DISSERTATION

LOW TEMPERATURE EFFECTS ON THE TRANSCRIPTOME OF *YERSINIA PESTIS* AND ITS TRANSMISSIBILITY BY *OROPSYLLA MONTANA* FLEAS

Submitted by

Shanna K. Williams

Department of Microbiology, Immunology & Pathology

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Doctoral Committee:

Advisor: Brad Borlee
Co-Advisor: Scott Bearden

Ken Gage
Mark Stenglein
Shawn Archibeque

ABSTRACT

LOW TEMPERATURE EFFECTS ON THE TRANSCRIPTOME OF *YERSINIA PESTIS* AND ITS TRANSMISSIBILITY BY *OROPSYLLA MONTANA* FLEAS

Yersinia pestis, the causative agent of plague, is primarily a rodent-associated, flea-borne zoonosis. Transmission to humans is mediated most commonly by the flea vector, *Oropsylla montana*, and occurs predominantly in the Southwestern United States. In these studies, we hypothesized that *Y. pestis*-infected *O. montana* fleas held at temperatures as low as 6ºC could serve as reservoirs of the plague bacillus during the winter months in temperate regions with endemic plague foci. With few exceptions, previous studies showed *O. montana* to be an inefficient vector at transmitting *Y. pestis* at 22-23°C particularly when such fleas were fed on susceptible hosts more than a few days after ingesting an infectious blood meal. We examined whether holding fleas at sub-ambient temperatures (for purposes of these studies, ambient temperature is defined as 23°C) affected the transmissibility of *Y. pestis* by this vector. Colony-reared *O. montana* fleas were given an infectious blood meal containing a virulent *Y. pestis* strain (CO96-3188), and potentially infected fleas were maintained at different temperatures (6ºC, 10°C, 15°C, or 23ºC). Transmission efficiencies were tested by allowing groups of ~15 infectious fleas to feed on each of seven naïve CD-1 mice on days 1-4, 7, 10, 14, 17, and 21, 28, 35, and 42 post infection (p.i.). Fleas held at 6ºC, 10°C and 15°C were able to effectively transmit at every time point p.i. The percentage of transmission to naïve mice by fleas maintained at low temperatures was higher than for fleas maintained at 23ºC and indicates that

O. montana fleas efficiently transmit *Y. pestis* at low temperatures. Moreover, bacterial loads of flea cohorts maintained at temperatures of 6ºC, 10ºC and 15ºC were statistically higher than fleas maintained at 23ºC. In addition, whole transcriptomes of *Y. pestis* bacteria grown at 6ºC, 10°C, 15°C and 23ºC were analyzed to assess differential gene expression at each temperature to identify genes which may contribute to an increase in virulence or survivability of the plague pathogen at the lower temperatures when compared to ambient temperature. This is the first comprehensive study to demonstrate efficient transmission of *Y. pestis* by *O. montana* fleas maintained at temperatures as low as 6ºC. Our findings further contribute to the understanding of plague ecology in temperate climates by providing support for the hypothesis that *Y. pestis* is able to overwinter within the flea gut and potentially cause infection during the following transmission season. The findings also might hold implications for explaining the focality of plague in tropical regions where plague occurs in cooler environments, primarily located at higher elevations.

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YERSINIA PESTIS TRANSMISSION AND DISEASE

1.1. Historical Background and *Yersinia pestis* Pandemics

Plague has caused three large pandemics which were recorded in 541, 1347, and 1855 (Perry and Fetherston 1997). Plague is caused by the bacterium *Yersinia pestis* and is further subdivided into four biovars, which historically are based on their abilities to reduce nitrate and ferment glycerol: Antiqua (glycerol positive, and nitrate positive), Mediaevalis (glycerol positive, and nitrate negative), Orientalis (glycerol negative, and nitrate positive), and Microtus (glycerol positive, and nitrate negative) (Zhou, Han et al. 2004). Researchers hypothesized that each biovar, excluding microtus, has caused a specific pandemic based on their geographic location, as well as on historical records that indicated the geographic origin of the pandemics (Drancourt, Roux et al. 2004). Each pandemic caused devastating mortality of people and animals across nations and continents, resulting in more than 200,000 million deaths throughout plague history (Frith 2012). In the human plague pandemics, the primary form of disease was the bubonic form. After the bacteria migrates to the bloodstream, the pathogen quickly disseminates throughout the body leading to hemorrhaging and necrosis of the skin followed by septicemic plague infection that resulted in shock and death. If the bacterium spread from the blood to the lungs, pneumonic plague ensued, resulting in a highly fatal form of the disease and potential of direct transmission from human to human (Frith, 2012). The primary flea vectors for transmission from the rats to humans during the pandemics were primarily the Oriental rat flea, *Xenopsylla cheopis*, and the Northern or Eastern rat flea, *Nosopsyllus fasciatus*; in addition, the

human flea, *Pulex irritans*, and the dog and cat fleas, *Ctenocephalides canis* and *felis* played a role as secondary vectors. The plague pandemics successfully spread over long distances because infected fleas were carried on rats as well as humans that traveled along the trade routes at sea and overland. Also, the rats carrying *Y. pestis*-infected fleas infested grains and rice, trade merchandise and clothing, which further contributed to the successful spread of plague (Frith, 2012).

The first pandemic, known as the Justinian plague, which was named after Justinian I, the Roman emperor of the Byzantine Empire at that time, dates to A.D. 541-544, and began in Pelusium, Eqypt, and was caused by the Antiqua biovar of *Y. pestis*. The first great pandemic recorded people suffering from the characteristic bubonic and septicemic plague infections (Frith, 2012). The epidemic began in Ethiopia and quickly spread to Pelusium, Eqypt in 540, and moved west to Alexandria and east to Gaza, Jerusalem, and Antioch. In 541, ships carrying rats with *Y. pestis*-infected fleas resulted in the spread of plague along sea trading routes to both sides of the Mediterranean and arrived in Constantinople (current day Istanbul) (Frith, 2012). Once plague reached Constantinople, the pandemic reached its peak in the spring of 542 with 5,000-10,000 deaths per day occurring in the city and eventually killing over a third of the city's population (Frith, 2012). The vast quantities of bodies falling victim to plague, were too numerous to bury and were stacked high in the city's churches and city wall towers. From 542-546, plague rapidly spread through Asia, Africa, and Europe, killing nearly 100 million people. This pandemic led to a permanent change to the social makeup of the Western world, resulting in the demise of Justinian's reign and disruption of food production leading to an eight year famine (Frith, 2012). This social and economic disruption led to the end of the Roman rule and the formation of medieval Europe. Over the next 200 years, plague outbreaks occurred throughout

Europe and the Middle East and intermittent cycles in Europe occurred into the middle of the 8th century and plague did not re-emerge as a major epidemic until the 14th century. During the Justinian pandemic, death rates were as high as 15-40%, resulting in population losses reaching up to 50-60% between 541 to 700 A.D (Perry and Fetherston 1997).

During 1330 to 1346, plague spread from the steppes of Central Asia westward along the trade routes before successfully making its way to Sicily; therefore, starting what was known as the second pandemic covering much of the "known world" (Wagner, Keim et al. 2014). In 1347, plague was brought to the Crimea from Asia by the Tartar armies of Khan Janibeg, who was responsible for the siege of Kaffa, which was a Genoese trading town on the shores of the Black Sea. However, the siege was unsuccessful, and in an attempt of revenge, the bodies of plague victims were catapulted over the walls of Kaffa. Panic stricken Genoese traders fled Constantinople and crossed the Mediterranean to Messina, Sicily where the second great pandemic of Europe began (Frith, 2012). The Black Death of 1347 was the first major European outbreak of plague and marked the second great plague pandemic which occurred between the 14th to 18th centuries (Frith, 2012). The first major epidemic of the Black Death pandemic resulted in devastating effects killing 17-28 million European deaths and a 30 to 40% depopulation. People died so rapidly that proper burials and cremations did not occur, and corpses were thrown into large pits and decaying bodies lay in homes and in the streets (Perry and Fetherston 1997). The epidemic cycles of *Y. pestis* biovar Medievalis, were unruly and persistently occurred in 2-5 year cycles from 1361-1480. During the Black Death and subsequent epidemics, the importance of medical education and practices were impending. The only remedies were inhalation of aromatic vapors from herbs and flowers such as rose, theriaca,

aloe, thyme and camphor; however, a shortage of doctors occurred leading to fake doctors selling useless adornments that claimed a magical cure (Frith, 2012).

The pandemic led to great social and economic devastation and many whole families and villages would be completely obliterated by the disease. Crops were not being harvested and traveling and trade came to a halt. The normal upper and lower class divisions of people blurred leading to the emergence of a new middle class. Many new policies were beginning to be developed and introduction of clinical research, public health regulations, enforcement of these regulations, and development of hospitals to treat and isolate patients were on the rise (Wagner, Keim et al. 2014). These new measures are thought to play a role in the decline of the first and second pandemics, as well as potential theories of climate change, rodent populations, public health awareness and the life cycle of *Y. pestis* may have played a role in the decrease of disease incidence. Between 1347-1350, the Black Death killed 25 million people, which was one quarter of the population in Europe, and 25 million people in Asia and Africa (Perry and Fetherston 1997). Paris, Venice and Florence had higher mortality rates in which more than half of the population succumbed to plague infection. In 1361, a second plague epidemic spread through Europe killing another 10-20% of the population, and the Europe population did not recover to the pre-pandemic levels until the 16th century (Perry and Fetherston 1997). In 1374, another Black Death epidemic re-emerged in Europe and new public health strategies were employed. Public health procedures were initiated including quarantining infected individuals from healthy individuals, as well as preventing ships with known disease from landing at port. Doctors, who cared for sick individuals wore peculiar outfits to protect themselves from acquiring plague infection, which consisted of leather or oil cloth robes protecting their bodies from head to toe, leggings, gloves, hood, a wide brimmed hat which denoted their medical profession, and a beak-

like mask that had glass eyes and two breathing holes filled with aromatic herbs and flowers to ward off the evil. Doctors treated plague patients by trying to avoid contact and taking their pulse using a stick as well as issuing prescriptions for aromatic herbs inhalations by passing them through the door, and lancing buboes using knives which were several feet in length (Perry and Fetherston 1997, Drancourt, Roux et al. 2004, Gage and Kosoy 2005, Frith 2012). Plague continued to re-emerge causing small epidemics throughout the world. In 1665-1666, a large pneumonic plague outbreak occurred in Europe and England and reached a peak in 1665 when 7,000 victims were dying of plague infections each week in London. The epidemic resulted in the loss of 100,000 people in London's population and the subsequent Great Fire of London in 1666 likely led to the end of the epidemic which resulted in the rebuilding of timber and homes with brick and tile which disturbed the normal habitat for the rats and led to an overall reduction in infected fleas which, were in close vicinity of humans (Frith, 2012).

Lastly, the Chinese province of Yunnan in 1855, began what is known as the third plague pandemic. Once in Yunnan, plague quickly spread along the tin and opium routes before reaching the capital of K'umming in 1866, the Gulf of the Tonkin in 1867, and the Kwangtung province port of Pakhoi in 1882 (Frith, 2012). *Y. pestis* biovar Orientalis is thought to be responsible for the rapid spread of the disease throughout the southern coast of China. Plague reached ports on every continent from the successful transportation of infected rats which traveled the international trade routes on steamships (Frith, 2012). Hong Kong and Canton were soon affected by 1894 and advancing further to Bombay by 1898. During the Hong Kong epidemic which began in June 1894, two scientists, Alexandre Yersin and Shibasaburo Kitasato each declared the isolation of the plague organism. Kitasato was initially credited with the identification and isolation of the plague organism; however, the bacillus Yersin isolated,

correctly fit the description of *Y. pestis*, and Yersin further cured a plague patient in 1896 using antiserum he developed against the organism. Kitasato's description was different than Yersin's, and later speculated that his organism was potentially contaminated with pneumococcus which led to the difference in description. Yersin further discovered the link between plague and rats. Scientists Masanori Ogata, and Paul Louis Simond were each credited with identifying a correlation with fleas in plague transmission, which, they each discovered in 1897 during the Indian epidemic (Simond 1898, Perry and Fetherston 1997, Frith 2012).

With the discovery of the plague organism and better understanding of the important correlations with rats and fleas, many scientists began advancing studies trying to find a vaccine as well as determining efficacies of antibiotics. Animal models were used and the epidemiology and pathology of the disease were being investigated. The nomenclature for *Y. pestis* has changed throughout the years and was previously named *Bacterium pestis* up until 1900, when it was then named *Bacillus pestis* until 1923, followed by *Pasteurella pestis* and finally *Yersinia pestis* in 1970.

1.2. *Y. pestis* General Characteristics and Etiology

The genus *Yersinia* consists of 19 nomen species and is a member of the family *Enterobacteriaceae* (Duan, Liang et al. 2014). There are three species pathogenic to humans: *Y. enterocolitica*, *Y. pseudotuberculosis*, and *Y. pestis*, and one species pathogenic to fish: *Y. ruckeri*. *Y. enterocolitica*, and *Y. pseudotuberculosis* typically cause self-limiting food-borne infections, but not all strains are pathogenic to humans (Jaafar, Chettri et al. 2015). *Y. ruckeri* is responsible for causing enteric redmouth disease (ERM) in salmonid and trout species of fish

(Furones, Gilpin et al. 1993). The species causng the most acute disease in humans is *Y. pestis*, which is the etiologic agent of plague.

Plague is primarily a flea-borne zoonotic disease of rodents and their associated fleas. *Y. pestis* is a Gram-negative, non-motile, non-spore forming coccobacillus, measuring 0.5 to 0.8 µm in diameter and 1 to 3 µm long and exhibits bipolar staining with Giemsa, Wright's, or Wayson staining. Optimal growth conditions for *Y. pestis* are at 28-30°C on blood agar or MacConkey agar, but is capable of growth ranging from 4-40°C. Bacterial colonies will appear as gray-white to slightly yellow opaque raised, irregular "fried egg" morphology, as well as, as having the appearance of a "hammered copper" shiny surface. *Y. pestis*' optimal pH range for growth is between 7.2-7.6, but the organism is capable of surviving and tolerating pH extremes as low as 5 and as high as 9.6 (Brubaker 1969, Poland and Barnes 1979, Holt, Krieg et al. 1994, Poland, Quan et al. 1994, Perry and Fetherston 1997). The cell wall of *Yersinia* spp., exhibits a typical Gram-negative lipopolysaccharide (LPS) and lipid bilayer composition similar to that found in members of the family *Enterobacteriaceae.* The LPS structures of *Y. pseudotuberculosis* and *Y. entercolitica* are smooth forms, confer a smooth phenotype and are comprised of a complete lipid A-oligosaccharide core-O antigen polysaccharide, whereas as *Y. pestis* LPS displays Lipid A and core components but lacks extended O-group side chains and is characterized as having a rough phenotype. At temperatures above 33°C, *Y. pestis* synthesizes a carbohydrate-protein envelope known as the fraction 1 (F1) or capsular antigen, but does not retain a true capsule. *Y. pestis* is a lactose nonfermenter, and is urease and indole negative (Riedel 2005). The nutritional requirements for this organism vary contingent on the environment and temperature in which this bacterium is residing; however, *Y. pestis* must be grown in the presence of L-isoleucine, L-valine, L-methionine, L-phenylalanine, and glycine. Additionally, at 37°C, this pathogen has

nutritional requirements for biotin, thiamine, pantothenate, and glutamic acid. *Y. pestis* is a facultative anaerobe which lacks detectable adenine deaminase, aspartase, glucose 6-phosphate dehydrogenase, ornithine decarboxylase, and urease activities, but successfully demonstrates utilization of a constitutive glyoxylate bypass and unregulated L-serine deaminase expression to permit salvage of tetrahydrofolic acid (Brubaker 1969, Holt, Krieg et al. 1994, Perry and Fetherston 1997). Growth of *Y. pestis* is slow at 28°C, with a 1.25 hour generation time, and 24-48 hours is needed for colony formation on enriched media (Poland, Quan et al. 1994, Perry and Fetherston 1997).

Y. pestis is a genetically homogeneous organism considering its wide range of hosts and vectors. *Y. pestis* has only one serotype, one phage type, and four biovars. The four *Y. pestis* biovars are Antiqua, Orientalis, Mediavalis, and Microtus. Each biovar is based on restriction fragment length polymorphisms of the locations of the IS*100* insertion element, and each biotype exhibits different physiological or biochemical requirements based on the conversion of nitrate to nitrite as well as the fermentation of glycerol. The biotype Antiqua, which is believed to be responsible for the first pandemic (Wagner, Keim et al. 2014), is positive for the conversion of nitrate to nitrite and glycerol fermentation. Orientalis strains, which are responsible for the third pandemic, can convert nitrate to nitrite, but unsuccessfully ferment glycerol. Biovar mediaevalis, implicated in the second pandemic, can successfully ferment glycerol, but is incapable of converting nitrate to nitrite. Lastly, the Microtus biovar, which has not caused any pandemics and further was found to be avirulent in humans and guinea pigs but virulent in mice, can successfully ferment glycerol, but is unsuccessful in converting nitrate to nitrite (Zhou, Han et al. 2004). Each *Y. pestis* biovar displays equal virulence (excluding Microtus), and all cause the same disease manifestations in humans and animals (Brubaker 1969, Poland and Barnes

1979, Perry and Fetherston 1997). More recently, studies have discovered new ribotypes, which is a molecular technique used to differentiate bacteria based on unique DNA sequences, of the biovar Orientalis, which have undergone chromosomal rearrangements. These new ribotypes of biovar Orientalis have been isolated in Madagascar, Vietnam and India. It is still unclear whether these new ribotypes have acquired any selective advantages in a new environment (Ramalingaswami and Colleagues 1995, Riedel 2005).

In the laboratory, fully virulent strains of *Y. pestis* are processed and contained in biosafety level-3 (BSL-3) laboratories and can be manipulated safely using standard microbiological methods. When working with fully virulent cultures and specimens, researchers should process samples in class II space A2 or higher biosafety cabinets (BSC), as well as wear appropriate personal protective equipment (PPE), because of the risk of generating aerosols when working with high concentrations of this pathogen or when working with specific antibiotic resistant strains (Perry and Fetherston 1997).

1.3. Plague as a Bioterrorism Agent

Y. pestis is considered a Tier 1 select agent, and is ranked as a high priority agent that poses a potential risk to our national security for reasons such as the pathogen's capability to be easily disseminated and transmitted person-to-person, a high mortality rate, and if used in a case of intentional bioterrorism, it would cause public hysteria and possible collapse of the public health infrastructure (DHHS 2012, Service 2012). The use of *Y. pestis* as a bioweapon would result in significant devastation, aside from the severe threat to public health and safety, leading to mass causalities and greatly disrupting our economy and critical infrastructure (DHHS 2012).

Plague was first documented as being used as a biological warfare (BW) agent in 1346 during the siege of Kaffa, which is now known as Feodosia in Crimea, when the Tartars threw plague-infected corpses into the city causing an epidemic which contributed to the surrender of the city (Horn 2003). In 1940, *Y. pestis* was used as a biological weapon during World War II when Japanese airplanes flew over Chushien, Chekiang Province, China, as well as another identified chinesse city and released rice and wheat contaminated with *Y. pestis*-infected fleas. These two attacks resulted in plague successfully infecting and killing 121 people (Barras and Greub 2014).

Since *Y. pestis* is considered a Tier 1 select agent, treatment for plague is required to be stockpiled in case of bioterrorism or biowarfare attacks. *Y. pestis* successfully fits the specific criteria must be met in order for an organism to be considered an important select agent. For an organism to be considered a Tier 1 select agent, it must meet the following criteria: (1) the organism must be capable of dissemination by aerosol to produce an inhalational form of the disease (2) the organisms can be rendered stable in both liquid and dry forms within the aerosols and can successfully remain viable for long enough periods to cause disease (3) the majority of the population has never been exposed; therefore no natural immunity to infection would exist (4) high morbidity and mortality (5) person-to-person transmission can occur, and lastly, (6) the diseases produced are difficult to diagnose and treat, because signs and symptoms mimic many other common diseases (Schatzmayr and Barth 2013). Plague has been one of the most devastating epidemic diseases known to mankind, and with the presence and availability of plague around the world, the capacity for mass production, aerosol dissemination, high fatality rate of pneumonic plague, and the potential for rapid secondary spread, thus establishes plague as an ideal organism for the potential use as a biological weapon (Riedel 2005).

1.4. *Y. pestis* Evolution from *Y. pseudotuberculosis*

Y. pestis recently evolved from *Y. pseudotuberculosis*, a food and water-borne enteric bacterium, within the last 1500-20,000 years. Chromosomal DNA from the two species exhibit a very high degree of relatedness as established by DNA-DNA hybridization and identical 16S rRNA genes (Achtman, Zurth et al. 1999, Chain, Carniel et al. 2004, Hinnebusch 2005). These two *Yersinia* species share a conserved chromosome with ~97% nucleotide identity and ~75% shared protein coding genes (Chain, Carniel et al. 2004). Since these two species cause vastly different diseases, have different host preferences, and transmission routes, but have such similar genomes, this evolutionary relationship presents an intriguing area of study to better understand the molecular determinants responsible for the difference in disease severity, signs and symptoms. Reservoirs of *Y. pseudotuberculosis* include dogs, cats, cattle, horses, rabbits, deer turkey, and many more animals) soil, plants, insects and amoeba. *Y. pseudotuberculosis* infection typically occurs after ingesting contaminated food or water, in which the pathogen then colonizes the gastrointestinal tract. First, *Y. pseudotuberculosis* colonizes the Peyer's patches of the small intestine before disseminating to the liver and spleen. Human infection is characterized by gastroenteritis and self-limiting mesenteric lymphadenitis and diarrhea. The disease is usually self-limiting, but in immunocompromised individuals, this pathogen can be severe and sometimes fatal. *Y. pseudotuberculosis* is capable of surviving long periods outside of a mammalian host in soil, water or within amoeba. *Y. pseudotuberculosis* can also survive on varied nutrients and can retain metabolic capabilities, which have been lost in *Y. pestis* (Lambrecht, Bare et al. 2013, Martinez-Chavarria and Vadyvaloo 2015, Santos-Montanez, Benavides-Montano et al. 2015). In *Y. pestis*, most infections are acquired from either the bite of an infected flea, contact with infected tissues, or inhalation of respiratory droplets or aerosols.

After fleas feed on a highly bacteremic host, they obtain *Y. pestis* from the blood where the pathogen multiplies in the flea proventriculus and midgut eventually causing an occlusion in some flea species. This blockage will impede ingestion of a fresh bloodmeal and the bacteria will get regurgitated into the bite site transmitting the pathogen to the host. After the flea bites a mammalian host, *Y. pestis* colonizes the dermis of the host and will then come into contact with macrophages. The macrophages are permissive to the survival and replication of the pathogen, and *Y. pestis* rapidly migrates to the regional lymph node inflammation and formation of a bubo (Sebbane, Gardner et al. 2005, Gonzalez, Lane et al. 2015). Next, hematogenous spread to the spleen and liver occurs, leading to a septicemic infection and possible spread to the lungs leading to secondary pneumonic plague.

