SELECTIVE ENRICHMENT OF BURKHOLDERIA PSEUDOMALLEI OUTER MEMBRANE VESICLES FOR VACCINATION AGAINST MELIOIDOSIS

An Abstract

SUBMITTED ON THE EIGHTH DAY OF APRIL 2016

TO THE GRADUATE PROGRAM IN BIOMEDICAL SCIENCES

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

OF THE SCHOOL OF MEDICINE

OF TULANE UNIVERSITY

FOR THE DEGREE

OF

DOCTOR OF PHILOSOPHY

BY

NICOLE KIKENDALL

APPROVED: _____
Dr. Lisa A. Morici – Chair

_____
Dr. James B. McLachlan

_____
Dr. Kerstin Honer zu Bentrup

_____
Dr. Deborah Sullivan

_____
Dr. Lucy C. Freytag

Abstract

Selective Enrichment of *Burkholderia pseudomallei* Outer Membrane Vesicles for vaccination against melioidosis

By

Nicole Kikendall

Doctor of Philosophy in Biomedical Sciences

Tulane University

Professor Lisa A. Morici, Chair

Burkholderia pseudomallei (Bp) is the causative agent of melioidosis, a disease with a mortality rate of over 40%, and is a major public health concern in the endemic regions of Thailand and northern Australia. Bp is a resilient pathogen capable of surviving in diverse environments including soil, fresh and seawater, and plant and animal tissues for extended lengths of time. Bp is intrinsically resistant to antibiotics, which contributes to persistence and relapse in over 25% of melioidosis patients, and there is currently no vaccine. Our lab has previously shown that immunization with Bp outer membrane vesicles (OMVs) derived from Bp grown in Luria Burtani broth provides significant protection against melioidosis in mice. However, this protection was limited to the acute phase of infection and animals immunized with OMVs were unable to clear the bacteria. In this work, we show that by manipulating the growth media to

simulate various bacterial niches, including the natural hypertonic soil environment (NaCl-supplemented), the limited-nutrient host macrophage intracellular environment (M9CG minimal media), and quorum sensing conditions (QS-molecule supplemented), OMV protein content can be modified to include those proteins potentially important for *Bp* survival and may contribute to protection against chronic or persistent infection. Here, we characterize the composition of selectively enriched Bp OMVs and demonstrate that enriched OMVs are non-toxic and well-tolerated both *in vitro* and *in vivo*. Immunization of BALB/c mice with QS OMVs elicits significantly greater OMV-, CPS-, and LPS-serum IgG along with cell-mediated immune responses compared to mice immunized with LB OMVs. LB, M9CG, and QS OMV immunization provided equal protection against aerosolized Bp through the acute phase of infection, and M9CG OMV-immunized mice demonstrated fewer signs of morbidity and less weight-loss over the course of infection, indicating potential control of the bacteria. These results suggest that immunizing with OMVs selectively enriched with intracellular proteins may elicit the necessary immune responses to protect against persistent melioidosis.

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Dedication

This dissertation is dedicated to my parents and sister for their unconditional love and support. It is also dedicated to my dog Posen, for getting me through it all.

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Chapter 1

Introduction

Selective enrichment of naturally-derived outer membrane vesicles as a vaccine against melioidosis

Burkholderia pseudomallei

Burkholderia pseudomallei (Bp) is a Gram-negative, encapsulated, facultative intracellular pathogen that is the causative agent of melioidosis, a disease with pneumonic and septicemic clinical manifestations. Bp is also an aerobic, motile, non-spore forming bacillus found in soil and water within endemic regions. Bp has a worldwide distribution with outbreaks of melioidosis occurring in Australia, India, southern China and Southeast Asia, the Middle East, and Central and South America. Bp is hyperendemic to Thailand and northern Australia, and melioidosis is responsible for 40% of all sepsis-related deaths in northeast Thailand [1,2]. Bp was recently categorized as a Tier 1 Select Agent by the Department of Health and Human Services as it poses a significant threat to human and animal health and has the potential for distribution as an aerosolizable biothreat agent. Due to this categorization, Bp may only be handled under biosafety level 3 (BSL-3) containment conditions. *Burkholderia pseudomallei*'s virulence is related to its genetic and phenotypic resilience and adaptability to changing environments such as soil and water or the human host.

Burkholderia pseudomallei is genetically related to *Burkholderia mallei*, the causative agent of glanders, and *Burkholderia thailandensis*, which is highly attenuated in humans and animals [3]. Bp has a large genome consisting of two circular replicons, chromosome 1 and chromosome 2. Chromosome 1 is the larger of the two, consisting of 4.07 Mb and contains genes involved in core functions

associated with central metabolism, cell growth, nucleotide and protein biosynthesis, and motility, whereas the smaller chromosome 2, 3.17 Mb, includes those genes involved in accessory functions such as environmental adaptation and virulence [4]. Bp's genome contains a number of genes that promote survival under diverse conditions and also many that modulate bacterial pathogenicity and host-cell interactions. The genome contains 16 genomic islands that have been acquired by variable horizontal gene acquisition and encode prophages and large numbers of hypothetical proteins [5]. The acquisition of over 6% of its genome by horizontal gene transfer is important to Bp's evolution to survive as both a saprophyte and an intracellular human pathogen [4,6].

Bp's natural environment is the soil in grass and root-dense areas. The bacterium is most often isolated from moist, slightly acidic, nutrient-rich soil and typically lives and replicates in the rhizosphere, which is the region of soil surrounding the plant root. The soil in this location is modified by the uptake and excretion of substances by the plant itself. In a symbiotic relationship, the bacteria contribute to the fixation of nitrogen and promote phosphate uptake by the plant [7,8] while benefiting from the deposition of minerals and nutrients from the plant roots. Here in the root zone, at the interface of soil and water, is the preferred habitat for Bp. This leads to the formation of a biofilm, a complex structure of slowly growing bacteria controlled by the release of quorum-sensing molecules [9], which allows Bp to remain in the environment for long periods of time, even during the dry season. However, studies have shown that Bp growth and proliferation are dependent on high water content of the soil and culturable Bp is most commonly isolated from

muddy, moist, clay-rich, acidic soil and pooled surface water of similar composition [8]. The bacteria is able to survive in a non-culturable form in distilled water for long periods of time, which implies an aquatic lifestyle in which long-term carbon storage is able to support a low-rate metabolism [10]. During dry seasons the bacteria is found in deeper locations where the plant root extends down to ground water, whereas during the rainy, or monsoon seasons, Bp is isolated from soil much closer to the surface. There is a clear positive association between seasonal rain or extreme weather events and incidence of melioidosis cases when the bacteria is found only a few inches into the soil and may be dislodged or carried by the wind [11]. Those characteristics allowing survival in adverse environments will be further discussed and investigated in **Chapter 2**. Bp is a natural saprophyte and it has no requirement to pass through an animal in order to replicate [12]. However, it has been shown to have a range of mammalian hosts including the following: cattle, pigs, horses, dogs, cats, dolphins, primates, and humans[13] [14].

Human infection with Bp may occur through a number of methods, including inhalation of bacteria carried in the air, ingestion of soil or water containing large amounts of the bacteria, or inoculation of pre-existing cuts or abrasions upon contact with contaminated soil or water. Bp has a large repertoire of virulence factors enabling it to survive and thrive in a mammalian host. Bp secretes a number of exotoxins including enzymes contributing to tissue necrosis, proteases, lipases, catalases, and hemolysins. This repertoire includes a cytotoxin, Burkholderia lethal factor 1 (BLF1), that interferes with host helicase activity and contributes to infection [15,16]. Bp is a motile species with antigenically conserved flagella [17], which

aids in its ability to invade host cells [18]. Extracellular Bp is able to subvert the innate immune system and the complement cascade through its surface polysaccharides, lipopolysaccharide (LPS) and capsular polysaccharide (CPS). Bp LPS is strain dependent, with genotype A (typical) comprised of strains occurring in Thailand and Southeast Asia. LPS genotype B (atypical) occurs in approximately 16% of Australian strains [19]. LPS consists of lipid A, the bacterial endotoxin, which is embedded in the phospholipid bilayer of the bacterial outer membrane, the core oligosaccharide (KDO), and the antigenic O-polysaccharide (OPS). The LPS of Bp contains two distinct OPS moieties, types I and II [20]. Type II OPS is essential for Bp serum resistance and virulence in the host by resisting complement-mediated bacteriolysis [21,22]. The LPS of Bp is less pyrogenic than that of *E. coli* and while it retains a level of lethal toxicity and is mitogenic, is considered "weaker" than the LPS of other Gram negative bacterial due to its longer fatty acid chains and TLR4 signaling through the TRIF pathway instead of the MyD88 pathway [19]. In addition, the CPS prevents host serum bactericidal activity and opsonophagocytosis by blocking C3b deposition and activation of complement-mediated bacteriolysis [23,24].

Bp is able to adhere to and invade a number of cell types including phagocytic cell types such as macrophages, neutrophils, dendritic cells, and non-phagocytic cells. Studies have shown that Bp has a broad tropism for epithelial cells, adhering to alveoloar, bronchial, laryngeal, oral, conjunctiva, and cervical cells [25]. In addition, the LPS of Bp has low macrophage-activation activity [19] and contributes to the survival of Bp within phagocytic cells. Bp expresses a number of genes that aid in cellular invasion, such as two adhesion genes, *boaA* and *boaB*, that code for a

trimeric autotransporter adhesin TAA [26-28]. Bp also expresses FhaB3, or filamentous hemagglutinin, which shares homology with Bordetella pertussis FhB, an adhesin that facilitates attachment during host cell infection [29]. In addition, Bp expresses the gene *pilA* for Type IV pilus proteins that aid in adhesion to host cells, particularly in the respiratory tract [25] where the pilus participates in GM1 and GM2 receptor binding on epithelial cells [30]. EF-Tu is a conserved protein on the surface of many pathogenic bacteria, including Bp, and the interaction between surface binding proteins and EF-Tu facilitates tissue invasion [31]. Once adherence has taken place, the bacteria are taken-up by the host cells via phagocytosis or invasion by phagocytic or non-phagocytic cells respectively.

The Type III Secretion System (T3SS) is a highly adapted, but conserved virulence mechanism that is essential to Bp's ability to invade and survive in both phagocytic and non-phagocytic cell types. The T3SS apparatus is a highly organized macromolecular machine that consists of 25 proteins that oligomerize to form an export apparatus, a basal body that spans the inner and outer membranes of the bacteria, and an extracellular needle that pushes the translocon through the host cell membrane. This structure is also known as the "injectisome" [32] and can export virulence factors directly into the target host cell's cytoplasm in order to subvert cellular functions and allow for the invasion or uptake of the bacteria [33]. *Burkholderia pseudomallei* encodes three T3SS clusters, two of which are important for plant pathogenesis, while numerous studies have shown that Burkholderia secretion apparatus (Bsa) T3SS-3 is essential for intracellular survival and replication in a mammalian host [34,35]. The event of invasion requires the involvement

of several T3SS effector proteins, BopE, BipD, and BsaQ [36,37]. The effector proteins interact with T3SS chaperones in the bacterial cytoplasm and are shuttled through the needle structure in coordinated secretion. The translocators, BipB, BipC, and BipD localize at the tip of the needle complex and when contact is made with the host cells, the molecules form a pore in the host membrane. The injectisome may then deliver bacterial T3SS effector proteins directly into the host cell cytoplasm. BopE, one such effector, acts as a guanine nucleotide exchange factor for cell cycle regulators that control the actin network in the host cells [38]. In this way, BopE induces rearrangement of the subcortical actin cytoskeleton and facilitates membrane ruffling and allows the bacteria entry into the host cell.

Once the bacteria is internalized, it must rapidly adapt in order to survive the intracellular environment. When the bacteria are taken-up by the host cell by phagocytosis or invasion, the bacteria enter into primary phagosomes. The Bsa T3SS also plays a crucial role in Bp's escape from the endocytic vesicles. Several studies have shown that Bp is able to rapidly escape the phagosome to avoid degradation through lysosomal maturation by lysing the endosomal membrane. BopA is a T3SS effector molecule that activates escape from the vacuole along with the genetic regulators *bsaZ* and *bsaQ* [39]. Once free in the cytosol, bacteria replicate and activate BimA-mediated actin-based motility through its Type VI Secretion System (T6SS) for invasion of adjacent cells via membrane protrusions. However, some cytosolic bacteria may be sequestered in autophagosomes as a host cell defense. These bacteria-containing autophagosomes may recruit the autophagy marker protein LC3 in a process known as LC3-associated phagocytosis (LAP), which stimulates further

phagosomal maturation as opposed to the canonical autophagy process [40,41]. During maturation the recruitment of other proteins including LAMP1, a late endosome/lysosome marker, occurs, leads to the subsequent fusion of phagosomes with lysosomes, leading to bacterial killing. However, in the case of Bp, the bacteria is able circumvent this process and actually redirect the host response to benefit bacterial growth and replication. Studies have shown that during this process, secretion of the T3SS protein BopA is substantially increased and mediates colocalization of LC3 with the bacteria [39,40]. Thus, Bp sequesters LC3 autophagy effector proteins and renders them incapable of inducing phagosomal maturation. In a similar manipulation, it has been speculated that Bp may have a structural or secreted protein associated with its secretion systems that actually triggers autophagy targeted to host cellular components in order to increase production of host nutrient resources and facilitate bacterial intracellular replication [41]. Thus, Bp has several methods of highjacking the host defense responses and redirecting use of these resources for its own benefit.

The bacteria are able to reside and replicate unimpeded in the host cell cytosol, leading to severe pathogenesis in the host [42,43]. Cif_{BP} or CHBP, the Bp Cif homologue, is a cyclomodulin effector blocks the host cell cycle progression to prevent apoptosis of infected cells and allow the bacteria to replicate [44]. Additionally, Bp has developed methods to evade killing by reactive nitrogen intermediates (RNI), such as nitric oxide (NO) [45]. Inducible nitric oxide synthase (iNOS) is responsible for generating nitric oxide within macrophages, which is essential to host defense against intracellular pathogens. However, upon

macrophage uptake, Bp does not activate expression of iNOS or the production of NO [46]. Thus, by bypassing the RNI-generating responses, Bp is able to promote its own survival. While the mechanisms by which this occurs have yet to be fully elucidated, our lab has shown that Bp induces macrophage production of human prostaglandin E2 (PGE2), which suppresses the macrophage bactericidal functions associated with Arginase 2 expression and NO production [47]. This inherent ability to turn-off macrophage killing enables Bp to survive and replicate intracellularly for long periods of time.

Bp also utilizes a T6SS for intracellular survival. Bp encodes six T6SS, with T6SS-1 being essential for mammalian pathogenesis, sharing upstream regulators with the T3SS, BspR and BsaN [48,49]. Several studies have shown that T6SS-1 is not normally expressed during regular culture of Bp in media, however it is expressed when Bp is taken up by host cells and also when Bp is grown in minimal media formulated to simulate the intracellular environment [50,51] . The T6SS expression is negatively regulated by iron and zinc, suggesting that the divalent cation depletion occurring during the intracellular lifecycle may serve as a signal for the active expression of this system [52]. The Bp T6SS-1 mediates actin-based motility and cell-to-cell spread for bacterial survival, spread to distal sites, and avoidance of host defense systems [53].

The gene cluster consists of approximately 20 proteins involved in the assembly, structure, and function of the apparatus [54]. The T6SS acts in a similar manner as the T3SS by forming a syringe-like structure through which the effector proteins may be ejected. Hemolysin-coregulated protein (Hcp) and valine-glycine

repeat protein (Vgr) assemble into a tube-like structure with a cell-puncturing needle-like cap. Hcp-1 proteins comprise the largest portion of the apparatus, each one building upon the last to create the tube complex that spans the inner and outer membranes of the bacteria and extends into the host cell, with VgrG capping the structure's end. Characterization of VgrG showed that it harbors a diverse set of functional domains, including actin cross-linking, peptidoglycan degradation, lipase, and ADP-ribosyltransferases[55].

An important role of Bp's T6SS in pathogenesis is that of actin-based motility. Once the bacteria has escaped from the phagosome and entered the host cell cytosol, the regulator virAG is expressed. VirAG is required for expression of the T6SS and BimA protein, which is actively involved in the binding and polymerization of host actin by Bp [56]. BimA is expressed on one end of the bacteria where it binds monomeric host actin molecules and stimulates actin polymerization in an Arp2/3-independent manner, despite the incorporation of the Arp2/3 complex in Bp's actin tails [57-59]. The T6SS secretes a number of effector molecules and proteins other than BimA that could potentially be involved in actin-tail formation, including BimB, BimC, BimE, VirA, and VirG [60]. BimA localizes at one pole of the bacteria where the actin assembly takes place and facilitates the actin rearrangement into a "comet" tail formation, which is typical of actin-based motility, has been observed at a single bacterial pole, implying directional motility. Bacterial motility within the cytosol provided by the BimA-mediated actin polymerization facilitates cell-to-cell spread and invasion [42].

Studies further evaluating the function of T6SS-1 suggested that multinucleated giant cell (MNGC) formation, a hallmark of Bp infection, is mediated by a T6SS effector molecule by activating the molecular machinery involved in host macrophage fusion [61]. Further, a recent study showed that T6SS-1 VgrG1 protein is required for MNGC formation [62]. MNGC are formed when the outer membranes of host cells are connected in such a way that the internal cytoplasmic compartments of the cells are fused together, thus creating a single "giant cell". This requires the two cells to be in close contact to disrupt and reorganize their outer membranes to incorporate the phospholipids of the other cell and fuse together. Toesca, et al., showed that intercellular fusion and MNGC formation during Bp infection requires the function of VgrG5 to stimulate this fusogenic process and facilitates the intercellular spread of Bp. Through this process, Bp is able to create granulomas and isolated niches that allow for unimpeded bacterial replication, growth, and spread while avoiding host humoral immune responses [60].

Bp is able to rapidly spread around the body and to colonize organ tissues and systems distant to the site of infection. A recent study showed that infected dendritic cells (DC) act as a vehicle for dissemination and the migration of these DCs facilitates systemic spread of Bp [63]. It is known that the migration of DCs from the site of infection to secondary lymphoid organs is an important factor for the development of specific adaptive immune responses against Bp, however Williams et al. demonstrated internalization of *Bp* by epithelial DCs at the site of infection and subsequent association of disseminated *B. pseudomallei* within DC in secondary lymphoid organs. In addition, DCs within the spleen, but not those at the site of

infection or the associated draining lymph node, appeared to provide an intracellular niche for *B. pseudomallei* replication [63]. The ability to disseminate via DCs exemplifies Bp's ability to spread and colonize systemic tissues distant from the site of infection.

Bp's quorum sensing (QS) systems contribute to its ability to spread and establish chronic infection. QS is an inherent characteristic of bacterial pathogenesis, in which gene expression is regulated in a cell-density manner that provides a niche that allows the bacteria to circumvent the host defenses and promotes pathogenicity. Bacteria are able to coordinate the production of an array of phenotypic characteristics in accordance with cell population size by producing diffusible cell-to-cell signals [64]. Quorum sensing works as a cell-to-cell communication pathway that enables bacterial populations to coordinate gene expression in response to cell population and density. These systems function by the bacteria producing and secreting a signal molecule into the surrounding environment. As the bacterial population grows, the concentration of signal molecules increases until it reaches a threshold concentration, at which time it binds to and activates its receptor protein, which is produced in significantly smaller proportions. The detection of the QS signal molecule by the QS receptor triggers a physiological response in all members of the population, thus ultimately reprogramming gene expression throughout the population [65]. In this way, QS both positively and negatively regulates diverse functions in bacteria including conjugation, antibiotic synthesis and resistance, extracellular enzyme and exopolysaccharide production, expression of extracellular virulence factors, and

biofilm formation and regulation [66]. One such QS system is hhqABCDE, which codes for a biosynthetic pathway for the synthesis and recognition of 2-alkyl-4(1H)-quinolones (AQs). AQs act as co-inducers for binding and activating a transcriptional regulator for the expression of acute virulence factors in Bp, such as iron chelation, antimicrobial activity, and vesicle formation [67,68]. Another QS network is comprised of *lux*I homologues, which are responsible for N-acyl-homoserine (AHL) biosynthesis, and *lux*R homologues, transcriptional regulators that are activated by their cognate AHLs. *Bp* has a complex QS system with 3 luxI and 5 luxR homologues, including *pml*IR, and numerous AHLs including N-(3-hydroxydecanoyl)-L-homoserine lactone, N-(3-hydroxyoctanoyl)-L-homoserine lactone, N-(3-oxotetradecanoyl)-L-homoserine lactone, and the most abundant being N-octanoyl-homoserine lactone (C8-HSL) and N-decanolyl-homoserine lactone (C10-HSL) [69]. C8 and C10-HSL have both been found in Bp culture supernatants. The relative timing of synthesis and subsequent concentration of these signaling molecules plays a distinct role in *Bp*'s pathogenicity and disruption of the *Bp* QS system results in reduced bacterial virulence in an inhalational infection model [70].

Acyl-HSLs C8- and C10-HSL are secreted by multidrug efflux pumps, such as BpeAB-OprB. Efflux pumps such as this, AmrAB-OprA, and BpeEF-OprC are attributed with *Bp*'s intrinsic resistance to antibiotics due to their ability to discharge aminoglycosides, macrolides such as gentamycin, kanamycin, streptomycin, spectinomycin, tobramycin, neomycin, eythromycin, and clarithromycin, in addition to chloramphenicol, and trimethoprim [71]. Inhibition of BpeAB-OprB has been shown to be clinically advantageous because it enhances the

susceptibility of *Bp* to commonly used therapeutic antibiotics, and attenuates cell invasion and cytotoxicity [70]. BpeAB-OprB function is essential for production of a number of QS-regulated virulence factors including siderophores, phospholipase C, MprA, and for biofilm formation [72].

The QS system is also responsible for positive regulation of biofilm formation and controlled maturation, particularly the secretion of C8-HSL by BpeAB-OprB and production of its receptor BpsR in *B. pseudomallei* [73]. Bacterial biofilms are the optimum site for QS activation because it is here that natural cell populations are at their highest density. The Bp biofilm consist of microcolonies producing protective exopolysaccharride [74]. This production of biofilms allows the bacterial cells to evade the host immune system and actively protects Bp against antibiotic agents, allowing persistence within patients. Bp grown in an induced biofilm resisted all antimicrobial agents tested and a viable biofilm remained after 24 hours of antimicrobial exposure, showing that these antibiotic resistant phenotypes could contribute to long-term persistence of Bp and disease relapse[75]. The amount of biofilm produced by Bp is strain-dependent and affected by the growth conditions. For example, the addition of glucose enhances biofilm formation, leading the authors of this particular study to postulate that this may, in part, explain why diabetics with poor glycemic control are more susceptible to chronic and relapsing infections [76]. The creation of a biofilm in systemic tissues, such as the spleen or lungs, is important to Bp's ability to resist antibiotics and establish a persistent infection within melioidosis patients [77].

Bp expresses a complex repertoire of proteins and polysaccharides that enable it to survive in drastically different environments, such as the soil or an animal host, and rapidly adapt to changes within these environments, for example rainy and dry seasons or extracellular vs. intracellular lifestyles within a host. These factors, which are described above, include but aren't limited to the following list: flagella, LPS, CPS, exotoxins, Type IV pili, T3SS, T6SS, QS systems, antibiotic efflux pumps, and production of biofilms. The multitude of virulence factors enables Bp to establish both lethal acute infection and persistent chronic infections with potential for latent reactivation in melioidosis patients.

Host Immune Responses to *Burkholderia pseudomallei*

The innate immune system is the first line of defense against invading pathogens. Toll-like receptors (TLRs) are among the first to recognize the pathogen and initiate the signaling cascades that lead to immune response activation and subsequent clearance of the pathogen. Melioidosis patients have been shown to have an upregulation of TLR1, TLR2, TLR4, and TLR5 [78]. These TLRs are present on the surface of both monocytes and neutrophils, which are important in the early stages of controlling infection and both cell types are rapidly recruited to the site of infection [79,80]. TLR4 recognizes Bp LPS, and signals through MyD88 and TRIFF pathways to activate NF-κB for chemokine and cytokine production [81]. This interaction has been shown to drive the stimulation of cytokines in human melioidosis patients and this study showed that the innate immune response to Bp correlated most highly with the LPS response [82]. Recent experiments show that

wild-type (wt) Bp is able to survive and multiply inside human monocytes, whereas an LPS-mutant Bp strain is susceptible to host killing and this difference is mediated by the activation of IL-23 gene expression by wt Bp [83]. Wt Bp induced a significantly greater level of Il-23 secretion as compared to the LPS mutant and this difference strongly correlated with intracellular bacterial survival. These results implied that the increase in IL-23 may be important for the innate immune response during *B. pseudomallei* infection. Similarly, Bp flagellin activates TLR5 and Chantratita, et al., showed that TLR5 knockout mice are associated with impaired cytokine production and decreased survival through the acute phase of infection [84]. This indicates that an overstimulation and overproduction of inflammatory cytokines may be detrimental to the host during this early phase of infection. Due to the composition of its CPS, extracellular Bp is able to evade initial killing mechanisms by monocytes and neutrophils and can also evade C3b deposition and activation of the complement cascade. In order to survive the acute phase of infection with Bp, it is essential for the host to induce local rapid, but controlled, production of IFNγ. IFNγ activation of macrophages mediates subsequent phagocytosis by the macrophage, secretion of pro-inflammatory cytokines such as IFNγ, TNFα, IL-1β, and IL-6, and antigen-presentation to T cells [85]. In a murine model of infection, exposure to a more virulent strain of Bp led to more potent cytokine and chemokine innate immune responses, whereas infection with the less virulent strain had a lower level, but longer duration, of pro-inflammatory responses [86]. It was also noteworthy that the responses to the less virulent Bp were localized to the spleen, not the site of

infection or the associated draining LN, indicating that these bacteria were able to spread and colonize in systemic tissues.

As previously discussed, the resistance of Bp to complement means that effective clearance during the extracellular phase of infection is dependent upon opsonization by Bp-specific antibodies. LPS- and CPS-specific antibodies have been found to be opsonizing and promote polymorphonuclear (PMN) cells to kill extracellular bacteria [87,88]. Other Bp proteins that induce specific antibodies include flagellin, GroEL, BipD, Hcp-1, and OmpA [34,61,79,89-91]. In studies investigating infection in susceptible mouse strains strong Bp-specific IgM and IgG antibody titers correlate with survival [91]. In clinical investigations, melioidosis patient serum included IgG1, IgG2, and IgG3, with IgG1 the predominant subclass [92]. Due to the ability of Bp to invade host cells and replicate intracellularly, the role of antibody responses is critical during the acute phase of infection, but these responses are insufficient to completely clear bacteria and prevent persistent infection.

Studies in murine models suggest that T-cell mediated IFNγ responses are important for achieving protection against melioidosis [93]. Furthermore, strong combined $CD4^+$ and $CD8^+$ responses were associated with survival in patients with acute melioidosis [94]. Despite these implications, there is no correlation between HIV status and susceptibility to melioidosis, suggesting that $CD4^+$ T cells may have a more limited role while $CD8^+$ T cells may play a major part in protection [95]. Alternatively, a study in mice depleted of either $CD4^+$ or $CD8^+$ T cells prior to infection showed that $CD4^+$ responses, and not those of $CD8^+$ cells, were necessary for resistance to chronic infection [93]. Serum of melioidosis patients shows high

concentrations of $CD4^+$ T cell-induced proinflammatory cytokines and immunoregulatory cytokines along with granzymes A and B from CD8 T cells [96]. IFNγ, TNFα, and IL-12 have all been shown to contribute to a Th1 response against Bp infection, with the predominant cytokine produced being IFNγ. All three cytokines were shown to be essential for protection against acute disease and controlling bacterial burdens in host tissues [93]. Collection of bacterial burdens of Bp infected mice at day 35 post-infection demonstrated that those animals with the greatest combined antibody and T-cell immunity were able to successfully reduce the bacteria [89]. Experimental infection data suggests that an active combination of both humoral and cell-mediated responses is necessary to inhibit infection and promote bacterial clearance to prevent acute and chronic disease.

Melioidosis

Infection with Bp causes the disease known as melioidosis. The first case of human melioidosis was described in Burma by the pathologist Dr. Alfred Whitmore as a "glanders-like" disease in 1911 [97]. It wasn't until 1949 when cases began to be reported in Australian sheep and subsequent human patients in Queensland that cases of melioidosis began to be documented and tracked [98]. Melioidosis occurs in a wide range of animals with varying degrees of severity and disease manifestations. In Thailand melioidosis is the third leading cause of death due to an infection after HIV/AIDS and *Mycobacterium tuberculosis*, with a mortality rate over 40% and relapse occurring in up to 25% of patients [99]. In another highly endemic region, the tropical "Top End" of northern Australia, melioidosis is the most common cause of

fatal community-acquired bacteremic pneumonia occurring in approximately 50 in 100,000 people per year and a fatality rate of 20% [100]. Infection may occur through subcutaneous inoculation, ingestion, or inhalation of the bacteria. This normally occurs through contact with contaminated soil or water. The route of infection contributes to the exhibited symptoms and severity of disease [11]. The highest prevalence of melioidosis is observed in individuals with a high level of exposure to soil in regions where Bp is endemic, such as rice farmers, indigenous peoples, active service, miners, and travelers, with the highest incidence rates occurring during the wet seasons. The route and dosage of infectious material are important for the development of symptoms and the resulting severity of disease.

