (Chapter 6) was to develop a mucosal vaccine for *B. pseudomallei*. Within this Aim, CLDC was investigated as a potential mucosal vaccine adjuvant, and a variety of vaccine formulations were tested for protective efficacy including heat-killed bacteria, protein subunits, and two attenuated *B. pseudomallei* strains.

When delivered intranasally with heat-killed *B. pseudomallei,* CLDC proved to be an effective vaccine adjuvant, as all of the mice were protected from melioidosis beyond 40 d after lethal intranasal challenge. Non-adjuvanted killed bacteria alone protected less than half of the mice from acute disease and all eventually succumbed to long-term chronic melioidosis, illustrating the protective effect initiated by the CLDC adjuvant. CLDC complexed to a variety of *Burkholderia* protein subunits offered partial protection

from acute disease, but was far less protective than the adjuvanted killed bacteria. The longest term protection from lethal challenge, with more than half of the mice surviving beyond 100 d, was afforded by the attenuated *B. pseudomallei* ∆*purM* strain, Bp82. However, this highly effective protection appears to be unique to Bp82, as when Bp422, a ∆*asd* attenuated mutant, was tested for protective efficacy, protection began to wane just beyond 20 d following lethal *B. pseudomallei* challenge.

Since Bp82 has been demonstrated to be fully attenuated and well-characterized *in vivo* [28], and also been granted Select Agent exemption, this fully attenuated mutant holds great potential for use as a vaccine strain. One of the biggest challenges with many murine vaccine studies is the lack of long-term protection, however Bp82 demonstrated the longest sterilizing immunity of all of the vaccine formulations tested within these studies and of those reported recently in the literature [24]. This strain has great potential as a vaccine option for high-risk persons, similar to the *Francisella tularensis* live vaccine strains (LVS) approved for use in exposed laboratory workers and military personnel [29-30]. In fact, Bp82 is even less virulent than LVS when delivered intranasally to mice [17] and is one of the most attenuated *B. pseudomallei* strains characterized to date [4-7, 28]. This strain also showed partial protection from lethal pneumonic *B. pseudomallei* challenge following oral delivery, illustrating the diversity in delivery routes that merit further study.

7.4 References

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