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WASHINGTON UNIVERSITY IN ST. LOUIS

Division of Biology and Biomedical Sciences
Molecular Microbiology and Microbial Pathogenesis

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Dominant Suppression of Early Innate Immune Mechanisms by *Yersinia pestis*

by

Paul Ault Price

A dissertation presented to the
Graduate School of Arts and Sciences
of Washington University in
partial fulfillment of the
requirements for the degree
of Doctor of Philosophy

December 2011

Saint Louis, Missouri

ABSTRACT OF THE DISSERTATION

Dominant Suppression of Early Innate Immune Mechanisms by *Yersinia pestis*

by

Paul Ault Price

Doctor of Philosophy in Molecular Microbiology and Microbial Pathogenesis

Washington University in St. Louis, 2011

Professor William E. Goldman, Advisor

Disease progression of primary pneumonic plague is biphasic, consisting of a pre-inflammatory and a pro-inflammatory phase. Most lung pathogens induce a measurable inflammatory response within a few hours of infection, but *Yersinia pestis* is strikingly different: the first 36 hours of infection are characterized by rapid bacterial replication in the lung without generating appreciable levels of proinflammatory cytokines/chemokines, histological changes in infected tissue or outward disease symptoms. The ultimate goal of this thesis was to understand the processes that contribute to the pre-inflammatory phase of disease.

Upon inhalation of infectious droplets, *Y. pestis* can interact with a myriad of different cell types, but the initial bacterial / host cell interactions during pulmonary infection are largely uncharacterized. I found that *Y. pestis* cannot be lavaged from the lung during the initial stages of infection but is freely lavageable during the later stages of infection, indicating that *Y. pestis* adheres tightly to lung tissues during the pre-inflammatory phase of disease. Although recent studies have implicated Ail as the *Y. pestis* adhesin that mediates this interaction, my data suggest that Ail acts to stabilize the outer membrane, and the loss of Ail alters other outer membrane proteins. Interestingly, the reintroduction of two major *Yersinia* adhesins, *yadA* and *inv*, into *Y. pestis* reduces the

virulence of *Y. pestis* in both bubonic and pneumonic animal models of infection, offering an explanation as to why these genes were lost during the course of *Y. pestis* evolution.

I also developed a negative selection screen termed transposon site hybridization (TraSH) to identify bacterial factors required for the two different phases of *Y. pestis* pulmonary infection. I validated the technique by defining the essential genes for *Y. pestis* viability, which largely coincided with essential genes found in *Escherichia coli*. However, I was unable to identify any known virulence factors using TraSH following an intranasal infection, including genes involved in the Ysc type-three secretion system (T3SS), which are known to be essential for pulmonary infection, suggesting that there is global *trans*-complementation of attenuated mutants by wild-type *Y. pestis*.

Finally, using a co-infection model of pneumonic plague, I discovered that *Y. pestis* quickly creates a localized, dominant anti-inflammatory state that allows for the survival and rapid growth of both itself and normally avirulent organisms. In contrast, *Y. pseudotuberculosis*, the relatively recent progenitor of *Y. pestis*, and *K. pneumoniae*, another highly virulent lung pathogen, show no similar *trans*-complementation effect, which is unprecedented among other respiratory pathogens. Both the unbiased negative selection screen using a vast pool of *Y. pestis* mutants (TraSH) and the co-infection experiments revealed no selection against any known virulence genes, demonstrating the transformation of the lung into a globally permissive environment during the pre-inflammatory phase of pneumonic plague that allows not only wild-type *Y. pestis* to proliferate rapidly in the lung, but other microbes as well.

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I have had the unique opportunity to work at two wonderful institutions during my graduate studies, Washington University in St. Louis and the University of North Carolina at Chapel Hill. Each institution has presented me with wonderful mentors, colleagues and collaborators. I would like to thank Bill for his constant encouragement during the move from St. Louis to Chapel Hill, even when I was less than helpful at times. Moreover, I would like to thank Bill for the freedom to pursue the different avenues my project presented and the helpful discussions when all seemed wrong with my experiments. His efforts on my behalf have been more than many mentors normally offer. I am also grateful for his vision and insight into microbial systems, which have left a lasting impression on my scientific views and ideas.

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“How many valiant men, lovely ladies and handsome youths whom even Galen, Hippocrates and Aesculapius would have judged to be in perfect health, dined with their family, companions and friends in the morning and then in the evening with their ancestors in the other world?”

Boccaccio, G. (est. 1353) *The Decameron - Introduction*, trans. David Burr, unpublished. Used with permission.

Chapter 1

Introduction:

Background and Significance

Yersinia pestis: A historical perspective

In October 1347, Genoese trading ships put in at Messina Harbor in Sicily, Italy with crews of dead and dying men. The ships had come from the Black Sea port of Caffa (currently Feodosiyya) in the present day Ukraine. The diseased sailors had unfamiliar black swellings about the size of an egg in their groins and armpits. Blood and pus oozed from these swelling, and boils and black patches quickly formed on the rest of their bodies. The afflicted suffered severe pain and died soon after their symptoms became apparent. Before long, other sailors and merchants in Sicily contracted similar symptoms and also started dying. As the disease spread through the town, other symptoms appeared, a continuous fever and bloody sputum, along with the swellings. These new victims sweated profusely and coughed uncontrollable and died soon after becoming sick, often within three days or less, sometimes even within 24 hours (Tuchman, 1978). The disease quickly spread along trade routes and within three years had spread to the entire “known world”, leaving so much death and despair in its wake that people lamented that “this is the end of the world” (Tuchman, 1978).

This disease was plague, manifest in two of its forms: bubonic plague and pneumonic plague. *Yersinia pestis*, the etiological agent responsible for plague, is estimated to have killed more than 200 million people worldwide, although many estimates put the death toll significantly higher (Perry and Fetherston, 1997). Although there are numerous references to “plagues” in many ancient texts that may be attributable to *Y. pestis*, the first great plague pandemic, termed the Justinian Plague, arrived in the Byzantine Empire from East Africa and Egypt in 541 A.D. during the reign of Emperor Justinian I. The initial epidemic ravaged Constantinople from 541 to 544 A.D. and quickly

spread throughout the Middle East and Mediterranean regions. Additional eight- to twelve-year cyclic epidemics lasted until 700 A.D., gradually spreading from region to region until the plague had overrun North Africa, Europe, central and southern Asia and the Middle East. Although the starvation and political unrest that followed each plague epidemic likely played some role, from 541 to 700 A.D., 50% to 60% of the population in these areas had been lost, making the Justinian Plague one of the greatest plagues in history (Russell, 1976).

Over the next 600 years, periodic plague epidemics still occurred in central and southern Asia, whereas Europe largely avoided major plague epidemics. However, that changed when the Genoese trading ships introduced plague into Sicily in 1347 and ushered in the start of the second plague pandemic. The first epidemic (1347 to 1352 A.D.), appropriately known as the Black Death, killed an estimated 18 to 27 million Europeans or an estimated one-third of Europe's population. The death toll was just as great throughout Asia, where an estimated 65 million people are thought to have died directly or indirectly from plague during this same epidemic (McNeill, 1976; Twitchett, 1979). Cyclic epidemics, lasting anywhere from two to twenty years, and the resulting chronic depopulation continued to overwhelm Europe until the end of the 1600's and eventually resulted in many of social, political, economic, religious and even educational changes of the Middle Ages (Defoe and Hurlbut, 1895; Gottfried, 1983; Tuchman, 1978). Various theories have been presented as for the decline of both the first and second pandemics, including changes in public health policy (better use of quarantine both within and between population centers), weather (droughts), rodent populations and even with *Y. pestis* itself (Haensch *et al.*, 2010; Perry and Fetherston, 1997; Slack, 1989; Stenseth *et*

al., 2006; Tuchman, 1978). However, none of these theories alone can account for the decline.

The third and ongoing pandemic is believed to have started in the Chinese province of Yünnan in 1855. Troop traffic from a war between provinces eventually spread the disease to the southern coasts of China, reaching Hong Kong and Canton in 1894. By the turn of the century, steamships and trading vessels had disseminated the plague throughout the world, including to the United States and other parts of the New World for the first time. During the height of the pandemic, over a million people were dying each year in India alone, and an estimated 15 million people worldwide have died since the pandemic began (Echenberg, 2002). This current pandemic is in decline thanks to improved health policy and the advent of antibiotics. However, stable enzootic foci are still found on every major continent except Australia and Europe (Perry and Fetherston, 1997).

The infectious source of the plague was unknown until June 1894 when Louis Pasteur suggested that a young medical physician, Alexandre Yersin, go to Hong Kong to investigate the current plague outbreak. In a makeshift laboratory at the end a hallway just inside the morgue, Yersin was able to isolate *Y. pestis* from fluid aspirated from buboes of a patient who had recently died of the plague. He also injected the same fluid samples into guinea pigs, which subsequently died, and found that their organs were teeming with the same microorganism isolated from the deceased patient. The knowledge that rats had long been associated with the plague and his own observations with guinea pigs lead Yersin to correctly conclude that this microorganism, which infects both rats and humans, caused the plague (Gross, 1995). Although Shibasaburo Kitasato also

reported having isolated the plague microorganism during the same time frame, descriptions of his isolate, which were also isolated from a patient who died of the plague, resemble that of a contaminating pneumococcus more than *Y. pestis* (Perry and Fetherston, 1997).

Epidemiology of *Y. pestis*

The exact route of transmission between rats and humans remained elusive until Paul-Louis Simond, a French colonial Army physician sent by Pasteur to continue Yersin's work in Indochina in 1897, observed that humans seemed to only contract the disease when they came into contact with recently dead warm rats but not with dead cold rats. He postulated that the fleas (*Xenopsylla cheopis*), which leave dead rats when they become cold, might be responsible for the transmission between rats and humans. Upon microscopically examining the fleas, he observed the plague bacterium in the digestive tract of the fleas. Using an ingenious series of experiments involving suspending uninfected rats in cages over plague infected rats with or without fleas, Simond was able to conclude that fleas could transmit plague between hosts (Fig. 1-1) (Gross, 1995). Masanori Ogata is also credited with independently discovering the role of fleas in plague transmission that same year. Currently, there are over 30 species of fleas worldwide that are capable of transmitting *Y. pestis* between hosts (Perry and Fetherston, 1997).

Plague is a zoonotic disease, and although rats, both the urban rat *Rattus rattus* and the domestic rat *Rattus norvegicus*, were the major reservoirs during the first two pandemics and beginning of the third pandemic, the majority of human plague cases today are the result of cross-infections from other wild rodents such as prairie dogs, squirrels and marmots or larger mammals that have become infected such as cats (Perry

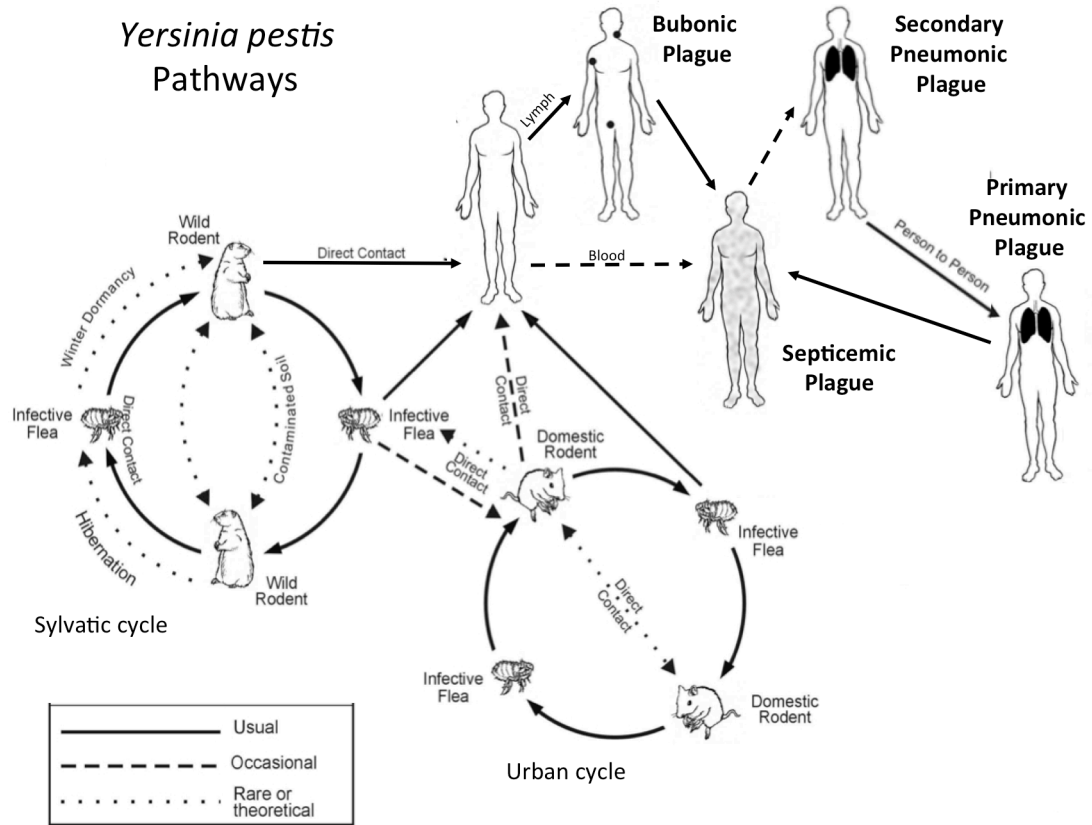


Figure 1-1: Transmission pathways for *Y. pestis*. This diagram describes the three major routes of transmission of *Y. pestis* between rodent and human hosts: bubonic, septicemic and pneumonic. Figure courtesy Neal R. Chamberlain, Ph.D., A.T. Still University/KCOM; adapted with permission (Chamberlain, 2004), subject to copyright restrictions.

and Fetherston, 1997). To date, *Y. pestis* has been associated with over 200 species of rodents worldwide, and its ability to infect arthropod vectors (fleas) and use them as a vehicle of transmission between susceptible hosts is unique among the *Enterobacteriaceae* (Hinnebusch, 1997). Human outbreaks therefore are often preceded by epizootic outbreaks, especially in places where plague is still endemic, including Madagascar, eastern and southern Africa, Southeast Asia, the western United States, Mongolia and northern China, Russia and central Asia. Currently, there are

approximately 2000 cases of plague reported to the World Health Organization (WHO) each year (WHO, 1999). WHO has also recently classified *Y. pestis* as a re-emerging disease after outbreaks in India, Malawi, Mozambique and east Africa (Schrag and Wiener, 1995).

During the 1910 Manchurian outbreak, Wu Lien-teh and Richard P. Strong noted that the sick plague patients arriving the hospital wards showed no signs of the classical bubonic plague. They also could find no evidence of the involvement of rats or fleas in this outbreak. Since the unresolved issue of transmission was critical in controlling the outbreak, both Strong and Wu independently performed a series of experiments where they held culture plates at certain distances from coughing or breathing patients and found the bacteria could be spread via coughing (respiratory droplets). They were able to recognize and describe the pneumonic form of plague transmission and helped institute the use of protective measures to protect against its spread. Much of the epidemiology and human pathology of pneumonic plague we have today is a result of their work during this epidemic (Chernin, 1989; Wu, 1995). Although the pneumonic form of transmission is still seen during isolated outbreaks today, it is very rare.

Clinical manifestations of *Y. pestis* infection

Human infection with *Y. pestis* is usually manifest as one of three clinical syndromes: bubonic, septicemic or pneumonic. As previously mentioned, the majority of cases are either bubonic or septicemic (Crook and Tempest, 1992), which result from the bite of a *Y. pestis*-infected flea. The clinical symptoms of bubonic plague include fever, chills, weakness, headache and swollen, tender lymph nodes (lymphadenopathy, i.e., buboes), which generally appear after a two- to eight-day incubation period. Buboes

result from unchecked bacterial growth in the regional draining lymph node and are most commonly associated with the inguinal and groin femoral lymph nodes. Bubonic plague is fatal in 40% to 60% of cases if left untreated.

Bacteremia, either directly from a flea bite (primary septicemic plague) or secondarily from an infected lymph node or lung (secondary septicemic plague), can lead to shock, bleeding from bodily orifices and/or death and blackening of tissues (gangrene) in a patient's extremities. The gangrene associated with septicemic plague inspired the nickname the Black Death during the 14th-century pandemic (Perry and Fetherston, 1997). Primary septicemic plague occurs in approximately 10% to 25% of plague cases and is characterized by patients with positive blood cultures but who lack a lymphadenopathy and pneumonia (Sebbane *et al.*, 2006). Both primary and secondary septicemic plague are clinically similar to most bacterial septicemias, commonly resulting in coagulopathy, meningitis and multi-organ failure. Septicemic plague is 100% fatal if not treated and 30% to 40% fatal even when treated with antibiotics (Perry and Fetherston, 1997).

The third manifestation of plague is primary pneumonic plague, which occurs when an individual inhales infectious respiratory droplets that are normally produced from humans or animals in the late stages of pneumonic plague by coughing. Conversely, secondary pneumonic plague develops as a complication of bubonic or septicemic plague when the infection disseminates to the lungs. Most naturally occurring cases of primary pneumonic plague are believed to have initiated from a case of secondary pneumonic plague, which occurs in approximately 12% of patients (Doll *et al.*, 1994). Although it is the least common form of the disease, pneumonic plague is the most rapidly progressing and most fatal form of plague. Primary pneumonic plague in humans begins as a

bronchopneumonia that is characterized by numerous bacteria and proteinaceous effusions in the small airways. The pneumonia then spreads rapidly, becoming lobar then multilobar within a few hours. Gross examination of the lungs reveals fluid in the upper and lower airways, fibrinous pleuritis, serosanguineous pleural effusions and hemorrhagic foci in the mediastinum. Histologic and microscopic examination of lung tissue reveals an acute pneumonia characterized by pulmonary alveolar flooding, pulmonary congestion and hemorrhaging, necrohemorrhagic foci, mediastinitis and extracellular bacteria in the alveoli but rarely seen in the interstitium (Davis *et al.*, 1996; Guarner *et al.*, 2002). Complete loss of alveolar architecture in necrotic foci, extensive neutrophilic infiltrate and fibrin deposition also occur (Doll *et al.*, 1994; Werner *et al.*, 1984). Death is normally due to respiratory failure and shock within four days post-infection. Pneumonic plague is 100% fatal if not treated within 24 hours from the onset of symptoms. Studies using non-human primates as a model of primary pneumonic plague show very similar gross and microscopic pathology (Davis *et al.*, 1996; Finegold, 1969). The mouse model of primary pneumonic plague infection also shows many of the same pathological features that have been seen in humans, non-human primates and cats (Chmura *et al.*, 2003; Davis *et al.*, 1996; Eidson *et al.*, 1991; Finegold, 1968; Watson *et al.*, 2001; Welkos *et al.*, 2002; Welkos *et al.*, 1997).

Biological warfare has been waged intermittently for more than 2500 years. The use of the *Y. pestis* as an agent of biowarfare is almost as old as the organism itself, although the techniques used to deliver and weaponize it have varied through the ages. They have gradually evolved from catapulting plague victims into besieged cities and dropping clay pots filled with infected fleas and flour (to attract rats) to more specialized

weapon systems (Robertson and Robertson, 1995). Three major attributes of *Y. pestis* contribute to fears of its intentional release as a weapon of bioterrorism: its well-documented pandemic capacity, its ability to be transmitted via aerosolized droplets and its significant morbidity and mortality in humans (Inglesby *et al.*, 2000). The isolation of multiple *Y. pestis* strains that have developed antibiotic resistance further emphasizes that *Y. pestis* remains a threat to public health (Galimand *et al.*, 2006; Galimand *et al.*, 1997; Guiyoule *et al.*, 2001). The Centers for Disease Control and Prevention (CDC) in the United States has therefore classified *Y. pestis* as a Category A Select Agent.

Molecular microbiology of *Y. pestis*

There are three *Yersinia* species that are pathogenic to humans, *Y. pestis*, *Y. enterocolitica* and *Y. pseudotuberculosis*. *Yersinia* species are facultative anaerobic, Gram-negative bacilli or coccobacilli. Although all three species are lymphotropic, *Y. enterocolitica* and *Y. pseudotuberculosis* are typically food-borne pathogens that cause a self-limiting limiting gastroenteritis that ranges from acute enteritis to mesenteric lymphadenitis (Cover and Aber, 1989; Huang *et al.*, 2006). All three species have an extended temperature growth range, 4°C to ~40°C, but grow optimally at 28°C. *Y. enterocolitica* and *Y. pseudotuberculosis* have an environmental niche, whereas *Y. pestis* is an obligate pathogen with limited abilities to survive outside its hosts. *Y. pestis* is auxotrophic for a number of nutritional requirements including several amino acids, biotin, thiamine, pantothenate, glutamic acid and other additional requirements that are only necessary for growth at 37°C. Several additional genomic lesions in metabolic genes alters carbon utilization in *Y. pestis*, which has been postulated as a mechanism of immune avoidance as many secondary bacterial metabolites are known pattern-associated

molecular patterns (PAMPs) recognized by the immune system (Brubaker, personal communication; Perry and Fetherston, 1997).

Currently, there are three recognized biotypes (or biovars) of *Y. pestis* (*antiqua*, *medievalis* and *orientalis*) based on their ability to convert nitrate to nitrite and to ferment glycerol. Another biovar, *microtus*, has recently been proposed based on a phylogenetic analysis of the genes necessary for these processes (Song *et al.*, 2004; Zhou *et al.*, 2004). Genome sequences are available for representatives of each biovar and show some level of heterogeneity and genome rearrangement between biovars and even between strains within a given biovar (Chain *et al.*, 2006; Deng *et al.*, 2002; Eppinger *et al.*, 2010; Garcia *et al.*, 2007; Morelli *et al.*, 2010; Parkhill *et al.*, 2001). Despite the heterogeneity, previous studies have shown that the different biovars, with the exception of *microtus*, do not significantly differ in their degree of virulence (Brubaker, 1991). Speculation based on the current geographical distribution patterns of descendant lineages and historical records has suggested that the biovar *antiqua* was mainly responsible for the first major pandemic, the biovar *medievalis* for the second pandemic and the biovar *orientalis* for the third and ongoing pandemic (Achtman *et al.*, 2004). However, recent molecular data from suspected plague cases from both the first and second pandemic suggest that all three pandemics were caused by *orientalis*-like *Y. pestis* strains (Drancourt *et al.*, 2004).

The genome of *Y. pestis* CO92 (the biovar *orientalis* strain most commonly used in the Goldman Lab) consists of a 4.65-megabase chromosome and 3 plasmids, pPCP1 (9.6 kilobases, kb), pCD1 (70 kb) and pMT1 (110 kb) (Parkhill *et al.*, 2001). Strong molecular evidence indicates that *Y. pestis* is a recently evolved clone of *Y. pseudotuberculosis* that may have originated in eastern Asia between 1500 and 20,000

years ago (Achtman *et al.*, 2004; Achtman *et al.*, 1999; Chain *et al.*, 2004; Morelli *et al.*, 2010). Indeed, the genomes of the two species are greater than 90% identical on average (some strains have an even greater percent identity), and their 16S rDNA nucleotide sequences are 99.7% identical (Chain *et al.*, 2004). *Y. pestis* has ~146 pseudogenes compared to *Y. pseudotuberculosis*, some of which, when restored, have detrimental effects on virulence in either the mammalian host or the flea (Bobrov *et al.*, 2011; Casutt-Meyer *et al.*, 2010; Chain *et al.*, 2004; Hinnebusch, 1997; Rosqvist *et al.*, 1988).

Indeed, evolutionarily recent adaptations in the *Y. pestis* genome are thought to have produced a pathogen that can be transmitted by different routes of infection, i.e., via arthropod vectors and human-to-human transmission via the aerosolization of respiratory droplets. Many of these same adaptations are thought to have substantially increased the virulence of *Y. pestis* towards its mammalian host. Although *Y. pestis* has also acquired two additional plasmids, pPCP1 and pMT1, that are required for full virulence, the presence of these plasmids alone cannot account for the markedly different clinical manifestations of these two nearly identical organisms (Chain *et al.*, 2004; Pouillot *et al.*, 2005).

Evolutionarily speaking (Gould and Lewontin, 1979), the highly virulent nature of plague may be due to the high levels of bacteremia ($\leq 10^6$ bacteria per milliliter blood) required to efficiently infect a flea (Hinnebusch, 1997). *Y. pestis* infects fleas poorly and is not readily transmitted back into its mammalian host after blockage of the flea proventriculus, dictating that a high level of bacteremia needs be established from a very low inoculum to maintain the natural transmission cycle. This situation favored the emergence and continued selection of a hypervirulent clone of *Y. pestis* that is able to

cause the high degree of severity associated with disease (Hinnebusch, 1997; Lorange *et al.*, 2005). Many of the evolutionary adaptations that changed a common enteric pathogen into a highly virulent pathogen are still unappreciated, and in depth comparative studies between *Y. pestis* and *Y. pseudotuberculosis* are necessary to gain a greater understanding of the pathogenic nature of *Y. pestis*.

Known virulence factors of *Y. pestis*

Even with the long history of this disease, relatively little is known about the pathogenesis of plague at the molecular level (Brubaker, 1991). Although there has been an increase in the amount of funding directed towards biodefense research and a subsequent increase in the number of publications related to *Y. pestis* pathogenesis, the majority of these studies have focused on only three virulence factors, albeit the three most predominant virulence factors: the Ysc-Yop type-three secretion system (Ysc-Yop T3SS) encoded on pCD1, the *pgm* locus encoded on the chromosome and the plasminogen activating protease Pla encoded on pPCP1. Other isolated studies have helped us gain insights into several other bacterial virulence factors although their mechanisms of action *in vivo* still need to be elucidated further.

All three *Yersinia* pathogenic to mammals harbor a nearly identical virulence plasmid that encodes the Ysc-Yop T3SS, designated pCD1 in *Y. pestis* and pYV in *Y. enterocolitica* and *Y. pseudotuberculosis*. The Ysc-Yop T3SS is absolutely essential for *Y. pestis* to cause disease in its mammalian host (Fig. 1-2). The Ysc-Yop T3SS consists of a needle apparatus whose basal body shares very high levels of homology with the basal body of flagellar apparatuses. The translocon, which is formed at the tip of the needle by *yopD*, *yopB* and *lcrF*, mediates the formation of a pore through which the effector

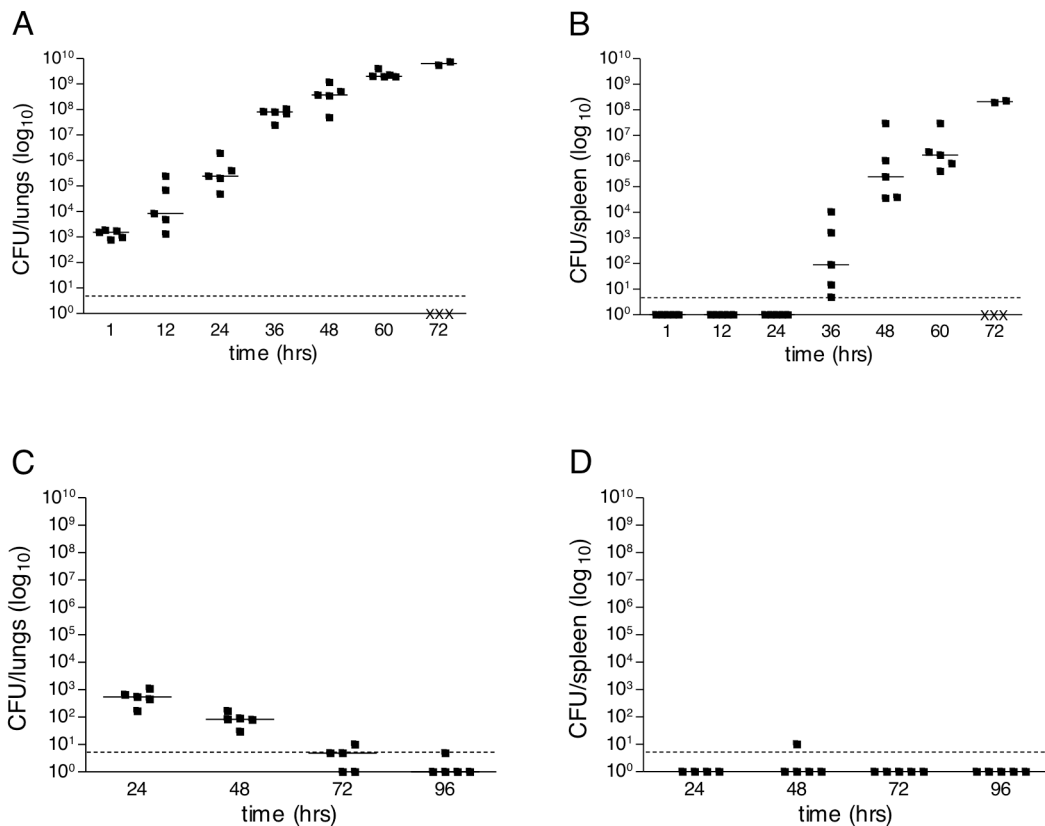


Figure 1-2: *Y. pestis* kinetics of pulmonary infection. The kinetics of infection for C57BL/6 mice intranasally inoculated with 10⁴ wild-type (A) or pCD1⁻ (B) *Y. pestis*. Colony-forming units (CFU) per tissue were determined at various time points of infection for lungs and spleen for both strains. Figure courtesy William E. Goldman, Ph.D. (Lathem *et al.*, 2005), subject to copyright restrictions.

proteins (*Yersinia* outer membrane proteins or Yops) enter the host cell directly from the bacterial cytoplasm (Cornelis, 2000; Cornelis, 2002; Costa *et al.*, 2010; Mueller *et al.*, 2005). Recent studies have also suggested that direct cytosol-to-cytosol translocation is not absolutely necessary (Akopyan *et al.*, 2011); however, this study fails to address the role of Pla (discussed below) in extracellular Yop cleavage or recent evidence that needle length is important in the translocation process (Mota *et al.*, 2005; Pouillot *et al.*, 2005).

The processes regulating translocation are not well understood; however, *in vitro* secretion of Yops can be achieved artificially by growing *Yersinia* under Ca⁺⁺ limiting

conditions, which also results in a bacterial growth restriction termed the low-calcium response (LCR). *In vivo* translocation of Yops into host cells is mediated through contact with the host plasma membrane (Pettersson *et al.*, 1996; Rosqvist *et al.*, 1994) and is tightly controlled by multiple feedback mechanisms from both inside the bacterium and the host cell (Cornelis, 2002; Dewoody *et al.*, 2011; Pettersson *et al.*, 1996). The translocated effector proteins (YopE, YopH, YopJ/YopP, YopK, YopM, YopO/YpkA and YopT) have been extensively studied both *in vitro* and *in vivo* and are known to alter the host cell cytoskeleton, inhibit phagocytosis, inhibit platelet aggregation and inhibit the production of proinflammatory cytokines. The cumulative effect of their individual actions results in an inhibition of host immune responses (Cornelis, 2002).

Although many of the known virulence factors are found on the three virulence plasmids, there are also several chromosomally encoded virulence factors (Perry and Fetherston, 1997). The most extensively studied of these factors are encoded in the *pgm* locus, a ~102kb region of DNA bounded by repeated copies of *IS100*, which are thought to allow for loss of the *pgm* locus by homologous recombination (Fetherston *et al.*, 1992). Although other genes within the *pgm* locus may contribute to infection, the locus can be subdivided into two distinct phenotypic regions (Jenkins *et al.*, 2009; Perry and Fetherston, 1997; Pujol *et al.*, 2005). The first region contains the *hms* genes, which are responsible for the production of a poly- β -1,6-N-acetyl-glucosamine extracellular polysaccharide matrix that is essential for biofilm formation (Lillard *et al.*, 1997). Polysaccharide production also produces a red pigmentation phenotype when *Y. pestis* is grown at 26°C on agar plates containing Congo red dye. Efficient *Y. pestis* biofilm formation is responsible for blockage of the flea proventriculus but is not required for

mammalian infection; however, its overproduction can be detrimental to mammalian infection (Bobrov *et al.*, 2011; Hinnebusch, 1997; Hinnebusch *et al.*, 1996).

The second region has a high G+C content and is therefore considered to be a high pathogenicity island (HPI). The HPI encodes for proteins used in the synthesis and utilization of the siderophore yersiniabactin. Mutants that prevent yersiniabactin production or utilization are severely attenuated in the subcutaneous route of infection due to the inability of HPI mutants to acquire iron (Bearden *et al.*, 1997). This defect can be overcome by simultaneously injecting iron sulfate during subcutaneous inoculation with the bacteria or into the mouse peritoneally, demonstrating one mechanism *Y. pestis* uses to overcome nutritional immunity (Perry and Fetherston, 1997; Weinberg, 1975). However, during pneumonic plague, the virulence defects of strains lacking the *pgm* locus cannot be fully explained by the loss of yersiniabactin production or utilization, suggesting that additional factors encoded on the *pgm* locus are involved in pathogenesis (Fetherston *et al.*, 2010; Lee-Lewis and Anderson, 2010).

The small plasmid pPCP1 is unique to *Y. pestis* and encodes the genes for the plasminogen activator protease (*pla*), the bacteriocin pesticin (*pst*) and a pesticin immunity protein (*pim*). Pla is an omptin cysteine protease that is not required for bacterial replication at the site of a subcutaneous inoculation but is important in bacterial dissemination, presumably because of its ability to cleave plasminogen into plasmin, which then digests fibrin clots that restrict bacterial dissemination (Sodeinde *et al.*, 1992; Welkos *et al.*, 1997). Conversely, during pneumonic plague, Pla is required for bacterial replication and subsequent pathology in the lungs but not for bacterial dissemination (Lathem *et al.*, 2007). Pla has also been shown to act as an adhesin/invasin to tissue

culture cells when expressed in *E. coli*; however, *Y. pestis* strains that lack Pla do not show a decrease in adherence or invasion into tissue culture cells, and its role as an adhesin has yet to be demonstrated *in vivo* (Kienle *et al.*, 1992; Lahteenmaki *et al.*, 2001). Identifying the protease substrates of Pla *in vivo* will be essential in determining its role in pathogenesis.

The plasmid pMT1 encodes the genes for the F1 antigen (*cafI*) and murine toxin (*ymt*). CafI forms a proteinaceous capsule-like structure on the outside of the *Y. pestis* when the bacteria are grown at 37°C under oxygen limiting conditions (Perry and Fetherston, 1997). The requirement of CafI in pathogenesis is disputed in the literature; however, very few natural isolates lack the genes encoding *cafI*, suggesting that there is a natural selection in for its maintenance (Meka-Mechenko, 2003; Sebbane *et al.*, 2009; Winter *et al.*, 1960). Recent evidence suggests that the role of CafI in pathogenesis is dependent on the animal model that is used in the study (Weening *et al.*, 2011). The capsule has been shown to inhibit phagocytosis by macrophages in tissue culture, but it may have additional roles in masking PAMP recognition during *Y. pestis* infection (Du *et al.*, 2002; Weening *et al.*, 2011). Additionally, CafI has been widely used as a candidate for vaccine development (see below).