The evolution of *Y. pestis* consisted of horizontal gene transfer and acquisition of two specific plasmids, pPCP and pMT1, and the new function of the *hms* chromosomal genes (Chain, Carniel et al. 2004, Hinnebusch 2005). For *Y. pestis* to evolve and be capable of surviving within the unique lifestyle inhabiting the flea gut as well as mammalian hosts, gene acquisition was essential for adaptation and survival. In addition to the 70 kb virulence plasmid, pCD1, which is found in all pathogenic *Yersinia* spp., *Y. pestis* acquired two unique plasmids that encode a variety of virulence factors. First, the 9.5 kb virulence plasmid, pPCP1, encodes the plasminogen activator pla, and is a putative invasin that is essential for virulence subcutaneously. Lastly, the 100-110 kb plasmid, pMT1, encoding murine toxin Ymt, is essential for bacterial colonization in the flea gut, and the F1 capsular antigen which is necessary for transmission and survival of plague (Parkhill, Wren et al. 2001). The F1 capsular protein is expressed at mammalian temperatures (37°C) and not at flea temperatures (>26°C), and is thought to play a role in evasion of phagocytosis by the host immune system (Du, Rosqvist et al. 2002). The

acquisition of the chromosomal *hms* genes are important in synthesis of extracellular matrix, which is required for biofilm formation within the flea (Jackson and Burrows 1956, Moore and Brubaker 1975, Lucier and Brubaker 1992, Perry and Fetherston 1997, Achtman, Zurth et al. 1999, Hinnebusch 2005). The high relatedness among *Y. pestis* and *Y. psuedotuberculosis* led to the proposal of each being subspecies, but has since been rejected by medical microbiologists because *Y. pestis* causes a fatal bubonic flea-borne infection, and *Y. psuedotuberculosis* is caused by a fecal-oral route of transmission, and rarely leading to death. Virulence differences between the two species is still unclear, but the acquisition of these two plasmids by *Y. pestis* is thought to play a role in the increased virulence capabilities (Brubaker 1991, Achtman, Zurth et al. 1999). More recently, one study proposed that regulatory RNA's, which are produced in coordination to produce distinct factors, are necessary for host adaptation and virulence in response to different environments; therefore, leading to distinct pathogenicity between *Y. pestis* and *Y. pseudotuberculosis* (Martinez-Chavarria and Vadyvaloo 2015).

Atypical *Y. pestis* Pestoides strains have been isolated in Central Asia which differ from wild-type *Y. pestis* by their ability to ferment rhamnose and melibiose, as well as lacking the small plasmid encoding the plasminogen activator (pla) and pesticin, and variants of the virulence plasmid pMT1. These atypical strains are thought to be a derived strain from the most ancient lineage of *Y. pestis*, and differences in the strains are a result of strain specific rearrangements, insertions, deletions, single nucleotide polymorphisms, and a unique distribution of insertion sequences (Garcia, Chain et al. 2007).

The dramatic pathogenic difference between *Y. pestis* and *Y. pseudotuberculosis* has increased interest in elucidating the exact factors responsible for the differences in virulence and pathogenesis (Martinez-Chavarria and Vadyvaloo 2015). Cornelis et al, discovered a shared

virulence factor, the Yop-Ysc Type three secretion system (T3SS), which is a plasmid-encoded virulence factor and shared among the two species encoding proteins necessary for the injectosome structure required to deliver Yop effector proteins into the cytosol of the host cells. The T3SS, is required for infection to enable the bacteria to subvert host immune function (Cornelis 2002, Martinez-Chavarria and Vadyvaloo 2015). Whole genome studies have been performed to examine differences between the two species at the DNA level based on phylogenetics and determined that genomic rearrangements occur, including gene loss and acquisition occurs in *Y. pestis* isolates more frequently than its predecessor, *Y. pseudotuberculosis* (Achtman, Zurth et al. 1999, Morelli, Song et al. 2010, Martinez-Chavarria and Vadyvaloo 2015). To better understand differences in gene expression between the two species, transcriptional studies must be performed. To date, very few comparative transcriptional studies have been performed in order to determine the transcriptional regulatory differences between *Y. pestis* and *Y. pseudotuberculosis* which would increase insight on the regulatory networks and virulence evolution of each pathogen (Heroven and Dersch 2006, Zhan, Han et al. 2008, Martinez-Chavarria and Vadyvaloo 2015).

1.5. *Y. pestis* Life Cycle

Plague exists in nature in enzootic or maintenance cycles between wild rodents and their associated fleas. Over 90% of human plague infections are caused by the bite of an infected flea. *Yersinia pestis* has the ability to cause disease in fleas, rodents and humans. The primary carriers of the pathogen are the Oriental rat flea, *Xenopsylla cheopis*, and infected rodents. *X. cheopis* was first reported responsible for parasitizing rats during the 19th century and in Egypt which caused the spread of plague because of the fleas on the rats carried on cargo ships (Gratz 1999).

In the United States, the primary carriers of the pathogen are the California ground squirrel/rock squirrel flea, *Oropsylla montana*. Other fleas and rodents, as well as lagomorphs have also been shown to play a role in plague outbreaks. Evidence of *Y. pestis* in the Artiodactyla, Carnivora, Hyracoidea, Insectivora, Marsupialia, and Primate families have been identified, indicating almost all mammals are capable of acquiring *Y. pestis* infection; however, birds, reptiles and amphibians are thought to be resistant to *Y. pestis* infection. Indirect roles in the spread of plague could be the result of mammals and birds which prey on plague hosts; therefore enabling movement of *Y. pestis*-infected fleas' between areas (Gage and Kosoy 2005). Male and female fleas blood feed on rodents and can successfully ingest the pathogen and later can transmit disease to naïve hosts. Plague has two cycles for transmission, the sylvatic cycle, which is also known as the pre-human cycle that occurs in wild rodents and fleas. This cycle continues in the wild rodents until a rodent die-off occurs or the fleas find a new food source, such as domestic rats or rodents. Once a domestic rodent or animal is bitten by a flea and becomes infected, the urban or demic transmission cycle begins. This is when humans are most at risk, and infected fleas can then bite humans and successfully transmit disease (Gage, Lance et al. 1992). Plague, is thought to be constitutively transmitted between partially resistant rodent hosts and their fleas in enzootic or maintenance cycles. Occasionally, changes in the environments such as those related to climate, an increase in the abundance of fleas, and/or increase in susceptible rodent hosts can lead to a plague epizootic. Epizootics are characterized by large rodent die-offs and the infected fleas begin seeking alternative hosts, and this is when humans have the highest risk for acquiring plague infection. Understanding the factors that results in plague epizootics is important in order to decrease the chance of human infections. More recently, the trophic cascade hypothesis has been suggested, indicating a correlation between increased precipitation,

that allows for greater plant growth leading to more rodent food which increases the host populations, resulting in a greater risk of an epizootic occurring (Gage and Kosoy 2005). Other models looking at precipitation and temperature correlations have been developed and found that increased precipitation leads to increased host and flea populations; therefore increasing the probability of acquiring plague infection. However, temperatures above >32.5°C, decrease the risk of acquiring plague infections and flea survival decreases. Studies have suggested that higher temperatures unfavorably affects bacterial blockage in the fleas, and leads to decreased risks of an epizootic occurring (Cavanaugh 1971, Enscore, Biggerstaff et al. 2002, Gage and Kosoy 2005). Many other factors play a role in the likelihood of a plague epizootic occurring, such as heterogeneity in *Y. pestis* strains, diversity of rodent populations, host immune status, genetic makeup of hosts, species of the flea vector, mechanism of transmission, mutations in bacterium or host immune cells, and interactions of *Y. pestis* with other pathogens (Pollitzer and Meyer 1961, Gage and Kosoy 2005, Gage, Burkot et al. 2008). Currently, it is still unclear where *Y. pestis* is maintained in between epizootics or during the inter-epizootic periods.

In rare instances, humans have become infected through contact of infected rodents or animals. Human to human transmission can also occur if the bacterium is aerosolized by coughing or sneezing of a pneumonically infected individual or by direct physical contact of an infected person or animal. Indirect contact, such as by touching contaminated soil; although highly unlikely, can be a source of infection. It has also been documented that consuming raw or undercooked meat (camel or goats) can be a source of plague transmission to humans (Leslie, Whitehouse et al. 2011). The human body louse, *Pediculous humanus corporis,* has also been shown to transmit *Y. pestis*, but clearly is not a significant vector for plague transmission (Ayyadurai, Flaudrops et al. 2010).

1.6. *Y. pestis* Reservoirs

1.6.1 Mammalian Hosts:

The natural vertebrate reservoir of *Y. pestis* is thought to be the rodent host. Over 203 different rodent species or subspecies are reported as being naturally infected with plague. Also playing a role as a reservoir, are the lagomorphs, in which 14 different species have been shown to be naturally infected with *Y. pestis* (Gage and Kosoy 2005). Many variables factor into the ability of a small mammal serving as a plague reservoir. For instance, the mammals must be capable of being infected with *Y. pestis* and circulating the pathogen at high enough bacterial levels in their blood to successfully infect feeding fleas to successfully continue the plague transmission cycle. In rare cases, some rodent hosts will become highly bacteremic and succumb to infection; but in most cases, the rodents remain somewhat resistant to the pathogen and never develop severe plague illness (Gage and Kosoy 2005). Important rodent hosts are typically densely parasitized by one or more important flea vectors; increasing the potential of disease spread. Rodents living in burrows containing large numbers of fleas, results in intimate interactions allowing plague pathogen to successfully cycle among the rodent and flea populations (Poland and Barnes 1979, Nelson, Madon et al. 1986, Pavlov, Mokrievich et al. 1996, Gage and Kosoy 2005). Many factors will determine rodent susceptiblility to plague infection. Differences among rodents species, such as genetic differences within individuals or populations, age, breeding status, immune and physiological status, and the season of the year all play a role in host susceptibility to plague infection (Gage and Kosoy 2005, Krasnov, Shenbrot et al. 2006, Hubbart, Jachowski et al. 2011). It is thought that rodent populations contain a mixture of hosts which are resistant to infection and a subset that are more susceptible to infection. Other

studies have speculated that some rodents may act as chronic carriers of *Y. pestis*, and successfully maintain infection between transmission seasons or epizootics (Williams, Harrison et al. 1975, Williams and Cavanaugh 1983, Gage and Kosoy 2005). Other studies have speculated that susceptible rodents become infected before they hibernate in the winter months, and successfully maintain infection until they reawake in the spring months (Pollitzer and Meyer 1961, Gage and Kosoy 2005). Plague infection and differences in host responses will lead to different outcomes depending on the rodent species infected, for example, plague infection in gerbils, marmots, deer mice, and California voles, are highly variable and infection may go unnoticed, whereas plague infection in highly susceptible hosts such as the black-tailed prairie dogs, will result in devastatation of the entire colony during an epizootic (Kartman 1963, Poland and Barnes 1979, Poland, Quan et al. 1994, Gage, Ostfeld et al. 1995, Gage and Kosoy 2005). The true number of rodent species that may be important accidental reservoirs of plague is unknown. Plague animal hosts are typically classified as either enzootic or maintenance hosts or as epizootic or amplification hosts. In the maintenance hosts these rodents are considered to be somewhat resistant to plague infection and epizootics are not commonly observed. In contrast to the effects when *Y. pestis* is introduced into a more susceptible rodent colony, or when rodent colonies or individuals overlap from two different populations or species, it results in an epizootic in the more susceptible colony. Classification of plague reservoirs is difficult with the large number of rodents, lagomorphs and other small mammals that are considered as occasional or common hosts of plague. Reservoir hosts are primarily characterized based on their susceptibility of plague infection, and this is difficult because susceptibility among the same species or within a single geographic location can be highly variable in susceptibility to plague infection. In addition, the virulence of the *Y. pestis* strain and the possibility of that strain

changing over time, could be factors affecting the appearance of epizootics. Most natural plague foci have been successful for years, therefore indicating that the natural reservoir populations must be somewhat resistant to plague infection or be able to survive infection, which has been seen in some species where the pathogen is capable of circulating with little observable host mortality (Baltazard 1960, Gage, Ostfeld et al. 1995, Gage and Kosoy 2005).

1.6.2 Other Potential Reservoirs:

Speculation about potential *Y. pestis* reservoirs has been deliberated for almost a century. One major unknown in the plague cycle is how it persists during inter-epizootic periods or the time between major epizootics or die-offs. Many studies have looked into other potential plague reservoirs such as, soil, long-term persistence in fleas, hibernating hosts, or survival within amoeba, in soil nematodes, or flea feces (Hirst 1953, Pollitzer 1954, Baltazard, Karimi et al. 1963, Mollaret 1963, Darby, Hsu et al. 2002, Gage and Kosoy 2005, Drancourt, Houhamdi et al. 2006, Bazanova, Nikitin et al. 2007, Bizanov and Dobrokhotova 2007, Eisen, Petersen et al. 2008, Boegler, Graham et al. 2012, Jones, Vetter et al. 2013, Williams, Schotthoefer et al. 2013). After many years of investigating the epidemiology and ecology of *Y. pestis*, much is still unknown about the strategies the pathogen uses to survive in its natural cycle.

1.7. *Y. pestis* and Fleas

In the insect order, *Siphonaptera*, ~2,500 species and subspecies of fleas exist, comprising 220 genera and 15 families. Around 80 flea species, which are associated with ~200 species of wild rodents have been found to be infected with *Y. pestis* (Pollitzer 1954, Lewin, Hertwig et al. 1998). Fleas are small wingless bloodsucking insects that feed on warm-blooded

animals. Flea bodies are small (1/16"), hard, polished, and covered with many hairs and short spines directed backward. The compressed bodies of the fleas, which are flattened side to side effectively allows them to easily maneuver through the hair on their hosts' bodies. An adult flea contains mouthparts consisting of stylets, which are used to pierce the skin of the host animal for sucking blood from the host (Gage, Maupin et al. 1997). Flea legs are long and adapted for jumping to aid in facilitation of host acquisition. The fleas jumping aptitude along with their laterally compressed bodies, well-developed sense organs, and haematophagous routine all contribute to the ability to successfully transmit *Y. pestis* (Gage, Maupin et al. 1997). Flea transmission efficiencies greatly varies among the different flea species, and some fleas which are capable of consuming an infectious bloodmeal are unsuccessful in being able to transmit the pathogen (Wheeler and Douglas 1945, Burroughs 1947). Approximately, 250 flea species have been reported as possessing naturally occurring *Y. pestis* infections; however, only a small number of these fleas are considered to be efficient vectors for transmitting *Y. pestis*. For a flea to be an efficient vector, they must consume a blood meal from a bacteremic host, live long enough for the bacteria to multiply to sufficient numbers to guarantee transmission, and successfully transmit *Y. pestis* to a susceptible host at concentrations adequate to cause infection (Gratz 1999, Eisen, Bearden et al. 2006, Eisen, Borchert et al. 2008, Eisen, Eisen et al. 2009). Adult fleas are obligate blood-feeding ectoparasites and most live in close association with their hosts. Blood meal storage, digestion, and adsorption all occur in a simple, non-compartmentalized midgut, which is composed of a single layer of columnar epithelial cells and basement membrane. A valve, known as the proventriculus, is found at the base of the esophagus, and guards the entrance to the midgut and is important in the transmission mechanism. The proventriculus of the flea is composed of densely packed rows of inward-

directing spines that are coated with a layer of cuticle. When a flea feeds, the proventricular valve opens and closes by means of muscles contracting in unison with three sets of pump muscles which are located in the flea's head to force the blood into the midgut and to prevent backflow (Hinnebusch 2005). Digestion of the blood meal begins a few hours after ingested and occurs by hemolysis and complete liquefaction of the blood components, and digestion is fully completed within a few days (Hinnebusch 2005). When a flea feeds on a bacteremic host, *Y. pestis* enters the flea midgut as individual planktonic cells and resides in the digestive tract; and once *Y. pestis* reaches the midgut, the bacteria do not adhere or invade the midgut epithelium (Engelthaler and Gage 2000, Lorange, Race et al. 2005). Persistence or maintenance of infection within the flea midgut depends on the ability of *Y. pestis* to form aggregates or clumps of bacteria. The bacterial aggregates will form within the first few days after infection and continue multiplying during the first week. The harsh biochemical and physiological environment of the flea digestive tract is still uncertain; however, the digestive milieu of the flea midgut is thought to be a hostile environment and few pathogens are found to persist in fleas. The only known flea-borne pathogens include *Bartonella henselae*, which causes cat-scratch fever and bacillary angiomatosis, *Rickettsia typhi* and *Rickettsia felis*. *Francisella tularensis* is also thought to be transmitted occasionally by fleas through mechanical means (contaminated mouthparts); but the importance of fleas as vectors for transmission of this pathogen is still unclear. Lastly, myxomatosis, a pox virus in rabbits has been attributed to transmission by fleas; although this is thought to be more of a mechanical transmission mechanism (Bahmanyar and Cavanaugh 1976, Beard 1988, Beard, Butler et al. 1990, Chomel, Jay et al. 1994).

The flea gut physiology and associated environmental conditions in the digestive tract have been reported as having a pH of 6 to 7, but other parameters such as osmotic pressure and

redox potential are still unknown (Wigglesworth 1984). Insect gut epithelium secretes a variety of digestive enzymes such as trypsin, chymotrypsin, amino- and carboxypeptidases, cathepsins, lysozymes, glycosidases, and lipases (Fehlbaum, Bulet et al. 1994, Meister, Braun et al. 1994). The flea innate immune response to *Y. pestis* infection has not been characterized, but may resemble the immune responses of other insects such as ticks or mosquitoes. Once *Y. pestis* is in the flea midgut, it has been shown to be resistant to flea midgut cationic antimicrobial peptides, specifically at lower growth temperatures (Bengoechea, Brandenburg et al. 1998, Reddin, Easterbrook et al. 1998, Anisimov, Dentovskaya et al. 2005). When fleas feed on mammalian hosts; the blood is primarily comprised of proteins and lipids. The lipids serve as the major energy source for hematophagous arthropods.

In 1914, Bacot and Martin first described mechanism of *Y. pestis* transmission by fleas, after these authors observed masses of *Y. pestis* filling the lumen of the proventriculus and obstructed blood flow; leading to flea starvation and the flea repeatedly attempting to feed, dislodging and flushing the mass of bacteria into the bite site, resulting in the successful transmission of *Y. pestis* to the mammalian host (Bacot and Martin 1914, Bacot and Martin 1914). In 1915, Bacot later amended the transmission mechanism, and proposed that fleas with only partial bacterial obstructions in their proventriculus were more efficient transmitters (Bacot 1915). These partially blocked fleas are still capable of pumping blood into the midgut, but some blood and *Y. pestis* is still able to back flow into the mammalian host. This change in the mechanism was important, because some highly efficient vectors of plague transmission do not readily form a blockage in the proventriculus but can become partially blocked (Burroughs 1947, Pollitzer 1954, Pollitzer 1954). Biological transmission, which requires *Y. pestis* colonization in the flea midgut, was thought initially to be the only reliable mechanism or early-phase

transmission; however, other studies have found evidence of mechanical transmission in which high levels of bacteria are found to survive on the flea mouthparts between feedings allowing for successfully transmission to the mammalian host (Burroughs 1947, Eisen, Bearden et al. 2006).

Not all flea species are efficient in successfully transmitting *Y. pestis*. Flea gut structure and environment is very important in bacterial growth. The flea digestive tract mainly contains the esophagus, proventriculus, midgut and hindgut (Hindson, McBride et al. 2005, Hinnebusch 2005). The flea midgut, which contains a layer of epithelial cells that cover the interior surface, is the main location where the flea digests and absorbs the blood meal. The proventriculus, the pear shaped valve linking the midgut and esophagus, contains packed inward-directing spines lying on the interior, which aid in digestion of the blood meal where the spines coated in cuticle are capable of lysing the blood cells. During blood meal digestion, the proventriculus is tightly closed in order to prevent any blood meal from leaking out of the midgut (Hindson, McBride et al. 2005, Hinnebusch 2005). In order to be an efficient and competent plague vector, the flea must be able to ingest the plague organism with its blood meal, and survive long enough for the pathogen to multiply and be transfered to an animal or human host in concentrations high enough to cause an infection. In the United States, at least 28 flea species have been identified as plague vectors, but when vector competence has been investigated experimentally, the vector competence of the fleas was found to differ immensely (Eisen, Eisen et al. 2009). Flea species found to be inefficient plague vectors were the cat flea (*C. felis*) and the human flea (*Pulex irritans*) (Laudisoit and Beaucournu 2007, Laudisoit, Leirs et al. 2007). One study indicated that fleas in which the bacterium can successfully form a blockage are more efficient plague transmitters (Mury, Paskewitz et al. 2004). Interestingly, *O. montana,* the flea responsible for the majority of plague infections in the United States, rarely becomes blocked by *Y. pestis*;

whereas the most important plague vector worldwide, *X. cheopis*, is a very efficient plague vector capable of forming bacterial blockages in the flea proventriculus, and has been implicated in earlier plague pandemics (Eisen, Eisen et al. 2009, Eisen and Gage 2009).

1.8. *Oropsylla montana*

O. montana, is the primary plague vector in the United States, and is found west of the Rocky Mountains in the United States and Mexico (Hubbard, 1968). More specifically, this vector is located in the Pacific Coastal region, the Rocky Mountain region and the four corners region (Colorado, Utah, New Mexico, and Arizona) of the United States (Traub 1972). This flea most commonly parasitizes ground squirrels in the genus *Spermophilus*, such as the California ground squirrel, *Spermophilus beecheui* and the rock squirrel, *S. variegatus* (Traub 1972). *O. montana* primarily lives in the nests of ground squirrels located in a non-urban setting, and is rarely found feeding on other rodents (Beard, Maupin et al. 1992, Beard, Rose et al. 1992). Rodent nests inhabited by these fleas are composed of twigs and leaves, and occasionally cotton or upholstery stuffing (Beard, Maupin et al. 1992, Beard, Rose et al. 1992). *O. montana* has been found to be most abundant on ground squirrels at temperature which are lower than 18.44°C (Lang, 1996). The hosts of this flea vector may become infected with plague from the flea after hibernation and after returning to burrows (Lang, 1996). *O. montana* fleas are nidicolous parasites, which will get on the host and immediately feed and then quickly get off. The flea's capability to quickly get on and off the host is enhanced by the flea's specialized jumping machinery. (Roberts, Leavitt et al. 1986).

Studies by Eskey and Haas, Wheeler and Douglas, and Burroughs, found *O. montana* to be a poor vector in the transmission of *Y. pestis* (Eskey 1938, Eskey and Haas 1940, Wheeler and

Douglas 1945, Burroughs 1947, Wheeler, Suyemoto et al. 1956). Their studies found only 20% of fleas would become infected with *Y. pestis* when feeding on diseased hosts, and only 10% of the fleas were capable of transmitting the pathogen to naïve guinea pigs. More recently, studies have found *O. montana* to be a highly competent plague vector, which can become infectious almost immediately after consuming an infected bloodmeal. These difference may have been contributed to differences in the flea colonies used in the studies in addition to differences in laboratory methods and procedures which may have resulted in the observed outcomes (Eisen, Bearden et al. 2006). Furthermore, this vector is capable of becoming infected with *Y. pestis*, and remaining infected and transmitting to naïve mice out to day 21 days post infection (Williams, Schotthoefer et al. 2013).

1.9. Flea (Insect) Immunity

Insects protect themselves from invading pathogens by possessing a functional innate immune system. The insect innate immune system is a fast acting and transient system comprised of organs composed of different types of cells, as well as numerous amounts of cells, which circulate freely in the hemolymph (Vilmos and Kurucz 1998, Lemaitre and Hoffmann 2007). Insect innate immunity contains three different mechanisms of defense, which include the columnar epithelial cells and peristalsis of the gut, which acts as a physical barrier against colonization. Next, are the humoral immune barriers, which include osmotic stress, digestive enzymes, lysozymes and two inducible components: the production of antimicrobial peptides (AMP) and the secretion of reactive oxygen species (ROS) and nitric oxide in the gut. Lastly, is the cellular immune system, which consists of phagocytosis, nodule formation, encapsulation, and melanization in the hemocoel (Lemaitre and Hoffmann 2007).

