DEVELOPMENT OF MODEL SYSTEMS FOR THE VECTOR-HOST-PATHOGEN INTERFACE OF BUBONIC PLAGUE

A Dissertation presented to the Faculty of the Graduate School
at the University of Missouri

In partial fulfillment
Of the Requirements for the Degree

Doctor of Philosophy

By
DAVID M. BLAND

Dr. Deborah Anderson, Dissertation Advisor

December 2013

The undersigned, appointed by the Dean of the Graduate School, have examined the dissertation entitled

DEVELOPMENT OF MODEL SYSTEMS FOR THE

VECTOR-HOST-PATHOGEN INTERFACE OF BUBONIC PLAGUE

Presented by David M Bland

A Candidate for the Degree of Doctor of Philosophy

And hereby certify that in their opinion it is worthy of acceptance.

_____

Professor Deborah M. Anderson

_____

Professor Brenda T. Beerntsen

_____

Professor Charles R. Brown

_____

Professor Mark A. Daniels

_____

Professor David W. Stanley

This work is dedicated to friends and family who have supported me throughout my academic career and personal adversities. It is also dedicated in loving memory of Richard L. “Doc” Kieft and Andrew Kuebrich. Two people who inspired me though their enthusiasm for life and their devotion to the endeavors they loved. The party must go on, but their presence is sincerely missed.

ACKNOWLEDGEMENTS

I would like to first acknowledge and thank my advisor, Dr. Deborah Anderson, for providing me with the knowledge, guidance, and resources required to ask questions about the *Y. pestis* life cycle that were truly interesting to me. Her insight and positive reinforcement over the past 5 years was instrumental in the generation of this research and any professional success that may be found in the future. I thank her for her tireless effort and willingness to leave the office door open.

I acknowledge and thank my dissertation committee for their encouragement to think critically about my research and for fruitful scientific discourse. I acknowledge Dr. Deborah Anderson, Dr. Brenda Beerntsen, Dr. Charles Brown, Dr. Mark Daniels, and Dr. David Stanley.

I acknowledge and thank past and present members of the Anderson lab for productive scientific discussions, technical assistance, constructive support, and all the extra hours required to propel BSL-3 animal experiments to their conclusion. Towards all or some of the aforementioned aid, I acknowledge Dr. Nick Eisele, Dr. Yuqing Chen, Dr. Kristen Peters, Dr. Hanni Lee-Lewis, Dr. Andrew Kocsis, Dr. Ami Patel , Miqdad Dhariwala, Joshua Willix, Dr. Paul Anderson, and Eric Coate.

I acknowledge and thank Dr. Joe Hinnebusch for technical assistance, flea work protocols, and *Xenopsylla cheopis* fleas. *Yersinia*-Flea research at the University of Missouri-Columbia would not be possible without his assistance.

I acknowledge and thank Michael Rood of the L.A. County Department of Public Health for his invaluable assistance in the collection of fleas from Downtown Los Angeles.

I acknowledge and thank Dr. Melanie Marketon for providing the KIM6 *Y. pestis* strains needed for the flea immunity research. In addition I would like to acknowledge Dr. Marketon and her lab for agreeing to share their *Drosophila* data and graphs.

I acknowledge and thank Drs. Herb Schweizer and Greg Phillips for providing the mini-Tn7 plasmids and I acknowledge and thank Dr. Kenneth Gage for providing *O. montana* fleas.

Finally, I acknowledge and thank friends & family for their support throughout the graduate school process.

TABLE OF CONTENTS

LIST OF ILLUSTRATIONS

Figure Page

LIST OF TABLES

ABSTRACT

Bubonic plague infections begin in the dermis when the pathogen is introduced by a flea during a routine blood feed. Several barriers separate *Yersinia pestis* from its replicative niche, including phagocytic cells in the dermis and the refractory midgut environment of the vector. For this flea-borne disease, very little is known about the genetic factors that influence the establishment of infection in the flea midgut, the mechanism of transmission to naive hosts, trafficking of bacteria to the mammalian lymph node, or survival in disparate environments. Despite its lethality and the discovery of antibiotic resistant isolates, no licensed plague vaccine has been developed for use in the U.S. or Western Europe. Even a single cell of *Y. pestis* can initiate a lethal case of bubonic plague. Modern pandemics have originated from the endemic maintenance of flea and rodent interactions, as such, an improved understanding of genetic determinants that contribute to *Y. pestis* persistence, virulence, and transmission is warranted. In order to achieve this goal, we have generated improved genetic tools for studying mammalian pathogenesis of bubonic plague. In addition, we have developed and improved multiple model systems for comprehensive studies of the *Yersinia pestis* life cycle. Furthermore, we identified bacterial genetic factors that influenced survival and virulence in both mammalian and insect hosts. Ideally, the data provided will allow researchers to acquire consistent and reliable data about the *Y. pestis* life cycle that may ultimately improve epidemiological modeling and prevention of disease. Holistic and comprehensive research directed at the host-vector-pathogen interface will likely lead to development of methods for controlling vector-borne pathogens, like *Yersinia pestis.*

CHAPTER 1

THE VECTOR-BORNE PATHOGEN, *YERSINIA PESTIS*

Adapted from: Bland DM and Anderson DA "Imaging Early Pathogenesis of Bubonic Plague: Are Neutrophils Commandeered for Lymphatic Transport of Bacteria?" *MBio.* 2013.

1.1 Bubonic Plague and Mammalian Pathogenesis

Yersinia pestis is a highly virulent and potentially lethal zoonotic bacterium that survives within the flea vector and is transmitted to mammals via an infectious bite. *Yersinia pestis* is the causative agent of plague, and is considered a Tier 1 Select Agent by the U.S. Centers for Disease Control and Prevention. This particular bacterium colonizes the flea midgut and is transmitted to mammals after forming an infectious biofilm that lodges in the proventriculus of the flea (1). Once deposited in the dermal layer of the mammalian host, the bacteria will migrate to the draining lymph node, establish a replicative niche, and eventually spread systemically, causing multi-organ failure and death of the host. *Y. pestis* can invade epithelial cells and survive and replicate inside macrophages, but tissue damage and disease are primarily caused by its rapid extracellular growth and toxicity to host cells (2, 3). This rapid growth and toxicity to the host is the cause of the inflammatory-based enlargement of draining lymph nodes. These infected lymph nodes, which are often black in coloration, are known as buboes. As such, this mode of disease is aptly named bubonic plague.

One of the defining characteristics of *Y. pestis* is that it is able to inhibit the host innate immune response as well as limit inflammation until the later stages of disease (4). The initial anti-inflammatory response is believed to be mediated, at least in part, by the type III secretion system (T3SS) (5). Following adherence of bacteria to host immune cells, the type III secretion system delivers effector proteins, collectively known as Yops, to the host cell cytosol. These Yops prevent phagocytosis, alter immune function, and eventually induce apoptosis of the target cell. When grown at low temperature and in the flea, the T3SS is poorly expressed, requiring 37°C for maximal transcriptional induction. The molecular mechanism and timing of how this transition occurs *in vivo* have not been well characterized, and it appears likely the initial interactions with host cells would proceed with bacteria vulnerable to immune activation. Furthermore, *Y. pestis* LPS is immunostimulatory at low temperature, and must undergo a biosynthetic change at 37°C that provides stealth and attenuation of toll-like receptor signaling such that inflammation can be controlled. The functioning of the T3SS and alterations made to LPS are believed to be the major contributors to the initial anti-inflammatory phase of the infection.

Y. pestis can infect multiple tissues, with continual bacterial growth at the inoculation site, as well as seeding of primary and secondary immune tissues followed by rapid bacterial growth at these sites (6). Once an inflammatory response is initiated by tissue injury, as well as recognition of *Y. pestis* pathogen associated molecular patterns (PAMPs), neutrophils and monocytes are recruited from the peripheral blood (7). Neutrophils are by far the largest recruited cell population and quickly migrate to the site of infection where they are believed to mediate bacterial clearance. Inflammatory monocytes also enter infected tissue from the blood, where they mature to carry out

macrophage cell functions in host defense: including bactericidal activity, tissue repair, and antigen presentation (8). In addition, resident dendritic cells routinely traffic to the lymph node to display antigen to B and T cells. The T3SS is known to be capable of injecting effectors into all of these host cell types (9). *In vivo*, *Y. pestis* preferentially targets phagocytic cells for injection of Yops, thus preventing their activation (10, 11).

When *Y. pestis* is phagocytosed, the T3SS is believed to be largely inactive when bacteria are in the phagolysosome. Whether this is due to an environment that down-regulates expression of the T3SS or because the translocation pore cannot assemble across the phagosomal membrane is not known (12). It is therefore likely, that these host cells are activated because of a soluble anti-inflammatory signal induced by the activity of the T3SS on other cells rather than a direct effect of Yop injection by intracellular bacteria. This interpretation is consistent with data from the lung model, whereby extracellular $T3SS^+$ *Y. pestis* establish an anti-inflammatory state that is permissive for growth of avirulent $T3SS^-$ bacteria (5).

Yersinia pestis is non-motile but invasive, with an extracellular protease (Plasminogen activator or Pla) that is essential for its ability to cause infection from peripheral routes. Pla is a broad spectrum protease and adhesin that likely cleaves multiple targets such as fibrinogen and plasminogen and enhances bacterial adhesion to host extracellular matrices (13). Expression of Pla is essential for the development of bubonic plague, as mice infected by Pla^- strains typically don't develop histopathology or lymphatic bacterial burdens characteristic of bubonic plague, but indeed appear to be shuffled directly to the septicemic form of the disease (14). It has been previously speculated that *Y. pestis* uses Pla to bind to cell surface receptors on macrophages and

dendritic cells as a means to traffic to the draining lymph node (15, 16). However, recent and more convincing data suggests that neutrophils with altered programming may carry live bacteria to the lymph node (17). The mechanism whereby this would occur is not clear, as neutrophils are not known to express lymph node homing receptors (18). Nevertheless, prevailing models of bacterial trafficking to the primary lymph node involve intracellular bacterial transport via the lymphatic system or extracellular vascular dissemination.

Bubonic plague is extremely infective; a flea bite that transmits even one bacterium to the human host can ultimately lead to lethal bubonic plague. Symptoms of bubonic plague in humans develop within 2-6 days of infection and include fever, headache, chills, lethargy, and the characteristic swollen lymph nodes (19). This is typically the result of being bitten by an infected flea, however, bubonic plague can also develop from accidental exposure via needle stick in experimental settings. The frequent development of secondary septicemia causes significant complications for the patient, with mortality rates as high as 50-60% (19). These rates are probably exacerbated in the clinical setting by the application of antibiotics that are ineffective against *Y. pestis.* However, when individuals know they have been exposed to plague, prophylactic treatment with tetracycline antibiotics is exceptionally effective. One of the more concerning aspects of plague, as well as all bacterial pathogens, is the identification and isolation of multi-drug resistant strains (20). In addition, there is no licensed vaccine for plague, necessitating a need to identify novel therapeutics for all forms of the disease. Together, these factors have made the U.S. Centers for Disease Control and Prevention

label *Y. pestis* a Tier 1 Select Agent, due to concerns over its potential use as a bioterrorism threat (21)

1.2 Flea-Borne Transmission and Evolutionary Perspectives.

The primary vectors for plague, fleas, are wingless insects belonging to the order Siphonaptera. Adults are blood-feeding ectoparasites that feed on live birds and mammals, with the majority parasitizing rodents. The plague life cycle results in stable maintenance of *Y. pestis* as an endemic infection of wild rodent populations in many parts of the world, including the United States (22). When a mammal becomes infected, and *Y. pestis* enters the host's blood stream, a flea can take an infectious bloodmeal. Titers of bacteria in the bloodstream of the infected host must be considerably high ($10^6 \leq$ CFU/ml), when the host is in the terminal stages of disease, in order for the flea to become infected. This is believed to be necessitated by the relatively small volume of blood a flea ingests, but may also be dependent on the flea's natural resistance to microbial challenge.

The traditional "Blocked" or "Late Phase" mechanism by which fleas can transmit plague requires that the bacteria form a biofilm within the insect digestive tract (1, 23). The biofilm, a layered bacterial mass, is mediated by secretion of a surface-associated exopolysaccharide that will eventually block the flea's esophagus and prevent the uptake of new bloodmeals (24). This biofilm will develop between 1-3 weeks post infection. The flea, unable to become satiated, will make repeated, unsuccessful attempts to feed on a host. During these feeding events, some of the bacteria will be regurgitated from the biofilm and into the skin, perpetuating bubonic plague (25). The most abundant world-wide vector for plague, *Xenopsylla cheopis*, is readily blocked by *Y. pestis*

biofilms, and up to 50% of these fleas have been observed to form transmissible infections in the laboratory (26, 27). However, over 20 different species of flea in North America are believed to be capable of plague transmission (28). To date, only *X. cheopis* has been demonstrated conclusively to transmit the infection through the "blocked proventriculus" method.

More recently, a second transmission mechanism, or "Early Phase" transmission, has been established, that does not require a bacterial biofilm, but can only occur shortly after flea infection (23, 29). Early phase transmission has been demonstrated under experimental conditions *in X. cheopis* as well as the North American flea *Oropsylla montana*. Researchers have shown that during the first four days following a blood meal spiked with a high concentration of *Y. pestis*, fleas are almost as efficient as transmitting plague as those producing transmissible infections from biofilm-mediated esophageal blockages. The most prevalent hypothesis for this phenomenon is that the flea's mouthparts may be contaminated with *Y. pestis* for a narrow window of time. However, there is currently no experimental evidence to support the mechanical transmission hypothesis. Early phase transmission proponents claim this provides a more plausible explanation for the rapid epizootic outbreaks that are observed in prairie dog populations in the U.S., where close to 100% of the population is wiped out within 6-8 weeks (30). Currently, the evidence suggests that both transmission mechanisms are mutually compatible; however, they do complicate the epidemiological modeling of plague.

The factors that contribute to making a flea efficient at transmitting plague bacilli remain poorly understood. Established efficiencies of *Y. pestis* transmission amongst laboratory *X. cheopis* populations can vary greatly (23, 26, 27, 31). Furthermore, much

of what is currently known about transmission of *Y. pestis* was obtained during the early portions of the 20th century when little information on its virulence was known (31). It is likely that contributions to a transmissible infection are made by the flea's immune system, anatomy of the digestive tract, the insect microbiome, as well as environmental conditions. However, these are unlikely to be the sole contributors, as nothing is known about genetic differences that may alter vector responses to infection.

Yersinia pestis evolved from the enteric pathogen, *Yersinia pseudotuberculosis,* recently in evolutionary terms, within the last 20,000 years. During this timeframe, the bacterium has acquired enhanced virulence capabilities in mammalian hosts, as well as, the ability to survive and propagate it's transmission from the flea vector. One of the reasons *Y. pestis* is thought to have evolved its hypervirulence is that the flea vector is only capable of delivering small infectious dosages in the blocked model. On average, less than 100 CFU of *Y. pestis* are delivered into the host dermis from a blocked *X. cheopis* flea (27). Additionally, acquisition of the infection by a naïve flea requires a high-titer bacteremia in the mammalian host (28). This situation would impose evolutionary pressures to select for bacteria that rapidly replicated and entered the host blood stream to perpetuate transmission.

The two major bacterial genetic elements that have been shown to have a direct influence on bacterial survival within the flea midgut are *Yersinia* Murine Toxin (*ymt*) and the Hemin storage (*hms*) locus. Ymt is encoded on the *Yersinia* virulence plasmid pMT1, Ymt has phospholipase D activity that protects the bacteria from an unknown antibacterial mechanism of the flea midgut (32). *Y. pestis* that lack Ymt are unable to survive in the flea midgut. The biofilm, composed of exopolysaccharide, is produced by

the hemin storage genes (33, 34). This operon is located within the pigmentation locus (*pgm*) of *Y. pestis*. It is called the pigmentation locus because the *hms* operon is also responsible for the uptake of the dye and heme analog, Congo Red, on solid media. The *pgm* locus is an unstable genetic element and can be lost during routine laboratory passage. However, *hms* retention can be rapidly screened for by growing *Y. pestis* on media supplemented with Congo Red. If the *pgm* locus has been lost, colonies will not take up Congo red and will appear white. Interestingly, all flea borne isolates retain the *pgm* locus, suggesting that retention of the *hms* operon is selected for during the plague life cycle (35-38). Laboratory strains lacking Hms can colonize the flea midgut for a short period of time, but they are unable to produce a biofilm or transmit via the blocked model of disease. In addition to acquiring the expression of Hms and Ymt, a number of subtractive genetic modifications have allowed *Y. pestis* to persist within the flea midgut, including the loss of acute oral toxicity to fleas, as well as, inactivation of insecticidal ABC toxin genes (39, 40).

While *Yersinia spp.* receive plenty of attention for their virulence to mammalian species, it's important for researchers to remember that this genus appears to be more closely related to members of the Enterobacteriaceae that are associated with invertebrates (*Photorhabdus, Serratia*) rather than vertebrates (*Escherichia, Salmonella*) (41). *Y. pestis* genes that do not appear to have functionality in the mammalian host may be relevant to the vector-borne life cycle of disease.

1.3 Plague Research Models: Concerns and Limitations

A number of *In vivo* model systems have been developed to study the plague life cycle and the strategies it uses to cause disease. Many of these models are well

established and provide insights about host responses, bacterial gene regulation, disease pathology, and vaccine suitability for plague. Within plague research models there are limitations as to how well data can be applied and interpreted for naturally occurring environments and alternative hosts. Addressing the inadequacies in existing model systems and improving safety for users is crucial for generating data that is relevant to disease progression and transmission.

More recently, principal investigators must be wary of dual use research of concern (DURC). DURC has been defined as research that is generated for legitimate purposes but has a high risk of being used for nefarious intentions (42). Experiments that generate bacterial strains that have resistance to therapeutically valuable antibiotics are among DURC concerns. As such, the CDC limits antibiotics that can be used for research with fully virulent *Y. pestis*. Many of the genetic tools used for the study of bacterial virulence factors employ selectable markers, such as chloramphenicol and kanamyicin, which are not practical due to DURC considerations. Currently, ampicillin is the only selectable marker approved by the CDC, and alternative selectable markers for *Y. pestis* genetic systems are limited. Complicating the matter is that many researchers perform their genetic manipulations at biosafety level 2 (BSL) within *Y. pestis* strains lacking the virulence plasmid that encodes the T3SS. A recombinant version of the virulence plasmid that contains an ampicillin resistance cassette is subsequently reintroduced into the strain in order to restore virulence and perform *in vivo* studies at BSL-3. This strategy hinders a plague researcher's ability to generate mutant *Y. pestis* strains and subsequently restore gene function through plasmid complementation.

Due to the lack of alternative selectable markers, new genetic systems should be developed for use with BSL-3 designated *Y. pestis* strains. The lysine biosynthesis pathway is involved in the production of diaminopimelic acid (DAP), an indispensable peptidoglycan cross-linking component in Gram-negative bacteria, and the essential amino acid lysine. These factors make the lysine biosynthesis pathway an attractive target for a bacterial selectable marker system, whereby lysine biosynthesis can be restored from a recombinant plasmid. Bacterial lysine auxotrophs can be grown in lab culture when DAP is supplemented in the growth medium, and these mutants have previously been employed successfully as genetic systems in other Gram-negative bacteria. In addition, these strains provide a user-safety advantage as they are avirulent to users due to the bacteria's inability to scavenge DAP from mammalian and insect hosts.

To date, research involving *Yersinia*-insect interaction has been relatively limited in scope and size. Veterinary entomology that focuses on plague is an emerging field that has promise for improving epidemiological modeling of plague, as well as, identifying proteins that are crucial for disease transmission (43). While the most direct and obvious model is using the flea itself, a few other *in vivo* and *in vitro* systems have been developed to attempt to model the plague life cycle. The nematode, *C. elegans,* has been used as a model to study biofilm formation in a number of bacterial species. *Y. pestis* is capable of forming biofilms on the mouthparts of *C. elegans,* which can be tracked through fluorescent microscopy (44). Increased biofilm formation decreases the ability of the roundworms to feed. The decreased survival of the organism can be used as an indirect measurement of biofilm formation (45, 46). However, this model only focuses

on genes that influence biofilm formation and does not mimic stresses imposed by the arthropod midgut environment. It is unlikely that bacterial gene regulation of *Y. pestis* on the nematode mouth parts is analogous to regulation utilized within the flea midgut, which limits this model's usefulness.

Ideally, researchers model the organism that is actually employed for dissemination of the pathogen. In this case, the flea is the most obvious choice for studying plague. However, there are limitations on the research aims that can be addressed within the flea. While there are approximately 80 species of flea have been found to be infected with *Y. pestis* in nature, only *X. cheopis* has been conclusively demonstrated to consistently perform blocked transmission (47). Mechanisms of transmission and host genomic comparisons between other flea species need to be examined to understand how the majority of fleas harbor and transmit plague. Currently, no flea genome has been sequenced. This makes it challenging to study the host response to microbial infection through gene specific analysis and/or knockdown. Furthermore, there are no genetic tools to generate transgenic fleas. This makes determining the importance of host genes for generating transmissible infections difficult. This knowledge gap may be addressed by utilizing a *Drosophila* larval model of infection. Numerous transgenic fruit flies have been generated and are readily available through the *Drosophila* Genomics Resource Center (Bloomington, Indiana). In addition, our collaborators have demonstrated that fruit fly larvae can ingest and stably maintain plague in the digestive tract for 4 days. While this window is relatively short, it can help identify both bacterial and insect genes that are significant during the critical early stages of midgut infection.

The influence of genetic drift on the capacity of fleas to generate transmissible *Y. pestis* infections remains unexamined. Researchers of plague-insect interactions in the U.S. use fleas that are derived from inbred stocks which were established decades ago. In *Drosophila* species, it has been demonstrated that inbred populations have altered competitive and reproductive fitness relative to their outbred counterparts after, on average, 180 generations (48, 49). In animal models of research, strict regulations exist for the generation number of established inbred mouse lines, such as BALB/c. This is due to concerns over the influence of inbreeding on experimental outcomes. While the biological systems of arthropods tend not to be as multifaceted as mammals, a large degree of genetic complexity exists and the influence of inbreeding has been documented in model insect systems (48-50). Therefore, it is likely that selective pressures imposed by laboratory rearing conditions have created genetically homogenous flea populations with altered capacities to generate transmissible infections. The influence of genetic drift on experimental outcomes may cause flea researchers to reassess how flea colonies are maintained within the lab, determine whether outbred genetic diversity must be reintroduced into existing lab colonies, and question whether currently accepted transmission efficiencies truly reflect what occurs in natural flea populations.

1.4 Considerations and Research Aims

Vector transmission of infectious disease is a research priority for the Departments of Health and Human Services, Homeland Security and Defense, and the Centers for Disease Control and Prevention (51). Little is known about mechanisms of long term survival and transmission of flea-borne pathogens, yet recent emergence and re-emergence of flea borne bacterial infections (*Bartonella* and *Yersinia*, respectively) have been documented (52, 53). Of particular concern is the recent evolution of *Yersinia*

pestis to acquire the flea life cycle, which added enhanced virulence, prolonged survival between mammalian outbreaks, and the potential for horizontal gene exchange (54). Together, not only is *Y. pestis* a current threat to human and animal health, but it gives us a glimpse of what could emerge in the future. It is essential that we understand mechanisms of flea transmission and mammalian virulence in order to prevent outbreaks of antibiotic resistant, highly virulent infection (20). As such, this dissertation addresses the following research aims:

- Develop genetic tools and model systems to improve our understanding of plague pathogenesis and flea-borne infection.
- Identify bacterial virulence factors and surface localized proteins that are important for *Y. pestis* survival in diverse environments.
- Determine the genetic contributions of the flea vector that influences stable maintenance of plague in the insect digestive tract.

Developing model systems to understand plague pathogenesis and disease transmission mechanisms is critical for the epidemiological modeling of plague. Identification of genetic elements needed throughout the plague life cycle may help in developing novel therapeutic strategies. We propose that fleas infected with *Y. pestis* can be used as a safe model system to develop a broad understanding of microbial-arthropod interactions. Research directed at the host-vector-pathogen interface will likely lead to development of methods for controlling vector-borne diseases.

1.5 References

1. **Hinnebusch BJ, Erickson DL.** 2008. *Yersinia pestis* biofilm in the flea vector and its role in the transmission of plague. Curr Top Microbiol Immunol **322:**229-248.

2. **Straley S, Harmon P.** 1984. Growth in mouse peritoneal macrophages of *Yersinia pestis* lacking established virulence determinants. Infect Immun **45:**649-654.

3. **Cowan C, Jones H, Kaya Y, Perry R, Straley S.** 2000. Invasion of epithelial cells by *Yersinia pestis*: Evidence for a *Y. pestis* specific invasin. Infect Immun **68:**4523-4530.

4. **Sebbane F, Gardner D, Long D, Gowen BB, Hinnebusch BJ.** 2005. Kinetics of disease progression and host response in a rat model of bubonic plague. Am J Pathol **166:**1427-1439.

5. **Price P, Jin J, Goldman W.** 2012. Pulmonary infection by *Yersinia pestis* rapidly establishes a permissive environment for microbial proliferation. Proc Natl Acad Sci USA **109:**3083-3088.

6. **Guinet F, Ave P, Jones L, Huerre M, Carniel E.** 2008. Defective innate cell response and lymph node infiltration specify *Yersinia pestis* infection. PLoS ONE **3:**e1688.

7. **Kolaczkowska E, Kubes P.** 2013. Neutrophil recruitment and function in health and inflammation. Nat Rev Immunol **13:**159-175.

8. **Shi C, Pamer E.** 2011. Monocyte recruitment during infection and inflammation. Nat Rev Immunol **11:**762-774.

9. **Marketon MM, DePaolo RW, DeBord KL, Jabri B, Schneewind O.** 2005. Plague bacteria target immune cells during infection. Science **309:**1739-1741.

10. **Fields K, Nilles M, Cowan C, Straley S.** 1999. Virulence role of V antigen of *Yersinia pestis* at the bacterial surface. Infect Immun **67:**5395-5408.

11. **Marketon M, DePaolo R, DeBord K, Jabri B, Schneewind O.** 2005. Plague bacteria target immune cells during infection. Science **309:**1739-1741.

12. **Zhang Y, Murtha J, Roberts M, Siegel R, Bliska J.** 2008. Type III secretion decreases bacterial and host survival following phagocytosis of *Yersinia pseudotuberculosis* by macrophages. Infect Immun **76:**4299-4310.

13. **Lahteenmaki K, Virkola R, Saren A, Emody L, Korhonen TK.** 1998. Expression of plasminogen activator pla of *Yersinia pestis* enhances bacterial attachment to the mammalian extracellular matrix. Infect Immun **66:**5755-5762.

14. **Sebbane F, Jarrett CO, Gardner D, Long D, Hinnebusch BJ.** 2006. Role of the *Yersinia pestis* plasminogen activator in the incidence of distinct septicemic and bubonic forms of flea-borne plague. Proc Natl Acad Sci U S A **103:**5526-5530.

15. **Zhang P, Skurnik M, Zhang SS, Schwartz O, Kalyanasundaram R, Bulgheresi S, He JJ, Klena JD, Hinnebusch BJ, Chen T.** 2008. Human dendritic cell-specific intercellular adhesion molecule-grabbing nonintegrin (CD209) is a receptor for *Yersinia pestis* that promotes phagocytosis by dendritic cells. Infect Immun **76:**2070-2079.

16. **Zhang SS, Park CG, Zhang P, Bartra SS, Plano GV, Klena JD, Skurnik M, Hinnebusch BJ, Chen T.** 2008. Plasminogen activator Pla of *Yersinia pestis* utilizes murine DEC-205 (CD205) as a receptor to promote dissemination. J Biol Chem **283:**31511-31521.

17. **Shannon JG, Hasenkrug AM, Dorward DW, Nair V, Carmody AB, Hinnebusch BJ.** 2013. *Yersinia pestis* subverts the dermal neutrophil response in a mouse model of bubonic plague. mBio **4:**e00170-00113.

18. **Saeki H, Moore AM, Brown MJ, Hwang ST.** 1999. Cutting edge: secondary lymphoid-tissue chemokine (SLC) and CC chemokine receptor 7 (CCR7) participate in the emigration pathway of mature dendritic cells from the skin to regional lymph nodes. J Immunol **162:**2472-2475.

19. **Perry RD, Fetherston JD.** 1997. *Yersinia pestis*--etiologic agent of plague. Clinical microbiology reviews **10:**35-66.

20. **Welch TJ, Fricke WF, McDermott PF, White DG, Rosso ML, Rasko DA, Mammel MK, Eppinger M, Rosovitz MJ, Wagner D, Rahalison L, Leclerc JE, Hinshaw JM, Lindler LE, Cebula TA, Carniel E, Ravel J.** 2007. Multiple antimicrobial resistance in plague: an emerging public health risk. PLoS ONE **2:**e309.

21. **Bland DM, Eisele NA, Keleher LL, Anderson PE, Anderson DM.** 2011. Novel genetic tools for diaminopimelic acid selection in virulence studies of *Yersinia pestis*. PLoS ONE **6:**e17352.

22. **Friggens MM, Parmenter RR, Boyden M, Ford PL, Gage K, Keim P.** 2010. Flea abundance, diversity, and plague in Gunnison's prairie dogs (*Cynomys gunnisoni*) and their burrows in montane grasslands in northern New Mexico. Journal of wildlife diseases **46:**356-367.

23. **Eisen RJ, Wilder AP, Bearden SW, Montenieri JA, Gage KL.** 2007. Early-phase transmission of *Yersinia pestis* by unblocked *Xenopsylla cheopis* (Siphonaptera: Pulicidae) is as efficient as transmission by blocked fleas. J Med Entomol **44:**678-682.

24. **Rempe KA, Hinz AK, Vadyvaloo V.** 2012. Hfq regulates biofilm gut blockage that facilitates flea-borne transmission of *Yersinia pestis*. J Bacteriol **194:**2036-2040.

25. **Jarrett CO, Deak E, Isherwood KE, Oyston PC, Fischer ER, Whitney AR, Kobayashi SD, DeLeo FR, Hinnebusch BJ.** 2004. Transmission of *Yersinia pestis* from an infectious biofilm in the flea vector. J Infect Dis **190:**783-792.

26. **Burroughs AL.** 1947. Sylvatic plague studies: The vector efficiency of nine species of fleas compared with *Xenopsylla cheopis*. The Journal of hygiene **45:**371-396.

27. **Lorange EA, Race BL, Sebbane F, Joseph Hinnebusch B.** 2005. Poor vector competence of fleas and the evolution of hypervirulence in *Yersinia pestis*. J Infect Dis **191:**1907-1912.

28. **Chouikha I, Hinnebusch BJ.** 2012. *Yersinia*--flea interactions and the evolution of the arthropod-borne transmission route of plague. Current opinion in microbiology **15:**239-246.

29. **Vetter SM, Eisen RJ, Schotthoefer AM, Montenieri JA, Holmes JL, Bobrov AG, Bearden SW, Perry RD, Gage KL.** 2010. Biofilm formation is not required for early-phase transmission of *Yersinia pestis*. Microbiology **156:**2216-2225.

30. **Wimsatt J, Biggins DE.** 2009. A review of plague persistence with special emphasis on fleas. Journal of vector borne diseases **46:**85-99.

31. **Kartman L, McManus AG, Quan SF.** 1956. Studies on *Pasteurella pestis* in fleas. IV. Experimental blocking of *Xenopsylla vexabilis hawaiiensis* and *Xenopsylla cheopis* with an avirulent strain. Experimental parasitology **5:**435-440.

32. **Hinnebusch B, Rudolph A, Cherepanov P, Dixon J, Schwan T, Forsberg A.** 2002. Role of *Yersinia* murine toxin in survival of *Yersinia pestis* in the midgut of the flea vector. Science **296:**733-735.