Although the majority of virulence factors currently studied are required for mammalian infection, Ymt has been shown to be important in flea infections. Ymt is a phospholipase D (PLD) that was originally identified for its ability to cause septic shock in mice but has recently been shown to be dispensable for mammalian infection (Hinnebusch *et al.*, 2000). Ymt has been shown to protect *Y. pestis* in the flea gut from cytotoxic digestion products of digested blood (Hinnebusch *et al.*, 2002). The acquisition

of Ymt by *Y. pestis* may have been a determining factor in allowing for *Y. pestis* to use an arthropod vector as a route of transmission.

The genes encoding the dominant adhesins/invasins found in *Y. enterocolitica* and *Y. pseudotuberculosis*, *inv* and *yadA*, are pseudogenes in *Y. pestis* (Simonet *et al.*, 1996; Skurnik and Wolf-Watz, 1989). Intimate contact with the host plasma membrane is thought to be required for use of the Ysc-Yop T3SS, and because the two predominate *Yersinia* adhesins are lacking in *Y. pestis*, many studies have looked for additional adhesins in *Y. pestis* that are required for T3SS translocation. Many potential adhesins have been identified, most of which are chromosomally encoded. One potential adhesin is encoded by the chaperon-usher operon *psa* (PsaA is also known as the pH6 antigen), which encodes a pilus that is thermally induced at low pH. When induced, the pilus mediated adhesion to tissue culture cells, and mutants lacking *psaA* are attenuated in mouse models of infection (Cathelyn *et al.*, 2006; Leo and Skurnik, 2011).

Autotransporters are another class of potential adhesins that are able to mediate adhesion when overexpressed in *E. coli* (Lawrenz *et al.*, 2009; Yen *et al.*, 2007). Specific autotransporters (YadBC and YapE) are attenuated in mouse models of infection when inoculated subcutaneously but not when inoculated intranasally (Andrews *et al.*, 2010; Forman *et al.*, 2008; Lawrenz *et al.*, 2009).

Ail is another adhesin that was originally discovered and characterized in *Y. enterocolitica* (Miller and Falkow, 1988; Miller *et al.*, 1989). There are four Ail homologs in both *Y. pestis* and *Y. pseudotuberculosis* but only one is *Y. enterocolitica*, all of which show a high degree of homology to each other and to OmpX in *E. coli* (Kolodziejek *et al.*, 2007). Although there are four Ail homologs in *Y. pestis*, only one

homolog (YPO2905) is produced at appreciable levels and is able to mediate the functions ascribed to Ail (Bartra *et al.*, 2008). When *ail* is expressed in *E. coli*, it significantly increases the attachment of *E. coli* to fibronectin on tissue culture cells (Felek and Krukoniš, 2009; Tsang *et al.*, 2010). In addition, levels of attachment to tissue culture cells and Yop translocation are significantly reduced in *ail* deletion mutants of *Y. pestis* (Felek *et al.*, 2010). In addition to its adhesive capabilities in *Y. pestis*, Ail also mediates resistance to complement and antimicrobial peptides such as polymyxin B (Bartra *et al.*, 2008). There are differing reports as to the requirement of Ail in pathogenesis; however when an *ail* mutant was engineered in fully virulent *Y. pestis* background it was attenuated in an intranasal mouse model of infection (Bartra *et al.*, 2008; Felek and Krukoniš, 2009; Kolodziejek *et al.*, 2010).

Pathogenesis of *Y. pestis*

Several mouse models of plague infection have been developed to study the pathogenesis of *Y. pestis*. A subcutaneous route of inoculation is used to simulate bubonic plague, and intranasal and aerosol routes of inoculation are primarily used simulate primary pneumonic plague. Although intravenous routes of inoculation are frequently used in literature to study plague pathogenesis, these routes are primarily used to study attenuated forms of *Y. pestis* such as strains lacking the *pgm* locus, which are unable to cause disease when inoculated subcutaneously or intranasally. Transcriptional profiling of *Y. pestis* grown in fleas or broth culture before infection has shown that fleas are able to prime several mammalian virulence factors, suggesting that there are certain caveats that accompany any artificial route of inoculation (Vadyvaloo *et al.*, 2010).

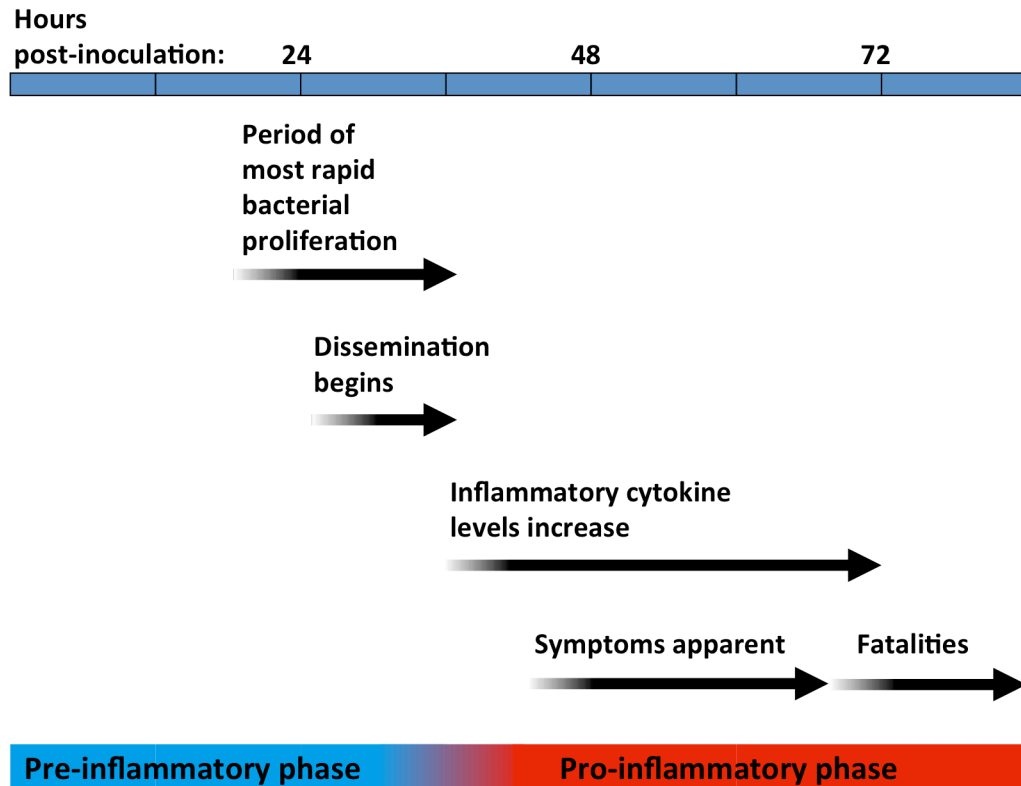


Figure 1-3: Progression of primary pneumonic plague in mice.

Using an intranasal mouse model of infection, Lathem *et al.* have shown that the pneumonic plague syndrome progresses in two distinct phases as defined by host immune responses and disease pathology (Fig. 1-3)(Lathem *et al.*, 2005). The initial 36 hours of infection are characterized by rapid bacterial replication in the lung without generating appreciable host immune responses, histological changes in infected tissue or obvious disease symptoms. Levels of proinflammatory cytokines/chemokines in the lung during this pre-inflammatory phase of infection were similar to those seen in uninfected mice (Lathem *et al.*, 2007; Lathem *et al.*, 2005). These observations have been corroborated using other animal models of infection including rats and non-human primates (Agar *et al.*, 2009; Agar *et al.*, 2008; Bubeck *et al.*, 2007; Koster *et al.*, 2010).

In contrast, an abrupt switch occurs at approximately 36-48 hours post-inoculation (hpi) when the second phase of disease begins. This pro-inflammatory phase is marked by the upregulation of pro-inflammatory cytokines, consolidation of the airways by a neutrophilic response and the onset of physical symptoms (Lathem *et al.*, 2005). Progression into this pro-inflammatory phase marks a critical turning point during infection that invariably results in death if not treated promptly. The mechanistic basis for the timing and the trigger(s) of the dramatic switch in host response remains unknown. Recently, Comer *et al.* reported a similar biphasic response during bubonic plague and noted a correlation between the onset of inflammation and bacteremia (Comer *et al.*, 2010).

Biphasic disease progression during plague highlights the complex nature of the interactions between *Y. pestis* and its host. Two current models have been suggested for the biphasic nature of disease. The first model is based on the observation that the switch to the pro-inflammatory phase of disease occurs concurrently with our ability to detect *Y. pestis* in the blood and/or spleen (Fig 1-3). This model implies that *Y. pestis* is able to maintain a localized immunosuppressive environment at the site of infection or in the draining lymph node, but upon dissemination to the bloodstream, an immune cascade is initiated, resulting in robust neutrophil migration into the lungs and lymph nodes and creating the characteristic inflammatory lesions that develop between 36 and 48 hpi (Personal observations and Comer *et al.*, 2010).

The second model in the literature suggests that there is a delay in the shift from Yop-mediated apoptosis, which is caspase-3 dependent and non-inflammatory, to Yop-mediated pyroptosis, which mediated by inflammasome and caspase-1 activation and

results in inflammation. The delayed shift may be responsible for the delayed inflammatory response (Bergsbaken and Cookson, 2009). For a macrophage to undergo inflammasome-mediated pyroptosis, it needs to first become activated. Since activated macrophages are not present until later in infection, there is a delay in the inflammatory response. Central to this model is the inflammasome, which is a component of the innate immune system and consists of an oligomeric protein complex that assembles upon activation by bacterial or viral signals to promote the maturation of the inflammatory cytokines IL-1 β and IL-18 (Reviewed in Martinon *et al.*, 2009). Indeed, several studies have recently been published demonstrating *Yersinia*-induced inflammasome activation (Bergsbaken and Cookson, 2007; Brodsky *et al.*, 2010; Zheng *et al.*, 2011). However, these studies were performed mostly with *Y. pseudotuberculosis* or attenuated strains of *Y. pestis*, and therefore, the *in vivo* relevance of inflammasome activation for *Y. pestis* pathogenesis or in the biphasic nature of disease progression remains unknown.

Macrophages are frequently used to study many of the molecular mechanisms involved in *Yersinia* pathogenesis, in particular *Y. pestis* pathogenesis. Phagocytic cells, including macrophages, neutrophils and dendritic cells, have been shown to be the predominant cell types with which *Yersinia* interacts *in vivo*, defined by the injection of T3SS effectors (Durand *et al.*, 2010; Köberle *et al.*, 2009; Marketon *et al.*, 2005). These studies also show that the interaction with phagocytic cells is preferential over other cell types including B-cells and T-cells. Initial interactions with macrophages and/or dendritic cells are thought to mediate the trafficking of *Yersinia* to regional lymph nodes (Pujol and Bliska, 2005). During these initial interactions, the enteric *Yersinia* are intracellular as they pass through M cells lining the intestinal wall (Clark *et al.*, 1998; Marra and Isberg,

1997) and extracellular as they replicate in different tissues (Balada-Llasat and Mecsas, 2006; Bergman *et al.*, 2009; Simonet *et al.*, 1990). However, the need for a transient intracellular lifestyle in *Y. pestis* pathogenesis is debated, as the vast majority of bacteria appear to be extracellular later in infection (Pujol and Bliska, 2005). *Y. pestis* can replicate inside macrophages *in vitro* (Pujol and Bliska, 2003) and can be found inside macrophages *in vivo* (Finegold, 1969; Lukaszewski *et al.*, 2005; Meyer, 1950); however, the necessity of a niche for intracellular replication remains controversial. Differences in the initial growth temperature (26°C vs. 37°C) seem to dictate resistance to phagocytosis *in vivo* (Burrows and Bacon, 1956), suggesting that flea-transmitted *Y. pestis* (26°C inoculation) or bubonic plague may have a brief intracellular lifestyle, whereas person-to-person transmitted *Y. pestis* (37°C inoculation) or pneumonic plague may not. Thus, while the interaction between *Y. pestis* and phagocytic cells plays an important role in *Y. pestis* pathogenesis, the nature of their interaction *in vivo* needs to be elucidated further.

Compared to other Gram-negative bacteria, the delayed inflammatory response of plague is unusual (Bubeck *et al.*, 2007). As mentioned previously, the Ysc-Yop T3SS and several effector Yops have been implicated in suppressing cytokine and chemokine production during plague infection. However, *Y. pestis* possesses other mechanisms of avoiding innate immune mechanisms. Lipopolysaccharide (LPS) with tetra-acylated forms of lipid A weakly activates Toll-like receptor 4 (TLR-4)-mediated inflammation compared to LPS with hexa-acylated forms of lipid A. *Y. pestis* primarily produces hexa-acylated lipid A when grown at 26°C but converts to tetra-acylated lipid A at 37°C (Kawahara *et al.*, 2002; Rebeil *et al.*, 2004). However, when a strain of *Y. pestis* was engineered to produce hexa-acylated lipid A at 37°C, it was more than 100,000-fold and

350-fold attenuated in mouse models of bubonic plague and pneumonic plague, respectively (Montminy *et al.*, 2006; Smiley, 2008). The attenuation phenotype is associated with increased levels of TNF- α and is dependent on TLR-4, an innate immune receptor for LPS (Montminy *et al.*, 2006). These results suggest that wild-type *Y. pestis* evades innate immune detection in part by avoiding TLR-4-mediated immune activation. These results are consistent with observations that increased cytokine levels during the initial stages of infection are detrimental to *Y. pestis* and to the other *Yersinia* species (Autenrieth *et al.*, 1994; Barton *et al.*, 2007; Bohn *et al.*, 1994; Nakajima and Brubaker, 1993).

Another possible mechanism for decreased immune activation is secreted LcrV, which activates TLR-2-mediated IL-10 production, producing an anti-inflammatory effect on innate immune cells (DePaolo *et al.*, 2008; Reithmeier-Rost *et al.*, 2004; Viboud and Bliska, 2005). However, these studies have primarily been performed with other *Yersinia* species and have not correlated well with *Y. pestis* pathogenesis when examined directly (Philipovskiy *et al.*, 2005; Reithmeier-Rost *et al.*, 2007). In addition, IL-10 production is not apparent when analyzed *in vivo* during pneumonic or bubonic plague (Bubeck *et al.*, 2007; Comer *et al.*, 2010; Lathem *et al.*, 2005). This example highlights the growing body of evidence that suggests that slight genetic differences between different *Yersinia* species may impact the function of virulence factors and ultimately dictate the differences in disease outcome.

The *pgm* locus has long been associated with virulence defects in *Y. pestis*, especially when inoculated subcutaneously. These defects have largely been attributed to the HPI that encodes for the biosynthesis and bacterial utilization of yersiniabactin.

However, Paauw *et al.* have recently shown that the production of reactive oxygen species (ROS) by neutrophils, monocytes and macrophages is blocked by yersiniabactin because it reduces iron availability to these cells (Paauw *et al.*, 2009). Iron functions as a catalyst during Haber-Weiss reactions, which generate ROS. The reduction in ROS by innate immune cells likely leads to decreased bacterial killing and decreased neutrophil infiltration, as the production of hydroxyl radicals has been shown to promote neutrophil infiltration into the lung (Brandes and Kreuzer, 2005). These observations may explain the disparity between the virulence defects of mutants lacking yersiniabactin biosynthesis genes and mutants lacking iron utilization genes during pneumonic plague (Fetherston *et al.*, 2010). The combination of decreased bacterial killing and decreased neutrophil influx during early stages of infection may also contribute to the biphasic nature of *Y. pestis* disease progression.

The dynamics of host/pathogen interactions are inherently complex and highly individualized for different pathogens and for different hosts. The dynamic interaction between *Y. pestis* and its host highlight the multifaceted nature of these interactions, as evidenced by the biphasic disease progression during plague. Ultimately, a number of synergistic mechanisms, both those discussed here and those less appreciated, likely contribute to the extraordinary virulence of *Y. pestis*.

Diagnosis, treatment and prevention of *Y. pestis*

Laboratory diagnosis of *Y. pestis* can be achieved through both bacteriologic and serologic methods from samples derived from a variety of different sources including lymph node aspirates, cerebrospinal fluid, urine and sputum. In smears, *Y. pestis* appears as a pleomorphic Gram-negative rod (1.0-2.0 x 0.5 μm) and may appear as single cells or

in small clusters (Perry and Fetherston, 1997). *Y. pestis* can be cultured on many solid and liquid culture mediums; however, growth is slow and visible colonies appear after 48 hr. Colonies are normally smooth and opaque with round or irregular edges. Colonies may also have a raised center with a flat periphery or “fried egg” appearance when viewed under magnification (Smego *et al.*, 1999). The optimal growth temperature for *Y. pestis* is 28°C, and if plague is suspected, all diagnostic cultures should be made in duplicate, one for growth at 28°C and one at 37°C. *Y. pestis* is classified as a oxidase-negative, catalase-positive, urea-negative, indole-negative, non-lactose fermenter (Smego *et al.*, 1999).

Antibody based methods, including direct immunofluorescence and serologic assays, can also be used to diagnose *Y. pestis* infection (Guarner *et al.*, 2002; Smego *et al.*, 1999). Many of these assays use or detect antibodies directed against the F1 antigen of *Y. pestis*, which is relatively abundant and immunogenic; anti-F1 antibodies appear after the first week of infection. Paired serum samples are normally taken, one during the acute infection and another during postconvalescence. Enzyme-linked immunosorbent assays (ELISA) are normally performed to detect F1 antigen or the serum levels of anti-F1 antibodies. A four-fold difference in titers between the paired samples is considered positive for *Y. pestis* infection, and a titer greater than 1:10 for a single serum sample is also indicative of infection (Perry and Fetherston, 1997). Serum samples from suspected plague patients are routinely analyzed at the CDC because most microbiology clinical laboratories are not equipped to definitively diagnose *Y. pestis* (Personal experience!!).

The mortality rate of plague is very high if untreated. Therefore, effective and timely antibiotic treatment is critical for recovery. Streptomycin, gentamycin, tetracycline,

doxycycline and chloramphenicol are the commonly used, but fluoroquinolones have also been shown to be effective in animal studies and may be used in treatment (Perry and Fetherston, 1997; Smego *et al.*, 1999). The antibiotic administered is often determined on a case-by-case basis; precautions should be taken in cases where non-life-threatening complications may arise, such as with pregnant or child-aged patients. Multiple antibiotics are routinely administered intravenously and intramuscularly during the initial treatment and then orally after a patient is stabilized. Prophylactic use of antibiotics (doxycycline or ciprofloxacin) is recommended for people exposed to plague patients especially those who come within six to ten feet of persons with pneumonic plague (Inglesby *et al.*, 2000). In addition, suspected plague patients should be maintained in isolation, and attending staff should use appropriate personal protective equipment until at least two after the start of antibiotic treatment (Inglesby *et al.*, 2000).

During the Black Death, plague patients who survived were effectively resistant to reinfection and oftentimes were the only people willing to succor those afflicted with the disease (Tuchman, 1978). This suggests that it should be possible to develop vaccines that confer protection by priming the adaptive immune system against plague. Whole-cell killed vaccines were commonly used in the late 1800's to vaccinate against a wide variety of bacterial and viral infections, including bubonic plague. However, side effects were common and multiple boosters were necessary to confer only partial protection. A live attenuated vaccine, EV76, which lacks the *pgm* locus, has also been widely used in humans in some plague endemic areas of the world, but is not currently available (Williamson, 2001).

The protective efficacy for both these vaccines was thought to be associated with the development of antibodies against the F1 antigen (Titball and Williamson, 2004), and purified F1 antigen has been shown to be more effective in protecting against plague challenge than the whole-cell killed vaccine. However, vaccines that exclusively target F1 antigen have been unsuccessful and have lead to F1 mutations during infection (Friedlander *et al.*, 1995). In addition, these vaccines also do not confer protection against pneumonic plague. Conversely, F1/LcrV-based subunit vaccines have shown promise against both pneumonic and bubonic plague challenge in cynomolgus macaques (Williamson *et al.*, 2011). Additional multi-antigen subunit vaccines are also being developed, and multiple efforts are underway to modify the antigens, adjuvants and delivery platforms needed to help improve the efficacy of sub-vaccines against *Y. pestis* (Reviewed in Smiley, 2008).

Conclusions

Although the relative incidence of plague is currently relatively low, epizootic foci are prevalent worldwide. The recent epidemics in India in 1994 and in the Republic of Congo in 2006 call attention to the resurgent capabilities of this disease when environmental and political conditions are favorable (Perry and Fetherston, 1997; WHO, 2006). There is also a heightened threat of the use of *Y. pestis* as an agent of bioterrorism, which would not only cause severe mortality in its victims but also cause widespread panic and civil unrest in affected populations. Thus, a more complete mechanistic understanding of disease progression, including virulence factors and host-mediated events involved in *Y. pestis* pathogenesis, is needed to identify appropriate therapeutic options.

Scope of the Project

Although our understanding of primary pneumonic plague is increasing, most studies have concentrated on the end stages of the disease when disease pathology is evident. Very few studies have examined the early events of primary pneumonic plague (≤ 36 hours post-inoculation) when mice are largely asymptomatic despite the large bacterial burden in their lungs. There are also no significant changes in lung architecture or in inflammatory cytokine profiles during these early stages. In addition, the bacterial factors that facilitate the biphasic nature of this unusual disease progression are relatively unknown for pneumonic plague. Therefore, my project has two major goals: characterize the initial bacterial/host interactions *in vitro* and *in vivo* (Chapter 2) and develop a broad, unbiased microarray-based negative selection technique called transposon site hybridization (TraSH) to identify virulence factors involved in both the pre-inflammatory and pro-inflammatory phases of disease (Chapters 3-4). Thus, Chapter 2 will describe the *in vitro* and *ex vivo* adhesion and invasion characteristics of *Y. pestis* and the models developed to study these interactions. The adhesins required for these interactions are also examined in Chapter 2. The development of the TraSH methodology and its use in identifying essential genes *in vitro* and virulence factors *in vivo* will be the focus of Chapter 3. The majority of my studies have focused on verifying and characterizing the unusual results from the TraSH negative selection screen (Chapter 4). Although not included as part of this work, in Chapter 5, I have included the abstracts for collaborative studies in which I have participated. The final chapter (Chapter 6) will provide overall conclusions and implications of this work on *Y. pestis* pathogenesis.

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Chapter 2

Characterizing early bacterial/host interactions during primary pneumonic plague

Abstract

Pneumonic plague is the deadliest form of plague caused by *Y. pestis*. After the inhalation of infectious droplets, *Y. pestis* can interact with a myriad of different cell types, but the initial bacterial-host cell interactions during *Y. pestis* pulmonary infection are largely uncharacterized. I adapted our animal model of pneumonic plague to specifically examine the interactions between *Y. pestis* and lung tissues. I show that *Y. pestis* cannot be lavaged from the lung during the initial stages of infection, but is freely lavageable during the later stages of infection. Multiple outer membrane proteins appear to specifically bind to tissue culture cells, implying that adhesion is mediated by more than one protein. Recent studies have implicated Ail as an adhesin in *Y. pestis*, but I found that Ail functions more to confer membrane stability and the proper integration of proteins into the outer membrane than as an adhesin. Finally, the reintroduction of two major *Yersinia* adhesins, *yadA* and *inv*, into *Y. pestis* reduces the virulence of *Y. pestis* in both bubonic and pneumonic animal models of infection, offering an explanation as to why these genes were lost during the course of *Y. pestis* evolution.

Introduction

Pneumonic plague is the deadliest, most rapidly progressing form of plague caused by *Y. pestis*. The onset of symptoms in humans normally occurs within 48 to 60 hours and is marked by sudden chills, high fever, severe cough, chest pain, dyspnea and production of copious amounts of watery, frothy and oftentimes bloody sputum (Davis *et al.*, 1996). Within the next 24 to 36 hours (3-4 days post-infection), pneumonic plague patients develop a severe pneumonia and usually succumb to respiratory failure or acute respiratory insufficiency and/or septic shock. Meanwhile, the sputum and aerosols produced by coughing are teeming with bacteria, which are easily transmitted to anyone within six to ten feet of the infected individual.

This, however, represents the terminal stages of pneumonic plague. In stark contrast, the initial 36 to 48 hours of infection are characterized by a lack of appreciable host immune responses, histological changes in infected tissue or obvious disease symptoms, classifying pneumonic plague as a bi-phasic disease syndrome. The potent anti-inflammatory activity of the Ysc-Yop T3SS likely contributes to the relative quiescence of early infection; however, *Y. pestis* lacks the two major adhesins found in both *Y. enterocolitica* and *Y. pseudotuberculosis* that are thought to be required for Ysc-Yop T3SS function (Leo and Skurnik, 2011). Additional uncharacterized adhesins likely mediate the intimate association required for the translocation of Yops.

To cause disease, *Y. pestis* needs to circumvent the innate immune mechanisms of the lung, including the mucociliary clearance provided by secretory goblet cells and ciliated cells, phagocytosis by alveolar macrophages resident in the large and small airways and a myriad of proteins and peptides that have anti-microbial and immunomodulatory activities (Suzuki *et al.*, 2008). In particular, the lung epithelium

plays a critical role in recognizing infection through pattern recognition receptors (PRRs), triggering localized inflammatory responses via the production and release of cytokines and chemokines, recruiting monocytes by upregulating cell-cell adhesion molecules and controlling the infection through the increased secretion of antimicrobial proteins and peptides. However, very little or nothing is known about how *Y. pestis* interacts with the lung epithelium or the consequences of those interactions early during the infectious process before macrophages and neutrophils are recruited to the site of infection. Characterizing these early interactions is essential in understanding early events in primary pneumonic plague. This chapter will outline the work I have done to characterize bacterial/host interactions *in vitro* and *in vivo* and my efforts to identify unknown *Y. pestis* adhesins.

Results and Discussion

In vitro Y. pestis adhesion and invasion tissue culture model

Upon entering the lung, *Y. pestis* has the opportunity to interact with a variety of cell types before it reaches the small airways. The pseudostratified and columnar tracheobronchial epithelium, which provide the major mechanisms for the mucociliary clearance, consist of ciliated cells, secretory goblet cells and microvilliated cells. The bronchioles consist of cuboidal epithelium and secretory Clara cells, which are the major epithelial cells. Finally, the alveolar epithelium or small airways consists almost exclusively of alveolar type I cells and alveolar type II cells, with about 95% of the alveolar epithelium consisting of alveolar type I cells (squamous alveolar cells), which provide for gas exchange in the lung. Although alveolar type II cells (septal cells) comprise 60% of the cells lining alveolus, because of their shape they cover a much smaller surface area than alveolar type I cells (<5%). Alveolar type II cells are responsible for the production and secretion of surfactant, a group of phospholipids and small hydrophobic proteins that reduce alveolar surface tension. The ability of the lung epithelial surface to mount an immune response and its role during infection is only beginning to be truly appreciated (Hippenstiel *et al.*, 2006). Pneumonic plague is primarily a disease of the small airways (Lathem *et al.*, 2005); therefore, I established an *in vitro* model system to help determine the molecular mechanisms involved in these early pathogen-host cell interactions in the small airways.

I elected to use A549 cells (ATCC #CCL-185), which are a human alveolar type II pneumocyte carcinoma cell line that has been used extensively as a model of the small airway epithelium (Liu *et al.*, 2006). Using an MOI of 10 bacteria to 1 cell (MOI 10:1), I found that *Y. pestis* adheres to and invades these cells. On average, 17.5% of the

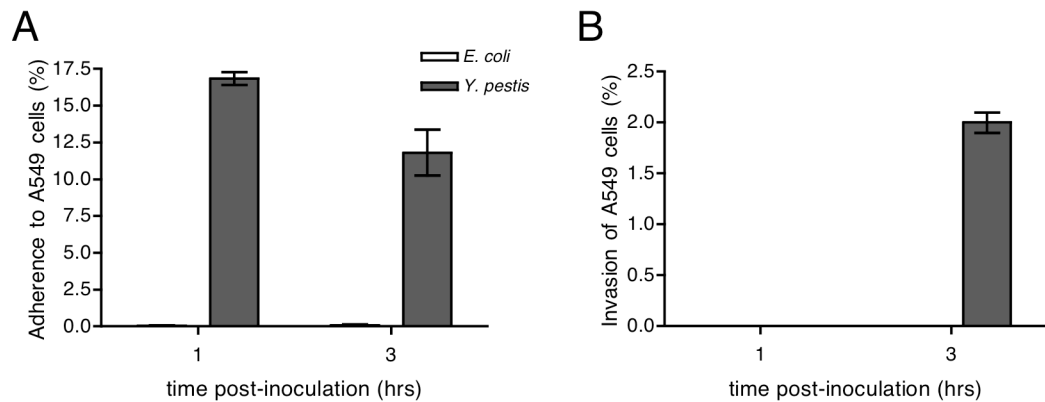


Figure 2-1: Tissue culture model of *Y. pestis* adherence and invasion. (A) Adherence of *Y. pestis* and *E. coli* to A549 cells at various time points post-inoculation. Bacteria were inoculated at an MOI of 10:1 (bacteria:cells). Each bar represents the mean percent adherence relative to the inoculum. (B) Invasion of *Y. pestis* and *E. coli* into A549 cells at various time points post-inoculation as determined by gentamicin protection assays. Bacteria were inoculated at an MOI of 10:1. At the indicated time point (1 or 3 hr), the cells were incubated with gentamicin to kill extracellular bacteria. Each bar represents the mean percent invasion relative to the inoculum. Error bars represent standard error of the mean (SEM).

inoculating bacteria were able to adhere to A549 cells at 1 hour post-inoculation (hpi) and 12.5% at 3 hpi, whereas only approximately 2-3% of these bacteria were able to invade the A549 cells at 3 hpi as determined by gentamycin protection assays (Fig. 2-1A-B).

This appears to be specific for *Y. pestis* because *E. coli* was unable to adhere to or colonize this cell line. Similar observations were seen for 6 and 12 hpi (data not shown).

Finally, I used hamster tracheal epithelial (HTE) cells, which are a homogeneous primary cell culture that have characteristics of both small and large airway epithelial cells

(Baseman *et al.*, 1980; Goldman and Baseman, 1980), to show that *Y. pestis* adheres to and invades these cells in a similar manner (data not shown). These results demonstrate

that *Y. pestis* is able to adhere to epithelial tissue culture cells *in vitro*, and although they are able to invade these cells, the frequency is very low.

In vivo Y. pestis adhesion and invasion model

Our laboratory has characterized the progression (kinetics) of disease using a C57BL/6J mouse model of infection (Lathem *et al.*, 2005). As part of these same studies, we were able to determine that approximately 85% of the bacteria at 48 hpi could be extracted from the lung by bronchoalveolar lavage (BAL). However, in attempts to extract bacteria from the lung by BAL at 2 hpi to examine *Y. pestis*-interacting cell types, I found that BAL could extract only approximately 10% of total bacteria in the lungs. Using a series of different time points, I found that as the infection progressed BAL can extract an increasing larger number of bacteria (Fig. 2-2A). The percent of lavageable bacteria correlates almost directly with the number of bacteria in the lung. Additional experiments show that this phenotype is not dependent on the size of the initial inoculum, the Ysc-Yop T3SS (pCD1) or known putative adhesins of *Y. pestis* (Fig. 2-2B). This observation tends to be specific for *Y. pestis* because over 75% of Δbvg *B. pertussis* and over 50% *K. pneumoniae* can be lavaged from the lung at 4 hpi (Fig. 2-2B). Interestingly, similar numbers of bacteria are present in the lungs for both the 1×10^5 inoculum at 2 hpi and the 1×10^4 inoculum at 12 hpi, yet 4-fold more bacteria can be extracted by BAL from mice at 12 hpi. These data suggest that the ability of *Y. pestis* to remain in the lungs during a BAL is more dependent on time post-inoculation than the number of bacteria.

A bacterium's surrounding environment often determines its expression profile, including genes that encode virulence factors. Growth of *Y. pestis* in the flea has been shown to induce a state in which *Y. pestis* is more resistant to innate immune defenses when inoculated into a mouse (Vadyvaloo *et al.*, 2010). To test whether my observations were the result of broth-grown bacteria, I designed a series of experiments in which I inoculated mice with bacteria harvested by BAL from mice that had been intranasally

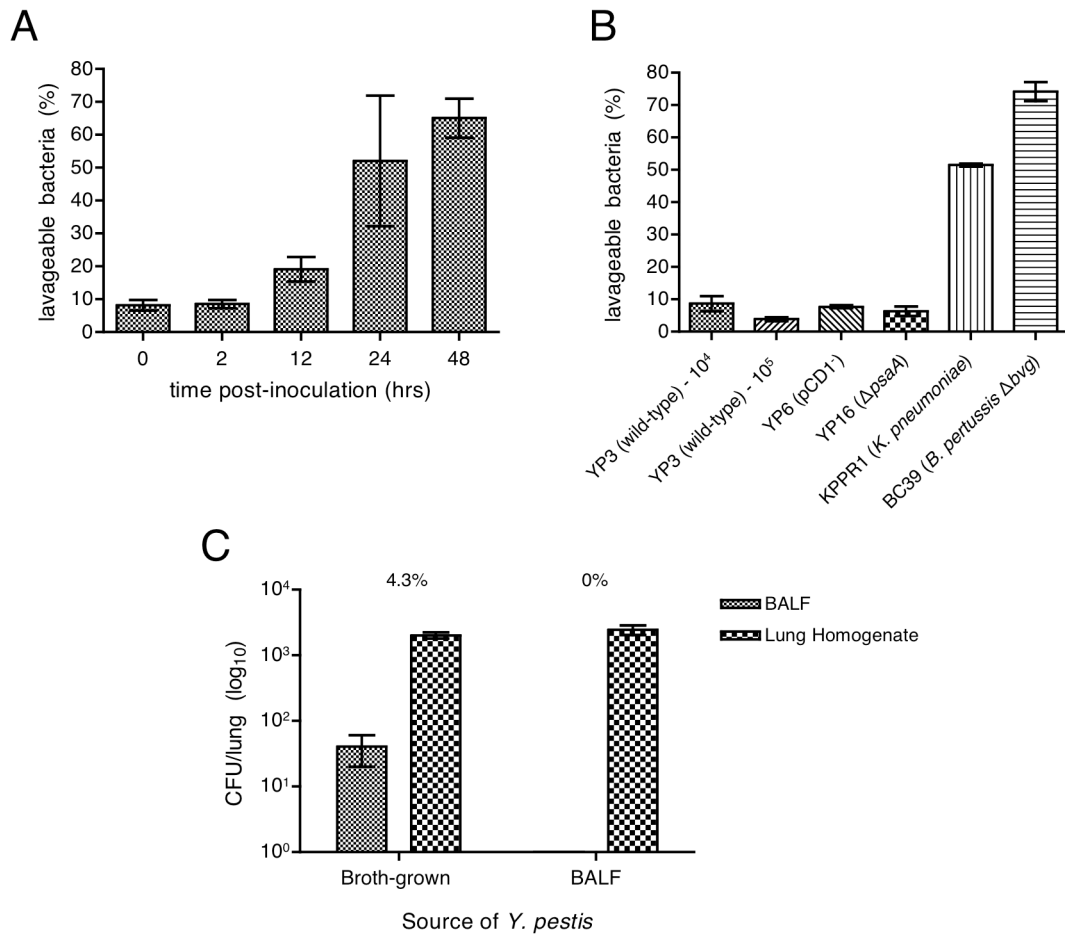


Figure 2-2: Adherence of *Y. pestis* to lung tissues during pulmonary infection. (A) Time course for lavagable bacteria from the lungs of intranasally infected C57BL/6J mice. CFU per lung of lavagable bacteria were determined at various time points post-inoculation. (B) Percent lavagable bacteria were compared for wild-type *Y. pestis* (YP3), pCD1⁻ mutants (YP6), ΔpsaA mutants (YP16), *K. pneumoniae* (KPPR1) and Δbvg *B. pertussis* (BC39). The size of the initial inoculum was also evaluated, 1x10⁴ vs. 1x10⁵. (C) Percent lavagable bacteria were compared for wild-type *Y. pestis* that had been pre-grown in broth or lavaged from the lungs of plague infected mice (48 hpi). (A-C) Each bar represents the mean percent lavagable bacteria from five mice. Percentages were calculated by comparing CFU recovered from BALF and lung homogenates. Error bars represent SEM.

inoculated 48 hours previously, when the bacteria are predominantly lavagable. The kinetics of infection, gross pathology and time to death for these “naturally” infected mice were similar to mice infected with broth-grown bacteria (data not shown). However,

when I performed BAL experiments at 4 hpi, I was unable to detect bacteria in the bronchoalveolar lavage fluid (BALF) (Fig. 2-2C). These data suggest that there may be subtle changes the infectious state of *Y. pestis* upon natural transmission. Although the ultimate outcome of the infection is the same, natural transmission may alter the initial infection, which may possibly lower the LD₅₀. These observations need to be examined in more detail to determine whether a “natural” infection alters the initial dynamics of pneumonic plague.