When an insect vector ingests pathogenic bacteria, pattern-recognition molecules will interact with the pathogen-associated molecules, (e.g. lipopolysaccharides in gram-negative bacteria; teichoic acids in gram-positive bacteria) and will result in stimulation and initiation of the insects' immune responses (Mury, Paskewitz et al. 2004). To date, multiple pattern-recognition proteins in insects have been identified: beta-1,3-Glucan-recognition protein/gram-negative-bacteria-binding protein, C-type lectin, hemolin and peptidoglycan-recognition protein, which is primarily synthesized in the fat body and hemocytes. Once the insect pathogen recognition receptors (PRRs) recognizes the pathogen associated molecular pattersn (PAMPs) of the invading pathogen, the humoral and cellular insect responses are triggered (Meister, Braun et al. 1994, Meister, Lemaitre et al. 1997).

Another important insect immune component are antimicrobial peptides (AMPs), which play an important role in protection and are found widespread in plants, insects and vertebrate hosts. An individual insect is capable of producing 10-15 AMPs, each having a different antimicrobial target (Meister, Braun et al. 1994, Meister, Lemaitre et al. 1997). AMPs are typically less than 10kD, hydrophobic, and recognize the acidic phospholipids on the membrane of bacteria or other pathogens. Insects express antimicrobial peptides in the hemolymph as early as 2 hours after septic injury (Meister, Braun et al. 1994, Meister, Lemaitre et al. 1997). Five different families of AMP are found in *Drosophila*: cecropin, defensin, drosocin, diptericin and attacin, plus two additional antifungal peptides, drosomycin and metchnikowin (Butler, Bate et al. 1999). In a study examining the transcriptome of the salivary glands of the flea vector, *X. cheopis*, an AMP family defensin gene was identified (Wang, Bian et al. 2015). AMP expression is regulated by way of two signaling pathways the Toll and immune deficiency (Imd) pathways, which control the transcription factor nuclear factor kappa B (NF-κB) homologs

(Silverman and Paquette 2008). After consuming blood meals, mosquitoes and the blood sucking fly *Stomoxyscalcitrans,* were found to secrete antimicrobial peptides to the gut lumen (Dimopoulos, Richman et al. 1997, Lehane 1997, Lehane, Wu et al. 1997). An immune reaction site for *Drosophila*, as well as the Tsetse flies is the proventriculus, which showed increased levels of antimicrobial peptides, nitric oxide, and reactive oxygen species when compared to that of the midgut; however, it is still unknown if this occurs in fleas (Tzou, Ohresser et al. 2000, Hao, Kasumba et al. 2003, Hao, Liu et al. 2003). *Y. pestis* localizes in the flea digestive gut and does not enter the hemocoel or salivary glands, and unlike other haematophagous insects, fleas do not form a chitinous peritrophic membrane around the bloodmeal (Hinnebusch, Perry et al. 1996). Evidence suggests that most *Y. pestis* flea interactions occur in the flea digestive gut, specifically the midgut, which has a pH around 6-7; however, much is still unknown about the flea gut environment (Wigglesworth 1984). In studies examining yeast and *E. coli* oral infected *Drosophila* identified dual oxidase enzymes, which are capable of producing reactive oxygen species (ROS) and catalases that can remove excess ROS were found to be consecutively induced (Won JL, 2005). In insects, the gut Imd/AMP immune response is required to fight bacteria that are resistant to ROS, but not to combat organisms which are ROS-sensitive (Won, Im et al. 2005). Production of ROS by insects is considered their first-line defense, which protects the epithelial cells from high numbers of bacteria, followed by the secondary Imd response, which becomes activated when the initial response unsuccessfully clears the infection. ROS are natural byproducts of normal oxygen metabolism but also produced by enzymes such as NADPH oxidase in phagocytes, as well as dual oxidases in epithelial cells. Known ROS include superoxide anion, hydroxyl radicals, and hydrogen peroxide, and these also can be converted to compounds with even higher antimicrobial activities, such as hypochlorous acid and

hypothiocyanite by peroxidase enzymes in mammals (Carlsson, Herrmann et al. 1984, Carlsson, Hofling et al. 1984, Weiss, Belisle et al. 1989, Weiss, Post et al. 1989). An important requirement for insect protection against microbes is the production of ROS, as well as the ability of the host cells to produce enzymes necessary for removing any excess ROS, such as superoxide dismutase (SOD) and catalase, in order to protect themselves from damaging host tissues (Carlsson, Herrmann et al. 1984, Carlsson, Höfling et al. 1984). It has been found that antioxidants, such as N-acetyl-cysteine, ascorbic acid and uric acid can be used to successfully reduce the oxidative stress in the insect midgut; but results in enhanced pathogen growth (Macleod, Darby et al. 2007, MacLeod, Maudlin et al. 2007). Much is still unknown about the production of ROS in the flea vector, but many studies have highlighted the importance of ROS in other insects and their role on pathogen survival within insect vectors.

1.10. *Y. pestis* Transmission Mechanisms

Studies have been performed for years trying to establish the role of fleas in plague transmission. If was first suggested in 1897 that fleas could potentially transmit *Y. pestis* (Simond 1898, Pollitzer 1954, Pollitzer 1954, Pollitzer and Meyer 1961, Gage and Kosoy 2005). This led to further studies and the theory of the proventriculus blocked model mechanism of transmission, which was first described by Bacot and Martin in 1914 (Bacot and Martin 1914, Bacot and Martin 1914). This model of biological transmission was proposed to be the dominant paradigm for flea-borne plague transmission and suggested that after fleas consume a host blood meal containing *Y. pestis*, the pathogen multiplies and begins colonizing in the flea midgut. This bacterial aggregation will then spread to the flea proventriculus, and further block any subsequent blood meals from flowing into the midgut. This blockage can form is a little as five

days after consumption, but typically occurs after 13-21 days (Mury, Paskewitz et al. 2004). *Y. pestis* blockage in the flea requires formation of a bacterial biofilm, which is defined as a dense association of bacteria cells coated with an extracellular matrix, often attached to a surface (Jarrett, Sebbane et al. 2004). *Y. pestis* only produces biofilm at flea temperatures ranging from 21-28°C, and biofilm is not produced at mammalian temperatures (37°C) (Jones, Lilliard et al. 1999). When a bacterial blockage occurs, the flea will have no fresh blood in the midgut, and begin to starve, and consequently they repeatedly attempt to feed. During the repeated attempts to acquire a fresh blood meal, the blockage prevents the blood from entering the flea midgut and the flea esophagus becomes distended with blood, the blood meal will partially return back to the biting site of the hosts; potentially dislodging bacteria from the blockage and transmitting plague to the host (Bacot and Martin 1914, Bacot and Martin 1914). This model of transmission, was later revised by Bacot when he determined that fleas which were only partially blocked by a bacterial aggregation were more effective transmitters. Fleas were capable of surviving longer since the partial blockage allowed for part of the blood meal to pass the proventriculus and reach the midgut where it could be further digested. However, regurgitation of *Y. pestis* was still possible from the pumping of the flea proventriculus during feeding (Bacot 1915). This revised transmission model provided an explanation to better explain why some flea species are good transmitters of plague, but are incapable of becoming completely blocked by bacterial biofilm. Another potential mechanism of plague transmission is a mechanical transmission route in which flea mouthparts could become contaminated with viable *Y. pestis* after feeding on an infected host. After the flea goes on to take a subsequent blood meal from a new host, the bacteria; which have survived on the mouthparts, would be transferred into the new bite site, transmitting plague to the host (Hinnebusch 2005). Recent studies have further investigated the partial-blocked flea

transmission mechanism and found that the flea vector *O. montana* can successfully transmit plague during the early-phase time points; which are the days before *Y. pestis* forms even a partial bacterial blockage in the flea (Eisen, Bearden et al. 2006). This study suggested a different model of biological transmission in which a proventricular blockage is not necessary (Eisen, Bearden et al. 2006). This mode of transmission has also been observed in several other flea species including the Oriental rat flea, *X. cheopis,* prairie dog flea, *O. hirsuta,* prairie dog flea, *O. tuberculata cynomuris,* cat flea, *Ctenocephalides felis,* mouse flea, *Aetheca wagneri* (Eisen, Bearden et al. 2006, Eisen, Borchert et al. 2008, Eisen, Holmes et al. 2008, Wilder, Eisen et al. 2008, Wilder, Eisen et al. 2008). This early-phase transmission mechanism has also been suggested as being the mechanism used by fleas during rapidly spreading *Y. pestis* epizootics (Eisen, Bearden et al. 2006). During the early-phase period, *Y. pestis* colonizes in the flea gut, where the pathogen successfully survives and multiplies for subsequent transmission. To date, multiple transmission factors and virulence factors of *Y. pestis* have been identified; however, it is still unknown if any flea factors contribute to the success of this transmission mechanism. Further identification of these potential factors would give additional insight on the exact mechanism of bacterial survival and transmission within the flea.

1.11. *Y. pestis* Transmissioin Factors

Transmission factors of *Y. pestis* are genes important for survival within the flea and subsequent transmission to a mammalian host. Survival of *Y. pestis* in the flea midgut depends on the plasmid encoded, *Yersinia* murin toxin (Ymt), which has phospholipase D (PLD) activity. In early studies, Ymt was discovered to be toxic to mice and rats by causing vascular collapse leading to death and was categorized as a virulence factor (Schar 1956, Schar and Meyer 1956).

Ymt is an important for pathogen survival in the flea midgut during digestion of the blood meal, but the agent and mechanism by which the PLD protects the bacterium is still unknown (Hinnebusch, Rudolph et al. 2002). Transmission of *Y. pestis* by fleas is unique since transmission occurs by regurgitation from the digestive tract; whereas many other arthropod-borne pathogens are transmitted through the vector saliva after dissemination to the salivary glands. *Y. pestis* infection in the proventriculus of the flea, greatly depends on the ability of *Y. pestis* to form a biofilm on the surface on of the proventricular spines (Darby, Hsu et al. 2002, Jarrett, Sebbane et al. 2004). Once the bacterial biofilm begins to grow and multiply, blockage formation occurs and makes possible obstruction of normal blood feeding. If complete blockage of the proventriculus ensues, then the flea will eventually starve to death after tenaciously attempting to feed on a mammalian host, while dislodging bacteria into the host and leading to potential transmission. The *Y. pestis* temperature dependent *hmsHFRS* genes are required for biofilm synthesis and formation of the polysaccharide extracellular matrix (ECM) (Bacot 1915, Hinnebusch, Perry et al. 1996). After ingestion of a *Y. pestis*-infected blood meal by a flea vector, the temperature shift (37°C to ~26°C) is detected by the pathogen and triggers *Y. pestis* to begin gene regulation for survival and adaptation inside of the vector (Perry and Fetherston 1997). *Y. pestis* biofilm formation in the flea involves a complex, multistage process which involves synthesis and transport of the biofilm ECM, which is regulated by the bacterial second messenger c-di-GMP (Hengge 2009). The intracellular levels of c-di-GMP depend on the opposing activities of GGDEF-domain diguanylate cyclase (DCG) enzymes that synthesize c-di-GMP and EAL-domain phosphodiesterase (PDE) enzymes that degrade it. Two functional DGC genes found in *Y. pestis* are *hmsT* and *y3730*, and one functional PDE gene, *hmsP* (Bobrov, Kirillina et al. 2011, Sun, Koumoutsi et al. 2011). The *Y. pestis pla* gene, encoding the

plasminogen activator (Pla), is located on the 9.5-kb plasmid known as pPCP1 (Sodeinde and Goguen 1988, Sodeinde, Sample et al. 1988, Hinnebusch 2005). This gene encodes a surface protease, which has been shown to result in increased *Y. pestis* dissemination and tissue invasion (Korhonen, Kosonen et al. 2004). Pla has been deemed essential as a transmission factor for flea-borne spread of *Y. pestis*, and shown to greatly increase bacterial dissemination following subcutaneous injection, a mode of inoculation which mimicked the flea-borne transmission route of infection (Sodeinde, Subrahmanyam et al. 1992, Hinnebusch 2005). Other bacterial adaptations occurring inside of the flea gut are genes involved in the uptake and catabolism of dipeptides, oligopeptides, and the L-glutamate group of amino acids. However, genes for hexose carbohydrate uptake, glycolysis, and fermentation are under-expressed in the flea environment. *Y. pestis* relies primarily on oligopeptides and select amino acids as its major carbon and energy source during persistent infection within the flea midgut. The *Y. pestis* PhoP-PhoQ gene regulatory system has also been found to be upregulated when inside the flea midgut, resulting in LPS modification and other outer membrane components further confering protection against cationic antimicrobial peptides (Vadyvaloo, Jarrett et al. 2010).

1.12. *Y. pestis* Virulence and Pathogenesis

Y. pestis pathogenesis involves complex sets of virulence genes which enable this pathogen to evade the host immune system and aid in disease progression. Many of these virulence genes are tightly regulated and expression of virulence components is largely dependent on the organism's response to the environment in which it is residing (flea or mammalian host). Many of the virulence genes among the pathogenic *Yersinia* spp. are conserved, however, a number of the enteropathogenic *Yersinia* virulence genes are

dysfunctional in the *Y. pestis* lineage through mutations or insertion sequence interruptions. In mammalian hosts, pathogen-associated molecular patterns (PAMPs) are highly conserved molecular structures which are specific to microbes and allows the host innate immune system the ability to identify between self and pathogenic cells by using pattern recognition receptors (PRRs) (Elvin and Williamson 2000, Li and Yang 2008). Different PRR's will react to specific PAMP's and activate immune cells to directly induce expression of a variety of genes involved in innate and adaptive immunity designed to fight off foreign invaders. Once the host PRR's have activated the complement pathway of the innate immune system, this elicits the induction of specific cytokines such as interleukin-1β (IL-1β), IL-6, tumor necrosis factor (TNF). Production of chemokines increases to induce the inflammatory response, which starts recruitment of host neutrophils to the site of infection as well as initiating activation of macrophage cells to begin killing the pathogen. *Y. pestis* evades killing by the host innate immune system by being introduced directly into the primary host barrier through the bite of an infected flea, where the pathogen then encounters phagocytic host cells such as polymorphonuclear leukocytes (PMNs), neutrophils, dendritic cells and macrophages (Li and Yang 2008). It is possible that the majority of the plague bacilli may be killed by neutrophils; however, *Y. pestis* is a facultative intracellular pathogen and is capable of successfully infecting and surviving within the phagolysosomes of macrophages. *Y. pestis* is thought to use specific host surface associated molecules such as CCR5 molecules in order to recognize macrophages and upon entrance into these cells, the bacterium begins to proliferate and produce multiple virulence factors. This is followed by release of *Y. pestis* into the extracellular compartment, where it then disseminates systemically while exhibiting phagocytosis resistance (Elvin and Williamson 2000, Li and Yang 2008).

When *Y. pestis* replicates inside the macrophage, the F1 capsular antigen is expressed and forms capsule-like structure around the bacterial cell. This capsule is an essential virulence factor and confers resistance to phagocytosis by macrophages and neutrophils by inhibiting receptor binding that would normally allow phagocytic cells to engulf the bacterial cells. Also induced in *Y. pestis* while residing in the acidic environment of the phagolysosome of the macrophages, is PsaA (pH 6 antigen), which encodes a fimbrial structure that eliminates interactions of the bacterial cells with macrophages and prevents further recognition and destruction by the host immune system (Huang, Chu et al. 2002, Makoveichuk, Cherepanov et al. 2003, Huang and Lindler 2004).

One important PRR hosts use to detect pathogens is Toll-like receptor 4 (TLR4), which recognizes the LPS in the outer membrane of Gram-negative bacteria. The recognition of LPS by the host, allows for a swift response to infection, as well as inducing host immune signaling; however, in order for the bacterial LPS to trigger a signficant immune response, the LPS component lipid A fatty acid acyl side chains need to range from 12 to 14 carbons in length. Since *Y. pestis* displays rough-type (R) LPS, which lacks O-antigen, *Y. pestis* is capable of altering expression of LPS components resulting in modification of LPS structure in response to host-specific environments. Temperature shifts are largely responsible for the different LPS structures and Lipid A variations of *Y. pestis,* which also are regulated by the PhoP-PhoQ system (Prior, Hitchen et al. 2001, Rebeil, Ernst et al. 2006, Knirel, Dentovskaya et al. 2007). More specifically, when *Y. pestis* is inside the flea gut at 21-26°C, the pathogen forms a typical hexa-acylated Lipid A, which if introduced into a mammalian host can activate immune signaling and specific cytokines such as TNF-α, IL-1, IL-6, and IL-8. However, once a temperature change in the environment is detected, and the bacteria enter a mammalian host, *Y. pestis* forms a tetra-

acylated Lipid A, which is found to be non-stimulatory to the host pathogen recognition receptors, TLR4, TLR2 and TLR9, and allows the pathogen to successfully prevent the induction of the inflammation responses by the signaling pathways induced by hexa-acylated Lipid-A-containing LPS (Montminy, Khan et al. 2006, Telepnev, Klimpel et al. 2009). Changes in the LPS of *Y. pestis* not only prevent activation of the innate immune response, such as activation of macrophages and secretion of proinflammatory cytokines, but also prevents the activation and maturation of dendritic cells (DCs) which are required for successful induction of the host's adaptive immune system (Dziarski 2006, Dziarski and Gupta 2006, Montminy, Khan et al. 2006, Rebeil, Ernst et al. 2006). To survive and replicate successfully in blood for transmission between the flea and mammalian host, *Y. pestis* possesses serum resistance to host complement-mediated lysis. *Y. pestis* is resistant to complement at temperatures typical for the bodies of both flea (26°C) and mammals (37°C). However, in other pathogenic *Yersinia spp*., *Y. pseudotuberculosis* and *Y. entercolitica* are only resistant to complement at 37°C and this function is found to be mediated by YadA, Ail and LPS. Since *Y. pestis* does not express YadA, the LPS of *Y. pestis* is thought to play a role in serum resistance because of elevated amounts of N-acetylglucosamine (Anisimov, Dentovskaya et al. 2005).

In the pathogenic *Yersiniae*, one hallmark feature is the ability of the organism to scavenge iron from its host using the siderophore called yersiniabactin (Ybt). The chromosomally located *ybt* genes are clustered on a high-pathogenicity island (HPI) located in the 102 kb pigmentation or *pgm* locus. Also located within the *pgm* region of *Y. pestis* is the *hms* locus which is responsible for bacterial biofilm formation and flea proventricular blockage. *Y. pestis* biofilm formation is involved in maintenance of infection in the flea, however, its necessity for efficient transmission of the pathogen from the flea vector to mammalian hosts has

been subject of much debate (Fetherston and Perry 1994, Parkhill, Wren et al. 2001, Vetter, Eisen et al. 2010).

In the three human pathogenic *Yersinia* spp., (*Y. entercolitica*, *Y. pseudotuberculosis*, and *Y. pestis*), resides the pCD1 virulence plasmid, which carries the low calcium response (LCR) regulon genes, components of the *Yersinia* T3SS, and associated effector proteins known as *Yersinia* outer proteins (Yops) (Shao 2008). The V-antigen or LcrV is a multifunctional protein that works in coordination with the T3SS, and is involved in formation of the tip complex of the T3SS needle as well as activation and secretion of translocation effector proteins by binding to the negative regulator LcrG and works in conjunction with YopB and YopD for successful delivery of Yops into eukaryotic cells. LcrV also plays a role in cell-to-cell adhesion by becoming exposed externally on bacterial cell surfaces allowing easy adhesion to mammalian cells (Lawton, Erdman et al. 1963, Cornelis and Wolf-Watz 1997, Pettersson, van der Biezen et al. 1999, Pettersson, Holmström et al. 1999). Once LcrV is secreted into the extracellular milieu, it usurps TLR2 and CD14 resulting in the release of IL-10 by host immune cells and subsequent suppression of the production of certain cytokines such as TNF-α and IFN-γ (Sing, Roggenkamp et al. 2002, Sing, Rost et al. 2002, Sodhi, Sharma et al. 2004, Sodhi, Sharma et al. 2005). The T3SS is important in host immune evasion, by formation of a needlelike structure on the surface of the pathogenic *Yersinia* spp., which then interacts with host target immune cells, such as macrophages, dendritic cells, and neutrophils, and quickly enables injection of six different Yop proteins which interfere with phagocytosis and other innate host cell responses as well as the adaptive inflammatory cascade leading to target cell apoptosis. Yop proteins (YopH, YopT, YopE, and YopO) are involved in preventing phagocytosis by macrophages and neutrophils by interfering with host cell actin regulation by Rho GTPases. YopH has also been linked to

suppressing the production of reactive oxygen intermediates by macrophages and polymononuclear cells (PMNs) (Green, Hartland et al. 1995).

More recently, virulence factor SurA, or survival protein A, which is a chaperone protein involved in the biogenesis of outer membrane proteins (OMPs) was found to be necessary in the virulence and invasion of *Y. pestis*. SurA exhibits peptidyl-prolyl isomerase (PPIase) activity, making it part of the parvulin group of PPIases. SurA was found to play a significant role in infection and intracellular replication in epithelial cells, as well as persistence in an *in vivo* infection model (Lazar and Kolter 1996, Rouviere and Gross 1996, Southern, Scott et al. 2016).

1.13. *Y. pestis* Geographical Distribution and Incidence

Plague has a worldwide distribution and is found on every continent except Australia and Antartica, and has caused epidemics in Africa, Asia, Europe, and South America. The World Health Organization (WHO) predicts between 1000-2000 reported plague cases each year, but the true number of cases is likely much higher (Biggins and Kosoy 2001). According to the WHO, Brazil, Democratic Republic of the Congo, Madagascar, Myanmar, Peru, United States of America, and Vietnam have reported plague cases every year for the last 44 years (WHO, 2004). Plague mortality rates, specifically in developing countries, are hard to predict because some cases are never correctly diagnosed or reported; however, the WHO cites mortality rates to be around 8-10%, but studies suggest rates are much higher in plague endemic regions (WHO, 2004). In the United States between 1900 and 2012, 1006 confirmed or probable human plague cases occurred with over 80% presenting in bubonic form (Kugeler, Staples et al. 2015). Human plague cases in the U.S. between 1950-2012, ranged from 1-17 cases per year, and usually average ~7 cases per year. Plague infections occur in people of all ages, and have infected

humans as young as infants, and individuals as old as 96 years old; however, 50% of plague cases have been found to infect people ages 12–45 years old. Plague occurs in both men and women, with a slightly more frequent occurrence in men than women, which is thought to be attributed to increased outdoor activities that put the men at a slightly higher risk of acquiring infection. With successful use of antibiotics to treat human plague infections, the overall number of human plague deaths has decreased greatly from what was observed between 1900-1941, which was the pre-antibiotic era. During the pre-antibiotic era, mortality among plague infected individuals in the United State was 66%; whereas with the use of antibiotics, the mortality rate was greatly reduced and by 1990-2010 the overall mortality rate decreased to 11%. Even with antibiotic treatment, plague infected individuals are still at risk of dying from the disease; although the chances are lower for people with the bubonic form of the disease compared to the septicemic or pneumonic plague forms. The actual plague mortality rate in developing countries is difficult to assess because relatively few cases are reliably diagnosed and reported to health authorities (CDC 1995, CDC 1997, CDC 1999).