Inhalation remains an important mode of acquisition. This was first observed in studies of U.S. soldiers returning from Vietnam with helicopter crews presenting high incidence and a long incubation period for activation, up to 62 years in one case [101]. This long incubation period was how melioidosis became known as "the Vietnamese time bomb". Also, the correlation of high numbers of cases associated with pneumonia with periods of heavy rainfall indicates that inhalation may be the chief mode of acquiring disease during extreme weather events in endemic areas. As previously mentioned, due to the risk of developing severe disease through inhalation, Bp is also considered a biothreat agent with the potential for spread through aerosolization [102]. Ingestion of Bp has also been recorded as a method of developing melioidosis, particularly in cases following extreme weather events, such as tsunamis, with contamination of drinking water likely as the source [10,103]. However, inoculation of bacteria through pre-existing cuts or abrasions appears to

be the most common mode of acquisition. In highly endemic regions, such as Thailand, it is common for agricultural workers, particularly rice farmers, to have poor foot protection and subsequent wounds on their feet while wading in contaminated soil, mud, and water during planting and harvesting [104]. Zoonotic transmission has also been associated with contact with pre-existing wounds in veterinarians and livestock caretakers [97]. There are also a number of risk factors that make one more susceptible to acquiring disease from Bp including diabetes mellitus, alcoholism or excessive alcohol consumption, chronic pulmonary disease, chronic renal failure, or immune suppression. One or more of these risk factors is present in over 80% of melioidosis patients regardless of location, route of infection, or disease presentation [105].

Melioidosis has an incubation period of 1-21 days post initial infection with a mean of 9 days for signs and symptoms of disease to develop. Pneumonia is the most common presentation and is involved in over half of all cases. Lung involvement occurs after the spread of bacteria following inoculation or by direct infection of the epithelial layer by infectious inhalation. Respiratory symptoms include coughing, breathlessness, and pleuritic chest pain and typically show evidence of alveolar infiltrates within 48 hours of admission [106]. Sepsis syndrome results more commonly from Thailand strains than those occurring in Australia, with melioidosis responsible for 20% of community-acquired septicemias in Thailand [11]. Sepsis typically includes fever, rapid heart and respiratory rates, low blood pressure, and abnormal white blood cell counts, and when progressed to severe sepsis includes organ dysfunction. Septic shock, defined as the presence of

sepsis with related organ dysfunction and persistent hypotension that is unresponsive to fluid therapy, was present in 21% of patients in a 20-year study conducted in Darwin, Australia [100,106]. This same study showed that bacteremia, or the presence of bacteria in the bloodstream, correlated with higher mortality rates and was present in 40% of cases. Patients who were bacteremic had a mortality rate of over 20%, while the rate of those who presented with non-bacteremic melioidosis was only 7%. The mortality rate in patients with septic shock was 50% [100].

Other symptoms of melioidosis include skin infections, osteomyelitis, neurological effects, and abscess formation in organ tissue. Skin and soft tissue infections are common and tend to be rapidly progressive, as they are typically the result of hematogenous spread. Infections within the bone and joints are rare and may be related to the previously mentioned pre-existing risk factors. Infection of the central nervous system occurs in a number of animal species, including humans and focal suppurative infections of the CNS occurred in up to 4% of patients [3]. Encephalomyelitis, characterized by brain stem encephalitis and flaccid paralysis, is rare but associated with significant morbidity and mortality [11]. Melioidosis can be associated with single or multiple purulent nodules or abscesses located in nearly any or all organ tissues. Infection may spread from the initial site of infection and affect many different organ or tissue systems including the following: mycotic aneurysms, mediastinal infection, corneal ulcers and subconjunctival abscesses that spread to the sinuses, myocardial abscesses and endocarditis, thyroid abscesses,

and scrotal abscesses [11,100]. The most common sites of infection and abscess formation are the lungs, spleen, or liver and their associated lymph nodes.

It is also important to note the differences in disease manifestation depending upon the region of endemicity. Two important clinical differences between patients in Australia compared to those in Thailand is the high incidence of genitourinary involvement in Australia and suppurative parotitis in Thailand. Prostatic abscesses occur in 18% of male patients in Australia and are much more commonly seen in those men presenting with chronic infection (exhibiting symptoms for longer than two months). Alternatively, Thailand had a negligible rate of prostatic abscesses. However, acute suppurative parotidis occurs in up to 40% of pediatric cases in Thailand whereas there were no recorded instances in Australian patients [11]. The differences in disease manifestation and the range of symptoms are important for clinicians to account for when distinguishing melioidosis from other infections, particularly in endemic regions where disease is acquired through a number of different routes.

Melioidosis is notoriously hard to diagnose and is known as “the great mimicker” due to its range of symptoms that resemble those of other diseases and the difficulty in isolating it from patients, as subclinical melioidosis is prevalent in endemic areas. Diagnosis depends upon a range of factors including: bacterial strain, dose, route of infection, and the presence of previously described risk factors [107]. Obtaining an active culture from any clinical sample is the preferred method of diagnosis. Polymerase chain-reaction (PCR) is also an accepted method but is much less sensitive, particularly when using blood samples [108]. Also, serological testing

alone is inadequate for proper diagnostics due to the high rate of seropositivity in highly endemic regions [3,106]. Prognosis is typically associated with response to therapy and indicators of organ failure due to infection. Markers of organ dysfunction, including leukopenia, hepatic dysfunction, renal dysfunction, and metabolic abnormalities on admission appear to predict mortality [109].

Due to Bp's previously described inherent antibiotic resistance, therapeutic treatment for melioidosis is quite extensive and involves two separate phases. Bp is resistant to treatment with most common therapeutics, including the following: first, second, and third generation cephalosporins, aminoglycosides, penicillins, and polymyxin. The initial intensive phase requires 10-14 days of ceftazidime, meropenem, or imipenem administered intravenously. Ceftazidime is a drug that inhibits DNA and protein synthesis and is excluded from extraction by the amrAB-oprA efflux pump [110,111]. This is immediately followed by the oral eradication phase, which consists of 3-6 months of oral trimethoprim-sulfamethoxazole (TMP-SMX) or, in pediatric cases or those in pregnant women, amoxicillin-clavulanate. However, amoxacillin-clavulanate has a much higher rate of relapse and is reserved for those patients whom are unable to use TMP-SMX [101,109]. Even after apparently successful treatment, relapse is common and occurs in up to 25% of cases with a mortality rate similar to that of initial infection [112]. The mean time of relapse of infection was eight months after initial diagnosis, but cases have been documented of reactivation occurring many years later. In the vast majority of cases, relapse is due to the reactivation of the original infecting strain, with only 6% of cases occurring due to infection with a different strain [100]. Relapse is associated with poor adherence to

eradication therapy, improper use of antibiotics, such as doxycycline or amoxicillin-clavulanate, during this phase, or a shortened period of intensive phase treatment [108]. Compliance with such an extensive treatment regimen is difficult in the rural areas where infection is most common, with many patients living in remote communities where clinical observation and follow-up are unrealistic. The natural resistance of Bp to a growing number of antibiotics and the personal and economic costs of extensive treatment highlight the need for the development of an effective vaccine against melioidosis.

Melioidosis Vaccine Candidates

There is currently no vaccine available for prevention of melioidosis. Development of new vaccines against melioidosis require a thorough understanding of the pathogenesis of infection and the characteristics of naturally occurring immune responses which develop following environmental or experimental exposure to the organism. A successful vaccine against Bp may require the induction of antibodies, $CD4^+$, and $CD8^+$ T cells to multiple bacterial antigens in order to protect against both acute and chronic infection. There are currently many different vaccine formulations being investigated in pre-clinical trials. This list of potential vaccine candidates may be divided into the following four categories: killed whole cell, live attenuated, subunit, and the alternative approaches or those candidates that do not fall into one of the other larger categories, such as outer membrane vesicles (OMVs), which will be discussed here in subsequent sections.

Killed whole cell vaccines are relatively simple and inexpensive to produce. These consist of whole pathogens that have been heat or chemically killed. Studies have shown that immunization with killed whole cell bacteria is able to provide effective protection against various bacterial diseases including *Bacillus anthrasis*, *Bordetella pertussis*, and *Yersinia pestis* [113]. In this way, the vaccine is able to stimulate the immune system with a range of naturally expressed antigenic targets, but the bacteria is unable to replicate in the host. This provides a relatively high level of specific immunity, particularly to the CPS, LPS, and peptidoglycan of bacteria, but often requires multiple administrations to achieve the necessary responses to achieve protection. These vaccines are able to elicit a robust antibody response, but stimulate much weaker cell-mediated responses [114,115]. As a result of the inactivation process, these are less immunogenic and may necessitate the addition of an adjuvant in order to enhance the initial responses. Additionally, vaccine site reactions are also a potential problem with this type of vaccine, but this is generally attributed to use of adjuvant. This type of vaccine is safe for use in immune compromised individuals.

Bp has been tested as a heat-inactivated whole cell vaccine in various doses and routes and has been shown to protect against intraperitoneal (i.p.) challenge in mice [116]. This study also showed that antibody titers against CPS and LPS did not correlate with protection and the possible mechanism of protection provided by this vaccine may be $CD4^+$ T-cell dependent, but cell-mediated responses were not evaluated in this study. However, a previous study showed that only live vaccines were able to elicit the Th1 responses necessary for bacterial clearance while the

killed preparations were unable to do so, and all heat-killed-Bp immunized mice succumbed to infection within 5 days of intravenous challenge [117]. Henderson et al. built upon this knowledge and showed that intranasal (i.n.) immunization with heat-killed Bp combined with a liposomal adjuvant, cationic lipid-DNA complex (CLDC), provided superior immunity against lethal pulmonary challenge as compared to immunization with the killed bacteria alone [118]. In a more recent study comparing heat and paraformaldehyde killed Bp preparations, mice immunized with the chemically inactivated bacteria showed greater survival to day 30 against a lethal intraperitoneal challenge and both preparations showed little to no added benefit with the addition of an adjuvant [119]. This vaccine type has shown across these studies that it is capable of eliciting a strong antibody response; however, there needs to be further research into the induction of cell-mediated responses necessary for long-term protection and to combat disease progression.

Live attenuated vaccines are often capable of stimulating robust humoral and cell-mediated immune responses with only a single dose and are able to induce protective immunity in humans [120]. This makes them easy to produce and administer. These are able to replicate *in vivo* and thus stimulate immune responses against antigens expressed at various stages of disease. However, while this is an advantage of this class of vaccine, it is also one of its greatest weaknesses. There is concern over the risk of reversion to wild-type, uncontrolled replication, and infection caused by an attenuated strain. This is of particular concern in immune compromised individuals, who are at greatest risk of acquiring melioidosis, and live attenuated vaccines are contraindicated in this population.

Despite these concerns, there have been multiple studies investigating a number of Bp mutant strains as potential vaccines. 2D2 is an auxotroph for branched chain amino acids, produced by mutating the *ilv*I gene in Bp that encodes the large subunit of the acetolactate synthase enzyme [114]. This strain was unable to persist in vivo and provided significant, albeit incomplete, protection against intraperitoneal challenge with wt Bp by the same route as immunization [121]. Another auxotrophic mutant, strain 13B11, this one for *aroB*, was also able to be cleared from the tissues of immunized mice; however, it was unable to induce long-term protection against virulent challenge and all mice eventually succumbed [90,122]. In similar fashion, biosynthetic pathway mutants, *purN*, *purM, hisF, pabB,* and *lipB*, have all shown limited protection against virulent challenge in mice [80,123]. None of the candidates were able to induce sufficient protection against development of chronic infection. In addition, mutants of secretion system apparatuses, such as *bipD* , *bopA* and *bopB*, were able to provide only partial protection against systemic challenge and resulted in residual bacteria in various tissue [34]. Bp82 is a purine biosynthesis mutant *(purM*$^{-}$) that has exhibited an impaired capacity to replicate intracellularly even in immunocompromised animal models [124]. As a result, this strain has been approved by the CDC for use under BSL-2 conditions. Immunization with Bp82 gave significant protection against acute infection with reduced tissue bacterial burdens; however, vaccine-induced protective immunity was found to be independent of both $CD4^{+}$ and $CD8^{+}$ T cells, which may be necessary for protection against latent or chronic infection [90].

Two strains from genetically related species, *B. mallei* and *B. thailandensis* have also been investigated for heterologous protection against Bp. In a cross-protection study of acute inhalational melioidosis with *B. pseudomallei*, immunization with a live *B. mallei tonB* mutant, TMM001, which is deficient in iron acquisition, showed significant protection [125]. Despite this, mice were unable to clear the immunization strain TMM001. Similarly, immunization with live *B. thailandensis* E555, a strain expressing an antigenic capsule similar to that of Bp, induced significant protection against a lethal i.p. Bp challenge [126].

Recent research has shown a shift to subunit vaccines in order to increase the safety of administration and to induce immune responses specifically to potential protective antigens. These vaccines incorporate specific molecules and proteins derived from the bacteria, as opposed to using the bacteria in its entirety, which can result in a range of protective abilities and duration [127,128]. These vaccines may be complicated or costly to produce and they may require a combination of antigens along with supplementary adjuvant.

There have been several different immunogenic proteins in Bp that have been identified as potential candidate vaccine antigens. First, flagellin is known to be an important antigenic molecule [18]. One study examined the response to vaccination with plasmid DNA encoding the flagellin gene *fliC*. Immunization led to significant protection through the acute phase of infection (7 days) against intravenous challenge [129]. In addition, a study demonstrated that passive immunization of Bp flagellin-specific antiserum was able to protect diabetic rats from challenge with a heterologous Bp strain [17].

Another potential candidate is LolC, a Bp outer membrane protein associated with ATP-binding cassette (ABC) transport system [130]. Mice immunized with recombinant LolC (rLolC) protein exhibited an enhanced survival rate of up to 80% after six weeks post-infection [131]. Further investigation showed that immunization with rLolC coupled with immune-stimulating complex and CpG oligodeoxynucleotide (ODN) stimulated a Th-1 type immune response, which is necessary for bacterial clearance and long-term protection. In this same study, recombinant PotF protein, a periplasmic biding protein from the PotFGHI system, provided significant, but partial, protection against i.p. Bp challenge. However, it is not clear whether sterile immunity was achieved in the surviving mice and it is unknown whether either antigen is able to elicit long-term protection.

The outer membrane protein A (OmpA) family of proteins has been shown to be immunogenic and thus, have been investigated for inclusion in a subunit vaccine. Administration of Omp3 or Omp7 provided significant, but incomplete, protection against Bp challenge [132]. Related to the OmpA results, BALB/c mice immunized with recombinant Omp85 demonstrated enhanced survival time in addition to reduced bacterial burdens in tissues as compared to unvaccinated controls {Su:2010cy}. However, in all three cases, bacteria were isolated from survivor tissue, indicating that bacteria were not completely cleared and could lead to a latent infection.

Hcp proteins (previously described components of T6SS) have been identified as another set of subunit vaccine candidates. Six recombinant Hcp proteins (Hcp1–Hcp6) were tested for their ability to confer protection against i.p. challenge with Bp [61]. This study showed that mice immunized with rHcp2 protein

exhibited a survival rate of 80% out to 42 days post-infection, but these survivors did have bacteria isolated from the spleen at the study endpoint. However, vaccination using Hcp1, Hcp3, and Hcp6 only provided 50% protection in challenged mice after 42 days of infection, and no bacteria were found in the spleen of the survivors immunized with Hcp1 and Hcp6. This information implies that it may be necessary to combine antigenic factors in order to provide protection through the acute phase and to establish sterilizing immunity.

Among the most promising and well-characterized candidates to date are the surface polysaccharide antigens, LPS and CPS. Both are ideal candidates as there are a limited number of LPS O-antigen and CPS isolates expressed by Bp. Mice immunized with purified CPS and LPS stimulated strong antibody responses and significantly extended the survival time after systemic challenge [133]. A number of studies have shown that monoclonal and polyclonal antibodies specific for OPS and CPS can be used to passively immunize mice against lethal challenges of *B. pseudomallei* [87,134,135]. More recently, two glycoconjugates composed of OPS or CPS covalently linked to a common carrier protein were constructed, and evaluated against Bp infection [126,136]. The OPS glycoconjugate was unable to protect against acute infection and mice were heavily burdened with bacteria in all evaluated organ tissues. However, the CPS glycoconjugate provided significant protection to day 21 post-challenge. These mice had significantly lower tissue bacterial burdens than unimmunized mice, but sterilizing immunity was not achieved. When combined with rLolC, the CPS glycoconjugate provided enhanced protection and lower bacterial burdens than either rLolC or the CPS glycoconjugate alone [136].

While all classes of vaccines have promising candidates, it is important to note that no candidate has yet been able to achieve complete protection and meet all requirements for disease prevention in both the native population in endemic regions and against use of Bp as a biothreat agent. Given the abundance of risk factors for developing disease and the inability to easily treat Bp, development of a safe and effective vaccine capable of inducing both the humoral and cell-mediated immune responses necessary for bacterial clearance and long-term protection is essential. Based upon previous studies, it is important to consider using a combination of a number of antigenic factors that are expressed by Bp over the course of infection.

Outer Membrane Vesicles

Outer membrane vesicles (OMVs) are non-viable spherical structures derived and secreted from the surface membranes of the parent cell in order to interact with and influence the surrounding environment. Formation and release of vesicles is a process that is conserved in prokaryotes and eukaryotes, including Gram-negative and Gram-positive bacteria, archaea, parasites, and fungi [137,138]. Here, the focus will be on the OMVs of Gram-negative bacteria.

Vesicles are produced by the bacterium during the normal growth cycle. This occurs by the bulging of the outer membrane LPS leaflet in areas lacking membrane-peptidoglycan bonds away from the underlying peptidoglycan layer and periplasmic space in response to envelope stressor signals. This bubble-like structure pinches off and separates from the parent cell, resulting in a double-

membrane enclosed vesicle composed of outer membrane components with an inner lumen containing periplasmic and cytoplasmic proteins and lipids [139]. The resulting vesicles range between 20-250nm in diameter and may include a variety of virulence factors [140]. Gram-negative bacteria release vesicles in response to environmental stresses and can use vesicles in quorum sensing or as communication devices, decoys, or even to directly mediate the entry of the included virulence factors into host cells and may be involved in horizontal gene transfer [141,142]. However, these structures are non-viable and non-replicating. Because OMV composition is similar to that of the parent cell, vesicles are able to stimulate host immune responses. Isolation preparations of OMVs secreted from Gram-negative bacteria are enriched in several different TLR agonists, including LPS and flagellin [143,144]. These are able to stimulate murine macrophages to secrete pro-inflammatory cytokines *in vitro* [145]. Vesicles are immunogenic and depending upon the content, may stimulate adaptive immune responses that are protective during infection [146]. Due to their multi-component nature and size distribution, vesicles may also be taken up by host immune cells for antigen processing and presentation [147]. This allows for the presentation of native protective antigens by antigen presenting cells to prime the host immune system against the parent bacteria. In this way, OMVs may serve as an ideal platform for vaccine development.

OMV based vaccines have been used to protect against systemic serogroup B *Neisseria meningitidis* disease. There have been several approved formulations of OMV vaccines against meningococcal disease. It was discovered that naturally produced OMVs derived from acapsular N. meningitidis were highly immunogenic,

but had to undergo detergent extraction of lipooligosaccharide to render them non-toxic [148]. The initial wt OMV vaccine produced for general use was VA-MENGOCOC-BC, which was developed in Cuba to address an ongoing MenB epidemic from 1987-1989 [149]. MtenBVac, another wt OMV formulation, was developed in Norway to similarly combat a strain-specific epidemic [150]. Both wt OMV vaccines were most effective when used against epidemics of a homologous strain. When used against endemic disease or outbreaks due to a heterologous strain or a combination of a number of different strains, the level of effectiveness was too low to justify the cost of administration to the general public. Additionally, it was established that multiple doses of this vaccine formulation would be required for initial protection and a booster dose is required to ensure long-term protection, especially in those who receive their initial vaccine series as young infants [151].

Internationally licensed MeNZB consists of deoxycholate detergent extracted OMVs from strain B:4:P1.7b,4 NZ98/254, containing outer membrane proteins and small amounts of LPS, co-administered with Alum [152]. This formulation was originally developed in Norway to combat an outbreak, but was not used there as the epidemic had ended before the vaccine was approved for use. However, in New Zealand, it was administered to the population as a short-term measure to reduce risk during an ongoing epidemic from 2004-2008 [153]. Protection provided by this formulation was limited to strain-specific immune-dominant porin protein (PorA) [154]. While the vaccination strategy lead to a significant and rapid decrease in cases during this particular epidemic, the inability to protect against heterologous strains (such as groups A, C, Y, W135, and other strains of group B) and the necessity of

three administrations limits the use of this vaccine to outbreaks of the parent strain [155]. While MenB wt OMV vaccines can target a local clonal outbreak, it is apparent that due to the diversity of infectious strains and antigenic targets, this strategy may need to be re-evaluated and manipulated in order to achieve a universal vaccine against meningococcal group B disease. More recently, the US FDA granted accelerated approval of Bexsero or 4CMenB (Novartis) for immunization of adolescents and young adults during meningococcal outbreaks [156]. This formulation consists of four immunogenic components including three recombinant surface exposed proteins combined with OMVs derived from the New Zealand outbreak strain, NZ98/254 [157]. While this approval is promising for OMV vaccine formulations, the long term protection provided has yet to be demonstrated.

Our lab has previously demonstrated that an OMV vaccine consisting of naturally shed, native Bp OMVs was protective against melioidosis in mice [158,159].These vesicles were derived from Bp strain 1026b grown to late-log/early stationary phase in Luria-Bertani (LB) broth. These vesicles contained both LPS and CPS. The vesicles underwent phenotype characterization by liquid chromatography mass-spectometry (LC/MS) for protein content and transmission electron microscopy (TEM) for size and shape determination. Vesicles were found to be 50-250 nm diameter spherical structures comprised of an electron dense center surrounded by a double membrane and numerous antigenic proteins. These OMVs were non-toxic and non-replicating *in vitro* and *in vivo.* This study demonstrated that subcutaneous immunization of BALB/c mice provides significant protection

against lethal aerosol challenge and vaccinated mice displayed OMV-specific serum antibody and IFNγ cytokine production [159].

In a separate study, Bp1026b OMV immunization provided significant protection against systemic challenge with a heterologous strain, Bp K92643 [158]. This immunization induced Bp-specific serum IgG and IgM that, when passively transferred, provided protection against subsequent i.p. challenge with Bp strain K96243. In addition, our lab has demonstrated the safety and immunogenicity of this Bp OMV vaccine in nonhuman primates at escalating doses [160]. This study shows that immunization of rhesus macaques with Bp OMVs generates humoral immune responses to protective protein and polysaccharide antigens, particularly OMV-, LPS-, and CPS-specific plasma IgG, without associated toxicity or reactogenicity. However, the protective efficacy of a Bp OMV vaccine in a non-human primate model has not yet been investigated.

Despite the promising data achieved by these studies, immunized survivors of the murine challenge studies had persistent Bp infection at the study endpoint, which indicates development of a chronic or latent infection. These results emphasize the need to stimulate both humoral and cell-mediated immune responses against relevant Bp antigens in order to achieve sterile immunity.

Selective Growth Media and Protein Enrichment

Our previous work using OMVs naturally shed by LB-grown Bp showed significant, but incomplete protection against aerosol challenge [159]. As previously described, these OMVs contain multiple virulence factors, including LPS, CPS, and

surface and secreted proteins. However, Bp grown in rich lab culture medium, such as LB, behaves very differently than when the bacteria is residing in its natural environment, such as the soil or plant rhizosphere, or when it has infected host cells. Bp's phenotypic plasticity and variable expression of virulence factors contribute to its survival under these diverse and austere conditions. Understanding the regulation and expression of virulence factors that are expressed differentially depending on the environment is imperative to initiating the necessary immune responses to prevent infection and clear the bacteria. Studies have shown that the composition and components of Gram-negative OMVs can be manipulated by inducing environmental stressors [161]. The approach described herein manipulates Bp's regulation of protein expression by simulating Bp's natural soil environment, the host macrophage intracellular environment, and quorum sensing conditions during OMV production. This approach will be discussed in greater detail in Chapter 2.

Briefly, as previously stated, the bacterium is most often isolated from moist, slightly acidic, nutrient-rich soil with an unusually high salt content. Proteins associated with Bp survival under salt-stress include those involved in drug resistance, intracellular stress, motility, chemotaxis, and secondary metabolism, such as antibiotic and surfactant biosynthetic pathways [162]. Similarly, comparisons of Bp strains derived from the soil to those isolated from a mammalian host showed distinct phenotypes with changes in morphotype, flagella number and composition, and surface proteins important in pathogenesis [163]. Thus, Bp likely expresses a very different proteome in the soil and during initial host encounter compared to the

proteome expressed during intracellular infection and survival in the host. Consequently, we chose to first investigate OMVs isolated from Bp grown in hypertonic media.

In addition, Bp grown in a rich media, such as LB, does not express virulence proteins necessary for intracellular survival such as T3SS or T6SS. Thus, OMVs extracted from LB culture may lack important T cell antigens necessary to induce T cell effector responses and prevent chronic infection. M9CG is a limited-nutrient minimal media that will be used to induce OMV expression of intracellularly-expressed antigens necessary for Bp intracellular survival [52]. Thus, we next tested OMVs derived from Bp grown in this minimal nutrient media.

Finally, as described previously, acyl-HSLs, C8- and C10-HSL, are secreted by multidrug efflux pumps, such as BpeAB-OprB which are attributed with Bp's intrinsic resistance to antibiotics. BpeAB-OprB function is essential for production of a number of QS-regulated virulence factors including siderophores and phospholipase C and for biofilm formation and controlled maturation, which actively protects Bp against antibiotic agents [70]. These antibiotic resistant phenotypes likely contribute to long-term persistence of Bp and disease relapse, so it may be important to direct an immune response to these phenotypes as well.

Growing Bp in LB broth allows us to isolate and extract vesicles shed during bacterial growth. However, these vesicles are likely lacking important virulence factors that are tightly regulated in response to environmental changes during Bp's lifecyle. By altering the growth media in order to naturally upregulate the expression of proteins involved in pathogenesis, the content and composition of the

naturally shed OMVs will be enriched with these proteins and potentially lead to better immune protection.

Summary and Hypothesis

Melioidosis is responsible for 40% of all sepsis-related deaths in northeast Thailand and is the third most common cause of death from infectious disease in that region. Melioidosis has a mortality rate of over 40% and recurrent symptoms and persistent infection are common. Melioidosis is caused by the Gram-negative soil-dwelling bacilli Bp, which is found worldwide but is hyperendemic in northeast Thailand and northern Australia. Bp is a very resilient pathogen capable of surviving diverse environments including soil, fresh and seawater, and plant and animal tissues for extended lengths of time. Bp's phenotypic adaptability and plasticity are mediated by a robust and tightly regulated repertoire of virulence factors. Bp is intrinsically resistant to antibiotics, which contributes to persistence and relapse in over 25% of melioidosis patients. There is currently no vaccine available and traditional vaccine strategies have failed to elicit complete protection, which will likely require the induction of antibodies, $CD4^+$ and $CD8^+$ T-cells. We have previously shown that parenteral immunization with naturally derived Bp outer membrane vesicles (OMVs) provides significant, but incomplete, protection against lethal sepsis and pneumonic melioidosis in mice. OMVs are constitutively shed by Gram-negative bacteria and consist of periplasmic, cytoplasmic, and outer membrane components including surface and secreted proteins. The incomplete vaccine-mediated protection against Bp infection may be due to an absence of essential protective antigens in OMVs. OMVs are purified from Bp grown in a nutrient rich culture medium (LB), which is not representative of Bp's environmental or host niche. By manipulating the growth media to simulate various

important environmental conditions, including the natural hypertonic soil environment, the limited-nutrient host macrophage intracellular environment, and quorum sensing conditions, we aim to alter the protein content of OMVs to include those proteins necessary for Bp survival under these conditions. ***We hypothesize that vaccination with these selectively-enriched OMVs will induce protective antibody and cell-mediated immune responses necessary to protect mice from both acute and chronic infection.***

Specific Aims

<u>Aim 1:</u> Characterize the composition of OMVs produced under hypertonic stress, nutrient limitation, and quorum sensing conditions.

1.1 Evaluate the composition of OMVs purified from *Bp* grown in hypertonic culture media to simulate the natural soil environment compared to those produced in LB.

1.2 Characterize the composition of OMVs purified from *Bp* grown in a minimal media intended to mimic the macrophage intracellular environment compared to those produced in LB.

1.3 Investigate the composition of OMVs purified from *Bp* grown in media supplemented with autoinducers C8-HSL and C10-HSL compared to those produced in LB.

<u>Aim 2:</u> Evaluate the safety and immunogenicity of OMVs derived from Bp grown in media simulating important environmental conditions compared to those produced in LB.

2.1 Determine the safety *in vitro* and *in vivo* of OMVs derived from Bp grown in differential media.

2.2 Assess the immunogenicity of enriched OMVs by using a murine immunization model and examining antibody responses by serum ELISAs and cell-mediated immune responses by flow cytometry.

<u>Aim 3:</u> Investigate the protective efficacy of OMVs produced in selective media compared to those produced in LB against a Bp infection in mice.

3.1 Assess the protective efficacy of *Bp* OMVs purified from selective media compared to OMVs produced in LB as measured by survival of vaccinated mice over 21 days following lethal Bp challenge.