33. **Hinnebusch B, Perry R, Schwan T.** 1996. Role of the *Yersinia pestis* hemin storage (hms) locus in the transmission of plague by fleas. Science **273:**367-370.

34. **Jarrett C, Deak E, Isherwood K, Oyston P, Fischer E, Whitney A, Kobayashi S, DeLeo F, Hinnebusch B.** 2004. Transmission of *Yersinia pestis* from an infectious biofilm in the flea vector. J Infect Dis **190:**783-792.

35. **Fetherston J, Schuetze P, Perry R.** 1992. Loss of the pigmentation phenotype in *Yersinia pestis* is due to the spontaneous deletion of 102kb of chromosomal DNA which is flanked by a repetitive element. Mol Microbiol **6:**2693-2704.

36. **Hare J, McDonough K.** 1999. High-frequency RecA-dependent and -independent mechanisms of Congo Red binding mutations in *Yersinia pestis*. J Bacteriol **181:**4896-4904.

37. **Achtman M, Morelli G, Zhu P, Wirth T, Diehl I, Kusecek B, Vogler A, Wagner D, Allender C, Easterday W, Chenal-Francisque V, Worsham P, Thomson N, Parkhill J, Lindler L, Carniel E, Keim P.** 2004. Microevolution and history of the plague bacillus, *Yersinia pestis*. Proc Natl Acad Sci **101:**17837-17842.

38. **Auerbach R, Tuanyok A, Probert W, Kenefic L, Vogler A, Bruce D, Munk C, Brettin T, Eppinger M, Ravel J, Wagner D, Keim P.** 2007. *Yersinia pestis* evolution on a small timescale: comparison of whole genome sequences from North America. PLoS One **2:**e770.

39. **Erickson DL, Waterfield NR, Vadyvaloo V, Long D, Fischer ER, Ffrench-Constant R, Hinnebusch BJ.** 2007. Acute oral toxicity of *Yersinia pseudotuberculosis* to fleas: implications for the evolution of vector-borne transmission of plague. Cell Microbiol **9:**2658-2666.

40. **Pinheiro VB, Ellar DJ.** 2007. Expression and insecticidal activity of *Yersinia pseudotuberculosis* and *Photorhabdus luminescens* toxin complex proteins. Cell Microbiol **9:**2372-2380.

41. **Qi M, Sun FJ, Caetano-Anolles G, Zhao Y.** 2010. Comparative genomic and phylogenetic analyses reveal the evolution of the core two-component signal transduction systems in enterobacteria. J Mol Evol **70:**167-180.

42. **Wolinetz CD.** 2012. Implementing the new U.S. dual-use policy. Science **336:**1525-1527.

43. **Lawrenz MB.** 2010. Model systems to study plague pathogenesis and develop new therapeutics. Frontiers in microbiology **1:**119.

44. **Atkinson S, Goldstone RJ, Joshua GW, Chang CY, Patrick HL, Camara M, Wren BW, Williams P.** 2011. Biofilm development on *Caenorhabditis elegans* by *Yersinia* is facilitated by quorum sensing-dependent repression of type III secretion. PLoS Pathog **7:**e1001250.

45. **Styer KL, Hopkins GW, Bartra SS, Plano GV, Frothingham R, Aballay A.** 2005. *Yersinia pestis* kills *Caenorhabditis elegans* by a biofilm-independent process that involves novel virulence factors. EMBO reports **6:**992-997.

46. **Sun YC, Koumoutsi A, Darby C.** 2009. The response regulator PhoP negatively regulates *Yersinia pseudotuberculosis* and *Yersinia pestis* biofilms. FEMS Microbiol Lett **290:**85-90.

47. **Pollitzer R.** 1952. Plague studies. 7. Insect vectors. Bulletin of the World Health Organization **7:**231-342.

48. **Latter BD, Mulley JC.** 1995. Genetic adaptation to captivity and inbreeding depression in small laboratory populations of *Drosophila melanogaster*. Genetics **139:**255-266.

49. **Latter BD, Mulley JC, Reid D, Pascoe L.** 1995. Reduced genetic load revealed by slow inbreeding in *Drosophila melanogaster*. Genetics **139:**287-297.

50. **Kneeland Kea.** 2012. Genetic variation in field and laboratory populations of the spined soldier bug, *Podisus maculiventris*. Entomologia Experimentalis et Applicata **143:**120-126.

51. **Peters N, Dixon D, Holland S, Fauci A.** 2008. The research agenda of the National Institute of Allergy and Infectious Diseases for antimicrobial resistance. J Infect Dis **197:**1087-1093.

52. **Gundi VA, Billeter SA, Rood MP, Kosoy MY.** 2012. *Bartonella spp.* in rats and zoonoses, Los Angeles, California, USA. Emerg Infect Dis **18:**631-633.

53. **Erickson DL, Jarrett CO, Callison JA, Fischer ER, Hinnebusch BJ.** 2008. Loss of a biofilm-inhibiting glycosyl hydrolase during the emergence of *Yersinia pestis*. J Bacteriol **190:**8163-8170.

54. **Hinnebusch BJ.** 2005. The evolution of flea-borne transmission in *Yersinia pestis*. Curr Issues Mol Biol **7:**197-212.

CHAPTER 2:

NOVEL GENETIC TOOLS FOR DIAMINOPIMELIC ACID SELECTION IN VIRULENCE STUDIES OF *YERSINIA PESTIS*

As found in: David M. Bland, Nicholas A. Eisele, Lauren L. Keleher, Paul E. Anderson, and Deborah M. Anderson "Novel Genetic Tools for Diaminopimelic Acid Selection in Virulence Studies of *Yersinia pestis" PLoS One (2011)*

Contributions: DB, DA, and NE conceived and designed experiments. Bacterial mutants and genetic tools generated by DB, PA, NE, and LK. All experiments and analysis performed by DB and DA except Figures 2.7 and 2.8 performed and analyzed by NE, DA, and LK. Manuscript written by DB and DA.

2.1 Abstract

Molecular studies of bacterial virulence are enhanced by expression of recombinant DNA during infection to allow complementation of mutants and expression of reporter proteins *in vivo*. For highly pathogenic bacteria, such as *Yersinia pestis*, these studies are currently limited because deliberate introduction of antibiotic resistance is restricted to those few which are not human treatment options. In this work, we report the development of alternatives to antibiotics as tools for host-pathogen research during *Yersinia pestis* infections focusing on the diaminopimelic acid (DAP) pathway, a requirement for cell wall synthesis in eubacteria. We generated a mutation in the *dapA-nlpB(dapX)* operon of *Yersinia pestis* KIM D27 and CO92 which eliminated the expression of both genes. The resulting strains were auxotrophic for diaminopimelic acid and this phenotype was complemented by *dapA* in single and multi-copy systems. *In*

vivo, we found that plasmids derived from the p15a replicon were cured without selection, while selection for DAP enhanced stability without detectable loss of any of the three resident virulence plasmids. The *dapAX* mutation rendered *Y. pestis* avirulent in mouse models of bubonic and septicemic plague, which could be complemented when *dapAX* was inserted in single or multi-copy, restoring development of disease that was indistinguishable from the wild type parent strain. We further identified a high level, constitutive promoter in *Y. pestis* that could be used to drive expression of fluorescent reporters in *dapAX* strains that had minimal impact to virulence in mouse models while enabling sensitive detection of bacteria during infection. Thus, diaminopimelic acid selection for single or multi-copy genetic systems in *Yersinia pestis* offers an improved alternative to antibiotics for *in vivo* studies that causes minimal disruption to virulence.

2.2 Introduction

Yersinia pestis is the causative agent of plague and is a recently evolved pathogen (1, 2). Due to its ability to undergo genetic flux from loss of genetic content and acquisition of DNA by horizontal transfer, *Y. pestis* evolved from a mild gastro-instestinal pathogen to one that rapidly induces high titer sepsis in mammals in order to promote its transmission and environmental survival in fleas (3). Many biovars of *Y. pestis* exist, varying between one another by significant changes, including plasmid acquisition, while even within biovars strains differ due to numerous point mutations, often in non-coding sequences (4, 5). Isolation of multi-antibiotic resistant *Y. pestis* from human plague patients has been reported in two independent cases, both of which were due to the acquisition of different multi-drug resistant plasmids, highlighting a potential public health concern for the evolution of drug resistant plague (6-8). This, combined with its hypervirulence in humans and mammals, stable maintenance in the environment between outbreaks, and the potential for rapid spread among humans, makes *Y. pestis* a potential reemerging threat to public health.

Heightened concern over highly pathogenic microbes such as *Yersinia pestis* has led to a surge in plague investigations, from basic mechanisms of pathogenesis to the development of novel vaccines and therapeutics. Yet, currently available gene expression and gene knockout tools used for attenuated *Yersinia* strains rely on the introduction of antibiotic resistance, which is restricted in the virulent isolates, thereby limiting the potential output of this surge in research activity. In this work, we addressed this shortfall and report the adaptation of standard genetic tools for metabolic, rather than antibiotic, selection.

Biosynthesis of lysine has become an increasingly used anti-bacterial target as it provides essential protein (lysine) and cell wall (meso-diaminopimelic acid) components, thereby inhibiting bacterial growth by two mechanisms (9). Mammals are unable to synthesize lysine and lack diaminopimelic acid, therefore the presence of a functional lysine biosynthetic pathway is essential for bacterial growth in mammalian hosts. Like antibiotics, this property has been explored as a mechanism for selection of bacteria carrying recombinant plasmids during infection. For example, *Salmonella typhimurium* lacking *asdA* (aspartate dehydrogenase) is unable to synthesize diaminopimelic acid and therefore is avirulent in a mouse model of disease (10, 11). Growth of this mutant is dependent on exogenous diaminopimelic acid or on the plasmid expression of *asdA* allowing for its selection *in vivo*. In *E. coli*, deletion of *dapA, B, C, D* and *E* confer diaminopimelic acid auxotrophy that can be used to select for recombinant DNA (12). Selection systems involving *dapB* (dihydropicolinic acid reductase) have been reported for other Gram negative pathogens, such as *Burkholderia pseudomallei*, thus it appears there are multiple genetic targets to block this highly conserved metabolic pathway (13, 14).

In this work, we explored the utility of diaminopimelic acid selection in *Yersinia pestis* for single and multi-copy expression of recombinant DNA. In *Y. pestis*, the genes encoding *dapB, C, D* and *E* are duplicated with two copies of each present in the chromosome (15). However the gene encoding *dapA*, a dihydropicolinic acid synthetase, is present in another chromosomal location, found in single copy, and is therefore predicted to be necessary for an early step of the pathway for biosynthesis of diaminopimelic acid. In *Y. pestis*, as well as many other bacteria, *dapA* is annotated as

the first gene of an operon that includes *nlpB/dapX*, an outer membrane lipoprotein that is not essential for growth (16). Here we show that null mutation of the *dapAX* operon results in diaminopimelic acid (DAP) dependent growth and an avirulent phenotype in mouse models of plague. Growth without DAP could be restored by supplying *Y. pestis dapA* in single or multiple copies and retention of plasmids could be achieved *in vivo* during murine infection. Complementation of the *dapAX* mutation *in vivo* required the introduction of both genes *in trans*, either in single or multiple copy, and this restored the development of plague to near wild type levels. We also report the development of dap-selectable DsRed reporter assays that enable identification of bacteria during infection. Together the data demonstrate the utility of the diaminopimelic acid biosynthetic pathway as a viable alternative to antibiotic selection for expression of recombinant DNA during experimental models of plague.

Table 2.1 Bacterial Strains used in this study.

Y. pestis Strains	Key Properties	Reference
KIM D27	Pgm^- Lcr^+; KIM 5 derivative	[18]
KIMD27-1003	KIM D27; Missing *dapA* promoter and entire *dapA* ORF, generated with pCVD442-dapAX	This Study
KIMD27-1011	KIM D27*dapAXatt*Tn7::*dapA*; *dapA* transposition downstream of glmS of the KIMD27-1003 parent strain	This Study
KIMD27-1012	KIM D27*dapAXatt*Tn7::*dapAX*; *dapAX* transposition into the attTn7 site of the KIMD27-1003 parent strain	This Study
KIMD27-1013	KIM D27*dapAXatt*Tn7::*dapAX DsRed*; *dapAX* and *cysZ-DsRed* transposition into the *att*Tn7 site of the KIMD27-1003 parent	This Study
KIMD27-1014	KIM *D27dapAXatt*Tn7::*dapAX Tomato*; *dapAX* and *cysZ-Tomato* transposition into the *att*Tn7 site of the KIMD27-1003 parent	This Study
CO92	Pgm^+ Lcr^+	[33]
CO92-1008	CO92; Missing *dapA* promoter and entire *dapA* ORF, generated with pCVD442-dapAX	This Study
CO92-1009	CO92*dapAXatt*Tn7::*dapAX*; *dapAX* transposition into the *att*Tn7 site of the CO92-1008 parent strain	This Study
CO92-1010	CO92*dapAXatt*Tn7::*dapAX DsRed*; *dapAX* and *cysZ-DsRed* transposition into the *att*Tn7 site of the CO92-1008 parent strain	This Study
CO92-1011	CO92*dapAXatt*Tn7::*dapAX Tomato*; *dapAX* and *cysZ-Tomato* transposition into the *att*Tn7 site of the CO92-1008 parent strain	This Study

doi:10.1371/journal.pone.0017352.t001

2.3 Materials and Methods

Bacterial strains and growth conditions. All strains used were taken from frozen stocks and streaked for isolation onto heart infusion agar (HIA) plates. The plates used for *Y. pestis* CO92 were supplemented with 0.005% Congo Red and 0.2% galactose to select bacteria that retain the pigmentation locus (17). For bubonic plague challenge, a single red pigmented colony was used to inoculate heart infusion broth (HIB) and grown 18-24 hrs at 26°C, 120 rpm. All handling of samples containing live *Y. pestis* CO92 was performed in a select agent authorized BSL3 facility under protocols approved by the University of Missouri Institutional Biosafety Committee. *Y. pestis* KIM D27, a non-pigmented strain originally isolated by Robert Brubaker, was routinely grown fresh from frozen stock on HIA, followed by aerobic growth at 26ºC in HIB overnight prior to use in experiments (18). Where indicated, ampicillin (100μg/ml) was added to media for selection of plasmids. For growth of *dapA* mutant *Y. pestis*, 400 μg/ml diaminopimelic acid (DAP) was added to liquid or agar media.

E. coli DH5α and JM109 served as cloning strains for construction of recombinant pACYC177 (New England Biolabs, Ipswitch, MA) based plasmids (19, 20). *E. coli* S17-1λpir (21) served as cloning strain for Ori-R6K based plasmids, including the suicide vector and the mini-Tn7 vectors (22, 23). *E. coli* strains were grown in LB media for propagation. For cloning purposes, ampicillin (100μg/ml) was added to the media for selection.

Plasmids and dapAX complementation. *Yersinia pestis dapA* and *dapAX* were amplified from *Y. pestis* KIM D27 by PCR. pACYC177 was modified by replacement of the

kanamycin resistance gene with that of *dapA* using restriction sites HindIII and SmaI. The resulting plasmid no longer conferred kanamycin resistance but still retained ampicillin resistance and was used for complementation studies. The suicide vector, pCVD442 *dapA*, was constructed by amplifying 1,000bp upstream of the *dapA* promoter and 1,000bp downstream of the *dapA* stop codon (22, 24). These DNA fragments were amplified by PCR and ligated into the XbaI and SphI sites of pCVD442 using EcoRI as a linker between upstream and downstream DNA segments. Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs (Ipswich, MA). Promoterless DsRed was amplified from pDsRed Monomer (Clonetech, Mountain View, CA) and cloned into pACYC177 for use in the promoter trap screen. For Tn7 transposition, *dapA* or *dapAX* was amplified by PCR and cloned into the SmaI and SpeI sites of pUC18R6KT mini-Tn7 (23).

Animals. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. This protocol was approved by the Animal Care and Use Committee of the University of Missouri. All efforts were made to minimize suffering of the animals. Female BALB/c or C57BL/6 mice, 6-8 weeks old, were purchased from Charles River Laboratories (Wilmington, MA). During bubonic plague challenge, mice were maintained in select agent approved containment facilities at the University of Missouri. All infected mice were monitored regularly by daily weighing and assignment of health scores. Animals that survived to the end of the 14 day observation period or were identified as moribund (defined by severe ataxia) were euthanized by CO_2 asphyxiation followed by bilateral

pneumothorax, methods approved by the American Veterinary Medical Association Guidelines on Euthanasia.

Plague challenge. *Y. pestis* CO92, grown as described above, was diluted in sterile PBS to the indicated dose just prior to use for challenge experiments. For bubonic plague, bacteria were delivered in 100µl volume by subcutaneous injection in BALB/c mice (LD50 = 1 CFU) (25). Actual dose and retention of the pigmentation locus were determined by plating in triplicate on HIA with congo red. For intravenous challenge involving non-pigmented *Y. pestis* KIM D27, bacteria were diluted in sterile PBS and delivered by tail vein injection of 100µl (LD50 = 100 CFU) (26). For intranasal challenge involving non-pigmented *Y. pestis* KIM D27, mice were pre-treated by intraperitoneal injection of 500µg Fe^{+2} ($FeCl_2$). All animals subcutaneously or intranasally infected with *Y. pestis* were first lightly anesthetized by isoflurane inhalation. Animals were observed for recovery from anesthesia following the procedure and returned to housing.

Competitive Index. This method was performed as previously described (27). Wild type *Y. pestis* KIM D27 with or without recombinant pACYC*dapA* were combined in a 1:1 ratio (doses ranging from 1,000 to 13,000 CFU each strain) and injected intravenously into BALB/c or C57BL/6 mice in a 100µl volume. Four days post infection, mice were euthanized, and spleens were harvested, homogenized in PBS and plated in duplicate on HIA (all bacteria) and HIA + amp (plasmid-bearing bacteria). To calculate plasmid loss, bacterial colony forming units (CFU) recovered without amp selection were subtracted from the CFU recovered with amp selection and percentages of each found in the spleen were determined. The Competitive Index (C.I.) is defined as: % Amp^r Recovered/%

Amp^r Input. For statistical analysis, the ratio of amp^r to total CFU recovered was compared with the ratio of amp^r to total CFU in the inoculum.

Yersinia promoter trap screen. Primers with abutted restriction sites were used to amplify the open reading frame of DsRed-Monomer (Clonetech, Mountain View, CA) which was subsequently ligated into pBR322 (New England Biolabs, Ipswitch, MA) in place of the tetracycline resistance gene. *Y. pestis* KIM D27 genomic DNA was digested with RsaI and 100-1,500bp DNA fragments were ligated directly upstream of DsRed. Colonies were screened in *E. coli* DH5α for DsRed expression, and those that gave the strongest signal were transformed into *Y. pestis* KIM D27 and checked for DsRed fluorescence. One plasmid from this screen, pRsaI-2.1, was further characterized by sequence analysis, followed by sub-cloning into pDB2 (pUC18R6KT + *dapAX*). The resulting plasmid was then used for transposition into *Y. pestis* KIM D27*dapAX* to insert DsRed and *dapAX* into the chromosome.

DsRed expression assay. Three independent colonies of *Y. pestis* KIM D27, KIM D27pRsaI-2.1, and KIMD27.1013 were grown at 26°C and 37°C in HIB. 100µl of each culture was added to a 96-well plate and analyzed on a plate reader for absorbance (600nm) and DsRed fluorescence (544/590nm). Measured fluorescent values were then divided by OD600 to normalize to cell number.

Macrophage assay. Macrophages were prepared as previously described (28). Briefly, $1x10^6$ biotinylated macrophages were plated in DMEM supplemented with 5% FBS and infected with the indicated strains at an MOI of 10 for 5 hours. Gentamicin was present in the media at 100μg/ml to kill extracellular bacteria. Cells were fixed with 4% paraformaldahyde then stained with DAPI and streptavidin conjugated to Alexa Fluor-488 (Invitrogen, Carlsbad, CA) and analyzed by confocal microscopy.

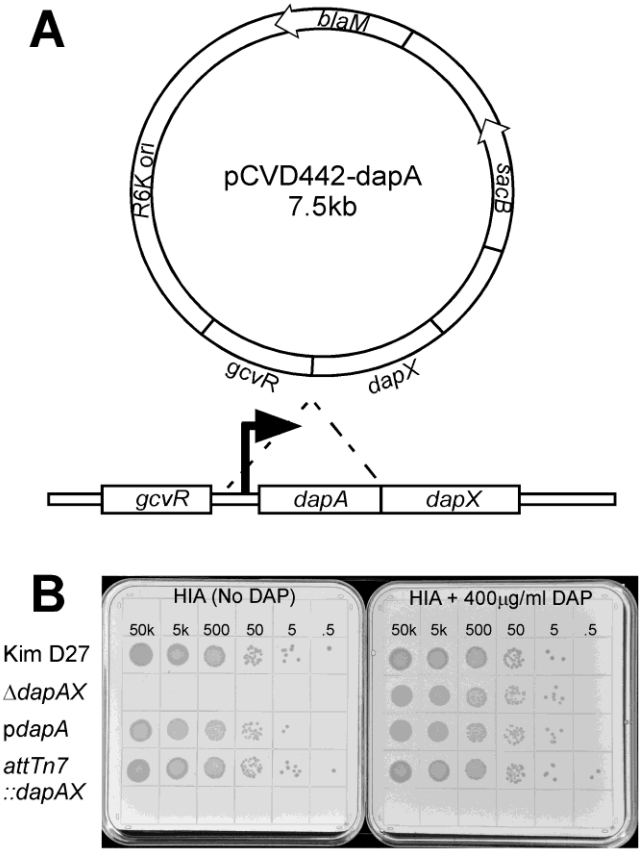

Figure 2.1 ***Construction of the dapAX mutation results in DAP dependent growth***. The *dapA* promoter and open reading frame were deleted by homologous recombination using the plasmid pCVD442 (A). Overnight cultures of the indicated strains (the *dapAX* mutant supplemented with 400μg/ml DAP) were serially diluted 10 fold in HIB (no DAP) and plated on HIA with or without DAP (B). Plates were incubated at 26°C for 48 hrs.

Statistical Analyses. Data from the competitive index were tested for difference from a given proportion using prop test from R (29). Briefly, the proportion of amp^r to total CFU in the recovery was tested for a difference from the proportion of amp^r to total CFU in the inoculums using alpha = 0.05. Survival data was evaluated by Cox proportional

hazards survival analysis or Kaplan-Meier Log Rank test. DsRed expression data were evaluated by one way ANOVA.

2.4 Results

2.4.1 Deletion of dapAX in Y. pestis results in DAP auxotrophy

Diaminopimelic acid (DAP) is a component of the cell wall that provides cross linking of peptidoglycan in many Gram negative bacteria including *Yersinia pestis*. Previous work showed that disruption of the metabolic pathway for biosynthesis of DAP renders *E. coli* unable to grow in media lacking diaminopimelic acid (12). Thus, standard laboratory media such as heart infusion agar, blood base agar and Luria agar cannot support growth of mutants lacking essential genes of the diaminopimelic acid biosynthetic pathway. A search of the *Y. pestis* genome revealed that many genes are duplicated, but one gene required for an early step in the biosynthetic pathway, dipicolinate synthetase or *dapA*, was present in single copy (15). We therefore generated a suicide vector designed to delete the promoter and open reading frame of *dapA* in *Yersinia pestis*, which is predicted to delete the expression of two genes, *dapA* and *nlpB* (*dapX*), likely present in an operon (Figure 2.1). Homologous recombination of the deletion construct was introduced by pCVD442 into the wild type, non-pigmented *Y. pestis* strain KIM D27 and resulted in a mutant strain that was unable to grow on plates without DAP supplementation or expression of *dapA in trans* (Figure 2.1B, data not shown). Deletion of *dapA* was confirmed by PCR, and the absence of *dapA* and *dapX* mRNA was observed by reverse transcriptase PCR of mRNA purified from stationary phase cultures (data not shown).

We next characterized the *Y. pestis* KIM D27 *dapAX* strain for growth characteristics in laboratory media with and without DAP. The *dapAX* strain was unable to grow in broth media without DAP, either at 26°C or 37°C and this was restored by supplying the wild type *dapA* gene in either single or multiple copies (Figure 2.2). However, the *dapAX* mutant grew normally when DAP was added to the culture media. Following removal of DAP from the media, *Y. pestis* KIM D27*dapAX* viability sharply declined 6 hrs later indicating depletion of DAP is rapidly lethal to the bacteria (data not shown). Together the data suggest that the absence of DapA confers dependency on supplemental diaminopimelic acid for growth.

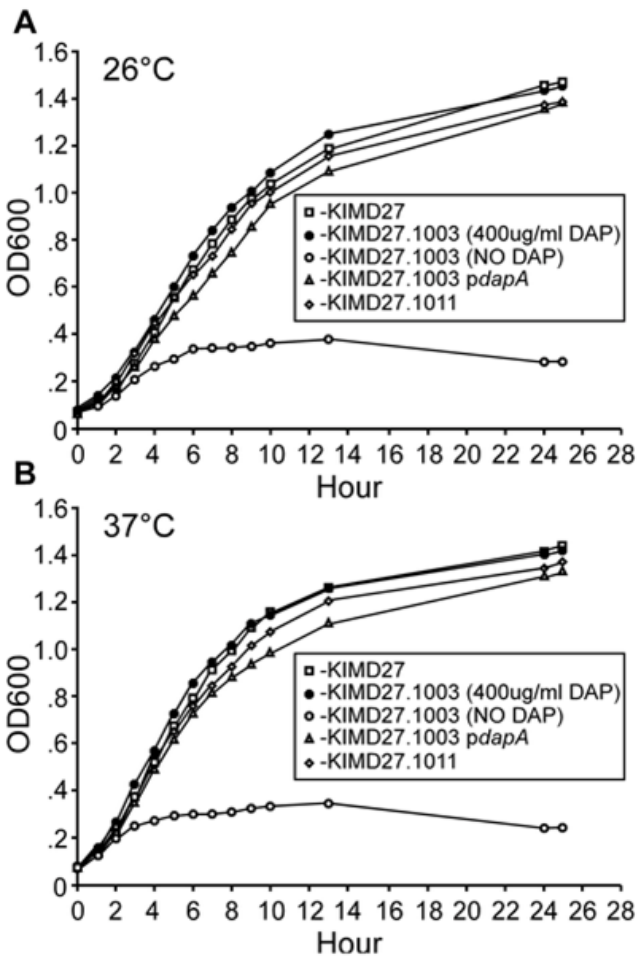

Figure 2.2 ***DAP independent growth is restored by expression of dapA in single or multi copy*****.**Wild type KIM D27, and isogenic strains KIM D27-1003, KIM D27-1003p*dapA*, or KIM D27-1011 were grown overnight in HIB at 26°C, then diluted to an OD600 of .075 and grown for 25 hrs at 26°C (A) or 37°C (B) with shaking at 130 rpm, monitoring OD600 as indicated. KIM D27-1003 strain with no DAP supplementation was washed 3X in sterile PBS to remove excess DAP from the overnight culture. Data are representative of 3 independent experiments.

2.4.2 Diaminopimelic acid selection is functional in vivo in mouse models of plague

We tested for diaminopimelic acid selection during a mouse model of septicemic plague. In this model, pACYC *dapA* was introduced by electroporation into *Y. pestis* KIM D27 *dapAX*, and the resulting strain was used to infect BALB/c mice by intravenous injection. Whereas the *dapAX* mutant was avirulent, with $>10^6$ fold increase in dose required for a lethal infection in this model, the pACYC *dapA* complemented strain had substantial restoration of virulence and an estimated LD50 of 30,409 CFU

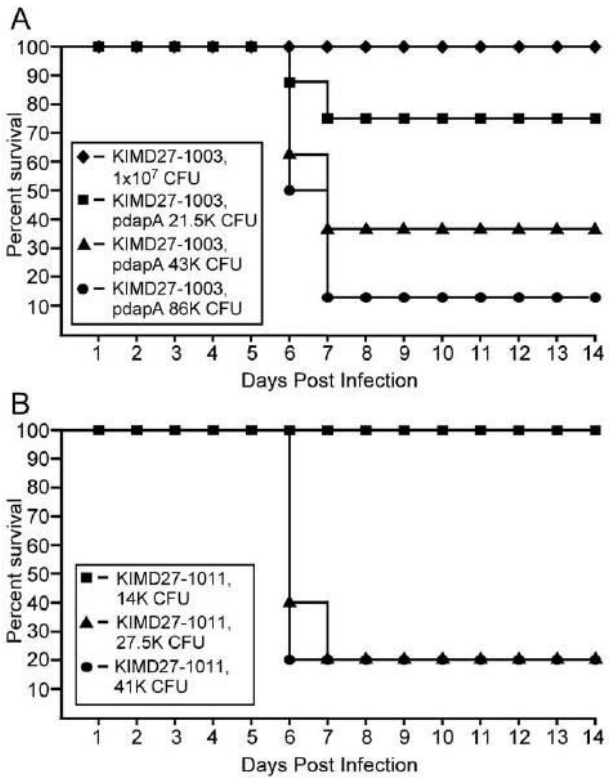

Figure 2.3 Expression of *dapA* on a multi-copy plasmid partially restores virulence.
Y. pestis KIM D27-1003 (*dapAX*) either with or without p*dapA* (A), or with *dapA* inserted into the *att*Tn7 site of *Y. pestis* KIM D27 (KIM D27-1011) (B) were grown overnight in HIB either with or without 100 µg/ml ampicillin, then diluted in sterile PBS to the indicated doses. 100 µl was injected intravenously into the tail vein of BALB/c mice (n = 8 per group for A; n = 5 per group for B). Survival was monitored for 14 days. The observed 50% lethal dose (LD50) was calculated as 30,409 (p*dapA*) and 20,804 (KIM D27-1011) by the method of Reed and Muench [27].

(~300XWT) (Figure 2.3). To investigate whether or not the *dapA* plasmid was stably maintained *in vivo*, we isolated bacteria from the spleens of BALB/c mice infected with 20,000 CFU of *Y. pestis* KIM D27*dapAX*pACYC-*dapA* on day 4 post-infection, when each mouse showed signs of acute disease. Colonies isolated from these spleens were tested by PCR to verify the presence of all three *Y. pestis* virulence plasmids in addition to the plasmid expressing *dapA*. PCR analysis of 81 colonies from each mouse verified a

Table 2.2 DAP selection *in vivo* does not cause instability of resident virulence plasmids.

Virulence plasmid	Gene	PCR Positive Colonies	% Retention
pCD1	*lcrH*	81/81	100%
	yopB	81/81	100%
pPCP1	*pla*	81/81	100%
	pst	81/81	100%
pMT1	*caf1*	81/81	100%
	ymt	81/81	100%
pACYC*dapA*	*bla*	81/81	100%

doi:10.1371/journal.pone.0017352.t002

high degree of retention of all three virulence plasmids as no plasmid loss was seen (Table 2.2). These results strongly suggest that p15a plasmids can be selected *in vivo* without loss of other *Y. pestis* virulence plasmids. However, since the pACYC-*dapA* strain was unable to fully restore virulence to wild type levels, we sought to further characterize the impact of p15a plasmids on the virulence of wild type *Y. pestis*.