Worldwide plague distributions are impacted by certain landscapes and climatic conditions for which *Y. pestis* is able to establish itself successfully. In the 1900s, *Y. pestis* was introduced into the United States at the Port of San Francisco, California by urban rats that spread the disease to the sylvatic rodent hosts. Despite improved sanitation methods which decreased human plague cases, the pathogen was able to spread and establish itself east to the 100th meridian (Eisen and Gage, 2011; (Eskey and Haas 1940, Adjemian, Bustos et al. 2007, Adjemian, Foley et al. 2007, Morelli, Song et al. 2010). Within the western United States, plague has established itself in enzootic cycles, which are randomly distributed (Eisen and Gage 2012). Many studies have examined numerous variables to determine the ecological niches of

plague and identify areas that may have potentially higher risks for human exposures or potential infection within the plague-endemic regions.

Studies have focused on landscape, vegetation, and climate variables that could either inhibit or aid in the persistence and survival of *Y. pestis* and have been focused in the African and North American regions (Collinge, Simirskii et al. 2005, Davis, Klassovskiy et al. 2007, Eisen, Enscore et al. 2007, Eisen, Lowell et al. 2007, Nakazawa, Williams et al. 2007, Neerinckx, Peterson et al. 2008, Eisen, Griffith et al. 2010). In sub-Saharan Africa, plague foci were positively correlated with elevation above 1300 meters, evaporation potential and transpiration by plants, mean daily temperature range and annual rainfall, making the ecological landscape of sub-Saharan Africa a prime location for *Y. pestis* transmission (Neerinckx, Peterson et al. 2008). Key plague risk predictors in Uganda and Tanzania were related to elevation and vegetation when focusing on local spatial scales. In Eastern Tanzania, plague was found to be located at areas at higher elevation (1200-2000 m) and areas in which seasonal vegetation changes (Neerinckx, Peterson et al. 2008). Interestingly, in northwestern Uganda, the risk of plague was increased in areas of higher elevation (>1300 m) than areas of elevations below 1300 m with less vegetation and increased moisture (Eisen, Griffith et al. 2010). Studies focusing on plague in the four corners region of the United States, found human risk of plague exposure increased in areas which were at 2300 m in elevation, and had specific vegetation types such as pinon-juniper or Ponderosa pine, in areas with higher moisture (Eisen, Enscore et al. 2007, Eisen, Lowell et al. 2007, Eisen, Reynolds et al. 2007, Eisen, Wilder et al. 2007). Plague distributions and endemic areas are directly correlated with temperature and rainfall patterns. Over 95% of the worldwide distribution of plague cases are reported in areas in which the average temperatures exceed 13°C, and most plague outbreaks are in regions where the

temperature ranges from 24°C to 27°C. Plague epidemics are found to diminish when the temperature is above 27°C. Plague epizootics are found to occur sporadically among the known plague foci and the important contributors of plague epizootics are still poorly understood. (Davis 1953, Davis 1953, Cavanaugh and Marshall 1972, Cavanaugh and Williams 1980, Gage and Kosoy 2005, Gage, Burkot et al. 2008). In plague enzootic regions in the western United States and Kazakhstan, quantitative models were used to determine that an increase in moisture levels prior to a transmission season are conducive for epizootic activity; however, elevated temperatures during the transmission season caused a decrease in epizootic activity (Parmenter, Yadav et al. 1999, Enscore, Biggerstaff et al. 2002, Stapp, Antolin et al. 2004, Stenseth, Samia et al. 2006, Stapp, Salkeld et al. 2008, Brown, Ettestad et al. 2010). The trophic cascade hypothesis was then created suggesting elevated precipitation increases the vegetation, and therefore enhances the food sources for the harborage of the small rodent hosts. The small rodent populations will increase and the flea infestations will also rise, leading to a higher probability that the contact rates between infectious fleas and susceptible hosts will rise and lead to increased probability of a potential epizootic (Parmenter, Yadav et al. 1999, Enscore, Biggerstaff et al. 2002, Lorange, Race et al. 2005, Eisen, Eisen et al. 2006, Eisen, Bearden et al. 2006). More recently, a study examining areas outside of the normal plague distribution in the United States, located *Y. pestis* in areas east of the known active plague endemic locations. This study examined *Orosyslla tuberculata* and *Oropsylla hirsuta* fleas, which were collected from black-tailed prairie dogs in five national parks east of the known distribution of active plague across the northern Great Plains and tested the fleas for the presence of *Y. pestis*. Fleas were found to be infected with *Y. pestis*, suggesting that sylvatic plague likely occurs across the range of black-

tailed prairie dogs and indicates that plague foci in the United States could have a much greater distribution than previously believed (Mize and Britten 2016).

1.14. *Y. pestis* Clinical Disease Features

Plague infections in humans can present in three main manifestations: bubonic, septicemic, and pneumonic forms. Other more rare forms have also been reported such as pharyngeal, meningeal, cellulocutaneous, pestis minor, asymptomatic, and abortive forms (Meyer 1942, Pollitzer 1954, Pollitzer 1954, Meyer 1961, Marshall, Ouy et al. 1967, Poland 1977, Hovette, Burgel et al. 1998, Arbaji, Kharabsheh et al. 2005).

The most commonly occurring plague form is bubonic. Bubonic plague infections occur most frequently by the bite of an infected flea or less frequently as a result of handling infected animals or their tissues. When an infected flea bites a human or animal, the bacterium gets regurgitated into the bite site. In humans, the bacteria is then capable of entering the lymphatic system which drains interstitial fluid, and *Y. pestis* then begins replicating in the nearest lymph node, and subsequently stimulates sever hemorrhagic inflammation that causes the lymph nodes to become inflamed and can increase to the size of an egg. This enlargement of the lymph node is clinically known as a "bubo", associated with the bubonic plague form. Buboes are typically found in the groin, neck and armpits. Bubonic plague typically manifests within 2-6 days after initial infection with *Y. pestis*, and patients will also exhibit sudden onset of illness characterized by headache, shaking, chills, fever, malaise, and pain in the affected regional lymph node. Gangrene can also develop in the extremities, which is where, according to some, the name, Black Death was established (Pollitzer 1954, Pollitzer 1954, Poland, Quan et al. 1994)(Plague Manual, 1999a)

Next, septicemic plague occurs when *Y. pestis* is introduced into the bloodstream, causing the signs and symptoms of Gram-negative septic shock. This form can either be primary or secondary to bubonic plague. The disease is contracted primarily through the bite of an infected flea or rodent, but like bubonic plague, it is rarely contracted through an opening in the skin or by cough from another infected individual. After reaching the bloodstream, *Y. pestis* can replicate to very high numbers, causing severe sepsis, and experience symptoms such as, fever, chills, weakness, abdominal pain, shock, and bleeding underneath the skin or in other organs. Once the pathogen has caused bacteremia, it is then capable of advancing to other organs systems, and has the potential of progressing to secondary pneumonic plague, plague meningitis, plague endophthalmitis, hepatic or splenic abscessed, or generalized lymphadenopathy. Prompt treatment with antibiotics greatly reduces the mortality rate in patients to 4-15% (Meyer 1950). If a patient with septicemic plague is not treated promptly with proper antibiotics, patients can die on the same day clinical symptoms appear (Meyer 1950, Butler 1983, Butler 1994, Butler 2009).

Lastly, pneumonic plague, which is the most severe form of the disease, can present as either a primary or secondary form of infection. Primary pneumonic plague occurs when the plague bacterium is contained in infectious respiratory droplets or is aerosolized and then inhaled into the lungs of a susceptible host. Secondary pneumonic infections can result from the progression of bubonic or septicemic plague infections. Common symptoms include fever, headache, weakness, shortness of breath, chest pain, cough, bloody or watery sputum, confusion, cyanosis, shock and death. The incubation period for pneumonic plague is typically 2-4 days, however, in some cases it can be as little as a few hours after initial acquisition of the pathogen. Without proper treatment or diagnosis, this infection is 100% fatal within 1-6 days after

pathogen acquisition (Hoffman 1980, Ratsitorahina, Chanteau et al. 2000, Ratsitorahina, Rabarijaona et al. 2000).

1.15. *Y. pestis* Diagnosis and Treatments

For timely diagnosis of human plague infections, the first step is for a health care worker to evaluate signs and symptoms. If a plague infection is suspected, the healthcare provider will takes samples of the suspect patient's blood, sputum, or lymph node aspirate, and each sample will be sent to the laboratory to be further evaluated. Once the laboratory receives the sample, preliminary results will be completed in less than two hours; and official laboratory confirmation will take around 24 to 48 hours to allow ample time for bacterial growth in culture. If plague infection is suspected in the patient, antibiotic treatment will begin as soon as samples are collected (Plague Manual, 1999a).

Diagnosis of bubonic plague-like symptoms are commonly mistaken for other infections, such as staphylococcal or streptococcal lymphadentitis, mononucleosis, cat-scratch fever, tick typhus, tularemia and other causes of acute lymphadenopathy. Depending on the location of the enlarged lymph node, or bubo, other diagnoses can be mistakenly made such as appendicitis, enterocolitis or an inguinal hernia. If the intrathoracic lymph nodes are targeted during plague infection, severe complications such as severe deep cervical adentitis can occur leading to tracheal displacement and obstruction of the patient's airway. In people who are exhibiting signs of septicemic plague, such as gastrointestinal discomfort, abdominal pain, nausea, vomiting and diarrhea, it is common for clinicians to diagnose the illness as non-specific sepsis syndrome or Gram-negative sepsis. Other potential issues making plague diagnostics challenging results are related to laboratory protocols, miscoding of automated diagnostic machines, or interpretations

by laboratorians. For example, if Gram stains are prepared either from blood smears or lymph node aspirates, improperly decolorized, then the bacterium will appear as a Gram-positive diplococcus when examined under the microscope. Also, automated bacterial identification devices may not code for *Y. pestis*; therefore, resulting in misidentification of the pathogen, often as closely related *Y. pseudotuberculosis*. Diagnosis of pneumonic plague can also be perplexing and the symptoms can be mistaken for pneumococcal, streptococcal, *Haemophilus influenzae*, anthrax, tularemia, *Legionella pneumophila*, letptospirosis, or hantavirus pulmonary syndrome. If lymphadentitis is observed in the the regional lymph nodes in plague-endemic regions, then clinicians usually properly suspect plague or tularemia pneumonia infection, which could be a secondary infection following infection by a cutaneous route (Plague Manual, 1999a).

In order for a sample from a plague suspected patient to be considered a laboratory positive, the isolation and identification of *Y. pestis* from the clinical specimens or a rise in antibody titers from serum samples must be confirmed. Routine testing includes specimens for smear and culture such as whole blood, aspirates from suspected buboes, pharyngeal swabs, sputum samples or tracheal washes from plague suspected pharyngitis or pneumonia, and cerebrospinal fluid from patients with suspected meningitis. Bubo aspirates are collected after an injection of 1-2 ml of saline using an 18-22 gauge needle. Microbiological media used to culture media include Brain-heart infusion broth, sheep blood agar, or MacConkey agar. Each smear is examined with Wayson or Giemsa stain and Gram stain, and further evaluated by performing a direct fluorescent antibody assay, which is an anti-F1 antibody examination. Acute phase serum samples will be tested for detection of antibodies to *Y. pestis* for serological confirmation, and subsequently, convalescent phase serum samples will be collected at 4-6 weeks post infection for further validation. If the suspected plague patient succumbs to infection, appropriate tissues such

as lymph nodes, liver, spleen, lung and bone marrow will be collected for culture, immunohistochemical staining, and fluorescent antibody testing and samples are then sent to the laboratory either fresh or frozen on dry ice, as well as in the appropriate transport media such as Cary-Blair (Plague Manual, 1999a).

When examining blood collected from *Y. pestis* infected patients, the white blood cell (WBC) counts typically range from 12,000-25,000/µl blood, with a large amount of immature polymorphonuclear cells (Butler, Bell et al. 1974). When bipolar staining is conducted, the polymorphonuclear leukocytes and the plague Gram-negative bacilli will be found in the sputum of plague infected patients. Patients infected with pneumonic plague will have X-ray's which exhibit patchy bronchopneumonic infiltrates, as well as, segmented or lobar consolidation of bilateral diffuse infiltrates of acute respiratory distress syndrome. In septicemic plague patients, *Y. pestis* will be identified in the stained peripheral blood smear or buffy-coat smear (Butler, Bell et al. 1974). For diagnosis of plague meningitis, cerebrospinal fluid (CSF) is examined and plague infected patients exhibit CSF with increased white blood cell counts with predominance of PMN's. The characteristic bipolar staining of *Y. pestis* either by Wayson or Giemsa staining, is not unique to this pathogen; therefore, is not an ideal indicator of disease and further tests are needed for confirmation of a plague positive sample. For a confirmed plague positive sample, *Y. pestis* must be successfully isolated from cultures of body fluids and/or tissues (Quan 1987, Chu, Katakura et al. 1999, Chu, Park et al. 1999, Chu, Tomita et al. 1999). In infected patients, the *Y. pestis* organism will be successfully isolated with the collection of three blood samples over a 45 minute period before treatment begins. When grown on agar for 48 hours at 28°C, *Y. pestis* appears as greyish-white, translucent colonies which are 1-2 mm in diameter. After 48-72 hours of incubation, *Y. pestis* colonies are raised and have an irregular hammered copper appearance

(Butler, Mahmoud et al. 1977, Chu, Katakura et al. 1999, Chu, Park et al. 1999, Chu, Tomita et al. 1999). *Y. pestis* culture positives are further confirmed by performing a specific bacteriophage lysis assay specific to the F1 capsular antigen. Automated bacteriological test systems can also be used to confirm positive plague colonies; however, misidentification of isolates can occur if the systems are improperly programmed (Wilmoth, Chu et al. 1996, Tourdjman, Ibraheem et al. 2012).

If plague colonies are not isolated from culture, *Y. pestis* infection can be tested for evidence of seroconversion events by performing passive hemagluttination assays (PHA) or ELISA. Any titers exhibiting changes of four-fold or greater to the *Y. pestis* F1 capsular antigen indicates a positive sample which has resolved a *Y. pestis* infection (Butler, Mahmoud et al. 1977, Chu, Katakura et al. 1999, Chu, Park et al. 1999, Chu, Tomita et al. 1999). PHA specificity of a positive test requires further confirmation by performing the F1 antigen hemaggluttination inhibition (PHI) assay which tests for any cross-reactivity (Chu, Katakura et al. 1999, Chu, Park et al. 1999, Chu, Tomita et al. 1999). Most plague patients will seroconvert between one and two weeks after the onset of disease; however, a few patients have been found to seroconvert five days after the onset of illness. Seroconversion events in some patients can take up to three or more weeks after the onset of disease and in rare cases, some patients do not seroconvert (Butler, Mahmoud et al. 1977). Patients who began early antibiotic treatment, are found to have delayed seroconversion. After seroconversion occurs in the patient, positive serological titers will decrease gradually over the next few months or years. Another assay commonly used in early stages of plague infection is the enzyme-linked immunosorbent assay (ELISA), which detects IgM and IgG antibodies and can also be used for antigen capture. (Chu, Katakura et al. 1999, Chu, Park et al. 1999, Chu, Tomita et al. 1999).

Plague infections can be successfully treated with antibiotics; however, it is important to begin antibiotic treatment as soon as possible after laboratory specimens are taken. In patients with suspected pneumonic plague infection, antibiotics should be given as soon as possible, preferably within 24 hours of the first symptoms. Specific antimicrobial therapy of plague patients is determined by the form and stage of the disease. Aminoglycosides such as streptomycin and gentamicin are most effective against *Y. pestis* in the pneumonic form of the disease (Byrne, Welkos et al. 1998) (Plague Manual, 1999a). For the treatment of bubonic and septicmic plague infections, chloramphenicol is considered the drug of choice; as well as for the treatment of invasive *Y. pestis* which has entered tissue spaces as seen in plague meningitits, pleuritis, or endophthalmitis since other drugs are incapable of crossing specific blood brain barriers. (Meyer 1950, McCrumb, Mercier et al. 1953, Meyer 1961, Becker, Poland et al. 1987). Tetracyclines can be used in treatment of patients with uncomplicated plague infections; as well as in adjunct with other antibiotics. Sulfonamindes have been used in plague infections; however, studies have determined that patients treated with this antibiotic results in higher mortalities, increased complications, and longer fevers (Butler, Mahmoud et al. 1977, Butler 1983, Boulanger, Le Maire et al. 1996). More recently, fluoroquinolones such as ciprofloxacin have been used in the treatment of plague infections. Ciprofloxacin is a bactericidal antibiotic with broad spectrum activity against most Gram-negative bacteria and many Gram-positive bacteria (Lemaitre, Ricard et al. 2012). Studies looking at the benefits of combination antibiotic therapy for treatment of plague have been shown to be more effective in eradication of *Y. pestis* specifically when given ciprofloxacin and gentamicin in combination (Lemaitre, Ricard et al. 2012). Penicillins, cephalosporins and macrolides are ineffective in the treatment of plague. During plague infections, supportive therapy is often needed to manage plague complications, in

addition to dealing with potential Gram-negative sepsis (Wheeler and Bernard 1999). It is important to monitor and manage potential septic shock in plague patients in order to prevent multiple organ failure, adult respiratory distress syndrome (ARDS) and disseminated intravascular coagulopathy (Plague Manual, 1999a). For treatment of pregnant women or children, gentamicin is the preferred antibiotic for plague infections; however, aminoglycosides have been shown to be effective for treatment. Streptomycin is ototoxic and nephrotoxic to fetuses and chloramphenicol has risk of causing "grey baby" syndrome and bone-marrow suppression so these classes are avoided in pregnant women and children (Plague Manual, 1999a). If any individuals were in close proximity or in contact with infected plague patients, exposed to *Y. pestis* infected fleas, had direct contact with body fluids or tissues of an infected mammal, or exposed during a laboratory accident, then prophylactic therapy is recommended (CDC 1996). Preferred antimicrobials for patients potentially exposed to plague include tetracyclines, chloramphenicols, or an effective sulfonamide, and in 2012, the U.S. Food and Drug Administration (FDA) licensed the use of ciprofloxacin (levofloxacin) to prevent and treat pneumonic and septicemic plague. In areas of suspected plague or the veterinary or hospital settings, patient care precautions must be followed and procedures such as proper protective equipment (PPE, such as gowns, gloves, masks, goggles, etc) should be worn to protect individuals from contracting plague infection (Plague Manual, 1999a).

Currently, no FDA licensed plague vaccine is available; however, plague vaccines are in development but not expected to be commercially available in the immediate future (CDC 1982, CDC 1996). Vaccine developers have focused on two subunits of *Y. pestis*: LcrV, which is a protein at the tip of the type III secretion needles, and F1, which is the fraction 1 capsular antigen (Quenee, Berube et al. 2010). LcrV and F1 are currently the only candidates for plague subunit

vaccines. The U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID) developed the F1-V fusion protein, which tethers the two antigens as a translational hybrid into a single molecule (Heath, Anderson et al. 1998). The fusion protein was designed to enable a one-protein-manufacturing process, which is considered an advantage over purifying two different polypeptides (Heath, Anderson et al. 1998, Williamson 2001). Worldwide, formalin-killed *Y. pestis* and live attenuated vaccines have been developed, but with some weaknesses. The live attenuated vaccine is highly reactogenic and not licensed for human use. The killed whole cell vaccine, also reactogenic, provides poor protection against pneumonic plague and immunization requires multiple follow-up doses of the vaccine. With such adverse reactions to vaccination, these vaccines were only given to individuals which are in close proximity of the bacterium, such as laboratorians or veterinarians (CDC 1982, CDC 1996, Titball and Williamson 2004)

1.16. *Y. pestis* Surveillance and Control

Plague activity in the United States is often difficult to detect. The limited evidence of plague transmission occurring at most times especially between epizootics. By contrast, there are occasional epizootics that result in highly visible die-offs of certain rodent species and it is during these times that the disease can be relatively easy to detect. In the United States, prairie dogs (*Cynomys spp.*) suffer the most dramatic die-offs with up to 98% dying in plague-affected populations (Biggins and Kosoy 2001). Documenting rodent die-offs is an efficient and low-cost way to monitor plague dynamics, however they are often detected only after an epizootic already has begun and does not serve as an early warning system. Monitoring plague exposure, or seroprevalence, through active surveillance of other animals that can act as sentinel species is a proven method for monitoring plague dynamics but is very expensive, time consuming, resource

intensive and difficult to maintain. The time required for analysis of serum samples also can be sufficiently long and the results are of little use by the time thtey are received many weeks to months later. In the United States, animal-based plague surveillance is performed by multiple state health departments at the local level, as well as through research universities, the Centers for Disease Control and Prevention and the US Department of Agriculture (USDA). The USDA Animal and Plant Health Inspection Wildlife Services' National Wildlife Disease Program (NWDP) also conducts continual plague surveillance in regions with documented plague activity in the US, which is primarily restricted to areas west of the 100th meridian (IHR, 2005).

1.16.1 Plague Surveillance:

For successful plague prevention and control, a surveillance program must be developed to allow for up-to-date information on the incidence and distribution of the pathogen. A surveillance program is beneficial when timely information is obtained from the collection and analysis of human animal samples, and the useful interpretion of clinical epidemiological data, and epizootiological data on *Y. pestis*. A successful surveillance program will contain the same information that has been collected over many years, in order to detect any changes that may lead to a potential plague outbreak in that area. Once this long-term information has been collected, it will be beneficial in multiple ways such as: prediction of areas in which humans and rodent epizootics could potentially occur; identification of the most common zoonotic sources of human infection; identify the most important rodent and flea species which could be harboring *Y. pestis*; identification of the hosts and flea vectors which are potential targets for control measures; assess the effectiveness of plague prevention and control strategies; identification of local ecological factors or human activities which may lead to increased human plague exposure

risks; and detection of trends in the epidemiology and epizootology of plague in that specific area (Gage, Dennis et al. 1999). The standardization of surveillance reports is crucial so the same information is recorded for each case; resulting in a database that can be combined with rodent and flea vector surveillance data to design the optimal prevention and control strategies. In reports involving human cases, more information must be collected such as: basic patient information, clinical observations and treatment, laboratory results, and results of the epidemiological and environmental investigations (Gage, Dennis et al. 1999).

1.16.2 Plague Control:

Since *Y. pestis* is a flea-borne disease of rodents, control measures for this disease is based on the ability to control the rodent reservoirs and flea vector populations. Control measures are extremely important during outbreaks when rodent die-off occurs, leading to starving fleas questing for new mammalian hosts. Identifying endemic plague foci is important in assessing locations of higher risk of outbreaks. After the plague foci have been identified, information should be gathered such as the seasonality of past outbreaks, the rodent reservoirs, and the flea vectors in the area. Also of importance in these areas is to gather baseline data which effect the type of control measures that will be administered if an outbreak did happen to occur. Such baseline information would include: insecticide susceptibility of the flea vectors in the area, flea population densities, and rodent host/reservoirs densities. Gathering this yearly baseline information would lead to a successful surveillance system, detecting inclines or declines in the numbers of fleas and rodents, in order to identify potential plague epizootics before they occur (Gage, Dennis et al. 1999). Other information of importance would be ecological and climate change data of the areas, giving insight on factors that would alter the

vegetation leading to an increase or decrease in food sources for the endemic rodent populations which directly correlates with flea abundance (Gage, Dennis et al. 1999).

Many concepts exist regarding controlling the transmission of the *Y. pestis* pathogen from one reservoir animal to another or from animals to humans. Most commonly, control of the flea vector is believed the most important step to be taken. Previous studies have concluded, that when designing plague control methods, the flea is the primary and initial target in controlling plague, and the rodents, which harbors the fleas, is secondary (Gordon, Isaacson et al. 1979). When controlling plague after a human plague case, it requires direct attack of the plague foci where the infection was acquired; which first requires confirmation of the pathogen presence in the area. Next, isolation of the plague infected patient and any contacts the patient may have come into contact with during infection is necessary to prevent additional human infections. Lastly, it is important to manage and control plague in the area by use of disinfectants and evacuation of people, in addition to killing fleas with insecticides to which the fleas are known to be highly susceptible to (Gordon, Isaacson et al. 1979, Gage, Dennis et al. 1999).