3.2 Determine the bacterial clearance following challenge in immunized animals by assessing morbidity by weight loss and collecting tissue bacterial burdens.

3.3 Investigate the effector functions of immune responses against Bp with a serum bactericidal assay and CD4+ and CD8+ T cell co-cultures using serum and splenocytes from OMV immunized animals.

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Chapter 2

Specific Aim 1

Characterize the composition of Bp OMVs produced under hypertonic stress, nutrient limitation, and quorum sensing conditions

Introduction

Bp represents unique challenges to vaccine development due to the presence of phenotypically diverse Bp strains, multiple infection routes, ambiguous and multifaceted disease manifestations, and the ability to develop chronic or persistent infection. As described in **Chapter 1**, a successful vaccine will likely require a number of conserved antigens that can direct immune responses against multiple strains and reduce the possibility of escape variants. Bp OMVs have the capacity to incorporate a number of naturally-expressed antigens and virulence factors and provide a multivalent strategy for vaccination against melioidosis. Our lab has previously demonstrated that immunization with OMVs derived from LB-grown Bp (LB OMVs) provides significant protection against lethal aerosol challenge with a homologous strain and against IP challenge with a heterologous strain in BALB/c mice [1] [2]. Immunization of mice with LB OMVs provided antibody-mediated protection against rapidly lethal sepsis by inducing robust OMV-, LPS-, and CPS-specific serum IgG and IgM antibody responses. Passive transfer of the OMV-immune sera also protected naïve mice against acute disease following challenge [1]. However, complete protection and sterilizing immunity were not achieved against inhalational melioidosis and surviving animals maintained viable bacteria in systemic tissues, indicating that the antibody responses induced by the LB OMVs was not sufficient to completely eradicate the bacteria following challenge [1]. Due to Bp's intrinsic ability to reside intracellularly, it may be necessary to induce cell-

mediated immunity in addition to humoral immunity in order to prevent acute disease and facilitate the clearance of bacteria to protect against chronic infection. In order for OMVs to elicit the immune responses necessary for protection, their cargo should be enriched with virulence determinants that are specific to both extracellular and intracellular life stages of Bp.

The LB OMVs used for immunization in our previous studies were derived from Bp grown in LB broth. LB is a synthetic, lab-formulated nutrient rich media utilized for the rapid growth of bacteria. However, Bp alters its metabolic activity depending upon its natural habitats, whether in the soil, inside eukaryotic cells, or in complex communities such as biofilms. It is very likely Bp's metabolism and protein expression in these environments greatly differs from that exhibited under laboratory-constructed nutrient-rich conditions [3]. Bp undergoes a number of rapid phenotypic changes when adapting to changing environments during infection [4]. Bp, a soil-borne bacteria, may be transmitted to mammalian hosts through a number of routes, where it may then lead to rapidly lethal disease or maintain a chronic infection. This represents Bp's ability to express different proteins important for its soil lifecycle as well as during active infection and persistence in a host. By incorporating a range of these tightly-regulated proteins in an OMV vaccine, we may be able to elicit both humoral and cell-mediated responses to virulence determinants in the early and late stages of infection. Herein, we investigate the use of selective growth media to upregulate the expression of proteins and virulence factors that may be unique to transmission, intracellular, or biofilm stages of infection.

First, as Bp exists as a saprophyte in tropical regions, we hypothesized that it was important to simulate the soil environment to influence Bp protein expression at the time of human exposure or infection. Bp is capable of tolerating a number of physical factors, pH changes, osmolarity, and chemicals, which leads to unusual persistence in soil and water [4]. Studies have shown that the soil containing Bp in endemic regions is typically the moist surface soil with high water and clay content, is unusually acidic, and slightly salinated [5] [6] [7]. Vellasamy et al. showed that soil-derived strains of Bp have distinct differences from mouse-derived strains including increased biofilm formation, decreased flagellin expression, different LPS structures, and altered secretion of extracellular enzymes [8]. A proteomics study examining alterations in the secreted proteins under salt stress compared to normal growth in LB showed 37 uniquely expressed proteins including metabolic enzymes, transcriptional and translational regulators, potential virulence factors, chaperones, drug resistance proteins, and transport regulators [9]. These proteins are important for bacterial growth and survival and show important roles in an adaptive response to its environment. Relatedly, Bp grown under salt stress invades lung epithelial cells (A549 cell line) more efficiently and exhibited greater antibiotic resistance as compared to wild-type LB-grown Bp [10]. In addition, the relationship between increased sodium chloride concentration and susceptibility to bacterial infection has been described in cystic fibrosis patients [11-14]. By growing Bp in media with a high salt content similar to those levels in northeastern Thailand or endemic coastal regions, it may be possible to identify those proteins and virulence factors

important to transmission or invasion in humans and to express those factors in an OMV vaccine to induce better protective responses.

Once transmission has occurred, the ability to survive and replicate intracellularly is essential to disease progression in melioidosis. The intracellular environment is drastically different than that of the soil or host extracellular milieu. As described in **Chapter 1**, Bp must alter its expression in order to avoid detection by the host immune system and also grow under limited nutrient conditions. The Bp chromosomes display distinct transcriptional landscapes depending upon growth conditions, with those associated with rich media (LB) grouped together and segregated independently from those associated with nutrient-limitation (minimal media) [15]. This distinction in gene regulation highlights the profound influence of external nutrient conditions on Bp's expression profiles. Several characteristics are unique to intracellular survival within a human host, including expression of the T3SS-1, T6SS-1, and metal ion acquisition systems. The expression of the T3 and T6SS are tightly regulated by the bacteria and are strictly activated in a temporal manner for intracellular suvival and replication. T3SS-1 is essential for escape from the lysosome and works in a pump-like manner to export bacterial effectors into the host cell, enabling bacterial escape into the macrophage cytoplasm. The T6SS-1 regulon is then activated for cell-to-cell spread and multinucleated giant cell (MNGC) formation. These two systems require activation cues associated with the intracellular environment. Sun et al. found that bacterial T6SS-1 genes were poorly transcribed during growth in rich media, but are significantly up-regulated following internalization by host cells [16]. Minimal media (M9) was formulated to

simulate the nutrient-limited, metal poor, slow-growing host cell environment [17]. Further, Burtnick and Brett describe defined minimal media conditions that activate T6SS-1 expression during in vitro growth of Bp, which consisted of M9 with added glucose and casamino acids. This media was found to mimic the signals required for activation of intracellularly expressed antigens and upregulates the expression of surface proteins that are necessary for intracellular survival [18], such as T3SS-1 and T6SS-1. One of the main components of M9 formulation is the low levels of metal ions. The intracellular environment has a lack of metals and the bacterial genes regulating metal ion acquisition systems are specifically induced following uptake by macrophages [19]. The study by Burtnick and Brett, among others, also found that both iron and zinc inhibit expression of T3SS and T6SS and have detrimental effects on intracellular survival [20] [18].

Growing Bp in a minimal media, M9CG, should enrich the OMV cargo with virulence determinants specific to the intracellular stage of infection. These proteins may be essential targets for T cell responses against intracellularly replicating Bp and their inclusion in an OMV vaccine may enhance its ability to eradicate persistent bacteria [21] [20] [1] [22].

One of the putative virulence factors of Bp is quorum sensing, as previously described in **Chapter 1**. QS is an inherent characteristic of bacterial pathogenesis, in which gene expression is regulated in a cell-density manner. The bacteria are able to coordinate the production of an array of phenotypic characteristics in accordance with cell population size by producing diffusible cell-to-cell signals, acyl-homoserine lactones (AHLs). In this way, QS both positively and negatively regulates diverse

functions in bacteria including conjugation, antibiotic synthesis and resistance, extracellular enzyme and exopolysaccharide production, expression of extracellular virulence factors, and biofilm formation and regulation [23].

Bp AHLs, C8- and C10-HSL, are secreted by multidrug efflux pumps, such as BpeAB-OprB [24]. Efflux pumps such as these are attributed with *Bp*'s intrinsic resistance to antibiotics due to their ability to discharge aminoglycosides, macrolides, chloramphenicol, and trimethoprim. Inhibition of BpeAB-OprB has been shown to be clinically advantageous because it enhances the susceptibility of *Bp* to commonly used therapeutic antibiotics, and attenuates cell invasion and cytotoxicity [25]. BpeAB-OprB function is essential for production of a number of QS-regulated virulence factors including siderophores and phospholipase C and for biofilm formation [26]. The QS system is also responsible for positive regulation of biofilm formation and controlled maturation. Bacterial biofilms are the optimum site for QS activation because it is here that natural cell populations are at their highest density. Production of biofilms actively protects Bp against antibiotic agents. Studies have shown that growing Bp in biofilm stimulating conditions significantly increases antimicrobial resistance. One such study showed Bp grown in biofilm-inducing conditions resisted all antimicrobial agents subsequently tested [27]. Similarly, Gilbert et al. showed biofilm bacteria can be up to one thousand times more resistant to antimicrobial agents than their planktonic counterparts [28]. This drug resistance mechanism occurs from the upregulation of antimicrobial factors, such as export pumps, when biofilm formation genes are stimulated by quorum sensing- signaling molecules [27]. The ability and proclivity of Bp to form and maintain biofilms may

explain its high rate of antibiotic resistance, persistence, and relapse in melioidosis patients [29]. Enriching OMVs with QS-regulated proteins such as BpeAB-OprB and other antibiotic efflux pumps, by growing Bp under QS conditions may allow targeting of host T cell responses to the Bp factors involved in resistance and biofilms and antibiotic efflux pumps in order to eliminate evasive or persistent bacteria.

Herein, the composition of OMVs derived from Bp grown under three different conditions simulating the natural environments of infection was characterized along with our original Bp OMVs grown in LB (LB OMV) for comparison. These conditions include: hypertonic stress, nutrient limitation, and quorum sensing. We hypothesize that by growing Bp in various lifecycle environments, Bp will naturally shed OMVs that will be enriched in important virulence factors and proteins with tightly regulated expression that were not seen in Bp OMVs extracted from LB and will contribute to inducing the necessary immune responses to achieve sterilizing immunity and prevent both acute and chronic melioidosis.

Materials and Methods

Bp strain for OMV production- Bp82 is a highly attenuated purM mutant of the clinical strain Bp1026b. Bp82 was chosen for its exclusion from Select Agent regulations and its ability to be used under Biosafety Level II conditions [30]. Bp82 was created by deleting the *purM* gene, which encodes phosphoribosyl formylglycinamide cycloligase, an enzyme which catalyzes the reaction producing

adenine and thiamine precursors. The ΔpurM is unable to produce a functional PurM enzyme and is an adenine auxotroph; however, supplementation with adenine and thiamine restore growth to levels indistinguishable from those observed for wild-type 1026b [30]. Bp82 has been extensively tested in several different highly immunocompromised animal strains with no reversion to virulence or persistence detected [31]. Bp82 was used for all OMV preparations.

Media preparation- Three different environmental growth conditions were evaluated for selective enrichment of naturally shed Bp OMVs, including endemic soil, host intracellular, and quorum sensing conditions. As it is impossible to isolate or extract OMVs from Bp grown in soil, our research focused on the unusually high salt content of endemic regions. Bp82 was grown to late-log/early stationary phase in LB Miller broth (Fisher) supplemented with 1.5% NaCl (Sigma). In order to simulate a host intracellular environment, Bp82 was grown in M9CG media consisting of M9 minimal salts agar (BD Difco) supplemented with 0.4% glucose (Sigma) and 0.5% casamino acids (Amresco). Quorum sensing conditions were replicated by growing Bp in LB broth for 8 hours and then supplementing the growth media with C8-HSL and C10-HSL (Cayman) and resuming growth for approximately 8-10 hours.

OMV extraction and purification- OMVs were prepared as previously described (Kuehn) with minor modifications as demonstrated in Figure 2.1. Bp strain Bp82 (provided by Dr. Herbert Schweizer, Colorado State University) was

streaked for isolation from a glycerol stock onto a Pseudomonas isolation agar (PIA) plate and incubated for 48-72 hours at 37°C. 35ml of LB Miller broth supplemented with 100μg/ml adenine hydrochloride (Sigma) and 5μg/ml thymine hydrochloride (Sigma) (LB+) was inoculated with 1 colony of Bp82 and incubated at 37°C, shaking at 233 rpm, for 18 hours. A 1:100 dilution of this initial culture into 3L broth was incubated at 37°C with shaking 233rpm until growth reached late-log/early stationary phase (OD 4.5-5.0$_{600nm}$). The secondary culture consisted of 3L of LB+ broth supplemented with 1.5% NaCl, 3L M9CG media, or 3L of LB+ broth with C8-HSL or C10-HSL (Cayman) added after 8 hours of growth. The different secondary culture medias represent the differentially enriched OMV growth environments. Once late-log/early stationary phase has been reached (approximately 18-20 hours), whole bacterial cells were pelleted by centrifugation (8,500rpm, 30 min, 4°C) using RC5C Beckman Coulter centrifuge and SLA-1500 fixed-angle rotor. Supernatants were removed and filtered through a 0.22μm polyethersulfone (PES) membrane (Millipore) to remove remaining bacteria or large bacterial fragments. The bacterial pellets were decontaminated and discarded. A small (500μL) sample was drawn from each bottle of filtered supernatant and plated on PIA plates to confirm the absence of bacterial contamination.

OMVs were precipitated from the media by incubating the filtered supernatants with 1.5M ammonium sulfate (Fisher Scientific) overnight, 4°C, with gentle stirring. Solutions were centrifuged (11,000rpm, 1 hr, 4°C) for collection of OMVs. Supernatants were discarded by vacuum suction. Crude OMVs, which appear as a brown/white smear, were resuspended and collected in 4mL 70% sucrose

(Sigma) in 10mM Tris-HCL pH 7.4, resulting in 5ml of crude OMV in approximately 60% sucrose. For the purification step, this 5ml solution is gently overlayed with consecutive layers of 5mL 55%, 5mL 50%, 4mL 45%, 4mL 40%, and 4mL 35% Sucrose in 30mM Tris-HCL pH 8.0 in a 26.3mL ultra-centrifuge bottle (Beckman Coulter). Density gradient centrifugation was performed using a Beckman Coulter Optima XL-100K ultracentrifuge with a Type 50.2Ti rotor at 41,200rpm, 3 hr, 4°C, for separation of flagella and other soluble materials from the intact vesicles. The resulting fractions were collected in equal volumes from the top and stored at 4°C. The remaining pellet was discarded, as it consists of extracellular proteins and flagellar contaminants.

To evaluate the quality of the purified OMVs, the presence of protein within each fraction was assessed by precipitating protein out of solution using tri-chloroacetic acid (TCA, Sigma). 20% TCA was added to 500μL samples of each fraction at a 1:4 dilution and imcubated for 10 min, 4°C. Samples were then centrifuged at 13,000rpm, 5 min, RT using a tabletop centrifuge. Supernatants were removed by vacuum suction. The resulting pellets were washed two times with 200μL cold acetone (Sigma) and centrifuged at 13,000 rpm, 5 min, RT. The pellets were then dried for 5 min to evaporate any remaining acetone. Pellets were then resuspended in 20μL 1x Laemilli loading sample buffer (Bio-Rad) containing 100mM DTT (Sigma) and boiled for 7-10 min. Each precipitated sample was then evaluated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-FAGE) using a Precision Plus Dual Colored Pre-stained Protein molecular ladder (Bio-Rad) in the first lane for comparison purposes. In order to visualize proteins, the gel was

stained with coomassie blue stain (250mL methanol, 250 mg coomasie blue powder, 50mL acetic acid, 200mL dH2O) overnight, RT, with gentle rocking and destained with a destaining solution (75mL acetic acid, 400mL methanol, 525mL dH2O) for 4 hours, RT. Fractions showed distinct banding patterns based upon the amount of contaminants within each fraction. Those samples that had the least amount of contaminants, as seen by distinct banding patterns and not protein smears-which indicate bacterial cell fragments, were pooled and subjected to ultracentrifugation to obtain a purified OMV pellet. The fractions were centrifuged at 41,200rpm, 16-18 hr, 4°C using a 26.3mL ultracentrifuge tube and a 50.2Ti rotor in the Beckman Coulter Optima XL-100K ultracentrifuge. The resulting purified OMV pellet was resuspended in 500μL- 1mL sterile saline. 10% of each purified preparation was plated on LB plates to confirm purity and the absence of contamination. OMV concentrations were determined by a Bradford Protein Assay (Bio-Rad). Purified OMV preparations were stored at -20°C until further evaluation was performed. OMV preparations were designated by the growth conditions: LB OMV, NaCl OMV, M9CG OMV, and QS OMV.

Transmission electron microscopy- A small volume (1-2 μL) of each purified OMV preparation was added onto a copper coated formvar grid (Electron Microscopy Sciences or EMS) and allowed to adsorb for approximately 10 minutes. Once the sample dried, the grid was washed twice by touching the stained surface of the grid to a drop of sterile filtered autoclaved dH2O and blotted dry with filter paper each time. The surface of the grid was then quickly stained (approximately 5

seconds) with 20μL 1% Uranyl acetate stain (EMS), blotted dry, and then incubated 1 full minute with 20μL more of the 1% Uranyl acetate stain. The grid was then blotted and allowed to fully dry prior to storage or imaging. Imaging was performed on a FEI Tecnai G2 F30 Twin TEM located in the Tulane University Coordinated Instrument Facility (CIF) under the guidance of Dr. Jibao He. The final purified enriched OMV preparations were evaluated using multiple fields of view under magnifications ranging from 17,500x to 31,000x in order to confirm the presence of OMVs and confirm the absence of cellular contaminants, such as flagella or bacterial fragments.

Determination of CPS or LPS presence- The presence of CPS and LPS in the enriched OMVs was determined by Western blot, using a monoclonal antibody specific for Bp CPS, MCA147 [32](provided by Dr. Burtnick, University of South Alabama) or a monoclonal antibody specific for Bp O-polysaccharide, Pp-Ps-W [33] (by Dr. Aucoin, University of Nevada School of Medicine). Ten μg of each OMV preparation, Bp82 LB, NaCl, M9CG, and QS along with acapsular B. thailandensis lysate (negative control for capsule) were separated by SDS-PAGE using a 4-20% gradient polyacrylamide gel (BioRad). The proteins were then transferred to a nitrocellulose membrane (Invitrogen) using the iBlot Gel Transfer System (Invitrogen) and blocked with 1.5% BSA (Sigma) in TBS-T (50mL 10xTBS, 450mL dH2O, 0.5mL Tween-20) 1hr, RT, with gentle rocking. The membrane was then washed 3x with TBS-T and incubated overnight, 4°C, rocking gently with MCA147 IgG3 (1:1000 dilution) or Pp-Ps-W IgM (1:500 dilution) in TBS-T. Following this

step, the membrane was washed 3x with TBS-T and then incubated with goat anti-mouse HRP-conjugated IgM secondary antibody (Pierce, 1:1000 dilution) for CPS detection or goat anti-mouse IgM for LPS detection, both for 1 hr at RT. The membrane was then washed 3 final times with TBS-T and developed according to the manufacturer's instructions using Opti-4CN substrate kit (BioRad).

Liquid Chromatography/Mass Spectrometry (LC/MS)- In order to determine the protein composition of each enriched OMV preparation as compared to those produced in LB, we utilized LC/MS and compared the findings to the published *Burkholderia pseudomallei* K96243 proteome [34]. All LC/MS was performed by Dr. Chau-Wen Chou, Department of Chemistry Proteomics and Mass Spectrometry Facility (PAMS), University of Georgia. 100μg of each OMV preparation was briefly run by SDS-PAGE until the band reached the stacking portion of the gel. These bands were then cut out of the gel and shipped to Dr. Chou for evaluation. Briefly, gel bands were manually cut into very small pieces and incubated for 20 minutes with 25mM ammonium bicarbonate in 50% acentonitrile. Proteins were then digested with 1μg/sample Trypsin in 25mM ammonium bicarbonate 16 hr, 37°C. Peptides were extracted by incubating the samples with 100μL extraction buffer (0.1% formic acid in 50% acentronitrile aqueous solution) for 20 minutes, briefly spinning the samples, and collecting the supernatant. This was then followed by a 20 min incubation with 100% acetonitrile. Samples were then dehydrated by Eppendorf Vacufuge and resuspended in 10μL 0.1% formic acid with 2% acetonitrile. Samples were then run on a ThermoScientific Orbitrap Elite mass spectrometer for high

resolution and high mass accuracy analysis. It is coupled with a nano HPLC, in order to analyze more complex protein mixtures. Results were provided as raw data, which was searched against the Bp proteome through the Basic Local Alignment Search Tool (BLAST) search engine (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

HCP-1 expression determination- The presence of HCP-1 in the Minimal media enriched OMVs was determined by Western blot, using a rat-HCP-1 serum [18] (provided by Dr. Mary Burtnick, University of South Alabama) as the primary antibody. Ten μg each of Bp82 LB and M9CG purified OMV preparations were separated by SDS-PAGE using a 4-20% gradient polyacrylamide gel (BioRad). The proteins were then transferred to a nitrocellulose membrane (Invitrogen) using the iBlot Gel Transfer System (Invitrogen) and blocked with 1.5% BSA (Sigma) in TBS-T (50mL 10xTBS, 450mL dH2O, 0.5mL Tween-20) 1hr, RT, with gentle rocking. The membrane was then washed 3x with TBS-T and incubated overnight, 4°C, rocking gently with rat-HCP-1 serum (1:1000 dilution) in TBS-T. Following this step, the membrane was washed 3x with TBS-T and then incubated with goat anti-rat IgG HRP-conjugated secondary antibody (Abcam, 1:2000 dilution) for 1 hr at RT. The membrane was then washed 3 final times with TBS-T and developed according to the manufacturer's instructions using Opti-4CN substrate kit (BioRad).

Results

Bp82 naturally sheds OMVs when grown in selective media- B. pseudomallei strain Bp82 produced OMVs when grown to late log/early stationary phase in all media tested. OMVs were successfully extracted and purified according to the described method (**Figure 1.1**) from Bp82 grown in LB broth supplemented with 1.5% NaCl, M9CG minimal media, and LB broth supplemented with C8-HSL and C10-HSL. Bp82 grown in broth supplemented with salt and the minimal media took several hours longer to reach late log/early stationary phase, 18-20 hours compared to 16-18 hours for Bp82 grown in LB or LB+C8/C10-HSL. Crude vesicles from each preparation underwent density gradient purification to concentrate the vesicles and to remove flagellar and cellular contaminants. Final concentrations of each 3L preparation were determined by Bradford protein assay to be approximately 0.5-1.0mg/ml. The presence of purified OMVs in the final preparations was confirmed visually by TEM (**Figure 1.2**). Vesicles ranged in size from 50-250 nm in diameter; however, those derived from Bp grown in LB+1.5% NaCl were noticeably smaller ranging from 20-150 nm in diameter. The NaCl OMVs visually had more flagellar fragments remaining in the purified preparations as compared to the OMVs derived from all other medias tested (**Figure 1.2B**). These results have been consistent in subsequent OMV extractions and purifications.

OMVs extracted from Bp82 grown in selective media contain LPS and CPS- The presence of LPS in each of the OMV preparations was determined by Western blotting. Ten micrograms of each preparation were probed with Pp-PS-W

monoclonal antibody, specific for Bp LPS. Each OMV preparation was run alongside a molecular weight ladder and 10ug of LB OMVs for comparison (**Figure 1.3**). The NaCl OMVs show a slightly different banding pattern to that of the LB OMVs (**Figure 1.3A**), with distinctive bands at 75kD, 40kD, and 35kD. The distinctive LPS "smear" banding pattern is also much lighter for the NaCl and M9CG OMVs, despite equal protein concentrations being evaluated. The M9CG OMVs showed a similar, though fainter, banding pattern to the LB OMVs (**Figure 1.3B**). QS OMVs show a nearly identical LPS banding pattern to that of LB OMVs; however, it is missing the 12kD band that was present in both the LB and M9CG OMVs (**Figure 1.3C**). The presence of CPS in each preparation was also detected by Western blotting. Similarly, ten micrograms of each preparation (A) LB OMV (B) NaCL OMV (C) M9CG OMV (D) QS OMV (E) Bt OMV (acapsular negative control) were probed with MC147 monoclonal antibody, specific for Bp CPS. CPS band is approximately 200kD. As shown by **Figure 1.4,** CPS is present in all Bp OMV preparations, but is not present in the Bt OMV negative control as expected.

Protein content of OMVs is dependent upon Bp growth environment- The purified OMV preparations from each selective media, including LB, were first analyzed by SDS-PAGE for protein profiles. **Figure 1.5** shows 10μg of each purified OMV preparation along with 10μg LB OMVs run alongside a molecular weight standard. The profiles for each preparation are unique, as seen by the varying banding patterns. In order to identify the differences in protein expression induced by the altered growth medias, OMV preparations were then analyzed by LC/MS. The

contents include membrane, cytosolic, periplasmic, and some ribosomal proteins, many of which are conserved across all preparations, as shown in **Table 1**. There are also numerous proteins expressed in the OMVs derived from Bp grown in selective media that are not expressed in the LB OMVs and are unique to their growth conditions, as further described in **Figure 1.6**, **Table 1**. There are also a number of proteins that are present in all three of the selectively enriched OMVs that are absent from LB OMVs. These include PhoPQ pathogenicity-related family protein, related to PqA protein [35], and FlgM, a flagellar biosynthesis anti-sigma factor protein involved in prevention of filament expression and chemotaxis [36]. As shown by **Figure 1.6**, the NaCl OMVs contained 61 proteins, M9CG OMVs had 106, and QS OMVs expressed 74 proteins not contained in LB OMVs. These included a number of porins and hypothetical proteins in addition to the virulence factors mentioned above that are present in the other enriched OMVs. Potential pathogenesis-related proteins contained in OMV preparations, both unique and shared proteins, are described in **Table 1**. NaCl OMVs also contained unique proteins including osmolarity response regulators and environmental effector proteins, including fimbrial proteins important to attachment pili formation [37], and bacteriolytic entericidin B-like protein that is activated in the stationary phase of growth under high osmolarity conditions [38]. In contrast, M9CG had a large number of unique proteins involved in T3SS, T6SS, and intracellular survival. These include Rhs element Vgr and HCP, which are essential components of the T6SS structure [18], thioredoxin (TrxB), which promotes intracellular replication and facilitates intracellular survival [39], and phosphoglycerate kinase (Pgk) a known pathogenesis

factor that has previously been investigated as a vaccine candidate [40]. QS OMVs had an abundance of hypothetical proteins and lipoproteins, but these also expressed MucD, involved in biofilm production and involved with the T3SS [41,42], beta-lactamase precursors which are involved in bacterial resistance to beta-lactam antibiotics, and important transcriptional regulators such as bacterial regulatory lysR, which regulates a diverse set of genes, including those involved in virulence, metabolism, quorum sensing and motility [43]. The diversity of proteins expressed in the OMVs appears to correlate with the environmental requirements in which they were derived, implying selective enrichment is possible by manipulating the bacterial growth conditions.

HCP-1 expression is unique to M9CG OMVs- The major component of the T6SS is HCP-1, which comprises the transport tube (**Figure 1.7**), and expression of HCP-1 is representative of T6SS functioning in the cell. Expression of HCP-1 exclusively in M9CG OMVs was determined by LC/MS, as shown in **Table 1**, and confirmed by Western blot (**Figure 1.8**).

Discussion

OMVs extracted from Bp grown in LB have shown potential as a vaccine against lethal Bp challenge but have, as yet, been unable to induce sufficient humoral and cell-mediated responses likely necessary for sterilizing immunity and protection against chronic melioidosis [1,44]. The development of persistent infection following challenge may be due to the absence of T cell antigens or virulence factors specific to the intracellular or persistent stages of infection within the OMVs that

were used to immunize, potentially as a result of OMV extraction from Bp grown in a synthetic, nutrient-rich media. Here, we investigated the ability of Bp grown in selective media intended to simulate the different environmental conditions of Bp to shed vesicles enriched in virulence factors and proteins that are specific to bacterial survival under those conditions, which are not present in OMVs derived from Bp grown in LB. All of the conditions tested yielded Bp OMVs with individually unique expression profiles but also contained important conserved virulence determinants.

The conditions selected for enrichment were determined to be representative of three drastically different phases of Bp growth and infection. These included a hypertonic endemic soil-like environment, intracellular host macrophage, and quorum sensing conditions that were simulated by supplementing LB with 1.5% NaCl, using a minimal media supplemented with glucose and casamino acids, or supplementing LB with AHL molecules, respectively. OMVs from Bp grown in unsupplemented LB were used as a wt control for comparison purposes. We were successfully able to extract and purify OMVs from all conditions.

The vesicles maintained a standard double membrane enclosed particle, important for cargo transportation and a consistent size of approximately 50-250nm. The physical size distribution of OMVs is important for vesicle uptake by host antigen presenting cells and subsequent immune activation [45]. The smaller particles, 10-200nm, are easily endocytosed by macrophages, dendritic cells, and B cells, where they are degraded and loaded on to MHC class II molecules that can present antigens to $CD4^+$ T helper cells [46]. For larger OMVs, 150-300nm, fusion with host cell membranes may also occur and allow the deposit of cargo directly into the

cell cytosol possibly leading to antigen processing in the cytoplasm and cross-presentation on MHC class I molecules, which present antigens to $CD8^+$ T cells [47,48]. While the size range for NaCl OMVs was smaller than that for LB or the other conditions, they still fall within the ranges necessary for host cell uptake. Additionally, the NaCl OMVs retained a larger amount of flagellar components following the standardized purification process. This is not unexpected as it has been shown that Bp grown under salt stress significantly increase expression of motility proteins, particularly flagella [49]. As a TLR5-agonist, flagellin is immunodominant and large amounts are not desirable in a vaccine formulation, so NaCl OMVs may require further optimization of the purification gradient or repetition of this process to remove excess flagella pieces.