2.4.3 Diaminopimelic acid selection is necessary for plasmid retention in vivo.

To understand the effects of p15a plasmids on the virulence of *Y. pestis*, we performed a competition experiment to determine if pACYC-*dapA* impaired growth *in vivo*. Towards this end, BALB/c or C57BL/6 mice were challenged by intravenous injection of 10^3-10^4 CFU wild type *Y. pestis* KIM D27 mixed in a 1:1 ratio with wild type bacteria expressing pACYC *dapA*. Following 4 days post-infection, mice were euthanized and bacteria in the spleens were enumerated. KIM D27 cells harboring pACYC *dapA* compared to those without plasmid were identified by plating bacteria on media

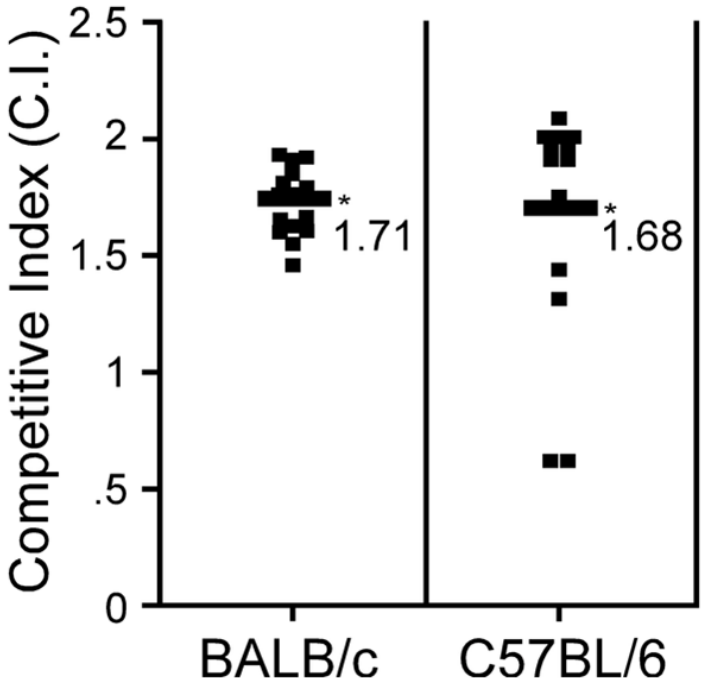

Figure 2.4. ***pACYC177 imposes a biochemical burden on Y. pestis in vivo.*** *Y. pestis* KIM D27 with or without p*dapA* was grown overnight in HIB with or without, respectively, ampicillin. An approximately 1:1 ratio of each strain was mixed and delivered by intravenous injection into the tail vein of BALB/c (A) or C57BL/6 (B) mice. On day 4 post-infection, when many of the mice were moribund, spleens were harvested and bacterial titer was determined for strains with and without plasmid by plating serial dilutions on HIA and HIA+amp. The Competitive Index (C.I.) is defined as the ratio of recovered bacteria from mouse spleens divided by the ratio in the inoculum. Scores less than one indicate the plasmid-bearing strain was less fit than its counterpart within an individual mouse. After no significant difference between experiments were detected, data were pooled from 3 independent experiments with groups of 4–5 mice, and a total of 15 (BALB/c) and 13 (C57BL/6), respectively, were analyzed. Data were tested for difference of proportion using R giving $p<0.0001$.

with and without ampicillin. The percentages of plasmid carrying strain recovered from the spleen were compared to the input values to calculate the competitive index (CI) (Figure 2.4). Statistical significance of data from infections of BALB/c and C57BL/6 mice was then evaluated by testing for a difference in proportion between input and recovery. Both strains of mice yielded similar results, and in nearly all mice, bacteria carrying the plasmid decreased in proportion after infection ($p<0.001$) and the corresponding CI was typically greater than 1 for bacteria without plasmid. In one experiment, we observed lower proportions, however if analyzed separately, this experiment still showed a significant decrease in proportion from inoculum to recovery. Together, these results suggest that carrying an additional plasmid, though it may not cause instability of other virulence plasmids, imposes a biochemical burden that either retards bacterial growth *in vivo* or causes it to be subject to curing during the infection.

We therefore also measured stability of pACYC177 and pACYC*dapA* during *Y. pestis* KIM D27 infection of BALB/c mice without selection. Bacteria harvested from the spleen on day 4 post-infection were monitored for loss of ampicillin resistance by plating on HIA with and without ampicllin. Results showed plasmid loss for both strains ranging from 1-4% with higher loss for the larger plasmid containing *Y. pestis dapA* (Table 2.3). Together the data indicate that p15a plasmids are cured during infection suggesting incompatibility with one or more virulence plasmids.

2.4.4 Single copy complementation of dapAX restores virulence.

Because of the biochemical burden imposed by plasmids, we aimed to develop methods for insertion of genes in the chromosome, for single copy, stable expression using DAP selection. Towards this end, we adapted the mini-Tn7 system for integration

of genes downstream of the *glmS* gene of Gram negative bacteria, which has been shown to be highly efficient in *Y. pestis* (23, 30). The *dapAX* operon was cloned into the multi-cloning site of pUC18R6KT which is flanked by *att*Tn7 transposition sites. The resulting plasmid, pDB2, and the helper plasmid encoding the transposase complex, pTNS2, were electroporated into *Y. pestis* KIM D27*dapAX*, and selected on HIA (no DAP). The complemented strain was verified by PCR to carry *dapAX* downstream of *glmS* rather than its original location on the chromosome (data not shown). To test complementation *in vivo*, we infected BALB/c mice with *Y. pestis* KIM D27*dapAX attTn7*::*dapAX* by intravenous injection and tested for survival at a dose equivalent to 1 LD50 of the wild type parent strain. Wild type KIM D27 IV challenge of approximately 45 CFU caused 60% lethality, and similarly, integration of *dapAX* by Tn7 transposition gave 40% lethality at low challenge dose (70CFU) suggesting single copy expression of *dapAX* is sufficient to fully restore virulence (Figure 2. 5A).

Table 2.3 p15a plasmid loss with no selection detected in spleens recovered from moribund mice.

Mouse	Total CFU[a] (from spleen)	Amp^S CFU	Plasmid Loss[b]
1- *pdapA*	100	5	5%
2- *pdapA*	100	2	2%
3- *pdapA*	80	3	3.75%
Combined	280	10	3.5%
1- *pACYC-177*	100	1	1%
2- *pACYC-177*	60	1	1.66%
3- *pACYC-177*	97	1	1.03%
Combined	257	3	1.17%

a: CFU: Colony forming units of *Y. pestis* KIM D27.
b: Amp^S/total CFU×100.
doi:10.1371/journal.pone.0017352.t003

We also tested whether DAP selection would work in the fully virulent *Orientalis Y. pestis* strain CO92. The *dapAX* mutation was generated by deletion of the promoter

and open reading frame for *dapA* using pCVD442 and homologous recombination as described above. The resulting strain was unable to grow on media without supplemental DAP (data not shown). The deletion was confirmed by PCR as well as retention of all three virulence plasmids and the pigmentation locus (data not shown). Because our results in KIM D27 suggested that both *dapA* and *dapX*/*nlpB* were required for virulence, we introduced both genes to determine if virulence could be restored in a bubonic plague model. Introduction of *dapAX* in single copy using the mini-Tn7 transposon restored growth in the absence of supplemental DAP. The *Y. pestis* CO92 *dapAX* mutant and *dapAX* Tn7::*dapAX* strains were then used to challenge BALB/c mice by

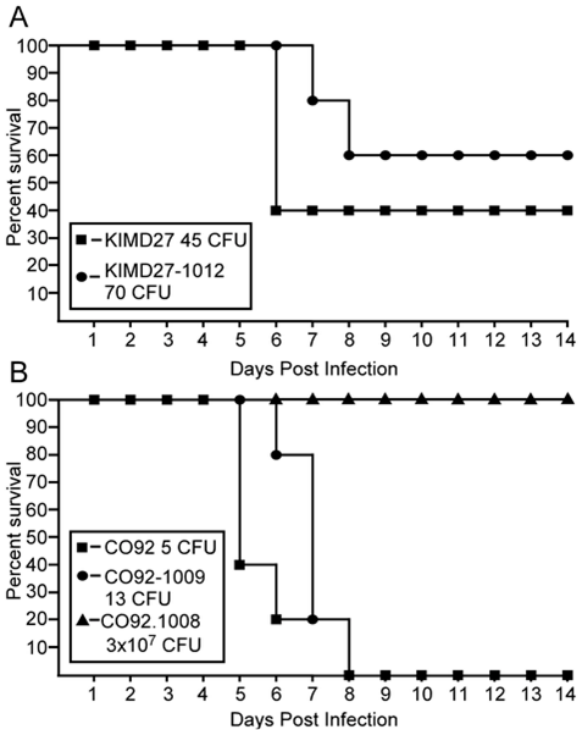

Figure 2.5 Complementation of the *dapAX* operon by chromosomal insertion restores virulence. (A) *Y. pestis* KIM D27 and KIM D27-1012 (*dapAX attTn7::dapAX*) were grown overnight, diluted to the indicated dose in sterile PBS and delivered by intravenous injection into the tail vein of BALB/c mice. (B) *Y. pestis* CO92, CO92-1008 (*dapAX*), and CO92-1009 (*dapAX attTn7*::*dapAX*) were grown overnight, diluted to the indicated dose in sterile PBS and delivered by subcutaneous injection into the left hind limb of BALB/c mice (n = 5 for all groups). Survival was monitored over 14 days for both models. No significant difference in survival was detected between wild type and *dapAX* complemented strains (p = 0.22 for KIM D27; p = 0.10 for CO92) using the Gehan-Breslow-Wilcoxon test.

subcutaneous injection in a bubonic plague model. In this model, insertion of *dapAX* by Tn7 transposition also appeared to fully complement virulence with 100% lethality caused by less than 15 CFU of either wild type or complemented strain (Figure 2.5B) (31). Histopathology of moribund mice indicated development of bubonic plague as lymph nodes taken from subcutaneously challenged mice on day 4 post-infection, showed severe hemorrhage and necrosis similar to wild type (Figure 2.6). Thus, with DAP as a selection for Tn7 insertion of genes in single copy, virulence could be restored indicating no significant impact on pathogenesis.

2.4.5 DAP selectable system for single copy detection of fluorescence in vivo.

To further develop single copy expression systems for virulence studies, we screened for constitutively active *Yersinia* promoters that could drive expression of the fluorescent protein DsRed that could be seen by microscopy in single copy. Towards this end, a library of *Y. pestis* KIM D27 DNA fragments (100-1,500bp) fused to a promoterless DsRed plasmid was generated in *E. coli*. Colonies were screened for expression of DsRed and those with the strongest signal were then tested for activity in *Y. pestis*. The strongest isolate, RsaI-2.1, could be identified on agar media as a red colony in both *E. coli* and *Yersinia* (data not shown). The insert was characterized by sequencing and the entire reporter cassette was sub-cloned into pDB2 upstream of *dapAX*, within the flanking *att*Tn7 sites. The DNA sequence revealed the presence of the *cysZ* promoter and first 178 codons of its open reading frame fused in frame to DsRed. In addition, we cloned the *cysZ* promoter from *Y. pestis* and used it to express the brighter DsRed variant, Tomato.

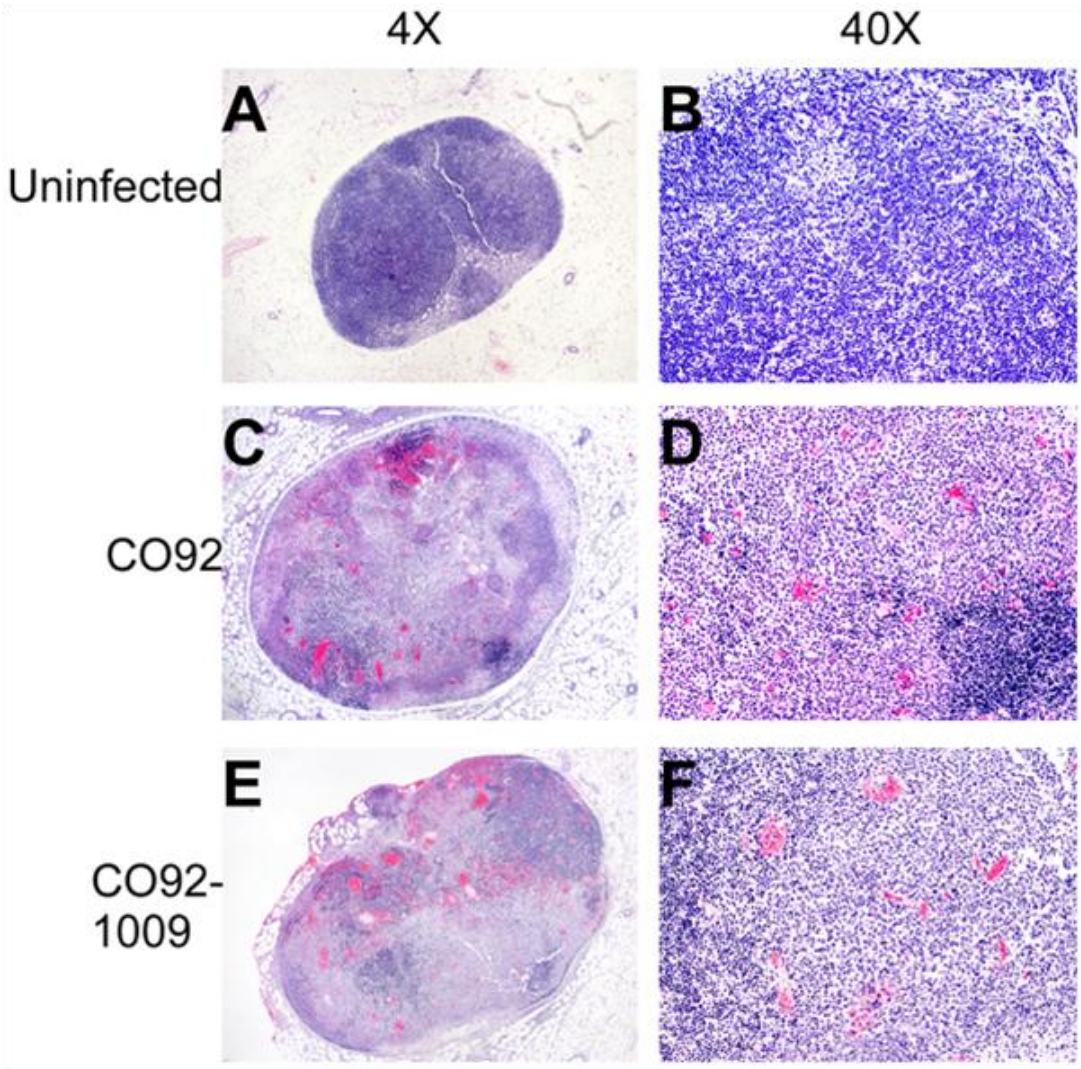

Figure 2.6 Development of fulminant bubonic plague is restored by*att*Tn7::*dapAX*.

Y. pestis CO92 and CO92-1009 (*dapAX att*Tn7*::dapAX*) were grown overnight at 26°C, diluted in sterile PBS to the indicated doses and delivered to BALB/c mice by subcutaneous injection. On day 4 post-infection, the left inguinal lymph node was removed, fixed in formalin, sectioned and stained with hematoxylin and eosin (H&E). (A–B) Uninfected; (C–D) CO92; (E–F) CO92-1009. Images are representative of tissues harvested from 5 mice in each group.

cysZ-DsRed and *cysZ*-Tomato reporters were introduced into *Y. pestis* KIM D27*dapAX* by Tn7 transposition and selected by growth on HIA without DAP supplementation. The resulting strain was confirmed by PCR (data not shown) and expression of DsRed was monitored in overnight cultures incubated at either 26°C or 37°C in HIB. The results showed strong expression of DsRed at both temperatures, with an increase at 37°C over 26°C (Figure 2.7). Tomato fluorescence was higher at both temperatures. Expression of DsRed or Tomato in this system did not have a significant impact on virulence compared to complementation with *dapAX* alone in an intranasal model of septicemic plague, as challenge doses of approximately 50X LD50 caused similar lethality (Table 2.4) (32). Similarly, expression of DsRed or Tomato in *Y. pestis* CO92 caused similar lethality when challenged with 50X LD50 compared to *dapAX* alone in a bubonic plague model.

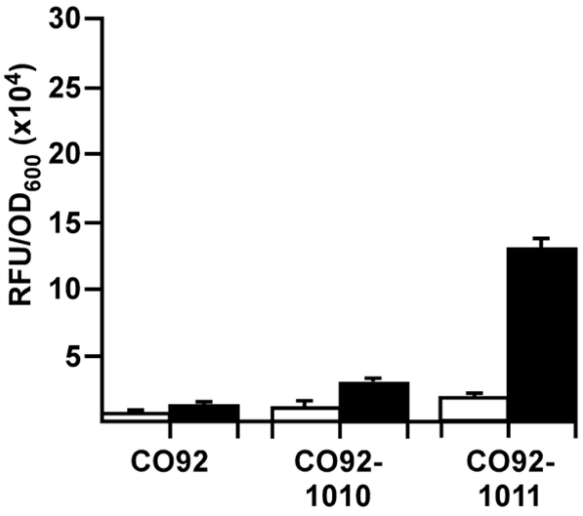

Figure 2.7. The *cysZK* promoter supports high level expression of fluorescence in single copy in *Y. pestis*. *Y. pestis* CO92 strains were grown overnight in HIB at 26°C (open bars) or 37°C (closed bars) and then analyzed on a 96-well plate. Relative fluorescent units (RFU) were measured on a plate reader at an excitation/emission spectra of 544/590 nm. Each value was normalized to the OD600 of the sample. To facilitate removal from the BSL-3 laboratory, 1 mL of culture was removed, fixed in 4% paraformaldahyde then resuspended in PBS. Error bars represent the standard error of the mean between three distinct overnight cultures.

Table 2.4 High level, constitutive expression of DsRed or Tomato causes minimal disruption to virulence.

Strain	Percent Survival	Challenge Dose
KIM D27	16.7 (1/6)	5.6×10^5 [a]
KIM D27pRsal2.1	0 (0/6)	5.3×10^5 [a]
KIM D27-1014 (*dapAX attTn7::Tomato*)	0 (0/9)	5.7×10^5 [a]
CO92	0 (0/8)	58[b]
CO92 -1010 (*dapAX attTn7::DsRed)*	12.5 (1/8)	66[b]
CO92-1011 (*dapAX attTn7::Tomato)*	12.5 (1/8)	60[b]

a: Challenge by intranasal instillation; mice pre-treated with 500 μg Fe^{+2} just prior to challenge; dose is equivalent to 50X LD50 for wild type KIM D27.
b: Challenge by subcutaneous injection; dose is equivalent to 50X LD50 for wild type CO92.
doi:10.1371/journal.pone.0017352.t004

We next tested expression during macrophage infections. *Y. pestis* KIM D27pRsaI-2.1 (multi-copy DsRed) or KIMD27.1013 (single copy DsRed) were grown at 26°C overnight, diluted in sterile PBS and added to biotin labeled RAW 264.7 macrophage-like cells. Infection was initiated by centrifugation, and after 30 min, gentamicin was added to kill extracellular bacteria. Cells were later fixed, labeled with streptavidin-Alexa Fluor 488 and stained with DAPI to enable fluorescent detection of macrophages, then examined by confocal microscopy (Figure 2.8). Expression of DsRed from this plasmid could readily be detected after 5 hrs infection, from both intracellular and extracellular bacteria. Tomato expression could also be seen by microscopy in single copy suggesting this expression system provides very high, constitutive induction of fluorescence in multiple environments. Together we have demonstrated the use of

diaminopimelic acid as a flexible selection system for *in vitro* and *in vivo* studies of *Yersinia pestis.*

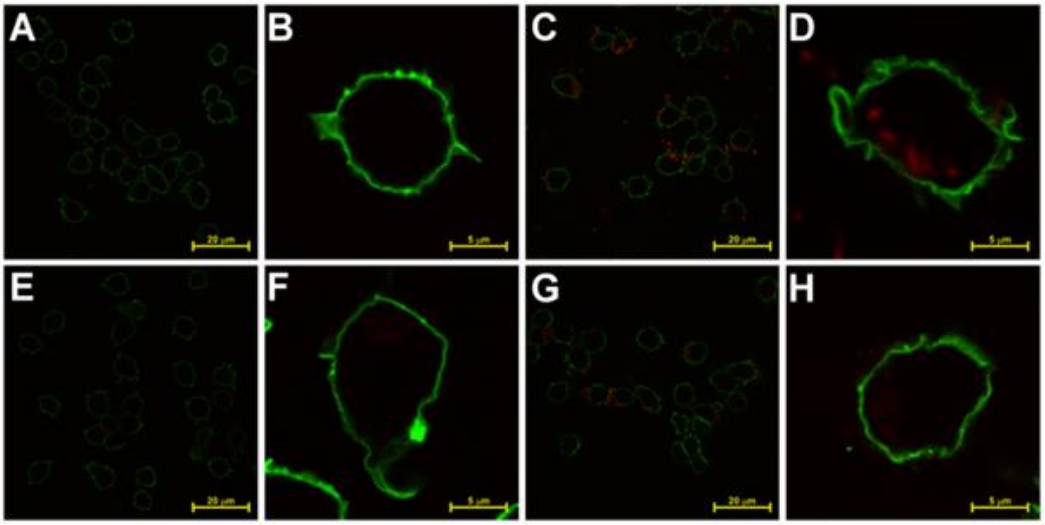

Figure 2.8 Detection of intracellular and extracellular bacteria by microscopy of single copy expression of *P_{cys}Tomato.* (A–B) *Y. pestis* KIM D27, (C–D) pRsaI-2.1, (E–F) KIM D27-1013 (*dapAX att*Tn7*::dapAX P_{cys}DsRed*), or (G–H) KIM D27-1014 (*att*Tn7*::dapAX cys-Tomato*) were grown overnight in HIB at 26°C, diluted 1:15 in HIB and grown for 2 hours, and then used to infect biotinylated RAW 264.7 macrophage-like cells at an MOI of 10 for 30 minutes. Cells were then fixed and stained with streptavidin conjugated to AlexaFluor 488 to indentify the host cell membrane. Samples were analyzed by laser scanning confocal microscopy.

2.5 Discussion

Research on highly pathogenic organisms such as *Yersinia pestis* has inherent limitations because of precautions required of genetic engineering. In particular, selection of recombinant DNA, either for retention of exogenous plasmids or to identify recombination events must be restricted to avoid the creation of antibiotic resistant strains that could compromise human treatment options. In this work we sought to establish a system for recombinant DNA expression in the highly pathogenic bacterium *Yersinia*

pestis based on metabolic rather than antibiotic selection. Our system targets the biosynthesis of the cell wall, similar to commonly used antibiotics that are effective against *Y. pestis*. Introduction of a null mutation in the *dapAX* operon caused growth dependence on diaminopimelic acid (DAP) for assembly of a functional cell wall. The resulting strain was highly attenuated for virulence in mouse models, and predictably will be in all mammalian species as well as fleas, none of which harbor pools of DAP. Unfortunately, the DAP selection system requires working in a mutant strain background which precludes its use on pre-existing strains. However, the benefits of switching to this approach are not limited to the ability to conduct experiments in a safer genetic background. Antibiotic selection in the mammalian or vector host is at best cumbersome, with a requirement for daily or more administration of drug, which may impact the outcome of infection. This introduces experimental risk, including safety concerns, reproducibility of dosing and other, perhaps unpredicted effects on the bacterium or host causing inherent variability and complications with interpretation. Thus, metabolic selection is superior to the introduction of antibiotic resistance for experimental models of infectious diseases.

The DAP system permits *in vivo* selection of plasmids, enabling the faithful study of gene expression by multi-copy plasmids, which has previously not been achieved for *Y. pestis*. To facilitate these studies, we have generated *dapAX* mutant strains in multiple *Y. pestis* backgrounds for use in all *in vivo* model systems, including both mammals and fleas (Table S1). In addition, we found that both genes in the *dapAX* operon contributed to virulence of *Y. pestis* in mouse models of bubonic and septicemic plague, thereby reducing the potential for spontaneous reversion of virulence. Though we have not yet

observed reversion of *dapA* mutant *Y. pestis* to DAP independent growth, it appears that *Yersinia* can acquire DAP from dead or dying bacteria when large populations are present (data not shown).

We reported the identification of a strong, likely constitutively active *Y. pestis* promoter, with similar activity in *E. coli*, that can drive detectable expression of a fluorescent reporter protein in laboratory media or during macrophage infection. CysZ is a conserved, non-essential gene that encodes an inner membrane protein involved in sulfate transport (33, 34). It is not surprising that sulfate transporter proteins would be highly abundant as this is a key nutrient for cells during all phases of growth. Other metabolite transporter genes have been used in expression vector systems, such as the *lac* operon. Though we and others have employed *lac* promoter constructs for high level expression of recombinant protein in *Y. pestis*, these promoter systems have not been strong enough for single copy use (Eisele, Keleher and Anderson, unpublished observations). Our screen identified optimized production of DsRed under conditions that minimized an impact to bacterial growth. Moreover, because *cysZ* is conserved in other Gram negative bacteria, it is likely that this technology may be broadly useful for pathogenesis research.

2.6 Acknowledgments

In memory of Malcolm Casadaban, to whom we are grateful for the ideas and helpful discussions that led to the development of this program. We are grateful to Dr. Craig Lewis for help with statistical analyses of competition experiments. We also wish to thank Drs. Herb Schweizer and Greg Phillips for providing the mini-Tn7 plasmids; Dr. Joe Hinnebusch for pCVD442; Kristen Peters for assistance with the intravenous model;

Dr. Hanni Lee-Lewis for help with the pathological analysis of disease and members of our laboratory for assistance with the BSL3 experiments. Histology services were provided by the University of Missouri Research Animal Diagnostic Laboratory (RADIL).

2.7 References

1. **Pollitzer R.** 1954. Plague. World Health Organization, Geneva, Switzerland.

2. **Chain P, Carniel E, Larimer F, Lamerdin J, Stoutland P, Regala W, Georgescu A, Vergez L, Land M, Motin V, Brubaker R, Fowler J, Hinnebusch B, Marceau M, Medigue C, Simonet M, Chenal-Francisque V, Souza B, Dacheux D, Elliott J, Derbise A, Hauser L, Garcia E.** 2004. Insights into the evolution of *Yersinia pestis* through whole-genome comparison with *Yersinia pseudotuberculosis*. Proc Natl Acad Sci **101:**13826-13831.

3. **Lorange E, Race B, Sebbane F, Hinnebusch B.** 2005. Poor vector competence of fleas and the evolution of hypervirulence in *Yersinia pestis*. J Infect Dis **191:**1907-1912.

4. **Achtman M, Morelli G, Zhu P, Wirth T, Diehl I, Kusecek B, Vogler A, Wagner D, Allender C, Easterday W, Chenal-Francisque V, Worsham P, Thomson N, Parkhill J, Lindler L, Carniel E, Keim P.** 2004. Microevolution and history of the plague bacillus, *Yersinia pestis*. Proc Natl Acad Sci **101:**17837-17842.

5. **Auerbach R, Tuanyok A, Probert W, Kenefic L, Vogler A, Bruce D, Munk C, Brettin T, Eppinger M, Ravel J, Wagner D, Keim P.** 2007. *Yersinia pestis* evolution on a small timescale: comparison of whole genome sequences from North America. PLoS One **2:**e770.

6. **Galimand M, Guiyoule A, Gerbaud G, Rasoamanana B, Chanteau S, Carniel E, Courvalin P.** 1997. Multidrug resistance in *Yersinia pestis* mediated by a transferable plasmid. New Eng J Med **337:**677-680.

7. **Guiyoule A, Gerbaud G, Buchrieser C, Galimand M, Rahalison L, Chanteau S, Courvalin P, Carniel E.** 2001. Transferable plasmid-mediated resistance to streptomycin in a clinical isolate of *Yersinia pestis*. Emerg Inf Dis **7:**43-48.

8. **Welch T, Fricke W, McDermott P, White D, Rosso M, Rasko D, Mammel M, Eppinger M, Rosovitz M, Wagner D, Rahalison L, LeClerc J, Hinshaw J, Lindler L, Cebula T, Carniel E, Ravel J.** 2007. Multiple antimicrobial resistance in plague: An emerging public health risk. PLoS One **2:**1-6.

9. **Hutton C, Southwood T, Turner J.** 2003. Inhibitors of lysine biosynthesis as antibacterial agents. Mini Rev Med Chem **3:**115-127.

10. **Curtiss Rr, Nakayama K, Kelly S.** 1989. Recombinant avirulent *Salmonella* vaccine strains with stable maintenance and high level expression of cloned genes *in vivo*. Immumol Invest **18:**583-596.

11. **Galan J, Nakayama K, 3rd CR.** 1990. Cloning and characterization of the *asd* gene of *Salmonella typhimurium*: use in stable maintenance of recombinant plasmids in *Salmonella* vaccine strains. Gene **94:**29-35.

12. **Bukhari A, Taylor A.** 1971. Genetic analysis of diaminopimelic acid- and lysine-tequiring mutants of *Escherichia coli*. J Bacteriol **105:**844-854.

13. **Rediers H, Bonnecarre`re V, Rainey P, Hamonts H, Vanderleyden J, De Mot R.** 2003. Development and Application of a *dapB*-Based *In Vivo* Expression Technology System To Study Colonization of Rice by the Endophytic Nitrogen-Fixing Bacterium *Pseudomonas stutzeri* A15. App Env Microbiol **69:**6864-6874.

14. **Norris M, Kang Y, Lu D, Wilcox B, Hoang T.** 2009. Glyphosate resistance as a novel select-agent-compliant, non-antibiotic-selectable marker in chromosomal, mutagenesis of the essential genes *asd* and *dapB* of *Burkholderia pseudomallei*. App Env Microbiol **75:**6062-6075.

15. **Parkhill J, Wren B, Thomson N, Titball R, Holden M, Prentice M, Sebaihia M, James K, Churcher C, Mungall K, Baker S, Basham D, Bentley S, Brooks K, Cerdeño-Tárraga A, Chillingworth T, Cronin A, Davies R, Davis P, Dougan G, Feltwell T, Hamlin N, Holroyd S, Jagels K, Karlyshev A, Leather S, Moule S, Oyston P, Quail M, Rutherford K, Simmonds M, Skelton J, Stevens K, Whitehead S, Barrell B.** 2001. Genome sequence of *Yersinia pestis*, the causative agent of plague. Nature **413:**523-527.

16. **Bouvier J, Pugsley A, Stragier P.** 1991. A gene for a new lipoprotein in the *dapA-purC* interval of the *Escherichia coli* chromosome. J Bacteriol **173:**5523-5531.

17. **Surgalla M, Beesley E.** 1969. Congo red-agar plating medium for detecting pigmentation in *Pasteurella pestis*. App Microbiol **18:**834-837.

18. **Brubaker R, Beesley E, Surgalla M.** 1965. *Pasteurella pestis*: Role of Pesticin I and iron in experimental plague. Science **149:**422-424.

19. **Hanahan D.** 1985. DNA Cloning: A practical approach., p. 109. *In* Glover D (ed.), vol. 1. IRL Press, McLean, VA.

20. **Yanisch-Perron C, Vieira J, Messing J.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103-119.

21. **Simon R, Priefer U, Pu¨hler A.** 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in gram negative bacteria. Biotechnology **1**.

22. **Donnenberg M, Kaper J.** 1991. Construction of an *eae* deletion mutant of Enteropathogenic *Escherichia coli* by using a positive selection suicide vector. Infect Immun **59:**4310-4317.

23. **Choi K, Gaynor J, White K, Lopez C, Bosio C, Karkhoff-Schweizer R, Schweizer H.** 2005. A Tn7-based broad-range bacterial cloning and expression system. Nat methods **2:**443-448.

24. **Richaud F, Richaud C, Ratet P, Patte J.** 1986. Chromosomal location and nucleotide sequence of the *Escherichia coli dapA* gene. J Bacteriol **166:**297-300.

25. **DeBord K, Anderson D, Marketon M, Overheim K, DePaolo R, Ciletti N, Jabri B, Schneewind O.** 2006. Immunogenicity and protective immunity against bubonic plague and pneumonic plague by immunization of mice with the recombinant V10 antigen, a variant of LcrV. Infect Immun **74:**4910-4914.

26. **Overheim K, DePaolo R, KeBord K, Morrin E, Anderson D, Green N, Brubaker R, Jabri B, Schneewind O.** 2005. LcrV plague vaccine with altered immunomodulatory properties. Infect Immun **73:**5152-5159.