Y. pestis can survive and replicate inside macrophages *in vitro* and *ex vivo*. Although *Y. pestis* is predominantly extracellular during later stages of infection, an intracellular lifestyle has been postulated to be important during the early stages of infection (Bosio *et al.*, 2005; Pujol and Bliska, 2003; Pujol and Bliska, 2005). However, many of these observations have been made *in vitro* with tissue culture cells or *in vivo* using *Y. pseudotuberculosis*. To test whether *Y. pestis* has an early facultative intracellular lifestyle during pulmonary infection, I performed *ex vivo* gentamicin protection assays on bronchoalveolar lavage fluid (BALF) from mice inoculated intranasally at 4 hpi. The results show that approximately 10% of the bacteria present in the BALF (~1% of the total bacteria in the lung) were resistant to gentamicin (Fig. 2-3A), implying that they are intracellular (Bosio *et al.*, 2005). I also adapted my *ex vivo* gentamicin protection assays to examine the intracellular nature of *Y. pestis* on a whole lung basis (Technique adapted from Justice *et al.*, 2006). Approximately 2-3% of *Y. pestis* were resistant to gentamycin at 4 hpi and 4-5% at 24hpi. In contrast, approximately 85% of *Y. pseudotuberculosis* were resistant to gentamycin at 4 hpi (Fig. 2-3B), suggesting that when *Y. pestis* is pre-grown at 37°C and inoculated intranasally, it

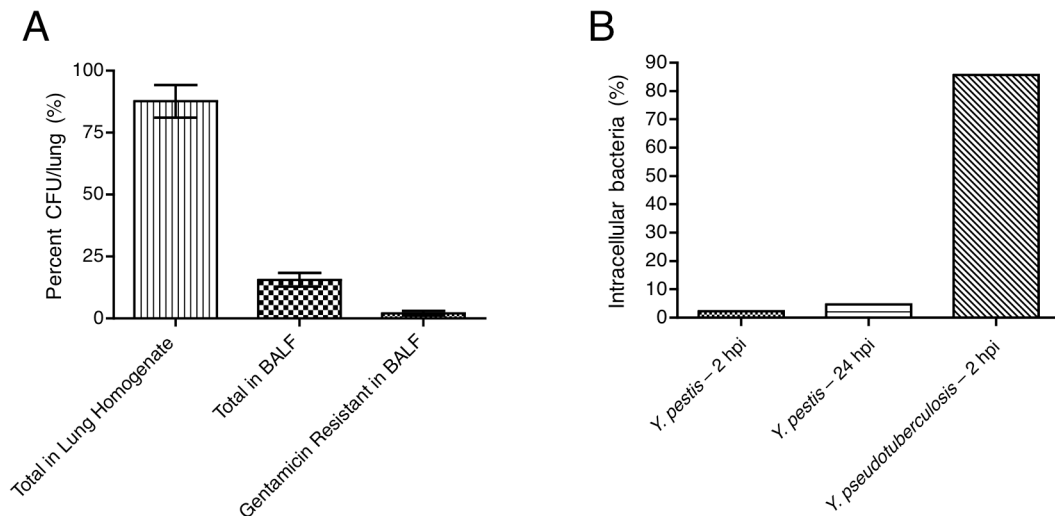


Figure 2-3: Determination of intracellular and extracellular *Y. pestis* during pulmonary infection. (A) Gentamicin resistant (intracellular) bacteria present in BALF. BALF from the lungs of intranasally infected C57BL/6J mice was treated with $100 \mu\text{g ml}^{-1}$ gentamicin or mock treated for 1 hr, and CFU per lung were then determined for lung homogenates and treated/untreated BALF. Percentages were determined from the total number of bacteria in both BALF and lung homogenates. Error bars represent SEM. (B) Percent intracellular bacteria were determined for wild-type *Y. pestis* (YP3) at 2 and 24 hpi and *Y. pseudotuberculosis* (BGY30) at 2 hpi. Each bar represents the mean percentage of intracellular bacteria for three mice. Percentages were calculated by comparing CFU recovered from gentamicin-treated lungs and mock-treated lungs.

remains predominately extracellular throughout the course of infection. However, the importance of the 2-3% of intracellular bacteria during infection is still unknown.

Together, these *in vivo* data indicate that the majority *Y. pestis* are able to adhere tightly to the extracellular surfaces of the lung during the initial stages of infection. In contrast, during the later stages of infection *Y. pestis* can be freely associated lung tissue, which may facilitate person-to-person transmission.

Potential candidates encoding *Y. pestis* adhesins

Adherence to host lung cells is an essential step in the infectious process because the mechanical properties of the mucociliary escalator clear bacteria and other foreign

particles from the lung. This mucociliary escalator plays an important part in maintaining the sterility of the lungs. However, many lung pathogens have adhesins that allow them to circumvent mechanical clearance. Additionally, the translocation of Ysc-Yop T3SS effector proteins (Yops) requires an intimate bacterial interaction with the host cell membrane. The major adhesins that mediate these processes for both *Y. pseudotuberculosis* and *Y. enterocolitica*, YadA and Inv, have been well characterized (Leo and Skurnik, 2011) but are inactivated in *Y. pestis*, suggesting that the major adhesin(s) required intimate host contact and Yop translocation in *Y. pestis* are unknown (Simonet *et al.*, 1996; Skurnik and Wolf-Watz, 1989).

Many studies have concentrated on identifying and characterizing adhesins in *Y. pestis*. The primary potential adhesins that have been studied include: Pla, which enables *E. coli* to adhere to and be internalized by HeLa cells (Lähteenmäki *et al.*, 2001); Pla2, a chromosomally encoded homolog of Pla; the *psa* locus, which is responsible for forming individual fimbrial strands when the bacteria are grown at pH 6 (Liu *et al.*, 2006); and Ail, which enables *E. coli* to adhere to tissue culture cells as well as conferring serum resistance (Bartra *et al.*, 2008; Felek and Krukoniš, 2009). However, *Y. pestis* mutants lacking both Pla and PsaA still show considerable adherence to and invasion into tissue culture cells, indicating the presence of additional surface molecules involved in adhesion and invasion (Kukkonen and Korhonen, 2004; Liu *et al.*, 2006). I generated deletion mutations for each of these genes and tested the resulting mutants for adhesion defects on A549 cells. As observed in the literature, none of these mutations showed decreased levels of *Y. pestis* adherence to A549 cells except *ail* deletion mutants (YPO2905) (data not shown). Phenotypes for *ail* deletion mutants are described below.

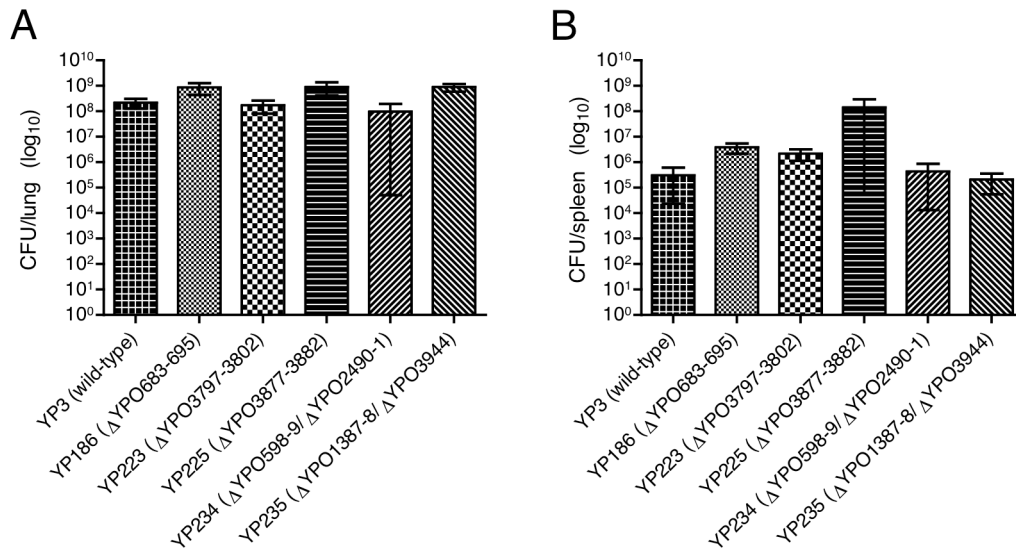


Figure 2-4: Evaluation of potential adhesion mutants in an intranasal animal model of infection. C57BL/6J mice were intranasally inoculated with wild-type *Y. pestis* or potential adhesion mutants [YP3 (wild-type), YP186 (ΔYPO683-695), YP223 (ΔYPO3797-3802), YP225 (ΔYPO3877-3882), YP234 (ΔYPO598-599/ΔYPO2490-2491) or YP235 (ΔYPO1387-1388/ΔYPO3944)]. CFU per lung (A) and spleen (B) were determined at 48 hpi. Each bar represents the mean CFU recovered from three mice. Error bars represent SEM.

The complete *Y. pestis* CO92 genome recently became available (Parkhill *et al.*, 2001). I used known adhesins from the literature to search for homologs in *Y. pestis* that may have adhesive properties. I identified seven genes/operons that encoded proteins with homologies to known adhesins: YPO598-599, Fha-like hemolysin/adhesin; YPO683-695, Tight adherence protein (Tad) operon; YPO1387-1388, Invasin-like adhesin; YPO2490-2491, Fha-like hemolysin/adhesin; YPO3797-3802, CfaA fimbrial operon; YPO3877-3882, Pilli assembly operon; and YPO3944, Invasin-like adhesin. I constructed deletion mutations in each individual operon and different combinations of operons that were homologous to each other using *lambda red*-based homologous recombination. C57BL/6J mice were infected intranasally with 1x10⁴ of each mutant and

ethanized at 48 hpi to examine gross pathology and bacterial burden. I did not observe any statistically significant differences in lung or spleen bacterial burdens or gross histopathology for any of these mutants compared to wild-type *Y. pestis* (Fig. 2-4A-B). Subsequent to these studies, Forman *et al.* showed that mutants lacking YPO1388 have an increased LD₅₀ when inoculated subcutaneously but not intranasally (Forman *et al.*, 2008). The differences in LD₅₀ between the different routes of inoculation emphasize the apparent necessity for specific virulence factors at particular sites of inoculation, where the repertoire of immune cells and their immunologic responses to infection can vary considerably.

Biotinylation of *Y. pestis* surface proteins

Pull-down assays are extensively used to identify specific protein-protein interactions in a complex mixture of proteins. This approach has been used to isolate biotinylated bacterial proteins that specifically interact with components on eukaryotic cell surfaces (Castañeda-Roldán *et al.*, 2006). For this technique, bacterial outer membranes are biotinylated using Sulfo-NHS-LC-Biotin, which readily dissolves in polar solutions and does not permeate cellular membranes, biotinylating only surface components. I used this technique to biotinylate *Y. pestis* surface proteins. Outer membrane fractions from biotinylated *Y. pestis* were then purified, solubilized and incubated with A549 cells for one hour. The cells were then extensively washed to remove any residual or loosely adherent proteins. Biotinylated bacterial proteins that remained adherent to the A549 cells were then detected on far-western blots that were probed with streptavidin-HRP. Four major bands (2 kDa, ~36 kDa, ~50 kDa and ~80 kDa) were present whole cell extracts were probed (Fig. 2-5A). Importantly, none of these

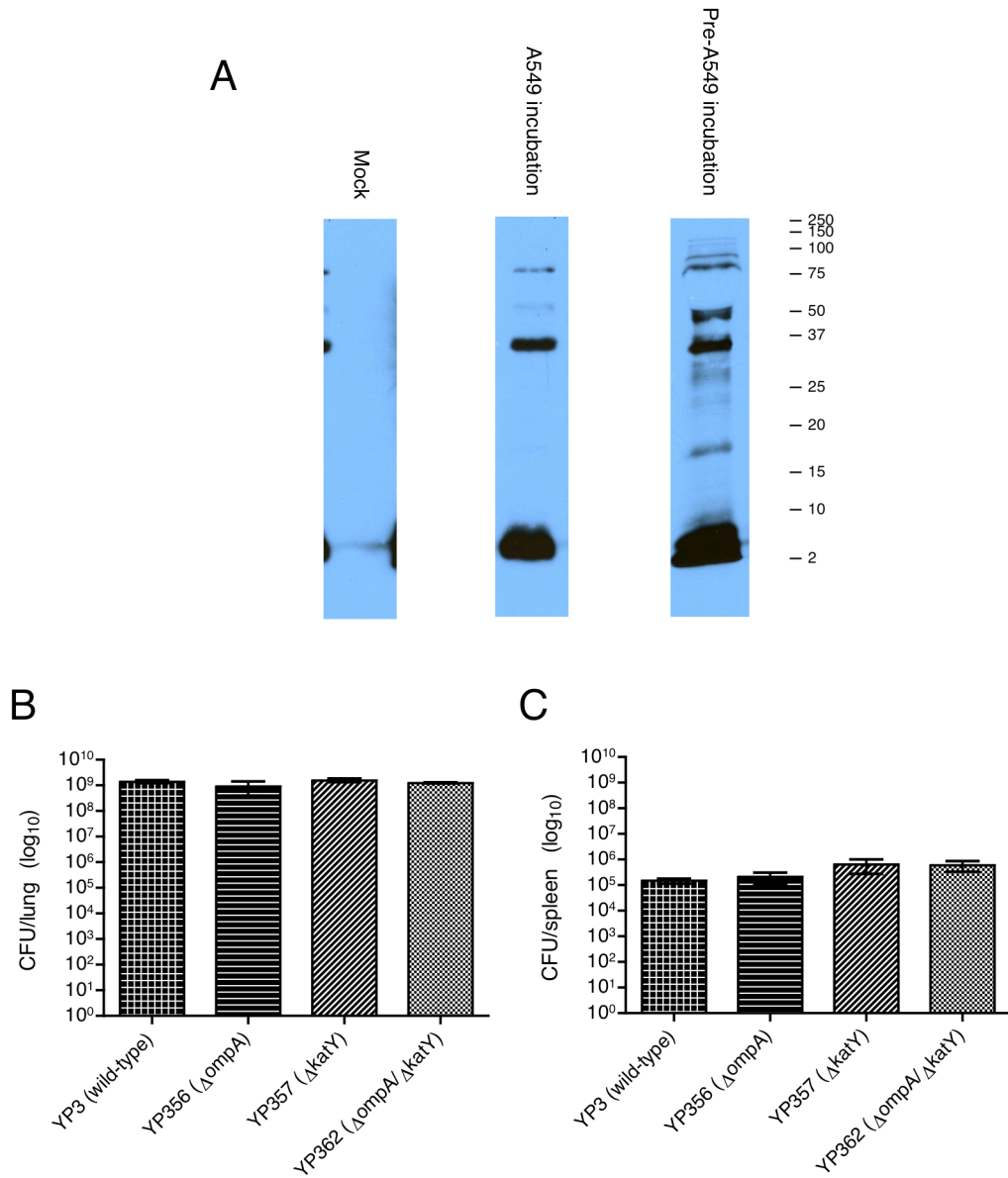


Figure 2-5: Biotinylation of *Y. pestis* surface proteins and evaluation of potential adhesins during infection. (A) Far-western blots of *Y. pestis* outer membrane proteins that were mock-biotinylated (lane 1) or biotinylated (lane 3 – 1/10 dilution). Biotinylated *Y. pestis* outer membrane proteins were then incubated with A549 cells for 1 hr (lane 2). (B-C) C57BL/6J mice were intranasally infected with wild-type *Y. pestis* or potential adhesion mutants [YP3 (wild-type), YP356 ($\Delta ompA$), YP357 ($\Delta katY$) or YP362 ($\Delta ompA/\Delta katY$)]. CFU per lung (B) and spleen (C) were determined at 48 hpi. Each bar represents the mean CFU recovered from three mice. Error bars represent SEM.

bands were the predicted molecular weight for any of the putative *Y. pestis* adhesins, including Ail, Pla or PsaA, indicating that they do not specifically bind to A549 cells under these assay conditions. However, when I used streptavidin affinity resins to specifically isolate these proteins, I was unable to isolate sufficient quantities for positive identification via mass spectrometry.

Fortunately, Myers-Morales *et al.* conducted a series of biotinylation experiments to identify possible protective antigens on the surface of *Y. pestis* (Myers-Morales *et al.*, 2007). From their data, I postulated that the ~36 kDa and ~80 kDa proteins from my biotinylation pull-down experiments could be OmpA and KatY, respectively. Therefore, I constructed $\Delta ompA$, $\Delta kayY$ and $\Delta ompA/\Delta katY$ *Y. pestis* mutants to determine their adhesive properties. Repeated biotinylation experiments with $\Delta ompA$ and $\Delta kayY$ pCD1⁻ mutants indicated that my assumptions were correct, as there was a corresponding loss of either the ~36 kDa or ~80 kDa band (data not shown) in the membrane fractions. Both $\Delta ompA$ and $\Delta kayY$ pCD1⁻ mutants were less autoaggregative when grown at 37°C (data not shown). In addition, OmpA has recently been implicated as an adhesin in certain strains of *E. coli* (Smith *et al.*, 2007). However, both individual mutants and an $\Delta ompA/\Delta katY$ double mutant were fully virulent when C57BL/6J mice were inoculated intranasally (Fig 2-5B-C). Interestingly, unlike the $\Delta ompA$ and $\Delta katY$ single mutants I generated in a pCD1⁻ background, the $\Delta ompA/\Delta katY$ double mutant in a pCD1⁺ background does not have an autoaggregation phenotype (data not shown). This would suggest that there might be additional adhesive proteins encoded on the pCD1 plasmid or at least induced by the presence of the T3SS. Together these data suggest that *Y. pestis* possesses multiple adhesive molecules. However, sufficient quantities of protein for mass

spectrometry identification from the pull-down assays are still needed to ensure that the ~36-kDa and ~80-kDa proteins are indeed OmpA and KatY and not other proteins with similar masses.

Enrichment and characterization of mutants unable to effectively colonize the lung

As described above, *Y. pestis* adheres tightly to lung tissue during a BAL (Fig 2-2A). I took advantage of this observation to devise an enrichment strategy for isolating mutants that are unable to effectively adhere to lung tissues and hence unable to efficiently colonize the lung. This strategy involved infecting mice with library of transposon mutagenized *Y. pestis* and lavaging the lungs of the infected mice two hours later. Bacteria that are lavaged from the lung are then used to infect additional mice. This process is repeated for three rounds of infection, thus, enriching for bacteria that are less adherent to lung tissues. One obvious caveat to this strategy is that it assumes that a single transposon insertion can reduce the adherence of *Y. pestis* to lung tissues enough to be enriched after multiple rounds of BAL. I performed these experiments using a *Himar-1*-based transposon system, and using high throughput sequencing technology, I determined the transposon insertion sites for the remaining mutants after three rounds of enrichment (described in Chapter 3) (Table 2-1).

Upon analyzing the list of genes with transposon insertions that were enriched in the mutant pool, I noticed a pattern in the type of enriched mutants. There were a large number of mutants that had transposon insertions in genes that are involved in sugar transport or sugar processing. Tim Brooks' lab has shown that adding certain types of oligosaccharides to *Y. pestis* before measuring adherence to tissue culture cells reduces the ability of *Y. pestis* to bind to cells (Thomas and Brooks, 2006). Based on these data, it is very likely that a large number of the enriched mutants in this pool are the result of

Table 2-1: Gene identification of enriched mutants. The gene ID, the relative number of sequences for a particular gene, the ratio of the mutant in the input vs. the output library and the annotation for each gene are included.

Gene ID	Sequences	Output/input ratio	Gene annotation
YPO2905	332522	5733.14	(<i>ail</i>) attachment invasion locus protein
YPO1715	136939	2794.67	(<i>ybjR</i>) probable N-acetylmuramoyl-L-alanine amidase
YPO2287	49533	2607.00	taurine transport system permease
YPO0655	35823	11.60	(<i>asoB</i>) involved in surface layer secretion
YPO1841	35238	419.50	(<i>flaV</i>) flagellar hook assembly protein
YPO1743	30203	54.32	(<i>aroP</i>) aromatic amino acid transporter
YPO4068	28837	6.43	(<i>mtlA</i>) Mannitol-specific enzyme IIABC of PTS transport
YPMT1.04c	25072	9.99	unknown function
YPO3991	24514	43.85	putative insulinase family protease
YPO1581	21639	3.27	(<i>rafA</i>) alpha-galactosidase
YPO3965	21247	4.47	hybrid two component system
YPO0698	19356	31.89	chaperone usher system
YPO2718	18490	1155.62	(<i>rnc</i>) ribonuclease III
YPO3992	18450	34.81	(<i>dctA</i>) C4-decarboxylate transport protein
YPO4082	17165	572.17	(<i>avtA</i>) pyruvate aminotransferase
YPO3987	17002	18.22	unknown function
YPO3016	15223	8.59	(<i>nanT</i>) sialic acid transporter
YPO3923	14610	7.09	heme receptor protein
YPO3129	13923	14.15	(<i>aefA/kefA</i>) potassium efflux system
YPCD1.29c	13909	1069.92	(<i>yopB</i>) T3SS translocon component
YPO4093	12609	40.41	putative haloacid dehalogenase-like hydrolase
YPO1254	12271	19.14	(<i>bglA</i>) 6-phospho-beta-glucosidase
YPO1440	11702	229.45	putative helicase IV
YPO0181	11580	5790.00	sodium dependent serotonin ABC transport protein
YPO0402	11396	52.28	PTS system, IIB component
YPO1661	10938	9.41	(<i>mgfB</i>) Mg(2+) transport ATPase protein B
YPO2289	10517	24.01	(<i>srfA</i>) <i>Salmonella</i> putative virulence factor
YPO0587	10334	41.17	(<i>yapG</i>) autotransporter
YPO3087	10320	245.71	(<i>ybaP</i>) conserved hypothetical protein
YPO3798	10313	5.27	fimbrial subunit precursor

increased levels of extracellular or periplasmic oligosaccharides inhibiting the actual adhesin.

For example, the mutant that were enriched the most after the *ail* mutants (see below) had transposon insertions in the gene *amiD*, which is involved in recycling peptidoglycan in the periplasm (Uehara and Park, 2007). An *amiDampG* deletion mutant

in *E. coli* releases large amounts of disaccharide-peptide subunits of peptidoglycan (also known as TCT) into the surrounding environment. *Y. pestis* C092 releases small amounts of TCT into the media (data not shown), but mutations in *amiD* may increase these levels. Disaccharides similar to those found in the disaccharide portion of TCT have been shown to inhibit *Y. pestis* adherence to tissue culture cells (Thomas and Brooks, 2006) and may explain why this mutant was enriched during the screen for non-adherent mutants. These findings suggest that the predominant adhesins in *Y. pestis* utilize certain mono- or polysaccharides found on lung tissues for adherence.

Mutants with transposon insertions in the gene *ail* (YPO2905) were highly enriched during the serial enrichment strategy. As described previously, *ail* mutants have a very distinct colony morphology (Kolodziejek *et al.*, 2007), and I initially used these observations to determine that mutants with transposon insertions in *ail* were being highly enriched. High throughput sequencing analysis of the entire pool of enriched mutants confirmed that mutants with transposon insertions in *ail* were by far the most enriched mutants in the pool. Ail is a 17 kDa outer membrane protein that has eight membrane-spanning amphipathic β -strands with four extracellular loops (Miller *et al.*, 1990; Plesniak *et al.*, 2011). The Ail homolog in *Y. enterocolitica*, when expressed in *E. coli*, has been shown to confer a high-level of attachment and invasion to tissue culture cells and to confer serum resistance (Bliska and Falkow, 1992). However, Ail homologs in *Y. pseudotuberculosis* and *Y. pestis* only have a slight adhesive phenotype (Felek and Krukonis, 2009; Yang *et al.*, 1996). A number of related proteins have been found in other organisms and despite their high degree of similarity, their ascribed functions vary widely (Baldermann *et al.*, 1998; Cirillo *et al.*, 1996; Mecsas *et al.*, 1995; Miller *et al.*,

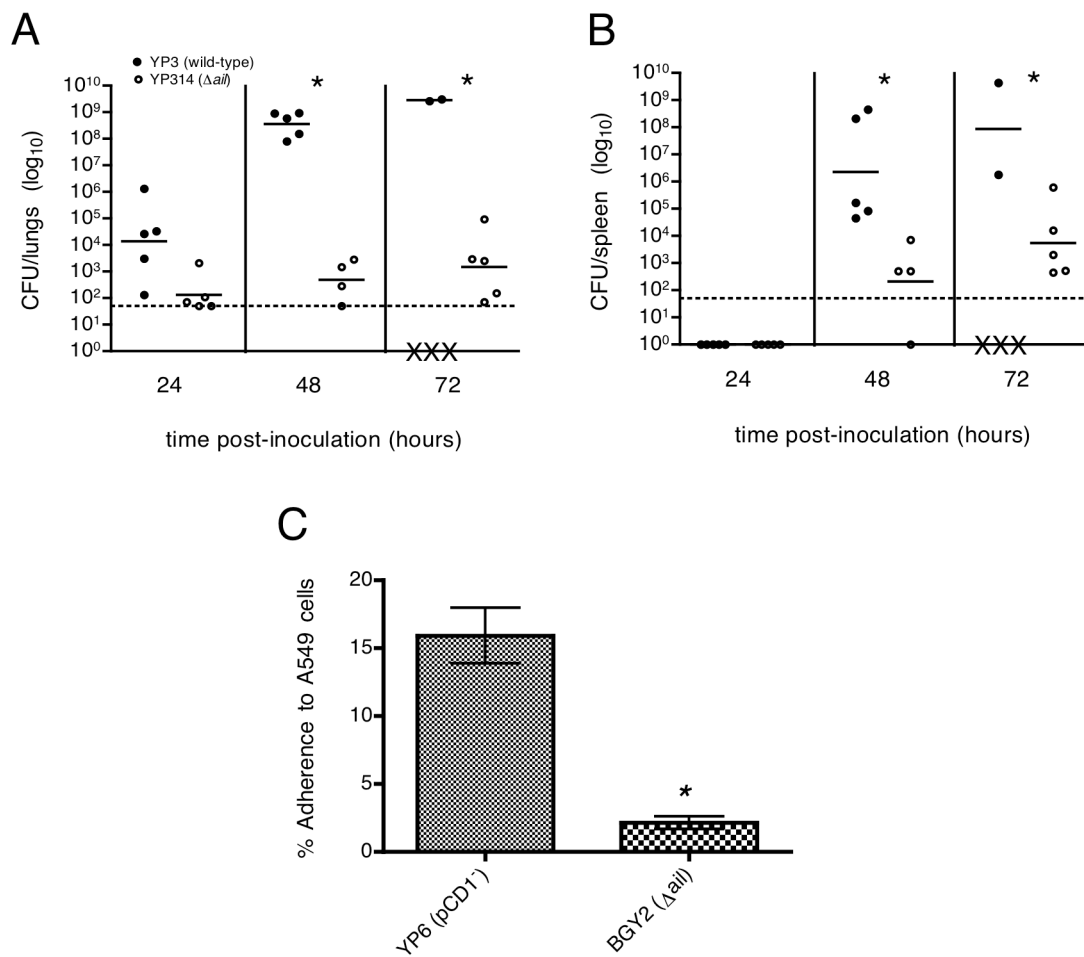


Figure 2-6: Kinetics of infection for a Δail *Y. pestis* mutant. (A-B) Kinetics of infection for wild-type *Y. pestis* (YP3 - solid circles) and Δail mutant (YP314 - open circles) infected intranasally in C57BL/6 mice. CFU per lung (A) and spleen (B) were determined at various time points of infection. Each bar represents the geometric mean for CFU recovered from five mice. The limit of detection is represented by a dotted line. (C) Adherence of pCD1⁺ *Y. pestis* and Δail pCD1⁺ mutant to A549 cells at 1 hpi. Bacteria were inoculated at an MOI of 10:1. Each bar represents the mean percent adherence relative to the inoculum. Error bars represent standard deviation (SD) from the mean. * $p \leq 0.05$.

1992). Although a few of these related proteins have minor phenotypes in their respective mouse models of infection when mutated, *ail* mutants in *Y. enterocolitica* or *Y. pseudotuberculosis* do not (Wachtel and Miller, 1995). On the other hand, when I constructed a clean *ail* deletion mutant using our standard *lambda red*- based homologous

recombination protocol, it was severely attenuated in our mouse model of pneumonic plague (Fig. 2-6A-B) and showed a 10-fold decrease in adhesion to A549 cells (Fig. 2-6C), which has subsequently been reported in the literature (Felek and Krukoniš, 2009; Kolodziejek *et al.*, 2010). However, it is unclear whether the adhesive phenotype or some other reported function of Ail such as serum resistance is responsible for this severe attenuation.

Each mutant I generate in the laboratory undergoes a series of tests to confirm that all the major virulence loci (pCD1, pMT1, pPCP1 and *pgm* locus) are present and that Ysc-Yop T3SS is functional. Upon testing my clean Δail mutant, I noticed that even though it had the pCD1 plasmid, it failed to fully undergo a classic low calcium response when grown at 37°C without supplemental calcium. Instead, the Δail mutant was able to grow quite readily on BHI-MOX plates at 37°C although not as well as pCD1⁻ mutants (data not shown). As reported in the literature (Felek and Krukoniš, 2009), my Δail mutant was able to secrete Yops, but the secretion profile for the Δail mutant was surprising more similar to that of a Δpla mutant than wild-type *Y. pestis* (Fig. 2-7A). The plasminogen-activating protease Pla normally cleaves extracellular Yops and hence, a Δpla mutant has a very distinct Yop secretion profile. Given that modifications in the structure of LPS can alter the activity of Pla (Suomalainen *et al.*, 2010), I performed several membrane stability assays on a Δail mutant in a pCD1⁻ background. As reported by Bartra *et al.*, my Δail mutant was 10-fold more susceptible to antimicrobial peptides (MIC for polymyxin B – YP6 - $\geq 250 \mu\text{g ml}^{-1}$ vs. YP314pCD1⁻ - $25 \mu\text{g ml}^{-1}$) and human serum compared to pCD1⁻ *Y. pestis* (data not shown)(Bartra *et al.*, 2008). Certain cationic dyes also inhibit bacterial growth, and growth in the dye is often directly dependent on

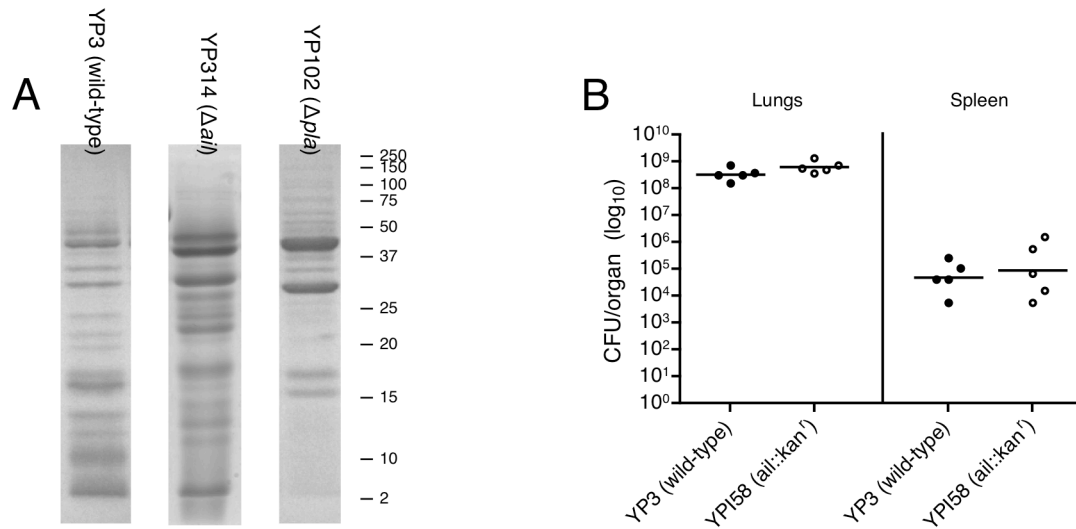


Figure 2-7: Characterization of Yop secretion profiles in wild-type and Δail mutant *Y. pestis*. (A) Yop secretion profile after 3 hr of growth in BHI-MOX media for wild-type *Y. pestis* (YP3 – lane 1), a Δail mutant (YP314 - lane 2) and a Δpla mutant (YP102 – lane 3). Supernatants were precipitated with trichloroacetic acid, run on a 12% SDS-PAGE gel and stained with coomassie blue. Standard molecular weight markers are indicated. (B) C57BL/6 mice were infected intranasally with wild-type *Y. pestis* (YP3 - solid circles) and Δail mutant (YPI58 - open circles). CFU per lung and spleen were determined at 48 hpi. Each bar represents the geometric mean for CFU recovered from five mice.

the degree of membrane stability (Bengoechea *et al.*, 1998). The Δail mutant showed increased susceptibility to crystal violet (MIC for crystal violet – YP6 - $\geq 64 \mu\text{g ml}^{-1}$ vs. YP314 pCD1⁻ - $16 \mu\text{g ml}^{-1}$) compared to wild-type pCD1⁻ *Y. pestis*. During assays used to test membrane stress, such as growing the bacteria on the detergents, I noticed that the unusual colony morphology of the *ail* mutant could be mimicked by growing wild-type pCD1⁻ *Y. pestis* on media containing 0.1% Triton-X (data not shown), suggesting that a decrease in membrane stability may be responsible for the colony morphology of the Δail mutant. In addition, growth of a Δail mutant at 37°C results in the release of 100-fold more LPS into the media compared to wild-type pCD1⁻ *Y. pestis*, which release can be

ameliorated by supplementing the media with calcium. These results further suggest that Ail is acting to stabilize the bacterial outer membrane because excess calcium helps stabilize interactions between LPS molecules in the outer membrane of Gram-negative bacteria (Alakomi *et al.*, 2003).