Previous studies investigating Bp OMVs as vaccine candidates showed that LPS and CPS are essential components in providing a robust antibody response and may be necessary for providing protection against acute infection [2,50,51]. Here, the OMVs derived under selective conditions all express CPS; however, our data suggests Bp expresses LPS to varying degrees depending upon environmental requirements. While, Bp LPS has been established as only weakly inflammatory, its mere presence is enough to stimulate the host innate immune response [52,53]. Additionally, LPS provides a barrier on the surface of the bacteria and inhibits uptake of soluble factors, which may be undesirable in soil-borne bacteria. Bp existing in the soil take in growth factors and nutrients from the plant rhizosphere, which may necessitate the downregulation of LPS expression similar to that seen in the NaCl OMVs.

The data demonstrates that OMVs derived from Bp grown under different lifecycle conditions exhibit very different protein profiles. However, there are also a number of important proteins shared across conditions including flagellar components, OmpC, an outer membrane porin important in diffusing small hydrophobic molecules, OmpA, EF-Tu, oxidoreductase, a number of molecular chaperones, SlyB, an outer membrane lipoprotein important in maintaining the outer membrane integrity, and MucD, a periplasmic serine protease, among others. As mentioned in **Chapter 1**, OmpA and EF-Tu are known virulence factors that are necessary for intracellular survival and tissue invasion, respectively. The various stress-responses induced by growth in the selective media lead to the expression of several proteins found in OMVs derived from all conditional medias that were not present in LB OMVs. This subset includes AhpC, which protects bacterial cells from reactive nitrogen intermediates and promotes intracellular survival [54] and BopE, a T3SS effector protein and important Bp virulence factor [55]. Another such protein is phoPQ-activated pathogenicity-related family protein that in *Salmonella typhi* is a two-component regulatory system activating many virulence genes and has been shown to confer resistance to antimicrobial peptides [56]. Likewise, there was a lot of overlap with protein expression occurring under one or two of the selective conditions, but not the others. This implies that many of the proteins expressed here are regulated by the various environmental stress conditions.

Finally, as expected, the data show vast differences in protein expression unique to each condition. NaCl OMVs exhibited an unusually large number of fimbrial proteins, which function as attachment pili for adhesion to the host cells.

NaCl OMVs also expressed a bacterial toxin peptide, bacteriolytic entericidin B-like protein, which is common in soil-borne environmental bacteria for regulation of growth and apoptosis [57,58]. These may be important factors to bacterial transmission and survival under growth-related stress. Alternatively, M9CG OMVs expressed a number of metal-binding proteins and metalloproteases, along with multiple T6SS proteins, including Vgr and HCP-1. Sun et al. found that BsaN, a T3SS regulator, controls expression of both T3SS effectors and T6SS regulators when grown in LB media, but during host intracellular growth, T6SS-1 expression is regulated by VirAG. This difference in regulation may explain why HCP-1 expression is not seen in LB OMVs, but is present in those grown in media simulating the intracellular environment [16]. However, it is now known that the T6SS is an essential component of Bp intracellular survival and is an important virulence factor, so its inclusion in a Bp OMV vaccine may be key to achieving T cell activation against intracellular Bp [59]. M9CG OMVs had the largest number of proteins identified by LC/MS overall; however, a large number of these were considered "hypothetical" and more extensive analysis of the Bp genome and protein functions will be required to properly describe their purpose. Bp grown under quorum sensing conditions shed vesicles with an abundance of porins and secretion system proteins involved in transport. One such protein is PqaA, which belongs to the YscC/HrcC family of T3SS outer membrane proteins and confers resistance to antimicrobial peptides [60]. This was expected, as quorum sensing systems are known to enhance antimicrobial resistance in bacteria [61] and this may be beneficial by including the proteins

necessary for that function in a vaccine, and thus prevent bacterial escape and persistent infection[62].

In conclusion, it is possible to direct protein expression and enrich naturally-derived OMVs simply by altering the bacterial growth media to simulate environmental changes. The resulting extracted OMVs have modified composition and include tightly regulated proteins and virulence factors not seen in those grown in a laboratory nutrient-rich media, such as LB. These results are important to understanding the effects of environmental conditions on bacterial protein expression and subsequent vesicle shedding and content. This information will contribute to formulating a Bp OMV vaccine containing sufficient antigens to induce the necessary antibody and T cell responses for complete protection.

Table 1: Putative and potential virulence factors in Bp82 OMVs, compared across preparations

Accession Number	Protein	LB	NaCl	M9CG	QS	Function	Reference
53719908	phasin-like protein					This protein of the phasin family is important for many bacterial strains, including Burkholderia pseudomallei, in accumulating polyhydroxyalkalnoic acid (PHA). Phasin proteins were found bound to granules from Burkholderia caryophvli, cepacia, and solanaceurum.	63
76818743	flagellar hook-associated protein 2					FliD. FliD is a flagellar capping protein, necessary for assembly of FliC monomers and formation of the flagellum's helical structure and part of B pseudomallei's T3SS. The protein's expression is negatively regulated by BsaN virulence regulon in B. pseudomallei.	64
386864666	outer membrane porin					OmpC. This protein is important for the diffusion of small hydrophilic molecules and is a porin-related export protein required for bacterial invasion into host cells.	65
53720307	chaperonin GroEL					GroEL is important for properly fonding proteins and is immunogenic. Patients with melioidosis develop a strong antibody response against GroEL, suggesting that the recombinant protein and its monoclonal antibody may be useful for serodiagnosis in patients with melioidosis and that the protein may represent a good cell surface target for host humoral immunity.	66
386860661	OmpA family outer membrane protein					OmpA-like domains have been shown to non-covalently associate with peptidoglycan. OmpA proteins are immunogenic in mice as well as melioidosis patients and have been further assessed as potential vaccine candidates against B. pseudomallei infection	67
740936897	elongation factor Tu, partial					(EF-Tu) GTP-binding proteins, EF-Tu is one of the most abundant proteins in bacteria, as well as, one of the most highly conserved. EF-Tu is membrane-associated and immunogenic during Burkholderia infection in the murine model of melioidosis. Active immunization with EF-Tu induced antigen-specific antibody and cell-mediated immune responses in mice.	68

53720358	oxidoreductase					Part of the thioredoxin superfamily, this protein is a homodimeric thiol-specific antioxidant (TSA) proteins that confer a protective role in cells by reducing and detoxifying hydrogen peroxide, peroxynitrite, and organic hydroperoxides. It has been investigated as a potential vaccine component.	9
740965308	elongation factor Tu, partial					See above for Ef-Tu function.	68
760220443	molecular chaperone GroES					Essential componenet combines with GroEL for bacterial growth	69
53720132	outer membrane protein A					See above for OmpA protein function.	67
53720597	lipoprotein					SlyB; SlyB is an outer membrane lipoprotein important in maintaining the integrity of the outer membrane in Burkholderia. SlyB mutants demonstrated increased sensitivity to EDTA and SDS and decreased siderophore production. SlyB Salmonella mutants that overexpressed the protein were less successful in growing in a magnesium poor environment, strongly suggesting that SlyB works as a negative feedback protein in the PhoP/PhoQ regulatory system.	70
76811732	outer membrane protein W					OmpW; It is a major antigen in bacterial infections and has implications in antibiotic resistance	19
490693230	translocation protein TolB					Members of this protein family are the TolB periplasmic protein of Gram-negative bacteria. TolB also interacts with the outer-membrane peptidoglycan-associated proteins Lpp and OmpA. The Tol-PAL system is required for bacterial outer membrane integrity. E. coli TolB is involved in the tonB-independent uptake of group A colicins (colicins A, E1, E2, E3 and K), and is necessary for the colicins to reach their respective targets after initial binding to the bacteria. It is also involved in uptake of filamentous DNA. Study of its structure suggest that the TolB protein might be involved in the recycling of peptidoglycan or in its covalent linking with lipoproteins. TolB is required for virulence in Salmonella typhimurium and Vibrio cholerae; its expression is regulated by Cl − ions in clinical isolates of Burkholderia cenocepacia.	71
126439636	hypothetical protein BURPS668_3636					poly-beta-1,6 N-acetyl-D-glucosamine export porin PgaA ; The PGA polysaccharide adhesin is a critical determinant of biofilm formation.	10

53723306	heat shock protein 20					small heat shock proteins (sHsp); sHsps are molecular chaperones that suppress protein aggregation and protect against cell stress, and are generally active as large oligomers consisting of multiple subunits. Transcription of the genes is regulated by a variety of stresses including heat, cold and ethanol.	72
121598480	serine protease, MucD					periplasmic serine protease, Do/DeqQ family; The ortholog in Pseudomonas aeruginosa is designated MucD and is found in an operon that controls mucoid phenotype.Members of this family are located in the periplasm and have separable functions as both protease and chaperone. This protein protects bacteria from thermal and other stresses and may be important for the survival of bacterial pathogens. The chaperone function is dominant at low temperatures, whereas the proteolytic activity is turned on at elevated temperatures.	73
53716718	lipoprotein					This protein contains two domains; one is the SYLF domain (also called DUF500), a novel lipid-binding module, and the other domain is a precursor to an acid shock protein superfamily. It is highly conserved from bacteria to mammals and is involved in membrane morphogenesis driven by actin polymerization.	74
53720482	hypothetical protein BPSL2874					BPSL2874 is a protein of unknown function; however, it contains a YceI-like domain. YceI is a highly conserved, secreted protein implicated in broad antibiotic resistance.	75
53719081	lipoprotein					OmpC; see above for OmpC function	65
386864735	outer membrane porin					OmpC; see above for OmpC function	65
126440935	lipoprotein					A ferrichrome outer membrane transporter. TonB dependent/Ligand-Gated channels prevents the free diffusion of small molecules thru the pore leading to active uptake of iron.	76
740977103	membrane protein SmpA, partial					SmpA. SmpA / OmlA family; Lipoprotein Bacterial outer membrane lipoprotein, possibly involved in in maintaining the structural integrity of the cell envelope and sometimes found adjacent to the OmpA domain	77
53716717	hypothetical protein BMAA0256					This protein has several nonspecific domains hits including DUF883 and ElaB, which codes for a membrane-anchored ribosome-binding protein, inhibits growth in stationary phase; quorum-sensing regulated expression in environmental B. cenopacia.	78

740961815	hypothetical protein					Hcp. Type VI protein secretion system component Hcp (secreted cytotoxin) is important for the immune response illicited by the T6SS. Hcp is both a T6SS substrate and part of the T6SS secretion tube, and the hexameric ring structure of Hcp is necessary for the successful T6SS tube assembly. Mutant proteins abolished the secretion of Hcp inside infected cells and the formation of multi-nucleated giant cells. Meliodosis patients were also found to have high titers of anti-Hcp antibodies; the protein's preferential binding to the surface of antigen-presenting cells, further demonstrate that protein's immunogenicity was also found to bind preferentially to the surface of antigen-presenting cells .	79
685721129	guanine nucleotide exchange factor BopE					BopE was the first component confirmed as a TTSS-secreted effector. BopE is a guanine nucleotide exchange factor that activates the host cell molecules Cdc42 and Rac1 and activation of these molecules leads to host cell actin rearrangement and membrane ruffling. It has been suggested that this activity of BopE is necessary for B. pseudomallei invasion.	55
497612727	x-prolyl-dipeptidyl aminopeptidase					Dipeptidyl aminopeptidases are enzymes involved in the posttranslational control of bioactive peptides; fungal dipeptidyl aminopeptidase	80
741010714	x-prolyl-dipeptidyl aminopeptidase					see above for function	80
53719150	hypothetical protein BPSL1516					BamB. Beta-barrel assembly machinery (Bam) complex component B and related proteins. BamB has been found to interact with BamA, which in turn binds and stabilizes pre-folded beta-barrel proteins; it has been suggested that BamB participates in the stabilization step	81

53720376	hypothetical protein BPSL2766					This hypothetical protein has unknown function, but does contain various domains. One domain is similar to a bacterial protein of unknown function (DUF881); This family consists of a series of hypothetical bacterial proteins. One of the family members from Bacillus subtilis is thought to be involved in cell division and sporulation.This protein is also similar to the BamD lipoprotein, a component of the beta-barrel assembly machinery essential for the folding and insertion of outer membrane proteins (OMPs) in the OM of Gram-negative bacteria. Transmembrane OMPs carry out important functions including nutrient and waste management, cell adhesion, and structural roles. BamD is the only BAM lipoprotein required for viability. Both BamA and BamD are broadly distributed in Gram-negative bacteria, and may constitute the core of the BAM complex.	81
685713863	phoPQ-activated pathogenicity-related family protein					This protein is similar to PqA and other PhoPQ related proteins.PhoPQ-activated pathogenicity-related protein ;Members of this family of bacterial proteins are involved in the virulence of some pathogenic proteobacteria.	56
386863036	chitinase					Chitin is degraded by a two step process: i) a chitinase hydrolyzes the chitin to oligosaccharides and disaccharides such as di-N-acetyl-D-glucosamine and chitobiose, ii) chitobiase then further degrades these oligomers into monomers. Common in environmental strains	82
686980740	flagellar biosynthesis anti-sigma factor FlgM					Anti-sigma-28 factor, FlgM ;FlgM binds and inhibits the activity of the transcription factor sigma 28. Inhibition of sigma 28 prevents the expression of genes from flagellar transcriptional class 3, which include genes for the filament and chemotaxis. Correctly assembled basal body-hook structures export FlgM, relieving inhibition of sigma 28 and allowing expression of class 3 genes. NMR studies show that free FlgM is mostly unfolded, which may facilitate its export. The C terminal half of FlgM adopts a tertiary structure when it binds to sigma 28. All mutations in FlgM that prevent sigma 28 inhibition affect the C-terminal domain and is the region thought to constitute the binding domain.	83
53716314	chitin binding domain-containing protein					Chitin binding domain; part of chitinase complex	82

741005500	beta-N-acetylhexosaminidase					Chitin-processing protein; part of chitinase complex	82
386860380	phenylacetic acid degradation protein paaN					paaN is a degraded protein product of phenylacetic acid. Phenylacetic acid degradation (PAD) proteins PaaZ (Escherichia coli) and PaaN (Pseudomonas putida) are putative aromatic ring cleavage enzymes of the aerobic PA catabolic pathway. PaaZ mutants were defective for growth with PA as a sole carbon source due to interruption of the putative ring opening system. This CD is limited to bacterial monofunctional enzymes.	84
126452465	OMP85 family outer membrane protein					YaeT. B. pseudomallei Omp85 is immunogenic and potentially able to induce protective immunity against melioidosis.	85
497617556	molecular chaperone DnaK, partial					chaperone protein DnaK ;Members of this family are the chaperone DnaK, of the DnaK-DnaJ-GrpE chaperone system. DnaK/DnaJ Chaperone Machinery of Salmonella enterica Serovar Typhimurium Is Essential for Invasion of Epithelial Cells and Survival within Macrophages, Leading to Systemic Infection	86
386863130	penicillin-binding protein 6					expressed by E. coli functions as a D-alanyl-D-alanine carboxypeptidase. Its precise function is unknown; van der linden showed PBP6 has a biological function clearly distinct from that of PBP5 and to suggest a role for PBP6 in the stabilization of the peptidoglycan during stationary phase	87
126458343	universal stress family protein					Usp: Usp is a small cytoplasmic bacterial protein whose expression is enhanced when the cell is exposed to stress agents. Usp enhances the rate of cell survival during prolonged exposure to such conditions, and may provide a general "stress endurance" activity.	88
740935803	malonic semialdehyde reductase, partial					functionally diverse family of oxidoreductases that catalyze a range of activities including the metabolism of steroids, cofactors, carbohydrates, lipids, aromatic compounds, and amino acids, and act in redox sensing. Also shown to contribute to QS gene expression in B. cenocepacia	89
705765759	ahpC/TSA family protein					peroxiredoxins; antioxidant enzymes that also control cytokine-induced peroxide levels and thereby mediate signal transduction in mammalian cell; Alkyl hydroperoxide reductase subunit C (AhpC) protects bacterial and human cells against reactive nitrogen intermediates	90

53720009	non-hemolytic phospholipase C					Phospholipase C ; melioidosis patients produce IgM against this PC-PLC protein, which was further characterized and shown have an additive effect on increasing the efficiency of Plc-2 to form plaques and contributes to Bp virulence.	91
760219919	preprotein translocase subunit SecA					BPSL3128; TatA; TatA; similar to TatE that is found in some proteobacteria; part of system that translocates proteins with a conserved twin arginine motif across the inner membrane; capable of translocating folded substrates typically those with bound cofactors; similar to a protein import system in thylakoid membranes	92
386865654	N-acetylmuramoyl-L-alanine amidase domain-containing protein					PGPR superfamily protein; promotes growth in salinated environments	93
760221512	alkyl hydroperoxide reductase					Thioreductase family, AhpC; upregulated in colony variants is associated with bacterial persistence and intracellular survival in pathogenic bacteria	10
740933032	membrane protein					BPSL0999; OmpA family transmembrane protein; see above for OmpA function	67
53718085	preprotein translocase subunit SecB					Protein transport chaperone	94
53719357	arginine deiminase					The arginine deiminase system in Pseudomonas aeruginosa was found to be unusually acid tolerant. The acid tolerance of the system allowed bacteria to survive potentially lethal acidification through production of ammonia to raise the environmental pH value.	95
53716140	succinate dehydrogenase iron-sulfur subunit					used for anaerobic respiration by Escherichia coli are structurally and functionally related membrane-bound enzyme complexes	96
760200234	peptidase					NlpD; shown virulence in Yersinia pestis	97
53720930	flagellar hook-associated protein					essential compenent of flagella, necessary for filament formation	98
53720130	hypothetical protein BPSL2520					putative exported protein; Diagnostic signature protein; Serodiagnostic antigen cross-reacts with convalescent sera	99
126675025	BipB					Plays a role in the bacterium-induced formation of multinucleated giant cell (MNGC), which is formed after host cell fusion, as well as in the intercellular spreading of bacteria and in the induction of apoptosis in macrophages. May act in concert with other effector proteins to induce fusion of host cell membranes.	100

53720946	lipoprotein					CsgG; Curli are functional amyloid fibres that constitute the major protein component of the extracellular matrix in pellicle biofilms. They provide a fitness advantage in pathogenic strains and induce a strong pro-inflammatory response during bacteraemia. Curli formation requires a dedicated protein secretion machinery comprising the outer membrane lipoprotein CsgG and two soluble accessory proteins, CsgE and CsgF.	101
126454430	competence lipoprotein ComL					ComL was found to co-purify with the outer membrane, directly interacting with the secretin PilQ. The ComL DNA binding properties and outer membrane localization suggest that this lipoprotein plays a direct role in neisserial transformation.	102
740954837	TonB-dependent receptor					In Gram-negative bacteria, high-affinity iron acquisition requires outer membrane-localized proteins that bind iron chelates at the cell surface and promote their uptake. Transport of bound chelates across the outer membrane depends upon TonB–ExbB–ExbD, a cytoplasmic membrane-localized complex that transduces energy from the proton motive force to high-affinity receptors in the outer membrane.	103
740934573	heat shock protein 90, partial					Heat shock protein 90 (Hsp90) is a molecular chaperone required for the stability and function of a number of conditionally activated and/or expressed signaling proteins, as well as multiple mutated, chimeric, or overexpressed signaling proteins, which promote cell growth or survival or both	104
685732488	putative gp27					bacteriophage functional VgrG homologue; the structure of the hexameric ring formed by Hcp protein (14) is similar to the gp27 trimer and that the sequences of at least 2 T6SS proteins are highly similar to the T4 phage tail components (baseplate protein gp25 and the tail tube protein	105
685696843	sicP binding family protein					T3SS export chaperone protein for cognate effector molecule	106
29726524	Chain A, Crystal Structure Of Catalase-peroxidase Katg Of Burkholderia Pseudomallei					KatG is QS-regulated protein; responsible for bacterial rice grain rot by Burkholderia glumae	107

740924102	lytic transglycosylase					peptidoglycan autolysin; up-regulated during host infection. LT reaction products have been demonstrated to play a role in infections. PG fragments in general elicit many of the general symptoms of bacterial infections such as fever (pyrogenicity), lack of appetite, and sleepiness (somnogenicity) and stimulate the NOD1 and NOD2 intracellular receptors.	108
53720900	S-adenosyl-L-homocysteine hydrolase					often used as target for mechanism-based and competitive inhibitors in anti-viral therapy	Liu, 1992
497624314	membrane protein					BPSL2704; OmpW; see above for OmpW function	90
740987408	hypothetical protein					BPSL2564; Omp W, see above for OmpW function	90
760210258	mechanosensitive ion channel protein MscS					Ion channel; active in regulating osmolarity of bacterial membrane	109
53723542	4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase					involved in the nonmevalonate terpenoid biosynthesis pathway; Detected only in virulent strains of F. tularensis, not in non- or less virulent strains	110
740930838	hypothetical protein, partial					BPSL0525;O-linked N-acetylglucosamine transferase regulated by RpoS subunit of RNA polymerase is a bacterial alternative sigma factor and major regulator important for response to a variety of stress conditions	111
740933003	peptidase S41, partial					S41 family, is a serine protease whose homologues in Gram-negative bacteria have been implicated in a range of biological functions, including pathogenesis	112
126442160	acetyl-CoA acetyltransferase					altered expression by salt stress; confers resistance toward a number of aminoglycoside antibiotics	49
740989003	type VI secretion protein					protein of unknown function; however, the T6SS is involved in pathogenesis of Bp	113
53717914	flagellar hook protein FlgE					See above for flagellar hook protein function	64
124381615	lipoprotein NlpD					The NlpD lipoprotein is the only factor encoded from the pcm locus that is essential for Y. pestis virulence	97
685736080	thermolysin metallopeptidase, catalytic domain protein					extracellular peptidase; Regulated by the cepIR Quorum-Sensing System in Burkholderia cenocepacia	78
740985393	flagellar L-ring protein FlgH					see above for Flagellar proteins	64
386863263	flagellar hook-associated protein FlgK					see above for flagellar hook-associated proteins	64
76818186	bacteriolytic lipoprotein entericidin B-like protein					induces programmed cell death of bacterial populations	58
740985341	chemotaxis protein CheW					involved in chemotactic signal transduction	114

126458493	CocE/NonD family hydrolase					up-regulated under planktonic growth was the CocE/NonD; hydrolase (a protein belonging to a family of aminopeptidases); typically found in soil-borne bacteria	115
53719705	osmolarity response regulator					response protein to hypo-osmolar conditions	
752521184	type VI secretion protein Rhs, partial					Rearrangement hotspot (Rhs); mediate intercellular competition, carry polymorphic C-terminal toxin domains (Rhs-CT/WapA-CT), which are deployed to inhibit the growth of neighboring cells. These systems also encode sequence-diverse immunity proteins that specifically neutralize cognate toxins to protect rhs+/wapA+ cells from autoinhibition. Exported by T6SS	116
760221062	iron transporter					iron transport protein	117
126452666	flagellar hook-associated protein FlgL					the basal body consists of four rings (L,P,S and M) surrounding the flagellar rod, which is believed to transmit motor rotation to the filament. The M ring is integral to the inner membrane of the cell, and may be connected to the rod via the S (supramembrane) ring, which lies just distal to it. The L and P rings reside in the outer membrane and periplasmic space.	118
740962478	TonB-dependent receptor					the function of the tonB gene is needed for energy-dependent transport processes mediated by the outer-membrane receptors for iron siderophore complexes	103
685798421	mucB/RseB family protein					alginate production in P. aeruginosa	119
760217996	H-type lectin domain protein					employ lectins to target host glycoconjugates for recognition and adhesion processes	120
760221062	iron transporter					Iron influx pump	117
126452666	flagellar hook-associated protein FlgL					see above for flagellar hook-associated proteins	64
740962478	TonB-dependent receptor					See above for Ton-B receptor function	103
760221402	thioredoxin					TrxA; virulence-associated protein that promotes intracellular replication and virulence	10
760217559	general secretion pathway protein GspG					The bacterial type II secretion (T2S) machinery delivers large folded proteins from the periplasm through the outer membrane to the extracellular environment.	121
740986062	cyclophilin, partial					Binds cyclosporins and cyclosporin analogs	122
740936864	BON domain protein, partial					A novel conserved protein region – the BON (bacterial OsmY and nodulation) domain – is found in the bacterial osmotic-shock-resistance protein OsmY,	123
685726459	Rhs element Vgr family protein					Essential component of T6SS	59

685736326	taurine catabolism dioxygenase TauD, TfdA family protein					soil survival protein	124
740931457	response regulator SirA, partial					SirA/HilA regulatory cascade is the primary regulon controlling enteropathogenic virulence functions in S. typhimurium.	125
740987967	metallo-beta-lactamase					Anti-biotic resistance	126
685734648	universal stress family protein A					environmental stress response and antibacterial resistance	88
740961813	EvpB family type VI secretion protein					T6SS effector molecule	127
740925154	beta-ketoadipyl CoA thiolase, partial					Thiolase 1 involved in degradative pathway; expression is significantly increased in B. cenocepacia following treatment with antibiotics and when the bacteria is grown under antibiotic resistant conditions	128
126455011	acid phosphatase AcpA					respiratory-burst inhibiting acid phosphatase homologous to acpA of Francisella, which contributes to intramacrophage survival and virulence	129
53716763	porin OpcP1					constitutes one of two parts of the major porin OpcPO, which is essential for antibiotic resistance and selective susceptibility in Burkholderia species	130
740993560	malate synthase					expression is controlled by quorum-sensing genes in B. cepacia	131
740960486	delta-aminolevulinic acid dehydratase					metal-sensitive heme-biosynthesis enzyme; necessary for survival in plant rhizosphere	132
705767187	BON domain protein					see above for BON domain protein function	123
740961148	thioredoxin reductase					Thioredoxin reductase is the only enzyme known to catalyze the reduction of thioredoxin and hence is a central component in the thioredoxin system, which, in the case of TrxA, is involved in intracellular replication and virulence.	10
740974145	phosphoglycerate kinase					Pgk, an important virulence factor in Brucella abortus; associated with birulence in Bp small colony variants	10
685732767	Rhs element Vgr family protein					See above for Rhs element Vgr family protein function	19
740970097	stress responsive protein					BPSL1428; environmental stress response protein	88
740943455	LuxR family transcriptional regulator					intracellular communication proteins essential to the Bp QS system	133
740960650	oxidoreductase					see above for oxidoreductase function	10

740931337	universal stress protein UspA, partial					UspA is involved in stress resistance regulation in pathogenicity and survival within the host. UspA makes an important contribution to the in vivo virulence of Salmonella in mice.	134
490301346	membrane protein					OmpW; see above for Omp W function	19
685801837	universal stress family protein					UspA; see above for UspA function	134
13649898	beta-lactamase precursor					antibiotic resistance; Beta-lactamases are enzymes produced by bacteria that provide resistance to β-lactam antibiotics such as penicillins, cephamycins, and carbapenems.	135
740979585	type IV secretion protein Rhs, partial					See above for Rhs element Vgr family protein function	116
740964823	universal stress protein UspA					UspA; see above for UspA function	134
124384857	ecotin					Ecotin, although present in only a small subset of genera, can inhibit a broad range of serine proteases including those typically associated with the innate immune system such as neutrophil elastase and cathepsin G. An Ecotin deficient B. pseudomallei strain is attenuated in a macrophage and a murine model of infection.	136
740979876	alginate lyase, partial					degradation of alginate; may provide survival advantage within colonies or competitive environment	137
752526605	membrane protein					FhaC; member of Omp85 family of proteins; hemolysin activator-like protein precursor, fhaC (BPSS1728) gene was significantly upregulated during intracellular infection	138
740974463	integrating conjugative element protein pill, pfgi-1					Common in rhizosphere-thriving bacteria, conjugative pili for transmission of mobile genetic elements	139
740967818	flagellin					virulence determinant, TLR-5 agonist; necessary for bacterial motility	140
740940213	beta-lactamase					See above for beta-lactamase function	135
685800313	metallo-beta-lactamase superfamily protein					See above for beta-lactamase function	135
685723194	transglycosylase SLT domain protein					Type IV pilus assembly and T2SS	141
760210213	protein tolQ					Involved in the TonB- independent uptake of group A colicins	142
740965554	BapC protein					T3SS secreted effector protein	143
740952941	flagellar basal body rod protein FlgG					essential component of flagella; see above	64
740933277	Hcp					see above for Hcp function	79
685791047	alkylhydroperoxidase AhpD family core domain protein					disulfide oxidoreductase partners of AhpC proteins, participate in oxidative defense system and are essential for intracellular survival	10

53718291	acyl-CoA dehydrogenase oxidoreductase					Acyl-CoA dehydrogenases are involved in the changes of bacterial membrane fluidity during salt tolerance; may play a role in response to high salt stress abd this role may be in modulation of the membrane layer when B. pseudomallei encounters high salt.	11
740936706	squalene--hopene cyclase					Squalene-hopene cyclase is important because its products, the hopenoids, are very much like sterols in eukaryotes in that they condense lipid membranes and reduce permeability. In the case of prokaryotes, they provide stability in the face of high temperatures and extreme acidity due to the rigid ring structures; Hopanoid production is required for low-pH tolerance, antimicrobial resistance, and motility in Burkholderia cenocepacia	144
685788854	cheB methylesterase family protein					chemotaxis receptor in bacterial chemosensing	145
685713146	bacterial regulatory helix-turn-helix, lysR family protein					Broad-type transcriptional regulator; LysR transcriptional regulation of penA and penR penicillnases, T3SS gene clusters, colony morphology and virulence genes	146
685791441	flagellar P-ring family protein					See above for flagellar protein functions	118
740942950	exopolysaccharide biosynthesis protein					EPS;immunogenic; sera from patients with septicemic melioidosis were tested against the affinity-purified exopolysaccharide and showed strong reactivity, which demonstrates the production of antibodies against this structure in humans and also indicates that the EPS is expressed in vivo	147
740933789	polyketide synthase, partial					this protein synthesizes polyketides, secondary metabolites, that may have pathogenic functions or properties in Bp	148
740958473	fimbrial protein					fimbriae are short pilus structures that allow bacteria to adhere to environmental surfaces and host cells, and pathogenic Bp has been shown to be more efficient than Bt in adhering to and invading host cells	149
685722491	hypothetical protein DP55_1535					T6SS protein	59

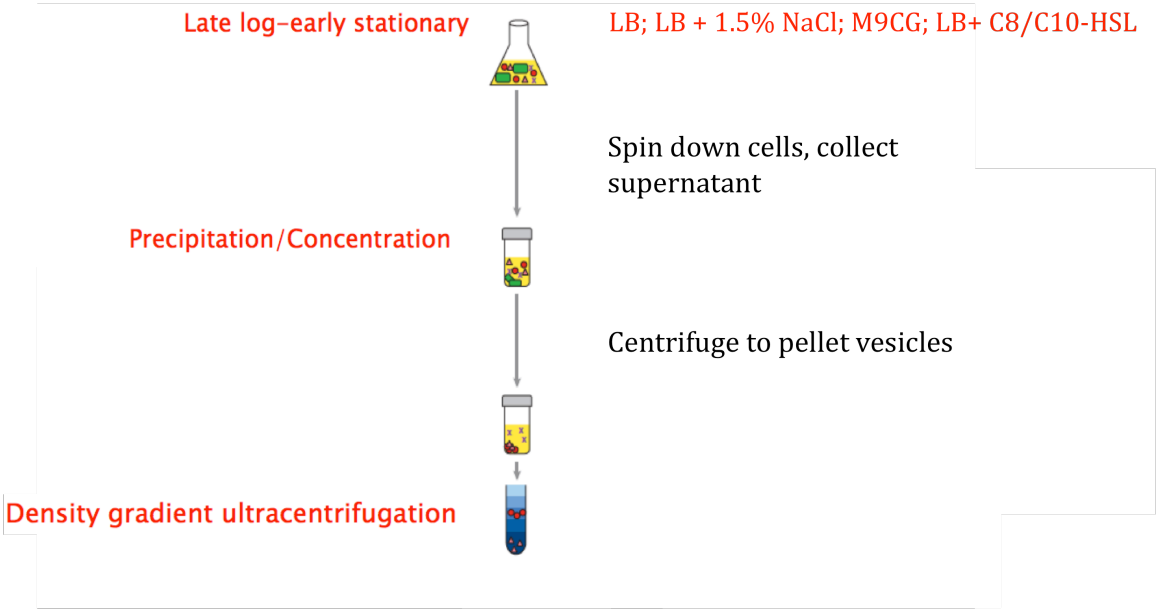

Figure 1.1 OMV extraction and purification from Bp82 grown in enriched media or LB. Bacteria were grown to late log, early stationary phase in appropriately enriched media. Cells were pelleted by low-speed centrifugation and pellets were discarded. Liquid supernatant was sterile filtered and proteins were collected by overnight ammonium sulfate precipitation followed by high-speed centrifugation. Vesicles were purified by density gradient ultracentrifugation.