27. **Logsdon L, Mecsas J.** 2003. Requirement of the *Yersinia pseudotuberculosis* Effectors YopH and YopE in Colonization and Persistence in Intestinal and Lymph Tissues. Infect Immun **71:**4595-4607.

28. **Eisele N, Anderson D.** 2009. Dual-function antibodies to *Yersinia pestis* LcrV required for pulmonary clearance of plague. Clin Vacc Immunol **16:**1720-1727.

29. **Team RDC** 2008, posting date. R: A language and environment for statistical computing. [Online.]

30. **Peters J, Craig N.** 2001. Tn7: smarter than we thought. Nat Rev Mol Cell Biol **2:**806-814.

31. **Anderson G, Leary S, Williamson E, Titball R, Welkos S, Worsham P, Friedlander A.** 1996. Recombinant V antigen protects mice against pneumonic and bubonic plague caused by F1-capsule positive and negative strains of *Yersinia pestis*. Infect Immun **64:**4580-4585.

32. **Lee-Lewis H, Anderson D.** 2010. Absence of inflammation and pneumonia during infection with non-pigmented *Yersinia pestis* reveals new role for the pgm locus in pathogenesis. Infect Immun **78:**220-230.

33. **Parra F, Britton P, Castle C, Jones-Mortimer M, Kornberg H.** 1983. Two Separate Genes Involved in Sulphate Transport in *Escherichia coli* K12. J Gen Microbiol **129:**357-358.

34. **Rückert C, Koch D, Rey D, Albersmeier A, Mormann S, Pühler A, Kalinowski J.** 2005. Functional genomics and expression analysis of the *Corynebacterium glutamicum fpr2-cysIXHDNYZ* gene cluster involved in assimilatory sulphate reduction. BMC Genomics **6:**121-138.

Chapter 3

CONSERVED OMP ACCESSORY FACTOR BAMC IS REQUIRED FOR EXTRACYTOPLASMIC STRESS RESPONSES DURING *YERSINIA PESTIS* INFECTION OF INSECT AND MAMMALIAN HOSTS

Contributions: David Bland and Deborah Anderson conceived and designed experiments. Bacterial mutants and genetic tools generated by David Bland. Experiments and Analysis performed by David Bland and Deborah Anderson. Manuscript was written by David Bland and Deborah Anderson.

3.1 Abstract

The β-barrel assembly machinery (BAM) complex of Gram-negative bacteria is composed of five subunits (BamA-E) that facilitate the insertion of β-barrel proteins into the outer membrane. BamA and BamD are highly conserved and essential for growth, while BamC is the most divergent member of the complex, with similarity amongst pathogenic Enterobacteriaceae. *Yersinia pestis* is one of the most pathogenic members of the Enterobacteriaceae family and the causative agent of plague, a flea-borne disease. In this work, we demonstrate that BamC is a virulence factor for *Yersinia pestis* that is required to cause systemic disease in murine plague models and is also involved in the colonization of fleas. As in other Gram-negative bacteria, loss of *Y. pestis bamC* causes little to no change in outer membrane protein (OMP) assembly during normal growth and a small increase in sensitivity to polymixin B. In contrast, under multiple conditions of extracytoplasmic stress, the survival of the *bamC* mutant was significantly reduced compared to the parent *Y. pestis* strain. Furthermore, the induction of the alternative

transcription factor σ^E in response to extracytoplasmic stress required BamC, and this led to increased surface expression of multiple OMPs. The *bamC* mutant was less virulent in mouse models of bubonic and septicemic plague and had reduced ability to grow in the midgut after maintenance blood feeding of the North American flea vector, *Oropsylla montana*. Together, the data support a role for BamC in promoting growth in adverse environments, where its activity is necessary for proper function of a subset of OMPs.

3.2 Introduction

Outer membrane proteins (OMPs), including porins and secreted autotransporters, are known to be important for bacterial metabolism and pathogenicity (1-3). These proteins adopt a β-barrel structure in the outer membrane where they mediate diverse processes such as adhesion, secretion, proteolysis or resistance to environmental stresses (4-8). The mechanism by which β-barrel proteins are folded in the periplasm and subsequently inserted into the bacterial outer membrane (OM) remains incompletely understood (9). However, it is well established that the outer membrane β-barrel assembly machinery (BAM) complex is required for OMP insertion (10, 11).

The BAM complex is composed of five proteins that have been co-purified from *E. coli*, BamA-E, of which BamA and BamD are essential for viability (12-15). BamA is a β-barrel protein located in the outer membrane where it functions to assemble other β-barrel proteins. BamB, C, D and E are outer membrane lipoproteins. BamB binds BamA directly and BamC, D and E form a complex in the periplasm that binds to BamA (16). All five proteins were required to reconstitute insertion of OmpT in proteoliposomes *in vitro (17)*. While BamD is absolutely required for OMP assembly *in vivo*, deletion of *bamB* or *bamE* causes a moderate reduction of OMPs during growth in laboratory media, as well as enhanced sensitivity to many classes of antibiotics (18, 19). BamC co-purifies with the BAM complex in a BamD-dependent manner, suggesting BamC may be an accessory factor for BamD (20). In contrast to *bamB*, deletion of *bamC* causes little to no decrease in OMPs and its function in the BAM complex of *E. coli* is not well understood (10). In *S. marcescens*, BamC regulates swarming motility, suggesting its function may be required under specific circumstances (21). Consistent with this hypothesis, BamC is

highly conserved amongst pathogenic *Enterobacteriaceae*, indicative of selective pressure for retention of *bamC* (22).

Yersinia pestis is a non-motile highly pathogenic member of the Enterobacteriaceae family and is the causative agent of the plague (23). Bubonic plague is a vector-borne infection due to the ability of *Y. pestis* to colonize the digestive tract of fleas and promote its transmission to mammalian hosts. Infection of fleas with *Y. pestis* occurs when they take a bloodmeal from a rodent or other mammal with terminal septicemic plague. The majority of the ingested bacteria are rapidly lost through defecation, and bacteria that remain can colonize the flea midgut where they can form an infectious biofilm that becomes lethal to the fleas over time (24). Fleas that carry *Y. pestis* can transmit the infection to mammals through two mechanisms. In early phase transmission, defined as less than 1 week post-infection, fleas can transmit bacteria at high frequency, through a currently unknown mechanism, during a subsequent bloodmeal (25). After about 7 days, a stable biofilm of *Y. pestis* can form in the midgut. When the biofilm lodges on the proventriculus of the flea, it occludes ingestion of blood and because of this blockage, bacteria are regurgitated into the bite site (26). Once inoculated in the skin or capillaries, *Y. pestis* migrates to the draining lymph node where it grows to high titer and spreads systemically. The elevated temperature of the mammalian host activates the expression of a number of surface-located β-barrel proteins that are required for immune evasion and bacterial growth in the blood (27-29). High titer bacteremia develops in the final stage of bubonic plague and is required for transmission to fleas (30).

Here we show that BamC is a virulence factor important to the vector and mammalian life cycles of *Y. pestis.* We found that BamC is required for bacterial growth in adverse environments during multiple stages of the *Y. pestis* life cycle. Furthermore, we show that induction of the extracytoplasmic stress factor σ^E is impaired during incubation in low pH providing a possible mechanism for attenuation of growth in macrophages and in the flea midgut. These findings suggest an explanation for selective pressure for retention of BamC in bacteria that colonize insect and/or mammalian hosts.

3.3 Materials and Methods

Bacterial strains and plasmids. All strains used were grown on heart infusion agar (HIA) plates and single colonies were used to inoculate heart infusion broth (HIB). The plates used for *Y. pestis* CO92 were supplemented with 0.005% Congo Red and 0.2% galactose to select for colonies that retain the pigmentation locus (*pgm*) (31). All handling of samples containing non-exempt strains of *Y. pestis* CO92 was performed in a select agent authorized BSL3 facility under protocols approved by the University of Missouri Institutional Biosafety Committee. *Y. pestis* KIM and CO92 strains were routinely grown fresh from frozen stock on HIA, followed by growth at 26ºC with aeration at 150rpm in heart infusion broth (HIB) overnight prior to use in experiments. Where indicated, ampicillin (50μg/ml) was added to media for selection of plasmids.

Y. pestis strains and recombinant plasmids used in this study are described in Table 4.1; *E. coli* strains are described in Supplemental Table 1. Restriction enzymes were purchased from New England Biolabs (Ipswich, MA). Recombinant DNA cloned

from *Y. pestis* was PCR amplified from CO92 pCD1$^-$ genomic DNA; primer sequences are listed in Supplemental Table 2.

Table 3.1 Bacterial strains and plasmids used in this study.

***Y. pestis* Strains/Plasmids**	**Key Properties**	**Reference**
Strains:		
CO92	*Pgm*$^+$ Lcr$^+$	(32)
CO92 pCD1$^-$	*Pgm*$^+$ Lcr$^-$	This Study
CO92*dapA-bamC*	CO92; Missing *dapA* promoter and ORF	(33)
CO92 *bamC1*	CO92*dapA-bamCattTn7::dapA*; *dapA* transposition into the *attTn7* site of the CO92ΔdapA-bamC parental strain	(33)
CO92 *bamC1 attTn7::dapA-bamC*	CO92*dapA-bamCattTn7::dapA-bamC*; *dapA-bamC* transposition into the *attTn7* site of the CO92ΔdapA-bamC parental strain	(33)
CO92*bamC2* pCD1$^-$	CO92; Missing the *bamC* ORF, pCD1$^-$	This Study
KIM D27	*Pgm*$^-$ Lcr$^+$; KIM5- derivative	(34)
KIMD27*bamC2*	KIM D27; Missing the *bamC* ORF	This Study
KIM6+	*Pgm*$^+$ Lcr$^-$	(35)
KIM6+ *bamC2*	KIM6+; Missing the *bamC* ORF	This Study
Plasmids:		
p*bamC*	pBR322, Ap^r, Constitutive expression of *bamC-FLAG* from the *lac* promoter	This Study
p*bamD*	pBR322, Ap^r, Constitutive expression of *bamD* from the *lac* promoter	This Study
p*rpoE*	pBR322, Ap^r, Expresses Tomato protein from the SigmaE (*rpoE*) promoter region (155bp upstream of the *rpoE* start codon)	This Study
p*dapA-bamC*	pBR322, Ap^r, Expresses Tomato protein from the *dapA-bamC* promoter region (162bp upstream of the *bamC* start codon)	This Study
pNull	pBR322, Ap^r, Tomato ORF without a promoter	This Study

Murine plague models. This study was carried out in strict accordance with the Animal Welfare Act and the NIH Guide for the Care and Use of Laboratory Animals. All animal procedures were approved by the Animal Care and Use Committee of the University of Missouri. Male and female BALB/c mice, bred from a colony maintained on campus, were originally obtained from Charles River Laboratories (Wilmington, MA). During bubonic plague challenge, mice were maintained in BSL-3 housing at the Laboratory for Infectious Disease Research at the University of Missouri. All infected mice were monitored daily for clinical signs of disease, including hunched posture, ruffled fur, progressively decreasing activity, and seizures, for 14 days post-challenge. Animals that exhibited pronounced neurologic signs and severe weakness were considered moribund and were humanely euthanized. Euthanasia was performed by CO_2 asphyxiation followed by cervical dislocation or bilateral pneumothorax, methods that are approved by the American Veterinary Medical Association Guidelines on Euthanasia.

For subcutaneous and intravenous plague challenge, isolated, single colonies were used to inoculate HIB and grown 18-24 hours at 26°C, 120 rpm. Bacteria were diluted in sterile PBS to the desired dose just prior to challenge; dose was verified by plating on HIA. Bacteria were recovered by mechanical disruption of indicated tissues followed by ten-fold serial dilutions in sterile PBS and plated on HIA with antibiotic selection where appropriate. The lethal dose (LD_{50}) was calculated according to the method of Reed and Muench following challenge with 3-4 doses, serially diluted 10-fold (36). Three independent trials for LD_{50} were performed.

Sedimentation Assay. Analysis of bacterial sedimentation was performed as described previously (37). Bacteria were grown overnight to stationary phase (OD_{600} of ~1.0),

vortexed, and placed in 15mL conical tubes. Cultures were maintained upright with minimal disturbance and optical density was recorded from 1mL of media removed from the top of the conical tube at the indicated time points. Measurements were compared to the initial OD_{600} to determine the percentage sedimentation.

Flea infections. *Oropsylla montana* fleas were maintained in a refrigerated incubator at 22°C with ~80% relative humidity (38). Fleas were reared in one gallon glass jars containing larval medium (3 parts saw dust to 1 part of an equal mixture of blood meal, dried milk, and mouse pellets). For infection, fleas were starved for 6 days and subsequently allowed to feed on 3-4mL of heparinized murine blood containing 10^8-10^9 CFU of *Y. pestis*. Following infection, fleas were anesthetized using a chill table set to 0°C and monitored under a dissection scope for signs of fresh blood in the esophagus or proventriculus. Fleas that did not acquire a *Y. pestis*-infected blood meal were removed from the study. Mechanical disruption of fleas for bacterial enumeration was performed using a bead beater and homogenates were plated on *Yersinia* Selective Agar (YSA) (Becton Dickinson Diagnostic Systems, Franklin Lakes, NJ).

Macrophage Assays. RAW 264.7 murine macrophages were maintained in Dulbecco's modified eagle medium (DMEM) with 10% FBS and 50μg/ml ciprofloxacin, 37°C, 5% CO_2. Prior to infection, macrophages were resuspended in 2ml of DMEM supplemented with 5% FBS. In the gentamicin protection assay, $5x10^6$ macrophages were seeded in a 12-well plate and challenged at an MOI of 10 with different *Y. pestis* $pCD1^-$ strains suspended in PBS. Plates were spun at 41xg for 5 minutes to promote cell contact, and incubated for 45 minutes at 37°C. After incubation, 50μg/ml of gentamicin was added to

each well to kill extracellular bacteria. Intracellular bacteria were recovered by lysing macrophages with 0.05% Triton X-100.

The capase-3 assay was performed using the Enzchek Caspase-3 assay kit #2 Z-DEVD-R110 substrate according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Briefly, $5x10^6$ macrophages were infected for 3 hours at an MOI of 5 with *Y. pestis* $pCD1^+$ strains. Cells were harvested, lysed and incubated with DEVD-R110 substrate. Percent caspase-3 activation was normalized to the KIM D27 parental strain.

Disc sensitivity Assay. Disc sensitivity assays were performed according to manufacturer's instructions (Becton Dickinson Diagnostic Systems). *Y. pestis* strains were grown at 37°C in duplicate HIA plates with 4 discs per plate. Antibiotic disc concentrations were as follows: 30µg nalidixic acid, 300 units polymyxin B, and 5µg rifampin.

Acidic Stress Assay. The acidic sensitivity assay was performed as previously described, viable bacteria were enumerated by plating on HIA (39). All samples were run in triplicate; percent recovery was determined relative to the amount recovered from PBS-treated control wells.

Fluorescence Reporter Assays. *Y. pestis* $pCD1^-$ strains carrying tdTomato reporter constructs were grown overnight and diluted to an OD_{600} of 0.05 in 4mL of HIB. Strains were grown for 24 hours at 37°C; media was supplemented with 150mM NaCl where indicated. Acidic growth conditions were achieved by titrating HIB to pH 6 and buffering media with 50mM 1,4- piperazine diethane sulfonic acid sodium salt (PIPES).

Relative fluorescence units (RFU) and OD_{600} were measured in duplicate using a plate reader. RFU was normalized to OD_{600}; fold-induction was determined by dividing the optical density normalized RFU value by the same value generated by the Null reporter construct ((RFU/OD_{600} of reporter construct)/(RFU/OD_{600} of null construct)).

Bacterial OMP preparation and Analysis. *Y. pestis* pCD1$^-$ strains were grown overnight and diluted to an OD_{600} of 0.1 in 20mL of HIB. Bacteria were grown for 6 hours to an OD_{600} of 0.6, centrifuged at 6120xg, resuspended in 10mL of protein buffer (100µM $MgCl_2$, 10µM HEPES, 2µM KCl) supplemented with 1 tablet of cOmplete Mini Protease Inhibitor Cocktail (Roche Diagnostics, Mannheim, Germany). Bacteria were lysed by two passages through a French press at 10,000 psi. Bacterial lysates were subjected to a low speed centrifugation (6120xg) for 10 minutes. Membrane proteins were enriched by ultracentrifugation for 30 minutes at 150,000xg (40, 41). Prior to loading on 12% SDS-PAGE gels, the Bicinchoninic Acid (BCA) protein assay was performed according to the manufacturer's instructions (Pierce Biotechnology, Rockford, IL). For mass determination, proteins were excised from the acrylamide gel and digested with trypsin. Samples were analyzed by Q-TOF LC MS/MS. Raw mass spectra were processed using the Agilent Qualitative Analysis software allowing extraction of MS and MS/MS data as a mascot generic format (MGF) file. The MGF files were uploaded to the MASCOT server and a search was conducted among Eubacteria sequence data.

Statistical Analysis. Data from all trials were analyzed for statistical significance using the tests indicated in the figure legends. All analyses were performed using the SigmaPlot v12.0 software (Systat Software Inc., Chicago, IL). Significance was defined as $p<0.05$.

3.4 Results

3.4.1 BamC is required for bacterial growth in the lymph node and dissemination during bubonic plague. BamC is encoded by the second gene in an operon (*dapA-bamC*) in many Gram- negative bacteria, including *Y. pestis*. We recently reported that BamC expression was required to fully restore virulence to *Y. pestis dapAbamC* mutants (33). To better understand the mechanism underlying this BamC-related attenuation, we characterized the virulence of *Y. pestis* CO92 *dapAbamC attTn7:dapA* (*bamC1*) strain in a murine bubonic plague model. BALB/c mice were challenged with 100 CFU (100x LD_{50} wild type) of the indicated strains of *Y. pestis* CO92 by subcutaneous injection and monitored for survival over 14 days. Loss of *bamC* caused attenuation of virulence and only 10% lethality was observed, compared to 100% lethality caused by wild type or BamC-complemented *Y. pestis* at this dose (Figure 3.1A). We constructed a second *bamC* mutant in the CO92 background where the *bamC* open reading frame was deleted by homologous recombination using pCVD442 (42). The resulting strain, *bamC2,* appeared to have a similar virulence defect when used to infect mice by subcutaneous injection (data not shown).

We determined the 50% lethal dose (LD_{50}) of *Y. pestis* CO92*bamC1* in the bubonic plague model by infecting groups of 10 mice with increasing challenge doses and monitoring for survival over 14 days. From these experiments, the LD_{50} of the *bamC1* mutant in BALB/c mice was calculated to be 469± 53 CFU, over 400-fold less virulent than the WT strain. Histopathology of lymph node and spleen recovered from moribund mice infected with WT or the *bamC1* mutant *Y. pestis* were similar and showed lesions that are characteristic of plague (Figure 3.7A-F). Bacterial titers in the draining

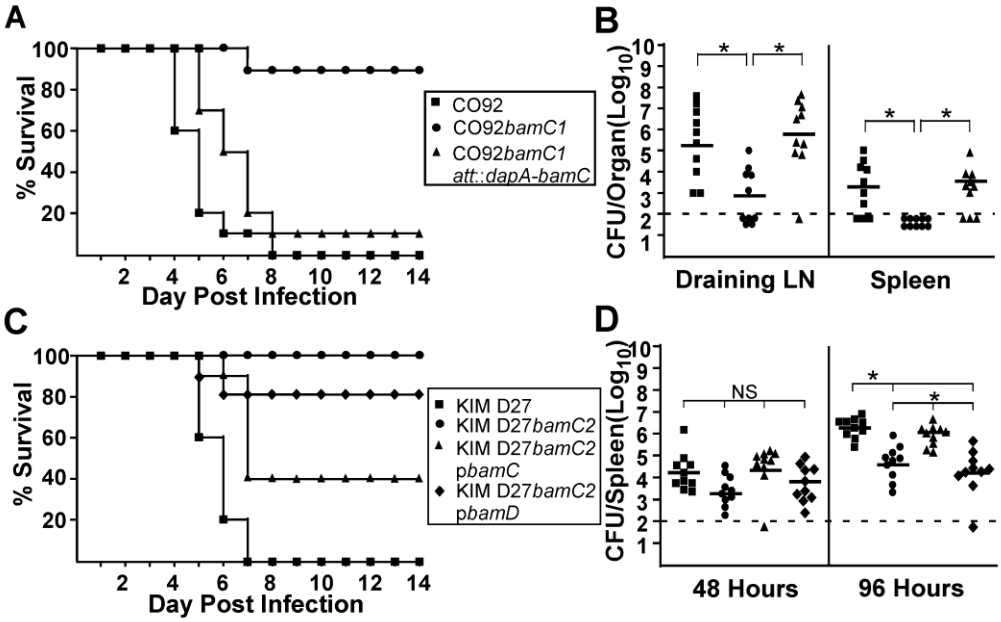

Figure 3.1 ***BamC is needed for virulence and bacterial dissemination.*** (A-B) BALB/c mice were challenged by subcutaneous injection with 100 CFU of the indicated strains of *Y. pestis*. (A) Survival was monitored over 14 days. Data shown were pooled from two independent experiments, n=10; statistical significance was analyzed by the Log Rank test followed by pairwise comparisons using the Holm-Sidak multiple comparisons test; *p<0.05. (B) Bacteria were enumerated from the draining lymph node and spleen at 48 hours post-infection. Data were pooled from 2 independent experiments, n=10; bars indicate median; statistical significance was evaluated by one-way ANOVA followed by Tukey's post test; *P<0.05. (C-D) BALB/c mice were challenged by intravenous injection with 1500 CFU of the indicated strains of *Y. pestis*. (C) Survival was monitored over 14 days. Data shown were collected in 2 independent experiments, n=10; statistical significance was analyzed by the Log Rank test followed by pairwise comparisons by the Holm-sidak multiple comparisons test; *p<0.05. (D) Bacteria were enumerated from the spleens at 48 and 96 hours post-infection. Data show the geometric mean and were pooled from 2 independent experiments, n=10; bars indicate median. Statistical significance was evaluated by one-way ANOVA followed by Tukey's post test; *P<0.05.

lymph node and the spleen were significantly reduced by 48 hours post-infection in the absence of BamC (Figure 3.1B). Complementation of BamC in single copy was sufficient to restore bacterial titers to those caused by infection with wild type bacteria. Together the data demonstrate a requirement for BamC in the early stages of bubonic infection, perhaps for growth or survival in the draining lymph node.

We also examined virulence of *Y. pestis* CO92*bamC1* in a mouse pneumonic plague model. BALB/c mice challenged by intranasal infection of the *bamC1* mutant were delayed in succumbing to disease, with a modest reduction of lethality compared to infection by the wild type strain (Figure 3.8). Histological analysis of lungs from

moribund mice indicated both WT and *bamC1* infected mice developed primary pneumonic plague (Figure 3.7). These results indicate that BamC is less important when bacteria are growing in the lung environment.

3.4.2 Over-expression of BamD does not restore virulence of Y. pestis bamC Previously, BamC was shown to interact with BamD in *E. coli*, suggesting BamC may be an accessory protein for this essential component of the BAM complex (9). We therefore sought to determine if over-expression of BamD could suppress the virulence defect of the *Y. pestis bamC2* mutant. *Y. pestis* KIM D27 *bamC2* is a deletion of the *bamC* open reading frame in the non-pigmented KIM D27 strain that was constructed by homologous recombination using the suicide vector pCVD442. While the KIM D27 strain is virulent by the intravenous route (LD_{50}=50-100 CFU), the *bamC2* mutant was attenuated in the septicemic plague model with growth defects in the liver and spleen (LD_{50}= 36,667 ± 3936, Figure 3.1C-D). The virulence defects of *Y. pestis* lacking *bamC* are therefore similar in the bubonic and septicemic plague models. We sought to determine if over-expression of BamD could suppress the *bamC2* virulence defect. When we expressed BamD from the *lac* promoter on a multi-copy plasmid in *Y. pestis* KIM D27 *bamC2*, the resulting strain appeared no more virulent in the septicemic plague model.

3.4.3 BamC is important for efficient colonization of fleas. We next studied the role of BamC in the flea life cycle of *Y. pestis*. Little is known about the vector-pathogen interactions in the midgut of *Oropsylla montana*, an important plague vector in North America, or other flea species due to the absence of a sequenced flea genome. We analyzed the life cycle of WT and *bamC2 Y. pestis* CO92pCD1$^-$ in *O. montana* following infection with $1x10^8$ CFU. Every 24 hours, groups of 6-10 fleas were individually

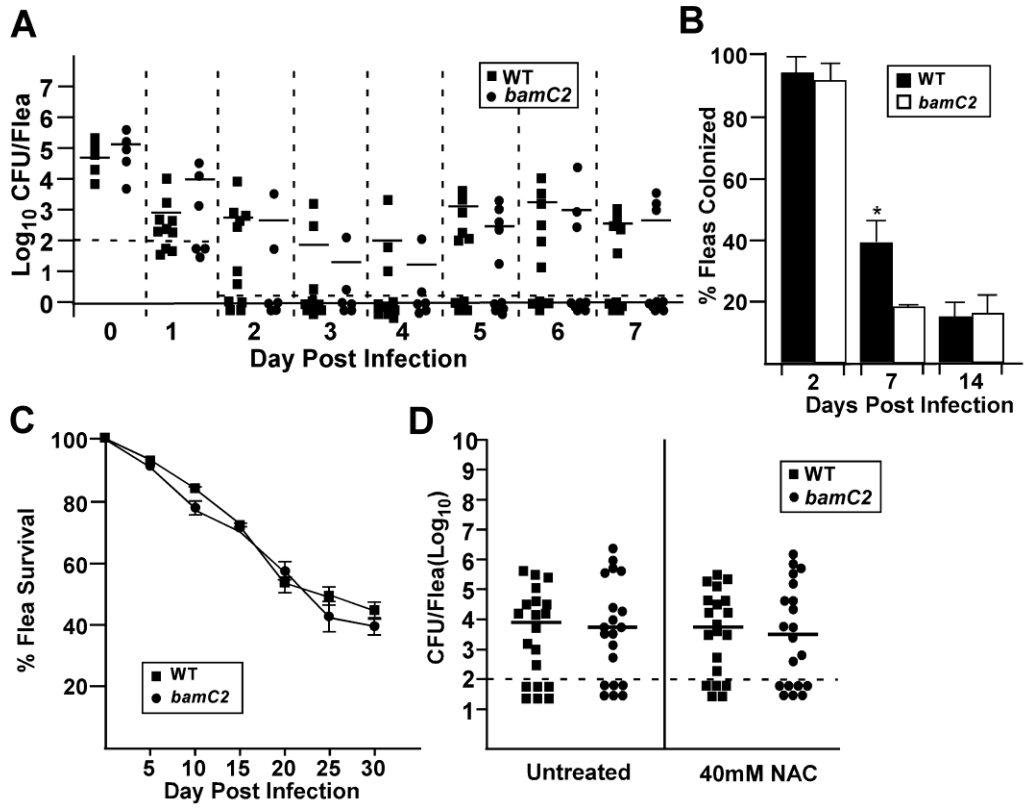

Figure 3.2 ***BamC is important for bacterial growth in fleas.*** (A) *Oropsylla montana* fleas were fed from a blood meal reservoir containing 10^8 CFU of the indicated strains. Groups of 6-10 fleas were individually homogenized every 24 hours and plated on *Yersinia* selective agar; solid lines indicate the average CFU recovered; dotted line indicates limit of detection. (B) Fleas were fed $1x10^8$ CFU from a bloodmeal reservoir of the indicated strains. On days 2, 7 and 14 post-infection, fleas were mechanically disrupted and homogenates were plated on *Yersinia* selective agar. Colonization was defined as >1 CFU recovered. Data shown were combined from two independent experiments (n=number of fleas in each group); error bars depict the standard deviation of the mean; data were evaluated using Student's *t*-test, *P<0.05. (C) *Y. pestis*-infected *O. montana* fleas were monitored for survival every 5 days; n indicates the number of fleas in each group. (D) Fleas were fed from a blood meal reservoir containing 10^9 CFU/ml of the indicated strains. For the treatment groups, the blood reservoir also contained 40mM n-Acetyl Cysteine (NAC). On day 2 post-infection, fleas were mechanically disrupted and homogenates were enumerated for bacterial burden on *Yersinia* selective agar; data were evaluated using Student's *t*-test; *P <0.05, ns=not significant.

homogenized and plated on *Yersinia* selective agar (43). Both strains initially infected at indistinguishable rates, and the mean CFU recovered from the flea midgut declined over days 1-4 but appeared similar between WT and *bamC* infections (Figure 3.2A). On day 5, fleas were given a maintenance bloodmeal and after that, both the WT and *bamC2* mutant appeared to grow due to the availability of nutrients, with increased median titers on days 6 and 7 compared to day 4. However, *Y. pestis bamC2* appeared to grow more

slowly and was more often cleared in fleas recovered after the bloodmeal. On day 7, the median CFU recovered from WT and *bamC* infected fleas appeared to be dropping. We repeated this experiment using a higher challenge dose and similar results were observed (Data not shown).

We quantified bacterial survival in the midgut and measured the percentage of fleas in which bacteria could be detected on days 2, 7 and 14 post-infection. Fleas were given maintenance bloodmeals on days 5 and 10 post-infection. Similar to the data described above, no differences in bacterial survival were observed on day 2, and most fleas remained infected (Figure 3.2B). In contrast, on day 7 post-infection, significantly fewer fleas harbored viable *bamC* bacteria compared to the WT infection. On day 14, however the percentage of fleas infected by WT bacteria had dropped and became indistinguishable from the *bamC* mutant. Infection with WT or *bamC2* mutant *Y. pestis* caused similar lethality of the fleas over the 30 day observation period, with a gradual decline in viability over time (Figure 3.2C). These data suggested that the differences in bacterial growth observed in the *bamC* mutant were not caused by changes in susceptibility of the flea to lethality during the course of the experiment. Overall, it appeared there was a population of bacteria that depended on BamC for survival or growth in ingested blood in the flea midgut.

Reactive oxygen species (ROS) production is stimulated after intake of bloodmeals as an anti-microbial defense mechanism in fleas (44). To determine if the *bamC* mutant was more sensitive to ROS, we performed an *in vivo* reactive oxygen assay. n-Acetyl-cysteine (NAC) is an antioxidant previously demonstrated to reduce ROS responses in the flea midgut when ingested with the bloodmeal (44). We used NAC to

determine if reduction of ROS would enhance growth of WT or *bamC2* mutant *Y. pestis* in fleas. To test if BamC was important for resistance to ROS in the midgut, fleas were infected with either the parental or mutant *Y. pestis* strain in blood supplemented with 40mM NAC which was previously shown to be bacteriocidal. Bacterial titers were measured and compared on day 2, prior to the development of biofilm to avoid any impact it may have on resistance to ROS. Supplementation with NAC did not influence overall bacterial titers of WT *Y. pestis* suggesting that ROS production does not hamper WT *Y. pestis* survival in the midgut (Figure 3.2D). Similarly, *Y. pestis bamC2* were also not affected by NAC treatment. Together these data suggest that the *bamC* mutant may have a growth defect in the flea midgut rather than increased sensitivity to clearance.