Strikingly, Δail mutants that have been passaged fewer times, i.e., where the kanamycin resistance cassette has not been resolved, are almost as virulent as wild-type bacteria (Fig. 2-7B). These data suggest that while Ail may not be in and of itself be an adhesin for *Y. pestis*, the loss of Ail in the outer membrane may lead to membrane instability and secondary compensatory mutations may affect other outer membrane proteins, such as Pla and putative adhesins. In agreement with this hypothesis, Pierson conducted a transposon mutant screen in *Y. enterocolitica* to identify genes whose inactivation resulted in increased Ail production and identified mutants with transposon insertions in genes involved in LPS biosynthesis (Pierson, 1994). Remarkably, many of these same genes, which are involved in O-antigen biosynthesis, are pseudogenes in *Y. pestis* (Parkhill *et al.*, 2001), suggesting that increased production of Ail may be compensating for the loss of membrane stability caused by the inactivation of certain LPS biosynthesis genes (Yethon *et al.*, 2000), whose inactivation, coincidentally, is required for the proteolytic activity of Pla (Kukkonen *et al.*, 2004; Pouillot *et al.*, 2005). Further investigation is needed to test this hypothesis, and genomic or proteomic comparisons between these two Δail mutant strains may be useful in determining which proteins in the outer membrane may act as the actual adhesins. These data also suggest that the severe animal phenotype seen for the Δail mutant lacking the kanamycin resistance cassette may be partially due to decreased Pla activity (Compare with Lathem *et al.*, 2007).

inv or *yadA* expression in *Y. pestis* and their *in vivo* effect on pathogenesis

The evolution of *Y. pestis* from *Y. pseudotuberculosis* involved the acquisition, loss and modification of several genes including predominant virulence factors. The slight modification of several genes has been implicated in virulence in *Y. pestis* including changes to the structure of LPS (Montminy *et al.*, 2006) and reductions in the secretion of a type-three effector (Zauberman *et al.*, 2009). As mentioned previously, the major adhesins for both *Y. pseudotuberculosis* and *Y. enterocolitica*, YadA and Inv, are both inactivated in *Y. pestis*. I introduced functional versions of these genes from *Y. pseudotuberculosis* into *Y. pestis* to determine whether their loss was the result of selective pressures or random mutation. These experiments were approved by the Biological Safety Officer and Institutional Biological & Chemical (IBC) Safety Committee at Washington University in St. Louis.

Previously, Rosqvist *et al.* reported that replacing the *Y. pestis* virulence plasmid (pCD1) with the virulence plasmid from *Y. pseudotuberculosis* (pYV) reduces the virulence of *Y. pestis* (Rosqvist *et al.*, 1988). They also showed that wild-type levels of virulence were restored when YadA was disrupted but failed to characterize these results further. I similarly reintroduced *yadA* into *Y. pestis* by exchanging the entire pCD1 plasmid for pYV to create a pCD1⁻ pYV⁺ mutant of *Y. pestis*. [Please note that the following observations were attributed to YadA only after the bulk of these analyses were completed; therefore the appropriate controls with defined pCD1⁻ pYV⁺ $\Delta yadA$ mutants were limited.] I infected C57BL/6J mice intranasally with a pCD1⁻ pYV⁺ mutant of *Y. pestis* and observed some unusual alterations in the disease course of the plague syndrome. Mice infected with pCD1⁻ pYV⁺ *Y. pestis* presented with physical signs of

disease much earlier than wild-type *Y. pestis*, where the animals showed the first signs of illness at 24-36 hpi compared to 36-48 hpi for wild-type-infected animals. In agreement with these observations, lung histology sections showed more neutrophil influx and lung consolidation at 48 hpi for pCD1⁻ pYV⁺ *Y. pestis* compared to wild-type bacteria (Fig. 2-8C-D). However, the bacterial burdens in the lungs during the time course of disease were not significantly different; however, there was a reproducible delay in dissemination to the spleen at 48 hpi for pCD1⁻ pYV⁺ *Y. pestis* (Fig. 2-8A-B). In addition, there was an increase in the time to death for mice infected with this mutant, which survived 12-24 hr longer than their wild-type controls (Fig 2-8E). I believe that these data indicate that lung pathology alone cannot fully explain the rapid time of death during primary pneumonic plague but that bacterial dissemination and the resultant multi-organ failure induced by septicemia may have a significant impact on the ultimate cause of death.

Mutants of *Y. pestis* that cause pathology earlier during infection are attenuated in a subcutaneous model of infection (Zauberman *et al.*, 2009). Given the increased and early onset of pathology in the lung for the pCD1⁻ pYV⁺ mutant, I used a subcutaneous inoculation to analyze the effect of YadA on bubonic plague. I inoculated C57BL/6J mice subcutaneously with pCD1⁻ pYV⁺ or wild-type *Y. pestis* and observed a decrease in colonization of the lymph nodes and a delay in dissemination to the spleen at 48 hpi (Fig. 2-9A-B). As the infection progressed, the size and weight of the lymph nodes from mice infected with pCD1⁻ pYV⁺ *Y. pestis* were significantly smaller than the lymph nodes from mice infected with wild-type *Y. pestis* (Fig 2-9C). Moreover, the average time to death for mice infected with wild-type and pCD1⁻ pYV⁺ *Y. pestis* was 96 and 192 hpi, respectively (Fig 2-9D). Interestingly, the lymph nodes of two moribund pCD1⁻ pYV⁺ *Y.*

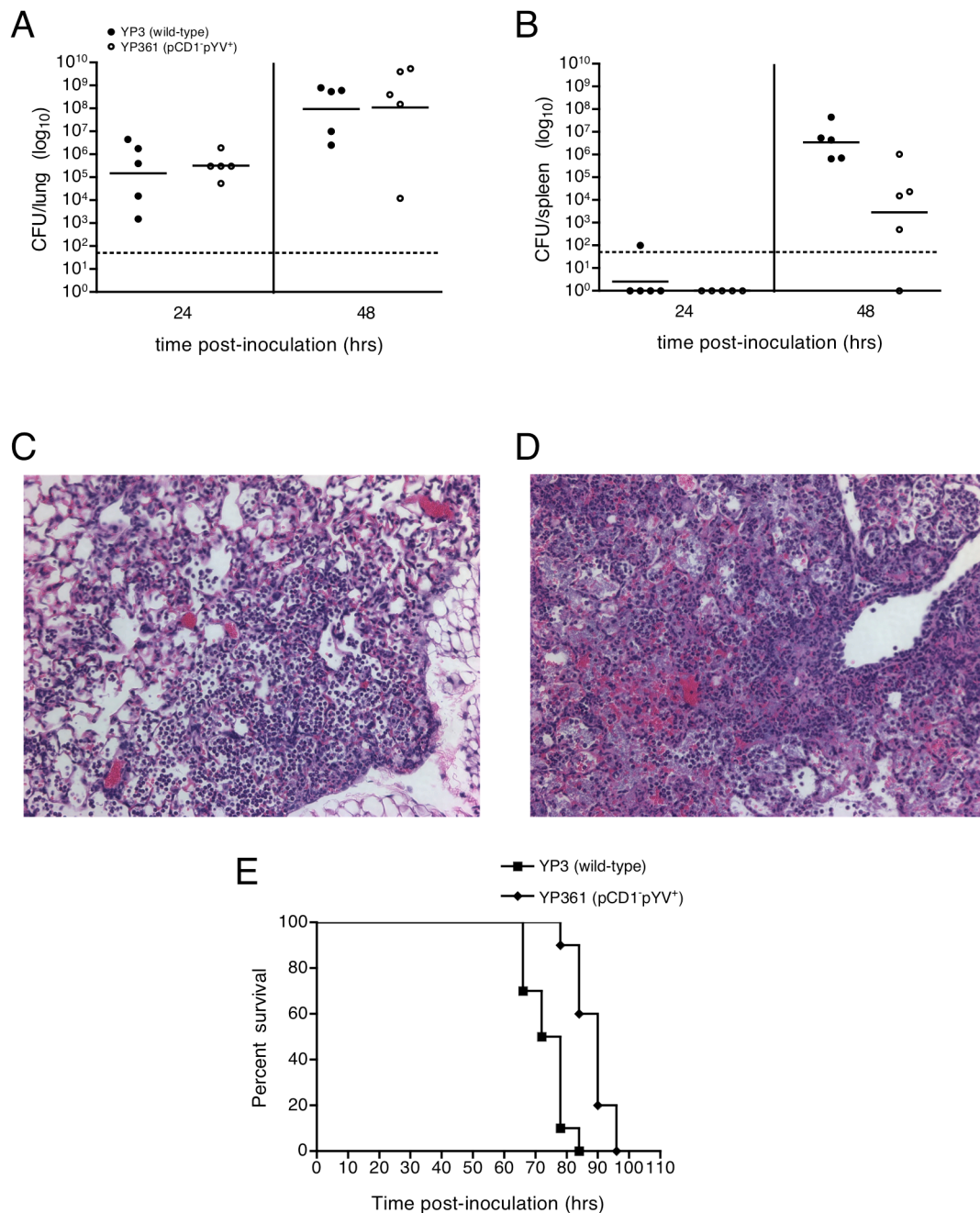


Figure 2-8: Pulmonary infection with pCD1⁻ pYV⁺ *Y. pestis*. (A-B) Kinetics of infection for wild-type *Y. pestis* (YP3 - solid circles) and pCD1⁻ pYV⁺ *Y. pestis* (YP361 - open circles) infected intranasally in C57BL/6 mice. CFU per lung (A) and spleen (B) were determined at various time points of infection. Each bar represents the geometric mean for CFU recovered from five mice. The limit of detection is represented by a dotted line. (C-D) Histology sections of paraffin embedded lungs stained with hematoxylin and eosin for mice intranasally infected with wild-type (C) and pCD1⁻ pYV⁺ *Y. pestis* (D). (E) Survival analysis of wild-type and pCD1⁻ pYV⁺ *Y. pestis* during pulmonary infection, in which the mean time to death was 75 and 90 hpi, respectively.

pestis-infected mice at 208 hpi showed limited signs of inflammation, and the terminal bacterial burdens in the lymph nodes and spleens of these mice were approximately two logs lower than wild-type-infected mice (84 hpi). In contrast, the lungs of these moribund pCD1⁻ pYV⁺ *Y. pestis*-infected mice were completely consolidated with bacterial burdens reaching approximately 5×10^{10} CFU (Fig. 2-9E). Further, I observed that symptoms of moribund pCD1⁻ pYV⁺ *Y. pestis*-infected mice were more consistent with the animals dying of a pneumonia and not septicemia, which is believed to be the ultimate cause of death during bubonic plague. Together, the data for the intranasal and subcutaneous infections suggest that the presence of YadA leads to an increase in pathology, which results in an altered immune response that is able to somewhat control the infection in the lymphatics and spleen but not the lungs.

Casutt-Meyer *et al.* have recently shown that *Yersinia* species induce the synthesis of neutrophil extracellular traps (NETs) upon contact with neutrophils. They also showed that *Yersinia* that express *yadA*, including when it is artificially overexpressed in *Y. pestis*, attach more readily to the collagen within NETs, which results in bacterial killing (Casutt-Meyer *et al.*, 2010). However, the plasminogen activating protease Pla cleaves the majority of YadA from the bacterial outer membrane when *yadA* is expressed at normal levels (Kutyrev *et al.*, 1999). I therefore constructed a Δ *pla* deletion mutant in a pCD1⁻ pYV⁺ *Y. pestis* background to test whether YadA and NET-induced killing would further reduce the virulence of a Δ *pla* mutant. Contrary to my expectations of more killing, I observed that the introduction of YadA into a Δ *pla* mutant was able to rescue three logs of bacterial replication at 48 hpi in the lungs of intranasally infected mice compared to Δ *pla* mutants or pCD1⁻ pYV⁺ Δ *pla* Δ *yadA* mutants (Fig. 2-10A). Although

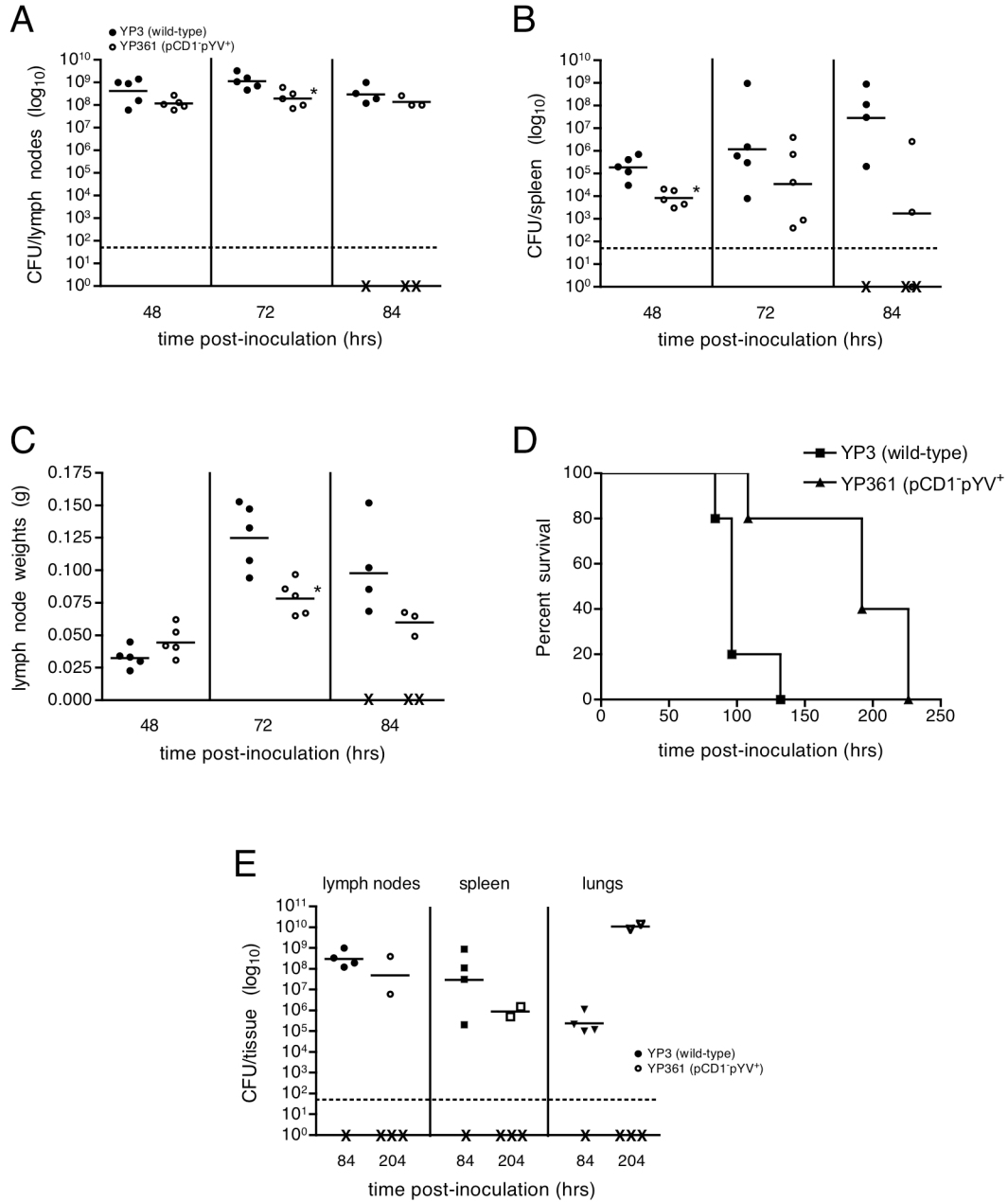


Figure 2-9: Bubonic infection with pCD1⁻ pYV⁺ *Y. pestis*. (A-B) Kinetics of infection for wild-type *Y. pestis* (YP3 - solid circles) and pCD1⁻ pYV⁺ *Y. pestis* (YP361 - open circles) in C57BL/6 mice infected subcutaneously. CFU per lymph nodes (A) and spleen (B) were determined at various time points of infection. (C) Lymph node weights taken from subcutaneously infected mice at various time points of infection. (D) Survival analysis of wild-type and pCD1⁻ pYV⁺ *Y. pestis* during subcutaneous infection, in which the mean time to death was 96 and 192 hpi, respectively. (E) Terminal organ burdens were taken from moribund mice infected subcutaneously with wild-type *Y. pestis* or pCD1⁻ pYV⁺ *Y. pestis* at 84 and 204 hpi, respectively. The limit of detection is represented by a dotted line. Each bar represents the geometric mean for CFU recovered from four to five mice. * $p \leq 0.05$.

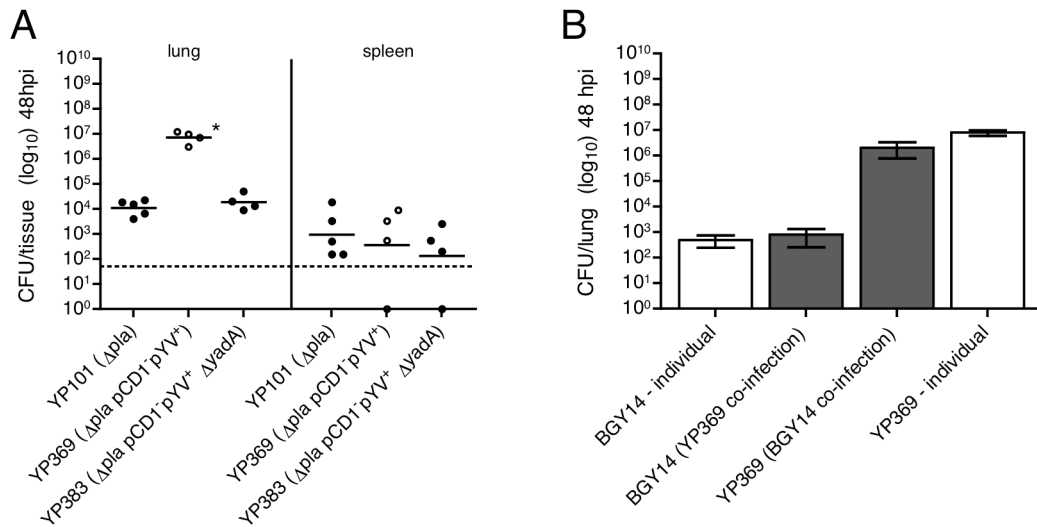


Figure 2-10: Pulmonary infection with Δpla pCD1⁻pYV⁺ and Δpla pCD1⁻pYV⁺ $\Delta yadA$ *Y. pestis*. (A) C57BL/6 mice infected intranasally with Δpla (YP102), Δpla pCD1⁻pYV⁺ (YP369) or Δpla pCD1⁻pYV⁺ $\Delta yadA$ (YP383) *Y. pestis*, and CFU per lung and spleen were determined at 48 hpi. Each bar represents the geometric mean for CFU recovered from four to five mice. The limit of detection is represented by a dotted line. (B) Individual intranasal infections (white bars) for pCD1⁻ (BGY14) or Δpla pCD1⁻pYV⁺ mutants or matched co-infections (gray bars) both strains in C57BL/6J mice. CFU per lung were determined at 48 hpi and strains were differentiated on BHI agar plates containing kanamycin. Error bars represent SEM. * $p \leq 0.05$.

the pCD1⁻ pYV⁺ Δpla mutants are able to partially restore bacterial replication in the lung, they are unable to trans-complement avirulent *Y. pestis* (See Chapter 4 and Fig. 2-10B). These findings suggest that the requirement of YadA for the virulence of *Y. pseudotuberculosis* and *Y. enterocolitica* in animals may have been compensated for by the acquisition of pPCP1, which encodes *pla*. The sensitivity of bacteria expressing *yadA* to NETs may provide an explanation as for why evolution selected for the inactivation of *yadA* in *Y. pestis*; however the timing of the acquisition of pPCP1 and the inactivation of *yadA* remains unknown. Additional experiments need to be performed to further investigate the evolutionary implications of these findings.

Similar to YadA, Invasin is also inactivated in *Y. pestis*. I reintroduced invasin into *Y. pestis* under the control of its native promoter, which shows maximal expression at 26°C and minimal expression at 37°C (Ellison *et al.*, 2003), to determine its contribution to *Y. pestis* virulence. As expected, *inv*⁺ *Y. pestis* was more invasive than wild-type *Y. pestis* in my tissue culture model of invasion when pre-grown at 26°C but not when pre-grown at 37°C (Fig. 2-11A). In my intranasal mouse model of infection, *inv*⁺ *Y. pestis* displayed similar kinetics of infection in the lung and spleen compared to wild-type bacteria (Fig. 2-11B-C). However, in my subcutaneous mouse model of infection, *inv*⁺ *Y. pestis* was unable to colonize the cervical lymph nodes as efficiently as wild-type *Y. pestis* at 48 hpi (Fig. 2-11D). Similar to YadA, the lymph nodes from *inv*⁺ *Y. pestis*-infected mice weighed significantly less than the lymph nodes from wild-type-infected mice (Fig. 2-11E). Organ weights are normally indicative of the level of inflammation, suggesting that *inv*⁺ *Y. pestis* mutants produce lower levels of inflammation during bubonic plague. As with YadA, the decreased ability to colonize the lymph nodes may explain why evolution selected for the inactivation of *inv* in *Y. pestis*.

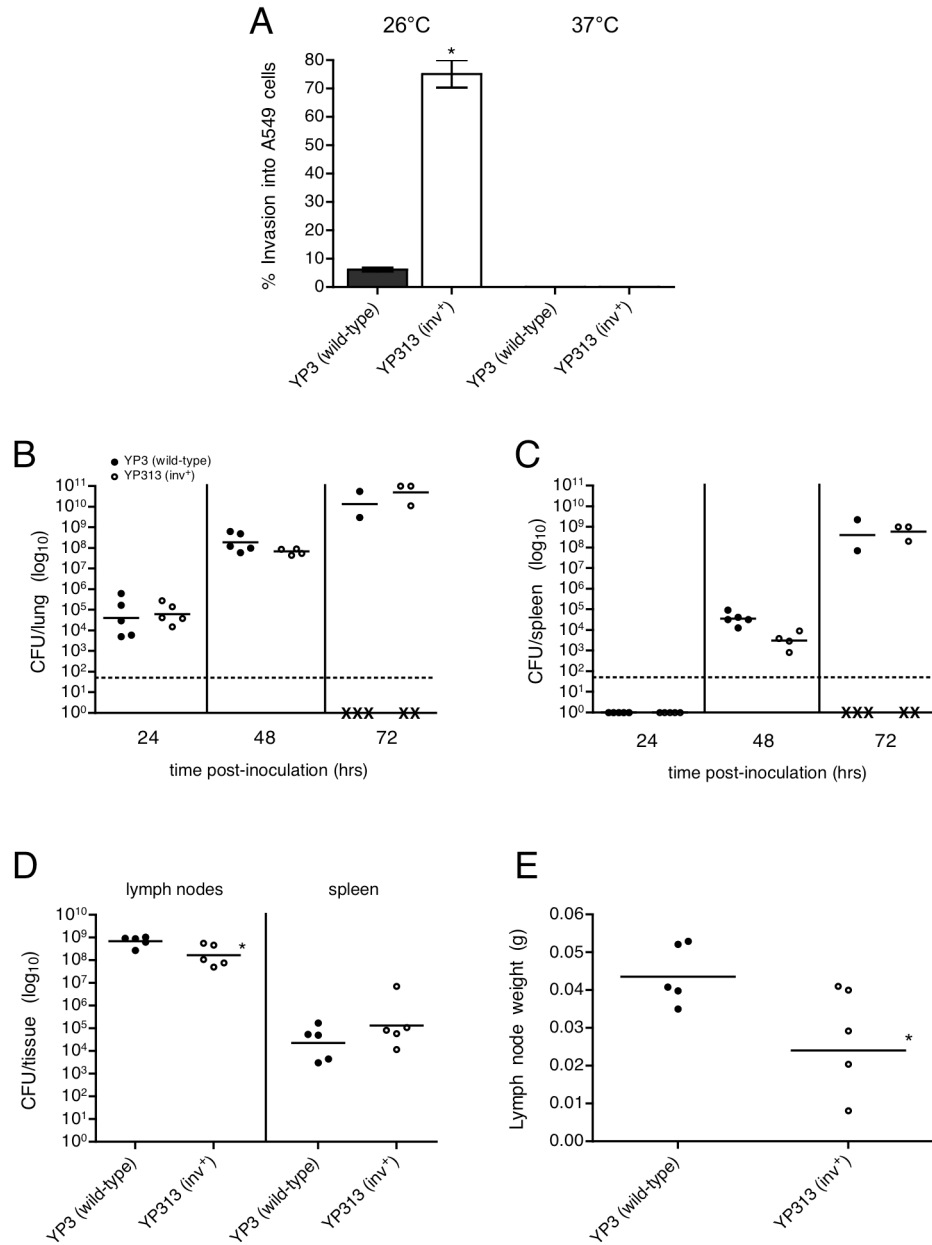


Figure 2-11: Analysis of *inv*⁺ expression on *Y. pestis* infection. (A) Invasion of wild-type and *inv*⁺ *Y. pestis* into A549 cells at 3 hpi as determined by a gentamicin protection assay. Bacteria were pre-grown at 26°C or 37°C and inoculated at an MOI of 10:1. At 3 hpi, the cells were incubated with gentamicin to kill extracellular bacteria. Each bar represents the mean percent invasion relative to the inoculum. Error bars represent SEM. (B-D) Kinetics of infection for wild-type *Y. pestis* (YP3 - solid circles) and *inv*⁺ *Y. pestis* (YP314 - open circles) in C57BL/6 mice infected intranasally (B-C) or subcutaneously (D). CFU per lung (B), spleen (C-D) and lymph nodes (D) were determined at various time points of infection. (E) Lymph node weights taken from subcutaneously infected mice at 48 hpi. The limit of detection is represented by a dotted line. Each bar represents the geometric mean for CFU recovered from five mice. * $p \leq 0.05$.

Conclusions

Adherence to tissues is considered one of the initial steps in the infectious process and is particularly important for bacteria that employ T3SS to subvert immune responses and promote virulence. *Y. pestis* adhesion to host tissues has been extensively researched *in vitro* on tissue culture cells, but many of these findings have not been corroborated *in vivo*. Here, I have established a model to examine *Y. pestis* adhesion to host tissues and show that *Y. pestis* adheres tightly to lung tissues early during mammalian infection and then becomes largely non-adherent (or adherent to freely lavageable cells such as neutrophils) during the later stages of infection. The dynamic shift in adherence to lung tissues may explain why person-to-person transmission of pneumonic plague has only been observed for patients in the late stages of infection (Chernin, 1989). I also show that *Y. pestis* is largely extracellular during early stages of pneumonic plague, indicating that a facultative intracellular lifestyle likely does not occur during pneumonic plague. My findings suggest that there are multiple potential adhesins that mediate adherence to lung cells; however, I was not able to specifically identify the exact nature of these adhesins. Additional research is needed to identify the adhesive components in *Y. pestis* that allow for intimate host cell contacts.

Y. pestis lacks the two dominant adhesins, YadA and Invasin, found in its ancestral strain, *Y. pseudotuberculosis*. I show that *Y. pestis* mutants in which *yadA* and *inv* expression has been restored are attenuated in our animal model of bubonic plague and cause a slightly altered form of pneumonic plague. Intriguingly, YadA is required for virulence in *Y. pseudotuberculosis* and *Y. enterocolitica* animal models of infection but detrimental to the virulence of *Y. pestis*. Strikingly, YadA can restore the bacterial

proliferation in the lung of Δpla deletion strains, suggesting that the acquisition of Pla and its proteolytic activity by early progenitors of *Y. pestis* may have compensated for the requirement of YadA during infection. Together these data corroborate theories that the *Y. pestis* genome has undergone (is undergoing) extensive remodeling to create the highly virulent pathogen currently found in the world today.

Materials and Methods

Reagents, bacterial strains and culture conditions

All chemicals, unless otherwise noted, were obtained from Sigma-Aldrich, Inc. (St. Louis, MO). Table 2-2 provides a description of bacterial strains, plasmids and primers used in Chapters 2, 3 and 4. The fully virulent, wild-type *Yersinia pestis* strain CO92 (YP3) and its plasmid cured derivative CO92 pCD1⁻ (YP6) were obtained from the U.S. Army, Ft. Detrick, MD. The presence or absence of pCD1, pMT1, pPCP1, and the *pgm* locus was confirmed by PCR for each strain prior to use. *Y. pestis* were routinely grown on brain-heart infusion (BHI) agar (Difco Laboratories, Detroit, MI) supplemented with at 26°C for 2-3 d. Liquid cultures of *Y. pestis* were grown in BHI broth (Difco Laboratories, Detroit, MI) at 26°C for 6-8 hr before being diluted to an OD₆₂₀ of 0.05-0.1 in 10 ml of BHI supplemented with 2.5 mM CaCl₂ in a 125 ml Erlenmeyer flask and grown overnight at 37°C or 26°C on a shaker set at 250 rpm. *Y. pseudotuberculosis* IP2666c and derivatives were obtained from the laboratory of Dr. Joan Mecsas. *Y. pseudotuberculosis* 32953 and *K. pneumoniae* subspecies *pneumoniae* and derivatives were obtained from the laboratory of Dr. Virginia Miller. *Y. pseudotuberculosis* strains were routinely grown at 26°C on LB agar for 2 d or in LB broth for 12-14 hr. *K. pneumoniae* strains were grown at 37°C for 10-12 hr on LB agar or in LB broth. For animal infections, *Y. pseudotuberculosis* and *K. pneumoniae* were grown in BHI broth as described above for *Y. pestis*. *B. pertussis* were grown on SSM agar supplemented with 10 ml l⁻¹ sheep blood at 37°C for 3-4 d or in SSM broth at 37°C for 24 hr. Media were supplemented with ampicillin (100 µg ml⁻¹), kanamycin (50 µg ml⁻¹), rifampicin (30 µg ml⁻¹), polymyxin B (25 µg ml⁻¹), Irgasan (2 µg ml⁻¹), streptomycin (50 µg ml⁻¹),

chloramphenicol (25 $\mu\text{g ml}^{-1}$), X-gal (50 $\mu\text{g ml}^{-1}$), magnesium oxalate (MOX, 20 mM MgCl_2 and 20 mM $\text{Na}_2\text{C}_2\text{O}_4$), 0.1% Triton-X or 2.5 mM CaCl_2 as required.

Construction of *Y. pestis* deletion strains

Y. pestis deletion strains were constructed using a modified *lambda red* recombination system described previously by Lathem et al. (Lathem *et al.*, 2007). Briefly, 500 bp upstream (first two primers) and 500 bp downstream (last two primers) of each gene was amplified by PCR using the following primer sets: $\Delta yadA$ (*yadA*(pYV)3', *yadA*(pYV)5'P1, *yadA*(pYV)3'P4 and *yadA*(pYV)5'); $\Delta YPO0598-599$ (YPO0598-599 KO3', YPO0598-599 KO5'P1, YPO0598-599 KO3'P4 and YPO0598-599 KO5'); $\Delta YPO1387-1388$ (YPO1387-8 3', YPO1387-8 5'P1, YPO1387-8 3'P4 and YPO1387-8 5'); $\Delta YPO3994$ (YPO3994 KO3', YPO3994 KO5'P1, YPO3994 KO3'P4 and YPO3994 KO5'); $\Delta YPO2490-2491$ (YPO2490-2491 KO3', YPO2490-2491 KO5'P1, YPO2490-2491 KO3'P4 and YPO2490-2491 KO5'); $\Delta YPO0684-695$ (YPO0684-695 3', YPO0684-695 5'P1, YPO0684-695 3'P4 and YPO0684-695 5'); $\Delta YPO3797-3802$ (YPO3797-3802 3', YPO3797-3802 5'P1, YPO3797-3802 3'P4 and YPO3797-3802 5'); $\Delta YPO3877-3882$ (YPO3877-3882 3', YPO3877-3882 5'P1, YPO3877-3882 3'P4 and YPO3877-3882 5'); Δail (YPO2905 3', YPO2905 5'P1, YPO2905 3'P4 and YPO2905 5'); $\Delta ompA$ (YPO1435 3', YPO1435 5'P1, YPO1435 3'P4 and YPO1435 5'); $\Delta katY$ (YPO3319 3', YPO3319 5'P1, YPO3319 3'P4 and YPO3319 5'); $\Delta yopH$ (*yopH* 3'+500, P1*yopH* 3'3, P4*yopH* 5'1406 and *yopH* 5'-500); $\Delta lacZ$ (YplacZUP-F, YplacZUP-R, YplacZDN-R and YplacZDN-F). The respective PCR products were gel-purified and combined in a SOE-PCR with a kan^R cassette flanked by FRT sites, which had previously been applied from the plasmid pKD13 (Datsenko and Wanner, 2000). The first and last primers in each set

were used in the SOE-PCR reactions. The resulting PCR products (upstream-FRT-Kan^R-FRT-downstream) were gel purified and transformed into YPI54 or BGY0, *Y. pestis* strains carrying pWL204, which were grown at 26°C in the presence of 10 mM arabinose to induce the *lambda red* recombinase genes. Putative recombinants were selected on BHI agar plates containing kanamycin. Recombinants were passaged on BHI agar plates containing 5% sucrose to cure pWL204. If required, the kan^R cassette was resolved by transforming the strains with pLH29, a plasmid carrying the FLP recombinase gene, or pSkippy, a plasmid carrying the FLP recombinase and *sacB* genes, and growing the strains overnight at 26°C in the presence of 1 mM IPTG. The overnight cultures were diluted and plated on BHI agar plates containing 5% sucrose to cure pSkippy or on BHI agar plates for the use of pLH29. Kan^S and amp^S recombinants were selected and in-frame deletions of the target gene were verified by PCR. For each strain, the presence of all the major virulence loci (pCD1⁺, pMT1⁺, pPCP1⁺ and *pgm*⁺) was confirmed by PCR. For stains containing multiple mutations, pWL204 was transformed into one mutant (first listed deletion) and the process was repeated as described above.

Construction of *Y. pestis* pCD1⁻ pYV⁺ strains

Y. pestis CO92 pCD1⁻ pYV⁺ strains were constructed by transforming the virulence plasmid from *Y. pseudotuberculosis* IP32953, designated pYV, into YP6, a pCD1⁻ strain of *Y. pestis*, and YP102pCD1⁻. Transformants were plated on BHI agar plates and incubated at 26°C for 24 hr. The transformants were then replica plated onto BHI agar plates containing magnesium oxalate (BHI-MOX) and incubated at 37°C (BHI-MOX) or 26°C (BHI) for an additional 24 hr. *Y. pestis* pCD1⁻ pYV⁺ recombinants (YP361) were selected for their ability to grow on BHI agar plates at 26°C but were

unable to grow on BHI-MOX plates at 37°C. PCR was also used to confirm the presence of pYV.