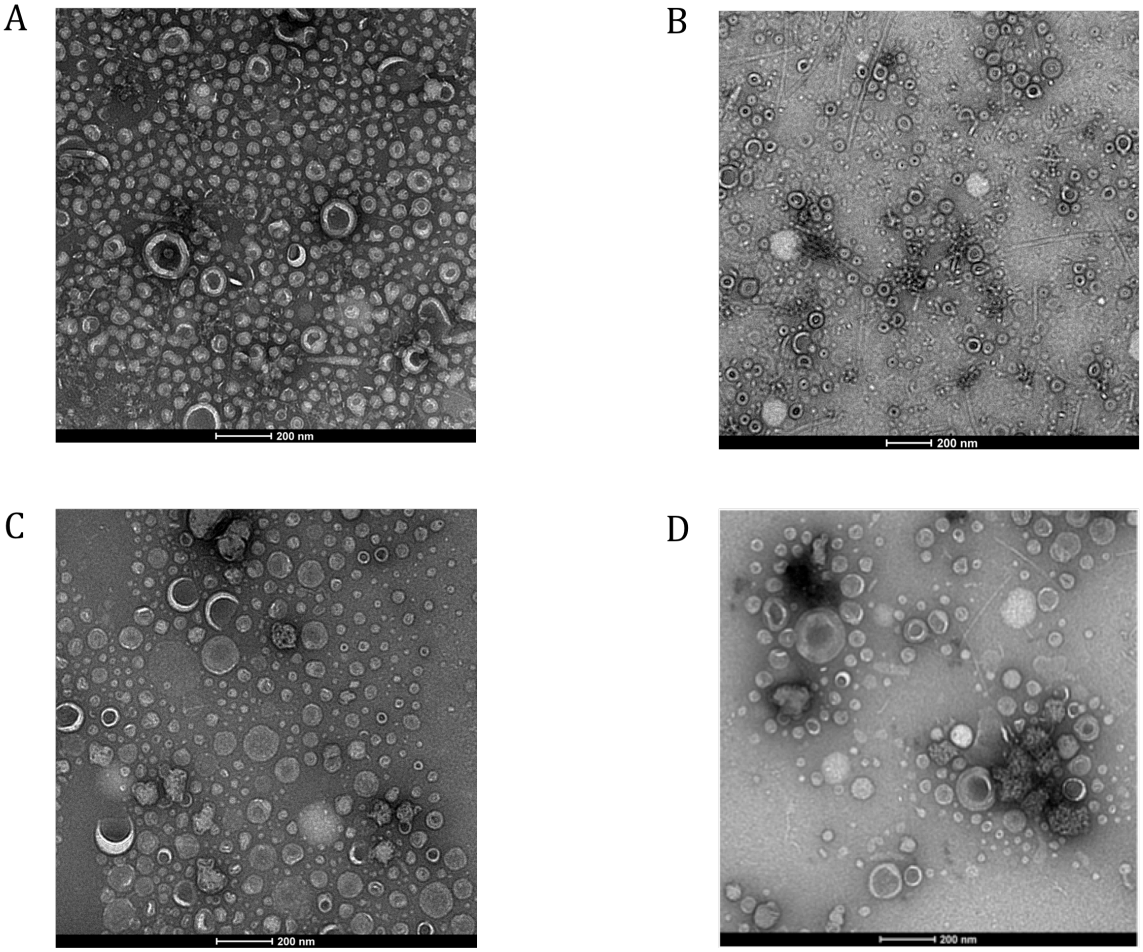

Figure 1.2 Visualization of purified enrichedOMVs by transmission electron microscopy. 1-2uL purified OMVs from each preparation were negatively stained with 1% uranyl acetate and imaged by TEM. OMVs show double membrane structures with an electron dense center and range between 25-250nm in diameter. (A) LB OMVs (B) NaClOMVs (C) M9CG OMVs (D) QS OMVs.
Magnification 31000x; Scale 200nm

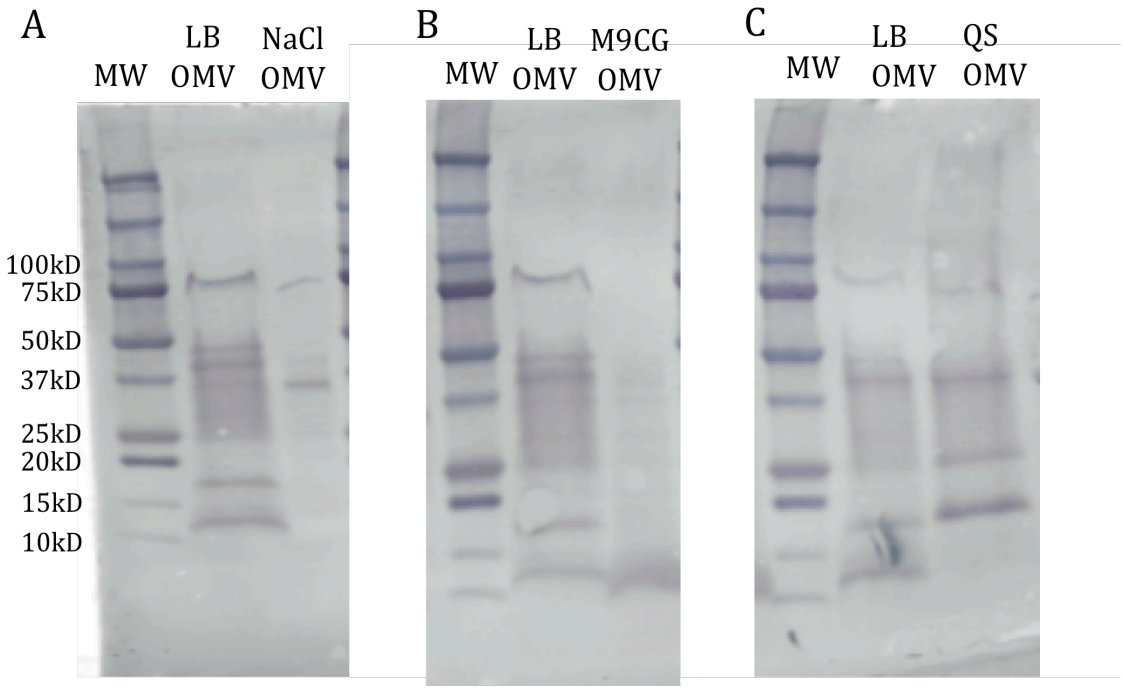

Figure 1.3 Enriched OMVs contain LPS. The presence of LPS in each of the enriched OMV preparations was determined by Western blot. Ten micrograms of each preparation (A)LB OMV vs. NaCl OMV (B) LB OMV vs. M9CG OMV (C) LB OMV vs. QS OMV were probed with Pp-PS-W monoclonal antibody, specific for Bp LPS.

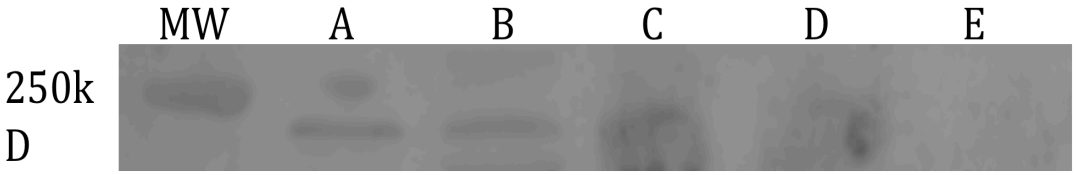

Figure 1.4 Enriched OMVs contain capsular polysaccharide. The presence of CPS in each of the enriched OMV preparations was determined by Western blot. Ten micrograms of each preparation (A) LB OMV (B) NaCL OMV (C) M9CG OMV (D) QS OMV (E) Bt OMV (acapsular negative control) were probed with MC147 monoclonal antibody, specific for Bp CPS. Band is approximately 200kD (Recksiedler-Zenteno, 2005).

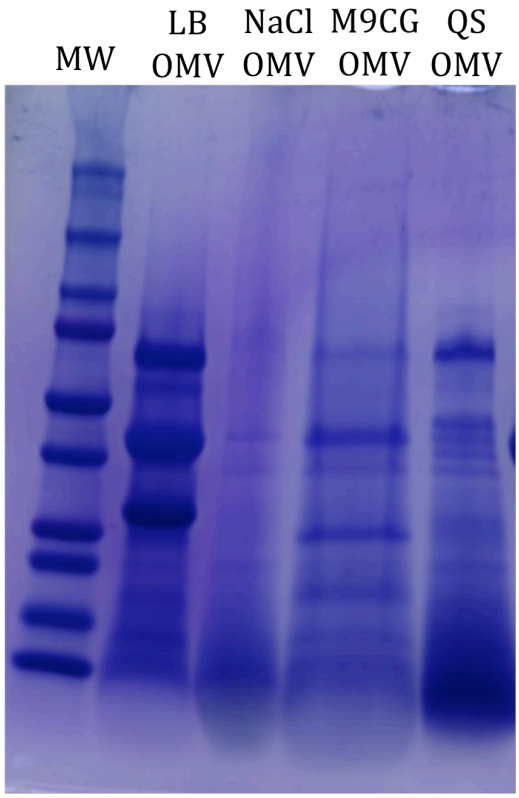

Figure 1.5 Purified enriched OMV protein banding patterns are unique to each preparation as shown by SDS-PAGE. Ten micrograms of each OMV preparation was run on a 4-20% agarose gradient and stained with Coomasie Blue for visual comparison of protein profiles.

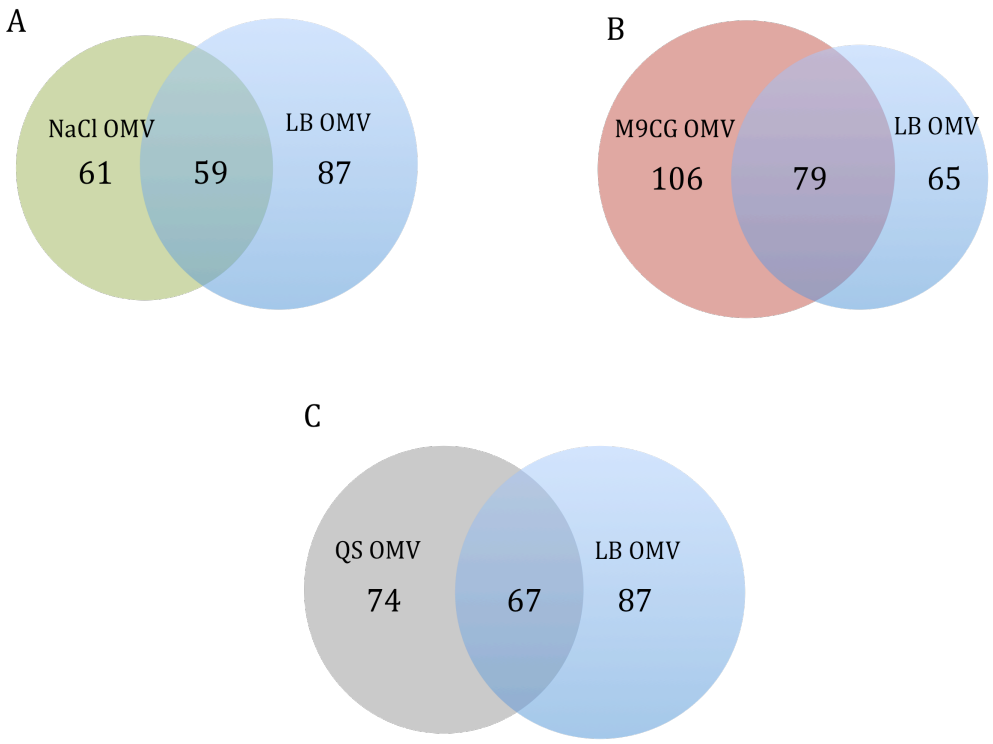

Figure 1.6 LC/MS data confirmed that OMVs grown in enriched media compared those grown in LB contained both shared and unique proteins. Venn diagrams of LC/MS determined shared and unique proteins of enriched OMVs compared to LB-derived OMVs only. (A) NaClOMVs (B) M9CG OMVs (C) Quorum Sensing OMVs

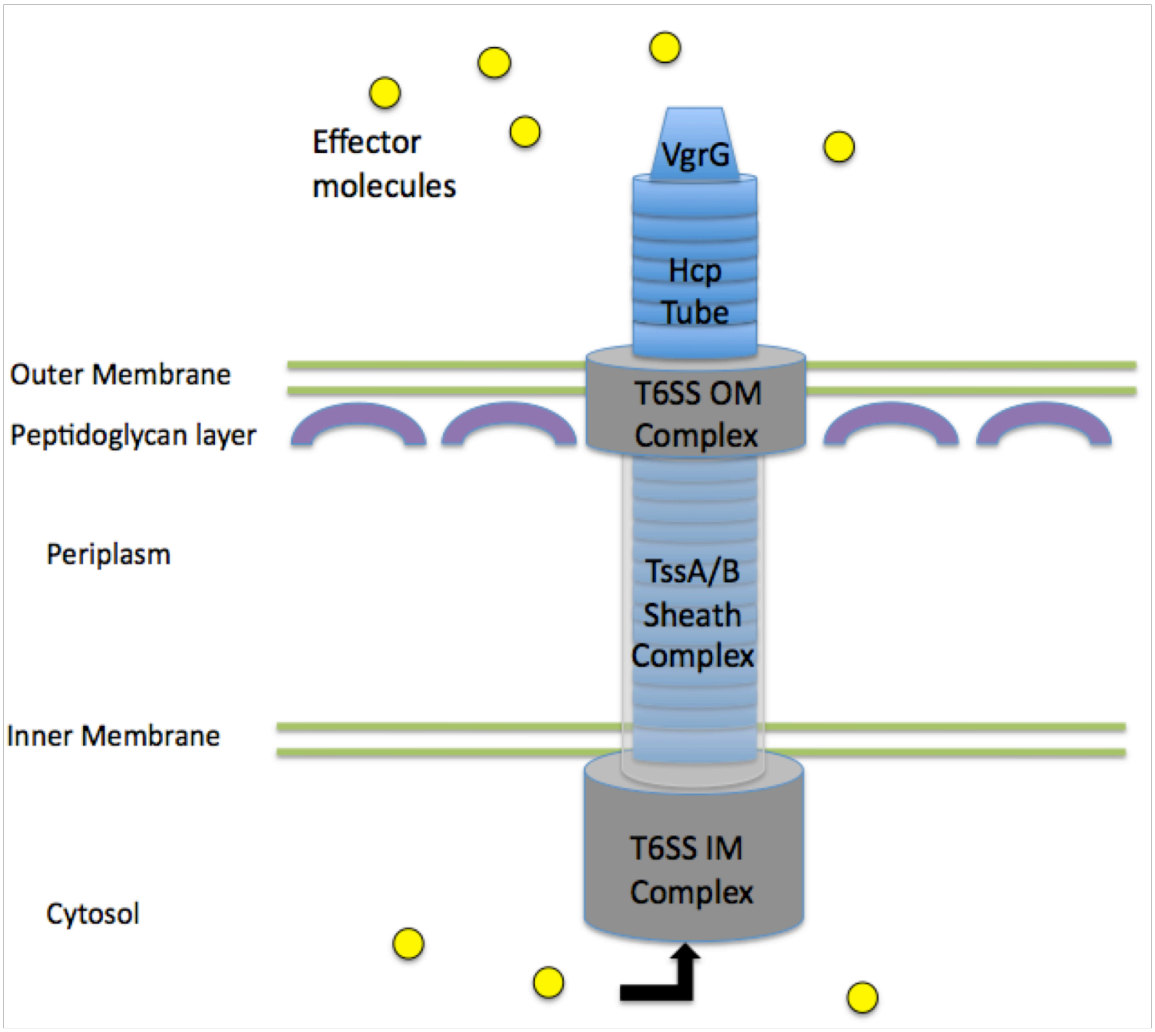

Figure 1.7 Hcp proteins comprise the largest portion of the T6SS apparatus and are a major virulence determinant. T6SS is composed of proteins forming a syringe-like structure composed of stacks of HCP proteins that traverse the membranes and is able to release effector molecules into the host cell.

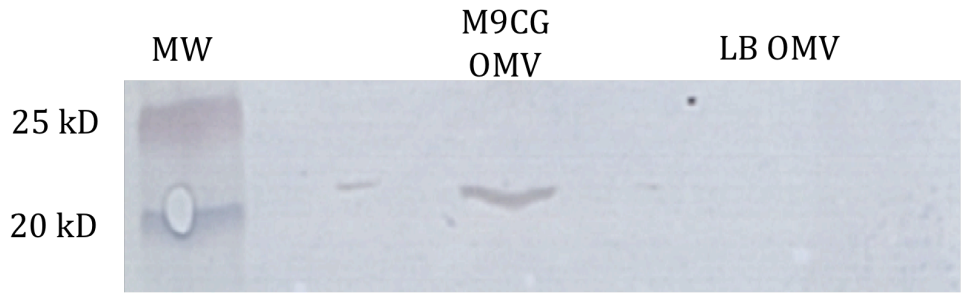

Figure 1.8 HCP-1 is representative of T6*SS* presence and confirms that T6*SS* is found in M9CG OMVs, not in LB OMVs. Presence of HCP-1 was confirmed by WB with rat-HCP-1 serum against 10µg each M9CG OMVs and LB OMVs. HCP-1 band is 21 kD. MW is molecular weight ladder (BioRad).

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Chapter 3

Specific Aim 2

Evaluate the safety and immunogenicity of OMVs derived from Bp grown in media simulating important environmental conditions compared to those produced in LB

Introduction

Safety of administration and lack of reactogenicity are of the utmost importance for vaccine formulation. This is particularly true for diseases, such as melioidosis, which are most commonly found in immune compromised populations [1]. This is also true for travelling military personnel who are uniquely susceptible to Bp infection as a result of higher exposure rates and little to no immunity to Bp, and for whom recovery time is limited due to occupational necessity [2,3]. Additionally, an effective vaccine will induce both a pathogen-specific humoral response and an expansion and maturation of pathogen-specific lymphocytes that will be able to target intracellularly infected phagocytes. Anti-LPS and anti-CPS antibody responses have been shown to provide passive protection against acute IP Bp challenge in mice, however this response was not sufficient to protect against chronic infection and only delayed time to death [4]. This data was contradicted in another study which showed these responses were not correlated with protection against IP challenge and only those animals with the highest combined antibody and cell-mediated immune responses survived [5]. Vaccination studies against melioidosis have further indicated that complete protection will likely require both robust antibody responses and sufficient T cell activation [5].

Killed whole cell Bp vaccines, which consist of whole pathogens that have been heat or chemically killed, provide a relatively high level of specific immunity, particularly to the CPS, LPS, and peptidoglycan of bacteria, but often require

multiple administrations to achieve the necessary responses for protection [6,7]. While killed whole cell vaccines are able to elicit a robust antibody response, they stimulate much weaker cell-mediated responses [8,9]. In order to enhance the initial responses, an adjuvant is often added to a vaccine formulation. This, in turn, leads to further safety issues and potential for adverse reactions at the vaccine site, such as redness and swelling. Similarly, live attenuated vaccines are contraindicated for use in immune compromised populations due to the possibility of reversion to wild-type, their ability to replicate *in vivo,* and the potential for infection. This is of particular concern with Bp, which is able to establish persistent latent infection [10]. One such example of a Bp live attenuated vaccine strain that has been extensively studied is the auxotroph Bp82 [11], which is also the strain our lab currently utilizes for OMV production. Immunization with Bp82 conferred significant protection against intranasal challenge and acute infection, with reduced tissue bacterial burdens but this protection was found to be independent of both $CD4^+$ and $CD8^+$ T cells which may be necessary for protection against latent or chronic infection [12]. Mice lacking in B cells were not protected but passive transfer of serum from Bp82-immunized mice provided partial protection to non-vaccinated animals, thus humoral immunity was found to be critically important for vaccine-induced protection against Bp [12].

OMVs provide an innovative vaccine platform that bypasses many of the safety concerns posed by other formulation types, while stimulating robust humoral and cellular immune responses. By enriching the OMVs with a multitude of virulence factors and outer membrane proteins necessary for intracellular survival

and persistence, Bp OMVs may also induce the antigen-specific cell-mediated immune responses necessary to target intracellularly infected cells and prevent chronic infection. OMVs deliver a comprehensive representation of the bacteria from which they are derived but are nonviable, nonreplicating, and noninfectious on their own. Thus, enriched Bp OMVs may be a safer alternative to current vaccine candidates, while maintaining the necessary immunogenic properties to provide protection. Bp OMVs derived from Bp grown in LB have previously been shown to be safe and well tolerated in mice and rhesus macaques and induce LPS and CPS-specific IgG and IgM responses [13,14]. However, LB OMV immunized mice were unable to clear the bacteria and animals maintained viable bacteria in systemic tissues [15]. The ability to achieve the necessary antigen-specific immune responses to induce sterilizing immunity through vaccination has been elusive. By enriching the Bp OMVs with a number of virulence factors and tightly-regulated proteins through the use of selective media, it may be possible to achieve antigen-specific $CD4^+$ and $CD8^+$ responses to target Bp-infected cells. As shown in **Chapter 2**, enriched Bp OMVs extracted from Bp grown in selective media contain a number of immunogenic components and proteins, including LPS (**Figure 1.3**), CPS (**Figure 1.4**), and the major component of the T6SS apparatus (HCP-1) (**Figure 1.8**), among other important protective antigens (**Table 1**). A number of these important factors that are represented in the enriched OMVs have previously been tested individually for use as subunit vaccine components against Bp. These include LPS, CPS, and HCP-1 [16]. Previous studies showed that while mice vaccinated with purified CPS and LPS eventually succumbed to infection following challenge, the immunization stimulated

strong antibody responses and significantly extended the survival time after systemic challenge [17]. As investigated in **Chapter 2**, all OMV preparations contained both CPS and LPS but with different expression profiles, which may affect antibody responses directed to these immunogenic components. Alternatively, researchers demonstrated that immunization with rHcp-1 only provided 50% protection in mice challenged with Bp, but were unable to obtain any viable bacteria in spleens from surviving mice [16]. This data indicates that T6SS proteins, particularly HCP-1, may be essential vaccine antigens for protection against chronic infection. Our LC/MS data (**Table 1**) confirmed the presence of HCP-1 in both M9CG and QS OMVs, while it was not detected in NaCl or LB OMVs. By immunizing animals with the different enriched OMV preparations, it may also be possible to induce robust antibody responses specifically targeted to these protective proteins and polysaccharides. Additionally, the inclusion of HCP-1 in the M9CG or QS OMVs may induce greater cell-mediated immune responses compared to the LB OMVs, and contribute to bacterial clearance. IFNγ production is indicative of a Th1 response, which has previously been shown to contribute to protection against Bp and may be necessary for preventing latent infection [18]. Here, splenocytes from OMV immunized mice were used to investigate $CD4^+$ and $CD8^+$ IFNγ production for the Th1 response.

Within this study, the serum IgG and IgM antibody responses against these known immunogenic components, along with the $CD4^+$ and $CD8^+$ IFNγ production were evaluated following immunization plus boost of enriched OMVs in a murine model. We hypothesized that mice immunized with OMVs enriched in a combination of tightly regulated survival proteins and virulence factors would

mount robust Bp antigen-specific serum IgG and IgM and have increased $CD4^+$ and $CD8^+$ IFNγ production as compared to mice immunized with LB OMVs.

Materials and Methods

Evaluation of OMV toxicity in vitro- The toxicity of enriched Bp OMVs from selective media were compared to E. coli OMVs, which are known to be toxic, by administration to RAW 264.7 murine macrophages (ATCC). Macrophages were plated at a density of 2.5 x 10^5 cells per well in complete cell media, consisting of DMEM (Gibco)+ 10% heat-inactivated FBS (Atlanta Biologicals) + 2% Na_2CO_3 (Sigma) + 1% antibiotic/ antimycotic (Gibco), in untreated 24-well plates (Fisher). Cells were allowed to adhere and grow to a confluent monolayer for 24 hours at 37°C with 5% CO_2. After 24 hours, media was removed from the wells and cells were washed with 1% PBS. Duplicate wells were treated with 10μg NaCl OMVs, M9CG OMVs, QS OMVs, 2μg E. coli OMVs, or untreated. Each treatment was prepared in 500μL/ well complete cell media, with untreated wells receiving complete cell media only. Cells were assessed for viability after 8 hours of treatment by visualizing with an inverted light microscope (Nikon, Tulane Department of Microbiology and Immunology Core Laboratory) on the 40x objective and viability staining with Trypan Blue (Sigma). Cells from each well were gently scraped from the well using a cell scraper (Fisher) and suspended in 0.04% Trypan Blue. Cell viability was assessed on a cell counter (Nexelcom Bioscience Cellometer, Tulane Department of Microbiology and Immunology Core Laboratory).