3.4.4 BamC is required for OMP assembly during extracytoplasmic stress Overall, it appears that BamC contributes to growth in the mammalian and flea life cycles of *Y. pestis*, either as a component of the BAM complex or as a surface exposed virulence factor. If BamC was functioning in the BAM complex in *Y. pestis,* then it is likely that insertion of OMPs would be defective in the *bamC2* mutant. To test this hypothesis, we examined membrane preparations from WT and *bamC2 Y. pestis* KIM D27. Both strains grow similarly in heart infusion media at 26°C and 37°C, and similar CFUs were recovered of WT or *bamC2* mutant at a given OD_{600} (Figure 3.3A) (33). In contrast, addition of 150mM NaCl to the media resulted in a small, but significant reduction in growth of the *bamC2* mutant. We generated membrane-enriched samples from WT, bamC2 and complemented strains from mid-log phase cultures grown in HIB or HIB + 150mM NaCl. Samples were normalized to an equivalent OD_{600} prior to SDS-PAGE analysis. The *bamC2* mutant appeared to harbor equal or even increased amounts of

membrane proteins compared to wild type (Figure 3.3B). However, upon the addition of NaCl, a global reduction in total protein was incorporated in the outer membrane of the *bamC2* mutant which was restored by plasmid expression of *bamC*. We re-analyzed these samples by SDS-PAGE following normalization to the same total protein levels and identified some of the proteins by Q-Time of Flight (TOF) mass spectrometry. OmpC, OmpA and Pla appeared to be reduced in the *bamC2* mutant compared to WT or the complemented strain (Figure 3.3C). In contrast, GroEL, an inner

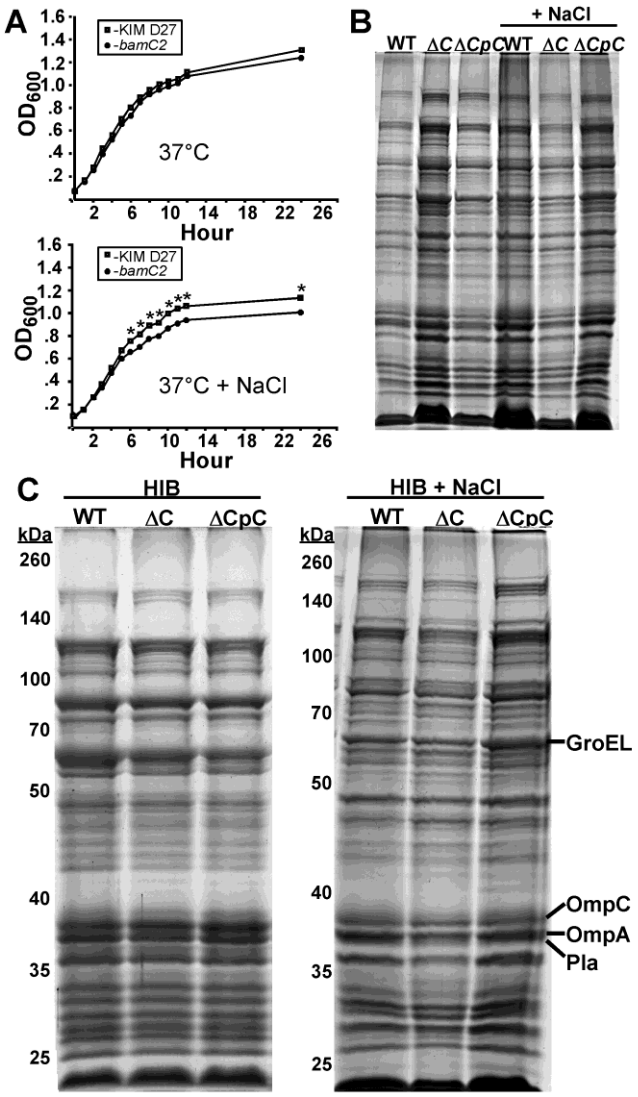

Figure 3.3 ***BamC is required for OMP assembly during extracytoplasmic stress.*** (A) Wild type or *bamC2* mutant *Y. pestis* KIM D27 cultures were grown in HIB with (top) or without (bottom) the addition of 150mM NaCl and growth was monitored for 24 hours at 37°C. Data shown was pooled from 3 independent experiments; statistical analysis of each time point was performed using the Student's *t*-test; *P < 0.05. (B-C) Coomassie stained SDS-PAGE of membrane proteins from bacteria grown with or without additional 150mM NaCl, protein samples were normalized to equivalent OD_{600} (B) or 50µg of total protein (C). Indicated proteins were identified by Q-TOF mass spectrometry. Data shown is a representative gel from 3 independent protein preparations.

membrane associated protein that was present in the sample, was not affected by increased salinity nor in the *bamC2* mutant suggesting there is no global effect on protein synthesis nor transport across the inner membrane in the *bamC2* mutant (45). Together the data suggest BamC may be involved in OMP assembly in high salinity environments.

We also compared the sensitivity of wild type and *bamC2* mutant *Y. pestis* strains to antibiotics. Rifampin and nalidixic acid, two antibiotics with cytoplasmic targets, were equally effective against $BamC^+$ and $BamC^-$ *Y. pestis* (Table 3.2). This suggests that the *bamC2* mutant does not have a general increase in membrane permeability. In contrast, polymixin B, an antibiotic that targets outer membrane lipopolysaccharide, was more effective against the *bamC2* mutant. Over-expression of BamC appeared to increase resistance to polymixin B. Polymixin B is a mixture of cationic lipopeptides that interact with LPS leading to increased membrane permeability (46). Increased sensitivity to polymixin B is therefore suggestive that LPS integrity may be disrupted in the *bamC2* mutant. LptD, which is essential for LPS assembly on the outer membrane, is a β-barrel protein that depends on the BAM complex for assembly (47, 48). Increased polymixin sensitivity may therefore be indicative that LptD depends on BamC for its assembly or function.

Table 3.2 Antimicrobial Disc Sensitivity Zone of Inhibition (mm)

Strain	Rifampin	Nalidixic Acid	Polymyxin B
KIM D27	14.33 +/-.78	33.5 +/- 1.73	15.09 +/- 1.04
KIM D27*bamC2*	14.58 +/- 1.08	35.5 +/- 1.24	21.45 +/- .93*****
KIM D27*bamC2pbamC*	14.5 +/- .9	32.17 +/- 1.34	14.91 +/- 1.04
KIM6+	13.17 +/- 1.03	36.17 +/- .58	13.09 +/- 1.04
KIM6+*bamC2*	13.5 +/- .9	35.83 +/- .58	20.55 +/- 1.29*****
KIM6+*bamC2pbamC*	13.67 +/- .78	35.17 +/- 1.03	9.64 +/- .81*****

Data was analyzed with Student's *t*-test versus the parental strain; *P<0.05

Auto-aggregation has been linked to virulence in a number of Gram-negative bacteria and is critical to the formation of biofilms (49-51). In order to assess this property and its dependence on BamC, we used a sedimentation assay. In the absence of BamC, *Y. pestis* more rapidly sedimented out of suspension compared to wild type and this phenotype was restored by expression of BamC from a plasmid (Figure 3.4A). This defect was observed in stationary phase bacteria that had been grown at 26°C and was not observed under normal growth conditions at 37°C likely due to the expression of the Caf1 capsule that forms at mammalian temperature(37). This result suggests that proteins involved in auto-aggregation do not depend on BamC for assembly.

One of the essential components of the type three secretion system (T3SS) is an OM β-barrel protein, YscC that is required for secretion of effector proteins into the host cell (4). Although it has not been tested if YscC assembly depends on the BAM complex, deletion of BamD in Salmonella results in reduced type III secretion consistent with dependency of one or more components of the T3SS on the BAM complex (19, 41).

Given the proposed function of BamC as an accessory factor of BamD, we tested the functionality of the T3SS in the *Y. pestis bamC1* mutant in a macrophage infection assay. Macrophage infection by *Yersinia pestis* results in apoptosis due to the translocation of YopJ by the T3SS (52, 53). The amount of caspase-3 activation correlates with the amount of YopJ translocation and caspase-3 detection has been used to quantify T3SS activity (54, 55). When we compared WT and *bamC1* infection of macrophages, no detectable difference in caspase-3 activation was observed (Figure 3.4B). This suggests that the T3SS is fully functional in the absence of *bamC* and YscC does not require BamC for assembly in the outer membrane. Overall, these assays suggest BamC function may be required in certain environments and/or for specific OMPs.

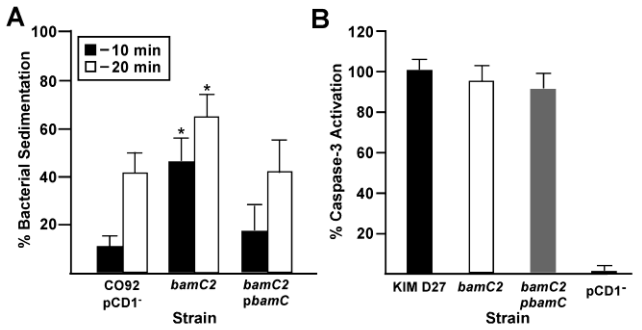

Figure 3.4 ***No role for BamC in auto-aggregation or type III secretion.*** (A) Bacteria were grown overnight to stationary phase, vortexed, and placed in 15mL conical tubes. Percent sedimentation was determined by measuring OD_{600} after 10 and 20 min incubation at room temperature. Error bars depicted indicate the standard error of the mean. (B) $5x10^6$ RAW264.7 macrophages were challenged at an MOI of 10 with the indicated strains in duplicate wells, gentamicin was added after 45 minutes and the infection continued for a total of 8 or 24 hours. Data shown was pooled from 3 independent experiments; error bars depicted indicate the standard deviation of the mean. All data was analyzed with a one-way ANOVA with Tukey's post test; *P<0.05.

3.4.5 BamC is required for activation of σ^E during extracytoplasmic stress. Given that the *bamC* mutant has an altered OMP profile when grown in high salinity media, we sought to determine if this phenotype was associated with induction of an extracytoplasmic stress response. Sigma E (σ^{24}) is a subunit of RNA polymerase induced when Gram-negative bacteria experience envelope stress or in response to accumulation of unfolded

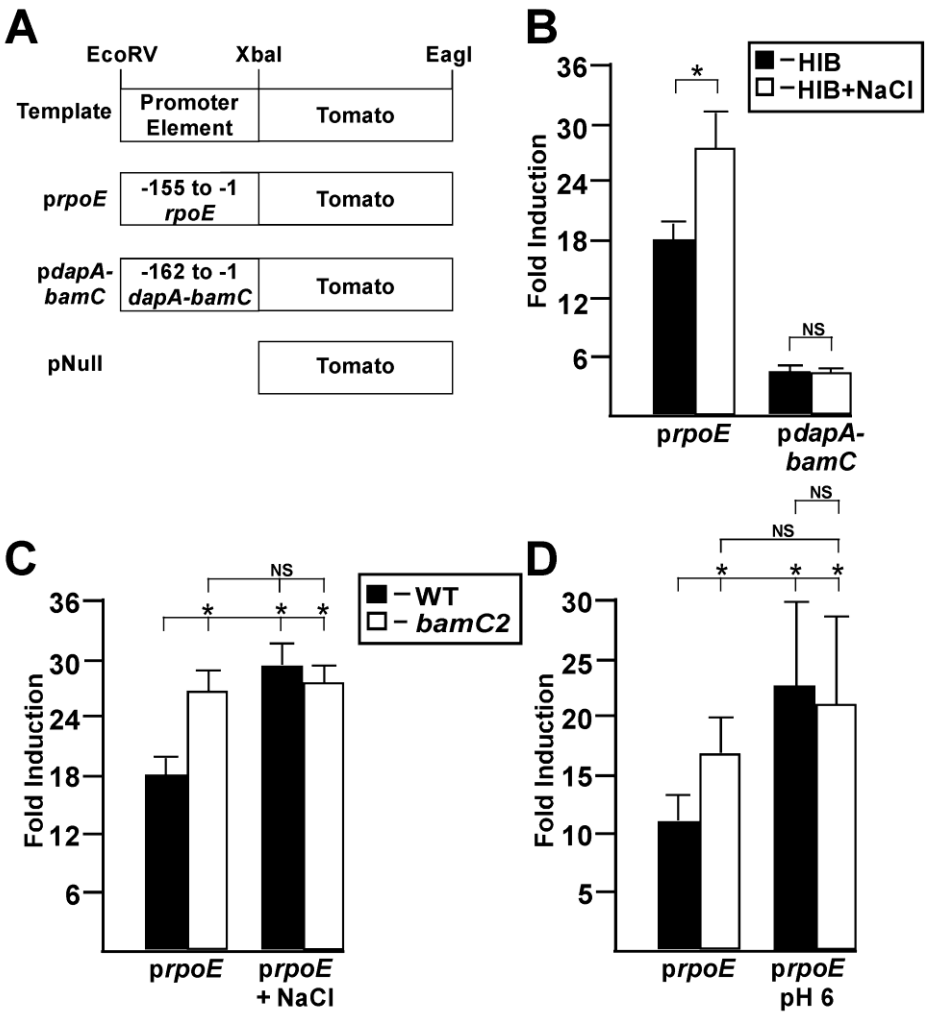

Figure 3.5 ***BamC is required for activation of σ^E during extracytoplasmic stress.*** (A) Diagram of fluorescent reporter constructs, each containing a different promoter element upstream of the fluorescent protein tdTomato, expressed in pBR322. *Y. pestis* KIM D27 WT (B) or WT and *bamC2* (C) strains carrying the indicated reporter constructs were analyzed by plate reader after 24 hours of growth. The relative fluorescent units (RFU) were normalized to the optical density (OD_{600}) of the bacterial culture. Shown is the fold induction compared to expression from the null promoter. Where indicated, 150mM NaCl was added to the media at time 0. Data shown is pooled from 7 independent experiments. Error bars depict the standard error of the mean. Statistical analysis was performed by one-way ANOVA followed by Tukey's post test; *P<0.05, NS=not significant.

proteins in the periplasm as a mechanism to control differential gene expression (56). In *E. coli*, deletion of BamB caused an increase in σ^E transcription suggesting induction of the periplasmic unfolded protein response (57). In addition, a putative σ^E binding site was identified in the *bamC* promoter in *E. coli*, however in *E. coli*, deletion of *bamC* did not induce expression of σ^E. We therefore examined induction of the σ^E response in the *bamC* mutant. Transcriptional reporters were generated by fusing the promoter of *rpoE* or *bamC* to the fluorescent protein tdTomato (Figure 3.5A). The σ^E response was induced by the addition of 150mM NaCl to the growth media (58, 59). Stationary phase *Y. pestis* induced the *rpoE* promoter 18-fold compared to a promoterless construct (Figure 3.5B). Upon incubation in high [NaCl], induction of the *rpoE* promoter to 27-fold over background was observed (Figure 3.5B). In contrast, NaCl did not induce expression of the *bamC* promoter, suggesting that *bamC* is not regulated by σ^E. The *rpoE* promoter was also induced to about 27-fold over background in *Y. pestis* bamC2 grown in untreated HIB, suggesting that, unlike *E. coli*, deletion of *bamC* in *Y. pestis* causes envelope stress or activates the unfolded protein response. No further induction of *rpoE* expression was observed in response to elevated [NaCl] in the *bamC2* mutant (Figure 3.5C). We tested another stress condition, that of low pH, and found that WT but not *bamC2* mutant *Y. pestis* induced more *rpoE* expression in low pH than in neutral media (Figure 3.5D). This data indicates that the *bamC2* mutant is unable to respond to acidic stress, similarly to a high salinity environment. Together, with the protein analysis, it appears that BamC may be involved in extracytoplasmic stress responses.

3.4.6 BamC is necessary for intracellular survival in RAW 264.7 cells. *Y. pestis* is an extracellular pathogen, but can survive and replicate in a *Yersinia* Containing Vacuole

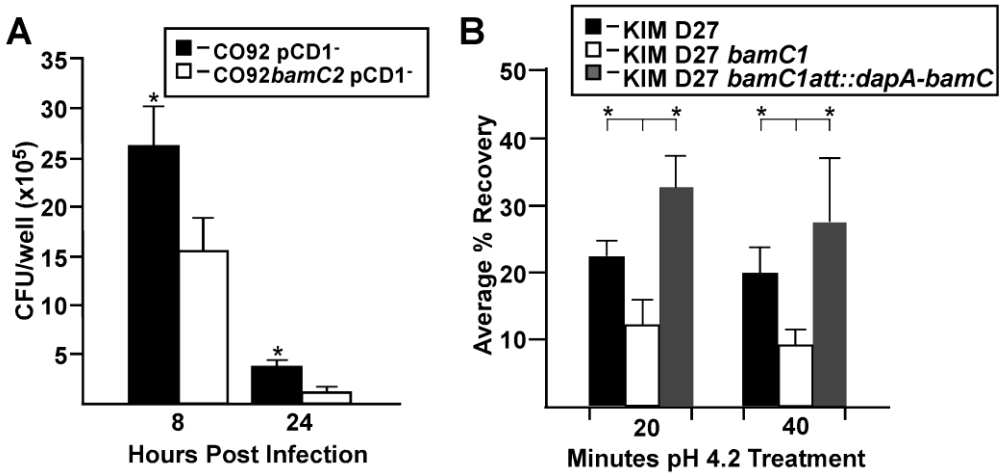

Figure 3.6 ***BamC is required for intracellular growth and survival in low pH.*** (A) $5x10^6$ RAW264.7 macrophages were challenged at an MOI of 10 with the indicated strains in duplicate wells, gentamicin was added after 45 minutes and the infection continued for a total of 8 or 24 hours. Data shown was pooled from 3 independent experiments; error bars depicted indicate the standard deviation of the mean. All data were analyzed with a one-way ANOVA with Tukey's post test; *p<0.05. (B) WT *Y. pestis* CO92pCD1-, *bamC* or *bamCpbamC Y. pestis* were grown overnight, washed with PBS, and resuspended in PBS pH 4.2 in triplicate and incubated at 26°C for the indicated times. Percent recovery was determined by comparing recovered CFU following pH 4.2 treatment to PBS pH7.0 control well. Data is pooled from 3 independent experiments. Error bars depict the standard error of the mean; statistical significance was evaluated by one-way ANOVA followed by Tukey's post test; *p<0.05.

(YCV) within macrophages and this intracellular stage is believed to be important for virulence (60). We therefore measured survival of the *bamC* mutant in macrophages. RAW 264.7 macrophages were infected with equal doses of *Y. pestis* KIM D27 or the *bamC* mutant (MOI=10), and after 45 min, gentamicin was added to kill extracellular bacteria, and the incubation was allowed to continue for 8 or 24 hours in order to determine intracellular survival. At 8 HPI, 40% fewer *Y. pestis bamC2* was recovered compared to WT, and this difference increased to 75% after 24 HPI (Figure 3.6A). The *bamC2* mutant was also tested for survival in low pH environments by incubation in PBS buffered at pH4.6 or 7.0. The results showed that the *bamC2* mutant was significantly more susceptible to an acidic environment compared to the wild type strain (Figure 3.6B). Expression of wild type BamC restored resistance to an acidic environment. Together, the data suggest that BamC is required for survival in macrophages and other

adverse microenvironments throughout the vector-mammal transmission cycle of *Y. pestis*.

3.5 Discussion

Growth assays, macrophage infections and protein gels support the hypothesis that BamC is an accessory factor that may only be required for optimal assembly of a subset of proteins under conditions of extracytoplasmic stress. We have shown that the absence of *bamC* causes mild to moderate growth defects under conditions of high salinity, low pH, and provided evidence that the function of some OMPs may depend on BamC more than others. Further, we have shown that the absence of *bamC* causes significant attenuation of virulence in the mouse bubonic and septicemic plague models as well as attenuation of growth in fleas. Together, the data provides further evidence of the co-evolutionary relationship in the development of the flea-mammalian transmission cycle.

We investigated the mechanism underlying the virulence defect of the *bamC* mutant in a variety of biological assays to ask the question whether a single protein or several proteins were responsible for bacterial growth in the flea and mammal. Respiratory infection of mice by *Y. pestis* proceeded nearly unaltered by loss of BamC consistent with the view that this environment is more favorable for bacterial growth than the skin or even blood. Many virulence factors have been identified, some of them β-barrel OMPs, that are more critical to infection by the subcutaneous route than the respiratory route. For example, PhoP is required for virulence in the bubonic plague model where a 75-fold increase in LD_{50} was measured after subcutaneous infection, but

no change was observed following respiratory infection(61). Compared to the 400-fold increase in LD_{50} observed in the *bamC* mutants, it appears likely that the reduced function of multiple virulence factors lead to the reduced pathogenesis we observed.

Polymixin B sensitivity appeared to correlate with BamC expression and we suggested that the OMP LptD, which catalyzes LPS assembly and is known to depend on the BAM complex for insertion in the outer membrane, might be dependent on BamC in *Y. pestis*. LPS is a major virulence factor for *Y. pestis* in mammals and fleas and it is likely to mutations that disrupt LPS could attenuate growth in both species either because the render the bacteria more sensitive to adverse environments or less evasive to innate immune recognition.

Gram-negative bacteria respond to the extracytoplasmic stress of adverse environments in part by inducing σ^E and the same response is activated when unfolded proteins or LPS accumulate in the periplasm. Induction of σ^E in the *bamC* mutant is consistent with the polymixin B phenotype and suggests that deletion of *bamC* causes an LPS assembly defect (57, 62). We provided evidence that BamC is important to responding to extracytoplasmic stress because the *bamC* mutant was unable to induce σ^E when bacteria were incubated in high salinity or low pH media. These data are in agreement with the recent report that the σ^E response is required for *Y. pseudotuberculosis* to survive low pH and other extracytoplasmic stresses (63). Moreover, σ^E was also recently shown to be required for the insect borne pathogen *Bartonella quintana* to adapt to the insect gut environment following transmission from its mammalian host (64). Our data provide a possible mechanism whereby adaptation of

these Gram-negative bacteria to adverse environments involves up-regulation of σ^E and OMP assembly.

BamC is highly conserved in pathogenic Gram-negative bacteria. Due to the evolutionary pressure on BamC function, a functionally equivalent protein, BamF, has also evolved in Alphaproteobacterial insect-borne human pathogens such as *Rickettsia.* BamF shares no sequence homology with BamC, but has been shown to associate with BamD suggesting functional similarity to BamC (22). In addition, the C-terminal domain of BamC has recently been demonstrated to be surface exposed in *E. coli* (20). Although the importance of the surface exposed region for BamC in particular and the BAM complex as a whole remains to be determined, these observations raise the possibility of BamC as a therapeutic target that might broadly protect against infection by Gram-negative bacteria. In support of this hypothesis, vaccination of mice with recombinant *Neisseria meningitidis* BamC was recently demonstrated to generate a protective antibody response against disease (65). Using antibodies or small molecules to target the surface exposed region of BamC may therefore be useful in disrupting the function of the BAM complex thereby reducing bacterial survival in multiple host environments.

Table 3.3 *E.coli* strains and Plasmid Construction

Strain/Plasmid	Key Properties	Reference
***E. Coli* Strains**		
S17-1	Sm^r Sp^r Tra^+; *pro thi hsdR recA*; chromosomal integration of RP4-2-Tc::Mu-*Kan*::Tn*7* λ*pir*+	(66)
DH5α	F^- , *lacZ*ΔαM15 *endA1 recA1 hsdR17*(r_M- m_K-) *supE44 thi-1 gyrA96* Δ(*lacZYA-argF*)U169	(67)
Plasmids		
pCVD442	Ap^r, R6K *Ori* and *sacB* counter selection; suicide vector	(68)
pCVD442-*bamC*	Contains 1000 base pairs up and downstream from the *bamC* open reading frame in order to promote homologous recombination.	This Study
pBR322	Cloning vector Ap^r Tet^r, ColE1 origin of replication	New England Biolabs
pBamC-Flag	Ap^r, expresses *bamC-FLAG* from the *lac* promoter (EcoRV); *bamC* inserted in place of Tc^r gene (EcoRV, EagI).	This Study
pBamD	Ap^r, expresses *bamD* from the *lac* promoter (EcoRV); *bamD* inserted in place of Tc^r gene (EcoRV, EagI).	This Study
p*dap*-Tomato	Ap^r, pBR322 Backbone, has a fragment of the dap ORF fused to the Tomato ORF via overlapping PCR and includes the introduction of an XbaI restriction site between the two DNA fragments. The entire DNA fragment was ligated into the EcoRV and EagI sites within the Tc^r cassette.	This Study
p*rpoE*	Ap^r, p*dap*-Tomato Backbone, Expresses Tomato protein from the SigmaE (rpoE) promoter region (155bp upstream of the *rpoE* start codon base pairs 3039217-372 on the CO92 genome Acession #: NC_003143) ligated into the EcoRV and XbaI sites.	This Study
p*dapA-bamC*	Ap^r, p*dap*-Tomato Backbone, Expresses Tomato protein from the *dapA-bamC* promoter region (162bp upstream of the *bamC* start codon base pairs 3420197-359 on the CO92 genome Acession #: NC_003143) XbaI site introduced between promoter and Tomato ORF. ligated into the EcoRV and XbaI sites.	This Study
pNull	p*dap*-Tomato Backbone, has the dap ORF fragment removed via restriction digest (XbaI and EcoRV) sticky ends were filled with DNA Polymerase I, Large (Klenow) Fragment and the construct was blunt end ligated resulting in a promoterless Tomato ORF.	This Sudy
pDsRed-monomer	Ap^r, *lac* driven DsRed expression, PCR template for the *lac* promoter.	Clontech
ptdTomato	Ap^r, *lac* driven Tomato expression, PCR template for the td Tomato ORF.	Clontech
pGEM-T easy	Ap^r, blue/white screen cloning vector	Promega

Table 3.4 Primers used for plasmid construction.

Name	Sequence (5' – 3')	Plasmid
BamC P1	TCT AGA CCA AGC GCT TAC AAC ACA GT	pCVD442*bamC*
BamC P2	GGG GCC GAA GCC CCT TTG GTT ATT TCC CTA AGA AGT TAC AGC	pCVD442*bamC*
BamC P3	GCT GTA ACT TCT TAG GGA AAT AAC CAA AGG GGC TTC GGC CCC	pCVD442*bamC*
BamC P4	GCA TGC GCA GGC GTT CCA TTT GGG TA	pCVD442*bamC*
F BamC	GGG ATA TCT TGA TGG CAA TAT CAT TGC A	pBR322-*bamC-FLAG*
R BamC-FLAG	GGC GGC CGT TAC TTG TCG TCA TCG TCT TTG TAG TCT TTT ATC GCG GTT GCG CTA GTC TG	pBR322-*bamC-FLAG*
F BamD	GAT ATC ATG ACG CGT ATG AAA TAT CTG GTG	pBR322-*bamD*
R BamD	CGG CCG TTA GGC TGG ATT GGC CGC AAT	pBR322-*bamD*
F *lac* promoter	AGA TAT CAG CGC CCA ATA CGC AAA CC	pBR322-*bamC-FLAG* and pBR322-*bamD*
R *lac* promoter	AGA TAT CTG TTT CCT GTG TGA AAT TGT	pBR322-*bamC-FLAG* and pBR322-*bamD*
dap-Tom P1	GAT ATC AGC GAA GAT TAA AAA TAT TGT TGC	p*dap*-Tomato, pNull
dap-Tom P2	GAT GAC CTC CTC GCC CTT GCT CAC CAT TCT AGA CAA ATT TCC CTA AGA AGT TAC AGC	p*dap*-Tomato, pNull
dap-Tom P3	GCT GTA ACT TCT TAG GGA AAT TTG TCT AGA ATG GTG AGC AAG GGC GAG GAG GTC ATC	p*dap*-Tomato, pNull
dap-Tom P4	CGG CCG TTA CTT GTA CAG CTC GTC CAT GCC	p*dap*-Tomato, pNull
F *rpoE* promoter	GAT ATC TAT AAG ATG TCT GAA TAA TAT TTG	p*rpoE*
R *rpoE* promoter	TCT AGA CCG AGG TGA ACT CTC CCG AAA	p*rpoE* p*dapA-bamC*
F *dapA-bamC* promoter	GAT ATC TCG CTC TTC CTG TCA TGC TCT	

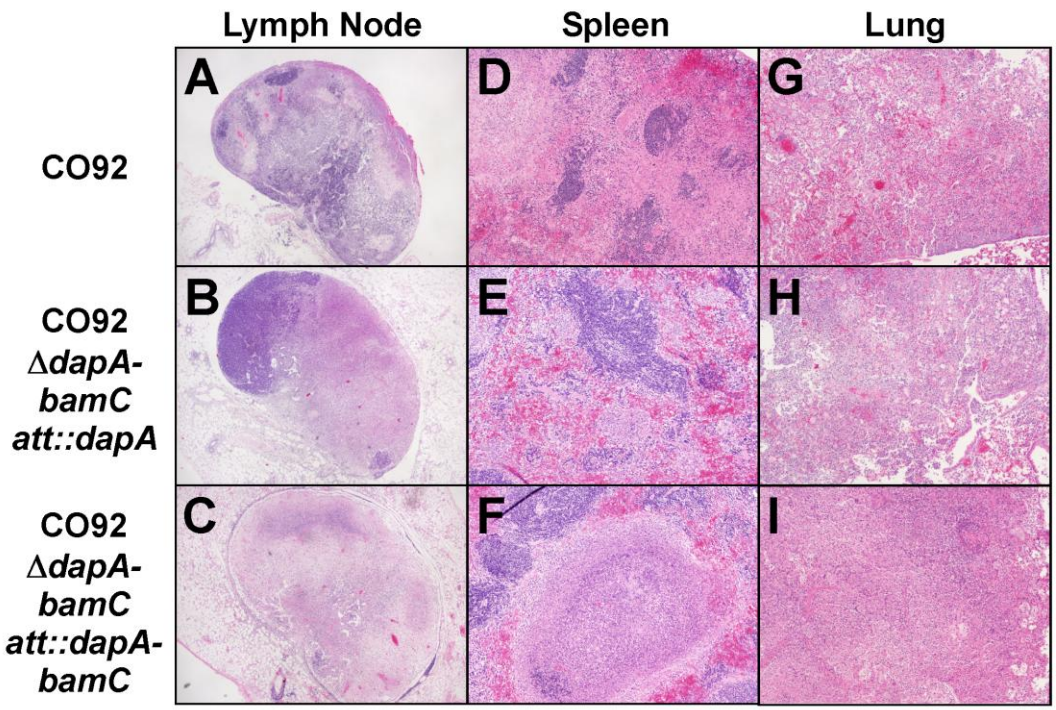

Figure 3.7 ***BamC deficient strains induce similar pathology to fully virulent Yersinia pestis in all routes of infection***. BALB/c mice (n=3-5) were challenged by subcutaneous (A-F) or intranasal (G-I) infection of 10x LD_{50} *Y. pestis* CO92, *bamC* or *bamC*-complemented strains. Inguinal lymph nodes (A-C), spleen (D-F) or lungs (G-I) were harvested from moribund mice, fixed in 10% formalin for 48 hours, followed by sectioning and staining with hematoxylin and eosin (H&E). Representative images are shown.

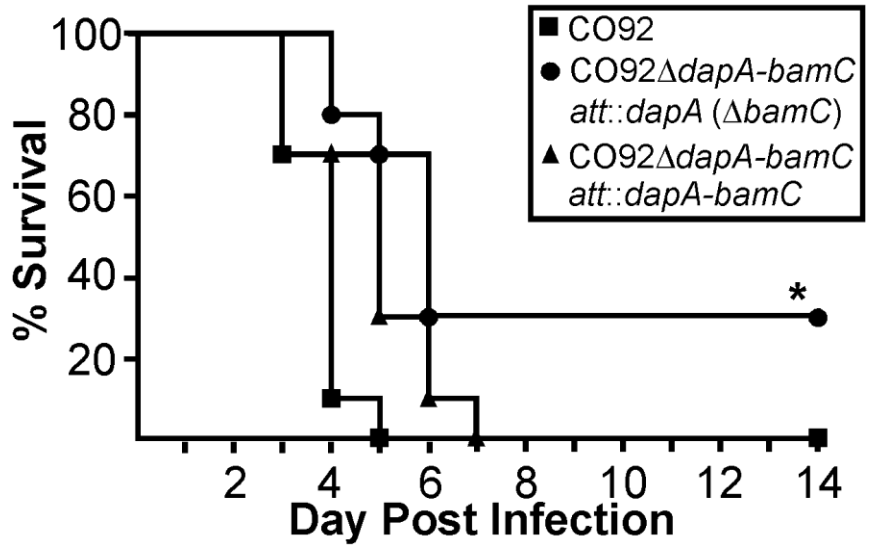

Figure 3.8 ***BamC deficient strains have a mild reduction of virulence in a pneumonic model of plague***. BALB/c mice were challenged by intranasal infection with 3000 CFU of the indicated strains of *Y. pestis.* Survival was monitored over 14 days. Data were pooled from two independent experiments, n=10. Statistical significance was evaluated by the Log Rank test; *$p<0.05$.