Construction of the *inv*⁺ *Y. pestis*

Y. pestis CO92 *inv*⁺ strains were constructed by co-transforming pPP53 and pTNS2 into wild-type *Y. pestis*, which inserted *inv* under the control of its native promoter into the Tn7 locus between the *glmS* and *pstS* genes (Choi *et al.*, 2005). *inv* was amplified from *Y. pseudotuberculosis* IP32953 using primers Inv-nativepromotor-*PstI* and Inv-Tn7-3' *BamHI* and ligated into the *PstI* and *BamHI* sites in pUC18R6K-mini-Tn7T-Kan to generate pPP53.

Construction of the *Y. pestis* *pgm*⁻ strain

Y. pestis CO92 *pgm*⁻ strains were selected for by passaging wild-type *Y. pestis* CO92 on BHI agar plates containing 0.02% Congo red. White colonies were selected and isolated for pure cultures. PCR was also used to confirm the loss of *pgm* locus.

Construction of pYV⁻ *Y. pseudotuberculosis* strains

Y. pseudotuberculosis IP2666c pIB1⁻ (BGY38) and *Y. pseudotuberculosis* IP32953 pYV⁻ (BGY21) were constructed by passaging the *Y. pseudotuberculosis* IP2666c pIB1⁺ (LL-89) and *Y. pseudotuberculosis* IP32953 (BGY30) on BHI-MOX agar plates and selecting large colony variants, indicating the loss of the virulence plasmid. The loss of the virulence plasmid was confirmed using PCR.

Construction of *Y. pseudotuberculosis* deletion strains

The Δ *lacZ* *Y. pseudotuberculosis* IP32953 strain was constructed using the modified *lambda red* recombination system, as described above.

Tissue culture adhesion and invasion assays

For both adhesion and invasion assays, 50,000 A549 cells were plated in 24-well plates. Before inoculation, the bacteria and A549 cells were washed three times with pre-warmed PBS, and the bacteria were resuspended in F12 media containing 10% fetal bovine serum (FBS) and 2mM L-glutamine. 5×10^5 bacteria in 250 μ l were added to the cells and incubated at 37°C for 1 hour. Cells were then washed three times with pre-warmed PBS and 0.5 ml of pre-warmed F12 was added for later time points. To harvest the bacteria, 1 ml of ddH₂O was added to the washed cells and incubated for 5 min at 37°C to lyse the cells. The bacteria were then serially diluted and plated on BHI or LB agar. Percent adherence was calculated by dividing the number of adherent bacteria by the number of bacteria in the inoculum. Gentamicin protection assays were performed similarly, except that at the designated time point 0.5 ml of F12 containing 100 μ g ml⁻¹ gentamicin was added to the cells and incubated for 1 hr. The cells were then washed, diluted and plated as described. The percent of intracellular bacteria was calculated by dividing the number of gentamicin resistant bacteria by the number of bacteria in the inoculum.

Biotinylation and isolation of outer membrane proteins

Y. pestis cultures were grown in BHI broth under standard conditions for a 37°C overnight incubation. 1 OD₆₂₀/ml of culture were washed three times and resuspended in phosphate buffered saline (PBS), pH 8.0. 100 μ l of a 2.0 mM EZ-Link sulfo-NHS-LC-biotin in ddH₂O were added to each sample. Mock-biotinylated control samples received 100 μ l ddH₂O. The samples were incubated on ice for 40 min and inverted every ten minutes to ensure homogeneous biotinylation. The samples were washed three times

with PBS containing 100 mM glycine to quench the reaction and remove excess biotin. The samples were then suspended in a lysis buffer (50 mM Tris pH 8.0) with protease inhibitor cocktail (Sigma). Total membrane and outer membrane fractions were fractionated as described by Vincent et al. (2006). Briefly, biotinylated and mock-biotinylated samples were lysed by French Press (14,000 PSI). Lysates were centrifuged at 10,000 g for 10 min at 4°C to remove any large particles or unlysed cells. Total membrane fractions were separated from periplasmic and cytoplasmic proteins by ultracentrifugation at 100,000 g for 60 min at 4°C. The inner membrane proteins were then solubilized in 50 mM Tris, pH 8.0 containing 1% Triton-X100, 20 mM MgCl₂ and protease inhibitor cocktail and separated from the outer membrane proteins by ultracentrifugation at 100,000 g for 60 min at 4°C. The pellet, which contains the outer membrane proteins, was then resuspended in 50mM Tris, pH 8.0 with protease inhibitor cocktail and 0.1% Tween-20. Samples were then sonicated using a Bransson 2200 for 30 min on ice. Biotinylated outer membrane proteins were then incubated with approximately 4x10⁶ A549 cells for 1 hr, washed six times with PBS and lysed with 1 ml ddH₂O containing protease inhibitor. Samples were then run on SDS-PAGE gels, transferred on a nitrocellulose membrane and probed with streptavidin-HRP.

Yop secretion profiles

Yop secretion profiles were analyzed by inoculating 0.2 OD₆₂₀ equivalents of an overnight *Y. pestis* culture into BHI-MOX broth. The cultures were then grown at 26°C for 2 hr in a 125 ml Erlenmeyer flask at 37°C on a shaker set at 250 rpm for 4 hours. OD₆₂₀ readings were then taken and 1 ml of supernatant was precipitated with trichloroacetic acid and washed with 100% acetone. Pellets were resuspended in 1M Tris

pH 9.0 to an OD-equivalent of 1OD 10 μ l⁻¹. Samples were run on a 12% SDS-PAGE gel and stained with “blue silver” coomassie (Candiano *et al.*, 2004).

Minimal inhibitory concentration (MIC) assays

Minimal inhibitory concentrations for crystal violet, polymyxin B and gentamicin were assayed using clinical standards, except that the bacteria were grown in BHI broth. Briefly, serial dilutions of BHI broth and antibiotic were assayed for microbial growth at 26°C. The last antibiotic concentration at which bacterial growth was inhibited was considered the MIC for a particular antimicrobial for that strain.

Endotoxin measurements

pCD1⁻ *Y. pestis* strains were grown overnight at 26°C and inoculated to an 0.2 OD₆₂₀ equivalent into BHI broth with or without calcium. The cultures were grown at 37°C for four hours and OD₆₂₀ readings were taken. Culture supernatants were then filter sterilized and used to quantitate endotoxin units. Uninoculated BHI broth was used as a baseline control. Endotoxin units were measured using Pyrochrome Chromogenic Endotoxin Testing Reagents (Associates of Cape Cod Incorporated, East Falmouth, MA).

Animals

All animals studies were approved by the Washington University Animal Studies Committee protocol #20060154 or the University of North Carolina in Chapel Hill Office of Animal Care and Use protocol #09-057.0-A. Pathogen-free, six- to eight-week-old C57BL/6J mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice were housed in a Techniplast Isocage containment system (Buguggiate, Italy) during all experiments. Mice were provided with food and water *ad libitum* and maintained on a 12-hr light/12-hr dark cycle at 25°C and 15% humidity. Bacteria were grown in BHI broth as

described above, and the optical density (OD₆₂₀) of the culture was used to estimate bacterial concentrations. Actual bacterial concentrations were determined by plating serial dilutions of the inoculum on BHI or LB agar plates. The bacteria were then washed in endotoxin-free phosphate buffered solution (PBS) and maintained at 37°C (intranasal infection) or 26°C (subcutaneous infection) prior to inoculation, unless otherwise indicated. Mice were anesthetized with a ketamine/xylazine solution (50 mg Kg⁻¹ / 10 mg Kg⁻¹) and inoculated with 20 µl or 100 µl of bacteria in PBS via an intranasal or subcutaneous route, respectively. Animals were monitored twice daily and sacrificed with an overdose of sodium pentobarbital (180 mg Kg⁻¹) at designated time points or when the animal was clearly moribund.

Animal infections

For both the individual infection and co-infection experiments (equal ratios of 1 mutant : 1 WT), groups of 3-5 mice were inoculated intranasally (i.n.) with 1 x 10⁴ CFU of *Y. pestis*, *Y. pseudotuberculosis*, *B. pertussis* and/or *K. pneumoniae*. Groups of 4-5 mice were inoculated subcutaneously (s.c.) in the front neck with 1000 CFU of *Y. pestis*. At the indicated times post-inoculation, mice were euthanized, lungs and spleen (after i.n. inoculation) or lymph nodes, spleen, and lungs (after s.c. inoculation) were surgically removed, weighed, and homogenized in 0.5 ml PBS. Serial dilutions for each organ were plated on BHI or LB agar as appropriate for growth. For co-infections, antibiotic sensitivity was used to differentiate between co-inoculated strains. Bacterial burdens are reported as CFU/tissue.

Animal survival

Groups of 10 mice were inoculated intranasally with 1×10^4 CFU or subcutaneously with 200 CFU of *Y. pestis*. Animals were monitored every 12 hr for survival. Animals that were clearly moribund were euthanized with an overdose of sodium pentobarbital and counted as having died in the next twelve hours. Animals inoculated intranasally with pCD1⁻ pYV⁺ *Y. pestis* were monitored every six hours starting at 60 hpi. The average time to death was calculated using Prism v4.0c software (GraphPad Software, Inc., La Jolla, CA).

“Natural” animal infections

The “natural” infection model of infection denotes animal infections in which mice were infected with bacteria harvested from the lungs of plague-infected mice. In brief, BAL was performed on a mouse showing sign of pneumonic plague at 48 hpi. BALF was maintained at 37°C and diluted to an appropriate concentration prior to infection, assuming that the bacterial burden of the lung was 5×10^8 . The actual inoculum was subsequently determined by plating the inoculum. Matched infections with broth-grown *Y. pestis* were conducted simultaneously.

Bronchoalveolar lavage (BAL) experiments

BAL experiments were performed as described in (Lathem *et al.*, 2005). In brief, groups of 3 mice were inoculated intranasally with 1×10^4 CFU of *Y. pestis*. At the designated time points post-inoculation, the mice were euthanized and the sternum and rib cage were surgically removed to provide sufficient space for the lungs to expand upon inflation. Tracheal cannulation was used to lavage the lungs three times with 1 ml PBS. BALF was centrifuged at 21,000 x g to pellet the bacteria and cell debris and then resuspended in 200 µl BALF. The remaining BALF was routinely tested for residual

bacteria, which were rarely encountered. The lungs were removed from the mouse and homogenized in 0.25 ml PBS. Serial dilutions were used to determine CFU/lungs.

Percent lavageable bacteria was calculated by dividing the CFU from BALF by the CFU from lungs and BALF. Results are reported as CFU/Organ or as percent lavageable bacteria.

Ex vivo gentamicin protection assays

Ex vivo gentamicin protection assays for BALF were performed on BALF from infected animals. In brief, gentamicin was added to BALF for a final concentration of 100 $\mu\text{g ml}^{-1}$ gentamicin. BALF was incubated at room temperature for 30 min and then pelleted (500 x g for 5 min) and washed three times. Mock treated samples were also conducted simultaneously. Percent survival, which is assumed to be the percent of intracellular bacteria, was calculated by dividing the CFU from BALF treated with PBS/gentamicin by the CFU from untreated BALF. *Ex vivo* gentamicin protection assays for entire lungs were adapted from protocols used for bladder tissue (Justice *et al.*, 2006). In brief, groups of 3 mice were inoculated intranasally with 1×10^5 CFU of *Y. pestis*. At the designated time points post-inoculation, the mice were euthanized and the sternum and rib cage were surgically removed to provide sufficient space for the lungs to expand upon inflation. Tracheal cannulation was used to inflate the lungs with 1 ml PBS/gentamicin or PBS alone for 30 min. The PBS/gentamicin or PBS was removed and the lungs were then lavaged three times with PBS to remove any residual gentamicin. The lungs were removed from the mouse and homogenized in 0.25 ml PBS. Serial dilutions were used to determine CFU/lungs. Percent survival, which is assumed to be the percent of intracellular bacteria, was calculated by dividing the CFU from lungs treated

with PBS/gentamicin by the CFU from lungs treated with PBS alone. Results are reported as CFU/lungs or as percent survival.

Enrichment of less adherent *Y. pestis* mutants

See Chapter 3 for the construction of the mutant library. A group of five mice was intranasally infected with 1×10^5 transposon mutants grown at 37°C overnight. At 2 hpi, the animals were euthanized and BALF was extracted and plated on BHI agar containing kanamycin. Lungs were also harvested to determine the percent adherent bacteria as described above. The plates were scrapped 24 hours later and extensively vortexed in 30 ml PBS. A 300 μ l sample was then inoculated into BHI broth and grown overnight at 37°C overnight. The process was repeated three times. The pool of mutants was analyzed as described in Chapter 3 using high throughput sequencing technology.

Histopathology

Groups of 3-5 mice were inoculated intranasally with 1×10^4 CFU of *Y. pestis*. Mice were euthanized at various time points post-inoculation with an overdose of sodium pentobarbital, and their lungs were inflated with 10% neutral buffered formalin via tracheal cannulation. The lungs were then removed from the animal and fixed for 96 hr in 10% neutral buffered formalin. All samples were negative for bacterial growth before being removed from the BSL-3 facility. The lungs were washed twice in PBS for 1 hr and then submerged in 70% ethanol. The lungs were then embedded in paraffin and 5- μ m sections were stained with hematoxylin and eosin. Images were viewed under an Olympus BX60 fluorescence microscope (Center Valley, PA), and Spot Imaging software (Diagnostic Instruments Inc., Sterling Heights, MI) was used to capture the images.

Statistical Analyses

Student's t-test was used to determine the statistical significance of animal and tissue culture experiments. The product limited (Kaplan-Meier) method was used to determine the median survival and statistical significance of animal survival experiments. All statistical analyses were calculated using Prism v4.0c software (GraphPad Software, Inc., La Jolla, CA).

Table 2-2. Bacterial strains, plasmids and primers used in Chapters 2, 3 and 4.

Strains	Genotype	Source/Reference
YP3	<i>Y. pestis</i> CO92 - pCD1 ⁺ pMT1 ⁺ pPCP1 ⁺ <i>pgm</i> ⁺	Laboratory Stock
YP6	<i>Y. pestis</i> CO92 pCD1 ⁻	Laboratory Stock
YP16	<i>Y. pestis</i> CO92 Δ <i>psaA</i>	(Cathelyn <i>et al.</i> , 2006)
YP30	<i>Y. pestis</i> pWL204	(Lathem <i>et al.</i> , 2007)
YP102	<i>Y. pestis</i> CO92 Δ <i>pla</i>	(Lathem <i>et al.</i> , 2007)
YP102pCD1 ⁻	<i>Y. pestis</i> CO92 Δ <i>pla</i> pCD1 ⁻	(Lathem <i>et al.</i> , 2007)
YP138	<i>Y. pestis</i> CO92 Δ <i>pla</i> carrying P _{N25} - <i>tetR</i> + P _{tet} - <i>pla</i> integrated in the <i>glmS</i> - <i>pstS</i> intergenic region	(Lathem <i>et al.</i> , 2007)
YP160	<i>Y. pestis</i> CO92 Δ <i>lacZ</i>	(Lawrenz <i>et al.</i> , 2009)
YP181	<i>Y. pestis</i> CO92 Δ YPO0598-599	This study
YP182	<i>Y. pestis</i> CO92 Δ YPO2490-2491	This study
YP184	<i>Y. pestis</i> CO92 Δ YPO3944	This study
YP186	<i>Y. pestis</i> CO92 Δ YPO0683-695	This study
YP221	<i>Y. pestis</i> CO92 Δ YPO1387-1388	This study
YP223	<i>Y. pestis</i> CO92 Δ YPO3797-3802	This study
YP225	<i>Y. pestis</i> CO92 Δ YPO3877-3882	This study
YP234	<i>Y. pestis</i> CO92 Δ YPO0598-599 Δ YPO2490-2491	This study
YP235	<i>Y. pestis</i> CO92 Δ YPO1387-1388 Δ YPO3944	This study
YP337	<i>Y. pestis</i> CO92 pGENRFP	This study
YP313	<i>Y. pestis</i> CO92 <i>inv</i> ⁺	This study
YP314	<i>Y. pestis</i> CO92 Δ <i>ail</i>	This study
YP356	<i>Y. pestis</i> CO92 Δ <i>ompA</i>	This study
YP357	<i>Y. pestis</i> CO92 Δ <i>kayY</i>	This study
YP361	<i>Y. pestis</i> CO92 pCD1 ⁻ pYV ⁺	This study
YP362	<i>Y. pestis</i> CO92 Δ <i>ompA</i> Δ <i>kayY</i>	This study
YP369	<i>Y. pestis</i> CO92 Δ <i>pla</i> pCD1 ⁻ pYV ⁺	This study
YP373	<i>Y. pestis</i> CO92 Δ <i>yopH</i>	This study
YP383	<i>Y. pestis</i> CO92 Δ <i>pla</i> pCD1 ⁻ pYV ⁺ Δ <i>yadA</i>	This study
YPI54	<i>Y. pestis</i> pWL204	This study
YPI58	<i>Y. pestis</i> CO92 <i>ail</i> :: <i>kan</i> ^R	This study
BGY0	<i>Y. pestis</i> CO92 pCD1 ⁻ pWL204	This study
BGY4	<i>Y. pestis</i> CO92 <i>pgm</i> ⁻	This study
BGY14	<i>Y. pestis</i> CO92 pCD1 ⁻ <i>lacZ</i> :: <i>kan</i> ^R	This study
BGY21	<i>Y. pseudotuberculosis</i> IP32953 pYV ⁻	This study
BGY23	<i>Y. pestis</i> CO92 pCD1 ⁻ pGEN222	This study

BGY30	<i>Y. pseudotuberculosis</i> IP32953	(Chain <i>et al.</i> , 2004)
BGY39	<i>Y. pseudotuberculosis</i> IP32953 $\Delta lacZ$	This study
BGY38	<i>Y. pseudotuberculosis</i> IP2666c pIB1 ⁺ Kan ^R	This study
LL-89	<i>Y. pseudotuberculosis</i> IP2666c pIB1 ⁺ yopH (NdeI) Kan ^R	(Fisher <i>et al.</i> , 2007)
MLF-13	<i>Y. pseudotuberculosis</i> IP2666c pIB1 ⁺ yopH (NdeI) $\Delta lacZ$ Kan ^R	(Fisher <i>et al.</i> , 2007)
KPPR1	<i>K. pneumoniae</i> subspecies pneumoniae - rifampicin-resistant, type 1 O antigen, type 2 polysaccharide capsule	(Lawlor <i>et al.</i> , 2005)
VK060	<i>K. pneumoniae</i> cpsB::Tn5_kan ^R	(Lawlor <i>et al.</i> , 2005)
BC39	<i>B. pertussis</i> Δbvg	(Beckett <i>et al.</i> , 2000)
DH5 α	<i>E. coli</i> DH5 α : <i>fhuA2</i> Δ (<i>argF-lacZ</i>)U169 <i>phoA glnV44</i> $\Phi 80$ Δ (<i>lacZ</i>)M15 <i>gyrA96 recA1 relA1 endA1 thi-1 hsdR17</i>	(Taylor <i>et al.</i> , 1993)
S17	<i>E. coli</i> S17-1 λ pir: <i>TpR SmR recA, thi, pro, hsdR-M⁺RP4: 2-Tc:Mu: Km Tn7</i> λ pir	(Priefer and Pühler, 1983)

Plasmids	Relevant features	Source/Reference
pGEN222	<i>gfp</i> expression and the <i>hok sok</i> post-segregation killing system for plasmid maintenance; amp ^R	(Galen <i>et al.</i> , 1999)
pGENRFP	<i>rfp</i> expression and the <i>hok sok</i> post-segregation killing system for plasmid maintenance; amp ^R	Gift from Mary Chelsea Lane and Harry Mobley
pKD13	Source of kan ^R cassette flanked by FRT sites; kan ^R	(Datsenko and Wanner, 2000)
pLH29	Plasmid carrying IPTG-inducible FLP recombinase; cm ^R	(Huang <i>et al.</i> , 1997)
pPP47	Plasmid carrying a <i>Himar1</i> -based transposon system with T7 promoters oriented towards the chromosome; kan ^R	This study
pPP53	pUC18R6K-mini-Tn7T-Kan carrying <i>inv</i> ; amp ^R kan ^R	This study
pSkippy	pFLP3 (Choi <i>et al.</i> , 2005) derivative carrying the FLP recombinase and <i>sacB</i> genes but lacking the tet ^R cassette; amp ^R	This study
pTnMod-RKm	Tn5 mini-transposon and transposase; kan ^R	(Dennis and Zylstra, 1998)
pUC18R6K-mini-Tn7T-Kan	mini-Tn7 transposon with a MCS and a kan ^R cassette flanked by FRT sites; amp ^R kan ^R	(Choi and Schweizer, 2006)
pWL204	Plasmid carrying the <i>lambda red</i> recombinase gene and <i>sacB</i> gene for sucrose counterselection; amp ^R	(Lathem <i>et al.</i> , 2007)

Primers	Sequence	Source/Reference
YplacZUP-F	CTTTCACCAAAATTCGCCAGCGGC	This study
YplacZDN-R	TGTTATAGCCGGTTATCAGTCGTTA	This study
YplacZDN-F	GGTCGACGGATCCCCGGAATGTGTGACGCTTATTTTTGTTATGTCTGAGG	This study
YplacZUP-R	GAAGCAGCTCCAGCCTACACAACCTGTTGGCAATACAAGGTGTAA GGCATCGG	This study
yopH 3'+500	ACTACATACACACTGGGAAAATCTC	This study
yopH 5'-500	GCATCCGTCGGTGGCCGTAGGCC	This study
P1yopH 3'3	GAAGCAGCTCCAGCCTACACCATGCTTCCCTCCTTAATTAATA CACGCC	This study
P4yopH 5'1406	GGTGCGACGGATCCCCGGAATAATGTAAATATTTATTCCTATGA GTAAATA	This study
YPO0598-599 KO3'	CACCACGAAGAGTAAACGAAGGC	This study
YPO0598-599 KO5'P1	GAAGCAGCTCCAGCCTACACCATGAGGAGTCCCTTCCCAAG	This study
YPO0598-599 KO3'P4	GGTCGACGGATCCCCGGAATGAAGAACCTCCTTTATGTAGTTTT ATT	This study
YPO0598-599 KO5'	CGTTGATGCTCCTTTGCTTCCAATG	This study
YPO1387-8 3'	CTGGCAACCGCCGCAAGTCACGGAG	This study

YPO1387-8 5'P1	GAAGCAGCTCCAGCCTACACCATGTTTAATATCCATAAATGCTA TTAGG	This study
YPO1387-8 3'P4	GGTCGACGGATCCCCGGAATGATCGATACCCGTCGTCCTTCAA	This study
YPO1387-8 5'	CCATCACCGACGTTCCCAAACCATGCC	This study
YPO3994 KO3'	GGTGCTTATGAAGGCTTGCGCATG	This study
YPO3994 KO5'P1	GAAGCAGCTCCAGCCTACACCATGGATACTTAACTCACAAATAG AAATGC	This study
YPO3994 KO3'P4	GGTCGACGGATCCCCGGAATAGTTTGAAGGGGATAACATGGCG	This study
YPO3994 KO5'	CTGCAACCCGTGGTAGAAAATGC	This study
YPO2490-2491 KO3'	GATTTCTTCAGTTTTTGTATTGTTTAGCTTGC	This study
YPO2490-2491 KO5'P1	GAAGCAGCTCCAGCCTACACCAAAGAGAATGCCTCGCCTCTGC	This study
YPO2490-2491 KO3'P4	GGTCGACGGATCCCCGGAATAATATGGATATGCTTTCAATATCA TATG	This study
YPO2490-2491 KO5'	CCGGATCGGTGTCTGGGATCTTACTG	This study
YPO0684-695 3'	CCCTCGACACCAAACTCCCTGTTACGG	This study
YPO0684-695 5'P1	GAAGCAGCTCCAGCCTACACCATCGTCAGACCTAATAGTGAGAT G	This study
YPO0684-695 3'P4	GGTCGACGGATCCCCGGAATGAAATTGACTTAAAAATCGATATT GACC	This study
YPO0684-695 5'	CGAGCAGGGTGATTTATGGCAATGCGAATG	This study
YPO3797-3802 3'	GGCCCCTGAATAAATCTGTGTGGCTCAC	This study
YPO3797-3802 5'P1	GAAGCAGCTCCAGCCTACACCATAAATACCTGATAGTCCTTTAT GTA	This study
YPO3797-3802 3'P4	GGTCGACGGATCCCCGGAATAATTTCAATGACGTTATTAATCGT TAG	This study
YPO3797-3802 5'	CAAGGATTATCTGCAGTTCGTTCCCTTCGC	This study
YPO3877-3882 3'	GCAACACTCGATAAGCGTTATCATACTCTTG	This study
YPO3877-3882 5'P1	GAAGCAGCTCCAGCCTACACCATTTAAACCCCGATGTAATCAT TG	This study
YPO3877-3882 3'P4	GGTCGACGGATCCCCGGAATAAGCACCTGGGGATTAATGAGCC	This study
YPO3877-3882 5'	CCAGAAGATGGTGCAGATGATAGTCCAGAAGG	This study
YPO2905 3'	CAACGATGAAGCGACCATTGAGCGTGTG	This study
YPO2905 5'P1	GAAGCAGCTCCAGCCTACACCATCCAGATTGTTATAACAATACC TAAT	This study
YPO2905 3'P4	GGTCGACGGATCCCCGGAATAACGTCTCCTAACCATGAAAAC C	This study
YPO2905 5'	GCGGGAGAGTAAACCAGCACCGAGC	This study
YPO1435 3'	GGTTACCCGAATTGACGGAAGATGATCG	This Study
YPO1435 5'P1	GAAGCAGCTCCAGCCTACACCATTTTACGCCTCATTATCATCCA AATAGGC	This Study
YPO1435 3'P4	GGTCGACGGATCCCCGGAATGCCAACAGCTGACTAAATCGCTT ATCTTG	This Study
YPO1435 5'	GAGCTCACGATGAAATATCAACAGCTGG	This Study
YPO3319 3'	CGCCCTACGCCTTGCCAATGTC	This Study
YPO3319 5'P1	GAAGCAGCTCCAGCCTACACCATATATAAGTCCCCTTTAACTAA AAGGCAAC	This Study
YPO3319 3'P4	GGTCGACGGATCCCCGGAATAACTAAATAGAGGGGAGGATTTA TCTTCC	This Study
YPO3319 5'	CCTGTCTGGAGATATATTCAATACTCCTCTATG	This Study
Inv-native promotor- <i>PstI</i>	GATCCTGCAGGTGTCATCAAGGCAACCATCAG	This Study
Inv-Tn7-3' <i>BamHI</i>	GATCGGATCCGTTATATTGACAGCGCACAGAG	This Study
TraSH-IR-3' <i>MfeI</i>	GATCCAATTGCTATCTGCTGACCGATGTTCCG	This Study

TraSH-IR-5'PmeI/Ascl	GATCGTTTAAACGGCGCGCCGACGGCCAGTGAATTGTAATACG AC	This Study
TraSH-5'Tase(Ascl)	GATCGGCGCGCCGGATTACACATGACCAACTTCG	This Study
TraSH-TnMod-PromII	GCGTAGAGGATCTGAAGATCAGCAGTTC	This Study
TraSH-Tase-PromII-GC	CACGAAATTCCTTTTTTCCATGAACGTTACCATGTTAGGCAGGT C	This Study
TraSH-Tase-PromII	GACCTGCCTAACATGGTAACGTTTCATGGAAAAAAGGAATTCG TG	This Study
TraSH-Probe-PCRadaptor	GTCCAGTCTCGCAGATGATAAGG	(Sassetti <i>et al.</i> , 2001)
TraSH-Probe-PCRtrans1	CCCGAAAAGTGCCACCTAAATTGTAAGCG	(Sassetti <i>et al.</i> , 2001)
TraSH-Probe-PCRtrans2	CGCTTCCTCGTGCTTTACGGTATCG	(Sassetti <i>et al.</i> , 2001)
TraSH-Probe-Lig2	AGTCTCGCAGATGATAAGGTGGTCTGGT	(Sassetti <i>et al.</i> , 2001)
TraSH-Probe-Lig1	CGACCACGACCA /3AmM/	(Sassetti <i>et al.</i> , 2001)
TraSH-cDNA-2ndstrand	GGGTCTAGAGACCGGGGACTTATC	This Study

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Chapter 3
Transposon site hybridization (TraSH)

Abstract

In vitro systems have been very useful in determining specific mechanisms of bacterial pathogenesis, but they often do not reflect the same physiological conditions found *in vivo*. However, the cost and appropriateness of *in vivo* animal models of infection often limit their use. Several negative selection techniques have been developed to help defray some of the costs associated with *in vivo* studies. I developed a negative selection screen termed transposon site hybridization (TraSH) to identify bacterial factors required for *Y. pestis* pulmonary infection. To validate the system, I defined the set of genes that are essential for *Y. pestis* viability. Similar to *E. coli*, 628 genes were considered essential using this technique. However, I was unable to identify any known virulence factors using TraSH, including genes involved in the Ysc type-three secretion system (T3SS), which are known to be essential for pulmonary infection. The finding imply that there is global *trans*-complementation of avirulent mutants by wild-type *Y. pestis*, emphasizing the need to use *in vivo* models to study *Y. pestis* pathogenesis.

Introduction

The virulence of bacterial pathogens is a complex, multifactorial process that requires the combined and coordinated activity of many gene products from both the bacteria and the host. To proliferate within a host, a pathogen needs to be able to circumvent the host immune system (both innate and adaptive) and obtain nutrients for itself. A variety of *in vitro* systems have been developed to simulate specific aspects of the infectious process, which have enabled researchers to study bacterial gene expression and the behavior of mutant strains in a controlled environment, which often reflects some physiological conditions found *in vivo* (DiRita and Mekalanos, 1989). These systems have been very useful in defining many mechanisms of bacterial pathogenesis, but they cannot accurately reproduce the complex nature of host-pathogen interactions found *in vivo*. A pathogen may encounter vastly different environments over the course of infection, each requiring a unique response. Consequently, genes that are deemed important during *in vitro* studies may not be important during an actual infection, and conversely, genes that seem unimportant in *in vitro* studies may play a vital role within a host.

On the other hand, animal models of infection can be very beneficial in determining many aspects of host-pathogen interactions. However, the costs associated with using such models and the difficulties in establishing informative and appropriate animal models of infection have limited their use in studying bacterial pathogenesis. Several methodologies have recently been developed to identify and study a wide array of bacterial virulence factors simultaneously in different animal models of infection. One such methodology uses negative selection to identify mutants that are unable to survive in

a host. Signature-tagged mutagenesis (STM) was an early derivative of this technology and was limited to studying between 50 and 100 mutants at one time (Reviewed in Chiang *et al.*, 1999). Negative selection screens, like STM, are ultimately limited to the technologies used to distinguish between the initial pool of mutants (input pool) and the surviving mutants after selection (output pool). Newer technologies have been developed to simultaneously study entire populations of mutants, encompassing transposon insertion mutants for every gene in the genome. Transposon site hybridization (TraSH) couples RNA amplification of the sites adjacent to transposon insertions with microarray analysis to identify mutants at a population level and can distinguish differences between two sets of mutant pools (input and output) with a high degree of accuracy (Sasseti *et al.*, 2001). This technique has already been used to study *Mycobacterium*, *Salmonella*, *Helicobacter*, and *Francisella* using animal models of infection (Lawley *et al.*, 2006; Salama *et al.*, 2004; Sasseti and Rubin, 2003; Weiss *et al.*, 2007). Currently, there is one partial STM negative selection screen that has been reported for bubonic plague, but there are none for pneumonic plague (Flashner *et al.*, 2004). Therefore, I have adapted TraSH negative selection technology to screen for mutants that are unable to survive during *Y. pestis* pulmonary infection.

Results and Discussion

Establishing TraSH for use with *Y. pestis*

STM uses the gain or loss of a sequence tag to identify whether a single mutant with a specific tag is enriched or lost from the output pool compared to the input pool. Pools of mutants are limited by the effectiveness and efficiency of differential hybridization used to determine the relative ratios of the sequence tag between the input and output pools. Sequencing the genomic DNA adjacent to the transposon can be then used to identify the site of the transposon insertion. In a somewhat different approach, TraSH combines high-density transposon mutagenesis and microarray mapping of pools of mutants to identify genes required for growth under certain conditions (Sasseti *et al.*, 2001). The ends of the transposon are engineered with T7 polymerase promoters facing into the chromosome, enabling the generation of short amplified RNA (aRNA) probes that correspond to the area immediately adjacent to the transposon insertion. For any given selection condition, the aRNA probes are used measure the relative abundance of transposon insertions per gene between input and output pools via microarray mapping. Changes in the relative abundance indicate a loss or enrichment of that particular gene under that selection condition.

I developed a *HimarI*-based transposon system with integrated T7 sites that can be mated into *Y. pestis* from *E. coli*. Using this transposon system, I generated a *Y. pestis* transposon insertion library consisting of 2.5×10^5 mutants using polymyxin B to counter select against *E. coli*. The high-density transposon insertion library corresponds to an approximately 1X coverage of every TA site in the chromosome, ensuring that there is a sufficient number of transposon insertions within the close proximity to the

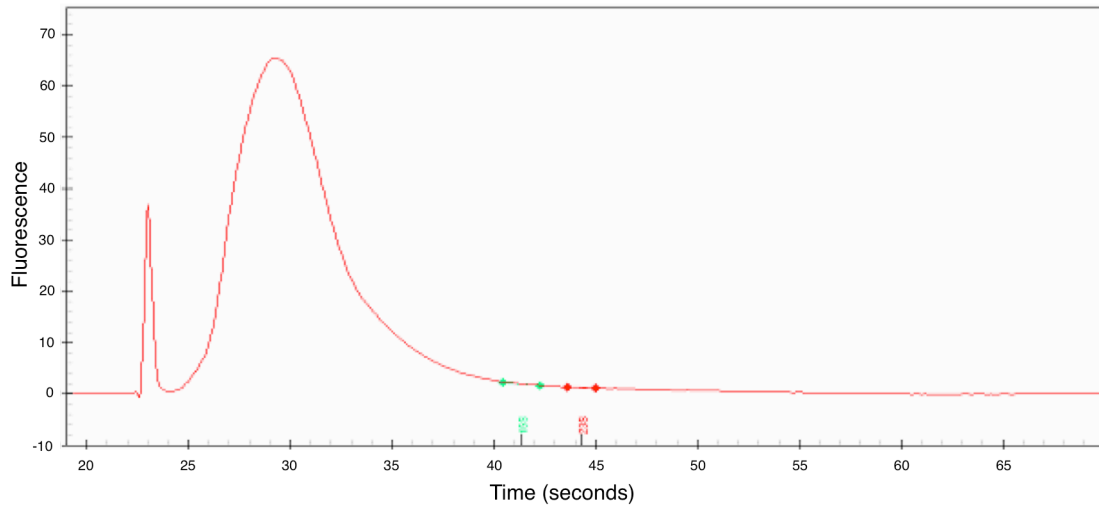


Figure 3-1: Experion RNA Analysis plot for aRNA TraSH probes. Microfluidic analysis of aRNA where the relative fluorescence (Y-axis), which measures the relative abundance of RNA, is measured over time (X-axis), which corresponds to the average length of the RNA in the sample. The time interval between 25 and 35 seconds roughly corresponds to aRNA species between 50 and 400 bp in length.

corresponding oligonucleotide sequences used on our custom microarray. However, DNA-based microarrays have traditionally been used to map transposon insertions in TraSH, and our custom build microarray is an oligonucleotide-based array that uses unique ORF-specific 70-mers. The oligonucleotides are designed to specifically recognize only antisense RNA or mRNA. Given that aRNA generated from the insertion site via *in vitro* T7 transcription will be both sense and antisense relative to the oligonucleotides on the microarray, I converted my aRNA to double-stranded complementary DNA (dscDNA) before hybridizing them to the array. Figure 3-1 shows an Experion RNA Analysis plot of aRNA probes before they were generated into dscDNA.