Many studies have looked into the control of flea vectors by use of insecticides, with the main purpose being to reduce the density of the rodent-flea vectors in human populated areas, as quickly as possible. Methods such as applying insecticide dusts to rodent runways or burrows is effective in controlling flea vectors, and when rodents cross the paths or enter or exit the burrows, they become brushed with the dust, which then gets spread all over their bodies during grooming, eventually killing the fleas. In urgent situations, liquid insecticides can be used for control of flea vectors in burrows and homes. In areas in which commensal rodents are found nesting in dwellings or buildings as seen in many tropical regions, insecticide dusts must be administered carefully to prevent contaminating foodstuffs, cooking utensils, and other personal

items. Some studies have looked at the use of bait boxes (food bait used as a rodent attractant) as well as fipronil tubes that coat the rodent with an insecticide; further killing the fleas that are parasitizing the host (Boegler, Atiku et al. 2014).

Flea control measures of wild rodent populations can be more difficult then commensal rodents, primarily because it is more challenging to locate the rodent burrows and runway and the populations are widely dispersed over an area. Fumigation methods are commonly used to control rodents, and this method kills very easy and fast, leading to immediate death of rodents and fleas. More recently, wild rodent fleas have been controlled using other methods such as insecticide applications by aircraft, or in and around burrows by power and hand dusters. Much concern about flea insecticide resistance and the environmental concerns of insecticides have arisen in the last few decades, leading to an increase in use of bait boxes. Bait boxes have been proven to be very efficient and the rodents carry the insecticide on their fur back to their nests, further killing the fleas on their body and in the nests. The bait boxes successfully kill the fleas, significantly reducing flea populations over a widely distributed area; however, this method is labor intensive and continual rebaiting and replenishing of the bait dust is a downfall (Kartman and Longergan 1955, Gage, Dennis et al. 1999).

One important step for designing a successful plague and flea vector control program is doing susceptibility tests to determine the best insecticide to use for flea control. Flea resistance to insecticides greatly impedes any plague control program. Previously, Dichlorodiphenyltrichloroethane (DDT) dust was the insecticide of choice, however with recent environmental concerns and resistant flea populations, other alternative compounds such as organo-phophorus, carbamate, pyrethroid, and insect growth regulator compounds are being used effectively for control of flea populations. More commonly used insecticides include fipronil,

imidacloprid, lufenuron, and pyriproxyfen, which all have shown to be effective in flea control (IHR, 2005)(Boegler, Atiku et al. 2014). One huge drawback of using insecticides for vector control programs is the high likelihood of vector resistance. Surveys of the flea species and the seasonal population densities should be monitored closely to identify the first signs of resistance in the population (Gage, Dennis et al. 1999).

When a plague outbreak or an epizootic in the commensal or sylvatic rodent hosts occurs, it is also important to control the rodent reservoirs. Foremost, flea vectors parasitizing the rodents must be controlled before controlling the hosts in order to prevent a high number of fleas carrying plague questing for new hosts and greatly increasing humans risk of plague infections. Once the flea populations have been reduced, control of the rodent reservoirs is carried out. For controlling rodents, acute and chronic rodenticides are used, each with pros and cons. Acute rodenticides, such as arsenic trioxide or Bromethalin, are advantageous because they kill the mice quickly, and the user can see the immediate effects. Next, they are very effective and no resistance occurs, in contrast to the anticoagulants in the chronic rodenticides in which resistance is a problem. Some cons of acute rodenticides are they can cause bait shyness, are very expensive, high concentrations must be administered and poor selectivity to target species leading to other potential animal deaths. In contrast, chronic rodenticides, such as anticoagulants and warfarin, have advantages over acute rodenticides, because they do not cause bait shyness, can be administered by a non-expert, are administered at a low concentrations can be used, and then are much more affordable. However, some disadvantages include not seeing bodies of dead rodents, non-selective target species, slow acting and possibility of anticoagulant resistance. Other rodent control methods include fumigants such as hydrogen cyanide or carbon monoxide. Fumigants are successful in killing rodents, as well as their ectoparasites, and can be used to

target rodents living in inaccessible areas or buildings, ship or burrow systems. One disadvantage of fumigants is they are very dangerous to the person applying them as well as to any people or animals in the area. One advantage however, is they are very fast acting and very efficient. Fumigants are required to be administered by experienced or well-trained individuals (Gage, Dennis et al. 1999).

1.17. Animal Models Used in Plague Research

1.17.1 Invertebrate models for studying transmission factors:

Many models to study plague have been developed in order to better understand interactions between *Y. pestis* and its hosts, potential antibiotic resistant strains, potential risk of plague as a bioweapon, and the development of plague vaccine candidates and prophylactic treatments (Galimand, Guiyoule et al. 1997, Inglesby, Dennis et al. 2000). Plague researchers have used animal models for over 100 years in order to study model insect and human infections. These plague models have allowed scientists to study different species and determine the progression of plague pathology and the effectiveness of vaccine candidates. Recently, specific models have been developed which allow scientists to study *Y. pestis* pathogenesis, such as interactions between the bacterium and the host cells, transmission from the flea vector, infection of the mammalian host, and host immune responses during infection (Lawrenz 2010).

Y. pestis is unique because it is the only enteric bacterium that uses an insect vector as its primary mode of transmission; therefore, invertebrate models are used to study transmission factors which are different than virulence genes which are needed for mammalian disease (Hinnebusch, Perry et al. 1996, Paskewitz 1997). Since the flea is the natural vector for *Y. pestis,*

it is the most logical system to use when studying *Y. pestis*-vector interactions and transmission. Most commonly used in the laboratory setting in the U.S., are the flea vectors *X. cheopis* and *O. montana*, which are both highly efficient vectors for transmission of *Y. pestis* to mammalian hosts (Hinnebusch 2005, Eisen, Bearden et al. 2006). For example, this model has been essential in studies examining the importance of the *Yersinia* murine toxin (Ymt) which was first described due to its toxicity to mice (Warren and Young 2005). However, later the *ymt* gene was shown to be unnecessary during mouse infection since the *ymt* mutants were still fully virulent (Drozdov, Anisimov et al. 1995, Hinnebusch, Perry et al. 1996). Even though the Ymt gene is not necessary as a mammalian virulence factor, it has been found to be essential as a transmission factor (Hinnebusch 2005). Studies by Hinnebusch et al, demonstrated using the flea model, that Ymt is required for colonization of the flea midgut, and *ymt*(−) mutants are rapidly cleared from the midgut before the bacteria block the proventriculus (Hinnebusch, Rudolph et al. 2002). Other studies, which have been useful to better understand *Y. pestis* transmission, examined the importance of biofilm production in the flea vector (Hinnebusch, Perry et al. 1996, Jarrett, Sebbane et al. 2004). Through use of the flea model, the researchers demonstrated that *Y. pestis* transmission efficiencies depend on factors that are not required for pathogenesis in the mammal. While the flea is the natural insect vector for *Y. pestis*, the model poses some difficulties that may limit its widespread use in *Y. pestis* studies. These include a requirement for dedicated and specialized facilities to work with fleas, relatively slow growth of the insects, and a risk for *Y. pestis* transmission from flea to human.

Next, a second invertebrate model that has been used to study *Y. pestis*-host interactions is the nematode *Caenorhabditis elegans* (Darby, Hsu et al. 2002, Joshua, Karlyshev et al. 2003, Darby, Ananth et al. 2005, Bartra, Styer et al. 2008). Benefits of the *C. elegans* model is the

simplicity of the nematode to maintain and rapid growth, as well as its ability to be easily tracked, making this a commonly used model system to study pathogenesis of multiple different microorganisms. *C. elegans* also has a chitinous cuticle which provides a somewhat similar substrate to the chitinous lining of the flea proventriculus (Darby, Hsu et al. 2002, Darby, Ananth et al. 2005). For *Y. pestis* studies, *C. elegans* grows at a similar temperature as *X. cheopis* (20–23°C), which is beneficial since many of the *Y. pestis* genes are temperature regulated genes and only expressed during flea infection. *Y. pestis* is also capable of formimg biofilms during *C. elegans* infection; which inhibits *C. elegans* ability to feed and leads to the decreased survival of the nematodes (Darby, Hsu et al. 2002, Joshua, Karlyshev et al. 2003). Mutational analysis confirmed that the *hms* genes were required for both biofilm formation and *C. elegans* death, and subsequent studies found that *C. elegans* can be used to identify new potential transmission factors such as the *gmhA* gene of *Y. pestis* which is involved in biofilm formation in *C. elegans* and is required for blockage of the flea proventriculus (Darby, Ananth et al. 2005). In addition, Styer and colleagues demonstrated that *Y. pestis* can kill *C. elegans* through a biofilm-independent mechanism by finding that the *Y. pestis* KIM5 strain, which lacks the *hms* locus and the ability to make biofilms, colonizes the nematode intestinal tract and causes a lethal infection (Styer, Hopkins et al. 2005). The pCD1 and pPCP1 plasmids were also found to not be required for death in *C. elegans,* indicating that the genes on these plasmids do not contribute to nematode colonization or death. These findings suggest that in addition to a model to identify *Y. pestis* factors important for the insect portion of the *Y. pestis* lifecycle, *C. elegans* may also be used to identify new pathogenesis factors involved in mammalian disease (Lawrenz 2010).

1.17.2 Mammalian models to study plague virulence and pathogenesis:

For plague research, many different animal models have been used to study plague pathogenesis including many rodent species as well as non-human primates (NHPs). However, rodent models are the most widely used because of factors such as affordability, small size, and availability of facilities to house and perform containment work. In studies such as those involving vaccines or other therapeutics, which require further testing and validation prior to FDA approval, NHPs are commonly used as the model system. Many different models are used for plague studies because of the wide range of susceptible hosts to plague infection; however, each model has its limitations that could influence a researcher's selection of which system is the best plague model to use for the intended study.

1.17.2a Mouse Model:

The most widely used animal model for plague research is the mouse model. Mouse genomes are well-characterized allowing for the capability of making different knock-out and transgenic mouse strains. The mouse is highly susceptible to plague infection and equally susceptible to different strains of *Y. pestis* (Butler 1983, Goodin, Nellis et al. 2007). Furthermore, the mouse model can be used for pneumonic and intravenous infection models, but is unable to be used for bubonic infection in studies using strains of *Y. pestis* lacking the *pgm* locus (Burrows 1956, Une and Brubaker 1984, Une and Brubaker 1984). Any *Y. pestis* strains lacking the pgm locus (*pgm*−) can be handled as a biosafely level-2 (BSL-2) organism and are exempt from select agent status. Conveniently for laboratories which do not have a BSL-3 space, plague studies using the mouse model can still be performed using *pgm*(−) strains which are attenuated, but unable to be used in transmission studies. Therefore, it is essential that

findings with *pgm*(−) strains, especially in the context of the host, be confirmed with fully virulent *Y. pestis* to ensure that the host does not react differently to the attenuated strain.

For models of bubonic plague infection, mice can be infected via subcutaneous (s.c.) or intradermal injection. Disease progression via both routes is similar to transmission from infected fleas (Jarrett, Sebbane et al. 2004). The LD50 during subcutaneous (s.c.) infection in this model is less than 10 colony forming units (CFUs) (Cathelyn, Ellison et al. 2007). After inoculation with ~102 cfu, *Y. pestis* can be detected in the draining lymph nodes as early as 24 hours post infection (Lawrenz 2010). The bacteria continue to replicate and reach high concentrations in these tissues, similar to human plague, but lymphadenopathy may not be seen until later stages of infection. In these models, systemic infection begins 12–24 hours after lymph node colonization and is evident by isolation of bacteria from the spleen at 48 hours post infection. Lung colonization or (secondary pneumonic plague) via systemic spread occurs after spleen colonization but is seen prior to the animals succumbing to infection. Death most commonly occurs between 3 and 5 days post infection (Cathelyn, Ellison et al. 2007, Lawrenz 2010).

For models of pneumonic plague infection, mouse models are typically infected by intranasal or aerosol inoculation and results in an infection similar to human pneumonic plague (Lathem, Crosby et al. 2005, Agar, Sha et al. 2008). The LD50 for intranasal infection with *Y. pestis* CO92 is approximately 10-fold less than for aerosol inoculation (~250 cfu vs. 2100 cfu), although only approximately 10% of the inoculum reaches the lungs by the intranasal inoculation route (Meyer 1950, Lathem, Crosby et al. 2005). Bacterial numbers in the lungs steadily increase between 1 and 72 hours post infection regardless of inoculation method. Lung pathology appears mild at 24 hours but drastically worsens over the course of infection, resulting

in large areas of consolidation and inflammation by 72 hours. As seen in primary human pneumonic plague, bacteria disseminate from the lungs of infected mice to colonize the spleen by 36 hours post infection. Despite spleen colonization, bacteria are not detected in the blood until 72 hours after aerosol inoculation, which is 24 hours later than from intranasal infection when concentrations were greater than 104 cfu/ml (Agar, Sha et al. 2008, Sha, Agar et al. 2008). In both aerosol and intranasal infection, mice typically succumb to pneumonic plague infection by 72–96 hours after exposure (Lathem, Crosby et al. 2005, Agar, Sha et al. 2008).

One finding from use of the mouse pneumonic plague model found that a biphasic immune response occurs during plague infection (Lathem, Crosby et al. 2005, Bubeck and Dube 2007, Agar, Sha et al. 2008). During the first 48 hours of infection, *Y. pestis* steadily grows in the lungs of infected mice in the apparent absence of recognition by the host immune system. Concurrent with systemic spread of the infection, a sudden and strong inflammatory response is mounted by the host, which includes induction of proinflammatory cytokines and chemokines and infiltration of PMNs into the lungs. Despite this intense response, *Y. pestis* continues to grow unaltered. Animal death results from the combination of lung damage and the uncontrolled host immune response (Lawlor, Daskaleros et al. 1987, Bubeck and Dube 2007). These findings support the hypothesis that *Y. pestis* actively modulates the immune response through effectors (Yops and LcrV) secreted by the type III secretion system encoded on the pCD1 virulence plasmid (Cornelis 1997, Viboud and Bliska 2005). Interactions between *Y. pestis* and the mammalian immune system are also commonly studied using mouse model systems as well as understanding host determinants which may contribute to resistance to *Y. pestis* infection (Li and Yang 2008, Smiley 2008, Smiley 2008, Turner, McAllister et al. 2008). Potential plague therapeutics are also initially tested using mouse models to study pathogenesis. Numerous

potential vaccines, including subunit, live-attenuated, killed, and DNA vaccine candidates, have been screened for efficacy in the mouse model, in addition to testing novel therapeutics, such as monoclonal antibodies, new antimicrobial compounds, and new adjuvants to augment vaccine efficacy (Uddowla, Freytag et al. 2007, Smiley 2008, Smiley 2008, Amemiya, Meyers et al. 2009, Eisele and Anderson 2009). One important point to note is the vast difference between mouse and human anatomy and physiology making a huge difference in the interpretations of therapeutic studies. When using a mouse model to study human plague, one variance to note is the difference among the respiratory tracts of mice and humans. For instance, mice have increased surface area in their nasal passages, and have only a single lobe in their left lung, lack bronchioles, are unable to cough in response to mechanical stimulation, and exhibit different immune potentials in the lungs (Irwin, Averil et al. 1993, Mizgerd and Skerrett 2008). Furthermore, the immune systems of mice are different than that of humans, and results from the mouse models studies must be further tested in a more closely related model such and NHPs before being administered for human use (Mestas and Hughes 2004).

1.17.2b Guinea pig model:

Since the early 1900s, guinea pigs have been a commonly used plague animal model and still used in many vaccines studies (Qu, Shi et al. 2010). Guinea pigs are highly susceptible to plague infections, (subcutaneous LD50 < 10, aerosol LD50 ~40,000), and subcutaneous infection results in the development of papules at the infection site and lymphadenopathy in the draining lymph nodes (Welkos, Davis et al. 1995, Titball and Williamson 2001). Comparable to human plague, guinea pigs will develop septicemic infection and succumb to disease within 14 days (Pollitzer 1954), however, the pneumonic guinea pig model can differ dramatically from the

mouse and NHP models, because aerosol infections which initiate a primary pneumonia in mice or NHPs may not cause primary pneumonia in the guinea pigs. *Y. pestis* introduced into guinea pigs by aerosol inoculation will initially colonize the cervical lymph nodes, indicating that infection originates from the upper respiratory tract. Lung infections can still occur, but is more delayed and appears as a secondary pneumonic infection as a result of dissemination through the blood (Strong, Banks et al. 1912, Meyer 1961). Another variance in the guinea pig model is the differences in susceptibility to different *Y. pestis* strains compared to other models, such as certain auxotrophic strains, in which the guinea pig models are highly resistant; whereas these strains are found to be virulent in mice and NHP's (Burrows 1960, Burrows and Bacon 1960, Burrows 1963, Meyer, Hightower et al. 1974, Meyer, Smith et al. 1974, Oyston, Russell et al. 1996). The guinea pig is also resistant to infection by *Y. pestis* strains lacking the F1 capsule; in contrast to being virulent in the mouse model; therefore, testing of attenuated mutants or any potential live vaccine candidates which are identified using the guinea pig model will have to be further tested using other small animal models before progressing to NHP studies (Burrows and Bacon 1958, Welkos, Davis et al. 1995).

1.17.2c Rat Model:

Throughout history, the rat has been linked to urban plague outbreaks of human infections. Rat genetics are well-characterized making this model very useful for research. Rats are susceptible to *Y. pestis* infection, but are not commonly used in pathogenesis studies because of variability in disease progression during infection. A previous study found that laboratory and wild rats have an increased resistance to *Y. pestis* infection when compared to other rodent models (Chen, Foster et al. 1974). The lethal does for rats is much higher (1,000 fold higher for

subcutaneous infection) when compared to the mouse model. Recently, the inbred Brown Norway strain of *Rattus norvegicus* has been found to mimic human plague, making this a reproducible model to study human plague infections, and disease manifestation in this rat strain closely simulates human infection therefore making this model useful in studying interactions in the draining lymph nodes during bubonic infection (Sebbane, Gardner et al. 2005). Disease manifestations in the rat model follow the same pattern that is seen in human bubonic plague. First, the proximal lymph nodes were infected in the rat, followed by the blood, spleen, and lastly, the distal lymph nodes. The draining lymph node was rapidly colonized by *Y. pestis*, within 6 hours post infection, and extracellular bacteria were visible by 24 hours post infection. As plague infection progresses, the bacterial numbers increase leading to lymphadenopathy, hemorrhage, necrosis, and other pathologies characteristic of human buboes. Lastly, the plague bacteria spread to the blood causing bacteremia, and disseminate to the spleen; which is typically seen in septicemic plague (Sebbane, Gardner et al. 2005).

The Brown Norway model has more recently been used as a model for pneumonic plague infections (Agar, Sha et al. 2009, Agar, Sha et al. 2009, Anderson, Ciletti et al. 2009). Infections were acquired by intranasal inoculation, and the LD_{50} for *Y. pestis* CO92 in the rat was comparable to that of mice (~200 cfu) (Anderson, Ciletti et al. 2009). Other studies further confirmed the intranasal LD_{50} for aerosol transmission was similar to that of mice ($\sim 1.6 \times 10^3$ cfu) (Agar, Sha et al. 2009, Agar, Sha et al. 2009). When rats are subjected to intranasal infection, rats appeared healthy until 36 hours post infection, in which they began to demonstrate a hunched posture. Next, the rats' condition quickly deteriorated and the animal succumbed to infection between 3 and 4 days post infection (Anderson, Ciletti et al. 2009). Lung inflammation in rats after aerosol inoculation could be observed by 24 hours; whereas, rats

which were infected by intranasal inoculation, or in the pneumonic mouse models, no lung inflammation was observed until 36 hours post infection (Lathem, Crosby et al. 2005, Agar, Sha et al. 2008). One unique element of the rat model is the capability of rat-to-rat transmission to occur (~33% transmission rate), which has never been demonstrated in mouse models (Agar, Sha et al. 2009, Agar, Sha et al. 2009). This transmission proposes that the rat may be used to model person-to-person transmission of primary pneumonic plague, as well as the examining lymph node disease between humans and the rat making this a relevant model to study pathogenesis in tissues (Sebbane, Gardner et al. 2005).

1.17.3 Non-human Primates (NHPs) models:

The most expensive but perhaps most relevant animal models are the non-human primates. Research using NHPs requires specialized facilities and experienced handlers. Because of this, limitations on the use of NHP models are imposed when studying *Y. pestis* pathogenesis (Patterson and Carrion 2005). The close relationship of humans to NHPs makes this an extremely important model when studying disease progression and manifestations of *Y. pestis*, as well as testing any potential plague vaccines or therapeutics. Most commonly used NHP species for studying plague are the *Rhesus macaques, Chlorocebus aethiops* (African green monkeys), and the *Cynomolgus macaques*.

1.17.3a *Rhesus macaques* model:

Pneumonic plague infection in the Rhesus macaques is very comparable to human infection with a very short incubation period and the first signs of infection are observed within 2 days after exposure (Speck and Wolochow 1957, Finegold 1968, Finegold 1968, Finegold,

Petery et al. 1968, Finegold 1969). The mean time to death for infected Rhesus macaques is approximately 6 days, and animals developed all stages of pneumonia during the course of infection. Although disease progression appears similar to humans, Rhesus monkeys are significantly more resistant to plague infection (Ehrenkranz and Meyer 1955, Speck and Wolochow 1957). The LD_{50} for *Y. pestis* in the Rhesus model can be as much as 10-fold higher for pneumonic infection, and several thousand-fold higher for bubonic infection when compared to the infectious doses of other NHP models or human infections. Furthermore, Rhesus macaques have demonstrated a tendency to develop a form of plague referred to as chronic pneumonic plague, which is very rarely seen in humans (Ransom and Krueger 1954). These differences in the Rhesus model should be considered when using this model to evaluate pathogenesis of plague or new plague therapeutics.

1.17.3b *Chlorocebus aethiops* (African green monkey) model:

The African green monkey is highly susceptible to plague infection, unlike the Rhesus model. The LD_{50} of *Y. pestis* appears to be slightly lower than the predicted doses for humans (~100 cfu), with a LD_{50} ~300 cfu by aerosol transmission and ~5–50 cfu by intradermal inoculation (Chen, Elberg et al. 1977, Powell, Andrews et al. 2005). Even with this increased susceptibility, disease manifestations and pathology in aerosol infected African green monkeys in a similar way to that of human pneumonic plague patients (Layton, Brasel et al. 2011). *Y. pestis* bacteria grow to high numbers in the lungs, which results in severe pneumonia, and dissemination to the blood around 2 days post infection. The African green monkeys also develop fever at various time points post infection; however, most fever cases were not observed until after bacteremia and approximately 24 hours before succumbing to infection. The mean

time to death for aerosol infected African green monkeys is approximately 4 days post exposure (Layton, Brasel et al. 2011).

1.17.3c *Cynomolgus macaques* model:

The Cynomolgus macaque model has been extensively used in the past for plague vaccine and pathogenesis studies. The Cynomolgus macaque is highly susceptible to aerosolized plague with the LD_{50} ~300 cfu's and the infected animal manifests clinical signs similar to human pneumonic plague, in which animals exhibit a fever as their first clinical symptom (Powell, Bishop et al. 1998, Powell, Andrews et al. 2005, Koster, Perlin et al. 2010). No additional clinical symptoms were observed until 24 hours post infection when the macaques showed signs of lethargy and anorexia; however, no respiratory distress or coughing were observed even though histology showed lung damage. The pathogen ultimately disseminated from the lungs to the liver and spleen through the blood stream (Koster, Perlin et al. 2010).