3.6 Acknowledgements

We thank Dr. Ken Gage and Dr. Rebecca Eisen for providing *O. montana* fleas that were used to start a laboratory-reared colony at the University of Missouri, and Dr. Joe Hinnebusch for providing protocols and helpful discussion. We also thank members of our laboratory for assistance with the select agent experiments. Histology staining was performed by IDEXX-RADIL (Columbia, MO) and pathological analysis of infected tissues was provided by Dr. Andrew Kocsis. Mass spectrometry was performed by the Charles Gehrke Proteomics Core facility at the University of Missouri. This work was supported in part by National Institutes of Health (NIH)/National Institute of Allergy and Infectious Diseases PHS award and the American Recovery and Reinvestment Act (R21AI074635) to DMA. The authors have no conflicts of interest related to this work.

3.7 References

1. **Bos MP, Robert V, Tommassen J.** 2007. Biogenesis of the gram-negative bacterial outer membrane. Annu Rev Microbiol **61:**191-214.

2. **Walther DM, Rapaport D, Tommassen J.** 2009. Biogenesis of beta-barrel membrane proteins in bacteria and eukaryotes: evolutionary conservation and divergence. Cellular and molecular life sciences : CMLS **66:**2789-2804.

3. **Knowles TJ, Scott-Tucker A, Overduin M, Henderson IR.** 2009. Membrane protein architects: the role of the BAM complex in outer membrane protein assembly. Nat Rev Microbiol **7:**206-214.

4. **Plano GV, Straley SC.** 1995. Mutations in yscC, yscD, and yscG prevent high-level expression and secretion of V antigen and Yops in *Yersinia pestis*. J Bacteriol **177:**3843-3854.

5. **Fetherston J, Kirillina O, Bobrov A, Paulley J, Perry R.** 2010. The *Yersinia*bactin transport system is critical for the pathogenesis of bubonic and pneumonic plague. Infect Immun **78:**2045-2052.

6. **Sodeinde OA, Sample AK, Brubaker RR, Goguen JD.** 1988. Plasminogen activator/coagulase gene of *Yersinia pestis* is responsible for degradation of plasmid-encoded outer membrane proteins. Infect Immun **56:**2749-2752.

7. **Yang Y, Merriam JJ, Mueller JP, Isberg RR.** 1996. The *psa* locus is responsible for thermoinducible binding of *Yersinia pseudotuberculosis* to cultured cells. Infect Immun **64:**2483-2489.

8. **Bartra SS, Styer KL, O'Bryant DM, Nilles ML, Hinnebusch BJ, Aballay A, Plano GV.** 2008. Resistance of *Yersinia pestis* to complement-dependent killing is mediated by the Ail outer membrane protein. Infect Immun **76:**612-622.

9. **Rigel NW, Silhavy TJ.** 2012. Making a beta-barrel: assembly of outer membrane proteins in Gram-negative bacteria. Current opinion in microbiology **15:**189-193.

10. **Wu T, Malinverni J, Ruiz N, Kim S, Silhavy T, Kahne D.** 2005. Identification of a multicomponent complex required for outer membrane biogenesis in *Escherichia coli*. Cell **121:**235-245.

11. **Ricci DP, Hagan CL, Kahne D, Silhavy TJ.** 2012. Activation of the *Escherichia coli* beta-barrel assembly machine (Bam) is required for essential components to interact properly with substrate. Proc Natl Acad Sci U S A **109:**3487-3491.

12. **Hagan CL, Silhavy TJ, Kahne D.** 2011. beta-Barrel membrane protein assembly by the Bam complex. Annual review of biochemistry **80:**189-210.

13. **Rossiter AE, Leyton DL, Tveen-Jensen K, Browning DF, Sevastsyanovich Y, Knowles TJ, Nichols KB, Cunningham AF, Overduin M, Schembri MA, Henderson IR.** 2011. The essential beta-barrel assembly machinery complex components BamD and BamA are required for autotransporter biogenesis. J Bacteriol **193:**4250-4253.

14. **Malinverni JC, Werner J, Kim S, Sklar JG, Kahne D, Misra R, Silhavy TJ.** 2006. YfiO stabilizes the YaeT complex and is essential for outer membrane protein assembly in *Escherichia coli*. Mol Microbiol **61:**151-164.

15. **Werner J, Misra R.** 2005. YaeT (Omp85) affects the assembly of lipid-dependent and lipid-independent outer membrane proteins of *Escherichia coli*. Mol Microbiol **57:**1450-1459.

16. **Hagan C, Kim S, Kahne D.** 2010. Reconstitution of outer membrane protein assembly from purified components. Science **328:**890-892.

17. **Hagan CL, Kim S, Kahne D.** 2010. Reconstitution of outer membrane protein assembly from purified components. Science **328:**890-892.

18. **Ruiz N, Falcone B, Kahne D, Silhavy T.** 2005. Chemical conditionality: a genetic strategy to probe organelle assembly. Cell **21:**307-317.

19. **Fardini Y, Chettab K, Grepinet O, Rochereau S, Trotereau J, Harvey P, Amy M, Bottreau E, Bumstead N, Barrow P, Virlogeux-Payant I.** 2007. The YfgL lipoprotein is essential for type III secretion system expression and virulence of *Salmonella enterica* Serovar Enteritidis. Infect Immun **75:**358-370.

20. **Webb C, Selkrig J, Perry A, Noinaj N, Buchanan S, Lithgow T.** 2012. Dynamic association of BAM complex modules includes surface exposure of the lipoprotein BamC. J Mol Biol **422:**545-555.

21. **Soo P, Wei J, Horng Y, Hsieh S, Ho S, Lai H.** 2005. Characterization of the *dapA-nlpB* genetic locus involved in regulation of swarming motility, cell envelope architecture, hemolysin production, and cell attachment ability in *Serratia marcescens*. Infect Immun **73:**6075-6084.

22. **Anwari K, Webb CT, Poggio S, Perry AJ, Belousoff M, Celik N, Ramm G, Lovering A, Sockett RE, Smit J, Jacobs-Wagner C, Lithgow T.** 2012. The evolution of new lipoprotein subunits of the bacterial outer membrane BAM complex. Mol Microbiol **84:**832-844.

23. **Fetherston J, Perry R.** 1997. *Yersinia pestis* -- etiologic agent of plague. Clin Microbiol Rev **10:**35-66.

24. **Hinnebusch BJ, Erickson DL.** 2008. *Yersinia pestis* biofilm in the flea vector and its role in the transmission of plague. Curr Top Microbiol Immunol **322:**229-248.

25. **Eisen R, Bearden S, Wilder A, Montenieri J, Antolin M, Gage K.** 2006. Early phase transmission of *Yersinia pestis* by unblocked fleas as a mechanism explaining rapidly spreading plague epizootics. Proc Natl Acad Sci **103:**15380-15385.

26. **Jarrett C, Deak E, Isherwood K, Oyston P, Fischer E, Whitney A, Kobayashi S, DeLeo F, Hinnebusch B.** 2004. Transmission of *Yersinia pestis* from an infectious biofilm in the flea vector. J Infect Dis **190:**783-792.

27. **Lathem W, Crosby S, Miller V, Goldman W.** 2005. Progression of primary pneumonic plague: A mouse model of infection, pathology, and bacterial transcriptional activity. Proc Natl Acad Sci **102:**17786-17791.

28. **Chauvaux S, Rosso M, Frangeul L, Lacroix C, Labarre L, Schiavo A, Marceau M, Dillies M, Foulon J, Coppee J, Medigue C, Simonet M, Carniel E.** 2007. Transcriptome analysis of *Yersinia pestis* in human plasma: an approach for discovering bacterial genes involved in septicaemic plague. Microbiol **153:**3112-3123.

29. **Bartra S, Gong X, Lorica C, Jain C, Nair M, Schifferli D, Qian L, Li Z, Plano G, Schesser K.** 2012. The outer membrane protein A (OmpA) of *Yersinia pestis* promotes intracellular survival and virulence in mice. Microb Path **52:**41-46.

30. **Lorange E, Race B, Sebbane F, Hinnebusch B.** 2005. Poor vector competence of fleas and the evolution of hypervirulence in *Yersinia pestis*. J Infect Dis **191:**1907-1912.

31. **Surgalla M, Beesley E.** 1969. Congo red-agar plating medium for detecting pigmentation in *Pasteurella pestis*. App Microbiol **18:**834-837.

32. **Anderson G, Leary S, Williamson E, Titball R, Welkos S, Worsham P, Friedlander A.** 1996. Recombinant V antigen protects mice against pneumonic and bubonic plague caused by F1-capsule positive and negative strains of *Yersinia pestis*. Infect Immun **64:**4580-4585.

33. **Bland D, Eisele N, Keleher L, Anderson P, Anderson D.** 2011. Novel Genetic Tools for Diaminopimelic Acid Selection in Virulence Studies of *Yersinia pestis*. PLoS One **6:**e17352.

34. **Brubaker RR, Beesley ED, Surgalla MJ.** 1965. *Pasteurella pestis*: Role of Pesticin I and Iron in Experimental Plague. Science **149:**422-424.

35. **Fetherston J, Lillard JJ, Perry R.** 1995. Analysis of the pesticin receptor from *Yersinia pestis*: role in iron-deficient growth and possible regulation by its siderophore. J Bacteriol **177:**1824-1833.

36. **Reed L, Muench H.** 1938. A simple method of estimating fifty percent endpoints. Am J Hyg **27:**493-497.

37. **Felek S, Muszynski A, Carlson RW, Tsang TM, Hinnebusch BJ, Krukonis ES.** 2010. Phosphoglucomutase of *Yersinia pestis* is required for autoaggregation and polymyxin B resistance. Infect Immun **78:**1163-1175.

38. **Burroughs AL.** 1947. Sylvatic plague studies: The vector efficiency of nine species of fleas compared with *Xenopsylla cheopis*. The Journal of hygiene **45:**371-396.

39. **Tidhar A, Flashner Y, Cohen S, Levi Y, Zauberman A, Gur D, Aftalion M, Elhanany E, Zvi A, Shafferman A, Mamroud E.** 2009. The NlpD lipoprotein is a novel *Yersinia pestis* virulence factor essential for the development of plague. PLoS ONE **4:**e7023.

40. **Fardini Y, Chettab K, Grepinet O, Rochereau S, Trotereau J, Harvey P, Amy M, Bottreau E, Bumstead N, Barrow PA, Virlogeux-Payant I.** 2007. The YfgL lipoprotein is essential for type III secretion system expression and virulence of *Salmonella enterica* Serovar Enteritidis. Infect Immun **75:**358-370.

41. **Fardini Y, Trotereau J, Bottreau E, Souchard C, Velge P, Virlogeux-Payant I.** 2009. Investigation of the role of the BAM complex and SurA chaperone in outer-membrane protein biogenesis and type III secretion system expression in *Salmonella*. Microbiology **155:**1613-1622.

42. **Philippe N, Alcaraz JP, Coursange E, Geiselmann J, Schneider D.** 2004. Improvement of pCVD442, a suicide plasmid for gene allele exchange in bacteria. Plasmid **51:**246-255.

43. **Ber R, Mamroud E, Aftalion M, Tidhar A, Gur D, Flashner Y, Cohen S.** 2003. Development of an improved selective agar medium for isolation of *Yersinia pestis*. Appl Environ Microbiol **69:**5787-5792.

44. **Zhou W, Russell CW, Johnson KL, Mortensen RD, Erickson DL.** 2012. Gene expression analysis of *Xenopsylla cheopis* (Siphonaptera: Pulicidae) suggests a role for reactive oxygen species in response to *Yersinia pestis* infection. J Med Entomol **49:**364-370.

45. **Bochkareva ES, Solovieva ME, Girshovich AS.** 1998. Targeting of GroEL to SecA on the cytoplasmic membrane of *Escherichia coli*. Proc Natl Acad Sci U S A **95:**478-483.

46. **Zavascki A, Goldani L, Li J, Nation R.** 2007. Polymixin B for the treatment of multidrug-resistant pathogens: a critical review. J Antimicrob Chem **60:**1206-1215.

47. **Wu T, McCandlish A, Gronenberg L, Chng S, Silhavy T, Kahne D.** 2006. Identification of a protein complex that assembles lipopolysaccharide in the outer membrane of *Escherichia coli*. Proc Natl Acad Sci U S A **103:**11754-11759.

48. **Chng S, Ruiz N, Chimalakonda G, Silhavy T, Kahne D.** 2010. Characterization of the two-protein complex in *Escherichia coli* responsible for lipopolysaccharide assembly at the outer membrane. Proc Natl Acad Sci U S A **107:**5363-5368.

49. **Kapperud G, Lassen J.** 1983. Relationship of virulence-associated autoagglutination to hemagglutinin production in *Yersinia enterocolitica* and *Yersinia enterocolitica*-like bacteria. Infect Immun **42:**163-169.

50. **Schommer NN, Christner M, Hentschke M, Ruckdeschel K, Aepfelbacher M, Rohde H.** 2011. *Staphylococcus epidermidis* uses distinct mechanisms of biofilm formation to interfere with phagocytosis and activation of mouse macrophage-like cells 774A.1. Infect Immun **79:**2267-2276.

51. **Al Safadi R, Abu-Ali GS, Sloup RE, Rudrik JT, Waters CM, Eaton KA, Manning SD.** 2012. Correlation between in vivo biofilm formation and virulence gene expression in *Escherichia coli* O104:H4. PLoS ONE **7:**e41628.

52. **Monack D, Mecsas J, Ghori N, Falkow S.** 1997. *Yersinia* signals macrophages to undergo apoptosis and YopJ is necessary for this cell death. Proc Natl Acad Sci **94:**10385-10390.

53. **Orth K, Xu Z, Mudgett M, Bao Z, Palmer L, Bliska J, Mangel W, Staskawicz B, Dixon J.** 2000. Disruption of signaling by *Yersinia* effector YopJ, a ubiquitin-like protein protease. Science **290:**1594-1597.

54. **Weeks S, Hill J, Friedlander A, Welkos S.** 2002. Anti-V antigen antibody protects macrophages from *Yersinia pestis*-induced cell death and promotes phagocytosis. Microb Path **32:**227-237.

55. **Eisele NA, Anderson DM.** 2009. Dual-function antibodies to *Yersinia pestis* LcrV required for pulmonary clearance of plague. Clinical and vaccine immunology : CVI **16:**1720-1727.

56. **Mecsas J, Rouviere PE, Erickson JW, Donohue TJ, Gross CA.** 1993. The activity of sigma E, an *Escherichia coli* heat-inducible sigma-factor, is modulated by expression of outer membrane proteins. Genes Dev **7:**2618-2628.

57. **Onufryk C, Crouch M, Fang F, Gross C.** 2005. Characterization of six lipoproteins in the sigmaE regulon. J Bacteriol **187:**4552-4561.

58. **Du H, Wang M, Luo Z, Ni B, Wang F, Meng Y, Xu S, Huang X.** 2011. Coregulation of gene expression by sigma factors RpoE and RpoS in *Salmonella enterica* serovar Typhi during hyperosmotic stress. Curr Microbiol **62:**1483-1489.

59. **Bianchi AA, Baneyx F.** 1999. Hyperosmotic shock induces the sigma32 and sigmaE stress regulons of *Escherichia coli*. Mol Microbiol **34:**1029-1038.

60. **Grabenstein J, Fukuto H, Palmer L, Bliska J.** 2006. Characterization of phagosome trafficking and identification of PhoP-regulated genes important for survival of *Yersinia pestis* in macrophages. Infect Immun **74:**3727-3741.

61. **Bozue J, Mou S, Moody KL, Cote CK, Trevino S, Fritz D, Worsham P.** 2011. The role of the phoPQ operon in the pathogenesis of the fully virulent CO92 strain of *Yersinia pestis* and the IP32953 strain of *Yersinia pseudotuberculosis*. Microb Pathog **50:**314-321.

62. **Taylor PL, Blakely KM, de Leon GP, Walker JR, McArthur F, Evdokimova E, Zhang K, Valvano MA, Wright GD, Junop MS.** 2008. Structure and function of sedoheptulose-7-phosphate isomerase, a critical enzyme for lipopolysaccharide biosynthesis and a target for antibiotic adjuvants. J Biol Chem **283:**2835-2845.

63. **Palonen E, Lindstrom M, Somervuo P, Korkeala H.** 2013. Alternative sigma factor Sigma E has an important role in stress tolerance of *Yersinia pseudotuberculosis* IP32953. App Env Microbiol **In press**.

64. **Abromaitis S, Koehler J.** 2013. The *Bartonella quintana* extracytoplasmic function sigma factor RpoE has a role in bacterial adaptation to the arthropod vector environment. J Bacteriol **195:**2662-2674.

65. **Pajon R, Yero D, Niebla O, Climent Y, Sardinas G, Garcia D, Perera Y, Llanes A, Delgado M, Cobas K, Caballero E, Taylor S, Brookes C, Gorringe A.** 2009. Identification of new meningococcal serogroup B surface antigens through a systematic analysis of neisserial genomes. Vaccine **28:**532-541.

66. **Simon R, Priefer U, Pu¨hler A.** 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in gram negative bacteria. Biotechnology **1**.

67. **Hanahan D.** 1985. DNA Cloning: A practical approach., p. 109. *In* Glover D (ed.), vol. 1. IRL Press, McLean, VA.

68. **Donnenberg M, Kaper J.** 1991. Construction of an *eae* deletion mutant of Enteropathogenic *Escherichia coli* by using a positive selection suicide vector. Infect Immun **59:**4310-4317.

Chapter 4

ARTHROPOD AND BACTERIAL GENETIC FACTORS INFLUENCE ESTABLISHMENT OF *Y. PESTIS* IN THE INSECT DIGESTIVE TRACT

Contributions: David Bland and Deborah Anderson conceived and designed flea experiments. Melanie Marketon's lab conceived and designed *Drosophila* experiments. Bacterial mutants provided by Melanie Marketon (Indiana University). Flea experiments and analysis performed by David Bland. *Drosophila* experiments and analysis performed by Melanie Marketon's lab. Manuscript written by David Bland. Model of flea infection generated by David Bland

4.1 Abstract

Yersinia pestis is the causative agent of plague, a highly virulent and potentially lethal zoonotic disease. This bacterium survives within the flea midgut and is transmitted to mammals when bacteria are regurgitated into the mammalian dermis. Over 20 different species of flea in North America are believed to be capable of plague transmission (1). However, the efficiency with which these fleas transmit the infection varies greatly (2-5). This variation is believed to be due to currently unidentified genetic differences in flea host species (6). Traditionally, the stable infection of the flea vector involves the growth of bacteria into a dense biofilm that lodges in the flea digestive tract and eventually blocks the esophagus, preventing future ingestion of blood meals (7). This mode of infection requires the direct interaction of bacteria with the insect midgut. As such, early establishment of a *Y. pestis* replicative niche in the midgut is likely a contributing factor for making fleas efficient vehicles for the transmission of plague. However, the genetic contributions of the insect midgut towards defense against

microbial incursion remain largely uninvestigated in the flea model of infection (8). Insect midgut immune responses are fairly well conserved. The production of reactive oxygen species (ROS) and antimicrobial peptides are common features of blood feeding arthropods (9). Researchers studying *Yersinia*-Flea interactions utilize flea strains that have been maintained in the lab for decades. The possibility exists that genetic drift in laboratory flea populations has influenced the *Y. pestis* life cycle. Here we show that *Y. pestis* mutants that are more sensitive to antimicrobial insult do not colonize the flea digestive tract as efficiently as their wild-type counterparts. This data is corroborated by using a *Drosophila* larva oral infection model, where transgenic fruit flies, defective for expression of conserved immune genes, carry increased bacterial loads. In addition, we demonstrate that North American inbred flea populations harbor greater concentrations of *Y. pestis* than their outbred counterparts. We propose that selective pressures imposed by laboratory rearing conditions have created genetically homogenous flea populations that have an altered capacity for allowing *Y. pestis* to colonize the flea midgut.

4.2 Introduction

All pathogens that are transmitted by blood feeding arthropods eventually interact with the insect digestive tract. The midgut is the first barrier to microbial establishment within the insect host (10). The majority of pathogens that are transmitted by mosquitoes, ticks, and biting flies must evade host immunity, cross the insect midgut, enter the hemoceol, and migrate to the salivary glands in order to be transmitted back to a mammalian host. *Y. pestis* is unique in that it does not enter the insect hemoceol and remains in close association with the flea digestive tract throughout the infection process (1). Therefore, evading the immune response of the flea midgut is likely the most critical step in a establishing a stable plague infection.

The majority of insect digestive tract immune mechanisms are well conserved across insect species (11, 12). Midgut epithelia of other blood feeding arthropods are known to secrete antimicrobial peptides in response to Gram-negative bacterial challenge (11, 13). In addition, *X. cheopis* fleas have been demonstrated to produce ROS in the midgut shortly after taking a bloodmeal (8). These innate immune responses are known to be vital for the successful establishment of microbes within the insect host (14). The lack of sequence data for flea immune genes also negates the possibility of using transgenic fleas to determine the importance of host responses for the colonization of *Y. pestis*. Therefore, development of alternative model systems is appropriate for establishing that host responses are influencing *Y. pestis* colonization in the flea midgut.

A number of bacterial genes have been identified that are known to be critical for resistance to ROS and antimicrobials in closely related bacterial species. The OxyR transcriptional regulator is activated upon encountering a hydrogen peroxide stressed

environment in Gram-negative bacteria (15, 16). *oxyR* is known to regulate the production of catalase and is critical for ROS stress responses (17). Without this gene, Gram-negative bacteria are more sensitive to the oxidative burst initiated by mammalian neutrophils. The *gmhA* gene encodes phosphoheptose isomerase, an enzyme that is needed for production of heptose (18). Heptose is an important sugar in lipopolysaccharide (LPS), a major structural component of a Gram-negative bacterium's outer membrane. Without GmhA production, bacterial LPS is truncated at the O-antigen site (19). This structural alteration is known to lead to increased antimicrobial susceptibility in E. *coli* (18). Furthermore, loss of GmhA has been implicated in reducing *Y. pestis* biofilm formation through *in vitro* assays and *on C. Elegans* mouth parts (20). Reduction in biofilm production and alteration in LPS structure may result in a synergistic loss of resistance to antimicrobial peptides in *ΔgmhA* strains. The PhoP-Q two component regulatory system is known to be important for *Y. pestis* to sense environmental changes and respond to stress (21). Similarly to the function of GmhA, interrupting PhoP signaling has downstream effects on LOS structure and biofilm formation (22). Loss of *Yersinia spp.* PhoP results in enhanced sensitivity to cationic antimicrobial peptides, including the insect midgut expressed peptide cecropin P1 (23-25). These bacterial mutants demonstrate characteristics that likely make them more susceptible to conserved midgut immune responses.

Arthropod model systems, such as *Drosophila*, have been the basis for much of our understanding of eukaryotic genetics. Numerous transgenic fruit flies exist, including those with mutations in genetic elements that control insect immune responses. In the absence of genetic tools for studying flea host responses, using fruit fly larvae may be a

viable alternative for studying early insect host responses to *Y. pestis* challenge. As such, we investigate the utility of a *Drosophila* larval system for examining early host responses to *Yersinia* infection. Knockouts of the critical *Drosophila* immune genes *imd* and *duox* exist are employed to achieve this goal. The immune deficiency (Imd) pathway is responsible for sensing and responding to microbial challenge through the secretion of antimicrobial peptides such as diptericin and cecropin A (26, 27). The dual oxidase (Duox) system is needed for production of ROS in the insect midgut (28).

Xenopsylla cheopis, or the oriental rat flea, is considered to be the most efficient vector for the transmission of plague throughout the world (7). While in the United States, *Oropsylla montana* is considered the main flea species that perpetuates the plague life cycle in rural prairie dog and ground squirrel populations (29, 30). However, *X. cheopis* is still endemic in urban rat populations in many of the larger U.S. cities. Despite these facts, direct comparisons between flea species capable of transmitting plague, such *as X. cheopis* and *O. montana*, are lacking. Comparing fleas from both urban and rural plague environments may improve our understanding of the *Y. pestis* life cycle. In order to establish what makes these two flea species efficient vectors, reliable data that closely mimics how these fleas generate transmissible infections in the wild needs to be obtained (31).

One of the barriers for generating data that closely mimics natural infection processes is genetic homogeneity in experimental animals. Many of the laboratory reared flea colonies previously employed for flea studies have been propagated for decades. Methodology associated with researching *Yersinia*-flea interactions has not considered the influence of genetically homogenous flea populations on microbial colonization of

the midgut. It has been established that inbreeding influences insect fitness and has the potential to alter host susceptibility to microbial challenge (32-34). In order to address this issue, a laboratory colony was established from an outbred *X. cheopis* population from Los Angeles, California in late 2012. The *X. cheopis* strain most frequently utilized in plague studies was isolated from Baltimore, Maryland over 30 years ago. Given the potential of genetic drift to alter experimental outcomes, the introduction of genetic variation to laboratory flea populations may be critical for translating mechanistic findings to epidemiological models of plague.

Here we show that *Y. pestis* strains lacking *oxyR*, *gmhA*, or *phoP* have reduced ability to colonize the *O. montana* midgut during the first week of infection. We establish that fruit flies are an alternative model organism for studying insect-*Yersinia* interactions. *Drosophila* larvae can be stably infected with *Y. pestis* and transgenic fruit flies lacking the immune genes *imd* and *duox* harbor higher bacterial titers than their wild type counterparts. Finally, we demonstrate that inbred *X. cheopis* and *O. montana* flea populations are more permissive to *Y. pestis* midgut establishment early during infection than their outbred counterparts. These differences between inbred and outbred strains have the potential to dramatically influence downstream transmission outcomes. Together the data demonstrates that both host genetic factors and bacterial resistance mechanisms are important for the stable colonization of plague in the flea midgut. In totality, the data supports standardizing flea rearing methodology to include frequent introduction of genetic diversity into experimental insect populations.

4.3 Materials and Methods

Bacterial strains and plasmids All strains used were grown on heart infusion agar (HIA) plates and single colonies were used to inoculate heart infusion broth (HIB). The plates used for *Y. pestis* KIM strains were supplemented with 0.005% Congo Red and 0.2% galactose to select for colonies that retain the pigmentation locus (*pgm*). *Y. pestis* KIM and CO92 strains were routinely grown fresh from frozen stock on HIA, followed by growth at 26ºC with aeration at 150rpm in heart infusion broth (HIB) overnight prior to use in experiments. KIM6+ *ΔphoP, ΔoxyR , ΔgmhA* strains were received from Melanie Marketon and were generated as described previously (25). The KIM6+ *ΔgmhA* strain was generated by removal of the *gmhA* open reading frame from the *Y. pestis* chromosome through homologous recombination using the lambda red system.

All *Y. pestis* strains harbor the plasmid pAH118, which expresses green fluorescent protein from a weak constitutive promoter and contains an ampicillin resistance cassette. Where indicated, ampicillin (100μg/ml) was added to media for selection of plasmids.

Flea infection Both *O. montana* and *X. cheopis* fleas were maintained in a refrigerated incubator at 22°C with ~80% relative humidity (2). Fleas were reared in one gallon glass jars containing larval medium (3 parts saw dust to 1 part of an equal blood meal, dried milk, mouse pellet mixture). For infection, fleas were starved for 6 days and subsequently allowed to feed on 3-4mL of heparinized murine blood containing 10^8-10^9 CFU of *Y. pestis*. Following infection, fleas were anesthetized using a chill table set to 0°C and monitored under a dissection scope for signs of fresh blood in the esophagus or proventriculus. Fleas that did not acquire a *Y. pestis*-infected blood meal were removed

from the study. Mechanical disruption of fleas for bacterial enumeration was performed using a bead beater. Bacterial enumeration was determined by plating in duplicate on HIA media with ampicillin (100μg/ml).

Oral Drosophila larvae infection. The procedure is adapted from the non-invasive infection method developed by Olcott et al (35). Adult flies were placed in collection chambers capped with ethyl acetate (EA) plates dabbed with small amounts of yeast paste, prepared with sterilized yeast grain and PBS water. After overnight incubation in the dark at 27°C, fresh EA plates and yeast paste were added and the flies were allowed to lay eggs for two hours to ensure synchronized development of the eggs. After this incubation period, the EA plates were removed and eggs were rinsed in a cell strainer with a 70 micron mesh filter. Eggs were then placed in 6-well dishes containing 2 ml of solidified Bacto-Agar per well, and wells were sprinkled with sterilized yeast pellets. The wells were sealed with a breathable membrane (Breathe-Easy, Diversified Biotech), and plates were stored at room temperature for two days. The following day (Day -1), *Y. pestis* strains in a freshly prepared yeast suspension (0.2 g killed yeast per 1.2 ml sterile phosphate buffered saline) were evenly distributed over the surface of the appropriate well to initiate infection. An equivalent amount of bacteria-free yeast suspension was applied to control wells. Bacterial suspensions were plated in order to verify the initial inoculation. Wells were then sealed and larvae were incubated at room temperature. For 3 days, 5 larvae were from each well were retrieved and surface-sterilized by washing once with 95% ethanol and sterile water. Individual larvae were homogenized in PBS and plated on *Yersinia* Selective Agar with appropriate antibiotics.

gltA PCR Screen Fleas were frozen for 5 minutes at -80°C and subsequently homogenized in 10µL of sterile water. 1µL of flea homogenate was added to a standard Taq DNA polymerase reaction (New England Biolabs). Primer sequences for *Bartonella* and *Rickettsia gltA* were utilized as described previously. Rick-F (5' GATTTTTTAGAAGTGGCATATTTG3')Rick-
R(5'GGKATYTTAGCWATCATTCTAATAGC3')
BartF(5'GCTATGTCTGCATTCTATCA3')Bart-
R(5'GATCYTCAATCATTTCTTTCC3').

Flea Measurements: Fleas were measured using an ocular micrometer with a 10x magnification lens. 20 Fleas (half male/ half female) were measured one hour after consuming a blood meal. The length was defined as the distance from the tip of the head to the tip of abdomen. Height was defined as the distance at its tallest point from the top to the bottom of the abdomen (near the center of the abdomen). Width was measured from above with the insect's mouth parts facing down at the widest point of the abdomen.

4.4 Results

4.4.1 Y. pestis strains susceptible to ROS and Antimicrobial peptides show decreased colonization of O. montana fleas. In order to determine if insect host response was important for *Y. pestis* colonization of the insect digestive tract, *O. montana* fleas were challenged with bacterial strains more susceptible to antibacterial stress. Fleas were fed blood meal spiked with $1x10^9$ CFU of the indicated strains of *Y. pestis*. Groups of 40 fleas were collected and mechanically disrupted for bacterial enumeration on days 1, 3, and 7 post infection in order to determine their infection status.