In vitro TraSH to identify essential genes in *Y. pestis*

Many genes encode for proteins that are essential in producing viable, replicative bacteria, such as genes encoding for ribosomal proteins, RNA and DNA polymerases,

general secretory proteins and proteins involved in cytokinesis. Bacteria cannot support transposon insertions into such genes and still be viable; hence, aRNA or dscDNA probes theoretically cannot be generated for essential genes. Therefore, to validate my system and approach, I comparatively hybridized dscDNA probes and sheared genomic DNA to identify genes that are essential for *Y. pestis* viability (“essential genes”). Any gene that showed more than a 2-fold reduction in hybridization signal compared to genomic DNA was considered to be essential. Using these criteria, I identified 628 genes as being essential for *Y. pestis* viability. Figure 3-2 shows a breakdown of essential genes based on their functional classification, and Table 3-1 shows the number of *Y. pestis* essential genes in specific sub-classifications and the fold change in the hybridization signal between sheared genomic DNA (input pool) and dscDNA probes generated from the transposon mutant library (output pool) for these genes. These data are in general agreement with similar studies that have been done to examine essential genes in *E. coli*, which identified 620 essential genes (Gerdes *et al.*, 2003).

Table 3-1: Essential *Y. pestis* genes required for growth divided into functional sub-classifications. The number of genes and the range representation between sheared genomic DNA (input pool) and dscDNA probes from the transposon mutant library (output pool). Negative values represent an underrepresentation.

Gene Classification	Number of genes	Fold Representation (Range)
Ribosomal protein synthesis	54	-2.00 □ > -100
DNA replication	11	-2.00 □ > -100
Surface polysaccharides and lipopolysaccharides	19	-2.00 □ > -100
General secretory proteins	16	-2.94 □ > -100
Cell Division	8	-2.50 □ -14.28

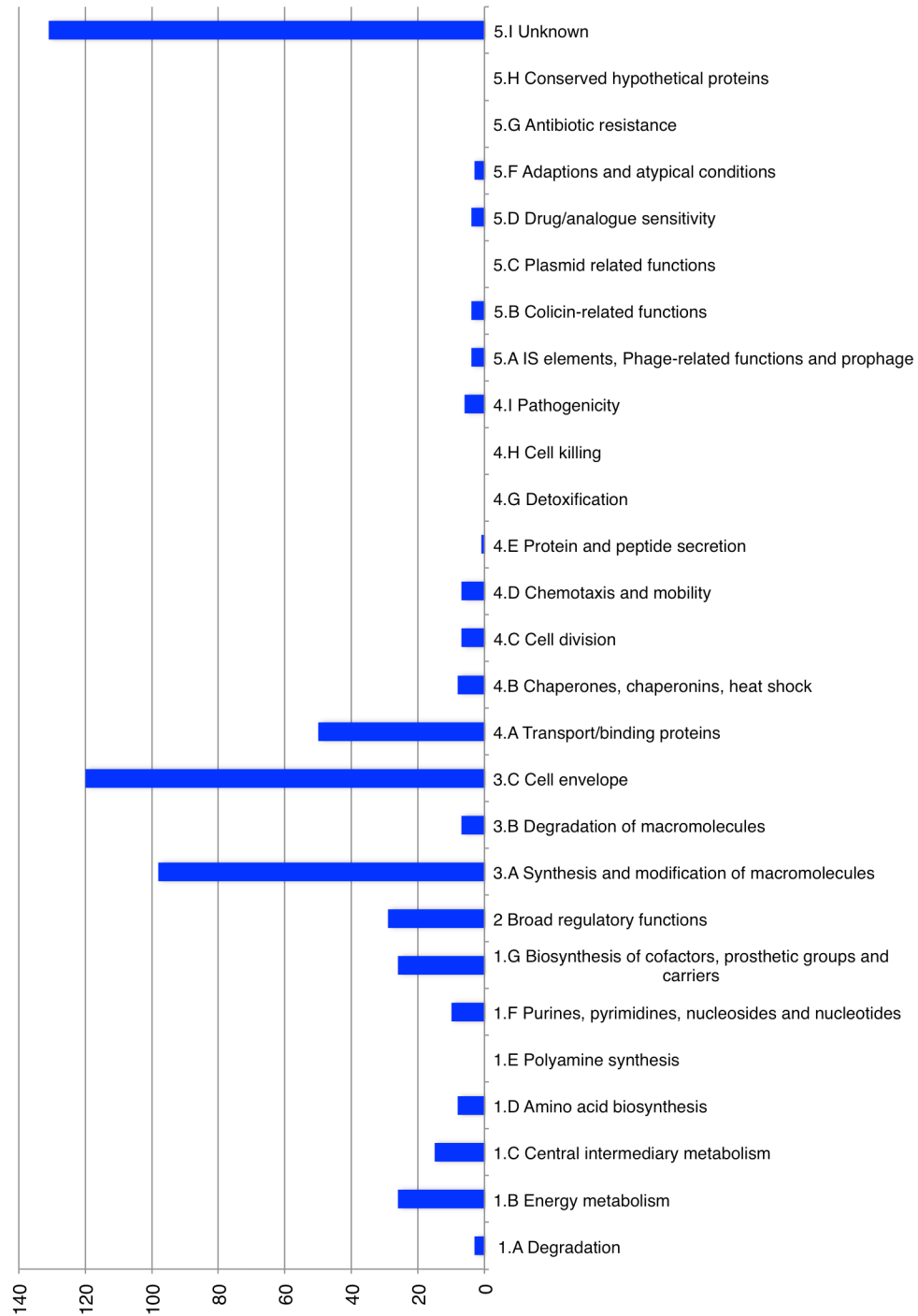


Figure 3-2: Functional classification of essential genes in *Y. pestis*: Comparative hybridization between the mutant library and sheared genomic DNA. Genes with a two-fold underrepresentation in the transposon insertion library were considered essential for *Y. pestis* viability.

Interestingly, YPO2490 but not YPO2491 was classified as an essential gene based on these studies. I had previously generated a clean deletion mutation in YPO2490-2491 (see Chapter 2), and this mutant showed no growth defects compared to wild-type bacteria (data not shown). Recently, Aoki *et al.* have discovered a two-partner secretion system that consists of three proteins, CdiA, CdiI and CdiB, that facilitate a process termed contact-dependent inhibition (CDI) (Aoki *et al.*, 2010; Aoki *et al.*, 2005). In this process, the C-terminus of CdiA inhibits the growth of or lyses other bacteria that come into contact with the bacteria that express CdiA on their surface. CdiI is a cytoplasmic protein that represses the inhibitory effects of CdiA. CdiB is a chaperone that aids CdiA in reaching the outer membrane. This system is present in *Y. pestis*, where YPO2490 corresponds to the CdiA protein, YPO2491 corresponds to CdiB protein and a small unannotated open reading frame downstream of YPO2490 corresponds to CdiI protein. *cdiA* and *cdiI* are driven by the same promoter, suggesting that a transposon insertion in *cdiA* would prevent *cdiI* expression. Therefore, in a pool of mutants, like my transposon insertion library, mutations in *cdiA* (YPO2490) but not *cdiB* (YPO2491) would result in a growth restriction, which may explain why I can generate individual mutations in this gene but random transposon insertions in a complex mutant library cannot be generated.

In vivo TraSH to identify virulence factors required for pneumonic plague

Having demonstrated the viability of this approach, I next used this approach to identify genes that contribute to virulence in an animal model of *Y. pestis* pulmonary infection (Fig. 3-3). Theoretically, mutants with insertions in important virulence factors should be present in the input pool (*in vitro* pool) but diminished or absent from the output pool (*in vivo* pool). Consistent with similar TraSH experiments (Sasseti and

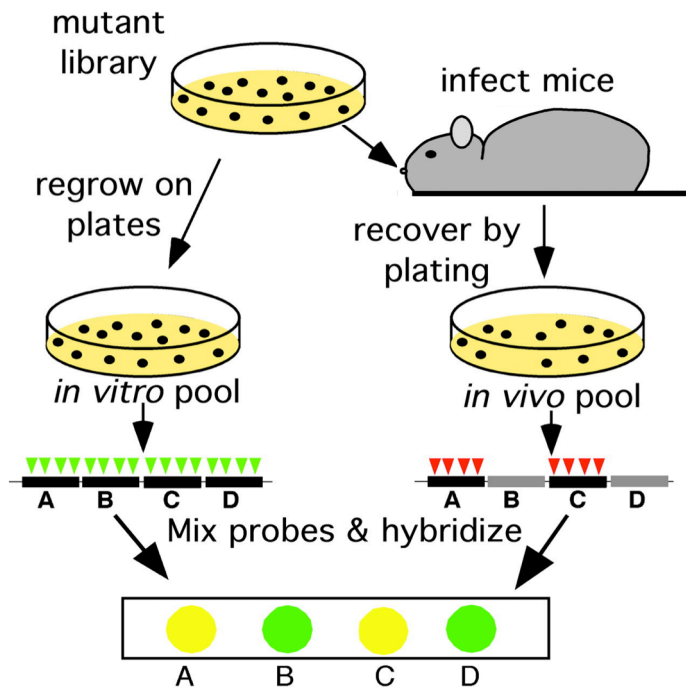


Figure 3-3: Scheme for Transposon Site Hybridization (TraSH). C57BL/6 mice are intranasally infected with 2×10^5 CFU from the transposon insertion library. *In vivo* pools are collected by plating lung homogenates, and *in vitro* pools are collected by plating the inoculum. TraSH probes are created by partially digesting genomic DNA, producing aRNA via *in vitro* T7 polymerase amplification reactions and comparatively analyzing the differences between the two pools by microarray mapping. Genes that have an increased fold representation in the *in vitro* pool compared to the *in vivo* pool based on hybridization signals represent genes specifically required for *Y. pestis* pulmonary infection. Figure courtesy Christopher M. Sassetti, Ph.D., UMass Medical School; adapted with permission (Sassetti and Rubin, 2003), subject to copyright restrictions.

Rubin, 2003), I infected two sets of 10 mice with 2×10^5 bacteria and harvested lungs from mice at 24 hpi and lungs and spleens from mice at 48 hpi. Lungs were homogenized and plated on BHI agar containing kanamycin to recover approximately 5×10^5 bacterial colonies per time point. Genomic DNA was extracted from multiple independent samples of the pooled bacteria and dscDNA probes were generated for each sample as described (See Materials and Methods and Sassetti *et*

al., 2001). The Microarray Core Facility at Washington University used the aRNA samples to generate dscDNA, which was then labeled and hybridized to microarray slides.

Overall the fold change between *in vitro* pool and *in vivo* pool was very small.

Only two genes showed more than a two-fold difference between pools, whereas 150

genes showed a fold difference between 1.5-fold and 2.0-fold. In contrast, my initial experiments with non-mutagenized genomic DNA and the mutant library showed between 2-fold and 100-fold differences in hybridization signals. Included in the 152 genes were a large number of metabolic genes but very few genes known to be essential for pathogenesis, in particular, genes encoding the type-three secretion system (T3SS) (Table 3-2 and Table 4-1). These data are very striking, and the full implications of these data are addressed in Chapter 4.

Currently, high-throughput sequencing technology is quickly replacing microarray analysis to determine the location and relative abundance of the transposon insertions for input and output pools. This approach has several advantages including the ability to determine the exact location of the transposon insertion without having to rely on probe-array hybridizations, which can vary from slide to slide. I adapted my protocols to use high-throughput sequencing technology to analyze the 24 hr time point. While many of the individual genes were different from the 48 hr data, the types of genes were similar, a large number of metabolic genes and very few genes known to be essential for pathogenesis (Table 4-1). The combined 24 and 48 hpi data suggest that wild-type-like *Y. pestis*, or mutants with transposon insertions in gene not required for virulence, are able to compensate for the virulence defects of mutants that have transposon insertions in known virulence factors. This type of global *trans*-complementation has never been documented in the literature and is discussed in great detail in Chapter 4.

Table 3-2: Genes showing greater than a 1.5-fold change in the hybridization signal between the *in vitro* pool and *in vivo* pool at 48 hpi. Negative values represent an underrepresentation in the *in vivo* pool.

Gene ID	Gene description	Fold Representation
YPCD1.02	transposase/IS protein	-1.70
YPCD1.38	(<i>tyeA</i>) Yop secretion and targeting control protein	-1.73
YPCD1.39	(<i>yopN</i>) Yop secretion control protein	-1.69
YPCD1.67	(<i>yopH</i>) putative secreted protein-tyrosine phosphatase	-1.85
YPCD1.97c	putative transposase	-1.61
YPO0034	putative membrane permease	-1.69
YPO0035	(<i>gltS</i>) sodium/glutamate symport carrier protein	-1.54
YPO0041	putative DNA ligase	-1.72
YPO0137	(<i>envZ</i>) osmolarity sensor protein	-1.63
YPO0147	conserved hypothetical protein	-1.54
YPO0260a	putative type III secretion apparatus	-1.53
YPO0275	conserved hypothetical protein	-1.64
YPO0282	(<i>hmuS</i>) hemin transport protein	-1.63
YPO0282	(<i>hmuS</i>) hemin transport protein	-1.69
YPO0308	putative oxidoreductase	-1.63
YPO0319	(<i>qor</i>) quinone oxidoreductase	-1.84
YPO0323	conserved hypothetical protein	-1.57
YPO0328	(<i>rhaD</i>) rhamnulose-1 phospahte aldolase	-1.72
YPO0352	putative lipoprotein	-1.58
YPO0369	conserved hypothetical protein	-1.50
YPO0369	conserved hypothetical protein	-1.52
YPO0373	(<i>ymr</i>) RNA-binding protein Hfq	-1.53
YPO0376	(<i>hflC</i>) hypothetical protein	-1.53
YPO0388	hypothetical protein	-1.95
YPO0433	hypothetical protein	-1.67
YPO0442	(<i>seB</i>) phosphoserine phosphatase	-1.69
YPO0470	(<i>nhaA</i>) sodium-proton antiporter	-1.66
YPO0483	putative LysE type translocator	-2.06
YPO0486	(<i>folA</i>) dihydrofolate reductase	-1.62
YPO0491	(<i>apaG</i>) CO ₂ resistance	-1.55
YPO0511	hypothetical protein	-1.73
YPO0580	(<i>uxaB</i>) tagaturonate reductase	-1.53
YPO0629	hypothetical protein	-1.51
YPO0641	hypothetical protein	-1.59
YPO0645	(<i>rpsU</i>) 30S ribosomal protein S21	-1.60
YPO0655	(<i>asoB</i>)-putative permease involved in surface layer secretion	-1.50
YPO0660	conserved hypothetical protein	-1.56
YPO0663	(<i>tolC</i>) outer membrane channel precursor protein	-1.50
YPO0680	putative DedA-family membrane protein	-1.66
YPO0748	hypothetical protein	-1.60
YPO0759	sodium:sulfate symporter-family protein	-1.52
YPO0760	conserved hypothetical protein	-1.58
YPO0782	hypothetical protein	-1.74
YPO0801	hypothetical protein	-1.63
YPO0802	(<i>cheD</i>) putative methyl-accepting chemotaxis protein	-1.99
YPO0806	prepilin peptidase	-1.57
YPO0859	sugar transport system permease	-1.57

YPO0949	putative glutaminase	-1.55
YPO0983	conserved hypothetical protein	-1.76
YPO0985	quorum-sensing transcriptional regulator	-1.69
YPO0989	<i>pseudogene (iucA)-E. coli aerobactin siderophore biosynthesis</i>	-1.51
YPO0998	hypothetical protein	-1.78
YPO1025	membrane-bound lytic murein transglycosylase A precursor	-1.79
YPO1091	putative prophage protein	-1.83
YPO1102	conserved hypothetical protein	-1.58
YPO1109	(<i>sdhC</i>) succinate dehydrogenase cytochrome b556 large membrane subunit	-1.59
YPO1116	(<i>sucD</i>) succinyl-CoA synthetase alpha subunit	-1.65
YPO1118	(<i>cydB</i>) cytochrome D ubiquinol oxidase subunit II	-1.61
YPO1119	conserved hypothetical protein	-1.60
YPO1129	conserved hypothetical protein	-1.59
YPO1158	conserved hypothetical protein	-1.74
YPO1211	(<i>eco</i>) ecotin precursor -serine protease inhibitor	-1.78
YPO1221	hypothetical protein	-1.53
YPO1240	putative prophage protein	-1.73
YPO1269	conserved hypothetical protein	-1.71
YPO1300	(<i>fruA</i>) PTS system, fructose-specific IIBC component	-1.51
YPO1305	(<i>psaC</i>) outer membrane usher protein PsaC precursor	-1.95
YPO1317	putative exported protein	-1.65
YPO1375	(<i>livR</i>) leucine-responsive regulatory protein	-1.55
YPO1395	(<i>msbA</i>) probable transport ATP-binding protein	-1.77
YPO1483	hypothetical protein	-1.55
YPO1535	putative iron-siderophore transport system, transmembrane component	-1.55
YPO1537	putative iron-siderophore receptor	-1.67
YPO1639	conserved hypothetical protein	-1.59
YPO1667	(<i>cheW</i>) chemotaxis protein	-1.57
YPO1669	YjgF-family lipoprotein	-1.52
YPO1689	putative lipoprotein	-1.70
YPO1708	putative chaperone protein	-1.57
YPO1723	exopolysaccharide lyase	-1.75
YPO1725	(<i>kduI</i>) 5-keto-4-deoxyuronate isomerase	-1.66
YPO1760	(<i>hpcR</i>) homoprotocatechuate degradative operon repressor	-1.55
YPO1843	(<i>fliA</i>) flagellar biosynthesis sigma factor	-1.51
YPO1906	(<i>fyuA/psn</i>) pesticin/yersiniabactin receptor protein	-1.54
YPO1934	putative LysR-family transcriptional regulatory protein	-1.58
YPO1937	L-asparagine permease	-1.66
YPO1938	probable deoR-family transcriptional regulatory protein	-1.54
YPO1969	putative IS100 transposase	-1.99
YPO1969	putative IS100 transposase	-1.65
YPO1974	putative chlorohydrolase	-1.57
YPO1977	<i>pseudogene</i> 3-oxoacyl-[acyl-carrier-protein] synthase III	-1.62
YPO2004	hypothetical protein	-1.67
YPO2064	(<i>pykA</i>) pyruvate kinase	-1.61
YPO2079	hypothetical protein	-1.69
YPO2080	conserved hypothetical protein	-1.60
YPO2124	putative prophage protein	-1.59
YPO2139	putative prophage protein	-1.56
YPO2158	conserved hypothetical protein	-1.70
YPO2200	hypothetical protein	-1.55

YPO2276	putative prophage protein	-1.51
YPO2306	putative amino acid antiporter	-1.78
YPO2328	conserved hypothetical protein	-1.64
YPO2329	(<i>ldhA</i>) D-lactate dehydrogenase	-1.67
YPO2346	conserved hypothetical protein	-2.07
YPO2364	IS21 element	-1.88
YPO2364	IS21 element	-1.66
YPO2405	hypothetical protein	-1.53
YPO2560	hypothetical protein	-1.58
YPO2567	(<i>pta</i>) phosphate acetyltransferase	-1.64
YPO2681	(<i>celD</i>) PTS system, cellobiose-specific IIA component-cel operon repressor	-1.67
YPO2691	(<i>kdpB</i>) putative potassium-transporting ATPase B chain	-1.56
YPO2725	conserved hypothetical protein	-1.68
YPO2759	putative mannose-resistant/Proteus-like fimbrial protein	-1.54
YPO2807	putative LysR-family transcriptional regulatory protein	-1.82
YPO2850	conserved hypothetical protein	-1.57
YPO2905	(<i>ail</i>) attachment invasion locus protein	-1.88
YPO2944	putative pili chaperone protein	-1.69
YPO2955	utative luxR-family regulatory protein	-1.56
YPO2958	(<i>sfuA</i>) iron(III)-binding periplasmic protein	-1.55
YPO2959	(<i>sfuB</i>) iron(III)-transport system permease	-1.59
YPO2988	conserved hypothetical protein	-1.56
YPO3241	conserved hypothetical protein	-1.60
YPO3263	probable sugar transporter	-1.50
YPO3330	putative sugar ABC transporter, ATP-binding protein	-1.54
YPO3331	putative sugar ABC transporter, permease protein	-1.96
YPO3368	hypothetical protein	-1.54
YPO3425	(<i>ppdD</i>) putative prepilin peptidase dependent protein D precursor	-1.61
YPO3454	(<i>nrdD</i>) anaerobic ribonucleoside-triphosphate reductase	-1.67
YPO3455	(<i>nrdG</i>) anaerobic ribonucleoside-triphosphate reductase activating protein	-1.77
YPO3472	putative sugar binding protein	-1.56
YPO3477	putative lipid carrier protein	-1.66
YPO3479	putative protease	-1.64
YPO3524	conserved hypothetical protein	-1.60
YPO3596	putative lipoprotein	-1.52
YPO3628	putative DNA-damage-inducible protein	-1.51
YPO3646	(<i>pcp</i>) outer membrane lipoprotein	-1.55
YPO3655	conserved hypothetical protein	-1.68
YPO3676	putative phage-related protein	-1.53
YPO3715	(<i>malF</i>) maltose transport system permease protein	-1.53
YPO3719	(<i>lysC</i>) lysine-sensitive aspartokinase III	-1.59
YPO3728	(<i>purH</i>) bifunctional phosphoribosylaminoimidazolecarboxamide formyltransferase/IMP cyclohydrolase	-1.57
YPO3738	hypothetical protein	-1.56
YPO3783	conserved hypothetical protein	-1.72
YPO3795	(<i>ugpA</i>) sn-glycerol-3-phosphate transport system, permease protein	-1.59
YPO3803	hypothetical protein	-1.57
YPO3849	(<i>hemC</i>) porphobilinogen deaminase	-1.81
YPO3861	(<i>rffH</i>) glucose-1-phosphate thymidyltransferase	-1.73
YPO3886	colicin (partial)	-1.83
YPO3902	putative magnesium chelatase family protein	-1.55

YPO3951	hypothetical protein	-1.72
YPO3954	(<i>gntT</i>) putative gluconate permease	-1.59
YPO3962	sugar-binding transport protein	-1.73
YPO3967	phosphate transport protein	-1.62

Conclusions

Our analysis of essential genes in *Y. pestis* required for viability indicates that the technical aspects of the technology used in the TraSH analysis are functioning as anticipated. However, the TraSH strategy relies on the fundamental assumption that during an infection with a pool of isogenic strains, it will be possible to select against single mutants that are attenuated. My experiments with TraSH using two different technologies and at two different time points of infection showed little or no selection against any known virulence factors including the T3SS genes, a surprise considering previous studies have demonstrated the importance of the T3SS in pneumonic plague (Lathem *et al.*, 2005). These data suggest that wild-type *Y. pestis* may establish a protective environment that allows avirulent organisms to prosper.

Although *in vitro* systems have been very important in determining many mechanisms in bacterial pathogenesis, they cannot truly replicate the complex nature of host-pathogen interactions found *in vivo* and often yield different findings from those seen *in vivo*. The global *trans*-complementation of avirulent mutants by wild-type *Y. pestis* highlights these differences, as *trans*-complementation is seen *in vivo* but not *in vitro* (G. Plano, personal communication). These data also emphasize the necessity of using animal models of infection and fully virulent organisms in the study of *Y. pestis* pathogenesis.

Materials and Methods

Reagents, bacterial strains, strain construction and culture conditions

See Chapter 2.

Construction of transposon insertion mutant library

The transposon mutant library was generated using the *HimarI*-based transposon system encoded on pPP47, which was constructed in multiple steps using the phagemid ϕ MycoMarT7 (Sasseti *et al.*, 2001) and pTnMod-RKm (Dennis and Zylstra, 1998). Briefly, pTnMod-RKm was digested with *BglII*, and blunt ends were generated using Klenow treatment. The resulting fragments were then digested with *EcoRI*, and the fragment corresponding to the RP4 origin of transfer was ligated to the *HimarI* transposon, which was previously amplified from ϕ MycoMarT7 using the oligonucleotides TraSHHimarIR5'(PmeI/AscI) and TraSHIR3'(MfeI), and transformed into *E. coli* S17. The *HimarI* transposon contains an R6K origin of replication, a *Kan*^R cassette and flanking T7 promoters oriented so as to promote transcription into the chromosomal DNA. The Tn5 promotor region (pTnMod-RKm) and the *HimarI* transposase (ϕ MycoMarT7) were independently amplified using the oligonucleotides TraSH-TnModPromII and TraSH Tase-PromII and TraSHTase-PromIIGC and TraSH5'Tase(AscI), respectively, and then joined together by SOE-PCR. The resulting PCR products were cloned into the AscI site adjacent to the *HimarI* transposon to create pPP47. *E. coli* S17 carrying pPP47 were mated with wild-type *Y. pestis* and plated on BHI agar containing kanamycin for *Y. pestis* selection and polymyxin B for *E. coli* counter-selection. Southern blot analysis of twenty randomly selected colonies indicated that each mutant contained a single transposon insertion (data not shown). There were

approximately 2.5×10^5 mutants in the transposon mutant library used in the subsequent experiments.

Transposon Site Hybridization (TraSH)

The transposon mutant library was used for both *in vitro* and *in vivo* experiments to identify genes required for bacterial growth on BHI agar and to identify genes required for infection during primary pneumonic plague, respectively. The protocols used in these experiments have been described previously (Sasseti *et al.*, 2001; Sasseti and Rubin, 2003). To analyze genes required for bacterial growth on nutrient rich sources, TraSH probes were generated as described below for the initial transposon mutant library and hybridized against sheared genomic DNA. *In vivo* selection against *Y. pestis* was then performed using C57BL/6J mice. Briefly, ten mice were inoculated with 2×10^5 CFU of the transposon mutant library. The *in vitro* pool was generated by replating the library on BHI agar. At 48 hpi, the mice were euthanized, and a subset of the surviving bacteria (5×10^5 CFU) was recovered from lung homogenates by plating on BHI agar plates containing kanamycin. Genomic DNA was isolated from pool, and modified TraSH probes were generated as described (Sasseti *et al.*, 2001). Briefly, genomic DNA was partially digested with *HinPI* and *MspI*. DNA fragments (500-2000bp) were gel-purified and ligated to adaptors generated with the primers TraSH-Probe-Lig1 and TraSH-Probe-Lig2. Ligated DNA was used as a template in two separate PCR reactions containing the primers TraSH-Probe-PCRadaptor and either TraSH-Probe-PCRtrans1 or TraSH-Probe-PCRtrans1. Gel-purified PCR products (250-500 bp) were used template for *in vitro* T7 transcription reactions (Ambion, Austin, TX). Template DNA was digested with TurboDNase (Ambion, Austin, TX), and the rRNA was purified and used to generate ds-

cDNA (Invitrogen, Carlsbad, CA) with the primers TraSH-Probe-PCRadaptor and TraSH-cDNA-2ndstrand. The *in vitro* and *in vivo* probes were then labeled with Cy3 or Cy5 and co-hybridized to the array as described previously (Lathem *et al.*, 2005). Probes were generated from each pool three times and analyzed on duplicate microarrays for a total of six microarray hybridizations per experiment. Two independent experiments were performed, yielding consistent results. The arrays were scanned following hybridization and analyzed using Partek Genomics Suite (Partek Inc., St. Louis, MO). Data for each experiment were analyzed using multiple testing correction after 3-way ANOVA and reported as the fold representation in the *in vivo* pool compared to the *in vitro* pool, where negative values indicate a relative underrepresentation in the *in vivo* pool.

Transposon site high-throughput sequencing

Genomic DNA was obtained as outline above and partially digested with *HinPI* and *MspI*. DNA fragments (500-2000bp) were gel purified and ligated to adaptors generated with the oligonucleotides TraSH-Probe-Lig1 and TraSH-Probe-Lig2. Ligated DNA was used as a template in a PCR reaction containing the primers TraSH-Probe-PCRadaptor and TraSH-Probe-PCRtrans3. The PCR products were cleaned and submitted to the UNC High Throughput Sequencing Facility, where 76-bp paired-end Illumina sequence runs were performed for each sample. Paired sequences that contained both the adaptor sequence and transposon sequence within 2kb of each other were mapped into the genome. The total number of sequence reads per gene and the ratio between the *in vitro* and the *in vivo* pool were determined for each gene. Genes that failed to have a minimum of ten sequence reads in the *in vitro* pool were discarded from the data set.

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Chapter 4

Dominant suppression of early innate immune mechanisms by *Y. pestis*

Abstract

Disease progression of primary pneumonic plague is biphasic, consisting of a pre-inflammatory and a pro-inflammatory phase. During the long pre-inflammatory phase, bacteria replicate to high levels, seemingly uninhibited by normal pulmonary defenses. Using a co-infection model of pneumonic plague, I discovered that *Y. pestis* quickly creates a localized, dominant anti-inflammatory state that allows for the survival and rapid growth of both itself and normally avirulent organisms. *Y. pseudotuberculosis*, the relatively recent progenitor of *Y. pestis*, shows no similar *trans*-complementation effect, which is unprecedented among other respiratory pathogens. Even an unbiased negative selection screen using a vast pool of *Y. pestis* mutants revealed no selection against any known virulence genes, demonstrating the transformation of the lung into a globally permissive environment during the pre-inflammatory phase of pneumonic plague.

Introduction

Most lung pathogens induce a measurable inflammatory response within a few hours of infection, but *Yersinia pestis* is strikingly different: the first 36 hours of infection are characterized by rapid bacterial replication in the lung without generating appreciable levels of proinflammatory cytokines/chemokines, histological changes in infected tissue or outward disease symptoms. This allows the organisms to multiply rapidly before the inflammatory response is fully activated, typically too late to control the infection. Thus, disease progression for primary pneumonic plague can be subdivided into two distinct phases, an initial pre-inflammatory phase followed by a pro-inflammatory phase, which is recapitulated in both mouse and non-human primate models of infection (Agar *et al.*, 2008; Bubeck *et al.*, 2007; Koster *et al.*, 2010; Lathem *et al.*, 2005). The absence of early pulmonary inflammation in response to *Y. pestis* could be due to the organisms avoiding detection by the immune system, a strategy employed by many pathogens; or it could be due to the organisms completely suppressing early innate immune responses, which has never been observed for bacterial pathogens. In this study, I evaluated the early consequences of *Y. pestis* respiratory infection and discovered that the organisms are responsible for a rapid and profound suppression of pulmonary defenses that allows not only these organisms to proliferate rapidly in the lung, but other microbes as well.

Results and Discussion

Trans-complementation of avirulent mutants by wild-type *Y. pestis*

Y. pestis exhibits decreased bacterial replication in the lungs of mice with a heightened state of immune activation induced by a latent herpesvirus infection (Barton *et al.*, 2007), suggesting that pre-existing or early inflammatory responses are able to control and/or mitigate disease progression. A *Y. pestis* mutant lacking YopH, an effector protein of the *Yersinia* Ysc type III secretion system (T3SS), is immunostimulatory and consequently growth-inhibited during respiratory infection (Cantwell *et al.*, 2010). Based on these studies, I hypothesized that the ability of *Y. pestis* to actively suppress early innate immune mechanisms may have a more significant role in primary pneumonic plague than any intrinsic non-stimulatory properties of the bacterium itself (Lathem *et al.*, 2007; Montminy *et al.*, 2006; Reithmeier-Rost *et al.*, 2007). I therefore designed a series of *in vivo* co-infection experiments to test the idea that *Y. pestis* produces a dominant immunosuppressive state in the lung. Using a genetically marked wild-type strain ($\Delta lacZ$ mutant of *Y. pestis* CO92 (Lawrenz *et al.*, 2009) -YP160) to quantitatively measure competition, I co-infected C57BL/6J mice with a *Y. pestis* $\Delta yopH$ strain to see if the immunostimulatory response to the mutant could be “suppressed” by the wild-type bacteria. After intranasal inoculation of a 1:1 mixture of wild-type and $\Delta yopH$ strains, I observed that wild-type bacteria were able to almost fully restore the *in vivo* replication defect of a co-infecting $\Delta yopH$ mutant (Fig. 4-1A). Consistent with my hypothesis, mice co-infected with both wild-type and $\Delta yopH$ strains produced lower cytokine levels at 24 hours post-inoculation (hpi) than mice infected with the $\Delta yopH$ mutant alone (Fig. 4-2).