The use of NHPs as models for human vaccine efficiency are still the most beneficial; however, a better understanding of how protective a human vaccine will be is extremely challenging. For example, multiple studies found that antibody titer directly correlated with successful protection; however, other studies have discovered that antibody titer may not guarantee successful vaccination and cellular responses should also be analyzed (Smiley 2008, Smiley 2008). In order for a model to successfully predict plague protection, further characterization of the NHP plague models, as well as immune responses to vaccines will have to be further assessed before a successful plague vaccine can be developed.

1.18. Detection Assays Used for *Y. pestis* Identification in Animals and Fleas

1.18.1 Rodents and other animal populations:

For *Y. pestis* identification in dead rodents or animal populations during or after die-offs or ratfalls, direct immunofluorescence assays, agglutination assays, enzyme-linked immuosorbent assays, or isolation of the organism in pure culture are the most commonly used detection methods. The best method for rapid and accurate detection for routine plague surveillance is direct immunofluorescence assays. Some advantages of this assay is its high sensitivity and specificity, when used appropriately with the necessary controls and plague-specific conjugates, and quick turn around time which is usually less than two hours. This assay detects the presence of the *Y. pestis* F1 capsular antigen. This assay is a useful tool specifically during emergency situations, when results can be relayed to local officials of positive test results on the same day as the specimens are received. Another advantage is *Y. pestis* can be detected in carcasses after the animal has been deceased for long periods from days to even weeks if samples are taken from the long bones, such as the femur, of the animal. The *Y. pestis*-Fraction-1 specific fluorescent antibody conjugates are made by hyperimmunizing rabbits with purified *Y. pestis*-Fraction 1 antigen, followed by collection of the high titer antibody from the rabbits, and finally conjugation of a fluorescent label. A conclusive diagnosis of plague in rodents relies primarily on isolated *Y. pestis* from the animal tissues, typically from the liver and spleen, and isolation of a *Y. pestis* isolate takes 48 hours; therefore, this method is a much more time consuming test, than the previously described direct immunofluorescence assay. Animal necropsies of the liver and spleen are streaked out on sheep blood agar (SBA) and incubated at 28°C for 48 hours, and observed for typical plague morphology. Colonies exhibiting typical plague morphology on the

SBA plates, are further confirmed by performing a specific bacteriophage lysis assay. This test is commonly used to identify bacteria which are grouped or subtyped since the bacteriophages are viruses adapted for specific host cells; which in turn helps identify the host strain. *Y. pestis* cells are sensitive to lysis by temperature-specific bacteriophage; therefore making this assay successful in identification and confirmation of positive *Y. pestis* samples (Plague Manual, 1999a).

1.18.2 Detection of *Y. pestis* in fleas:

In areas where recent rodent die-offs or rat-falls occur, fleas in the area may be collected and tested for presence of the plague pathogen. Fleas can be collected from rodent burrows by burrow swabbing or flagging. A burrow swab consists of a flexible steel cable or hard rubber hose with a piece of white flannel cloth attached to the end. This cable is used to power the cloth down the burrow hole; in which fleas will cling to the cloth. The cloth is then removed from the burrow, and if fleas are attached to the cloth, it will be placed in a plastic bag and transported back to the laboratory where the flags (cloths) will be placed in the freezer to kill the fleas. Each dead flea will then be placed individually in an Eppendorf tube, and Heart Infusion Broth (HIB) will be added to each tube. Fleas are then homogenized using a sterile teflon pestle in order to disrupt the gut contents where *Y. pestis* will be present. Each flea triturate will then be plated on SBA plates and incubated at 28°C for 48 hours. Any colonies with typical plague morphology will be further confirmed at *Y. pestis* by performing the phage lysis assay. Plague colonies can also be grown up in pure culture and passaged through a laboratory mouse for further confirmation of the plague pathogen. At the United States CDC, the standard procedure to prepare fleas for inoculation is to grind flea pools in a mortar and pestle and further suspend the

ground material in 2 ml of physiological saline. This suspension will then be used to inoculate mice subcutaneously (0.5 ml per mouse). Mice are then monitored for 21 days for signs of infection. Any sick or dead mice will then be necropsied to collected tissues which will be used for bacterial isolation. Any mice surviving out to day 21 post infection will be euthanized and serum tested for presence of *Y. pestis* infection. Other methods for *Y. pestis* detection include polymerase chain reaction (PCR), which is highly sensitive and reliable; however, false positives and contamination can be an issue (Plague Manual, 1999a).

1.19. Next Generation Sequencing (NGS) for Transcriptomics Analysis

Next generation sequencing (NGS) is a state of the art technology allowing researchers to investigate cellular states, physiology, and gene activity. NGS has quickly became the method of choice for transcriptional profiling experiments. In contrast to microarray technology, high throughput sequencing allows identification of novel transcripts, does not require a sequenced genome and avoids background noise associated with fluorescence quantification. Furthermore, unlike hybridization-based detection, RNA-seq allows genome wide analysis of transcription at single nucleotide resolution, including identification of alternative splicing events and post-transcriptional RNA editing events. NGS is commonly used for whole transcriptome sequencing and includes many applications such as simple messenger RNA (mRNA) profiling for discovery and analysis of the entire transcriptome, including both coding mRNA and non-coding RNA (ncRNA) (e.g., micro RNA, small RNAs) (Martin, Wang et al. 2012). These applications, as a group are termed RNA-seq, and have become popular because of the capability of exposing new genetic information that may have been missed by array-based platforms since no prior knowledge of the gene sequence is necessary. A transcriptome is defined as the whole set of

RNAs that are transcribed by the genome from a specific tissue or cell type at a specific developmental stage or under a certain physiological condition (Willingham, Dike et al. 2006, Jacquier 2009). RNA-seq uses data from millions of short sequence reads, which are aligned to a reference genome, and can be used to perform comparative transcriptomics analyses to get a global view of the cell's response to a given environment or stimulus. RNA-seq was initially used for discovery applications such as rare genes, splice junctions, and gene fusions, as well as, with novel or poorly studied organisms for which there was no good standard microarrays. More recently, RNA-seq has become more widely available and the overall costs have been decreasing. Specialty applications can also be performed using RNA-seq such as RNA editing and allele specific expression since the technology is sequenced based (Qi, Liu et al. 2011, Martin, Wang et al. 2012). While there are a variety of RNA-seq applications and protocols, most follow the basic strategy of isolation of total RNA, following removal of the ribosomal RNA (rRNA) since rRNA accounts for 95-98% of the transcriptome, and is beneficial to eliminate as much of the rRNA as possible prior to sequencing. Commercially available kits are commonly used for ribodepletion, and these work by using oligos which are complementary to known rRNA sequences to sequester the ribosomal RNA molecules. Following removal of rRNA, the remaining RNA is fragmented and adaptor sequences are ligated and hybridized to each RNA fragment, and first strand cDNA is synthesized. Next, the cDNA is amplified in order to generate the cDNA library. After the cDNA libraries are constructed, they are used as sequencing templates for NGS. Following sequencing, bioinformatics analysis is performed which maps each sequencing read to a reference genome, and further comparative analysis can be performed to determine differentially expressed genes (DEGs). NGS platforms can be used for transcriptome profiling, miRNA profiling, DNA-protein interaction studies using chromatin

immunoprecipitation (ChIP), and DNA methylation studies (Wang, Gerstein et al. 2009, Wang, Feng et al. 2010).

A significant part of transcriptome research is the bioinformatics analysis. The bioinformatics analyses of these studies involves massive amounts of data, in addition to the complexity of the transcriptome. A typical RNA-seq data analysis typically includes the following steps (i) Low-quality sequences produced from library construction or the sequencing process are trimmed and removed. (ii) If a reference genome is available, then the millions of short reads are mapped to the reference genome, the expression level of each transscript is calculated, and then differentially expressed genes (DEGs) across the samples are compared (Li, Dong et al. 2013). For studies in which no reference genome is available, de novo transcriptome assembly is performed from short RNA-seq reads and then assembled contigs are subjected to functional annotation, which requires extensive computer resources (Grabherr, Haas et al. 2011). Further specific analysis will be performed to investigate specific questions involved in transcriptomics, such as analysis of RNA editing and ncRNAs, discovery of novel transcripts, and correlation of transcriptome data to available genomic or epigenetic data (Li, Morgenroth et al. 2008, Li, Dong et al. 2013, Zhang, Wang et al. 2014). RNA-seq technology has created the ability to obtain a global view of the transcriptome in normal and pathological processes, or in different environmental conditions, which contributes to a greater understanding and novel insights on cellular functions and differential gene expression.

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CHAPTER II

Effects of Low Temperature Flea Maintenance on the Transmission of *Yersinia pestis* by *Oropsylla Montana*

Overview

Yersinia pestis, the causative agent of plague, is primarily a rodent-associated, flea-borne zoonosis maintained in sylvatic foci throughout western North America. Transmission to humans is mediated most commonly by the flea vector, *Oropsylla montana*, and occurs predominantly in the Southwestern United States. With few exceptions, previous studies showed *O. montana* to be an inefficient vector at transmitting *Y. pestis* at ambient temperatures, particularly when such fleas were fed on susceptible hosts more than a few days after ingesting an infectious blood meal. We examined whether holding fleas at sub-ambient temperatures affected the transmissibility of *Y. pestis* by this vector. An infectious blood meal containing a virulent *Y. pestis* strain (CO96-3188) was given to colony-reared *O. montana* fleas. Potentially infected fleas were maintained at different temperatures (6ºC, 10°C, 15°C, or 23ºC). Transmission efficiencies were tested by allowing up to 15 infectious fleas to feed on each of seven naïve CD-1 mice on days 1-4, 7, 10, 14, 17, and 21 post infection (p.i.). Mice were monitored for signs of infection for 21 days after exposure to infectious fleas. Fleas held at 6ºC, 10°C and 15°C were able to effectively transmit at every time point p.i. The percentage of transmission to naïve mice by fleas maintained at low temperatures (46.0% at 6ºC, 71.4% at 10°C, 66.7% at 15°C) was higher than for fleas maintained at 23ºC (25.4%) and indicates that *O. montana* fleas efficiently transmit *Y. pestis* at low temperatures. Moreover, pooled percent per flea transmission efficiencies for flea cohorts maintained at temperatures of 10ºC and 15ºC (8.67 and 7.87 percent, respectively) showed a statistically significant difference in the pooled percent

per flea transmission efficiency from fleas maintained at 23ºC (1.94 percent). This is the first comprehensive study to demonstrate efficient transmission of *Y. pestis* by *O. montana* fleas maintained at temperatures as low as 6ºC. Our findings further contribute to the understanding of plague ecology in temperate climates by providing support for the hypothesis that *Y. pestis* is able to overwinter within the flea gut and potentially cause infection during the following transmission season. The findings also might hold implications for explaining the focality of plague in tropical regions.

2.1. Introduction

Yersinia pestis, a Gram-negative bacterium and agent of plague, is a recently emerged clone of *Yersinia pseudotuberculosis* having evolved within the past 1,500-20,000 years (Achtman, Zurth et al. 1999). The distinct life cycles of these two organisms can likely be explained as a function of both genetic loss and the recent acquisition of the unique plasmids pPCP and pMT by *Y. pestis* (Ferber and Brubaker 1981, Chain, Carniel et al. 2004, Chain, Hu et al. 2006). In humans, the enteropathogenic *Y. pseudotuberculosis* promotes a relatively benign food-borne gastrointestinal illness while *Y. pestis* causes a severe, acute, often fatal disease that has been the cause of three historic pandemics including the Black Death which was responsible for millions of deaths in 14th century Europe .

Despite its historical human toll, plague is primarily a flea-borne zoonosis of rodents, though nearly all mammals are susceptible to infection with the highly virulent plague bacterium and some experience severe disease (Pollitzer 1954, Gage and Kosoy 2005). Plague is thought to persist in nature in enzootic or maintenance cycles involving transmission between rodents and their associated fleas (Gage and Kosoy 2005). Periods of enzootic transmission are characterized

by low to virtually undetectable levels of rodent mortality. Although this might be explained simply by low level but sustainable transmission between rodents and their fleas, the actual mechanism(s) of persistence of *Y. pestis* during enzootic or interepizootic periods is still a subject of much speculation (Gage and Kosoy 2005, Eisen and Gage 2009). However, under favorable conditions, which are likely to include certain abiotic environmental factors, such as those related to climate, as well as the abundances of both fleas and susceptible mammalian hosts (amplifying hosts), rapidly spreading epizootics can occur that often have devastating effects on local populations of rodents and, occasionally, other wildlife species. Periods of epizootic activity also can pose significantly increased risk for human plague, as infected fleas seek alternative hosts (Poland and Barnes 1979, Poland, Quan et al. 1994, Gage, Ostfeld et al. 1995, Gage and Kosoy 2005, Eisen and Gage 2009).

Transmission of *Y. pestis* typically occurs through the bite of an infectious flea that has fed on a heavily bacteremic host. Subsequent transmission to a naïve host has been reported to be dependent on the ability of the plague bacteria to colonize the midgut and proventriculus of the flea, eventually forming bacteria-laden masses large enough to occlude the proventricular valve separating the foregut from the midgut. This blockage phenomenon, which was first described by Bacot and Martin (Bacot and Martin 1914) in the flea *Xenopsylla cheopis* and quickly became the prevailing paradigm for flea-borne transmission of *Y. pestis,* prevents the flow of blood to the midgut resulting in starvation of the flea. Blocked fleas, though tenacious in their repeated attempts to feed, are typically unsuccessful in moving blood past the proventricular blockage. Consequently, blood and *Y. pestis* bacteria are ostensibly flushed back into the bite site thus infecting the parasitized host (Gage and Kosoy 2005). More recently, the molecular mechanisms responsible for blockage-dependent transmission of *Y. pestis* from flea to mammalian host have

been elucidated (Hinnebusch, Perry et al. 1996, Perry and Fetherston 1997, Hinnebusch 2005). *Y. pestis* forms a biofilm whose synthesis is temperature-dependent and reliant on the gene products of the *hms* (hemin storage locus) operon involved in the adsorption of exogenous hemin or the dye Congo Red and which comprises three gene loci (Perry, Pendrak et al. 1990, Hinnebusch, Perry et al. 1996, Perry and Fetherston 1997, Jones, Lilliard et al. 1999, Kirillina, Fetherston et al. 2004). Moreover, regulation of biofilm production occurs post-transcriptionally and synthesis of this polymeric β-1,6-N-acetyglucosamine-containing extracellular polysaccharide matrix appears optimal at ambient temperatures (23-28ºC) and thus conducive to the environment of the flea gut (Kirillina, Fetherston et al. 2004, Perry, Bobrov et al. 2004, Bobrov, Kirillina et al. 2008, Hinnebusch and Erickson 2008). Recently, Sun *et al.* (Sun, Koumoutsi et al. 2011) described the differential control of *Y. pestis* biofilm formation *in vitro* and in the flea gut by the DGCs HmsT and Y3730, respectively, with the latter exhibiting significantly greater involvement in blockage formation within the flea, *X. cheopis.* While biofilm-mediated proventricular blockage has been the prevailing paradigm for flea-borne transmission of plague for nearly a century, recent evidence has demonstrated that unblocked fleas are also capable of transmitting *Y. pestis* almost immediately after obtaining an infectious bloodmeal, with the time to transmission being largely determined by how soon an infected flea will take its next bloodmeal (Eisen, Bearden et al. 2006, Eisen, Wilder et al. 2007, Eisen, Borchert et al. 2008, Eisen, Holmes et al. 2008, Wilder, Eisen et al. 2008, Wilder, Eisen et al. 2008). This so-called early phase transmission (EPT) model, which focuses on the ability of unblocked fleas to transmit for a period of a few days post-infectious feeding (~ 4 days), may be a means of explaining the dynamics of rapidly moving plague epizootics (Eisen, Bearden et al. 2006). Moreover, EPT can occur in the absence of biofilm formation though biofilm likely is

required for long-term maintenance of *Y. pestis* infection in fleas (Vetter, Eisen et al. 2010 Vetter et al., unpublished data). Further, Schotthoefer et al. (2011b) observed that infectious *X. cheopis* fleas can successfully transmit *Y. pestis* during EPT at temperatures up to 30ºC, a temperature that equals or exceeds what has historically has been reported to result in blockage inhibition, low transmission rates, and higher clearance of infection (Kartman 1969, Cavanaugh 1971, Hinnebusch, Fischer et al. 1998, Schotthoefer, Bearden et al. 2011).

The relationship between climate, temperature and plague transmission has been studied for decades. Climatic conditions have been shown to influence the prevalence of plague with variations in temperature, relative humidity, vapor pressure deficits, and precipitation as factors either favoring epidemic spread of plague or causing its rapid decline with the latter being significantly influenced by temperatures that exceed 27-28ºC (Cavanaugh 1971, Cavanaugh and Marshall 1972, Montminy, Khan et al. 2006, Pham, Dang et al. 2009). Similarly, in the southwestern US, increased late winter-early spring precipitation (Parmenter, Yadav et al. 1999) or time-lagged increases in late winter precipitation correlated positively with increased human plague cases, while case declines were significantly impacted by the number of days exceeding threshold temperatures of 32 or 35ºC, depending on the area investigated (Enscore, Biggerstaff et al. 2002, Gage, Burkot et al. 2008). While recent studies have demonstrated successful transmission of *Y. pestis* by *X. cheopis* fleas at temperatures up to 30ºC during the early phase (days 1-4 p.i.) (Schotthoefer, Bearden et al. 2011) and late phase periods (days 7-28 p.i.) (Rouviere and Gross 1996), *X. cheopis*-mediated transmission of *Y. pestis* at low temperatures has met with limited success due either to increased mortality of this tropical flea species (Rouviere and Gross 1996) or prolonged extrinsic incubation periods (Kartman 1969). In the current study, we report on the successful transmission of *Y. pestis* by the temperate zone ground

squirrel flea *O. montana* maintained at temperatures as low as 6ºC over a period of 21 days p.i.. Exposure of fleas to sub-ambient temperatures is not without precedent as ground squirrel burrow temperatures reportedly have been measured at 15ºC (Telepnev, Klimpel et al. 2009). The implications of low temperature growth, survival, and persistence within the *O. montana* gut as a means of explaining the potential for *Y. pestis* to persist during the colder winter months and remain viable transmitters of the plague bacillus during the subsequent transmission season are discussed. We also discuss the implications of these findings for the potential role of fleas as reservoirs of *Y. pestis* and the maintenance of long-term plague foci in both temperate and tropical regions.

2.2. Materials and Methods

Yersinia pestis *growth conditions:*

The bacterial strain used in this study, CO96-3188 (Pgm+, pCD1+, pMT1+, pPCP1+) (Engelthaler and Gage 2000), is a fully virulent North American strain of *Y. pestis*. Culture stocks of CO96-3188 were maintained in heart infusion broth (HIB) supplemented with 10% glycerol and stored at -80°C. For artificial infection of fleas, *Y. pestis* CO96-3188 was grown in HIB and incubated at 28°C overnight for 14-16 h with shaking at 175 rpm.

Infection of fleas:

Colony-reared, *Oropsylla montana*, the California ground squirrel flea, were obtained from the Centers for Disease Control and Prevention/Division of Vector-Borne Diseases for artificial infection with *Y. pestis* and for transmission feeds on 6-8 week-old naïve, female Charles River (Charles River Laboratories International, Inc., Wilmington, MA) CD-1 outbred mice obtained

from the specific pathogen-free mouse colony maintained by the Division of Vector-Borne Diseases, Fort Collins, CO.

The artificial feeding procedure used to feed *O. montana* fleas an infected bloodmeal was performed as previously described (Eisen, Bearden et al. 2006). Briefly, *O. montana* male and female adult fleas were randomly collected and removed from an established colony and starved for 4 days at 23°C. An overnight culture of CO96-3188 was prepared by inoculating 60 ml of HIB with a 0.5 ml aliquot of bacterial glycerol stock and grown as described above. Bacteria were pelleted by centrifugation and resuspended in 40 ml of defibrinated Sprague-Dawley strain rat blood (Bioreclamation, Jericho, NY) pre-warmed to 37°C. Artificial feeders, each containing approximately 200 *O. montana* fleas, were prepared according to Eisen et al. (Eisen, Bearden et al. 2006) and fleas were allowed to feed for 1 h through mouse skins taken from euthanized, hairless SKH-1 mice (specific pathogen-free mouse colony maintained by the Division of Vector-Borne Diseases, Fort Collins, CO) on blood infected with 10^8-10^9 CFU ml^{-1} of *Y. pestis* CO96-3188. Three independent artificial infections of *O. montana* fleas were performed for each temperature. Fleas were then examined by light microscopy to determine whether fleas had consumed a potentially infectious bloodmeal. Fed fleas were collected and maintained in 25 cm^2 tissue culture flasks (Corning, Lowell, MA) at their respective treatment temperature (6°C, 10°C, 15°C, or 23°C) and held in acrylic dessication chambers (Thermo Scientific, Rockford, IL) containing beakers of saturated potassium chloride (KCl) solution to maintain relative humidity at 80-85%. Fleas which did not feed on the infected blood were discarded.

Flea-borne transmission to naïve mice:

Transmission feeds were performed on days 1-4, 7, 10, 14, 17, and 21 post infection (p.i.) using fleas maintained at each temperature to determine the relative efficiencies of transmission

of low temperature fleas compared to those maintained at 23ºC. Capsules were affixed to seven 6-8 week-old, naïve, specific pathogen free, CD-1, outbred mice (CDC, DVBID mouse colony, Fort Collins, CO), and pools of ~15 potentially infectious fleas were allowed to feed for 1 h (Eisen, Bearden et al. 2006). Fleas were then collected, and viewed by light microscopy to determine fed status as well as the sex of each flea in the pool. Fleas were stored individually in microcentrifuge tubes and placed at -80°C for later analysis to determine infection prevalence and bacterial loads. Exposed mice were held in individually ventilated cages (Tecniplast, Philadelphia, PA) at 27ºC and monitored for signs of infection (e.g. ruffled fur, lethargy, hunched posture, slow response to stimuli). At first sign of illness, mice were euthanized and later necropsied to detect *Y. pestis* infection by performing *Y. pestis* anti-F1 antigen direct fluorescent antibody assays (DFA) on liver and spleen smears. Positive transmission events were confirmed by specific bacteriophage lysis of bacterial colonies isolated from mouse tissues (Chu 2000). Though subjective in nature, monitoring for distinct signs of illness in exposed mice correlated well with the presence of *Y. pestis* organisms in mouse tissues following euthanasia and necropsy. There were no instances in which mice were euthanized and *Y. pestis* bacteria were not recovered. Time to death or time to euthanasia for individual mice for each time point and temperature also was recorded. Mice showing no signs of infection were held for up to 21 days p.i. before being euthanized. Animal procedures used in this study were approved by the Centers for Disease Control and Prevention, Division of Vector-Borne Diseases, Institutional Animal Care and Use Committee (Protocol #09-020).