Infected fleas were pooled into 4 different bacterial concentration groups: uninfected, $<10^3$, 10^3-10^5, and $>10^5$ CFU. Grouping categories were designed to provide insight into the bacterial dynamics of infection early in the plague colonization process. Strains lacking the hemin storage locus (Hms) were included to determine the importance of biofilm formation in the flea digestive tract. The hms locus is responsible for producing and secreting a poly-*β*-1,6-*N*-acetyl-D-glucosamine based exopolysaccharide (EPS) (36, 37). The EPS is critical for blockage based transmission and likely obstructs the clearance of midgut infection (38). After one day post infection, all bacterial strains capable of producing EPS, despite the flea host response, follow a colonization pattern where 75-90% of fleas harbor bacteria (Figure 4.1A). However, strains unable to generate biofilms (KIM6-, *ΔgmhA*-) have a significantly reduced colonization percentage. Roughly half of the fleas with EPS defective strains clear the infection during the first 24 hours. This suggests that biofilm production is important very early in the infection process for persistent infection of the flea. By day 3 post infection, strains that show enhanced susceptibility to antimicrobial peptides (*ΔgmhA* and *ΔphoP*), are more readily cleared from the flea midgut, as indicated by a reduction in the population bearing greater than 10^3 CFU of *Y. pestis.* Strains lacking EPS production are more rapidly cleared by day 3, as indicated by the reduction in overall colonization and the loss of flea populations harboring bacterial concentrations greater than 10^5 CFU. Interestingly, the *ΔoxyR* strain closely follows the WT infection pattern during the first 3 days of infection. However, by day 7 post infection, this strain appears to behave similarly to other strains defective for antibacterial resistance. It is known that in response to a bloodmeal, the flea midgut secretes ROS (8). In order to keep experimental groups alive for extended study,

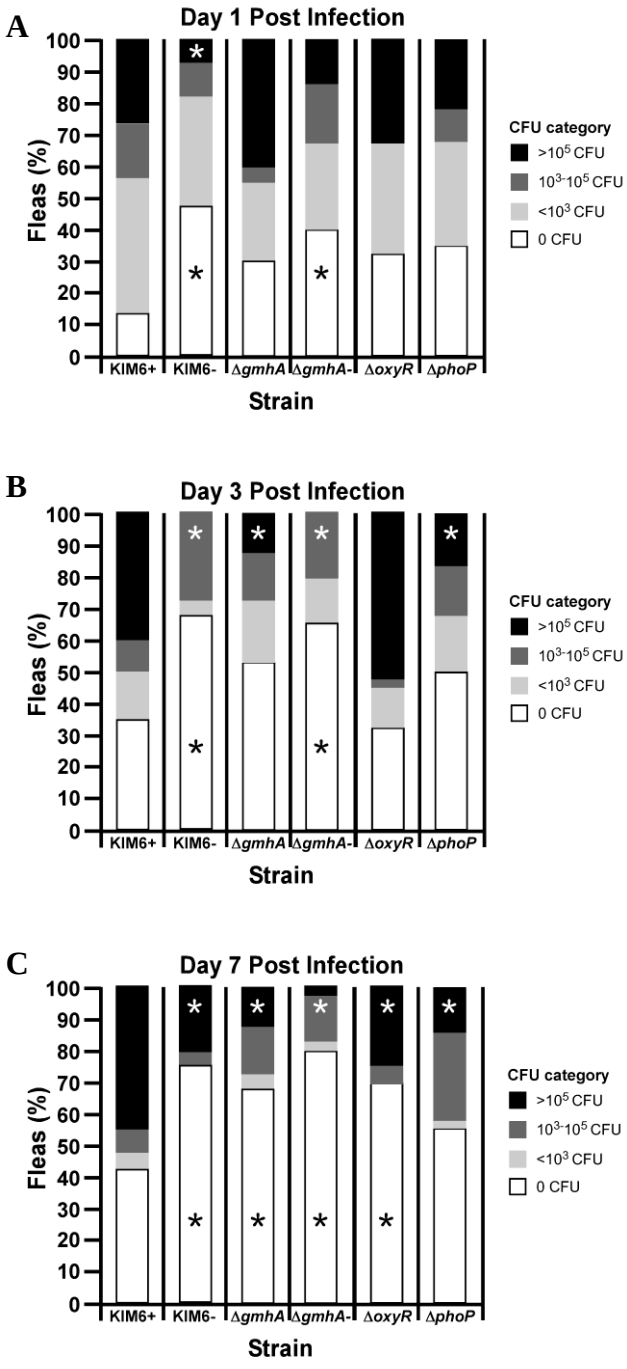

Figure 4.1 ***Resistance to antimicrobials and ROS is important for Y . pestis colonization of the flea midgut*** *Oropsylla montana* fleas were allowed to feed on blood spiked with $1X10^9$ CFU of the indicated *Y. pestis* strains. Strains designated with a "-" indicate the absence of the *hms* operon. On days 1 (A) 3 (B) and 7 (C) post infection, 40 fleas per bacterial strain were mechanically disrupted and enumerated on heart infusion agar. Fleas were grouped according to their bacterial burden and are represented as a percentage of the total population enumerated on that day. Data is analyzed with a Holm-Sidak Multiple Comparison One-way ANOVA. Significance is compared to the control group (KIM6+). Black asterisks = percentage of fleas infected. White asterisks = percentage of fleas with a large bacterial burden ($>10^3$ CFU). n = 40 P < .05

fleas are given a maintenance blood feed every 4 days. It is possible that upon initial infection, bacteria maintained at 37°C are primed for ROS resistance. Once maintained in the flea midgut for an extended period at lower temperatures (22-26°C), bacteria are more

susceptible to a secondary oxidative burst that is initiated by flea maintenance feeding events. By day 7, all bacterial strains have decreased ability to maintain a high titer infection. However, mutant strains have significant reduction in bacterial colonization, between 25-35% relative to the parental strain. Furthermore, fleas still colonized with mutant bacteria by day 7 are less likely to have a high bacterial burden ($>10^3$ CFU) compared to the wild type strain. It is important to note that wild type KIM6+ is the only strain capable of increasing the percentage of fleas bearing a high bacterial burden ($>10^5$ CFU) throughout the week long infection. The data indicates that maintaining a bacterial concentration less than 10^3 is not sustainable during the first week of infection. The majority of fleas in this bacterial concentration category appear to be capable of clearing *Y. pestis* during the first week of infection. This data is consistent with the notion that EPS production is critical for maintenance of plague beyond the first week of infection. Taken together, the data suggests that host antibacterial defenses influence *Y. pestis* midgut colonization early during the infection.

4.4.2 Midgut innate immune responses are important for Y. pestis colonization of Drosophila larvae

Using *Y. pestis* strains known to be more susceptible to antibacterial responses provides strong evidence for a flea host response, however, it does not directly link host genes to specific outcomes. In order to address this issue, a *Drosophila* larval model was developed to study early host immune responses to *Y. pestis* infection. Larvae were allowed to feed on yeast agar inoculated on day -1 with *Y. pestis.* Insects were collected for bacterial enumeration on *Yersinia* Selective Agar for 3 days following initial infection (day 0). Larvae can stably maintain 10^4 CFU of *Y. pestis* in their digestive tract, regardless of the initial bacterial seeding concentration, for up to 3 days post infection

(Figure 4.2A). To verify recovered bacteria was from larval colonization and not remnants of the initial innoculum, yeast agar plugs were enumerated to determine how long *Yersinia* could survive in the larval medium. While bacterial concentrations within the larvae digestive tract remain relatively constant, bacterial concentration in the yeast medium decreases over time (Figure 4.2B). By day 3, almost no bacteria can be recovered from the larval rearing medium. This data is suggestive that larvae aren't continuously clearing and subsequently refreshing *Y. pestis* in the digestive tract, but rather become persistently infected after the initial feeding events.

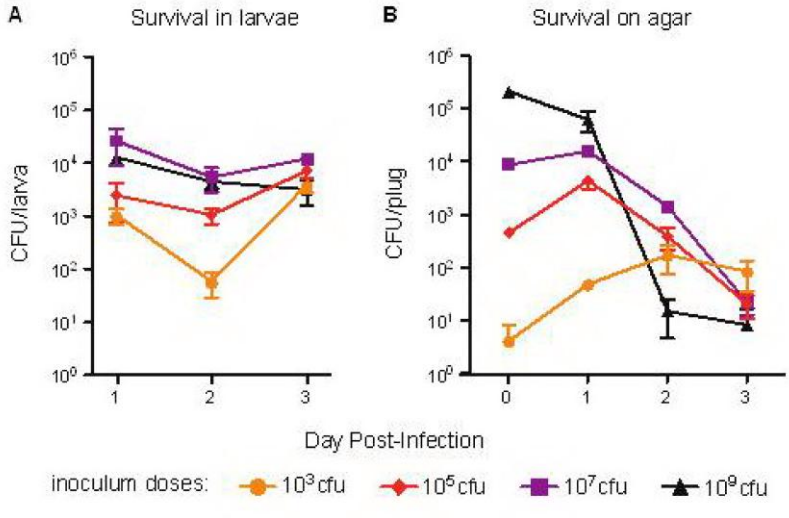

Figure 4.2 ***Y. pestis stably colonizes the Drosophila digestive tract through oral infection.*** Fruit fly larvae were allowed to feed on yeast agar seeded with the indicated doses of *Y. pestis* on day -1. (A) On each day, 5 larvae were mechanically disrupted and enumerated for bacterial burden on *Yersinia* selective agar. (B) Small sections of the yeast agar known as "plugs" were also enumerated for *Y. pestis* colonization. Shown is pooled data from 2 independent (Marketon)

In order to directly test if immune genes are important for plague colonization of the insect digestive tract, transgenic *Drosophila* larvae were infected with *Y. pestis* and bacterial titers were monitored for 3 days. Larvae lacking the *imd* gene, a genetic element needed for regulating the production of antimicrobial peptides, have increased bacterial burdens on day 2 and 3 post infection (Figure 4.3). Furthermore, wild type

larvae harboring *ΔphoP* bacterial strains have a significant reduction in bacterial burden throughout the infection. However, the bacterial parent phenotype can be rescued when *ΔphoP Y. pestis* is used to colonize *imd* deficient fruit flies. The generation of reactive oxygen species also appears to be important for host resistance to *Y. pestis* colonization. Larvae lacking expression of *duox*, a gene needed for producing oxygen radicals in the midgut, had significantly higher bacterial burdens for both wild type and *ΔoxyR Y. pestis* strains on day 3 post infection. In addition, the *ΔoxyR* strain had decreased bacterial burden in larvae that were capable of producing ROS at this same time point. Similarly to the data obtained in *O. montana* fleas, bacteria grown at 37°C seem to be primed for resistance to initial ROS host responses during the first 24 hours of bacterial infection. The data demonstrates that

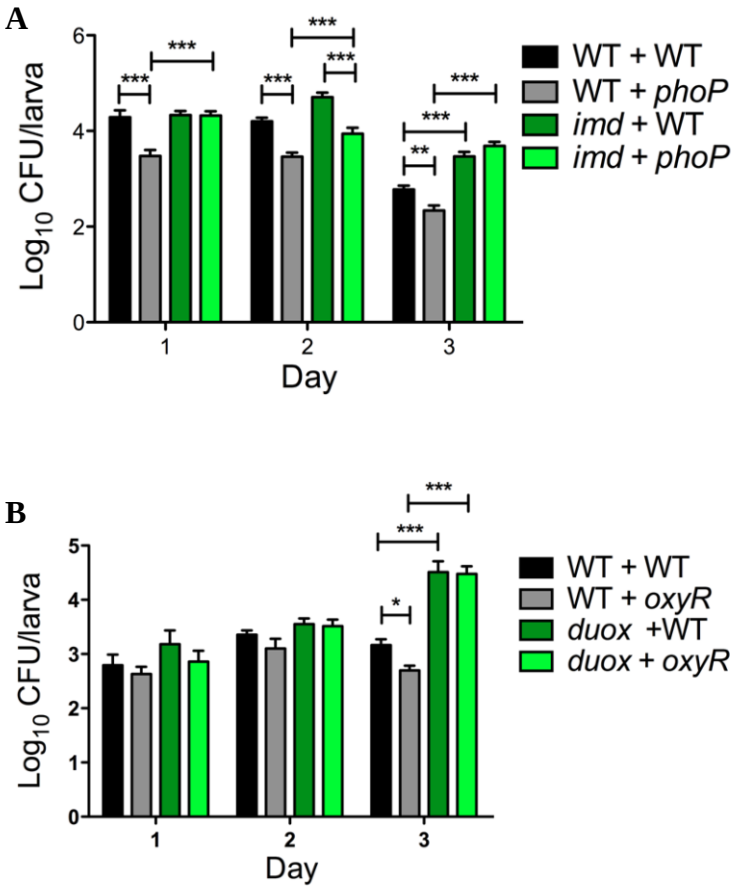

Figure 4.3 ***Knockout of host immune responses increases Y. pestis burden in the digestive tract.*** Transgenic fruit fly larvae lacking the imd (A) or duox (B) genes were challenged with 10^5 CFU of the indicated strains of *Y. pestis*. On each day, 5 larvae were mechanically disrupted and enumerated for bacterial burden on *Yersinia* selective agar. Shown is pooled data from 3 independent experiments. Data was analyzed using Student's t-test. * = p< .05, ** = p< .01, ***= p< .001. (Marketon)

Drosophila larvae can be stably infected with *Y. pestis.* Furthermore, this data provides the first evidence that a direct connection exists between insect midgut immune genes and the concentration of plague in the insect digestive tract.

4.4.3 Establishing outbred laboratory flea colonies

In order to determine if genetic drift is influencing *Y. pestis* colonization, an outbred *X. cheopis* population was established from fleas obtained from *Rattus norvegicus* in downtown Los Angeles. *X. cheopis* parasitizes rats inhabiting Los Angeles County, California. The L.A. county department of public health has a rat surveillance program to monitor flea burdens and the incidence of flea borne pathogens. With help from the L.A county department of public health, tomahawk traps were set with dog biscuits coated in peanut butter at two sites in downtown Los Angeles. Over the course of 6 hours, from dusk until 1 A.M. during late October, 12 rats were caught from two sites in downtown Los Angeles. Rats were combed for parasites and 26 fleas were recovered (~2.17 fleas/rat). Fleas were placed into a 50ml conical tube for overnight shipping back to Columbia, Missouri. While combing rats, users wore full tyvek suits with hoods and hepa-filtered power air purified respirators. These precautions were taken due to the presence of rats in Los Angeles county that previously tested seropositive for Seoul virus, a hantavirus with significant morbidity (39). Upon receiving the shipment, fleas were placed into a one gallon glass jar with 2 inches of larval rearing medium and stored in a refrigerated incubator maintained at 22°C with 80% relative humidity (RH). Fleas were fed bloodmeals 3 times a week to ensure their survival and maximize their reproductive success.

It is known that fleas from this area may be infected with *Bartonella spp.* and/or *Rickettsia typhi (40).* In order to ensure fleas were not infected with these pathogenic organisms, the population was propagated for 6 weeks and handled as if they may be infected with a flea borne pathogen using BSL-2 protocols. Primers were generated to PCR amplify the *gltA* open reading frame, a conserved metabolic gene encoding citrate synthase, for both *Bartonella* and *Rickettsia spp. (40, 41)*. Twenty fleas (approximately $1/4^{th}$ of the total population) were negative for amplification of both the aforementioned bacterial genus's *gltA* genes (Figure 4.4). Fleas were also screened via PCR for the presence of *Y. pestis* using primers specific for *hmsF*, as expected, all fleas were negative (Data not shown). While it is difficult to tell if fleas were infected at the time of collection, the rearing of fleas under laboratory conditions may be sufficient for the loss of these bacteria from the insect flora. Alternatively, providing a bloodmeal spiked with appropriate antibiotics may have been applied if fleas had tested positive for bacterial pathogens.

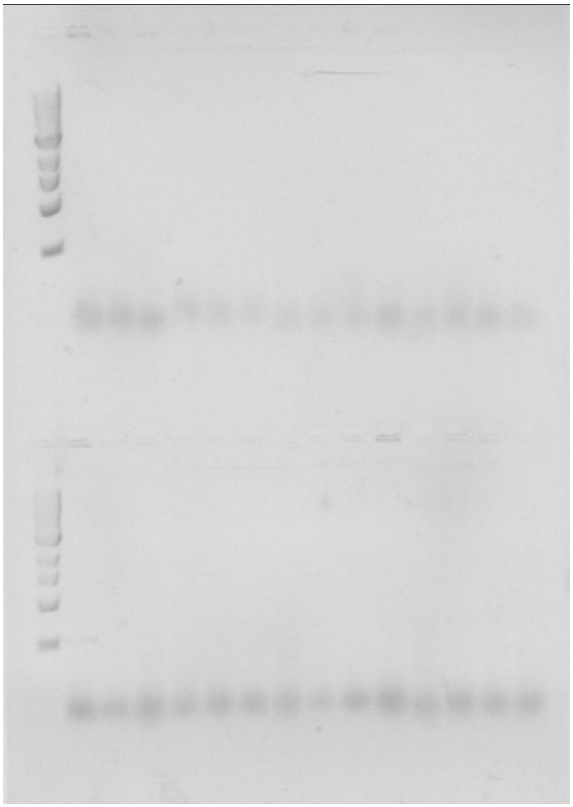

Figure 4.4 ***LA12 Fleas do not contain Bartonella or Rickettsia spp.*** Fleas were mechanically disrupted in 5ul of sterile ddH_2O. Flea homogenates were used in a Taq polymerase PCR reaction and reactions were loaded on a 1% agarose gel and were screened through electrophoresis for the presence of either *Batonella* (top) or *Rickettsia gltA* DNA. Each lane represents an individual flea. Lanes are designated by primers seen at the bottom of each lane. *gltA* amplicon = ~700bp. Shown on the left is a 1kb DNA standard.

4.4.4 Inbred flea populations are more permissive to Y. pestis colonization

Utilizing the newly established *X. cheopis* flea population, which will henceforth be referred to as the "LA12" strain, we sought to understand whether genetic drift had influenced the ability of *Y. pestis* to colonize the digestive tract. Strikingly, when we compared the inbred *X. cheopis* strain established from Baltimore, which will be referred to as BALT, to the LA12 strain, we noticed that the BALT fleas were noticeably larger when fed to repletion. In order to confirm this observation, fleas were measured using an ocular micrometer. The dimensions of the BALT fleas were significantly larger than their LA12 counterparts (Table 4.1).

Table 4.1 Flea Dimensions

Flea Strain	**Dimensions (mm)**		
	Length	**Height**	**Width**
***X. Cheopis* (LA12)**	1.93 ± .2	.74 ± .1	.47 ± .08
***X. Cheopis* (BALT)**	2.36 ± .31*	.96 ± .14*	.72 ± .15*
***O. montana* (OMT)**	2.09 ± .15*	.79 ± .09	.54 ± .07

n = 20, half male/female. Data was analyzed using a One-way ANOVA (Holm-Sidak vs. control). *$p < .05$.

As a population, BALT fleas are about 20% longer and taller and 30% wider than their LA12 counterparts. This data suggested to us that the BALT strain may be capable of taking larger infectious bloodmeals. In contrast, the *Oropsylla montana* fleas, which will be referred to as "OMT", are slightly longer than the LA12 strain but are not significantly taller or wider following a bloodmeal. Visibly, OMT fleas appear to be similar in size to the LA12 strain when fed to repletion. It is possible that larger blood meals may increase

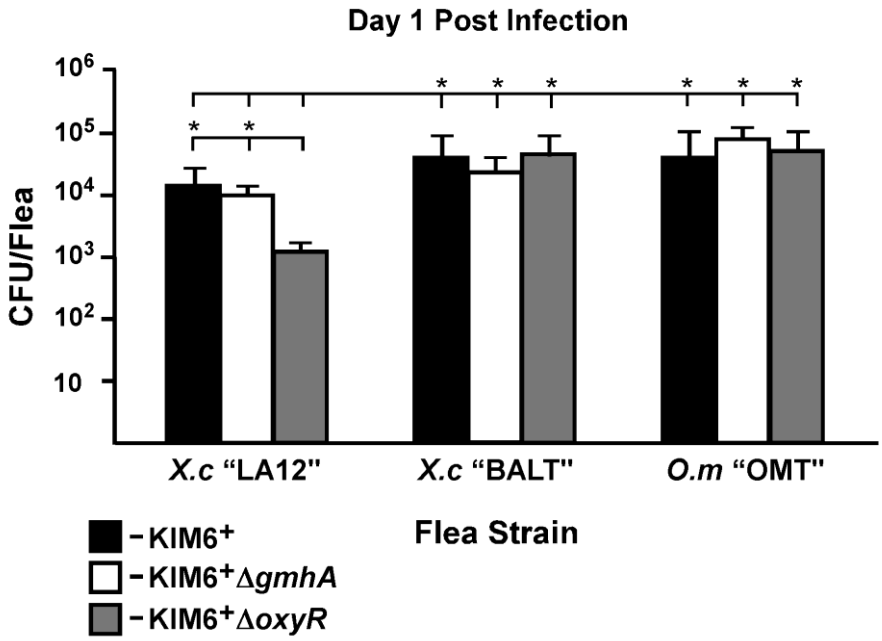

Figure 4.5 ***Inbred flea strains are more permissive to Y. pestis Colonization*** Fleas were allowed to feed on blood spiked with with $1x10^9$ CFU of the indicated strains of *Y. pestis*. On day one post infection, fleas were mechanically disrupted and enumerated on heart infusion agar with ampicillin. Data was pooled from 2 independent experiments. Bars represent groups of 12-26 fleas of the indicated species and recovery location. Fleas that did not harbor *Y. pestis* were eliminated from CFU analysis. *X.c.* = *Xenopsylla cheopis*. *O.m.* = *Oropsylla montana*. Limit of detection = 50 CFU. Ceiling of detection = 10^5 CFU. Data was analyzed using a One-way ANOVA with Holm-Sidak multiple comparison. $p < .05$

the challenge dose taken by the BALT strain. This bloodmeal discrepancy may contribute to plague persistence later during infection.

In order to determine if outbred strains were, overall, more permissive to colonization, LA12, BALT, and OMT fleas were infected with $1x10^9$ CFU/ml of KIM6, *ΔgmhA*, and *ΔoxyR* EPS positive strains of *Y. pestis*. Groups of fleas were enumerated for bacterial burden on day 1 post infection. Interestingly, both inbred strains, OMT and BALT, harbored significantly higher bacterial burdens than the outbred LA12 strain. While it is possible that the increase in bacterial burdens among the BALT fleas is related to the size of the infectious bloodmeal, the data for the OMT strain would refute that

hypothesis. There was no significant difference between OMT and LA12 flea dimensions, suggesting that the differences in bacterial recovery are independent of the size of the infectious bloodmeal. Furthermore, within the LA12 fleas, there was significantly less recovery of the *ΔoxyR* strain, more than one log reduction in CFU, relative to the parental *Y. pestis* strain. This phenotype contrasts with the inbred flea populations as there was no significant difference in bacterial recovery of *ΔoxyR* bacteria within BALT or OMT fleas.

Together, the data are consistent with the hypothesis that outbred fleas are more permissive to establishment of *Y. pestis* in the digestive tract early during infection. The data suggests that production of ROS and AMPs are important for colonization of the flea midgut early during infection. Furthermore, it would appear that the ROS response is more robust in the outbred LA12 fleas than their inbred counterparts.

4.5 Discussion

The genetic contribution of the flea host remains a major question for flea vector competence that has not been rigorously addressed at a functional level. While insect immune responses are fairly well characterized in other disease vectors, the flea host response and its importance for establishment of plague has been largely unexamined. Using strains that are more susceptible to cationic antimicrobials and reactive oxygen species, we conclusively show that there is decreased *Y. pestis* colonization of the flea midgut early during infection. Using these data, combined with what is already known about the flea life cycle of *Y. pestis*, we are able to develop a model of the first week of infection within the flea digestive tract (Figure 4.6).

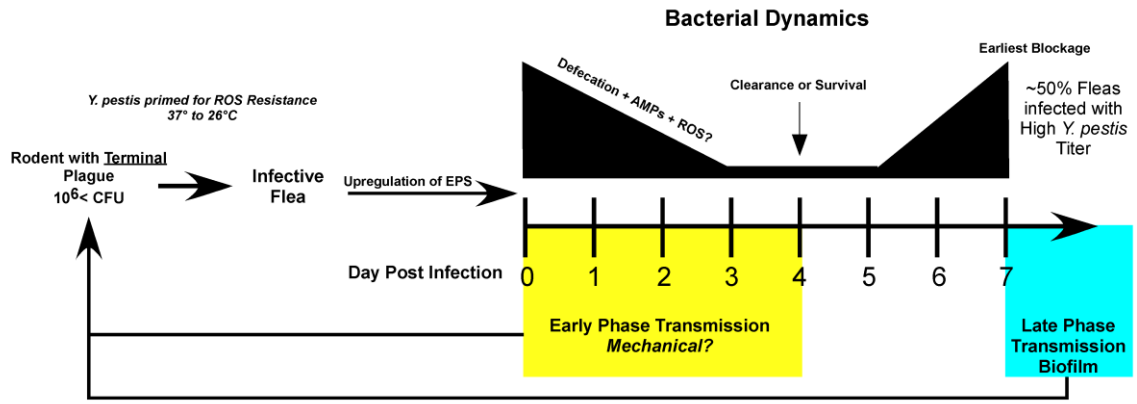

Figure 4.6 ***Model of the first week of flea midgut infection with Y. pestis*** The flea acquires an infectious bloodmeal by feeding on a mammal with a high titer of *Y. pestis* in the bloodstream. *Y. pestis* transitioning from the mammalian temperature (37°C) to the flea temperature (21-22°C) are resistant to reactive oxygen species (ROS) and induce expression of exopolysaccharide (EPS) in order to initiate bacterial aggregation. During the first 3 days of infection, bacterial concentrations decrease due to flea defecation and production of antimicrobial peptides (AMP)s. Fleas are capable of transmitting *Y. pestis*, through an unknown mechanism, during the first 4 days of infection. A large percentage of infected fleas are capable of clearing *Y. pestis* infection during days 3-5. Fleas that remain infected increase bacterial concentrations in the digestive tract through replication from days 5-7. Typically, around half of the flea population remains infected with *Y. pestis* by day 7. Once biofilm-mediated blockage occurs, fleas can transmit plague bacilli to a new mammalian host through the Late Phase mechanism.

Once a flea acquires an infectious bloodmeal from a rodent with terminal plague, the flea digestive tract is exposed to *Y. pestis*. Initially, *Y. pestis* transitioning from the mammalian temperature (37°C) to the flea temperature (21-22°C) are resistant to reactive oxygen species produced in the midgut. The temperature shift also induces expression of exopolysaccharide, which initiates biofilm formation by promoting bacterial aggregation. During the first 3 days of infection, the majority of bacteria appears to be cleared from the digestive tract. Likely due to the frequent defecation of recently fed fleas as well as the production of antimicrobial peptides. Also during this time frame, Early Phase Transmission can occur, possibly through a mechanical transmission mechanism. Days 3-5 represents a critical juncture whereby *Y. pestis* either persists and replicates in the digestive tract or is cleared by the flea. Fleas that remain infected increase bacterial concentrations from days 5-7, typically with about half of the population capable of

clearing the infection. From day 7 onward, blockage of the insect esophagus is a possibility. Once biofilm-mediated blockage occurs, fleas can transmit plague bacilli to a naïve host through the Late Phase mechanism, perpetuating the *Y. pestis* life cycle.

While antimicrobial resistance appears to be needed throughout the first week of midgut colonization, ROS resistance appears to follow a different infection pattern in OMT fleas and *Drosophila* larvae. The *ΔoxyR* strain has a colonization pattern that is indistinguishable from WT during the first 3 days of infection, however, after fleas receive a maintenance feed there is a dramatic reduction in bacterial colonization. This finding is interesting for a number of reasons. This data would suggest that bacteria grown at 37°C have been primed for resisting ROS during the initial infection, but are far more susceptible to the oxidative burst initiated by a new blood feed after multiple days of residing at lower temperatures (21-22°C) in the insect midgut. This phenotype is recapitulated in the *Drosophila* midgut where *ΔoxyR* titers are indistinguishable from wild type bacteria during the first two days of infection. Furthermore, it appears as though the overall ROS response by inbred strains may be dampened in inbred flea strains considering the fact that there is a significant increase in *Y. pestis* burden for BALT and OMT fleas compared to the LA12 strain. The LA12 strain appears to be significantly better at clearing *ΔoxyR* bacteria than both of the other inbred flea strains. The differences in flea size complicate the interpretation of this data. However, due to the fact that LA12 and BALT strains have similar sizes (and likely similar bloodmeal volumes), it seems plausible that flea inbreeding alters the natural course of *Y. pestis* infection. Further studies examining the day 0 flea burden are needed to address this issue.

We show that the *Drosophila* oral infection model is a viable alternative for studying the early host response to *Y. pestis*. This model identifies *imd* and *duox* as genetic elements that mitigate the establishment of plague in the insect midgut. In the future, once flea sequencing data becomes available, performing q-PCR studies on these genes over the course of the infection will allow us to better understand the flea immune response. The fruit fly model offers a clear advantage over *C. elegans* research models because the digestive tract is actively engaged in the infection process. Furthermore, genetic systems in *Drosophila* are far more advanced and abundant than in any other arthropod model system. This system may also be useful in addressing biological questions regarding the timing and importance of early biofilm formation. The factors that mediate initial adherence of bacteria to the flea digestive tract remain unknown. This system may be useful for determining if specific host cell receptors or bacterial proteins directly influence the midgut adherence process.

The influence of genetic drift on experimental flea colonies has not previously been examined. We show the first evidence that inbred flea populations have distinct differences in size as well as an ability to harbor increased concentrations of *Y. pestis*. Further experimentation is required to directly pinpoint the cause of this disparity between inbred and outbred flea populations, however the data is suggestive that researchers should consider generating genetically heterogeneous flea populations. This may be achieved through establishment of outbred colonies or through the introduction of external genetic information into existing populations through breeding. We have provided some simple techniques and practices to ensure the safe collection and establishment of outbred flea populations. These techniques may be useful for

introducing genetic heterogeneity into currently existing flea model systems and avoiding concerns associated with genetic drift. Alternatively, the development of new methodology for handling fleas may be useful for mitigating the influence of genetic drift. If eggs can be recovered, stored for extended periods, and subsequently induced to hatch prior to infection, this would provide a valuable tool for maintaining genetic heterogeneity. This practice would have the added benefit of normalizing the age of the fleas and may improve flea feeding efficiency of the initial infectious bloodmeal.

Considering *Y. pestis*'s unique characteristic of only interacting with the insect digestive tract, it seems to be an ideal candidate for modeling the insect gut response to prolonged microbial colonization. Here we make strides in identifying critical pathogen and host genetic factors that are important for establishment of plague bacilli in the insect midgut. In addition, improvements in our understanding of bacterial dynamics during the first week of flea colonization will allow us to ask specific biological questions about early and late phase transmission events. Furthermore, we identify limitations with existing flea model systems that researchers should take into consideration when designing experimental protocols and handling laboratory insect colonies. We hope this work encourages researchers to take a systems biology approach for studying the complexities of the *Y. pestis* life cycle.

4.6 Acknowledgements

We would like to acknowledge Dr. Joe Hinnebusch and Dr. Kenneth Gage for providing both *Xenopsylla cheopis* and *Oropsylla montana* fleas. We would especially like to thank Dr. Melanie Marketon for graciously contributing the *Y. pestis* strains that were critical for generating these studies. In addition we thank Dr. Marketon for providing the *Drosophila* bacterial enumeration data. We would also like to thank Michael Rood of the Los Angeles County Department of Public Health for his invaluable assistance trapping rats and the subsequent collection of the Los Angeles *X. cheopis* population.

4.7 References

1. **Hinnebusch BJ.** 2005. The evolution of flea-borne transmission in *Yersinia pestis*. Curr Issues Mol Biol **7:**197-212.

2. **Burroughs AL.** 1947. Sylvatic plague studies: The vector efficiency of nine species of fleas compared with *Xenopsylla cheopis*. The Journal of hygiene **45:**371-396.

3. **Kartman L, Prince FM, Quan SF.** 1956. Studies on *Pasteurella pestis* in fleas, comparative plague-vector efficiency of *Xenopsylla vexabilis hawaiiensis* and *Xenopsylla cheopis*. Bulletin of the World Health Organization **14:**681-704.

4. **Lorange EA, Race BL, Sebbane F, Joseph Hinnebusch B.** 2005. Poor vector competence of fleas and the evolution of hypervirulence in *Yersinia pestis*. J Infect Dis **191:**1907-1912.