Determination of OMV safety in vivo- Safety of the enriched OMVs in a small animal model was first tested in *Galleria mellonella* (wax moth larvae). *G. mellonella* are commonly used for in vivo toxicity studies as their immune response is dependent upon haemocytes, which are functionally homologous to mammalian phagocytes and are capable of engulfing bacteria and generating bactericidal compounds [19]. The wax worm has also been previously shown to be susceptible to Bp and used in Bp toxicity studies [20]. For our study, larvae were divided into groups of 10 per treatment and kept in petri dishes with sawdust and wax worm food (Waxwormkit.com). Treatments were as follows: 1.0μg each of NaCl OMV, M9CG OMV, QS OMV, or E.coli OMV as a positive control, or 10μL sterile saline as a negative control. OMVs were administered with sterile saline in a total volume of 10μL using a 1 mL syringe and 27 gauge needle (Fisher), injected into the left proleg in the second back segment. Larvae were only treated once and were observed for the first two hours post-treatment to account for death due to injection trauma and not the toxicity of the treatment itself and were replaced. Larvae were visually evaluated and scored for size (measured with a ruler for signs of shrinkage), color (white, cream, brown, black, or pupae), motility, and responsiveness to stimuli. Larvae were scored at two hours post-injection and every 24 hours thereafter. Larvae began to pupate after 4 days as a natural progression of life, however only those that were nonmotile and unresponsive to stimuli were considered dead. Percent survival was analyzed at day 6 post-treatment using a log-rank Mantel-Cox survival test.

In order to evaluate safety in a small mammalian animal model, female BALB/c mice (Charles River) 7-9 weeks old were administered OMVs by the subcutaneous (SC) route. Groups consisted of 5 mice each and were given 100μL total volume of 10μg LB, NaCl, M9CG, QS OMVs or saline only using 27 gauge needles, injected into the scruff of the neck. Saline only animals were used as a negative control, while LB OMVs have previously been shown to be non-toxic and safe in BALB/c mice and were used for comparison purposes [14]. Groups were injected two times, 21 days apart. Animals were observed for signs of reactogenicity at the site of injection, such as redness, swelling, or associated nodules, and toxicity or signs of disease, such as ruffled fur, huddling, or decreased behavior, for 48 hours post-administration as well as four weeks after the second treatment.

Characterization of Bp-specific antibody responses to OMV immunization- Serum was collected from mice treated with OMV preparations. Mice used for safety studies were euthanized for analysis of immune responses. Five BALB/c mice per group were immunized SC with 10μg LB, NaCl, M9CG, or QS OMVs in 100μL total saline, or 100μL saline alone, as a control. Animals were immunized on a prime-boost schedule with 21 days between each administration. Three weeks after the boost, animals were euthanized by CO_2 asphyxiation and blood was collected from each mouse by cardiac puncture. The blood was kept in individual serum collection tubes and allowed to clot for 30 minutes at room temperature prior to centrifugation at 9000g for 5 min in a tabletop centrifuge (Beckman Coulter). Serum was collected and stored at -80°C until assayed by ELISA.

Flat-bottomed ninety-six well plates were coated with 0.5μg/well LB OMV, Bp LPS (provided by Dr. Paul Brett, University of South Alabama), or Bp CPS (provided by Dr. Brett) in Carbonate Coating Buffer (0.1M sodium bicarbonate + 0.2M sodium carbonate + 0.1g sodium azide in 500mL autoclaved, sterile dH_2O), 100uL/well, incubated overnight at 4°C. Plates are then washed three times each with PBST (1x PBS + 0.05% Tween20). Two-fold serial dilutions of each serum sample were prepared in each column of the plates and incubated for 2 hours at room temperature. Plates were again washed three times with PBST and the secondary antibody was added. Each sample was screened with alkaline phosphatase conjugated rat anti-mouse IgG (1:300 dilution in PBST) (Sigma) and alkaline phosphatase conjugated goat anti-mouse IgM (1:2,000 dilution in PBST) (Sigma). The secondary antibodies were allowed to incubate for one hour at room temperature before plates were washed three times with PBST. Plates were developed with 100μL/well SIGMAFAST p-Nitrophenyl phosphate tablets (Sigma) dissolved in diethanolamine buffer (48.5mL diethanolamine + 0.5g Magnesium Chloride in 450 mL autoclaved dH_2O, pH 9.8). Plates were allowed to develop for 15-30 minutes, then reactions were stopped with 50μL 2M NaOH (Sigma) stopping solution. Plates were read at 405nm using a μQuant microplate reader (BioTek) and analyzed with Gen5 Software (BioTek). Results were expressed as the mean reciprocal endpoint titers for total serum IgG or IgM. Here, endpoint titer is defined as the greatest dilution yielding an optical density (OD_{405}) greater than three standard deviations above the mean OD405 for “blank” background wells. Further analysis was performed using one-way ANOVA by GraphPad Prism, comparing

serum values from mice immunized with enriched OMVs compared to serum from mice immunized with LB OMVs.

Protein-specific rHCP-1 ELISA was performed in a similar manner as above. However, rHCP-1 (provided by Dr. Mary Burtnick, University of South Alabama) was coated at 100ng/well. Following the coating incubation period and subsequent plate washing, plates were blocked with 2% BSA (Sigma) in PBST for 1 hour. Plates were then washed and serum samples were added as described above. The rHCP-1 ELISA was tested for IgG alone, using mean reciprocal endpoint titers for total serum IgG as final readout.

Evaluation of cell-mediated immune responses to OMV immunization- Splenocytes were harvested from immunized mice and single cell suspensions were obtained by rupturing the spleen and passing cells through a 70μm cell strainer (Fisher). Cells were washed with cold Hank's Buffered Saline Solution (HBSS) and centrifuging at 300xg, 5 min, 4°C. The wash was repeated. Pellets were resuspended in ACK lysis buffer (Invitrogen) for 3 minutes to lyse the red blood cells. The lysing reaction was stopped with cold HBSS and samples were centrifuged 1500x g, 10 min, 4°C. The leukocyte cells were washed with HBSS and centrifuged for a total of two washes. The resulting pellets were resuspended in complete RPMI (cRPMI, RPMI media + 10% FBS +1% antibiotic/antimycotic). Cells were counted and were plated at 2 x 10^6 cells/well in a final volume of 100μL cRPMI in a 96-well round bottom microtiter plate (Fisher). Splenocytes were then stimulated with 5μg of LB OMV in cRPMI. For a positive control, replicate wells were stimulated with 20μg/ml

(final concentration per well) of phorbol 12-myristate 13-acetate (PMA, Sigma) and 100ng/ml Ionomycin (Sigma) mitogens in cRPMI. As a negative control, unstimulated cells were incubated with cRPMI only. Incubation with stimulants occurred for 2 hours, 37°C, 5% CO_2. Next, Golgi plug was added 100μL/well (BD, stock concentration at 1μL to 1mL cRPMI) and incubated a further 6 hours, for a total incubation time of 8 hours. Cells were spun down in the plates by centrifuging at 300 x g for 5 minutes. Supernatants were removed and cells were washed with 200μL sterile PBS, spun, and flicked. Cells were resuspended in 50μl sorter buffer containing 5μg/ml Fc Block + 1 μl/ml Golgi plug, and incubated at room temperature for 10 minutes. Following this incubation, cells were stained with surface stain cocktail in a volume of 50μl sorter buffer added to the top of each well. Surface stain cocktail consisted of CD3 (PE-Cy5), CD4 (PerCP-Cy5.5), CD8 (ef450), CD44 (APC-ef780), and IFN-γ (PE) (all stains from eBioscience) combined in sorter buffer. Cells were incubated with stains for 20 minutes, room temperature, in the dark. Plates were washed with cold sorter buffer, centrifuged, and flicked a total of 2 times. Cells were fixed by resuspending in 100μl BD cytofix/cytoperm buffer and incubated 20 minutes at room temperature. Cold 1x BD Perm/Wash buffer was added at 150μl/well, spun, and flicked for 2 total washes. Cells were then resuspended in intracellular antibody cocktail in the same manner as the surface stain cocktail, with 10μl per well of intracellular stain cocktail. This was incubated overnight at 4°C in the dark. The following day cells were washed twice with 150μl Perm/Wash buffer, centrifuged, and flicked, and resuspended a final time in 200μl of sorter buffer and stored covered by a light-tight covering at 4°C until samples

were ready to be run by flow cytometry. To prepare for running, cells were resuspended and filtered through 100μm nylon mesh into a polystyrene round bottom tube. Flow cytometry was performed on a BD Fortessa (Tulane Department of Microbiology and Immunology Core Laboratory) and analysis was done using FlowJo analysis software.

Results

Selectively enriched OMVs are non-toxic in vitro- RAW murine macrophages survived treatment with enriched Bp OMVs up to 8 hours (**Figure 2.1**). Microscopy images obtained at 8 hours (40x magnification) showed that cells treated with NaCl OMVs (**Figure 2.1A**), M9CG OMVs (**Figure 2.1B**), or QS OMVs (**Figure 2.1C**) maintained their monolayer as well as untreated cells (**Figure 2.1E**). The wells treated with *E.coli* OMVs had noticeably fewer cells and a disrupted monolayer (**Figure 2.1D**). **Figure 2.1F** confirmed this by Trypan blue viability testing, which showed NaCl OMV treated (74% viability), M9CG OMV treated (65% viability), QS OMV treated (86% viability) were comparable to untreated cells (72% viability) after 8 hours of treatment. In contrast, the *E.coli* OMV treated cells only had 43% viability at the endpoint.

Enriched OMVs are safe and non-toxic in vivo- Galleria mellonella (n=10/group) injected with 1μg NaCl OMV, M9CG OMV, QS OMV, *E. coli* OMV, or saline alone were visually inspected for changes in color, motility, size, and response to stimuli for up to 6 days, with non-responsive (pupate or otherwise) considered

dead (**Figure 2.2**). At Day 6 post-immunization, 100% of NaCl OMV treated (**Figure 2.2A**), 80% M9CG OMV treated (**Figure 2.2B**), and 100% QS OMV treated (**Figure 2.2C**) larvae maintained their color, size, and motility, and responsiveness. This is in contrast to *E.coli* OMV immunized wax worms, of which only 20% did not undergo any physical change following treatment (**Figure 2.2D**). Treatment with enriched OMVs or saline alone was non-toxic, with 100% of larvae in each Bp OMV immunization group surviving through the study endpoint, while significantly fewer (40%) of *E. coli* treated animals survived to Day 6 (**Figure 2.2F**).

Following subcutaneous administration of LB OMVs, NaCl OMVs, M9CG OMVs, or QS OMVs to BALB/c mice, none of the animals showed signs of toxicity or reactogenicity resulting from the OMV formulation or injection, such as swelling or redness at the site of injection, huddled or hunched behavior, inactivity, ruffled fur, or excessive grooming at the injection site. The immunized animals behaved normally and there were no differences among groups compared to the animals receiving saline alone (data not shown).

Immunization with QS OMV stimulates greater Bp-specific antibody responses as compared to LB OMVs- There was no difference in OMV-specific serum IgG or IgM in mice immunized with LB OMVs as compared to mice immunized with NaCl or M9CG OMVs, however serum from BALB/c mice immunized with QS OMV demonstrated a significantly greater OMV-specific IgG response than LB OMVs (**Figure 2.3**). Similarly, animals immunized with QS OMVs induced significantly greater Bp CPS-specific serum IgG and IgM compared to animals immunized with LB

OMVs, but there was not a significant difference in Bp CPS-specific serum antibody between LB OMV immunized mice and those receiving NaCl or M9CG OMVs (**Figure 2.4**). As shown in **Figure 2.5**, serum from mice immunized with NaCl or M9CG OMVs had significantly less LPS-specific serum IgG as compared to mice immunized with LB OMVs, whereas mice immunized with QS OMVs produced significantly greater LPS-specific serum IgG. **Figure 2.5A** also shows that NaCl OMV immunized mice produced significantly less LPS-specific IgM compared to LB OMV immunized mice (**Figure 2.5B**). Saline immunized mice did not exhibit Bp-specific serum antibody reactive for the antigens used in these assays.

Immunization with enriched OMVs elicits equal serum IgG to Bp-specific rHCP-1 compared to immunization with LB OMVs- There was not a significant difference among animals immunized with different OMV preparations for rHcp-1 –specific serum IgG (**Figure 2.6**).

*Immunization with QS OMVs induces greater cell-mediated immune responses as compared to LB OMVs***-** We observed no differences among NaCl OMV immunized, M9CG OMV immunized, and LB OMV immunized IFNγ^+ $CD4^+$ or $CD8^+$ T cell numbers (**Figure 2.7**). However, splenocytes from mice immunized with QS OMVs had significantly more IFNγ-producing $CD4^+$ (**Figure 2.7A**) and $CD8^+$ T (**Figure 2.7B**) cells following restimulation with Bp OMVs compared to splenocytes from mice immunized with LB OMVs.

Discussion

Successful and protective vaccines should be both safe and immunogenic in populations that are most susceptible to a given disease. This is particularly important for melioidosis, which is often associated with patients who are immune compromised. The potential for infection, reactivation, or severe adverse side effects limits the use of live inactivated or killed whole cell vaccines in this population. Bp OMVs provide a nonviable, non-reactogenic vaccine alternative that have been shown to be safe in various animal models [13,14]. We hypothesized that OMVs enriched with tightly regulated antigens, virulence factors, or survival proteins would remain safe and non-toxic both *in vitro* and *in vivo* and would be as well tolerated as LB OMVs. Here, we show that enriched OMVs are non-toxic *in vitro* in murine macrophages and *in vivo* using *G. mellonella.* Enriched OMVs were also shown to be safe after subcutaneous administration in mice with no signs of reactogenicity or illness following immunization.

We predicted that OMVs enriched in immunoreactive antigens, including polysaccharides and selectively regulated proteins, would stimulate greater protective immunity than the previously evaluated LB OMVs. Antigen-specific antibody and $CD4^+$ and $CD8^+$ T cell responses in OMV-immunized mice were evaluated to investigate differences in immunogenicity in OMV vaccine preparations. OMV-specific IgG and IgM responses were similar among LB OMV-, NaCl OMV-, and M9CG-OMV immunized mice. However, QS OMV immunized mice had a significantly greater OMV-specific IgG. As seen in **Chapter 2**, QS OMVs had the most similar protein profile pattern compared to LB OMVs when equal protein

concentrations were evaluated. This increase in IgG may be explained if QS OMVs have increased expression and production of antigens shared with LB OMVs, which were used for coating for the OMV-specific IgG and IgM ELISAs. Similarly, there was significantly greater CPS-specific IgG and IgM in the serum of mice immunized with QS OMVs compared to that of mice immunized with LB OMVs. CPS is an important protective surface component for Bp, and may be increased under QS conditions. A study in *Burkholderia thailandensis* stated that QS activates two clusters of genes responsible for CPS production and may promote defense against antimicrobial factors [21]. This function may be preserved in genetically related Bp. The significant difference between LPS-specific IgG in LB OMV immunized mice and NaCl OMV immunized mice is unsurprising, as it was seen in **Chapter 2** that NaCl OMVs contain less LPS and present a different expression profile. LPS acts as a permeability barrier on bacterial surfaces, particularly to hydrophobic agents [22], some of which may be important for growth in the soil and in the environmental microbiome of plant roots where Bp is naturally found. The decrease in LPS production may provide an advantage for intake of growth factors in the soil, but limits the LPS-specific immunogenicity by NaCl OMVs. However, there was a significant increase in LPS-specific IgG in mice immunized with QS OMVs as compared to LB OMV immunized mice. Sawasdidolin, et al. showed that the LPS pattern profile was different when Bp is grown in QS conditions that induce biofilm formation [23], which was also seen in the QS OMV LPS protein pattern presented in **Chapter 2**. Their study of Bp biofilm mutants ultimately showed that the drug resistance mechanism of biofilm-forming bacteria is not by forming the biofilm

itself, but from the upregulation of certain factors when biofilm genes are stimulated by quorum sensing molecules [23]. Through the inclusion of these gene products in QS OMVs, it may be possible to target the immune response to these factors and inhibit future antibiotic resistant Bp infections and immune-evasion by persistent bacteria. However, despite the confirmed presence of Hcp-1 in M9CG OMVs and QS OMVs, there was not a significant difference in serum IgG to recombinant Hcp-1 compared to mice immunized with LB OMV or NaCl OMV preparations, which do not contain Hcp-1. It may require a greater concentration of these proteins than is currently present to elicit a significant antigen-specific response. The levels of rHcp-1-specific IgG in all animals was extremely low, indicating that expression of the T6SS may be more tightly regulated than expected and, in order to specifically target this system, it may be necessary to significantly upregulate Hcp-1 expression through genetic modifications. Despite this, immunization with QS OMVs induced immune responses that may contribute to long-term clearance of intracellular bacteria. IFNγ production, which is associated with a Th1 response, was significantly increased for mice immunized with QS OMVs compared to those immunized with LB OMVs. This indicates that a number of other antigenic factors specifically expressed in the QS OMVs may contribute to the increased $CD4^+$ and $CD8^+$ responses. This combination of factors implies that QS OMVs have enhanced immunogenicity as compared to LB OMVs. The results presented here are important for predicting potential protection provided by these formulations knowing that both humoral and cell-mediated immune responses are required for bacterial clearance and protection through against acute and chronic infection.

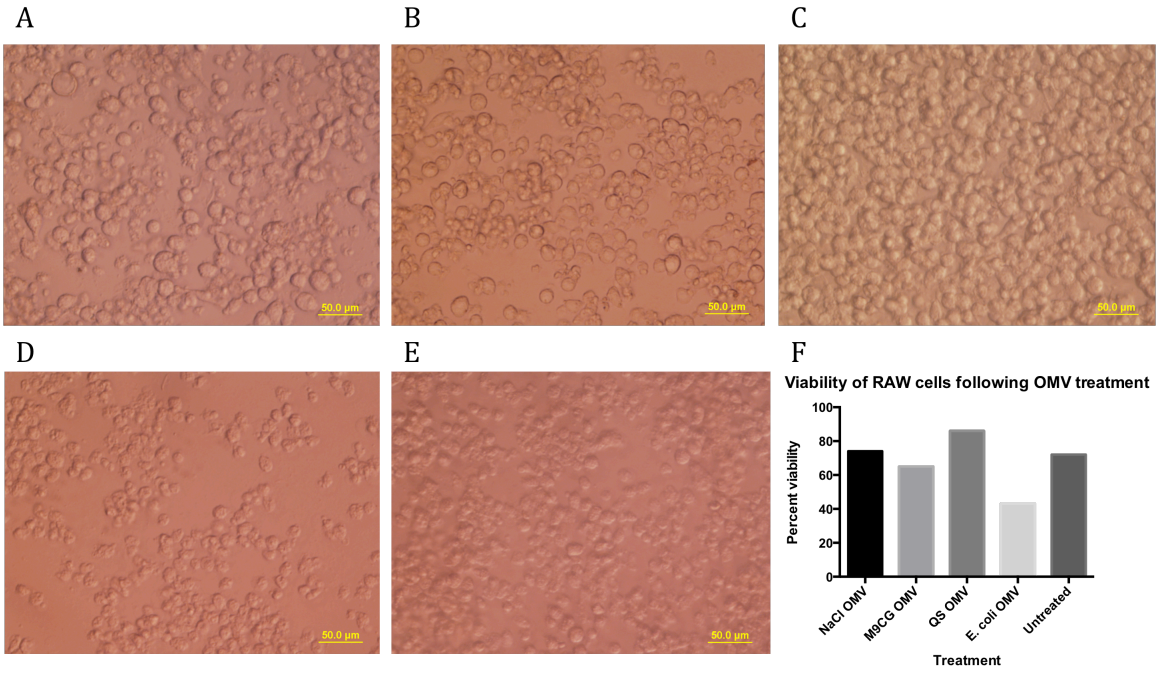

Figure 2.1 Enriched Bp OMVs are nontoxic *in vitro*. RAW murine macrophages were treated with 10 µg NaCL OMVs (A), M9CG OMVs (B), QS OMVs (C), 2 µg E. coli OMVs (D), or left untreated (E) for 8 hours. Microscopy images were obtained at 8 hours (40x magnification). Cells were scraped from each well and treated with Trypan Blue for viability assessment (F). Treatment with Bp OMVs was nontoxic to cells. Scale bar 50µm.

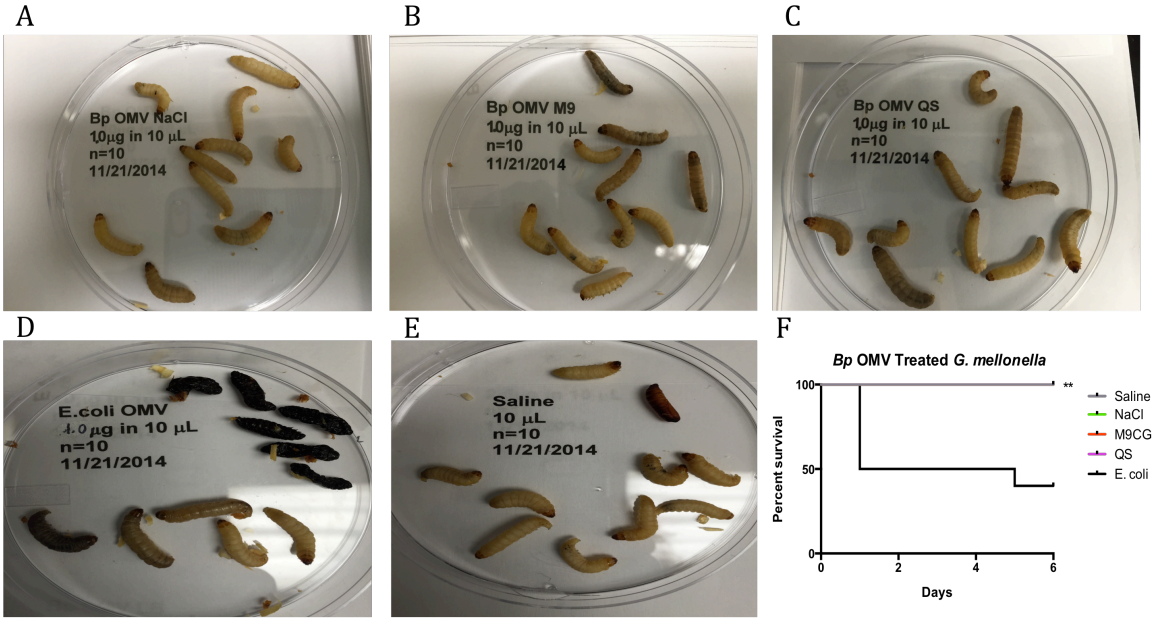

Figure 2.2 Low doses of enriched Bp OMVs are non-toxic in *Galleria mellonella* larvae. *G. mellonella* (n=10) were treated with 1µg NaCl OMV (A), M9CG OMV (B), QS OMV (C) E. coli OMV (D), or saline alone (E). Larvae were visually inspected every 24 hours, from Day 0 (2 hours post-injection) to Day 6, and were scored on color, motility, size, and responsiveness. Non-responsive (pupae or otherwise) were considered dead. Treatment with enriched OMVs was tolerated as well as saline, while significantly fewer (40%) of E. coli treated animals survived. Analysis performed by suvival analysis, log-rank Mantel-Cox test. *P<0.05

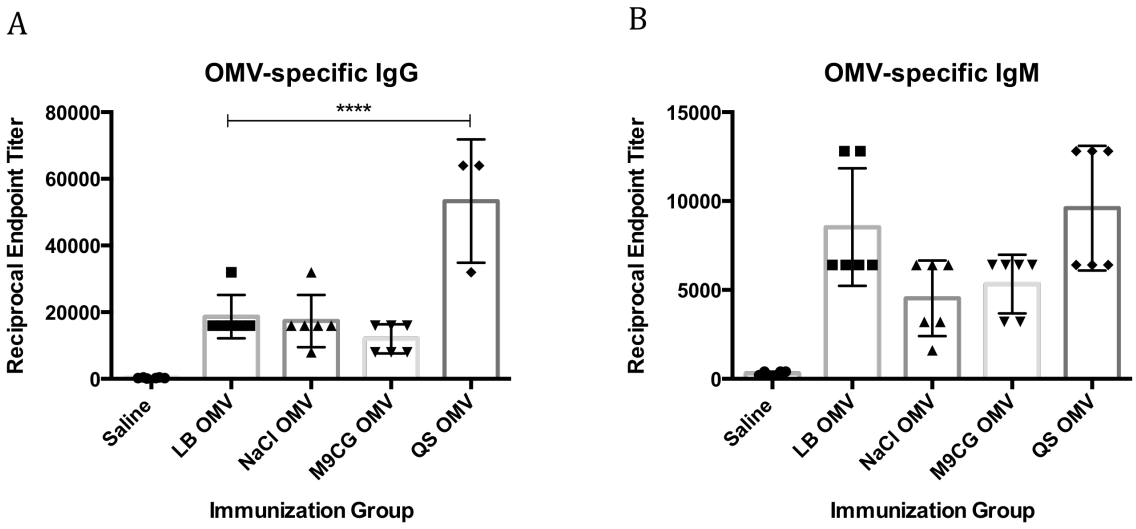

Figure 2.3 Immunization with QS OMV stimulates greater OMV-specific IgG as compared to LB OMVs. Bp OMV-specific serum IgG (A) and IgM (B) were measured by ELISA using 96-well plates coated with 0.5μg/well of purified LB OMVs. Serum from BALB/c mice (n=6) immunized with QS OMV had a significantly greater OMV-specific IgG response than LB OMVs; however, there was no significant difference in OMV-specific IgM production among OMV-immunized mice. ****P<0.0001 by one-way ANOVA and Dunnett's multiple comparisons test

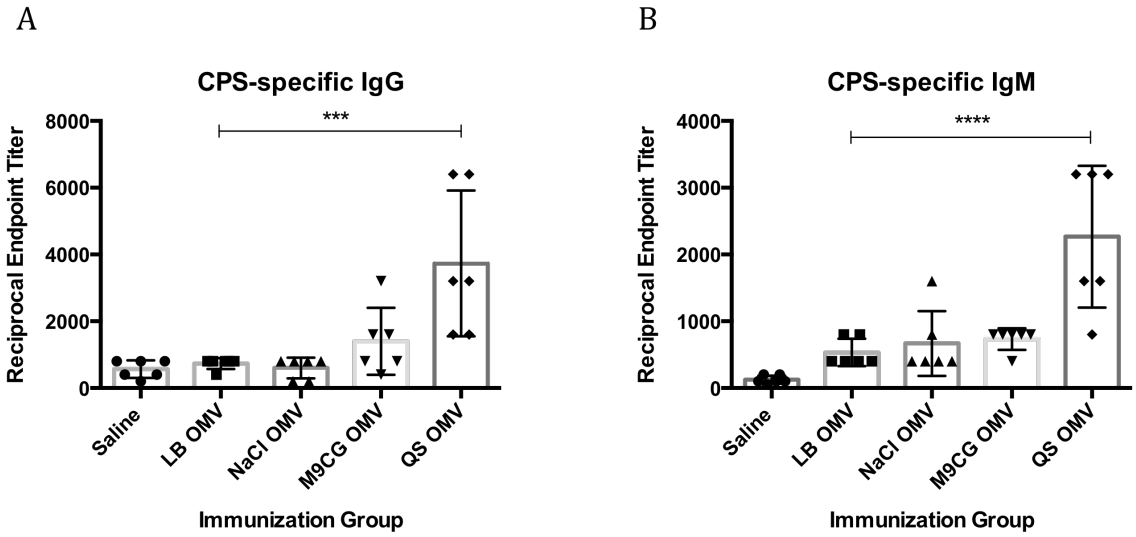

Figure 2.4 QS OMV vaccine induces greater CPS-specific antibody responses as compared to LB OMVs. Bp CPS-specific serum IgG (A) and IgM (B) were measured by ELISA using 96-well plates coated with 0.5μg/well of purified Bp CPS. Serum from BALB/c mice (n=6) immunized QS OMVs had significantly greater CPS-specific serum IgG and IgM as compared to mice immunized with LB OMVs. ***P<0.001 ****P<0.0001 by one-way ANOVA and Dunnett's multiple comparisons test

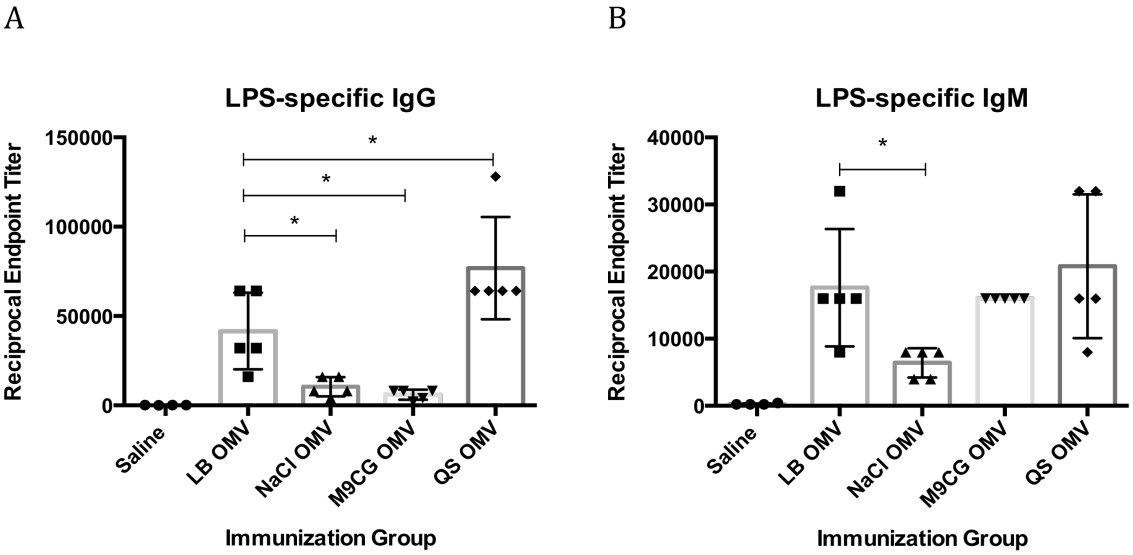

Figure 2.5 Immunization with enriched OMVs elicits significantly different Bp LPS-specific IgG and IgM responses as compared to immunization with LB OMVs. Bp LPS-specific serum IgG (A) and IgM (B) were measured by ELISA using 96-well plates coated with 0.5μg/well of purified Bp LPS. Serum from BALB/c mice (n=5) immunized with NaCl or M9CG OMVs had significantly less LPS-specific serum IgG as compared to mice immunized with LB OMVs. Mice immunized with QS OMV had a significantly greater LPS-specific IgG response than LB OMVs; Only NaCl OMV-immunized mice had significantly different LPS-specific IgM production. *P<0.05 by one-way ANOVA and Dunnett's multiple comparisons test

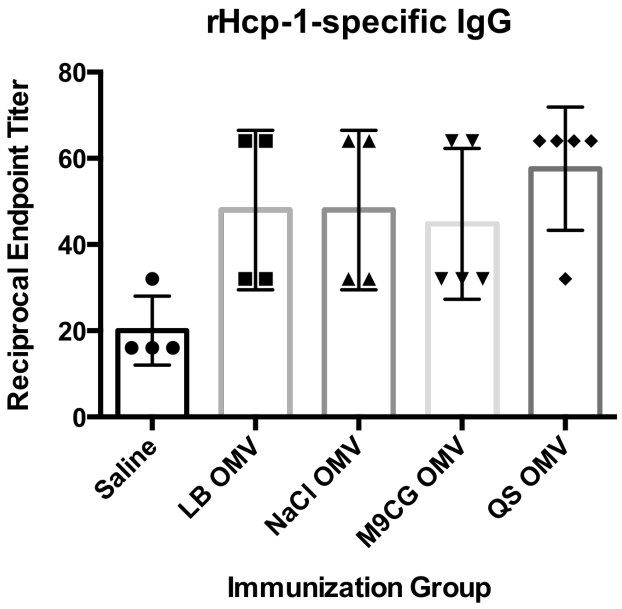

Figure 2.6 Mice immunized with enriched OMVs produce comparable serum IgG to rHCP-1, a T6SS protein, as compared to mice immunized with LB OMVs. rHcp-1-specific IgG were measured by ELISA using 96-well plates coated with 0.1μg/ well of recombinant TSSM or Hcp-1 proteins. There was no significant difference in protein specific serum IgG from BALB/c mice (n=5) immunized with enriched OMVs compared to mice immunized with LB OMVs.