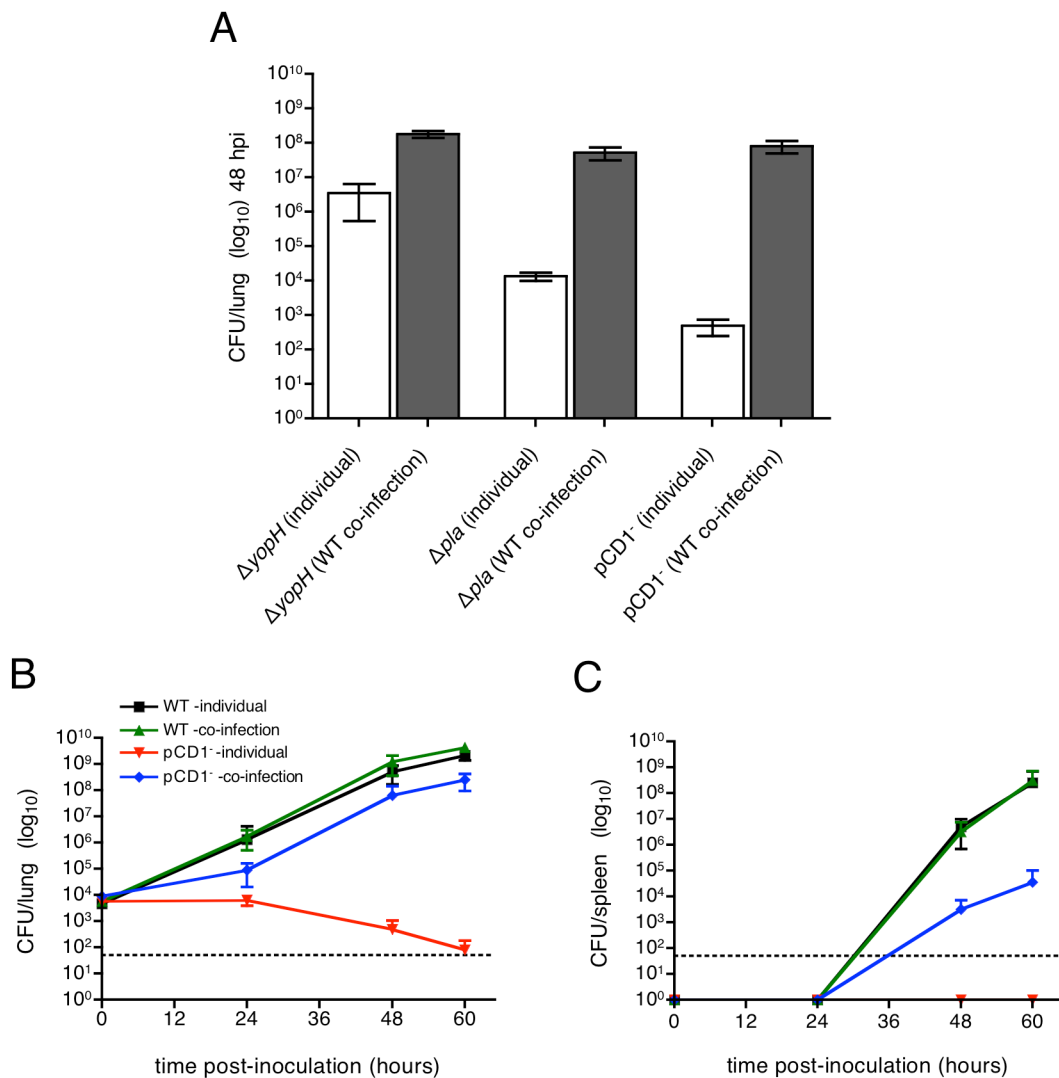


Figure 4-1: Wild-type *Y. pestis* is able *trans*-complement avirulent isogenic strains of *Y. pestis*. (A) Individual intranasal infections (white bars) for $\Delta yopH$ (YP373), Δpla (YP102), or pCD1⁻ (YP6) mutants or matched co-infections (gray bars) with wild-type *Y. pestis* in C57BL/6J mice. CFU per lung were determined at 48 hpi using BHI agar plates containing X-gal. All CFU differences between individual infection and corresponding co-infection are statistically significant ($p \leq 0.05$). (B-C) Kinetics of infection for pCD1⁻ mutants (YP6 - red), wild-type (YP160 - black) and a 1:1 co-infection of pCD1⁻ mutants (blue) and wild-type *Y. pestis* (green) infected intranasally. CFU per tissue were determined at various time points of infection for lungs (B) and spleen (C). Each bar (A) or point (B-C) represents the mean CFU recovered from five mice. The limit of detection is represented by a dotted line. Error bars represent SEM (A) and SD from the mean (B and C).

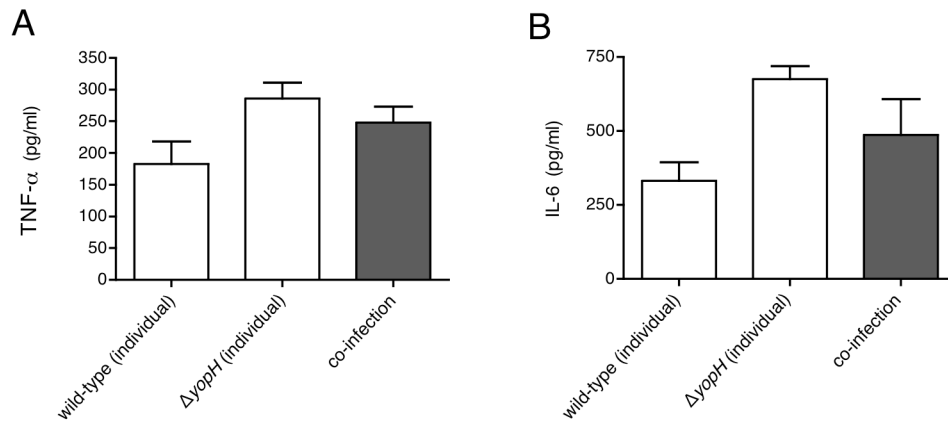


Figure 4-2: Cytokine production in response to individual and co-infections using wild-type *Y. pestis* and a $\Delta yopH$ mutant. Individual (white bars) and matched co-infections (gray bars) of C57BL/6J mice infected intranasally with $\Delta yopH$ (YP373) and/or wild-type *Y. pestis* (YP160). Whole lung homogenates were probed for (A) TNF- α and (B) IL-6 cytokine production using ELISA. Results are represented the mean cytokine levels (pg/ml) recovered from five mice. Error bars represent SD from the mean.

These results were somewhat unexpected based on previously published work with closely related *Y. pseudotuberculosis*, from which *Y. pestis* has rather recently evolved (Cover and Aber, 1989; Huang *et al.*, 2006). Respiratory co-infection with wild-type *Y. pseudotuberculosis* is unable to rescue bacterial replication defects of individual Yop mutants (Fisher *et al.*, 2007). All pathogenic *Yersinia* harbor a nearly identical Ysc-Yop T3SS, which directly injects into host cells a number of cytotoxins or effector proteins (Yops) that inhibit bacterial phagocytosis and other innate immune processes (Cornelis, 2002). Therefore, *Y. pestis*-specific evolutionary adaptations beyond the Ysc-Yop T3SS have produced a pathogen that is not only substantially more virulent than its immediate ancestor, but also able to compensate for the virulence defects of a co-infecting attenuated strain. I next tested the extent to which fully virulent *Y. pestis* could compensate for deficiencies of other attenuated isogenic mutants. In these respiratory co-

infections, wild-type *Y. pestis* could also compensate for (or “*trans*-complement”) the major virulence defects of both Δ *pla* and pCD1⁻ mutants (Fig. 4-1A), restoring 4-6 logs of bacterial growth capacity during the first 48 hpi.

The profound impact of the presence of wild-type *Y. pestis* on the fate of pCD1⁻ mutants was particularly surprising, since the pCD1 plasmid (commonly called the *Yersinia* “virulence plasmid”) encodes the entire Ysc-Yop T3SS and its virulence-associated Yop effectors. In our intranasal model of infection, pCD1⁻ mutants are severely attenuated (Lathem *et al.*, 2005), unable to proliferate in the lung and successfully cleared by innate immune mechanisms. Therefore, I examined the impact of wild-type *Y. pestis* on co-infecting pCD1⁻ mutants in more detail. Although the kinetics of bacterial replication progressed as expected during pulmonary infection with either wild-type bacteria or pCD1⁻ mutants when inoculated individually (Lathem *et al.*, 2005), growth of the pCD1⁻ strain increased substantially when co-inoculated at a 1:1 ratio with wild-type *Y. pestis*. As the infection progressed, this *trans*-complementation of pCD1⁻ mutant growth continued to increase, reaching 6-8 logs at 60 hpi (Fig. 4-1B). While we normally do not see pCD1⁻ mutants spreading to the spleen of mice following a pulmonary infection, I was able to detect 10⁴-10⁶ pCD1⁻ bacteria in the spleens of mice co-infected with wild-type *Y. pestis* at 60 hpi (Fig 4-1C).

Trans-complementation of avirulent mutants by wild-type *Y. pestis* during bubonic plague

The lung alveoli play a critical role in respiratory gas exchange, and inflammation-induced edema can lead to severe lung distress. Thus, the lung has many innate immune mechanisms, including the mucociliary escalator and alveolar

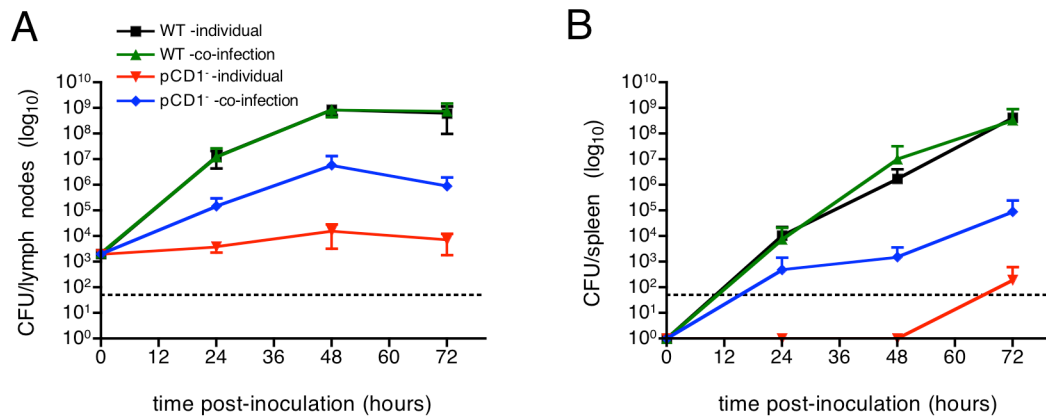


Figure 4-3: Wild-type *Y. pestis* is able *trans*-complement avirulent isogenic strains of *Y. pestis* during a subcutaneous infection. (A) Kinetics of infection for pCD1⁻ mutants (YP6 - red), wild-type (YP160 - black) and a 1:1 co-infection of pCD1⁻ mutants (blue) and wild-type *Y. pestis* (green) infected intranasally. CFU per tissue were determined at various time points of infection for lymph nodes (A) and spleen (B). Each bar represents the mean CFU recovered from five mice. The limit of detection is represented by a dotted line. Error bars represent SD from the mean.

macrophages, that allow for the clearance of inhaled microorganisms and foreign particles (Suzuki *et al.*, 2008). These systems most likely aid in the elimination of *Y. pestis* pCD1⁻ mutants, which are cleared without producing any appreciable inflammatory response from the host (Lathem *et al.*, 2005). To determine whether unique aspects of the lung's physiology contribute to the ability of *Y. pestis* to *trans*-complement avirulent isogenic strains, I also examined the kinetics of bacterial replication in a subcutaneous inoculation model that mimics the route of infection and lymphatic dissemination seen in bubonic plague (Fig. 4-3A and 4-3B). In the draining cervical lymph nodes, pCD1⁻ mutants were able to replicate 4-5 logs more by 48 hpi when co-infected with wild-type bacteria than when infected individually (Fig. 4-3A). Wild-type bacteria also facilitated the dissemination of pCD1⁻ mutants to the spleens of animals co-infected by the subcutaneous route (Fig. 4-3B). Although these results are not as pronounced as the phenotype I see during pulmonary infection, they reinforce my observations regarding a

dominant immunosuppressive effect of wild-type bacteria and demonstrate that the physiology of different organ systems may determine the extent of *trans*-complementation.

The life cycle of *Y. pestis* between flea and mammalian hosts suggests that there is differential expression of virulence determinates between the two hosts. Many *Yersinia* virulence determinates are thought to be regulated by temperature: *in vitro*, 26°C incubation can mimic the growth temperature in fleas just before mammalian infection, and 37°C incubation can mimic the growth temperature found in most mammalian hosts (Perry and Fetherston, 1997). I therefore tested these two pre-incubation conditions prior

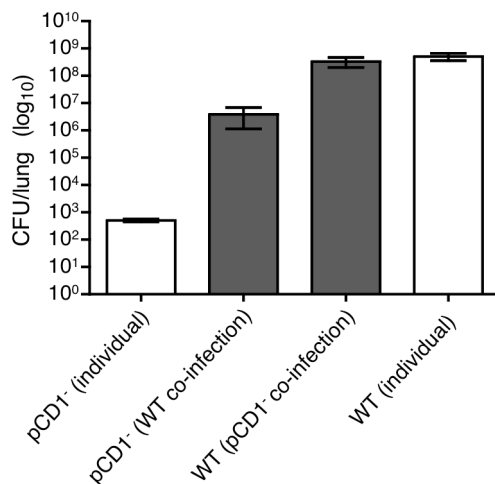


Figure 4-4: The ability to *trans*-complement avirulent isogenic strains is independent of initial growth temperature. Individual (white bars) and matched co-infections (gray bars) of C57BL/6J mice infected intranasally with pCD1⁻ mutants (YP6) and/or wild-type *Y. pestis* (YP160) pre-grown at 26°C. Each bar represents the mean CFU recovered from five mice. Error bars represent SEM.

to intranasal inoculation from 37°C to 26°C to determine whether the initial growth temperature would significantly alter the *trans*-complementation effect. C57BL/6J mice were individually or co-infected intranasally with pCD1⁻ and wild-type *Y. pestis* grown at 26°C or 37°C. The co-infection results were similar under both conditions. Figure 4-4 shows only *Y. pestis* grown at 26°C and data for the 37°C bacteria were similar to the data presented in Fig 1A. These data

indicate that the initial growth temperature of the bacteria does not significantly alter the ability of wild-type *Y. pestis* to *trans*-complement pCD1⁻ mutants.

Localization and timing of *trans*-complementation

In pneumonic plague, the earliest stages of inflammation appear as distinct foci, and this is also where most of the multiplying bacteria are observed (Lathem *et al.*, 2005). I wondered whether the *trans*-complementation effect is similarly localized, or whether it reflects immunosuppression of the entire pulmonary compartment? My first indication came from co-infection experiments in which I varied the ratios of avirulent to virulent *Y. pestis* (Fig. 4-5A). After the first 24 hpi, the levels of pCD1⁻ mutants are maintained at a constant ratio to the levels of wild-type *Y. pestis* (1 pCD1⁻ : 10 wild-type), independent of the initial ratio of pCD1⁻ to wild-type in the inoculum. The tight relationship between the growth rate of pCD1⁻ mutants and that of wild-type *Y. pestis* suggests that *trans*-complementation may be a more of a tightly controlled local effect rather than a global effect throughout the lung. In agreement with these observations were the results of pulmonary co-infections with *gfp*-expressing pCD1⁻ mutants and *rfp*-expressing wild-type *Y. pestis* (1:1). Co-localization of the green and red fluorescence revealed replicating pCD1⁻ mutants only in close association with wild-type *Y. pestis* in inflammatory foci, but not in regions of the lung where wild-type bacteria were not present (Fig. 4-5B). These data suggest that during the initial stages of infection, an avirulent mutant's proximity to fully virulent *Y. pestis* determines whether it will proliferate or be eliminated by the lung's innate immune mechanisms.

After the first 24 hpi, the levels of pCD1⁻ mutants are maintained at a constant ratio to the levels of wild-type *Y. pestis* (1 pCD1⁻ : 10 wild-type), independent of the

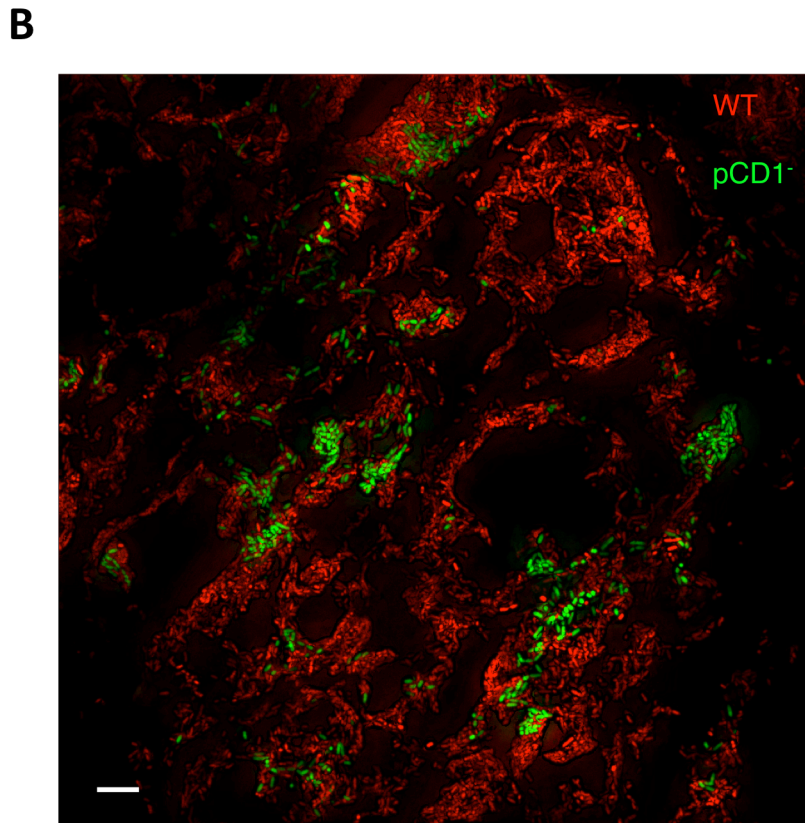
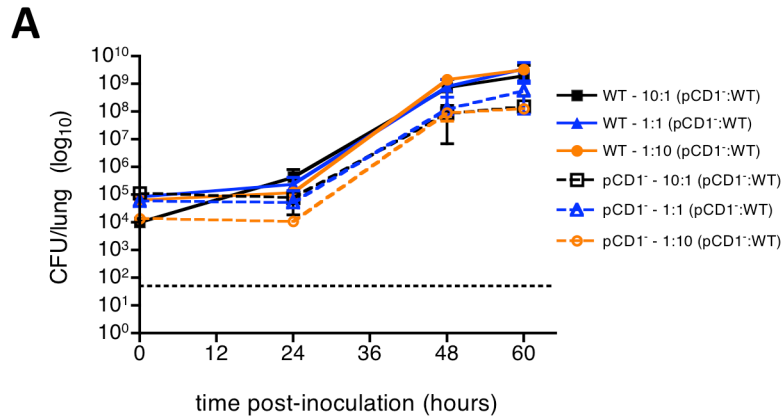


Figure 4-5: Relative numbers and distribution of virulent and avirulent *Y. pestis* during pulmonary co-infections. (A) Kinetics of infection for C57BL/6J mice intranasally infected with varying ratios of pCD1⁻ mutants (YP6) and wild-type *Y. pestis* (YP160) [10:1 (black), 1:1 (blue) and 1:10 (orange) pCD1⁻ : wild-type]. CFU per lung were determined at various time points of infection. Each point represents the mean CFU recovered from five mice. The limit of detection is represented by a dotted line. Error bars represent SD from the mean. (B) Mice were co-inoculated intranasally with *gfp*-expressing pCD1⁻ mutants (BGY19) and *rfp*-expressing wild-type *Y. pestis* (YP337). At 48 hpi, lungs were fixed and cryosectioned. Fluorescence deconvolution images were generated from 10- μ m sections. The image is representative of multiple independent experiments. Scale bar represents 10 μ m.

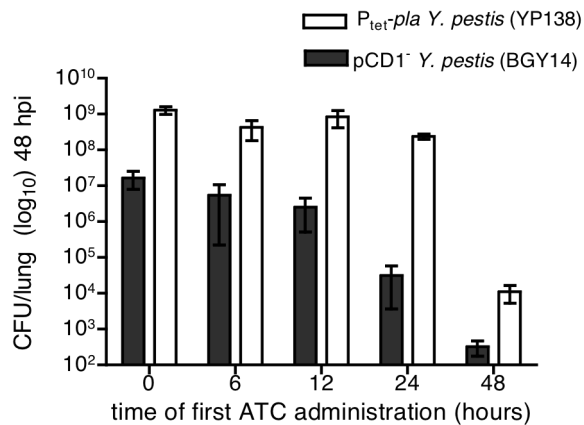


Figure 4-6: The first 24 hpi are critical in establishing *trans*-complementation. C57BL/6J mice were intranasally infected with pCD1⁻ mutants (BGY14 – gray bars) and ATC-inducible *pla* strain of *Y. pestis* (YP138). ATC was administered (2 mg kg⁻¹) at various time points and CFU per lung were determined at 48 hpi. Each bar represents the mean CFU recovered from three mice. Error bars represent SEM.

allows us to control the timing of a productive infection. These experiments show that the first 24 hpi are critical in establishing the *trans*-complementation effect, which is reduced by 3-4 logs if ATC is not administered until 24 hpi (Fig. 4-6). These data imply that the first 24 hours following infection are critical in controlling pCD1⁻ mutants during pulmonary infections and that wild-type *Y. pestis* are able to subvert these early innate immune responses.

Trans-complementation requires fully virulent *Y. pestis*

The (CDC) in the United States has classified *Y. pestis* as a Category A Select Agent because it causes significant morbidity and mortality in humans and its potential for release as a weapon of bioterrorism. Although Biosafety Level-3 laboratory

initial ratio of pCD1⁻ to wild-type in the inoculum. To examine the importance of the initial 24 hours of infection, I co-infected pCD1⁻ mutants with an engineered strain of *Y. pestis* in which *pla* is under the control of a tetracycline-

inducible promoter (Lathem *et al.*, 2007). Intranasal inoculation of the *pla*-inducible strain results in an unproductive infection until anhydrotetracycline (ATC) is administered to the animal, which

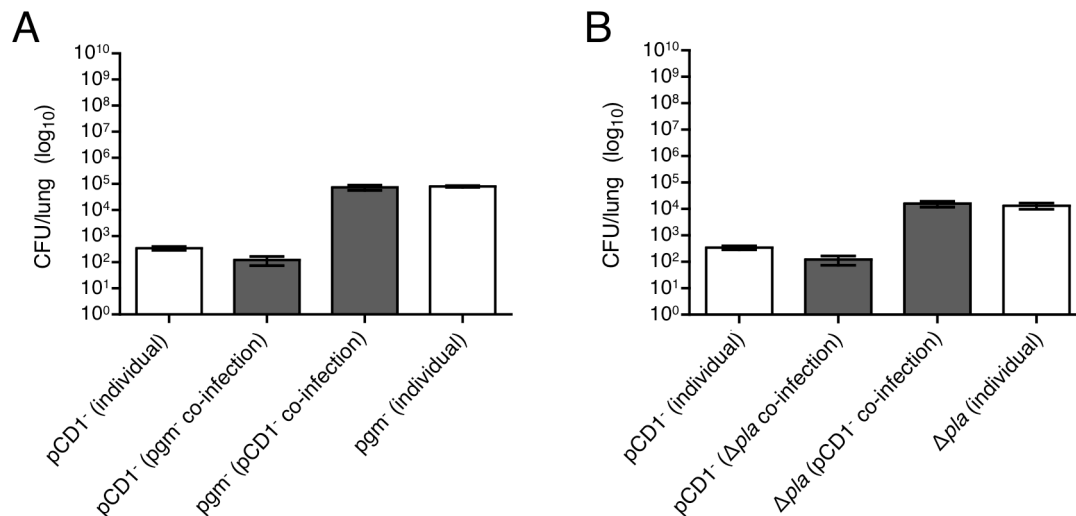


Figure 4-7: Attenuated *Y. pestis* strains are unable to *trans*-complement avirulent isogenic strains. (A) Individual (white bars) and matched co-infections (gray bars) of C57BL/6J mice infected intranasally with (A) pCD1⁻ mutants (BGY14) and/or *pgm*⁻ (BGY4) and (B) pCD1⁻ mutants (BGY14) and/or Δ *pla* (YP102). CFU per lung were determined at 48 hpi using BHI agar plates with or without kanamycin. Each bar represents the mean CFU recovered from four to five mice. The limit of detection is represented by a dotted line. Error bars represent SEM.

conditions are required for the study of fully virulent *Y. pestis*, two attenuated strains (*pgm*⁻ and pCD1⁻) are exempt from these restrictions and can be handled under normal laboratory conditions. The prospect of *trans*-complementation of raises a significant biosafety question: can two different attenuated strains mutually compensate for the virulence defects of each other to cause disease?

To address this concern, I performed a series of individual and co-infection experiments using different attenuated strains. I individually infected or co-infected C57BL/6J mice intranasally with *pgm*⁻ and pCD1⁻ mutants of *Y. pestis*. Both the *pgm*⁻ and pCD1⁻ mutant bacterial burdens in the co-infected mice were similar to the bacterial burdens in individually infected mice (Fig. 4-7A). Similar results were seen when Δ *pla* and pCD1⁻ mutants were examined in co-infection experiments (Fig. 4-7B). Additionally, I was unable to observe any differences in the onset of plague symptoms or in gross lung

pathology between mice individually infected with the *pgm*⁻ or Δ *pla* mutants and mice co-infected with *pgm*⁻ or Δ *pla* mutants and pCD1⁻ mutants (data not shown). These results demonstrate that *Y. pestis* strains must be able to cause significant disease in the lung in order to *trans*-complement other attenuated strains.

Trans-complementation is unique to *Y. pestis*

The ability of a microorganism to suppress the lung's innate immune system, effectively compensating for mutants missing a variety of virulence factors, is unprecedented among bacterial pathogens. In contrast to the *trans*-complementation phenomenon I have documented with *Y. pestis*, wild-type *Y. pseudotuberculosis* is unable to *trans*-complement isogenic mutants lacking the *Yersinia* virulence plasmid (designated pYV in *Y. pseudotuberculosis*) or pCD1⁻ mutants of *Y. pestis* during pulmonary co-infection (Fig. 4-8A and Ref. (Fisher *et al.*, 2007)). Similarly, wild-type *K. pneumoniae*, which can also cause an acute bacterial pneumonia, fails to *trans*-complement isogenic strains that have mutations in predominant virulence genes (such as a Δ *cpsB* mutant, which lacks the polysaccharide capsule) or pCD1⁻ mutants of *Y. pestis* (Fig 4-8B and Ref. (Lawlor *et al.*, 2005)). In addition, I have observed that pulmonary infection with wild-type *Y. pestis*, but not pCD1⁻ mutants, frequently leads to secondary bacterial infections. These bacteria, most likely introduced during the process of intranasal inoculation, can reach levels of 10⁷ bacteria/lung at 60 hpi in wild-type-infected mice (Fig. 4-9). Shibasaburo Kitasato likely provided the first documentation of rampant secondary

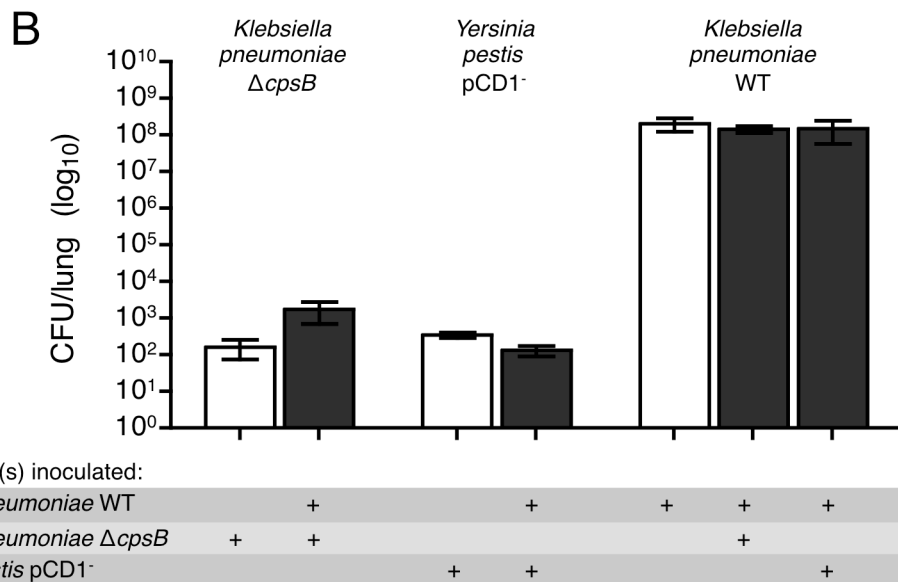
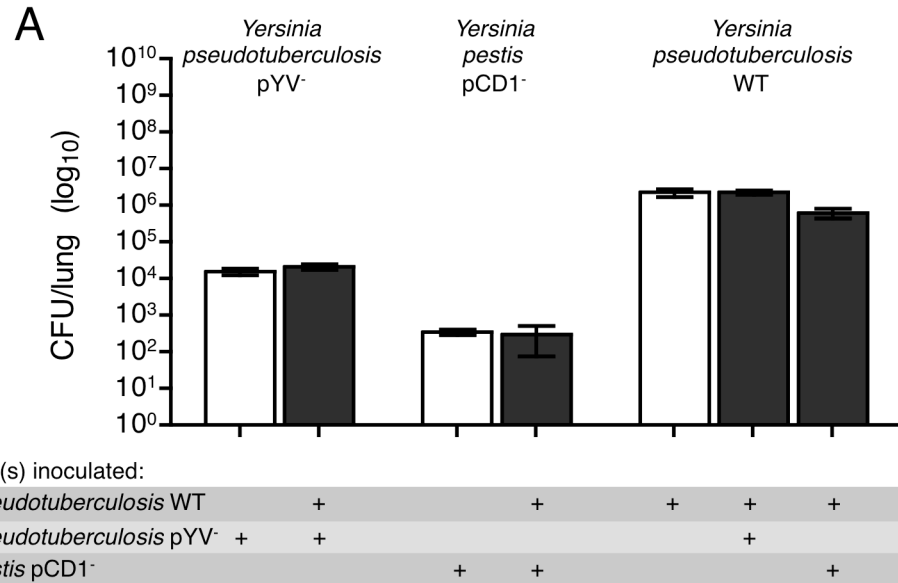


Figure 4-8: Specificity of bacterial *trans*-complementation during pulmonary infection. (A) *Y. pseudotuberculosis*, which harbors the same T3SS as *Y. pestis*, is unable to *trans*-complement in the lung. Individual intranasal infections (white bars) for pYV⁻ *Y. pseudotuberculosis* mutants (BGY38), pCD1⁻ *Y. pestis* mutants (BGY14-1) and wild-type *Y. pseudotuberculosis* (BGY30) and matched co-infections (gray bars) with wild-type *Y. pseudotuberculosis* in C57BL/6J mice. (B) *Klebsiella pneumoniae*, another pulmonary pathogen, is unable to *trans*-complement in the lung. Individual intranasal infections (white bars) for $\Delta cpsB$ *K. pneumoniae* mutants (VK060), pCD1⁻ *Y. pestis* mutants (BGY14-1) and wild-type *K. pneumoniae* (KPPR1) and matched co-infections (gray bars) with wild-type *K. pneumoniae* in C57BL/6J mice. (A and B) CFU per lung were determined at 48 hpi using LB or BHI agar plates with and without kanamycin. Error bars represent SEM.

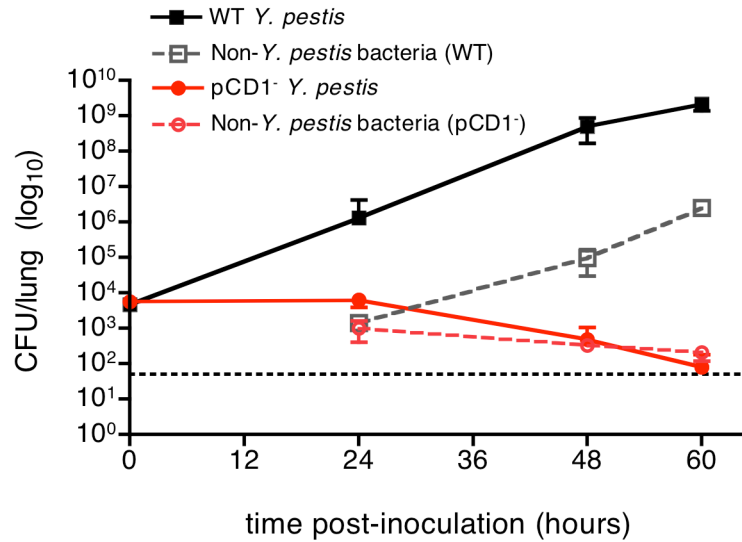


Figure 4-9: Wild-type *Y. pestis* creates a permissive environment that allows non-pathogenic bacteria to proliferate in the lung. Kinetics of infection for pCD1⁻ mutants (red) and wild-type *Y. pestis* (black) in C57BL/6J mice infected intranasally. CFU per lung were determined at various time points of infection on both BHI (*Y. pestis*) and BHI-MOX agar plates (non-*Y. pestis* bacteria). Each point represents the mean CFU recovered from five mice. The limit of detection is represented by a dotted line. Error bars represent SD from the mean.

infection when he originally published a description of the bacteria he thought caused the plague in 1894 but its described features resembled that of a pneumococcus (Perry and Fetherston, 1997). The rampant growth of secondary bacteria may necessitate the use of a broader spectrum of antibiotics during clinical treatment of pneumonic plague.

Some Yop effector proteins secreted by the plasmid-encoded Ysc-Yop T3SS are known to have anti-inflammatory mechanisms, and the Ysc-Yop system is required for all three *Yersinia* species to cause mammalian infection. Since I observed *trans*-complementation for *Y. pestis* but not *Y. pseudotuberculosis*, I tested whether this could be due to *Y. pestis*-specific evolutionary changes in the Ysc-Yop T3SS encoded on the virulence plasmid. I exchanged pCD1 in *Y. pestis* with its corresponding *Y. pseudotuberculosis* virulence plasmid (pYV), and this *Y. pestis* pCD1⁻ pYV⁺ strain still

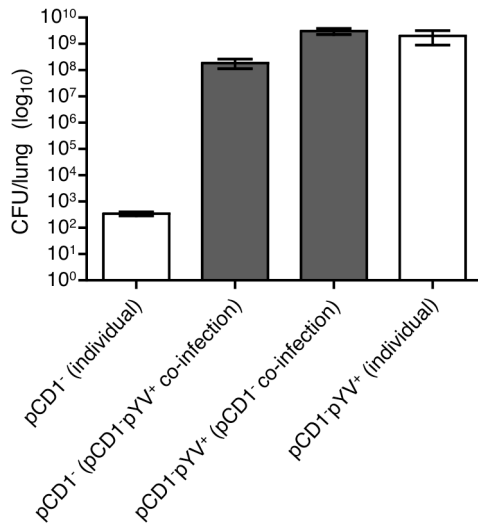


Figure 4-10: The ability to *trans*-complement avirulent isogenic strains is independent on the *Y. pestis*-specific T3SS. Individual (white bars) and matched co-infections (gray bars) of C57BL/6J mice infected intranasally with pCD1⁻ mutants (BGY14) and/or pCD1⁻ pYV⁺ *Y. pestis* (YP361). CFU per lung were determined at 48 hpi using BHI agar plates with/without kanamycin. Each bar represents the mean CFU recovered from four to five mice. Error bars represent SEM.

trans-complemented a *Y. pestis* pCD1⁻ mutant (Fig. 4-10). These data suggest that while the Ysc-Yop T3SS is necessary for a productive infection, subtle evolutionary adaptations to Ysc-Yop system during the evolution of *Y. pestis* are not solely responsible for the *trans*-complementation effect.