5. **Eisen RJ, Wilder AP, Bearden SW, Montenieri JA, Gage KL.** 2007. Early-phase transmission of *Yersinia pestis* by unblocked *Xenopsylla cheopis* (Siphonaptera: Pulicidae) is as efficient as transmission by blocked fleas. J Med Entomol **44:**678-682.

6. **Chouikha I, Hinnebusch BJ.** 2012. *Yersinia*--flea interactions and the evolution of the arthropod-borne transmission route of plague. Current opinion in microbiology **15:**239-246.

7. **Jarrett CO, Deak E, Isherwood KE, Oyston PC, Fischer ER, Whitney AR, Kobayashi SD, DeLeo FR, Hinnebusch BJ.** 2004. Transmission of *Yersinia pestis* from an infectious biofilm in the flea vector. J Infect Dis **190:**783-792.

8. **Zhou W, Russell CW, Johnson KL, Mortensen RD, Erickson DL.** 2012. Gene expression analysis of *Xenopsylla cheopis* (Siphonaptera: Pulicidae) suggests a role for reactive oxygen species in response to *Yersinia pestis* infection. J Med Entomol **49:**364-370.

9. **Gillespie JP, Kanost MR, Trenczek T.** 1997. Biological mediators of insect immunity. Annual review of entomology **42:**611-643.

10. **Houk EJ, Obie F, Hardy JL.** 1979. Peritrophic membrane formation and the midgut barrier to arboviral infection in the mosquito, *Culex tarsalis* Coquillett (Insecta, Diptera). Acta tropica **36:**39-45.

11. **Xu Q, Lu A, Xiao G, Yang B, Zhang J, Li X, Guan J, Shao Q, Beerntsen BT, Zhang P, Wang C, Ling E.** 2012. Transcriptional profiling of midgut immunity response and degeneration in the wandering silkworm, *Bombyx mori*. PLoS ONE **7:**e43769.

12. **Gakhar SK, Shandilya HK.** 2001. Midgut specific immune response of vector mosquito A*nopheles stephensi* to malaria parasite *Plasmodium*. Indian journal of experimental biology **39:**287-290.

13. **Lehane MJ, Wu D, Lehane SM.** 1997. Midgut-specific immune molecules are produced by the blood-sucking insect *Stomoxys calcitrans*. Proc Natl Acad Sci U S A **94:**11502-11507.

14. **Pakpour N, Akman-Anderson L, Vodovotz Y, Luckhart S.** 2013. The effects of ingested mammalian blood factors on vector arthropod immunity and physiology. Microbes Infect **15:**243-254.

15. **Flores-Cruz Z, Allen C.** 2011. Necessity of OxyR for the hydrogen peroxide stress response and full virulence in *Ralstonia solanacearum*. Appl Environ Microbiol **77:**6426-6432.

16. **Lau GW, Britigan BE, Hassett DJ.** 2005. *Pseudomonas aeruginosa* OxyR is required for full virulence in rodent and insect models of infection and for resistance to human neutrophils. Infect Immun **73:**2550-2553.

17. **Wei Q, Minh PN, Dotsch A, Hildebrand F, Panmanee W, Elfarash A, Schulz S, Plaisance S, Charlier D, Hassett D, Haussler S, Cornelis P.** 2012. Global regulation of gene expression by OxyR in an important human opportunistic pathogen. Nucleic Acids Res **40:**4320-4333.

18. **Taylor PL, Blakely KM, de Leon GP, Walker JR, McArthur F, Evdokimova E, Zhang K, Valvano MA, Wright GD, Junop MS.** 2008. Structure and function of sedoheptulose-7-phosphate isomerase, a critical enzyme for lipopolysaccharide biosynthesis and a target for antibiotic adjuvants. J Biol Chem **283:**2835-2845.

19. **Bauer BA, Stevens MK, Hansen EJ.** 1998. Involvement of the *Haemophilus ducreyi gmhA* gene product in lipooligosaccharide expression and virulence. Infect Immun **66:**4290-4298.

20. **Darby C, Ananth SL, Tan L, Hinnebusch BJ.** 2005. Identification of *gmhA*, a *Yersinia pestis* gene required for flea blockage, by using a *Caenorhabditis elegans* biofilm system. Infect Immun **73:**7236-7242.

21. **Oyston PC, Dorrell N, Williams K, Li SR, Green M, Titball RW, Wren BW.** 2000. The response regulator PhoP is important for survival under conditions of macrophage-induced stress and virulence in *Yersinia pestis*. Infect Immun **68:**3419-3425.

22. **Rebeil R, Jarrett CO, Driver JD, Ernst RK, Oyston PC, Hinnebusch BJ.** 2013. Induction of the *Yersinia pestis* PhoP-PhoQ regulatory system in the flea and its role in producing a transmissible infection. J Bacteriol **195:**1920-1930.

23. **Hitchen PG, Prior JL, Oyston PC, Panico M, Wren BW, Titball RW, Morris HR, Dell A.** 2002. Structural characterization of lipo-oligosaccharide (LOS) from *Yersinia pestis*: regulation of LOS structure by the PhoPQ system. Mol Microbiol **44:**1637-1650.

24. **Reines M, Llobet E, Llompart CM, Moranta D, Perez-Gutierrez C, Bengoechea JA.** 2012. Molecular basis of *Yersinia enterocolitica* temperature-dependent resistance to antimicrobial peptides. J Bacteriol **194:**3173-3188.

25. **Erickson DL, Russell CW, Johnson KL, Hileman T, Stewart RM.** 2011. PhoP and OxyR transcriptional regulators contribute to *Yersinia pestis* virulence and survival within *Galleria mellonella*. Microb Pathog **51:**389-395.

26. **De Gregorio E, Spellman PT, Tzou P, Rubin GM, Lemaitre B.** 2002. The Toll and Imd pathways are the major regulators of the immune response in *Drosophila*. EMBO J **21:**2568-2579.

27. **Tanji T, Hu X, Weber AN, Ip YT.** 2007. Toll and IMD pathways synergistically activate an innate immune response in *Drosophila melanogaster*. Mol Cell Biol **27:**4578-4588.

28. **Kumar S, Molina-Cruz A, Gupta L, Rodrigues J, Barillas-Mury C.** 2010. A peroxidase/dual oxidase system modulates midgut epithelial immunity in *Anopheles gambiae*. Science **327:**1644-1648.

29. **Stapp P, Salkeld DJ, Eisen RJ, Pappert R, Young J, Carter LG, Gage KL, Tripp DW, Antolin MF.** 2008. Exposure of small rodents to plague during epizootics in black-tailed prairie dogs. Journal of wildlife diseases **44:**724-730.

30. **Wilder AP, Eisen RJ, Bearden SW, Montenieri JA, Gage KL, Antolin MF.** 2008. *Oropsylla* hirsuta (Siphonaptera: Ceratophyllidae) can support plague epizootics in black-tailed prairie dogs (*Cynomys ludovicianus*) by early-phase transmission of *Yersinia pestis*. Vector Borne Zoonotic Dis **8:**359-367.

31. **Eisen RJ, Eisen L, Gage KL.** 2009. Studies of vector competency and efficiency of North American fleas for *Yersinia pestis*: state of the field and future research needs. J Med Entomol **46:**737-744.

32. **Latter BD, Mulley JC, Reid D, Pascoe L.** 1995. Reduced genetic load revealed by slow inbreeding in *Drosophila* melanogaster. Genetics **139:**287-297.

33. **Latter BD, Mulley JC.** 1995. Genetic adaptation to captivity and inbreeding depression in small laboratory populations of *Drosophila melanogaster*. Genetics **139:**255-266.

34. **Schlenke TA, Begun DJ.** 2005. Linkage disequilibrium and recent selection at three immunity receptor loci in *Drosophila simulans*. Genetics **169:**2013-2022.

35. **Olcott MH, Henkels MD, Rosen KL, Walker FL, Sneh B, Loper JE, Taylor BJ.** 2010. Lethality and developmental delay in *Drosophila melanogaster* larvae after ingestion of selected *Pseudomonas fluorescens* strains. PLoS ONE **5:**e12504.

36. **Bobrov AG, Kirillina O, Forman S, Mack D, Perry RD.** 2008. Insights into *Yersinia pestis* biofilm development: topology and co-interaction of Hms inner membrane proteins involved in exopolysaccharide production. Environ Microbiol **10:**1419-1432.

37. **Jones HA, Lillard JW, Jr., Perry RD.** 1999. HmsT, a protein essential for expression of the haemin storage (Hms+) phenotype of *Yersinia pestis*. Microbiology **145 (Pt 8):**2117-2128.

38. **Hinnebusch BJ, Perry RD, Schwan TG.** 1996. Role of the *Yersinia pestis* hemin storage (hms) locus in the transmission of plague by fleas. Science **273:**367-370.

39. **Smith HM, Reporter R, Rood MP, Linscott AJ, Mascola LM, Hogrefe W, Purcell RH.** 2002. Prevalence study of antibody to ratborne pathogens and other agents among patients using a free clinic in downtown Los Angeles. J Infect Dis **186:**1673-1676.

40. **Billeter SA, Gundi VA, Rood MP, Kosoy MY.** 2011. Molecular detection and identification of *Bartonella* species in *Xenopsylla cheopis* fleas (Siphonaptera: Pulicidae) collected from *Rattus norvegicus* rats in Los Angeles, California. Appl Environ Microbiol **77:**7850-7852.

41. **Stevenson HL, Labruna MB, Montenieri JA, Kosoy MY, Gage KL, Walker DH.** 2005. Detection of *Rickettsia felis* in a New World flea species, *Anomiopsyllus nudata* (Siphonaptera: Ctenophthalmidae). J Med Entomol **42:**163-167.

Chapter 5

SUMMARY AND FUTURE PERSPECTIVES

5.1 Summary

Here we present data that supports the stated aims of developing genetic tools for plague research, improving *Y. pestis* model systems, identifying plague virulence factors, and determining the insect host contribution towards permitting a stable midgut infection. First, we show that diaminopimelic acid selection can be used as a safe alternative to generating antibiotic resistant *Y. pestis* strains for *in vivo* virulence studies (1). Strains with a deficiency in the lysine biosynthesis pathway are unable to grow without supplementation of DAP in the growth media. Furthermore, DAP is an amino acid only produced by bacteria, rendering these strains avirulent in humans due to an inability to scavenge DAP from the environment. The DAP project also resulted in the generation of genetic tools for single copy expression of recombinant protein with thermally regulated promoters.

Researchers of microbial pathogenesis follow a set of criteria established by Dr. Stanley Falkow called Molecular Koch's postulates (2). These postulates state that when studying the function of a mutated gene on a pathogen's virulence *in vivo,* restoration of that gene's expression should restore any virulence defects observed when the gene is absent. Typically, this is achieved through cloning the gene of interest into a plasmid construct that also contains an antibiotic resistance cassette. The plasmid can then be transformed into competent mutant bacteria in order to restore expression of the gene of interest. Retention of these plasmids *in vitro* can be selected for by supplementing the

growth or experimental medium with the antibiotic that the plasmid provides resistance for. However, in animal models of disease, it is impractical to continuously provide antibiotics intravenously in order to promote retention of the pathogen's complementing plasmid. Furthermore, this scenario would almost certainly alter the host's resident microbiota, complicating the interpretation of experimental data.

We demonstrate, in the first research chapter, that plasmids are occasionally lost during the course of *Y. pestis* pathogenesis. Using a plasmid with a p15a replication of origin, we show that between 1-5% of bacteria recovered from mouse spleens on day 4 post infection experienced plasmid loss as measured by retention of ampicillin resistance. It is critical for data interpretation that expression of recombinant protein is maintained throughout the disease process. To remedy this, we generated genetic tools using DAP selection and the TN7 transposon system (3). This transposon system induces integration of recombinant DNA directly into the bacterial chromosome, in single copy, at a specific *att*Tn7 site (4). Single copy complementation of the *dapA* mutation is sufficient for restoration of wild type growth and virulence characteristics. Genetic information inserted in this manner is unlikely to be lost due to bacterial growth requirements for DAP and lysine as well as the infrequency of chromosomal reversion. If the insertion was somehow lost, bacteria would be unable to replicate and would rapidly be eliminated from the bacterial population.

The second research chapter investigates the role of the BamC lipoprotein in mammalian virulence and survival in the flea host. BamC is an accessory component of the BAM complex believed to be important for outer membrane biogenesis. Despite the essential function of the complex as a whole, BamC had largely been considered

dispensable due to a lack of growth phenotypes associated with *ΔbamC* strains in *E. coli*. We show that loss of BamC in *Y. pestis* causes a reduction in virulence in murine models of bubonic and septicemic plague and that it is also required for efficient colonization of the flea midgut. This is the first report of a surface exposed protein that is needed for both hosts of the *Y. pestis* life cycle.

Overall, *Y. pestis* BamC is important for bacterial survival during stress conditions *in vivo*. The BamC mutant shows enhanced sensitivity to cationic antimicrobials and acidic pH conditions. These phenotypes are corroborated by reduced survival of the *ΔbamC* mutant in murine macrophages, where the bacteria would encounter a low pH environment. However, extracytoplasmic stress sensitivity does not seem to result from an overall increase in membrane permeability for BamC deficient strains. The *ΔbamC* mutant's resistance to antibiotics with a cytoplasmic target is indistinguishable from the parental strain, yet this strain has significantly increased sensitivity to polymyxin B, which targets OM LPS. Together, the data suggests an OM specific structural inadequacy, with possible implications for LPS production. Under normal growth conditions, in a complete liquid medium, the *ΔbamC* strain incorporated decreased concentrations of OMPs into the bacterial OM. This is consistent with the observation that the extracytoplasmic stress sigma factor, σ^E, is upregulated in BamC deficient strains. Upregulation of sigma E is indicative of increased concentrations of unfolded or misfolded protein in the bacterial periplasm. This suggests BamC is needed either for efficient function of the BAM complex or that this protein is part of the extracytoplasmic stress regulatory network. When inducing osmotic stress by adding sodium chloride to the growth medium, it appears as though some subsets of OMPs are

more affected than others in the *ΔbamC* bacterial background. Furthermore, BamC has no significant homology with proteins of known function. These two facts, paired together, is an indication that BamC may have novel function in regulating protein incorporation within the BAM complex.

The BamC project, in totality, suggests that loss of this protein results in a decrease of multiple OM proteins. When these proteins are reduced in the OM, *Y. pestis* exhibits decreased virulence and colonization defects due to enhanced sensitivity to environmental stresses. However, whether this phenotype is the result of a change in global gene regulation or a decrease in BAM complex efficiency remains to be determined.

The final research chapter establishes that insect host responses influence *Y. pestis* colonization of the flea digestive tract. This chapter also improves the model systems used for studying the *Y. pestis* life cycle in the insect host. We demonstrated that bacterial strains which are more sensitive to antimicrobial insult are not as effective in colonizing the flea digestive tract. The data supports the notion that the flea midgut secretes antimicrobial peptides and ROS in order to try and limit microbial establishment. The *oxyR* strain did not appear to be sensitive to the initial ROS response in *O. Montana* fleas, however, after maintenance blood meal, the strain's ability to persist in the midgut was reduced. Considering the success of wild type *Y. pestis* in the flea digestive tract, the data is consistent with the notion that bacteria leaving the mammalian blood stream have been primed for resisting initial ROS responses within the flea midgut (5). Furthermore, *Y. pestis* has clearly undergone a number of genetic modification events that have improved its success in the flea midgut (6). Due to a lack of sequencing data and tools

for the genetic manipulation fleas, the *Drosophila* larval model provides an alternative system for understanding how *Y. pestis* colonizes the insect host. Not only does the oral infection model verify the postulated antimicrobial resistance defects in mutated bacterial strains, but it validates the role of insect host immune genes in mediating the Y. pestis infection process.

The role of genetic drift in influencing the colonization of flea populations with *Y. pestis* has not been previously examined. In order to study this concern, we established an outbred laboratory population of *X. cheopis* fleas from an urban rat focus. When we compared bacterial burdens between inbred and outbred strains, we found that inbred strains were more permissive to high bacterial titers. While it is true that the X. *cheopis* outbred strain has a larger body size, and therefore, may ingest greater concentrations of *Y. pestis* in the initial infectious bloodmeal, the inbred *O. Montana* strain did not have a significant size difference compared to the outbred LA12 fleas yet carried bacterial burdens that were indistinguishable from the inbred *X. cheopis* strain. Surprisingly, loss of bacterial *oxyR* expression did not reduce midgut bacterial burden in inbred flea populations. However, in the outbred LA12 strain, *Y. pestis* titers were significantly lower for the Δ*oxyR* strain. This is suggestive that ROS secretion may be compromised for inbred flea populations. To avoid these concerns in the future, we have provided methods for the safe collection and screening of fleas. These methods provide guidelines for re-establishing outbred populations of key flea species and generating populations of uncharacterized species, in order to study their capacity as a competent host for plague bacilli (7).

This project increases the tools at the disposal of those researching *Yersinia*-insect interactions. It also supports the standardizing of methods used for the handling of laboratory flea population. Together, this should improve the quality of data the can be acquired and applied to epidemiological modeling of plague and understanding insect midgut immune responses.

5.2 Future Directions

The genetic tools generated for use with diaminopimelic acid auxotrophs are valuable to *Yersinia pestis* researchers as an alternative selection system. While other non-antibiotic based selectable markers exists, such as those for heavy metal resistances, these systems do not provide the same degree of user safety and may cause additional health concerns due to the inevitable handling of toxic metals (8, 9). Despite the risks, investigating the viability of other alternative selectable markers is warranted. The *cysZ* promoter is a valuable genetic tool as its expression is both strong and thermally regulated in *Y. pestis*. This sulfate transport promoter allows researchers to induce expression of recombinant DNA in an environmentally conditional manner. Identifying new *Y. pestis* promoters that are regulated in response to specific environmental queues can be valuable for asking very specific questions about the *Y. pestis* life cycle.

The role of the BamC protein as a BAM complex accessory component remains incompletely understood. While it is clear that BamC interacts with the essential complex member BamD, this protein's function may be independent of OMP biogenesis. The significant loss of virulence associated with *Y. pestis ΔbamC* strains suggests that the protein is more important than previously believed for pathogenic bacteria. Interestingly, it is the only member of the BAM complex accessory proteins with a surface exposed

region. BamC's C-terminal domain has recently been demonstrated to be surface accessible (10). Our own studies using fluorescent microscopy and recombinant FLAG tagged BamC have suggested that antibodies can bind to the C-terminal domain of BamC without permeabilizing the bacterial membrane (Data not shown). Studies where recombinant BamC was expressed with C-terminal truncations would be useful in determining whether the surface exposed region has implications in *Y. pestis* virulence. If this is true, it may cause those researching the BAM complex to rethink the role of BamC as merely a stabilizing protein for the complex. It is possible that BamC may play a role in sensing the external environment in order to modulate the incorporation of OMPs through sigma factor regulation. If the aforementioned hypothesis is true, it would make targeting of BamC with neutralizing antibodies an attractive strategy for disease prevention. This would warrant *in vivo* studies where it could be determined if recombinant BamC or antibodies against specific BamC epitopes provided protection from subsequent *Y. pestis* challenge.

Many questions remain to be addressed with regard to flea-*Yersinia* interactions and the transmission of *Y. pestis* to mammalian hosts. The research indicates that the first 7 days of infection appear to be a critical time in determining whether a *Y. pestis* infection will be cleared or will be able to establish itself within the flea. By day 7 the majority of fleas still infected with *Y. pestis* have allowed the pathogen to establish itself in high titers ($>10^5$ CFU). It is important to note that one week post infection is the earliest time point that biofilm mediated blockage has been observed experimentally (11). Understanding bacterial and insect genes that modulate this early infection process may be critical for developing techniques to prevent the establishment and/or transmission of

Y. pestis. Ideally, the *X. cheopis* chromosome will be sequenced in the near future, however, an intermediary step would involve isolating flea RNA from midgut epithelial cells as the infection progresses. Generating a cDNA library of transcripts produced during the infection process should allow us to compare DNA sequences to closely related immune genes in other sequenced arthropods. This would make it possible for qPCR studies to be performed to study temporal dynamics of flea immune responses.

While the production of EPS is critical for early *Y. pestis* establishment in the digestive tract, combining the loss of EPS with a defect in antimicrobial resistance did not appear to increase bacterial clearance. This result may suggest that some percentage of the bacteria may never reach the midgut and strains defective for EPS production are more susceptible to host immune responses. Furthermore, in studies of early phase transmission, biofilm formation was not required for flea-borne transmission of plague during the first 4 days of infection (12). The possibility exists that there is an EPS-independent mechanism of flea colonization that may involve colonization of another area of the flea. It has been suggested that the flea mouthparts may become contaminated with *Y. pestis* and that bacteria can survive on the surface of chitin for a limited time. If this were true, it would provide a possible mechanism for early phase transmission of plague bacilli. *Y. pestis* present on the surface of the flea mouthparts, specifically the maxillary lacinae and the epipharynx, would repeatedly be in contact with dermal tissue during a feeding event. Mechanical transmission of disease through mouthpart contamination has been documented and even occurs in non-blood feeding arthropods (13-15). Preliminary studies into whether *Y. pestis* can survive on the surface of chitin and for how long may provide a starting point for studying this phenomenon. We

propose that screening the *Y. pestis* proteome for chitin binding proteins may be useful for understanding bacterial adherence to the flea. If the bacteria in early phase transmission originate from the flea digestive tract, the mechanism of its arrival in the host dermis remains elusive and may have more to do with the physiology of the flea bite rather than a directed strategy of the bacterium.

Ideally, researchers will establish a threshold for bacterial burden that denotes when transmission events can actually occur. One of the difficulties with this type of analysis is that bacterial enumeration and excision of midguts for fluorescent microscopic analysis result in termination of experimentally useful organisms. Bioluminescent imaging of bacteria in the flea midgut would allow researchers to rapidly identify infected fleas, roughly estimate the amount of bacteria within flea biofilms, and allow fleas positive for *Y. pestis* to continue the infection process without termination of the experimental insect (16, 17). This imaging process has been successfully employed during *in vivo* challenges with *Y. pestis* in murine experimental models of bubonic plague (18). Our data is suggestive that during the *Y. pestis* colonization process, by day 7, roughly half of the experimental flea population still harbors bacteria. This situation necessitates doubling the number of insects for experimental screening in order to ensure that an exact number of fleas will actually retain bacteria at any late stage time point. The number of fleas required for statistical significance will likely increase when studying arthropod attenuated *Y. pestis* mutants. Furthermore, bacterial dosages traditionally used for infecting fleas with *Y. pestis* in the laboratory are likely larger than the amount of bacteria that the mammalian blood stream can support before the animal succumbs to disease. Using infectious doses consistent with the limitations of the

mammalian bloodstream would likely lead to decreased bacterial colonization percentages of flea populations. Bioluminescent tracking would provide for the acquisition of precise colonization data for physiologically relevant doses. A practically unlimited number of fleas can rapidly be screened using this method of analysis, eliminating concerns of small sample sizes.

In totality, a key piece of information is missing from our understanding of the *Y. pestis* transmission cycle. There is strong evidence to suggest, given our current understanding of the *Y. pestis* infection cycle, that bubonic plague should be self-limiting. Development of bubonic plague is acute, progresses rapidly, and is almost always terminal for the mammalian host. In the insect host, *Y. pestis* eventually forms a biofilm that blocks the flea esophagus, preventing ingestion and ultimately leading to the death of the flea. If this is the entirety of the *Y. pestis* life cycle, how do plague bacilli still persist in the wild? There is no evidence to suggest that *Y. pestis* is capable of existing as a latent infection in mammalian or insect species to re-emerge at a later time with the correct biological trigger. There is limited evidence to suggest that *Y. pestis* survives environmentally, within the soil, however the mechanism of how contaminated soil would be reintroduced into either host currently lacks experimental demonstration (19, 20). As such, comprehensive examination of the *Y. pestis* life cycle is needed to address the missing piece of *Y. pestis* environmental stability.

5.3 Contributions to the Field

The DAP selection project provides safe and practical genetic tools for the study of plague pathogenesis. The system avoids concerns over *in vivo* plasmid loss to ensure expression of recombinant DNA throughout the disease process. Principle investigators

concerned with safety can use this system in order to reduce health risks for select agent users. The possibility exists that DURC concerns may cause stricter regulations on antibiotic use to be implemented on Tier 1 Select Agents by the CDC in the future, including eliminating the use of antibiotic resistance cassettes in fully virulent *Y. pestis (21)*. If this were to occur, this is one of the few developed and rigorously tested genetic systems for *Y. pestis* that would comply with the standards set by Koch's molecular postulates.

Data presented supports the notion that BamC important for *in vivo* survival of pathogenic Eubacteria in refractory host environments. We propose a new hypothesis for novel function of BamC in regulating insertion of specific protein subsets into the Gram-negative bacterial OM. Our data provides the first evidence of a surface exposed virulence factor that is important for bacterial survival in both mammalian and insect hosts. As a virulence factor capable of inducing a mammalian immunogenic response, BamC is a potential therapeutic target that may be broadly applicable to multiple Gram-negative bacterial pathogens (22).

The final chapter provides direct evidence that flea host immune responses play a role in *Y. pestis* colonization of the midgut. The data is consistent with a requirement for EPS production that likely reduces bacterial clearance early during infection due to biofilm mediated resistance to host immune response. BamC colonization data supports the notion that there are additional OMPs utilized by *Y. pestis* for survival in the flea midgut beyond those implicated in EPS production and Ymt mediated resistance. In addition, we provide evidence for the applicability of a *Drosophila* oral infection model for studying early *Y. pestis*-insect midgut interactions. Finally, we show that flea

inbreeding may cause laboratory flea populations to be more permissive to the colonization of plague, perhaps due to a dampening of innate immunity. Our studies support a strategy whereby flea researchers are proactive in promoting genetic heterogeneity in laboratory flea populations.

5.4 References

1. **Bland DM, Eisele NA, Keleher LL, Anderson PE, Anderson DM.** 2011. Novel genetic tools for diaminopimelic acid selection in virulence studies of *Yersinia pestis*. PLoS ONE **6:**e17352.

2. **Falkow S.** 1988. Molecular Koch's postulates applied to microbial pathogenicity. Reviews of infectious diseases **10 Suppl 2:**S274-276.

3. **Choi KH, Gaynor JB, White KG, Lopez C, Bosio CM, Karkhoff-Schweizer RR, Schweizer HP.** 2005. A Tn7-based broad-range bacterial cloning and expression system. Nat Methods **2:**443-448.

4. **Waddell CS, Craig NL.** 1989. Tn7 transposition: recognition of the attTn7 target sequence. Proc Natl Acad Sci U S A **86:**3958-3962.

5. **Vadyvaloo V, Jarrett C, Sturdevant DE, Sebbane F, Hinnebusch BJ.** 2010. Transit through the flea vector induces a pretransmission innate immunity resistance phenotype in *Yersinia pestis*. PLoS Pathog **6:**e1000783.

6. **Erickson DL, Waterfield NR, Vadyvaloo V, Long D, Fischer ER, Ffrench-Constant R, Hinnebusch BJ.** 2007. Acute oral toxicity of *Yersinia pseudotuberculosis* to fleas: implications for the evolution of vector-borne transmission of plague. Cell Microbiol **9:**2658-2666.

7. **Eisen RJ, Eisen L, Gage KL.** 2009. Studies of vector competency and efficiency of North American fleas for *Yersinia pestis*: state of the field and future research needs. J Med Entomol **46:**737-744.

8. **Shen Z, Han J, Wang Y, Sahin O, Zhang Q.** 2013. The contribution of ArsB to arsenic resistance in Campylobacter jejuni. PLoS ONE **8:**e58894.

9. **Sone Y, Nakamura R, Pan-Hou H, Itoh T, Kiyono M.** 2013. Role of MerC, MerE, MerF, MerT, and/or MerP in resistance to mercurials and the transport of mercurials in *Escherichia coli*. Biological & pharmaceutical bulletin.

10. **Webb CT, Selkrig J, Perry AJ, Noinaj N, Buchanan SK, Lithgow T.** 2012. Dynamic association of BAM complex modules includes surface exposure of the lipoprotein BamC. Journal of molecular biology **422:**545-555.

11. **Chouikha I, Hinnebusch BJ.** 2012. *Yersinia*--flea interactions and the evolution of the arthropod-borne transmission route of plague. Current opinion in microbiology **15:**239-246.

12. **Vetter SM, Eisen RJ, Schotthoefer AM, Montenieri JA, Holmes JL, Bobrov AG, Bearden SW, Perry RD, Gage KL.** 2010. Biofilm formation is not required for early-phase transmission of *Yersinia pestis*. Microbiology **156:**2216-2225.

13. **Graczyk TK, Knight R, Tamang L.** 2005. Mechanical transmission of human protozoan parasites by insects. Clinical microbiology reviews **18:**128-132.

14. **Tuppurainen ES, Lubinga JC, Stoltsz WH, Troskie M, Carpenter ST, Coetzer JA, Venter EH, Oura CA.** 2012. Mechanical transmission of lumpy skin disease virus by *Rhipicephalus appendiculatus* male ticks. Epidemiology and infection**:**1-6.

15. **Desquesnes M, Dia ML.** 2004. Mechanical transmission of *Trypanosoma vivax* in cattle by the African tabanid *Atylotus fuscipes*. Veterinary parasitology **119:**9-19.

16. **Francis KP, Yu J, Bellinger-Kawahara C, Joh D, Hawkinson MJ, Xiao G, Purchio TF, Caparon MG, Lipsitch M, Contag PR.** 2001. Visualizing pneumococcal infections in the lungs of live mice using bioluminescent *Streptococcus pneumoniae* transformed with a novel gram-positive lux transposon. Infect Immun **69:**3350-3358.

17. **Lane MC, Alteri CJ, Smith SN, Mobley HL.** 2007. Expression of flagella is coincident with uropathogenic *Escherichia coli* ascension to the upper urinary tract. Proc Natl Acad Sci U S A **104:**16669-16674.

18. **Nham T, Filali S, Danne C, Derbise A, Carniel E.** 2012. Imaging of bubonic plague dynamics by in vivo tracking of bioluminescent *Yersinia pestis*. PLoS ONE **7:**e34714.

19. **Ayyadurai S, Houhamdi L, Lepidi H, Nappez C, Raoult D, Drancourt M.** 2008. Long-term persistence of virulent *Yersinia pestis* in soil. Microbiology **154:**2865-2871.

20. **Eisen RJ, Petersen JM, Higgins CL, Wong D, Levy CE, Mead PS, Schriefer ME, Griffith KS, Gage KL, Beard CB.** 2008. Persistence of *Yersinia pestis* in soil under natural conditions. Emerg Infect Dis **14:**941-943.

21. **Frankel MS.** 2012. Regulating the boundaries of dual-use research. Science **336:**1523-1525.

22. **Thomas S, Luxon BA.** 2013. Vaccines based on structure-based design provide protection against infectious diseases. Expert review of vaccines **12:**1301-1311.

VITA

David M Bland was born in Springfield, Illinois in 1985 and is son to Raymond and Kimberly Bland and brother to Steven Bland. In 2003, David graduated from Springfield High School where he enjoyed playing travel ice hockey and running the 110m high hurdles in track & field. While attending Monmouth College in Monmouth, Illinois he held multiple leadership positions in the Zeta Beta Tau fraternity, enjoyed attending the Fighting Scot's football games, read the novels of Kurt Vonnegut, and became a live music enthusiast. In 2007, David received his Bachelor of Arts degree in Biology with departmental honors.

In the summer of 2008, David was accepted into the Molecular Microbiology & Immunology/Veterinary Pathobiology joint graduate program at The University of Missouri-Columbia and subsequently joined the lab of Dr. Deborah Anderson. After completion of his graduate work, David will begin postdoctoral research at Rocky Mountain Laboratories in Hamilton, Montana in the laboratory of Dr. Joe Hinnebusch. He will continue to research flea-borne transmission of plague.