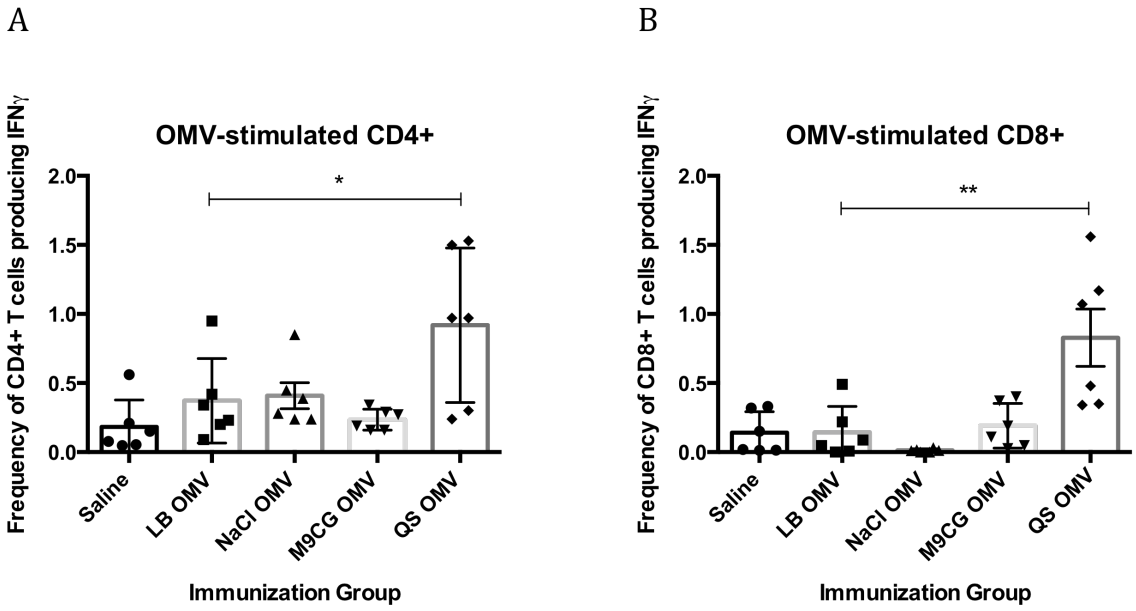

Figure 2.7 QS OMV vaccine induces greater cell-mediated immune responses as compared to LB OMVs. Splenocytes from BALB/c mice (n=6) immunized with OMVs or saline alone were restimulated with 5μg LB OMVs, PMA/Ionomycin (not shown), or unstimulated (not shown) and stained for analysis by flow cytometry. Splenocytes from mice immunized with QS OMVs had significantly more IFNγ-producing CD4+ and CD8+ T cells as compared to mice immunized with LB OMVs. *P<0.05, **P<0.01 by one-way ANOVA

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Chapter 4

Specific Aim 3

Investigate the protective efficacy of OMVs produced in selective media compared to those produced in LB against a Bp infection in mice

Introduction

The ultimate goals of a vaccine include protection against exposure and clearance of bacteria from the body. The protective efficacy of a vaccine should be tested in a model that reflects the anticipated route of natural human exposure, including as a lethal aerosolized biothreat agent. Melioidosis may result from exposure to Bp through a number of routes including inoculation, ingestion, or inhalation. The ability of Bp to infect through various routes poses a challenge to vaccine formulation and preclinical trials have shown that pulmonary infection by aerosolized Bp is particularly difficult to prevent [1]. Additionally, the populations most susceptible to developing melioidosis are immune compromised, which presents further constraints on vaccine formulation. Thus, an effective vaccine must overcome these challenges to protect against pneumonic infection and elicit the necessary immune responses to both kill extracellular bacteria and clear intracellularly-residing bacteria in order to prevent chronic infection.

Despite recent research efforts, there are currently no vaccine candidates against Bp that are able to provide protection against pneumonic melioidosis and elicit sterilizing immunity. Current preclinical vaccine candidates for Bp include live attenuated, killed whole cell, subunit, and OMV formulations. Killed whole cell formulations provide robust antibody responses but insufficient cell-mediated responses and are unable to provide complete protection [2,3]. Live-attenuated strains of Bp have been tested against challenge and are able to stimulate both humoral and

cell-mediated immune responses, providing significant protection against systemic challenge. However, none of the strains have been able to induce long-term protection against virulent challenge and all mice eventually succumb to infection or present with residual bacteria in various tissues [4-6]. Additionally, vaccines composed of live attenuated bacterial strains are contraindicated in immune compromised populations, which are the most at-risk of developing melioidosis. Of the Bp subunit vaccines, none have been able to elicit the necessary combination of humoral and cell-mediated immune responses to provide protection against chronic infection [7-10]. Our lab has previously demonstrated that an OMV vaccine consisting of naturally-shed, native Bp OMVs derived from Bp grown in nutrient-rich media, LB, provided significant protection against lethal aerosol challenge in mice [11]. However, despite the ability of LB OMV-immunized mice to display OMV-specific serum antibody and IFNγ cytokine production, this was not enough to provide sterilizing immunity and mice maintained viable bacteria [12]. The combination of data shows that no vaccine formulation has yet elicited the desired combination of protection and sterilizing immunity that is required for an efficacious vaccine against Bp.

Evidence provided by a study by Healey, et al. demonstrated that both antibody and cell-mediated immune responses are necessary for clearance of bacteria and protection against Bp infection in mice [13]. Bp-specific antibodies are required for clearance of extracellular bacteria and passive transfer of LPS- or CPS-specific antibodies protected mice from acute infection [14,15]. Further, effector T-cell functions are essential for protecting against intracellular bacteria and preventing immune evasion by persistent phenotypes. Another study showed that adoptive

transfer of antigen-specific $CD4^+$ T cells into SCID mice, which are extremely susceptible to infection, provided protection from systemic infection, thus suggesting that $CD4^+$ IFNγ-secreting T cells are required for protection [16]. Despite this information, the low $CD4^+$ T cell counts often observed in HIV/AIDS patients were not associated with increased susceptibility to Bp infection, which may imply a $CD8^+$ T cell contribution to protection as well [17-19]. It will be important to further determine the benefaction of antibodies and T cells from immunized animals to protection against acute or chronic Bp infection.

While the protective efficacy of our LB OMV vaccine formulation is promising, we believe it can be further improved by including proteins and virulence factors essential for bacterial survival in the environment or human host. The data presented in **Chapter 3** showed that some of these selectively enriched OMVs were able to elicit greater Bp-specific antibody, $CD4^+$ T cell, and $CD8^+$ T cell responses compared to LB OMVs. We hypothesize that immunization with these immunogenic selectively-enriched OMVs will induce the necessary antibody and cell-mediated immune responses to clear bacteria and protect against both acute and chronic infection.

Materials and Methods

Animals- Inbred female BALB/c mice (Charles River) 7-9 weeks of age were used in all experiments, as they are highly susceptible to Bp infection [20]. Animals were housed in microisolator cages and had access to sterile food and water *ad libitum.* Mice were euthanized by CO_2 inhalation. Animal use and handling protocols

were reviewed and approved by the Institutional Animal Care and Use Committee. Animals were transferred to the Tulane National Primate Research Center for aerosol exposure and infection studies.

Bacterial strains for challenge- Lethal aerosol challenge was performed using the virulent Bp1026b (BEI Resources) clinical strain. Bp1026b was streaked for isolation from a glycerol stock onto a Pseudomonas isolation agar (PIA) plate and incubated for 48 hours at 37°C. Prior to use, single colonies were selected from PIA plates and used to inoculate LB broth incubated overnight at 37°C, shaking at 217 rpm. Approximately 18 hours later, LB broth was inoculated with a 1:100 dilution of the primary overnight culture and incubated at 37C, shaking at 217 rpm, until log phase was reached (OD_{600nm}=0.7). All experiments utilizing live Bp 1026b were conducted at the Tulane National Primate Research Center (TNPRC) under BSL-3 containment conditions.

Immunization of BALB/c mice with Bp OMVs- BALB/c mice were immunized on a prime-boost schedule 21 days apart. Mice were immunized subcutaneously in the scruff of the neck with 100μL total saline plus 10μg LB OMV (n=30), NaCl OMV (n=15), M9CG OMV (n=15), QS OMV (n=15), $5x10^6$ CFU live Bp82 (n=10). Live Bp82 was plated on a PIA plate and allowed to grow for 48 hours at 37°C prior to use. A single colony was selected to inoculate LB broth supplemented with adenine (Sigma) and thiamine (Sigma) (LB^+), and was incubated overnight at 37°C, shaking at 233 rpm. The primary culture was diluted 1:100 in fresh LB^+ broth and allowed to

grow to log phase (OD600nm=6.0). Bacteria were spun down (12000 rpm) and supernatant was discarded. The pellet was resuspended in sterile saline and brought to a concentration of $5x10^7$ CFU/ml. Mice were subcutaneously administered $5x10^6$ CFU/mouse live Bp82. A sample was plated for CFU confirmation. Control mice were subcutaneously given 100μL saline alone.

Challenge by aerosolized Bp exposure- Thirty days following the final immunization, mice were challenged with Bp1026b grown in either LB or LB supplemented with 1.5% NaCl. The Bp1026b LD_{50} for aerosolized dose is 5-10 CFU/lung [21]. Here, mice were challenged with 20 CFU/lung, or 2-4 LD_{50} of Bp1026b. For the environmental-type challenge, Bp1026b grown in LB +1.5% NaCl, we were unable to achieve a lethal dose and mice received less than 10 CFU/lung, or approximately 1 or <1 LD_{50} of NaCl-Bp1026b. The inhaled dose was calculated based on the number of CFU in a sample of the experimental atmosphere at the time of exposure and the breathing rate of mice. Aerosol challenge occurred using whole-body inhalation exposure system. Mice were divided by immunization and challenge groups as follows: LB OMV (n=15), NaCl OMV (n=15), or saline (n=8) for NaCl-Bp1026b exposure, or LB OMV (n=10), M9CG OMV (n=10), QS OMV (n=10), live Bp82 (n=10), or saline (n=7) for Bp1026b exposure. Mice were placed in stainless-steel mesh cages (10 mice/cage) (5 cages/exposure run) inside exposure chambers in a Class III biological safety cabinet under negative pressure. Animals were not anesthetized, were unrestrained and able to move about normally. Small aerosol particles were generated with a three-jet Collison nebulizer (BGI, Inc.) fitted

with a fluid reservoir. The Collison nebulizer was operated at 18 psi (7.5lpm) and was flow-checked with a frictionless bubble meter (Gilabrator, Gilian Instrument Corporation) prior to nebulization to ensure the integrity of the rubber O-rings. Positive pressure fresh air was supplied to animals, which were observed throughout the exposure for signs of distress. Following challenge, mice were returned to microisolator cages and monitored for survival over 21 days. The primary endpoint for vaccine protective efficacy was survival of immunized animals compared to controls.

Assessment of morbidity- Animals were observed for signs of illness and morbidity over the course of the study. Clinical signs were noted and recorded on the day they arose and include hunching, ruffling of fur, neurological sequelae or loss of motor function in limbs. Morbidity was assessed by weight-loss. Weights were collected prior to challenge exposure, Day 7 post-challenge, and regularly throughout the study. Mice exhibiting severe clinical signs of infection or weight-loss greater than 20% were euthanized by CO_2 asphyxiation. Endpoint weight-loss was compared to a healthy, unchallenged group of mice.

Evaluation of mouse antibody effector functions following immunization- Bp82 was grown in 5 ml LB^+ for 16 hours, 37°C, shaking at 233rpm. After the growth period, the culture was diluted 1:100 in fresh LB^+ broth and allowed to grow 3 hours to mid-log phase (OD_{600}= 4.0). Bacteria were pelleted by centrifugation at 6000 x g for 10 min, at room temperature. Supernatant was decanted and the pellet was

resuspended in sterile LB^+ to adjust the bacterial concentration to $1x10^7$ CFU/ml. Mouse serum was pooled (n=5 mice/group) and heat inactivated at 56°C for 1 hour. Bacteria was plated at $1x10^5$ bacteria/well in a 96 well plate, with 20% guinea pig serum complement (22uL/well) (Millipore). Mouse serum was added at 50μL/well of 3-fold serial dilutions, starting at 1:2 total well dilution. Controls included the following in lieu of mouse serum: no-serum added (negative control) or anti-CPS antibody (MCA147, provided by Dr. Brett and Dr. Burtnick, University of South Alabama) (positive control). Each well was brought to 100μL total volume with sterile LB^+. Plates were incubated for 4-6 hours at 37°C. After incubation, supernatants were collected from each well and plated on PIA agar for CFU determination. Bactericidal activity was determined as the serum resulting in 50% reduction in CFU compared with that of wells containing no serum.

Analysis of T cell effector functions in immunized mice- RAW murine macrophages (ATCC) were plated in a 24 well plate, $5x10^5$ cells/well, in 500μL cell culture media (DMEM with D-glucose, L-glutamine, sodium pyruvate (Gibco)) and allowed to adhere overnight at 37°C, 5%CO_2. Bp82 was grown for 18 hours in LB^+, 37°C, 233 rpm. After 18 hours, bacteria were diluted 1:100 in fresh LB^+ and allowed to grow to log phase (OD600=4.0, approximately 3 hours), 37°C, 233 rpm. Final bacterial concentration was adjusted to $1x10^7$ CFU/ml in LB^+. Media was removed from RAW cell culture wells and macrophages were infected at an MOI 2:1 (bacterial concentration of $1x10^6$ CFU/well) and 100μL cDMEM. Bacteria was allowed to infect cells for 2 hours at 37°C, 5% CO_2. Cells were washed 3x with 1xPBS to remove

extracellular bacteria. Antibiotic media (1mg/ml Kanamycin sulfate (Gibco)) in cDMEM) was added to each well to kill any remaining extracellular bacteria and incubated for 3 hours, 37°C, 5% CO_2. Supernatant was removed and cells were washed 3x with 1x PBS. Plates were incubated for 4 hours, 37°C, 5% CO_2, with 500μL DMEM per well.

CD4+ and $CD8^+$ T cells were isolated from mice subcutaneously immunized with LB OMVs, M9CG OMVs, or saline alone. Briefly, mice were euthanized by CO_2 asphyxiation and spleens were excised. Spleens were stored in 5ml 1% RPMI (RPMI +1% FBS) media (Gibco, Sigma). Spleens were passed through a 70μm nylon cell strainer into a 50mL conical tube, rinsing the strainer with 5mL 1% RPMI. Splenocytes were centrifuged for 10 min, 460 x g, 4°C. Media was decanted and pellets were resuspended in 2mL ACK red blood cell lysis buffer (Invitrogen) with gentle mixing for 3 minutes. This reaction was stopped by adding 20mL 1% RPMI and samples were centrifuged at 1200 rpm for 10 min. Pellets were resuspended in 5 ml 10% RPMI. Cells were passed through a 40μm cell strainer to remove RBC debris. CD4+ T cells were isolated by negative selection following vendor instructions using a CD4 + T cell Isolation Kit by Miltenyi Biotec. For this kit, cells were counted, spun at 300 x g for 10 min, and resuspended in 40μL buffer per $1x10^7$ total cells. The provided Biotin-Antibody cocktail was added 10μL per $1x10^7$ cells and allowed to incubate at 4°C for 5 minutes. 30μL buffer and 20μL Anti-Biotin MicroBeads were added to each tube per $1x10^7$ cells, mixed, and incubated for 10 minutes at 4°C. $CD4^+$ T cells were isolated by passing the samples over magnetic columns using a MACS Separator, and collecting the unlabeled cells in the flow-

through as the enriched $CD4^+$ T cells. $CD8^+$ T cells were isolated by negative selection using EasySep Mouse $CD8^+$ T Cell Isolation Kit (StemCell Technologies). Briefly, viable cells were counted, spun at 300 x g for 10 min, and resuspended in HBSS + 2% FBS with 1mM EDTA (cell media) for $1x10^8$ total cells/mL in a 5 mL polystyrene tube. The kit-provided normal rat serum and EasySep Mouse CD8+ T cell Isolation Cocktail were added at 50μL each per mL of cells, mixed well, and incubated at room temperature for 10 minutes. Vortexed EasySep Streptavidin RapidSpheres 50001 were added to the mixture at 125 μL/mL of cells, mixed well, and incubated at room temperature for 5 minutes. The cell suspension was brought to a total volume of 2.5mL with cell media and mixed by pipetting. Tubes were placed in the magnet and allowed to incubate at room temperature for approximately 3 minutes. Supernatants in the tubes, while still in the magnet, were removed and collected in a new 5mL tube by single pipette draw from each tube. The magnetically labeled unwanted cells remained bound inside the original tube held by the magnet field of the EasySep Magnet and were discarded. Isolated $CD8^+$ T cells were contained in the tube of collected supernatant and were counted by the automated Cellometer.

Purified $CD8^+$ T cells were added to infected macrophage wells at a concentration of $1x10^6$ cells/mouse/well in duplicate and allowed to incubate for 16 hours, 37°C, 5% CO_2. Wells were observed at regular timepoints for sloughing off of macrophage monolayer. After 16 hours, cells were scraped from the first duplicate of wells and stained by Trypan Blue (Invitrogen) for viability counting. The second of duplicate wells was lysed with 200μL HBSS + 0.2% Triton (Sigma) and collected

in 1.5 mL tube for bacterial CFU plating on PIA agar plates. Plates were incubated at 37°C for 48 hours for colony growth.

Results

Efficacy of NaCl OMV immunization was inconclusive due to challenge with sublethal dose of Bp- Both immunized and control animals subjected to challenge with Bp1026b grown in LB + 1.5% NaCl survived to Day 21 post-exposure. Following exposure, it was determined by AGI and challenge dose calculations that mice were challenged with <1 $LD_{50.}$ At Day 6 post-exposure, one saline-immunized challenged mouse was sacrificed and lung burdens determined for confirmation of pulmonary infection. The burden was determined to be $3x10^4$ CFU. Remaining challenge animals were allowed to progress, however the majority of animals in this challenge group were able to survive to Day 21, or study endpoint. At the study endpoint, 100% NaCl OMV-immunized, 87% LB OMV-immunized, and 78% saline immunized animals survived (**Figure 3.1**). Surviving animals were weighed for an indication of morbidity. There was no significant weight-loss among LB OMV, NaCl OMV, or saline immunized mice compared to healthy, unchallenged mice (**Figure 3.2**). This data indicates that the challenge dose that these animals were exposed to was sublethal and animals were able to control infection up to Day 21 post-exposure.

Immunization with M9CG OMVs or QS OMVs provides equal protection to LB OMVs against acute, lethal Bp infection- Animals immunized with LB OMVs, M9CG

OMVs, QS OMVs, live Bp82, or saline alone were exposed to a lethal dose of $5LD_{50}$ aerosolized Bp1026b. M9CG OMV-immunized and live Bp82-immunized groups demonstrated 90% survival, LB OMV and QS OMV-immunized mice presented 80% survival to Day 21 post-exposure, whereas saline immunized mice succumbed at a significantly greater rate with 30% survival at study endpoint (**Figure 3.3**). M9CG OMV, LB OMV, and QS OMV immunization provided significant protection against lethal Bp challenge through the acute phase of infection.

M9CG OMV immunization provides significant protection against morbidity following lethal aerosol Bp challenge- Challenged mice were weighed at the study endpoint for signs of morbidity compared to the healthy, unchallenged group, which maintained weight over the course of the study. LB OMV, QS OMV, live Bp 82-immunized mice, along with the saline-alone group, presented significant weight-loss by Day 21 compared to the unchallenged group (**Figure 3.4**). However, only the M9CG OMV-immunized animals were able to maintain weight over the study and did not have significant weight-loss compared to the unchallenged mice.

Mice immunized with enriched OMVs possess serum bactericidal activity- Serum from mice immunized with Bp OMVs demonstrated bactericidal activity when added to live Bp82 culture with added guinea pig complement. Sera from all OMV immunized mouse groups, including LB OMV-immunized, exhibited bactericidal activity and had decreased CFU after 4 hours compared to wells receiving serum from saline-immunized mice or the control wells with bacteria and

complement alone (**Figure 3.5A**). LB OMV-immunized sera exhibited the least amount of bactericidal activity with 43%, whereas treatment with NaCl OMV immunized serum resulted in 66% reduction in CFU, M9CG OMV immunized serum killed 58% of bacteria, and QS OMV immunized serum reduced CFUs by 67% (**Figure 3.5B**). There was no significant difference between the OMV-immunized groups and the antibody control, which showed 71% bactericidal activity.

$CD8^+$ T cells from mice immunized with M9CG OMVs show effector function in co-culture with Bp-infected macrophages- We were unable to isolate sufficient numbers of $CD4^+$ T cells from immunized mice for the appropriate controls in our co-culture system (data not shown). There was not a significant difference in the percent of viable Bp82-infected RAW cells following treatment with $CD8^+$ T cells from M9CG OMV immunized mice compared to those treated with $CD8^+$ T cells from LB OMV immunized mice (**Figure 3.6A**). However, treatment of Bp82-infected RAW murine macrophages with $CD8^+$ T cells from M9CG OMV immunized mice resulted in a significant decrease in intracellular CFU compared to infected macrophages treated with T cells from mice immunized with LB OMV or saline alone. There was also a significant reduction in CFU for cells treated with $CD8^+$ T cells from M9CG OMV immunized mice compared to the Bp82-infected macrophages left untreated (**Figure 3.6B**).

Discussion

While natural infection of Bp may occur by ingestion, inoculation, or inhalation, it is important to note that Bp's designation as a Tier 1 Select Agent occurs as a result of it's ability to be aerosolized and cause acute pneumonic disease, which accounts for a significant portion of the 40% mortality rate of Bp infection in endemic regions [1]. A vaccine against melioidosis must be able to protect against aerosolized Bp and overcome its natural proclivity for persistence in those populations most at risk. To date, our lab's naturally-derived Bp OMVs extracted from Bp grown in rich media, LB, have demonstrated the most significant levels of protection against this route of infection [11]. However, this OMV vaccine formulation was unable to protect against chronic infection and surviving mice maintained viable bacteria in systemic tissues [11]. The inability of immunized mice to fully clear infection may be a result of insufficient immune effector functions. We hypothesized that vaccination with OMVs selectively enriched in virulence factors and proteins necessary for environmental, intracellular, or biofilm survival would induce protective antibody and cell-mediated immune responses necessary to protect mice from both acute and chronic infection and lead to the clearance of bacteria. Here, it is shown that M9CG OMVs and QS OMVs provide protection against acute aerosolized infection, M9CG OMVs protect against acute morbidity, and immunization with enriched OMVs elicits immune effector functions.

Unfortunately, we were unable to assess the protective efficacy of the NaCl OMVs against Bp aerosol exposure due to the animals receiving a sublethal

infectious challenge dose. Animals were able to clear the infection and survived to the study endpoint with no significant weight loss or clinical signs of infection. It has been shown previously that excess environmental NaCl may delay Bp growth and alter its protein expression and secretome [22] [23] [24]. This may have contributed to the decrease in CFU obtained in our challenge study. The addition of 1.5% NaCl to the culture media may also attenuate the virulence of Bp and lead to decreased infection or disease manifestation [25]. The study presented here was inconclusive, but presents interesting data that poses potential for further study in the future.

Alternatively, M9CG OMV and QS OMV immunization provided equal or slightly better protection against aerosolized Bp compared to immunization with LB OMVs or live attenuated Bp (Bp82) through the acute phase of infection. However, there were significantly reduced signs of morbidity in M9CG OMV immunized animals at Day 21 post-exposure. The M9CG OMV immunized animals were the only immunization group to exhibit reduced clinical signs of infection and did not have significant weight loss compared to the unchallenged healthy mouse group at the study endpoint. The M9CG OMV vaccine protected 100% of animals through Day 14 and 90% through Day 21, showing protective efficacy through the acute phase of infection but may also potentially lead to clearance of bacteria and prevent subsequent chronic infection. The inclusion of proteins strictly regulated by Bp for intracellular survival may be necessary to target host immune responses against persistent bacteria and lead to sterilizing immunity. Since our study had to be prematurely truncated, mice were only monitored for 21 days and we did not to evaluate protection against chronic infection as previously planned. Due to

restrictions on our work at this time, we were also unable to assess bacterial burden in surviving mice in order to determine bacterial clearance in immunized animals.

Data presented in **Chapter 3** showed that immunization with enriched OMVs was able to induce Bp-specific immune responses. However, it was important to investigate whether these Bp-specific antibody and T cell responses were functional against Bp. Serum from animals immunized with LB OMVs, NaCl OMVs, M9CG OMVs, QS OMVs, or saline alone was evaluated for bactericidal activity against Bp. Serum from mice immunized with any of the enriched-OMV formulations exhibited slightly more bacterial killing overall, resulting in decreased CFUs, than LB OMV immunized mice and were not significantly different from the anti-Bp CPS antibody control. All OMV-immunized, including LB OMV, mice possess greater serum bactericidal activity as compared to the saline immunized animals, which did not demonstrate any bacterial killing. This experiment indicates that immunization with NaCl OMVs, M9CG OMVs, or QS OMVs elicits production of anti-Bp serum antibodies with bacterial killing capabilities. Further, as shown in the co-culture assay, mice immunized with M9CG OMVs had $CD8^+$ T cells with cytotoxic effector function able to kill RAW macrophages intracellularly infected with Bp. The Bp-infected RAW cells treated with M9CG OMV-immunized mouse $CD8^+$ T cells had significantly fewer CFU compared to cells treated with $CD8^+$ T cells from LB OMV immunized or saline immunized animals. More particularly, there was significantly less bacteria in wells treated with M9CG OMV-immunized mouse $CD8^+$ T cells compared to the control, untreated-infected cells. The cytotoxic activity of $CD8^+$ T cells and bactericidal activity of antibodies elicited by vaccination with M9CG OMVs may contribute to the

significant reduction in signs of morbidity and the resulting protection against lethal exposure seen in these animals. $CD8^+$ T cell cytotoxic effector function was evaluated by T cell killing of the intracellularly-infected RAW cells, resulting in a subsequent loss of macrophage viability. Even though we did not see a significant difference in RAW cell viability following treatment with $CD8^+$ T cells from mice immunized with different OMV preparations, this may be a consequence of our method of viability testing. Here, viability was assessed by Trypan Blue staining and evaluated by a Cellometer Cell Counter. This method does not distinguish between live macrophages and the live T cells and, even though the wells underwent multiple washes to remove T cells prior to viability staining, it was noted that there may have been residual $CD8^+$ cells contaminating the sample and were included in the overall viability counts. Future studies of this type should utilize cell staining for different cell types, as well as live/dead staining, and determine the total numbers of live macrophages by flow cytometry. This will give a more accurate readout specific for macrophage viability following treatment. As shown in **Chapter 3**, immunization with enriched OMVs also induced $CD4^+$ T cell production of IFNγ, which has previously been shown to play an important role in protection against melioidosis [26], so it will be important to further evaluate the effector functions of these cells within this system as well. Unfortunately, we were unable to isolate a sufficient amount of $CD4^+$ T cells from the immunized mice to compare to our controls, so this test will need to be repeated in order to establish the role of antigen-specific $CD4^+$ T cells during Bp intracellular infection.