In Vitro Bacterial Viability Curve:

A 0.5 mL aliquot of frozen glycerol stock of strain CO96-3188 was used to inoculate 3 ml of HIB and grown for 16 h at 28°C and 175 rpm. Overnight cultures were diluted 1:50 into 15 ml

of fresh HIB to achieve a bacterial concentration of ~1 x 10^7 CFU mL^{-1}. Cultures were grown statically (in the absence of aeration) and maintained at 6°C, 10°C, 15°C, and 23°C. Cultures were inoculated in duplicate for each growth temperature. Aliquots from each tube were obtained at time 0 h and decimally diluted in saline, plated on blood agar containing 6% sheep blood (SBA) in duplicate, and incubated at 28°C for 48 h to determine initial bacterial concentrations. Broth cultures were incubated at their appropriate temperature for a total of 5 days, and aliquots of each culture tube were serially diluted and plated in duplicate on SBA at times: 0, 4, 8, 12, 24, 36, 48, 60, 72, 84, 96, 108, and 120 h. Bacterial colonies were enumerated and recorded to determine viable bacteria at each time point and incubation temperature.

Flea infection prevalence and bacterial loads:

Fleas were homogenized in 100 µL of HIB + 10% glycerol using sterile Teflon pestles and plated on SBA medium to determine infection status. The number of artificially fed fleas that were both infected and uninfected and which fed on naïve mice were used in determining the infection prevalence of fleas at each time point and incubation temperature. Bacterial loads of *Y. pestis*-infected fleas were estimated by decimally diluting individual flea lysates and plating on SBA media in duplicate. Per flea bacterial load estimates were used in calculating the median bacterial burden of fleas at each time point and temperature as well as computing the average of the log-transformed sum of bacteria within groups of fleas which fed on naïve mice at each time point and temperature (average logsum, described below).

Statistical Analyses:

Maximum likelihood estimates for percent per flea transmission rates were calculated by using the Microsoft Excel Add-In PooledInfRate v. 4.0 (Ransom and Krueger 1954), for each time point at each temperature. Estimates were based on the number of infected fleas that were

also determined to have fed on an individual mouse and whether subsequent transmission occurred in that mouse. Pooled percent per flea infection rates were also used to analyze the effect of day p.i. as well as temperature on transmission by using a pooled binomial regression algorithm in R (binGroup package, the Comprehensive R Archive Network (CRAN), http://cran.r-project.org/web/packages/binGroup/index.html).

Pooled percent per flea transmission rates for each temperature, total mouse transmission rates for each temperature, and bacterial viability curve analysis by temperature, time point, and bacterial counts were compared using one-way ANOVA. The average logsum of bacterial CFU of infected, fed fleas at each time point were compared between temperatures using one-way ANOVA with Dunnett's post-hoc control test. Logsum data comprise the log-transformed sum of the bacterial loads of all infected fleas that fed on an individual naïve mouse for a particular time point and temperature. Logsum calculations were determined for each group of fleas which fed on mice within a cohort (7 mice) for each time point at a particular treatment temperature. The bacterial logsum value to which each individual mouse within a cohort was exposed, were then averaged to obtain a single logsum value at each time point for all four temperatures. These averaged logsum values were then used in further statistical analyses. Differences among or between groups were considered statistically significant at $P < 0.05$. Contingency table analysis was also performed to determine differences in transmission efficiencies for each temperature and day p.i. Wilcoxon/Kruskal-Wallis tests (rank sums) were used to establish the significance of incubation temperature on i) *in vitro* bacterial viability, ii) average bacterial logsum data, iii) pooled percent per flea transmission rate, iv) mouse infection rate, and v) median flea bacterial loads. All statistical comparisons were performed using JMP statistical software (SAS Institute, Cary, NC).

2.3. Results

Effects of temperature on flea transmission efficiencies:

Y. pestis transmission was observed on each challenge day post infection (p.i.) using fleas maintained at each of the three low temperatures tested (6°C, 10°C, and 15°C); whereas no transmission was observed on day's 3 and 4 p.i. from fleas held at ambient temperature (i.e. 23°C) (Table 2.1). With the exception of results for the *in vitro* bacterial viability curve (see below), all references to temperatures and numerical notations for specific temperatures (eg. 10ºC) refer to the maintenance temperatures of infected fleas and not to temperatures at which mice were exposed. All mice used in this study were held at a constant temperature of 27ºC. Percent per flea transmission efficiencies, which estimate the number of infected fleas that were also determined to have fed on an individual mouse and whether subsequent transmission occurred in that mouse, were highest at 10°C. The lowest percent per flea transmission efficiency (1.02%) was exhibited at day 1 p.i. for 10º C, with transmission efficiency for the remaining eight time points ranging from 5.55-13.09%, with day 17 p.i. showing the highest observed percent per flea transmission efficiency (Table 2.1). Similarly, percent per flea transmission efficiencies were observed at 15°C ranged from 1.12-15.05%, excluding days 10, 14, and 17 which showed percent per flea transmission efficiency estimated at 100%). Percent per flea transmission efficiencies at 6°C ranged from 0.96-7.52% with successful transmission events observed on each challenge day (Table 2.1). Surprisingly, the lowest overall percent per flea transmission efficiencies were observed at 23ºC, the temperature at which most previous transmission studies for *O. montana* has been performed (Burroughs 1947, Eisen, Bearden et al. 2006, Eisen, Lowell et al. 2007). In the current study, percent per flea transmission efficiencies ranged from 0.00-3.45%, in which no successful transmission events were observed on days 3

and 4 p.i. (Table 2.1). Pooled percent per flea transmission rates, incorporating all days p.i., were 4.11 (95% CI, 2.83-5.84), 8.67 (95% CI, 6.43-11.65), 7.87 (95% CI, 5.82-10.58), and 1.94 (95% CI, 1.16-3.09) for 6,°C 10°C, 15°C, and 23°C, respectively. By pair-wise comparisons, significant differences in pooled percent per flea transmission rates were observed between temperatures, 6°C and 10°C ($P = 0.0170$), 10°C and 23°C ($P = 0.0022$), and 15°C and 23°C ($P = 0.0136$) (Figure 2.1). Pooled binomial logistic regression analysis by temperature incorporating simultaneous 95% confidence intervals for each time point also demonstrated significant differences between 6°C and 10°C, 10°C and 23°C, and 15°C and 23°C ($P < 0.05$). Logistic regression analysis by day p.i. showed no significant differences in pooled percent per flea transmission rate with the exception of day 1 versus day 14 p.i. ($P < 0.05$). Overall, the data reflect that pooled percent per flea infection rate was influenced by temperature. ($\chi^2 = 13.69$, df=3, $P = 0.0034$).

The number of successful transmission events directly correlated with the pooled percent per flea transmission rates. Late phase time points (day's 7-21 p.i.) showed the highest rates of transmission with days 10, 14, and 17 p.i. showing 100% transmission in all seven mice challenged at 15ºC (Table 2.1). Consistent with the 10°C percent per flea transmission efficiencies being the highest, the number of total transmission events that occurred at this temperature was also found to be the highest with 46 out of the 63 (71.4%) mice exposed succumbing to infection by *Y. pestis* (Table 2.1). Transmission events for days p.i. 2-21, ranged from 57.1-85.7%, with only a single successful transmission occurring on day 1 p.i. for 10ºC fleas. Transmission events at 15°C were also successful at each of the nine time points with the fewest number of events observed on day 4 and 100% transmission occurring on days 10, 14, and 17 p.i. (Table 2.1). Transmission rates to naïve mice ranged from 14.3-100% for fleas

maintained at this temperature. For *Y. pestis*-infected fleas maintained at 6ºC, 46.0% (29 of 63) of naïve mice challenged, successfully became infected and died, with each time point having successful transmission events (range, 14.3-71.4%). The lowest number of transmission events occurred at 23°C on days 3 and 4 with no successful transmission, and overall, only 25.4% (16 of 63) of mice exposed to fleas at this temperature resulted in mortal infection by the pathogen (range, 0-42.9%) (Figure 2.2). Flea maintenance temperature was a significant factor in mouse mortality ($\chi^2 = 13.52$, df = 3, $P = 0.0036$). Similar to pair-wise comparisons for pooled percent per flea transmission, statistical significance in the number of successful transmission events were found between 6°C vs. 10°C ($P = 0.0343$), 10°C vs. 23°C ($P = 0.0003$), and 15°C vs. 23°C ($P = 0.011$) (Figure 2.2). Moreover, binomial logistic regression analysis revealed flea maintenance temperature and day p.i. as significant predictors of the rate of mouse infection. Specifically, as day p.i. increased, the probability of a successful transmission event occurring also increased (all P values < 0.05).

Effects of temperature on* Y. pestis *bacterial loads in fleas:

Individually homogenized infected fleas maintained at each of the four temperatures which fed on exposed naïve mice were quantified to determine infection prevalence and bacterial loads. Though more female than male fleas were used in our study, no significant differences were found between male and female fleas with regard to feeding rate and infection prevalence and thus, sex of the flea was not used as a criterion in our study. Overall flea infection prevalence for each of the four temperatures tested and on each day p.i. ranged from 93.8-100%. The average number of fleas feeding on each of seven mice per time point and temperature (Table 2.1) yielded a range of 13.1-15.1 fed fleas per naïve mouse. No significant differences in the number of infected fleas that successfully took a blood meal from naïve mice were shown on any of the

challenge days or among temperatures (Table 2.1). When quantifying bacterial loads for each infected, fed flea, a significant difference was found between fleas maintained at 6°C, (2.45×10^6 median CFU per flea, averaged for each time point), when compared to fleas maintained at 10°C (1.03×10^6 median CFU per flea, averaged for each time point) ($P = 0.0181$), 15°C (5.96×10^5 median CFU per flea, averaged for each time point) ($P = 0.0026$) and 23°C (1.61×10^4 median CFU per flea, averaged for each time point) ($P = 0.0002$) (Figure 2.3). While fleas maintained at 23°C were found to harbor the lowest median number of bacteria, comparisons to average median bacterial loads for 10°C and 15°C were not statistically significant ($P = 0.0832$ and $P = 0.3159$, respectively) (Figure 2.3). However, our results indicate that temperature is an influential factor on median bacterial loads ($\chi^2 = 23.31$, df = 3, $P < 0.0001$).

To determine whether the total number of CFU calculated from all fleas within a cohort which were infected and fed on an individual naïve mouse, we employed average logsum data as a means to evaluate whether or not this parameter was affected by incubation temperature of the flea. Pair-wise comparisons of average logsum CFU per temperature and incorporating all time points show that values for fleas maintained at 6°C were significantly higher than those for 10°C ($P = 0.0231$), 15°C ($P = 0.0071$), and 23°C ($P < 0.0001$) (Figure 4). Moreover, average logsum values at 23°C were also significantly less than the determined values for 10°C ($P < 0.0001$) and 15°C ($P < 0.0001$) (Figure 2.4). Overall, temperature proved to be a significant factor with respect to logsum values ($\chi^2 = 22.06$, df = 3, $P < 0.0001$).

In vitro *bacterial viability curve:*

Based on observations on *in vivo* growth of *Y. pestis* within fleas at 6°C, 10°C, 15°C, and 23°C, and the patterns revealed in the results for median bacterial loads and logsum values, we chose to examine growth and viability of *Y. pestis in vitro* at the same temperatures. A five day

growth curve analysis measuring bacterial cell viability showed statistically higher peak growth at temperatures 10°C and 15°C when compared to 6°C ($P = 0.0121$ and $P = 0.0099$, respectively) and 23°C ($P = 0.0075$ and $P = 0.0060$, respectively) (Figure 2.5). Static growth (without aeration) at 6°C appears to be more linear and approaching stationary phase at the end of the 5 day sampling period, whereas 23°C had achieved peak growth by 24 h. Although pair-wise comparison of results by temperature indicate that *Y. pestis* grows more favorably *in vitro* at 10°C and 15°C when compared to 6°C and 23°C, a comparison of all temperatures incorporating all time points showed no statistical significance in overall growth of *Y. pestis* ($\chi^2 = 7.68$, df = 3, $P < 0.0531$) (Figure 2.5). Moreover, *in vitro* growth at 6°C does not appear to mimic *in vivo* bacterial loads as shown in Figures 3 and 4, however, the comparative trend of higher peak growth of *Y. pestis* at lower temperatures remains consistent when compared to growth at 23°C.

2.4. Discussion

Historically, it has been shown that increases in the mean monthly ambient temperature above 27.5ºC result in declines in epizootic and human plague activity (Pollitzer 1954, Schar 1956, Cavanaugh 1971, Cavanaugh and Marshall 1972, Enscore, Biggerstaff et al. 2002, Brown, Ettestad et al. 2010). These declines in transmission are thought to be associated with reductions in flea blockage or loss of infection (Kartman and Prince 1956, Kartman 1969, Hinnebusch, Fischer et al. 1998) however, Schotthoefer et al. (2011), recently demonstrated *Y. pestis* transmission by the flea *X. cheopis* at temperatures as high as 30ºC during the early phase transmission period (days 1-4 p.i.) (Schotthoefer, Bearden et al. 2011), as well as during late phase (days 7-28 p.i.) (Rouviere and Gross 1996), suggesting that other factors, such as threshold host or flea densities also could be involved.

Similar studies have not been performed at low temperatures. The aim of the current study was to examine the effect of low temperature incubation of *O. montana* (formerly *D. montanus*) fleas on transmission during both early and late phase periods. Kartman et al. (Kartman 1969) demonstrated survival and transmission of *X. cheopis* at 8.5ºC, however these fleas were held until blockage was observed which was beyond the extrinsic incubation period of the fleas held in the current study. We used *O. montana,* a temperate flea that rarely blocks (Burroughs 1947, Engelthaler and Gage 2000), to demonstrate transmission during early and late phase periods at sub-ambient temperatures. Our data clearly demonstrate that efficient flea-borne transmission of *Y. pestis* does occur at low temperature during both early and late phase periods and is particularly robust at 10ºC and 15ºC. Though the data do not provide a specific explanation for greater transmission as measured by mouse mortality from infected fleas held at sub-ambient temperatures relative to 23ºC, reasons for this may include higher median bacterial loads and bacterial logsum values of flea groups held at the lower temperatures that fed on individual mice (Figures 3 and 4, respectively). Though overall transmission events from infected fleas held at 23ºC was significantly lower than fleas held at 10ºC and 15ºC and differ substantially during EPT from data reported by others using *O. montana* (Eisen, Bearden et al. 2006), our study utilized CD-1 outbred mice which may have slightly different susceptibility characteristics. Moreover, Eisen et al. (2006) used only female fleas for transmission experiments. Additionally, bacterial load and logsum data were significantly lower at 23º in the current study. Furthermore, EPT studies by Vetter et al. (2010) using *O. montana* fleas and biofilm biosynthetic mutants also showed reduced mouse mortality numbers during these time points including one EPT p.i. time point in which no transmission was observed (Vetter, Eisen et al. 2010).

Percent per flea transmission efficiency data (Table 2.1 and Figure 2.1), a maximum likelihood estimate of transmission by infected fleas that also fed on a naïve mouse, clearly indicates the significance of low temperature incubation in promoting flea-borne transmission. Pooled percent per flea transmission efficiencies were highest at 10ºC and 15ºC. Additionally, binomial logistic regression analysis also showed an increased probability of flea-borne transmission over time particularly at 10ºC and 15ºC. Schotthoefer et al., demonstrated transmission in *X. cheopis* fleas held at 10ºC up to 14 days p.i. (Rouviere and Gross 1996), though these fleas showed much lower survivability than was observed in our study for *O. montana* fleas held at the same temperature at 14 days p.i. (data not shown). Since ground squirrel burrow temperatures of 15ºC have been described previously (Telepnev, Klimpel et al. 2009), it is possible that temperatures of 10-15ºC represent burrow conditions that are optimal for both bacterial growth and transmission potential by *O. montana* or the fleas of other burrow dwelling rodent hosts of plague.

Changes in lipopolysaccharide (LPS) structure for *Y. pestis* at lower temperatures may provide an alternative explanation to explain enhanced transmission at low temperatures by *O. montana* fleas (Anisimov, Dentovskaya et al. 2005, Han, Zhou et al. 2005, Knirel, Lindner et al. 2005, Knirel, Lindner et al. 2005). These changes may be more conducive to survival, growth, and transmission potential for *Y. pestis* within the flea, although Suomalainen et al. (2010) recently reported a dramatic reduction in LPS-dependent plasminogen activation by the Pla surface protease as a result of temperature-induced (20ºC) changes in the LPS of *Y. pestis* (Suomalainen, Lobo et al. 2010) from which one might conclude that dissemination of *Y. pestis* may be delayed and flea-borne transmission impaired at temperatures even lower than 20ºC. However, results of the current study suggest that the reduction in Pla protease activity is only

transient as transmission of *Y. pestis* by *O. montana* fleas maintained at 10ºC and 15ºC is significantly higher than at the other study temperatures and no significant differences were observed in mean time to death among transmission events across all temperatures (data not shown), It should also be noted that LPS biosynthesis by an alternative pathway has been suggested for *Y. pestis* grown at 6ºC resulting in a novel LPS structure (Knirel, Lindner et al. 2005). It would be of interest to know whether this novel *Y. pestis* LPS structure interacts with the Pla protease or fosters enhanced resistance to antimicrobial factors in the flea gut, although very little is known about immunity in fleas.

Due to significantly greater bacterial growth within the flea gut at lower temperatures (Figures 2.3 and 2.4), it may be likely that up-regulation of bacterial factors that contribute to growth and survival occurs at sub-ambient temperatures. Although a direct comparison cannot be made due to presumed differences in the growth enviroments of HIB media and the flea midgut, it is interesting to note that the *in vitro* static growth characteristics of *Y. pestis* at different temperatures showed peak growth at lower temperatures similar to the growth kinetic patterns observed *in vivo*. Conversely, physiological changes within the flea may favor maintenance and growth of the plague bacilli. Moreover, factors that contribute to resistance to infection or elimination of *Y. pestis* within the flea may be down-regulated or altered in function at lower temperatures. In a recent study, genes identified as being involved in the production of reactive oxygen species in response to *Y. pestis* infection in *X. cheopis* fleas (Collinge, Simirskii et al. 2005), may be less active or non-functional at lower temperatures. Toxic degradation products derived from the flea blood meal and from which *Y. pestis* is thought to be protected via expression of *ymt*, encoding a phospholipase D required for survival of *Y. pestis* in the flea, may be less abundant in fleas maintained at low temperatures thus reducing exposure of *Y. pestis* to

these compounds (Hinnebusch, Fischer et al. 1998). Moreover, likely induction of the cold shock protein stimulon (Han, Zhou et al. 2005) may act as a global regulatory mechanism for factors which enhance the growth and survival of *Y. pestis* within the flea at low temperatures. These factors will require further investigation and planned studies involving transcriptomic analysis of *Y. pestis* infected *O. montana* may reveal additional factors that contribute to enhanced growth and survival.

Increased growth and survivability of *Y. pestis* in the flea gut at low temperature may contribute to the transmissibility of overwintering fleas, particularly in burrow-dwelling fleas such as *O. montana*. Overwintering of *Y. pestis* infection in fleas have been previously reported for various species of fleas including ground squirrel fleas (Kartman, Quan et al. 1962, Baltazard, Karimi et al. 1963, Sing, Roggenkamp et al. 2002, Bazanova, Nikitin et al. 2007, Bizanov and Dobrokhotova 2007, Robinson, Telepnev et al. 2009, Anisimov, Dentovskaya et al. 2010). *O. montana* or other fleas overwintering as infectious fleas in burrows may transmit to hibernating hosts or hosts in late winter and early spring. Because low temperature incubation of infected fleas enhances the growth of *Y. pestis* (Figures 2.3 and 2.4), it would be of interest to know whether these conditions also promote partial or complete blockage and/or enhanced biofilm production within *O. montana*, a flea that rarely blocks at temperatures and time periods used in earlier studies (Burroughs 1947, Engelthaler and Gage 2000). Additional experiments, beyond the scope of the current study, will utilize reporter strains of *Y. pestis* to localize the bacteria within the flea gut. Biofilm biosynthetic mutants will be used to determine the contribution of exopolysaccharide production in the growth and survival of *Y. pestis* within the flea at low temperatures. The results of the current study have significant implications for the utility of fleas as reservoirs for plague from one season to the next as well as the focality of

plague in global endemic foci including those in tropical regions that occur at higher elevations and experience cooler temperatures. This is particularly true in the East African countries of Uganda and Tanzania where elevated plague risk is associated with vegetation and higher elevation, the latter being a factor in predictably cooler temperatures (Lawrenz 2010).

CHAPTER II REFERENCES

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Table 2.1. Transmission efficiency of infected fleas held at different temperatures during the early phase (days p.i. 1-4) and late phase transmission periods (days p.i. 7-21)

Temp (°C)	Days p.i.	Flea infection prevalence (%)	Average No. fed, infected fleas per mouse (total in treatment group)	No. of mice infected (exposed)	Percent per flea transmission efficiency (95% CI)
6	1	100	14.9 (104)	1 (7)	0.96 (0.06-4.76)
	2	98.1	14.7 (105)	4 (7)	4.99 (1.71-12.81)
	3	97.3	15.0 (108)	4 (7)	4.89 (1.68-12.56)
	4	99.0	14.9 (105)	3 (7)	3.38 (0.93-9.60)
	7	96.0	13.1 (106)	3 (7)	3.82 (1.06-10.78)
	10	98.1	14.7 (104)	2 (7)	2.08 (0.39-7.01)
	14	96.9	15.1 (109)	5 (7)	7.28 (2.85-18.37)
	17	99.0	14.7 (105)	2 (7)	2.06 (0.38-6.98)
	21	98.9	14.1 (106)	5 (7)	7.52 (2.94-18.96)
10	1	95.1	14.0 (105)	1 (7)	1.02 (0.06-5.04)
	2	98.1	15.0 (107)	6 (7)	10.04 (4.43-27.45)
	3	96.3	14.9 (106)	5 (7)	6.95 (2.75-17.23)
	4	100	14.9 (105)	6 (7)	9.97 (4.40-27.37)
	7	100	13.1 (106)	4 (7)	5.11 (1.74-13.21)
	10	98.7	14.4 (104)	4 (7)	8.27 (3.29-20.20)
	14	98.1	14.9 (106)	6 (7)	10.56 (4.81-27.69)
	17	98.1	14.9 (105)	6 (7)	13.09 (5.66-44.93)
	21	96.3	13.3 (97)	6 (7)	12.84 (5.80-34.27)
15	1	93.8	14.1 (103)	3 (7)	3.70 (1.02-10.53)
	2	99.0	14.9 (105)	2 (7)	2.05 (0.38-6.92)
	3	98.0	14.7 (105)	4 (7)	5.08 (1.73-13.17)
	4	95.1	13.4 (98)	1 (7)	1.12 (0.07-5.51)
	7	99.0	14.7 (99)	5 (7)	7.29 (2.85-18.39)
	10	98.9	14.1 (104)	7 (7)	(N/A)[a]
	14	96.4	15.1 (105)	7 (7)	(N/A)[a]
	17	97.4	14.4 (105)	7 (7)	(N/A)[a]
	21	97.1	13.6 (93)	6 (7)	15.05 (7.70-33.48)
23	1	99.0	14.4 (98)	2 (7)	2.11 (0.40-7.10)
	2	100	14.7 (104)	1 (7)	0.95 (0.06-4.71)
	3	100	14.9 (106)	0 (7)	0.00 (0.00-2.93)
	4	100	14.4 (102)	0 (7)	0.00 (0.00-2.87)
	7	100	14.3 (102)	3 (7)	3.30 (0.91-9.34)
	10	100	14.1 (101)	3 (7)	3.38 (0.94-9.51)
	14	99.0	14.1 (103)	3 (7)	3.36 (0.93-9.49)
	17	99.0	13.7 (102)	3 (7)	3.45 (0.95-9.85)
	21	98.1	14.0 (103)	1 (7)	0.98 (0.06-4.87)

[a]When all pools are positive, likelihood methods cannot be calculated. Likelihood estimates therefore do not exist in this case, indicated as N/A for these quantities.