Scope of *trans*-complementation as evidenced by transposon site hybridization (TraSH)

Finally, I wanted to employ a broad, unbiased approach to evaluate the scope of *trans*-complementation by *Y. pestis*. Negative selection screens are often used to identify and confirm virulence-associated genes in animal models of infection (Chiang *et al.*, 1999). These screens rely on the fundamental assumption that during an infection with a pool of isogenic mutants, it will be possible to select against and subsequently identify single attenuated mutants. I used the microarray-based negative selection technique known as transposon site hybridization (TraSH) (Sasseti and Rubin, 2003) to comprehensively characterize *trans*-complementation during experimental pneumonic plague (see Chapter 3). The typical expectation of TraSH is that it should reveal *in vivo* selection against mutants that lack key virulence factors; however, my *Y. pestis* co-

infection results predicted that I may not observe any *in vivo* selection against individual attenuated mutants in a mixed pool. Remarkably, all known *Yersinia* virulence genes implicated in pneumonic plague showed less than a two-fold negative selection *in vivo* (Table 4-1). In contrast, negative selection screens have been employed to identify and confirm virulence factors in other lung pathogens, including *Pseudomonas aeruginosa*, *Burkholderia pseudomallei*, *Burkholderia cenocepacia*, *Streptococcus pneumoniae* and *Mycobacterium tuberculosis* (Camacho *et al.*, 1999; Cuccui *et al.*, 2007; Hava and Camilli, 2002; Hunt *et al.*, 2004; Winstanley *et al.*, 2009; Zhang *et al.*, 2005).

Table 4-1: TraSH screen reveals global *trans*-complementation during primary pneumonic plague. Known genes required for primary pneumonic plague and their representation in the *in vivo* pool (high-throughput sequence (HTPS) reads or TraSH probes for the transposon mutant library at 24 or 48 hours post-intranasal inoculation into C57BL/6J mice, respectively) compared to the *in vitro* pool (HTPS reads or TraSH probes for the mutant library). Negative values indicate an underrepresentation in the output pool. n/d indicates data that were not determined by the analysis.

Gene	Reference	Fold Representation 48 hpi (microarray)	Fold Representation 24 hpi (HTPS)
<i>yopH</i>	(Bubeck and Dube, 2007)	-1.85	n/d
<i>yopD</i>	(Cornelis, 2002)	-1.33	n/d
<i>pla</i>	(Lathem <i>et al.</i> , 2007)	-1.32	-1.33
<i>lcrF</i> (T3SS)	(Perry and Fetherston, 1997)	-1.26	-1.63
<i>ail</i>	(Kolodziejek <i>et al.</i> , 2010)	-1.88	1.05
<i>psn</i> (Yersiniabactin)	(Fetherston <i>et al.</i> , 2010)	-1.54	n/d

Conclusions

The concept of *trans*-complementation has always been hypothesized as a potential caveat for negative selection screens. Examples of individual secreted proteins have been shown to produce this effect, however it has never been seen on a global level. The *in vivo* TraSH results suggest that *Y. pestis* is able to *trans*-complement all known virulence factors during a pneumonic co-infection with wild-type organisms. These observations suggest that *Y. pestis* is able to create a dominant, localized anti-inflammatory state early during lung infection, and this establishes a unique protective environment that even allows non-pathogenic organisms to prosper. *Y. pestis* rather recently evolved from a food-borne pathogen (*Y. pseudotuberculosis*) to become an arthropod-borne pathogen, and this is thought to have strongly favored the coevolution of increased virulence for mammals (Lorange *et al.*, 2005). Poor arthropod vector competence imposed the need to generate very high levels of bacteremia from very few inoculating bacteria in order to efficiently maintain the natural route of transmission between arthropods and their mammalian hosts. Thus, an early anti-inflammatory state at the site of infection would be biologically advantageous in ensuring that *Y. pestis* can replicate unfettered until its numbers are sufficient for transmission. When finally recognized by the immune system, the bacteria are at such a high titer that they trigger an overly robust inflammatory response. In the case of a lung infection, the switch from unrestricted bacterial growth to overwhelming inflammation results in rapid decline in pulmonary function and the corresponding fatalities associated with primary pneumonic plague.

Materials and Methods

Reagents, bacterial strains, strain construction and culture conditions

See Chapter 2.

Animals

All animals studies were approved by the Washington University Animal Studies Committee protocol #20060154 or the University of North Carolina in Chapel Hill Office of Animal Care and Use protocol #09-057.0-A. Pathogen-free, six- to eight-week-old C57BL/6J mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice were housed in a Techniplast Isocage containment system (Buguggiate, Italy) during all experiments. Mice were provided with food and water *ad libitum* and maintained on a 12-hr light/12-hr dark cycle at 25°C and 15% humidity. Bacteria were grown in BHI broth as described above, and the optical density (OD₆₂₀) of the culture was used to estimate bacterial concentrations. Actual bacterial concentrations were determined by plating serial dilutions of the inoculum on BHI or LB agar plates. The bacteria were then washed in endotoxin-free phosphate buffered solution (PBS) and maintained at 37°C (intranasal infection) or 26°C (subcutaneous infection) prior to inoculation, unless otherwise indicated. Mice were anesthetized with a ketamine/xylazine solution (50 mg Kg⁻¹ / 10 mg Kg⁻¹) and inoculated with 20 µl or 100 µl of bacteria in PBS via an intranasal or subcutaneous route, respectively. Animals were monitored twice daily and sacrificed with an overdose of sodium pentobarbital (180 mg Kg⁻¹) at designated time points or when the animal was clearly moribund.

Animal infections

For both the individual infection and co-infection experiments, groups of 4-5 mice were inoculated intranasally (i.n.) with 1×10^4 CFU of *Y. pestis*, *Y. pseudotuberculosis* and/or *K. pneumoniae* for individual and co-infections with equal ratios (1 pCD1⁻ : 1 WT) and 1×10^5 CFU of *Y. pestis* for infections with varying ratios (1 pCD1⁻ : 10 WT, 1 pCD1⁻ : 1 WT and 10 pCD1⁻ : 1 WT). Groups of 4-5 mice were inoculated subcutaneously (s.c.) in the front neck with 1000 CFU of *Y. pestis* for both individual and co-infections. For both *Y. pestis* and *Y. pseudotuberculosis*, *lacZ* mutants (Fisher *et al.*, 2007; Lawrenz *et al.*, 2009) were designated as my “wild-type” strain, and blue/white screening was used to differentiate between co-inoculated strains. Bacterial organ burdens were determined by plating bacteria on BHI or LB agar containing X-gal for experiments involving multiple *Y. pestis* or *Y. pseudotuberculosis* strains. For experiments involving *K. pneumoniae*, organ burdens were determined using LB agar with kanamycin (selection mutant) or without kanamycin. For mixed species experiments [*Y. pseudotuberculosis* or *K. pneumoniae* with *Y. pestis* pCD1⁻ *lacZ*::kan^R (BGY14-1)], organ burdens were determined using BHI agar with kanamycin (*Y. pestis* pCD1⁻ mutant selection) or without kanamycin. For experiments in which *pla* was induced with anhydrotetracycline (ATC), *Y. pestis* (YP138) was grown in the absence of ATC as described in Chapter 2 and inoculated intranasally into mice (three per time point). At the indicated time point, mice were injected intraperitoneally with ATC (2 mg kg⁻¹ diluted in PBS) to promote *pla* expression (Lathem *et al.*, 2007). ATC was then administered every twelve hours, except for the mice at the 6-hour time point, which received ATC at 6 hpi and then at 12 hpi. At the indicated times post-inoculation, mice were euthanized, lungs and spleen (after i.n. infection) or lymph nodes, spleen, and lungs (after s.c. infection)

were surgically removed, weighed, and homogenized in 0.5 ml PBS. Serial dilutions for each organ were plated on BHI or LB agar as indicated above. Bacterial burdens are reported as CFU/tissue.

Histopathology

Groups of 3-5 mice were inoculated intranasally with 1×10^4 CFU of a 1:1 ratio of *Y. pestis* [1 *gfp*-expressing pCD1⁻ (BGY23) : 1 *rfp*-expressing WT (YP337)]. Mice were euthanized at 48 hours post-inoculation (hpi) with an overdose of sodium pentobarbital, and their lungs were inflated with 10% neutral buffered formalin via tracheal cannulation. The lungs were then removed from the animal and fixed for 96 hr in 10% neutral buffered formalin. All samples were negative for bacterial growth before being removed from the BSL-3 facility. The lungs were washed twice in PBS for 1 hr and then in PBS-30% sucrose for 24 hr. Frozen sections were generated by freezing the lungs in Optimal Cutting Temperature (OCT) compound on dry ice for twenty minutes and then at -80°C overnight. Ten- μ m sections were mounted with Pro-Long Gold (Invitrogen, USA) and viewed under an Olympus BX60 fluorescence microscope (Center Valley, PA). iVision software v. 4.0.0 (BioVision Technologies, Exton, PA) used for Z-stack image collection and deconvolution.

Cytokine analysis

For the cytokine analysis, groups of 4-5 mice were co-infected or individually infected with 1×10^4 CFU of wild-type *Y. pestis* (YP160) and/or a $\Delta yopH$ mutant (YP373). At 24 hpi, the lungs were surgically removed and homogenized in 1 ml PBS containing protease inhibitor cocktail set III (Calbiochem, EMB Chemicals, Gibbstown, NJ). The homogenates were centrifuged at 21,000 rpm to remove the bacteria and cellular

tissues and then treated with a 1% sodium azide / 100 µg/ml gentamicin solution.

Samples were stored at -80°C until the ELISA analysis was conducted. All samples were tested for the presence of viable *Y. pestis* before being removed from the BSL-3 facility.

Commercially available IL-6 and TNF-α ELISA test kits (BD) were used according to the manufacture's instructions to determine cytokine levels for each lung sample. Mock-infected animals were used to control for baseline cytokine levels. Results are reported as pg/ml for each cytokine.

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Chapter 5
Collaborative studies

Abstract

“Primary pneumonic plague is transmitted easily, progresses rapidly, and causes high mortality, but the mechanisms by which *Yersinia pestis* overwhelms the lungs are largely unknown. We show that the plasminogen activator Pla is essential for *Y. pestis* to cause primary pneumonic plague but is less important for dissemination during pneumonic plague than during bubonic plague. Experiments manipulating its temporal expression showed that Pla allows *Y. pestis* to replicate rapidly in the airways, causing a lethal fulminant pneumonia; if unexpressed, inflammation is aborted, and lung repair is activated. Inhibition of *pla* expression prolonged the survival of animals with the disease, offering a therapeutic option to extend the period during which antibiotics are effective.”

Contribution

My main contribution to this study was the development of the *in vitro* plasminogen activation assays to confirm the protease activity of Pla in wild-type strains, strains carrying various inactive point mutants and the ATC-inducible Pla strains. I also helped generate the pCD1⁻ strains used for each of these mutants used in the *in vitro* plasminogen activation assays.

Lathem, W., Price, P., Miller, V., and Goldman, W. (2007) A Plasminogen-Activating Protease Specifically Controls the Development of Primary Pneumonic Plague. *Science* **315**: 509-513. Subject to copyright restrictions.

Abstract

“Cyclic di-GMP (c-di-GMP) is a signaling molecule that governs the transition between planktonic and biofilm states. Previously, we showed that the diguanylate cyclase HmsT and the putative c-di-GMP phosphodiesterase HmsP inversely regulate biofilm formation through control of HmsHFERS-dependent poly- β -1,6-N-acetylglucosamine synthesis. Here, we systematically examine the functionality of the genes encoding putative c-di-GMP metabolic enzymes in *Yersinia pestis*. We determine that, in addition to *hmsT* and *hmsP*, only the gene *y3730* encodes a functional enzyme capable of synthesizing c-di-GMP. The seven remaining genes are pseudogenes or encode proteins that do not function catalytically or are not expressed. Furthermore, we show that HmsP has c-di-GMP-specific phosphodiesterase activity. We report that a mutant incapable of c-di-GMP synthesis is unaffected in virulence in plague mouse models. Conversely, an *hmsP* mutant, unable to degrade c-di-GMP, is defective in virulence by a subcutaneous route of infection due to poly- β -1,6-N-acetylglucosamine overproduction. This suggests that c-di-GMP signaling is not only dispensable but deleterious for *Y. pestis* virulence. Our results show that a key event in the evolution of *Y. pestis* from the ancestral *Yersinia pseudotuberculosis* was a significant reduction in the complexity of its c-di-GMP signaling network likely resulting from the different disease cycles of these human pathogens.”

Contribution

My main contribution to these studies was to generate mutants in the CO92 background of *Y. pestis* and test them for c-di-GMP production. I also made all of the original observations in animal for all of the different mutants although only those in the Kim

strain background were used in the publication. I also generated additional strains that helped clarify the role of HmsT in c-di-GMP production that were not included in the publication but directly lead to observation that were included.

Bobrov, A.G., Kirillina, O., Ryjenkov, D.A., Waters, C.M., Price, P.A., Fetherston, J.D., Mack, D., Goldman, W.E., Gomelsky, M., and Perry, R.D. (2011) Systematic analysis of cyclic di-GMP signalling enzymes and their role in biofilm formation and virulence in *Yersinia pestis*. *Mol Microbiol* **79**: 533-551. Subject to copyright restrictions.

Abstract

“*Yersinia pestis* is a highly pathogenic Gram-negative organism and the causative agent of bubonic and pneumonic plague. *Y. pestis* is capable of causing major epidemics; thus, there is a need for vaccine targets and a greater understanding of the role of these targets in pathogenesis. Two prime *Y. pestis* vaccine candidates are the usher-chaperone fimbriae Psa and Caf. Herein we report that *Y. pestis* requires, in a nonredundant manner, both PsaA and CafI to achieve its full pathogenic ability in both pneumonic and bubonic plague in C57BL/6J mice. Deletion of *psaA* leads to a decrease in the organ bacterial burden and to a significant increase in the 50% lethal dose (LD₅₀) after subcutaneous infection. Deletion of *cafI* also leads to a significant decrease in the organ bacterial burden but more importantly leads to a significantly greater increase in the LD₅₀ than was observed for the Δ *psaA* mutant strain after subcutaneous infection of C57BL/6J mice. Furthermore, the degree of attenuation of the Δ *cafI* mutant strain is mouse background dependent, as the Δ *cafI* mutant strain was attenuated to a lesser degree in BALB/cJ mice by the subcutaneous route than in C57BL/6J mice. This observation that the degree of requirement for CafI is dependent on the mouse background indicates that the virulence of *Y. pestis* is dependent on the genetic makeup of its host and provides further support for the hypothesis that PsaA and CafI have different targets.”

Contribution

I generated the Δ *cafI* mutant strains used in these studies and made all of the original observations with the Δ *cafI* mutant during a pneumonic infection. Although not included in the publication, I was able to show that *cafI* was the only gene on the pMT plasmid that resulted in a loss of virulence in the C57BL/6 mouse model of infection.

Weening, E.H., Cathelyn, J.S., Kaufman, G., Lawrenz, M.B., Price, P., Goldman, W.E., and Miller, V.L. (2011) The dependence of the *Yersinia pestis* capsule on pathogenesis is influenced by the mouse background. *Infection and Immunity* **79**: 644-652. Subject to copyright restrictions.

Chapter 6
Conclusions and Future Directions

Summary of the Work

Project Goals – Before this project began, there was very little information in the literature about the early stages of *Y. pestis* pathogenesis, especially during the initial pre-inflammatory phase of disease. The overall goal of this project was to characterize the initial interactions between *Y. pestis* and lung tissues and to identify specific bacterial virulence factors that are involved in this process and in the overall pathogenesis of *Y. pestis*.

Y. pestis adherence to lung tissues

Adherence to host tissues is considered one of the earliest essential steps in bacterial pathogenesis. Although a number of studies have been conducted to examine adhesion to tissue culture cells, an *in vivo* model of *Y. pestis* adhesion has never been developed to study specific *Y. pestis*-host cell interactions. I therefore adapted our animal model of pneumonic plague to specifically examine the extent of interactions between *Y. pestis* and lung tissues and to compare these interactions to those the interactions between *Y. pestis* and tissue culture cells. In Chapter 2, I was able to demonstrate that over 90% of *Y. pestis* remains adherent to lung tissues at 2 hpi during BAL, whereas in my tissue culture model only 17% of *Y. pestis* remains adherent. These data imply that adherence to tissue culture cells does not necessarily mimic what is occurring *in vivo* and that *Y. pestis* adheres fairly tightly to lung tissues during the initial stages of infection. This tight adherence may help prevent mechanical clearance of *Y. pestis* from the lung by the action of the mucociliary escalator. In contrast, *Y. pestis* is freely lavageable during the later stages of infection, which may provide evidence for why *Y. pestis* is only transmitted person-to-person during this stage of the disease.

The high levels of adhesion during the initial stages of infection provide a means of enriching for less adherent mutants of *Y. pestis*. Ail was implicated as an adhesin in *Y. pestis* from this enrichment. Concurrent with these studies, Ail was found to mediate adhesion to tissue culture cells (Felek and Krukoni, 2009). However, my subsequent studies using Δail mutants suggest that Ail mediates membrane stability and/or the proper integration of proteins into the outer membrane and not direct adhesion. These studies along with the biotinylation studies imply that adhesion is likely mediated by more than one outer membrane protein. Given the possible role of Ail in maintaining membrane stability, the role of the outer membrane itself in mediating adhesion cannot be ruled out.

Aberrant adhesion to host tissues may by *Y. pestis* appears to be detrimental to *Y. pestis* pathogenesis. Reintroduction of two major *Yersinia* adhesins, *yadA* and *inv*, into *Y. pestis* reduces the virulence of *Y. pestis* in both bubonic and pneumonic animal models of infection. The exact mechanism of associated with this attenuation is unknown, but Casutt-Meyer *et al.* have shown that *yadA* expression in *Y. pestis* increases its susceptibility to neutrophil extracellular traps (NETs) (Casutt-Meyer *et al.*, 2010). However, the early and increased influx of neutrophils seen during pneumonic infection with *yadA*⁺ *Y. pestis* seems to have lead to increased bacterial burdens, not increased killing, implying that another mechanism of action beyond NET-mediated killing may be limiting *Y. pestis* pathogenesis when either *yadA* or *inv* is reintroduced into *Y. pestis*. Interestingly, the reintroduction of *yadA* into a Δpla mutant restores 3-4 logs of bacterial growth over a Δpla mutant during pulmonary infection. Given that YadA is required for full virulence in other *Yersinia* species (Durand *et al.*, 2010), Pla may be compensating for the lack YadA in *Y. pestis*. The combination of the compensation by Pla and the

liability of having expressing YadA during a bubonic infection may explain why evolution selected for mutants that lack these adhesins.

Transposon Site Hybridization (TraSH)

In vitro systems have been great at determining specific mechanisms in bacterial pathogenesis, but they often do not reflect the same physiological conditions found *in vivo*. Therefore, I developed a negative selection screen termed transposon site hybridization (TraSH) to identify bacterial factors required for *Y. pestis* pulmonary infection (Sasseti *et al.*, 2001). I validated the system in Chapter 3 by defining the genes that are essential for *Y. pestis* viability, which included 628 genes, similar to *E. coli*. However, I was unable to identify any known virulence factors using TraSH, including genes involved in the Ysc type-three secretion system (T3SS), which are known to be essential for pulmonary infection. These unusual data suggest that wild-type *Y. pestis* is able to globally *trans*-complementation of avirulent mutants in our animal model of pneumonic plague.

Trans-complementation by wild-type *Y. pestis*

Trans-complementation of avirulent bacteria by wild-type bacteria has been theorized to occur during co-infections (Chiang *et al.*, 1999), but the global nature of *trans*-complementation I see for *Y. pestis* has never been described. The *trans*-complementation effect may help explain the biphasic disease progression of primary pneumonic plague, where during the long pre-inflammatory phase, bacteria replicate to high levels, seemingly uninhibited by normal pulmonary defenses. Two different models may explain the *trans*-complementation and the pre-inflammatory phase of disease. The first model suggests that *Y. pestis* fails to elicit a sufficient immune response during this

phase of disease because it is naturally non-stimulatory, failing to trigger TLR-4- or TLR-2-mediated immune responses (Montminy *et al.*, 2006; Reithmeier-Rost *et al.*, 2007). A second model supposes that *Y. pestis* creates a dominant anti-inflammatory state that allows for the survival and rapid growth of both itself and normally avirulent organisms. Using a co-infection model of pneumonic plague in which known immune-stimulatory *Y. pestis* strains are inoculated along with wild-type bacteria, I discovered that *Y. pestis* quickly creates dominant immunosuppressive state in the lung, more consistent with the second model.

The protective environment is localized to the areas immediately surrounding wild-type *Y. pestis*, as evidenced by fluorescence microscopic examination of lung cryosections at 48 hpi. However, the overall size of these areas may be larger during the pre-inflammatory phase of disease, before the efflux of neutrophils into the lungs. Immune responses during the first 24 hpi appear to be critical in controlling the proliferation of avirulent bacteria, further suggesting that the pre-inflammatory phase of disease results from immunosuppression and not simply a failure to elicit an immune response.

Neither *Y. pseudotuberculosis*, the relatively recent progenitor of *Y. pestis*, nor *K. pneumoniae*, another bacterial lung pathogen, showed a similar *trans*-complementation effect, which appears to be unprecedented among other respiratory pathogens. Thus, the unbiased negative selection screen approach used in TraSH, which revealed no selection against any known virulence genes, further demonstrates the ability of wild-type *Y. pestis* to transform the lung into a globally permissive environment during the pre-inflammatory phase of pneumonic plague.

Model for *Y. pestis*-specific *trans*-complementation

Comparing evolutionary adaptations between *Y. pestis* and its recent progenitor *Y. pseudotuberculosis* may offer some insights into the mechanisms of *Y. pestis* pathogenesis, which ultimately culminate in the localized anti-inflammatory environment that allows for *trans*-complementation. Although the Ysc-Yop T3SS is undoubtedly important in this process, *Y. pestis*-specific virulence factors likely play a key role in enhancing immune suppression during the initial stages of disease and/or significantly altering the lifestyle of *Y. pestis* in host tissues to allow for increased pathogenesis. Figure 6-1 depicts my current model for the life style differences that allow *Y. pestis* but not *Y. pseudotuberculosis* to *trans*-complement *in vivo*. This model is based on differences in the initial intracellular lifestyle between the two species.

A facultative intracellular lifestyle has been described *in vivo* for *Y. pseudotuberculosis* following intragastric infection, enabling *Y. pseudotuberculosis* to be trafficked to the regional lymph nodes (Pujol and Bliska, 2005). I have also shown that *Y. pseudotuberculosis* also has an intracellular lifestyle soon after intranasal infection. In contrast, a facultative intracellular lifestyle for *Y. pestis* has only been postulated. However, using gentamycin protection assays, I have shown that *Y. pestis* is predominantly extracellular throughout the course of disease. Examples of individual bacteria inside phagocytes has been demonstrated, but their importance to *Y. pestis* pathogenesis is still unknown (Pujol and Bliska, 2005). Further, Δ *phoP* mutants that are attenuated in *in vitro* macrophage models of infection are not attenuated *in vivo*, whereas the same Δ *phoP* mutation in *Y. pseudotuberculosis* leads to attenuation in an intranasal animal model of infection (Bozue *et al.*, 2011). The intracellular lifestyle of *Y.*

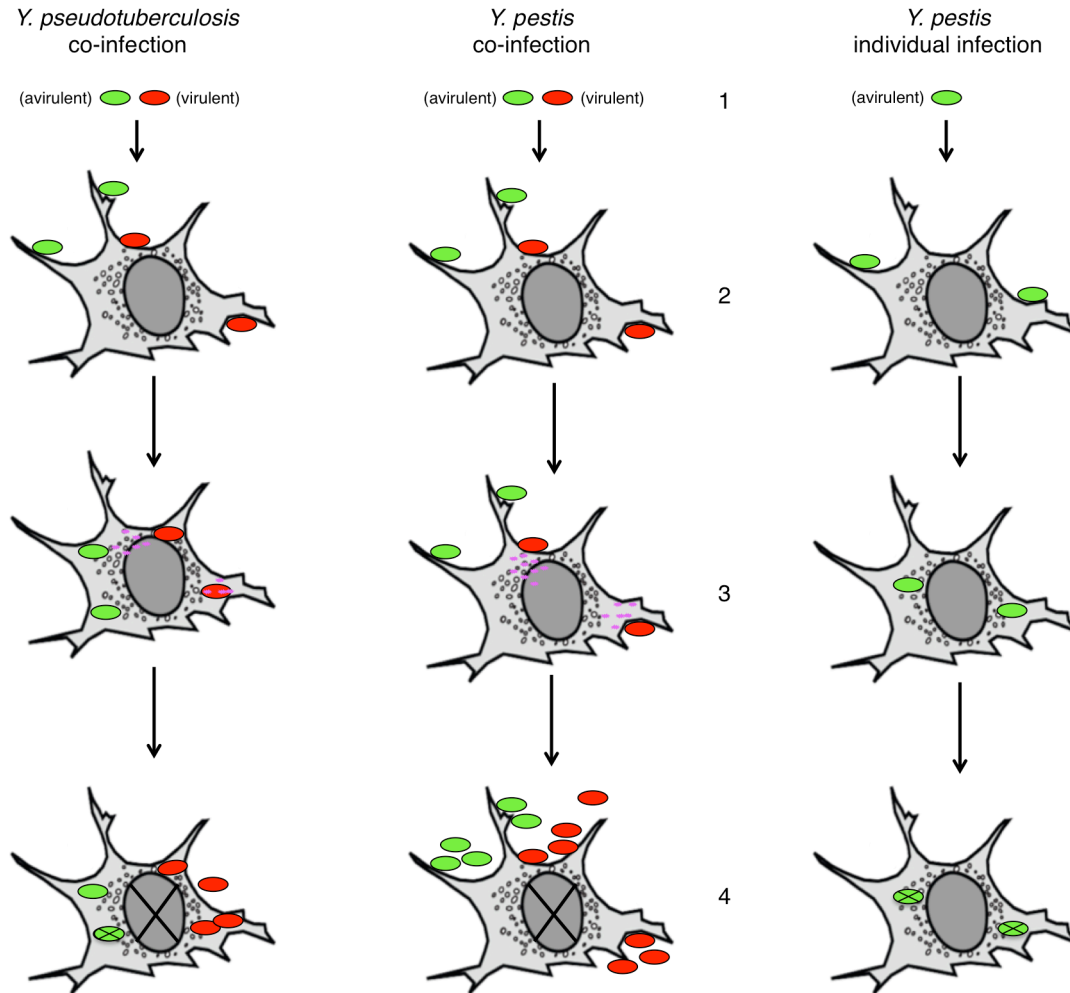


Figure 6-1: Model for *Y. pestis*-mediated *trans*-complementation. Wild-type *Y. pestis* (center red circles) mediates *trans*-complementation of attenuated *Y. pestis* strains (green circles) by replicating extracellularly early during infection. In contrast, *Y. pseudotuberculosis* (left red circles) has an initial intracellular lifestyle, failing to *trans*-complement attenuated bacteria. (1) Attenuated bacteria are inoculated individually (right) or co-inoculated with wild-type *Y. pestis* (center) or *Y. pseudotuberculosis* (left). (2) Soon after infection professional phagocytes (macrophages or dendritic cells) encounter the bacterial in the small airways of the lung. (3) Wild-type *Y. pestis* is able to prevent the phagocytosis of itself and co-inoculated attenuated mutants via combined activity of Pla, the proteinous capsule, Caf1, and Ysc-Yop T3SS (purple stars). *Y. pseudotuberculosis* is engulfed by phagocytic cells via a YadA-mediated mechanism. Conversely, attenuated *Y. pestis* are engulfed by macrophages when inoculated individually. (4) The harsh intracellular environment encountered inside macrophages limits the initial growth of *Y. pseudotuberculosis* before the macrophages undergo apoptosis. The macrophages are able to kill attenuated *Y. pestis* when inoculated individually and fail to undergo apoptosis, whereas wild-type *Y. pestis* and co-inoculated attenuated mutants are able to proliferate. Co-inoculated attenuated mutants will continue to require wild-type *Y. pestis* to proliferate over the entire course of the infection.

pseudotuberculosis is thought to be mediated by YadA and/or Inv, although there are differences between YadA- and Inv-mediated invasion that alter cellular responses to infection (Viboud and Bliska, 2005). The evidence that YadA is able to partially rescue a Δpla mutant during an intranasal infection further supports this idea. However, I still need to verify that the introduction of *yadA* into a Δpla mutant leads to an intracellular lifestyle. Although the exact proteolytic targets for Pla are unknown, it may enable *Y. pestis* to survive extracellularly over the entire course of infection.

Our model also relies on the assumption that the intracellular environment that *Y. pseudotuberculosis* encounters is likely a much harsher environment for survival than an extracellular environment, decreasing the survival or restricting the growth of attenuated bacteria upon internalization. Additionally, the levels of Yop translocation from internalized bacteria appear to be lower or less frequent than the levels from extracellular bacteria (Durand *et al.*, 2010), implying that extracellular bacteria have an enhanced ability to inhibit macrophage function. The harsher intracellular environment encountered by attenuated strains coupled with decreased Yop translocation by wild-type bacteria may prevent intracellular *Y. pseudotuberculosis* from achieving *trans*-complementation. Conversely, the extracellular nature of wild-type *Y. pestis* may enhance its ability to inhibit phagocytosis of both itself and attenuated strains, enabling both strains to proliferate. Although *Y. pseudotuberculosis* replicates primarily extracellularly during the later stages of infection (Balada-Llasat and Mecsas, 2006), a brief intracellular lifestyle may be sufficient to prevent *Y. pseudotuberculosis* from rescuing avirulent mutants, given that the first 24 hpi are critical in enabling *trans*-complementation (Chapter 4).

Overall, this model implicates the acquisition of pPCP1, which encodes *pla*, as a major advance in evolution of *Y. pestis* pathogenesis.

Conclusions

The biphasic disease progression of the pneumonic plague syndrome is unusual because it is characterized by rapid bacterial replication in the lung without generating appreciable levels of proinflammatory cytokines/chemokines, histological changes in infected tissue or outward disease symptoms. I have been able to show that this pre-inflammatory phase of disease is the result of a rapid and profound suppression of pulmonary defenses that ultimately allows not only these organisms to proliferate rapidly in the lung, but other microbes as well. These observations suggest that dominant, localized immunosuppression plays a greater role than the inherent non-stimulatory nature of *Y. pestis* in maintaining the pre-inflammatory during primary pneumonic plague.

Y. pestis rather recently evolved from the food-borne pathogen *Y. pseudotuberculosis* to become an arthropod-borne pathogen, and this is thought to have strongly favored the coevolution of increased virulence for mammals (Lorange *et al.*, 2005). Poor arthropod vector competence imposed the need to generate very high levels of bacteremia from very few inoculating bacteria in order to efficiently maintain the natural route of transmission between arthropods and their mammalian hosts. Thus, an early anti-inflammatory state at the site of infection would be biologically advantageous in ensuring that *Y. pestis* can replicate unfettered until its numbers are sufficient for transmission. The exact mechanistic details behind the increase immunosuppression at the early stages of disease are unknown; however, differences in the intracellular/extracellular lifestyle during the first hours 24 hours of infection may provide an insight into the evolution of *Y. pestis* from *Y. pseudotuberculosis*.

Future Directions

I have proposed a model for *Y. pestis*-mediated *trans*-complementation that relies on the fundamental assumption that *Y. pestis* remains extracellular and *Y. pseudotuberculosis* intracellular during the initial phases of infection. I have developed the techniques to examine this model directly and have shown that the majority of *Y. pestis* is extracellular, whereas *Y. pseudotuberculosis* is intracellular. However, the relationship between the *in vivo* roles of Pla and YadA needs further characterization. Understanding the partial compensation of a Δpla strain by YadA will aid in this characterization. These studies will also greatly aid in understanding the role of Pla during pulmonary infection. I have developed three plausible models that may account for the partial rescue.

The first model is similar to the intracellular/extracellular model presented in Figure 6-1. This model suggests that YadA-mediated internalization, which appears to be slightly mechanistically different from other means of internalization (Hudson *et al.*, 2005), protects bacteria during the initial stages of disease and bacterial replication. If Pla is acting to ensure survival in extracellular spaces and its loss decreased survival in extracellular spaces, this intracellular environment would now have a protective effect. Testing the cellular localization of a $\Delta pla yadA^+$ strain during pulmonary infection will help clarify this model.

The second model is fundamentally different than the first model. Yop translocation depends on both the binding of bacteria to host cells and activation of signal-transduction cascades within those cells, the later of which is triggered by ligand-host cell receptor binding (Mejía *et al.*, 2008). In *Y. pseudotuberculosis*, the activation of

signal-transduction cascades is mediated mainly by YadA- β 1 integrin binding and to a lesser extent Invasin- β 1 integrin binding. Both *yadA* and *inv* are pseudogenes in *Y. pestis* and hence cannot mediate Yop translocation via this signal-transduction cascade. Pla may engage other signaling cascades via proteolytic cleavage of cell surface components. Caulfield *et al.* have recently presented data to suggest that Pla readily cleaves Fas-ligand from the surface of cells to disrupt its signaling (Caulfield and Lathem, 2010). Cleavage of other cell-surface ligands by Pla may initiate the signaling cascades that restore Yop translocation. In this model, the unknown defect of a Δ *pla* mutant may simply be its inability to efficiently translocate Yop effectors into host cells. YadA would then restore the ability of the Δ *pla* mutant to trigger signal-transduction cascades and restore Yop translocation. Testing the *in vivo* translocation of a Δ *pla* mutant and a Δ *pla yadA*⁺ mutant may be able to address this model. The techniques required for these examinations are now widely used in the field, and I am currently adapting them for use in studying pneumonic plague (Durand *et al.*, 2010; Marketon *et al.*, 2005).

Pla has been shown to have adhesive properties that aid in attachment and invasion of tissue culture cells (Lähtenmäki *et al.*, 2001), independent of the proteolytic activity of Pla. The proteolytic activity of Pla is required for pulmonary infection (Lathem *et al.*, 2007), but the relevant proteolytic targets of Pla *in vivo* remain unknown. Pla cleaves after basic residues in proteins, leaving the positively charged end of the protein attached to the cell. Considering Pla can cleave the vast majority of proteins from the surface of *Y. pestis*, robust cleavage of surface proteins may result in a positively charged outer membrane that can adhere more readily to host cells. In this third model, YadA would be able to compensate for the loss of Pla and the charged outer membrane

by restoring the adherence to host cells. Analyzing the overall charge of the outer membrane using certain cationic and anionic dyes can be used to test this model (Bengoechea *et al.*, 1998a; Bengoechea *et al.*, 1998b).

The concept of global *trans*-complementation by *Y. pestis* lends itself to a myriad of different studies. Oddly enough, many of the most pressing studies involve defining the early events in pneumonic plague. Although not part of my dissertation, we are currently defining which cell types are actively translocated during the pre-inflammatory phase of disease and hope to continue these studies to also analyze the pro-inflammatory phase of disease. Simultaneously, we are monitoring changes in the host-cell repertoire in the lungs of mice during *Y. pestis* infection using flow cytometry. Although these studies may not identify the initial interacting cell types, they should allow us to determine which immune cells are responding to infection during the pre-inflammatory phase of disease. These studies should be able to open up new avenues of study and provide a wealth of information on the pre-inflammatory phase of disease.

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