The results shown here are important to understanding the factors necessary for a Bp vaccine to achieve optimal protection and sterilizing immunity. It may be necessary to include those proteins expressed during intracellular survival, such as those included in M9CG OMVs, to elicit the necessary immune cell effector functions to clear persistent bacteria and prevent chronic infection. These data will be important in the future for determining the necessary composition of effective vaccine formulations against intracellular bacteria, including Bp.

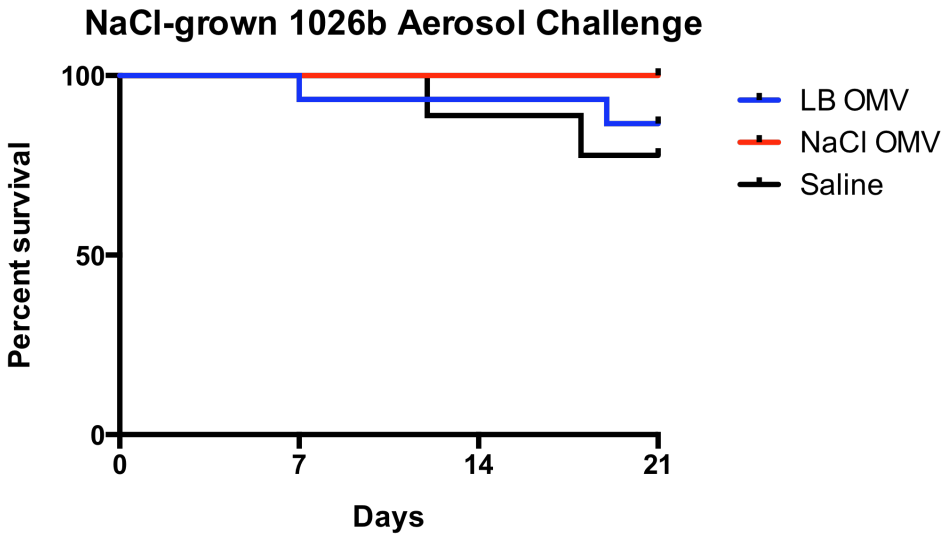

Figure 3.1 All mouse challenge groups survive a sublethal dose of Bp1026b grown in LB supplemented with 1.5% NaCl. Mice immunized with 10μg LB OMV, NaCl OMV, or saline alone survived a sublethal challenge with <10CFU/lung (1LD50) aerosolized Bp1026b grown in LB supplemented with 1.5% NaCl. 78% of saline-immunized control animals survived to Day 21 post-exposure, indicating the challenge dose of NaCl-Bp1026b was not lethal.

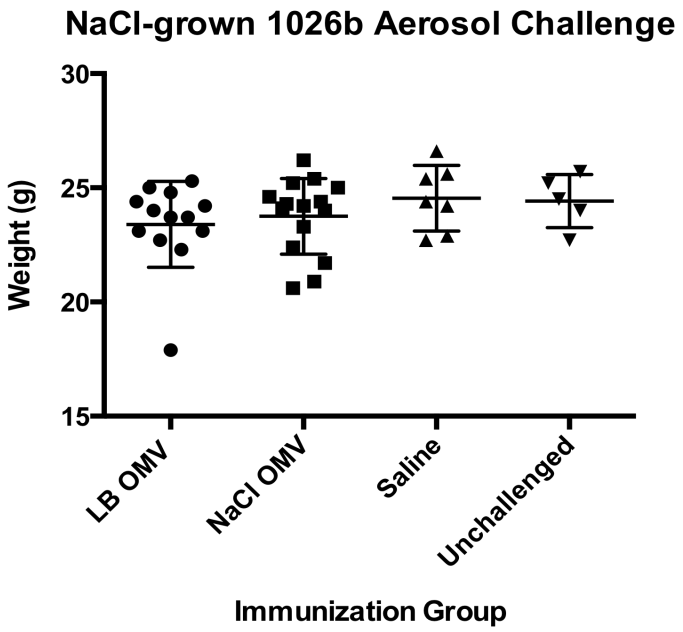

Figure 3.2 Animals show no weight-loss following sublethal challenge with Bp1026b grown in environmental conditions. Animals immunized with LB OMVs, NaCl OMVs, or saline alone did not show significant weight-loss, or signs of morbidity, at Day 21 post-aerosol exposure with Bp 1026b grown in LB supplemented with NaCl.

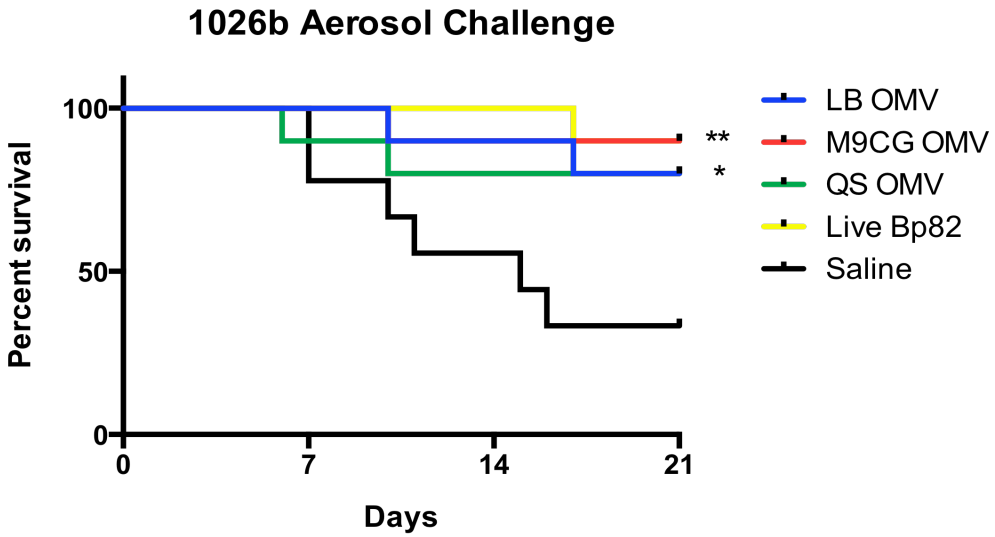

Figure 3.3 Animals immunized with enriched OMVs are significantly protected against lethal aerosol challenge. Animals (n=10/group) immunized with 10μg LB OMVs, M9CG OMVs, QS OMVs, CFU live Bp82, or saline alone on a prime-boost schedule were challenged with 5LD50 aerosolized Bp 1026b. M9CG OMV and Live Bp82-immunized mice had 90% survival while LB and QS OMV immunized mice had 80% to day 21 post-exposure compared to only 30% survival of saline immunized mice. *P<0.05, **P<0.01 by survival curve log-rank Mantel-Cox test

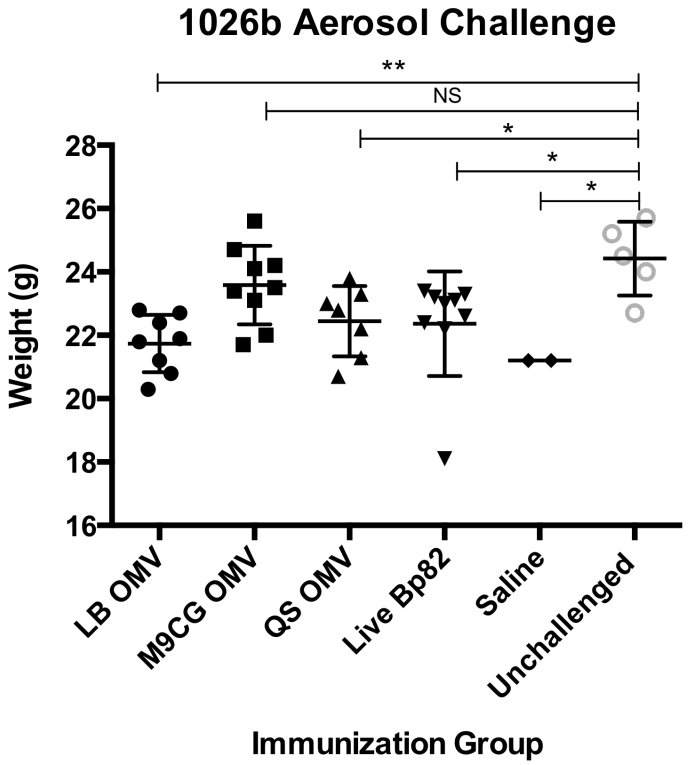

Figure 3.4 M9CG OMV immunized mice did not have significant weight loss following lethal Bp exposure. Animal morbidity following lethal aerosol challenge was assessed by weight loss (grams) at Day 21 post-exposure. All groups of challenged mice had significant weight-loss at Day 21 post-exposure as compared to unchallenged mice except mice immunized with 10μg M9CG OMVs. *P<0.05 by one-way ANOVA and Dunnett's multiple comparisons test

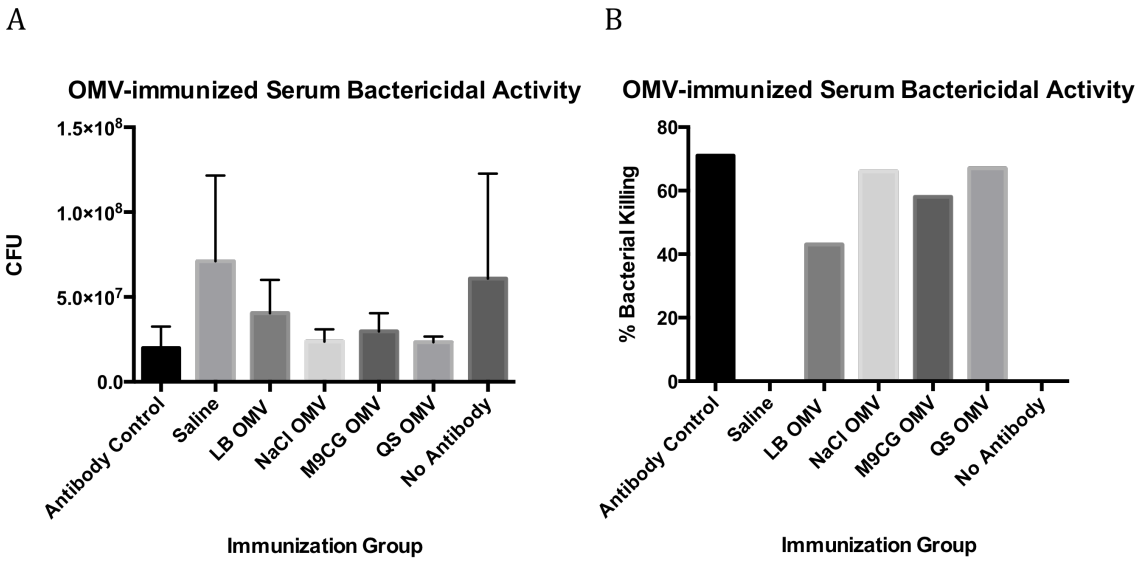

Figure 3.5 Mice immunized with Bp OMVs possess serum bactericidal activity. Serum from mice immunized with LB OMVs, enriched OMVs (n=5), or saline alone was diluted diluted 1:2 and compared to 3C5 Anti-Bp CPS antibody in wells with $1x10^5$ CFU live Bp82 and guinea pig complement. There was not a significant difference in total CFU counts comparing the No Antibody control with OMV-immunized serum (A). Despite this, mice immunized with NaCl OMVs exhibited a 66% reduction in CFU, M9CG OMVs demonstrated 58% bacterial killing, and mice immunized with QS OMVs showed 67%, while serum from mice immunized with LB OMVs reduced CFUs by 43%. Bactericidal activity of serum from mice immunized with enriched OMVs was not significantly different from the antibody control (71%) (B).

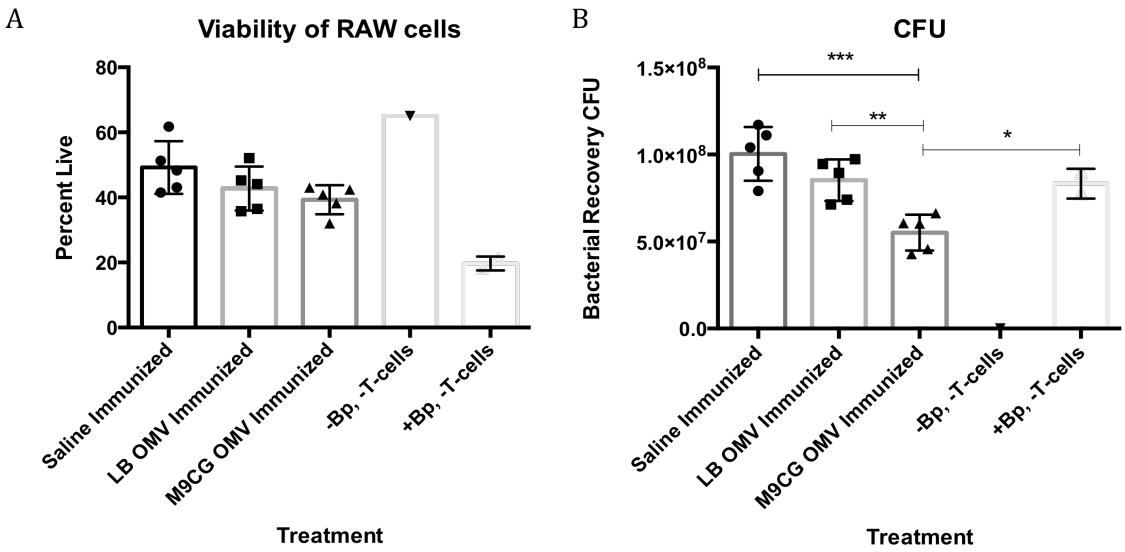

Figure 3.6 CD8⁺ T cells extracted from mice immunized with M9CG OMVs provide a significant reduction in intracellular bacteria in an ex-vivo co-culture system. There was no significant difference in viable RAW macrophages following treatment with CD8⁺ T cells extracted from mice immunized with LB OMVs, M9CG OMVs, or saline alone (n=5/group). Treatment with CD8⁺ T cells from M9CG immunized mice resulted in a significant decrease in intracellular bacteria by CFU compared to treatment with CD8⁺ cells from LB OMV immunized mice, saline immunized mice, or untreated (B). ***P<0.001, **P<0.01, *P<0.05 by one-way ANOVA and Dunnett's multiple comparisons test

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Chapter 5

Dissertation Conclusion

Burkholderia pseudomallei (Bp) is a Gram-negative, encapsulated, facultative intracellular pathogen that is the causative agent of melioidosis, a disease with pneumonic and septicemic clinical manifestations. Bp is also an aerobic, motile, non-spore forming bacillus found in soil and water with a worldwide distribution. Bp is hyperendemic to Thailand where melioidosis is the third leading cause of death due to an infection with a mortality rate over 40% and relapse occurring in up to 25% of patients [1]. In northern Australia, melioidosis is the most common cause of fatal-community-acquired bacteremic pneumonia occurring in approximately 50 in 100,000 people per year and a fatality rate of 20% [2]. Pneumonia occurs after the spread of bacteria following inoculation or by direct infection of the epithelial layer by infectious inhalation, is the most common presentation of melioidosis, and is involved in over half of all cases. Relapse is common even after what is considered to be successful treatment, occurring in up to 25% of cases, and has a mortality rate similar to that of initial infection [3]. Additionally, Bp has been categorized by the Department of Health and Human Services as a Tier 1 Select Agent due to its potential for distribution as an aerosolizable biothreat agent and because it poses a significant threat to human and animal health.

The virulence of Bp is related to its genetic and phenotypic resilience and adaptability to changing environments such as soil and water or the human host. Bp can be isolated in endemic regions from moist, slightly acidic, nutrient-rich soil from the plant rhizosphere. Human infection with Bp may occur through inhalation of

bacteria carried in the air, ingestion of soil or water containing large amounts of the bacteria, or inoculation of pre-existing cuts or abrasions upon contact with contaminated soil or water. Alternative to its saprophytic lifestyle, Bp also has a large repertoire of virulence factors that enable it to survive and thrive in a mammalian host. Extracellular Bp is able to subvert the innate immune system and the complement cascade through its surface polysaccharides, lipopolysaccharide (LPS) and capsular polysaccharide (CPS) [4]. Bp is then able to adhere to and invade a number of human cell types and resides and replicates intracellularly due, in large part, to its expression of T3SS-3 and T6SS-1, which leads to severe pathogenesis in the host [5] [6] . Bp is also capable of utilizing its quorum sensing (QS) systems to rapidly spread around the body, colonize tissues and systems distant to the site of infection, and establish chronic infection [7]. Activation of Bp's QS systems is also essential to the development and maintenance of biofilms, which actively protect Bp against antibiotic agents and cultivates persistent infection [8].

There is currently no vaccine available to prevent Bp infection. Development of an effective vaccine against melioidosis requires a thorough understanding of the pathogenesis of infection and the characteristics of the immune responses that develop following exposure. A successful vaccine against both acute and chronic Bp infection may require a combination of antibodies, $CD4^+$ T cells, and $CD8^+$ T cells responding to multiple Bp-specific antigens. Bp's resistance to complement during the extracellular phase of infection means that effective clearance following transmission is dependent upon Bp-specific antibody opsonization. In clinical investigations, surviving melioidosis patients had a predominant IgG response,

indicating its importance during human infection [9]. While the role of antibody responses is critical during the acute phase of infection, due to Bp's ability to invade host cells and replicate intracellularly, antibodies are insufficient to completely clear bacteria and prevent persistent infection. Mouse studies have confirmed this and suggest that T-cell mediated IFNγ responses are important for achieving protection [10] [11]. Additional investigations have shown that strong $CD4^+$ and $CD8^+$ responses were associated with survival in patients with melioidosis [12], thus implying that a combination of humoral and cell-mediated responses is required to prevent or clear Bp infection.

Given Bp's multitude of antigenic factors, intracellular survival proteins, and its inherent antibiotic resistance, development of a safe and effective vaccine capable of inducing both antibody and T-cell responses is essential. Our lab has previously demonstrated that immunization with an outer membrane vesicle (OMV) vaccine provided protection against acute melioidosis in mice [13] [14]. However, at the study endpoint, the immunized survivors of the murine challenge studies maintained persistent Bp in organ tissues, indicating development of a chronic infection [14]. This incomplete vaccine-mediated protection against Bp infection may be due to an absence of essential protective antigens in the OMVs. The vaccine formulation used in our previous studies consisted of naturally shed, native Bp OMVs derived from Bp grown in Luria-Bertani (LB) broth. LB is not representative of Bp's natural environmental or host niche and may limit the expression of virulence factors and intracellular survival proteins, thus resulting in insufficient immune activation and persistent infection following exposure.

The studies within this dissertation seek to build upon previous work that has shown that the composition and components of Gram-negative OMVs, including tightly regulated virulence factors and survival proteins, can be manipulated by inducing environmental stressors [15]. The approach described within this dissertation involves the manipulation of bacterial regulation of protein expression during OMV production by simulating the natural soil environment, the host macrophage intracellular environment, or quorum sensing conditions. As previously described, Bp grown in rich lab culture medium, such as LB, behaves very differently than when the bacteria is residing in its natural soil environment or when it has infected a host. For this work, I proposed that by altering the Bp growth media to upregulate the expression of proteins involved in pathogenesis, the content and composition of the naturally shed Bp OMVs will be enriched with these proteins and thus induce the necessary antibody and cell-mediated responses for improved protection against melioidosis and clearance of bacteria.

Aim 1 investigated the ability of Bp grown under hypertonic stress, nutrient limitation, and quorum sensing conditions to shed vesicles enriched in virulence factors and proteins that are specific to bacterial survival under those conditions. The differential Bp growth medias were established to be representative of three environmentally different infectious stages of Bp: transmission of a soil-dwelling bacteria (LB supplemented with 1.5% NaCl), intracellular infection (M9CG minimal media), and biofilm development (LB supplemented with C8-HSL and C10-HSL). It was determined that vesicles were naturally shed from Bp grown under all three of the conditions during log-phase growth and were able to be isolated and purified in

a similar manner to the previously tested LB-derived Bp OMVs, which served as the wild-type control for comparison purposes [13]. Purified vesicles maintained a standard size and double-membrane shape regardless of growth conditions.

The composition of OMVs derived from differential media was characterized and compared to the LB OMVs to determine whether protein enrichment through manipulation of environmental growth conditions was possible. Analysis for specific Bp antigens was performed by liquid chromatography/mass-spectometry and Western blotting. All enriched OMV preparations expressed CPS and LPS, both of which have been shown to be essential for inducing a robust antibody response and correlate with protection against acute infection [16]. While CPS was uniformly presented by enriched OMVs, LPS expression was affected by growth conditions and the NaCl OMVs had a different LPS expression profile by Western blot. This indicates that Bp may downregulate or alter LPS content while replicating in the soil, perhaps to facilitate easier uptake of nutrients and soluble materials from the surrounding earth.

Through this aim, it was also determined that the growth environment heavily influences Bp protein expression and subsequent OMV protein profiles, particularly for virulence factors and proteins involved in bacterial survival. A number of important proteins, including flagellar components, OmpC, an outer membrane porin important in diffusing small hydrophobic molecules, OmpA, EF-Tu, oxidoreductase, and molecular chaperones were shared across all conditions, including the wild-type LB OMVs. Several of these shared proteins are known virulence factors that are necessary for invasion and intracellular survival [17,18].

However, the various environmental stress responses induced by selective media lead to the expression of proteins unique to OMVs derived from conditional medias. NaCl OMVs exhibited an unusually large number of fimbrial proteins, which function as attachment pili for adhesion to the host cells. Alternatively, M9CG OMVs expressed multiple T6SS proteins, including Vgr and HCP-1. It was previously shown that the T6SS-1 is an essential component of Bp intracellular survival and is an important virulence factor with tightly-regulated expression [19]. QS OMVs had an abundance of porins and secretion system proteins involved in transport, such as PqaA, which is a T3SS outer membrane protein and confers resistance to antimicrobial peptides [20]. These results are essential to understanding the effects of the growth environment on bacterial protein expression and will contribute to formulating a Bp vaccine containing sufficient antigens to induce the necessary immune responses for complete protection.

Aim 2 sought to determine the safety and immunogenicity of OMVs enriched in virulence factors and antigenic proteins. Enriched OMVs were shown to be non-toxic *in vitro* through visualization and viability testing of murine macrophages up to 8 hours after OMV treatment. Enriched OMV preparations were also non-toxic *in vivo* in a non-mammalian animal model *G. mellonella,* and were safely administered in mice with no signs of reactogenicity or illness following subcutaneous immunization.

The immunogenicity of enriched OMVs was evaluated by testing serum and isolated T cells from mice immunized subcutaneously with 10μg LB OMV, NaCl OMV, M9CG OMV, QS OMV, or saline alone. Bp OMV-specific IgG and IgM responses were

similar among LB OMV-, NaCl OMV-, and M9CG OMV-immunized mice, all of which were significantly greater than saline alone-immunized mice. Additionally, mice administered QS OMVs had a significantly greater OMV-specific IgG compared to LB OMV immunized mice. Similarly, only the QS OMVs provided greater CPS-specific serum IgG and IgM compared to LB OMVs. The increases in antibody responses associated with QS conditions may be related to a study of Bp biofilm mutants, which showed that the drug resistance mechanism of biofilm-forming bacteria is from the upregulation of certain protective factors when biofilm genes are stimulated by quorum sensing molecules, such as C8-HSL and C10-HSL [21]. The inclusion of these gene products, including LPS, CPS, and antigenic proteins, in QS OMVs may enhance targeting of the immune response to these factors. This was further examined by flow cytometric analysis of $CD4^+$ and $CD8^+$ IFNγ production in immunized mice, which has been shown to contribute to protection in both mice and human cases of melioidosis [10]. Mice immunized with QS OMVs had significantly greater numbers of IFNγ-producing $CD4^+$ and $CD8^+$ T cells compared to mice immunized with LB OMVs, possibly as a result of increased expression of antigenic proteins in the QS OMV preparation. The combination of experiments performed in **Aim 2** showed that enriched OMVs are safe *in vitro* and *in vivo* and that QS OMVs exhibit increased immunogenicity as compared to LB OMVs.

Aim 3 investigated the protective efficacy of the enriched OMVs in a lethal aerosol challenge model. Animals were subcutaneously immunized on a prime-boost schedule 21 days apart with LB OMV, NaCl OMV, M9CG OMV, QS OMV, live Bp82, or saline alone. Thirty days following the secondary immunization, animals

were challenged with aerosolized Bp. The NaCl OMV-immunized animal group received a sublethal infectious challenge dose and animals were able to clear the infection and subsequently survived to the study endpoint with no significant weight loss or clinical signs of infection. Thus, we were unable to assess the protective efficacy in this group. M9CG OMV and QS OMV immunization provided equal protection compared to LB OMV immunization or immunization with live-attenuated Bp (Bp82) against acute lethal Bp challenge. Additionally, M9CG OMV immunized animals were the only immunization group that did not have significant weight loss compared to the unchallenged healthy mouse group and was able to maintain weight over the 21-day course of infection. However, our study was surreptitiously concluded on Day 21 post-exposure and we were unable to assess protection against chronic infection or bacterial burdens in surviving mice.

In order to evaluate the immune effector functions, serum from mice immunized with enriched OMVs was tested for bactericidal activity against live Bp. Serum from animals immunized with any of the enriched OMV formulations showed greater than 50% Bp-specific bactericidal activity, while LB OMV immunized serum had 43% bacterial killing. Additionally, we performed a co-culture assay with Bp-infected macrophages treated with T cells isolated from immunized mice. Treatment of infected macrophages with $CD8^+$ T cells from M9CG OMV immunized mice resulted in significantly less intracellular CFU than the untreated control cells. The functional immune responses elicited by immunization with M9CG OMVs may contribute to the observed decrease in morbidity and associated protection following Bp exposure in this group. These results lead to the assumption that

intracellularly expressed proteins and virulence factors may be essential vaccine antigen components for providing complete protection and bacterial clearance.

To summarize this dissertation work, we discovered that naturally derived OMVs may be selectively enriched with virulence factors and survival proteins through the manipulation of the bacterial growth environment, and these vesicles remain non-toxic *in vitro* and safe for administration *in vivo*. The enriched OMVs are immunogenic in a murine model and elicit Bp antigen-specific antibody and cell-mediated immune responses with QS OMV exhibiting the most significant increase in IgG, IgM, $CD4^+$, and $CD8^+$ responses as compared to LB OMVs. Immunization with M9CG and QS OMVs provided protection against lethal aerosol Bp challenge through 21 days; however, the protective efficacy study of NaCl OMVs was inconclusive. M9CG OMV immunization was also able to protect against morbidity through the acute phase of infection, by displaying no significant weight loss compared to healthy unexposed mice. Unfortunately, our studies were unexpectedly halted at day 21 and we were unable to determine tissue bacterial burdens in surviving mice. The ability of immunized animals to achieve sterilizing immunity should be investigated in the future, particularly for M9CG OMV immunized animals, which appeared to possibly clear infection by day 21 post-exposure. Additionally, mice immunized with enriched OMVs showed serum bactericidal activity and $CD8^+$ cytotoxic T cell function against Bp-infected macrophages. Future studies investigating enriched OMV immunization will further illuminate the effector functions of the humoral and T cell responses to Bp infection, particularly that of $CD4^+$ T cells known to be important to protection [11] [22].

The results shown here are important to understanding the factors necessary for a Bp vaccine to achieve optimal protection and sterilizing immunity. It may be necessary to include those proteins expressed during intracellular survival and/or biofilm maintenance and antibiotic resistance to elicit the necessary immune cell effector functions to clear persistent bacteria and prevent chronic infection. This work demonstrates that it is possible to alter protein expression without genetic modifications in order to provide multiple antigenic targets that are normally tightly regulated by the bacteria during human infection. It was shown here that inclusion of these proteins and antigenic factors in a vaccine formulation enhanced protection and lead to a reduction of morbidity in an animal model of infection. These are important considerations for vaccine development of Gram-negative intracellular bacterial infections. This is particularly true for Bp, which is classified as a Select Agent, commonly infects immune compromised populations, is extremely difficult to treat, and is able to establish latent infection. The data found herein may be essential to the future development of an efficacious vaccine against melioidosis.

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Biography

Nicole Kikendall was born January 9, 1984 to her mother, Carolyn, and father, Kevin Kikendall. She attended high school in Mt. Zion, Illinois where she had excellent teachers who encouraged her passion for learning. She graduated in the top of her class and proceeded to the University of Illinois at Champaign-Urbana. There, she majored in Molecular and Cellular Biology and graduated in May, 2006 with a Bachelors in Science. Upon graduation, she moved to Albuquerque, New Mexico for a job as a surgical services patient liaison for the Operating Room and Recovery departments at Kaseman Presbyterian Hospital. After obtaining that clinical experience, she took a position as a research technologist in the Immunology Core Laboratory of Dr. Julie Wilder at Lovelace Respiratory Research Institute, where she contributed to a number of vaccine and infectious disease studies. In August 2011, she began the Biomedical Sciences graduate program at the Tulane School of Medicine and joined Dr. Lisa Morici's lab in May 2012. She completed her dissertation work investigating an OMV vaccine against *Burkholderia pseudomallei* and will graduate with her PhD in Biomedical Sciences in May 2016.