Figure 2.1. Pooled percent per flea transmission rates by temperature

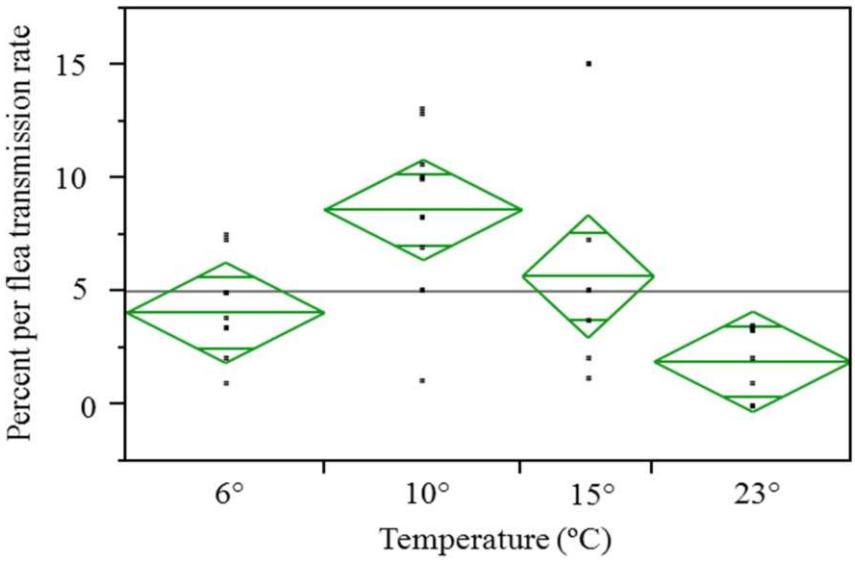

Figure 2.1. A one-way ANOVA analysis comparing pooled percent per flea transmission rates by temperature and including all experimental time points (represented by closed squares). Means are indicated by the center horizontal lines within each rhombus and parallel lines above and below the means denote the 95% confidence intervals (CI). The horizontal line extending from the Y axis to the right margin of the graph is the mean of response across all temperatures. The mean pooled percent per flea transmission rates were: 6°C (4.11; 95% CI, 2.83-5.84), at 10°C (8.67; 95% CI, 6.43-11.65), 15°C (7.87; 95% CI, 5.82-10.58), and 23°C (1.94; 95% CI, 1.16-3.09).

Figure 2.2. Mouse mortality at each temperature and time point

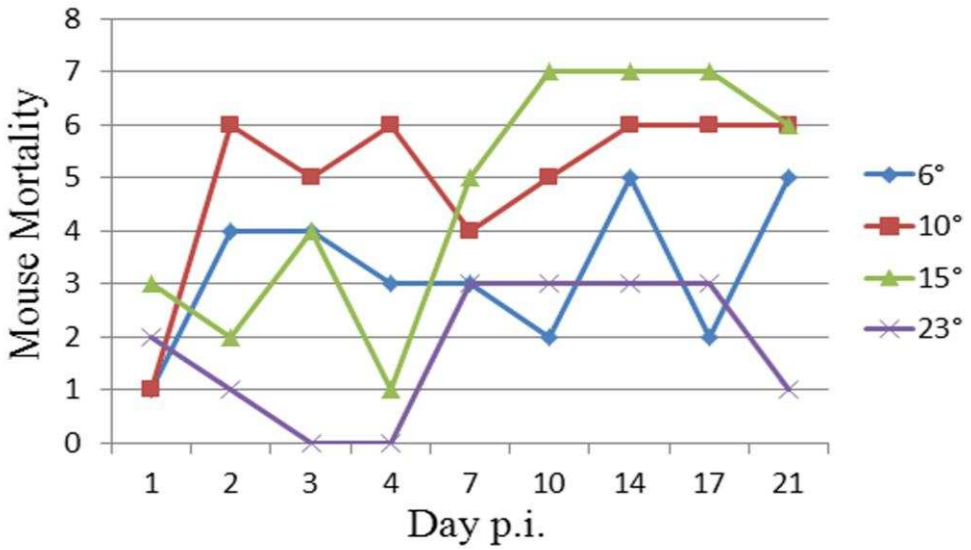

Figure 2.2. Graph showing the number of mice that succumbed to *Y. pestis* infection, or were euthanized and confirmed to be infected by *Y. pestis* by bacteriophage lysis of culture isolates from mouse tissues, for each temperature and day post infection. The X axis denotes days on which naïve mice (7 mice were used for each time point and temperature) were exposed to infected fleas (infected fleas were obtained from three independent artificial infections for each temperature) and the Y axis indicates mouse mortality (dead or euthanized mice).

Figure 2.3. Median CFUs/flea for each temperature and time point

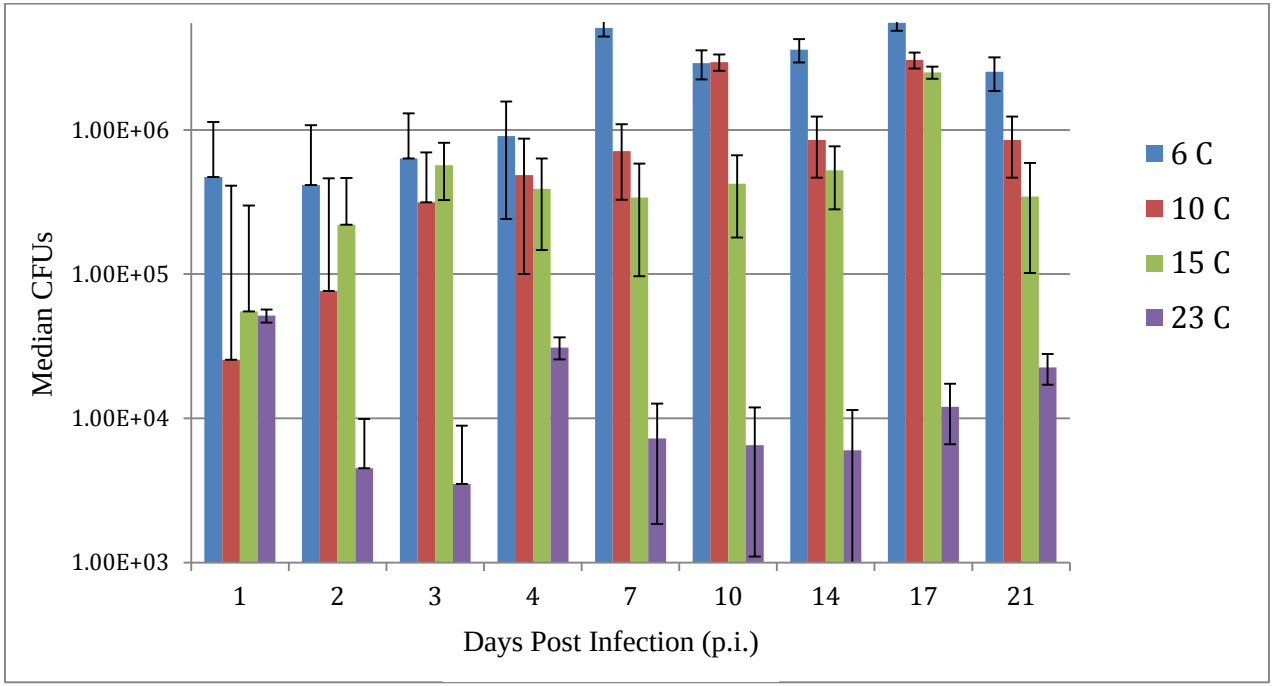

Figure 2.3. Histogram comparing median *Y. pestis* CFU per flea (Y axis) for temperatures 6°C, 10°C, 15°C, and 23°C and across all experimental time points denoted as days post infection (X axis). Sets of bars for each day post infection represent the average of the median CFU per infected, fed fleas for all temperatures at that time point.

Figure 2.4. Average log sum CFU's per temperature

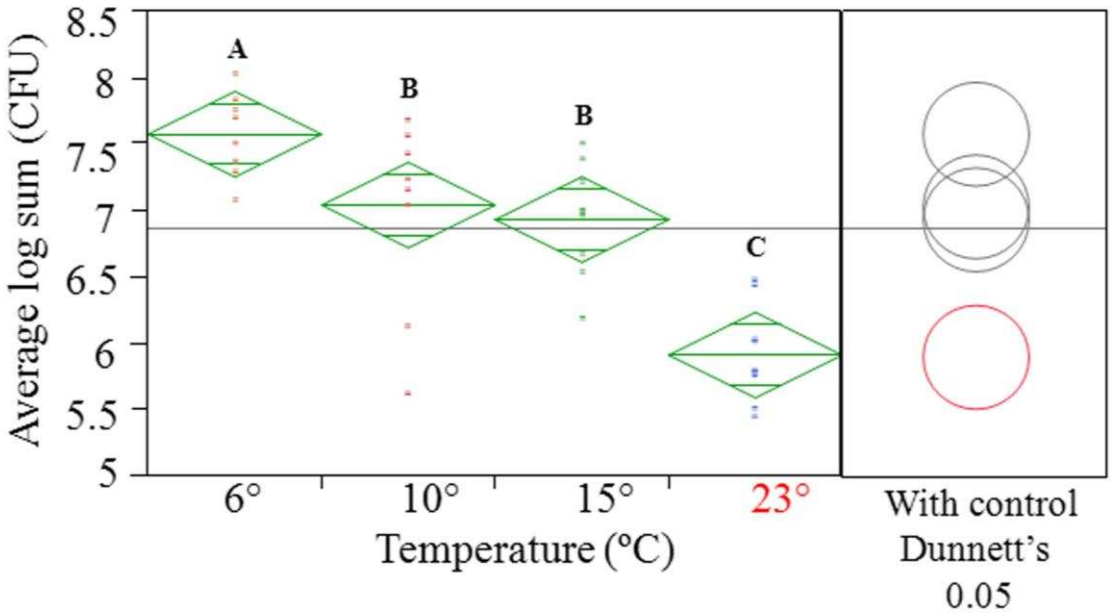

Figure 2.4 A one-way ANOVA analysis (left panel) with Dunnett's post-hoc control test (right panel) comparing the average logsum CFU's (Y axis) of infected, fed fleas maintained at each temperature (X axis) incorporating all time points. Diamonds not connected by the same letter are significantly different. Means are indicated by the center horizontal lines within each rhombus and parallel lines above and below the means denote the 95% confidence intervals (CI). The horizontal line extending from the Y axis to the right margin of the graph is the mean of response across all temperatures. The Dunnett's post-hoc control test (right panel) shows a significant difference between all three lower temperatures when compared to the control temperature (23°C), which is represented by the bottom circle in the right panel.

Figure 2.5. *In vitro* growth analysis of *Y. pestis* at 6°C, 10°C, 15°C and 23°C

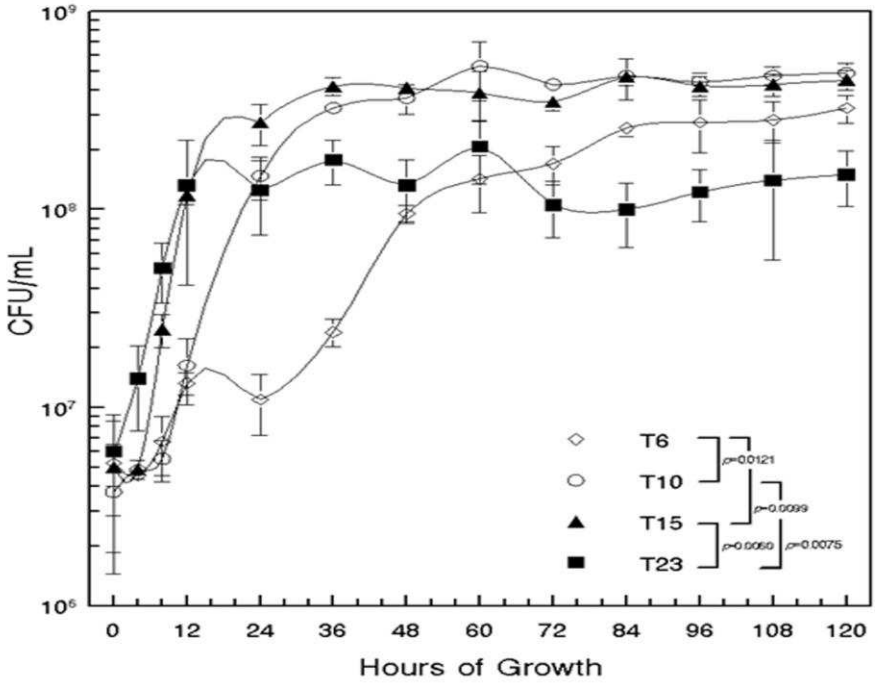

Figure 2.5. *In vitro* static growth of *Y. pestis* CO96-3188 over 5 days at different temperatures. Viable bacterial counts depicted as CFU/mL (Y axis) for a period of growth ranging from 0 h to 120 h (X axis) were determined by plating in duplicate from two independent experiments. Error bars for each sampling time point represent the standard deviation of two independent experiments. Pair-wise significant differences for mean growth between temperatures and inclusive for all time points were observed between 6ºC and 10º (*P*=0.0121), 6ºC and 15ºC (*P*=0.0099), 10º and 23ºC (*P*=0.0075), and 15º and 23ºC (*P*=0.0060).

CHAPTER III

Effects of Low Temperature on the Long-Term Transmissibility of *Yersinia pestis* by *Oropsylla montana*

Overview

Yersinia pestis is a zoonotic disease which cycles between fleas and rodent hosts. In the United States, the majority of human cases are associated with the flea vector, *Oropsylla montana*. To better understand plague ecology in temperate climates, our study tested the ability of infected fleas to live in soil and successfully transmit at temperature's mimicking rodent burrows. Fleas were fed an infectious *Y. pestis* bloodmeal, and further divided and maintained at one of four temperatures (6ºC, 10°C, 15°C, or 23ºC) in flasks containing autoclaved prairie dog burrow soil. Flea transmissibility of this pathogen was tested by feeding ~15 fleas weekly in capsules placed on seven naïve CD-1 mice. Fleas held at 6ºC were able to survive and effectively transmit out to 42 days p.i., as indicated by 87% overall mortality among mice fed upon by these fleas. Fleas held at 10°C and 15°C survived out to day 28 p.i. and transmitted *Y. pestis* to mice resulting in >90% overall mouse mortality. Fleas held at 23ºC, were unable to survive past day 11 p.i., therefore, transmission studies could only be conducted on day 7 p.i. A significant difference was found in flea mortality, as well as average bacterial loads between fleas held at sub-ambient temperatures when compared to 23ºC (χ^2=0.0012) on day 7 p.i.. Our study suggests that plague bacteria might overwinter in the flea gut and persist for weeks to months during other seasons in infected fleas living off-host in relatively cool rodent burrows. The ability of fleas to transmit *Y. pestis* to susceptible hosts after many days to weeks of incubation below ambient temperatures also demonstrates the potential of these insects to act as a reservoir of plague.

3.1. Introduction

The Gram-negative, rodent associated pathogen, *Yersinia pestis*, causes a zoonotic disease that has been implicated in three historic pandemics causing millions of deaths. *Y. pestis* is the etiologic agent of the disease most commonly known as plague, which is primarily a flea-borne disease of rodents. Plague is thought to persist in nature in enzootic or maintenance cylces involving transmission between rodents and their associated fleas (Gage and Kosoy 2005). *Oropsylla montana*, a ground squirrel flea commonly found on California ground squirrels and the closely related rock squirrel, is the vector most commonly associated with human plague transmission in the United States. Transmission of plague typically occurs through the bite of an infectious flea that has fed on a highly bacteremic host. Occasionally, epizootic periods or rapid spread of plague occurs, causing noticeable rodent die-offs and increasing human risks for plague infection from the susceptible rodent hosts succumbing to infection as their starved fleas begin questing to find new hosts (Eisen, Wilder et al. 2007). The potential mechanisms of plague maintenance during inter-epizootic periods have been studied extensively but are still poorly understood. Persistence of *Y. pestis* in soil has been suggested as a possible mechanism of interepizootic persistence as well as a potential factor describing plague foci. Much speculation regarding plague reservoirs exists in the literature, including such proposed mechanisms as low levels of transmission occurring between enzootic hosts and fleas (Gage and Kosoy 2005), survival of *Y. pestis* in soil (Mollaret 1963, Drancourt, Houhamdi et al. 2006, Boegler, Graham et al. 2012) or transmission of *Y. pestis* by contact of susceptible hosts with infected flea feces (Jones, Vetter et al. 2013). Some support for low level transmission between fleas and hosts during interepizootic periods exists, but the question of its importance relative to other mechanisms remains uncertain. Similarly, a report by Drancourt et al. (2006) discovered

that *Y. pestis* could remain viable and fully virulent after 40 weeks in soil suggesting a possible reservoir role for survival of *Y. pestis* in soils but Boegler et al. (2012) succeeded in infecting only one mouse out of 104 exposed to *Y. pestis*-contaminated soil. Although this finding demonstrates that transmission of *Y. pestis* to rodents through contact with contaminated soils is possible, the frequency at which such transmission occurs appears to be very low, making its significance uncertain. Survival of *Y. pestis* in flea feces for many weeks was first documented during the last pandemic in India but its significance as a reservoir mechanism has received little attention and has been dismissed by some.

Environmental conditions such as climate, temperature, and humidity play a pertinent role in the viability and transmission of this particular pathogen. Plague transmission and its relationship to climate and temperature (e.g. relative humidity, vapor pressure deficits, precipitation, or changes in temperature) has also been studied for decades and found to influence plague prevalence in ways which either favored epidemic spread of the pathogen at relatively moderate temperatures or significantly decreased its spread when temperatures surpassed 27-28ºC. This relationship has been observed in the plague endemic areas in the southwestern United States, in which an increase in late winter-early spring precipitation positively correlated with an increase in human plague cases. In contrast, human plague cases decline significantly when temperatures increased above 32-35 ºC for a given time period. In this study, we examined the effects of incubating infected fleas at sub-ambient temperatures, which mimic ground squirrel burrow temperatures during the winter months, (e.g. 6 ºC, 10 ºC, 15 ºC), on their ability to remain viable and successfully transmit *Y. pestis* to naïve rodent hosts. The implications of low temperature growth, survival, and persistence within the *O. montana* midgut could further explain the potential for *Y. pestis* to persist during the colder winter months

and remain viable in order to successfully transmit during the subsequent transmission season. Because *Y. pestis* is pathogenic for its flea vector and could be subject to attack by the fleas natural immune system we also measured midgut peroxide levels to provide some estimate of the innate immune responses of *O. montana*. Finally, we discuss the implications of these findings for the potential role of fleas maintained in soil of burrows as a potential reservoir of *Y. pestis* and the maintenance of long-term plague foci in both temperate and tropical regions.

3.2. Materials and Methods

Yersinia pestis *growth conditions:*

The fully virulent *Yersinia pestis* strain, CO96-3188 (Pgm+, pCD1+, pMT1+, pPCP1+), was used for artificial infection of fleas. Glycerol stocks of CO96-3188 were maintained in heart infusion broth (HIB) with 10% glycerol and overnight cultures were grown in HIB and incubated at 28°C overnight for 14-16 h with shaking at 180 rpm.

Infection of fleas:

Colony-reared, *Oropsylla montana* fleas were obtained from the Centers for Disease Control and Prevention/Division of Vector-Borne Diseases for artificial infection with *Y. pestis* and for transmission trials performed on 6-8 week-old naïve, female Charles River CD-1 outbred mice obtained from a specific pathogen-free mouse colony (Charles River Laboratories International, Inc., Wilmington, MA).

The artificial feeding procedure used to feed *O. montana* fleas an infected bloodmeal was previously described (Eisen, Bearden et al. 2006, Williams, Schotthoefer et al. 2013). Briefly, *O. montana* male and female adult fleas were randomly collected and removed from an established colony and starved for 4 days at 23°C. An overnight culture of CO96-3188 was prepared by

inoculating 60 ml of HIB with a 0.5 ml aliquot of bacterial glycerol stock and grown as described above. Bacteria were pelleted by centrifugation and resuspended in 40 ml of defibrinated Sprague-Dawley strain rat blood (Bioreclamation, Jericho, NY) pre-warmed to 37°C. Artificial feeders, each containing approximately 75 *O. montana* fleas, were prepared according to Eisen et al (6) and fleas were allowed to feed for 1 h through mouse skins taken from euthanized, hairless SKH-1 mice (specific pathogen-free mouse colony maintained by the Division of Vector-Borne Diseases, Fort Collins, CO) on blood infected with 10^8-10^9 CFU ml^{-1} of *Y. pestis* CO96-3188. Fleas were then examined by light microscopy to determine whether fleas had consumed a potentially infectious bloodmeal. Any fleas which did not feed on infected blood were discarded. Fed fleas were collected and maintained in 30 grams of autoclaved prairie dog burrow soil (Boegler, Graham et al. 2012) contained in 25 cm^2 tissue culture flasks (Corning, Lowell, MA) at their respective treatment temperatures (6°C, 10°C, 15°C, or 23°C). Fleas in these flasks were held for incubation in acrylic desiccation chambers (Thermo Scientific, Rockford, IL) containing beakers of saturated potassium chloride (KCl) solution to maintain relative humidity of 80-85%. The acrylic desiccation chambers containing the fleas were held in the dark within temperature controlled incubators and remained untouched until subsequent transmission feeds.

Flea-borne transmission to naïve mice:

Transmission feeds were performed on days 7, 14, 21, 28, 35*, and 42* (*for 6ºC fleas only) post infection (p.i.) using fleas maintained at each temperature to determine the relative efficiencies of transmission of infected fleas incubated at low temperatures compared to those maintained at 23ºC. Capsules were affixed to seven 6-8 week-old, naïve, specific pathogen free,

CD-1, outbred mice (Charles River Laboratory Inc.,), and pools of ~15 potentially infectious fleas were allowed to feed for 1 hour. Fleas were then collected, and viewed by light microscopy to determine fed status as well as the sex of each flea in the pool. Fleas were stored individually in microcentrifuge tubes and placed at -80°C for later analysis to determine infection prevalence and bacterial loads. Exposed mice were held in individually ventilated cages (Tecniplast, Philadelphia, PA) at 27ºC and monitored for signs of infection (e.g. ruffled fur, lethargy, hunched posture, slow response to stimuli). At first sign of illness, mice were euthanized and later necropsied to detect *Y. pestis* infection by performing *Y. pestis* anti-F1 antigen direct fluorescent antibody assays (DFA) on liver, spleen, or abscess smears. Positive transmission events were confirmed by specific bacteriophage lysis of bacterial colonies isolated from mouse tissues. In one instance the *Y. pestis* bacterium could not be recovered from a euthanized mouse, which showed signs of illness typical of *Y. pestis* infection on day 42 p.i. at 6 ºC. Mice showing no signs of infection were held up to 21 days p.i. before being euthanized after blood was taken by cardiac puncture from the anesthetized mice for serology testing to identify evidence of resolved infections using passive hemagglutination and inhibition tests (PHA/HI) for antibodies to *Y. pestis* F1 antigen. Animal procedures used in this study were approved by the Centers for Disease Control and Prevention, Division of Vector-Borne Diseases, Institutional Animal Care and Use Committee (Protocol #09-020 and #14-004).

Flea infection prevalence and bacterial loads:

Fleas were homogenized in 100 µL of HIB + 10% glycerol using sterile Teflon pestles and plated on SBA medium to determine infection status. The number of artificially fed fleas that were both infected and uninfected and which fed on naïve mice were used in determining the infection prevalence of fleas at each time point and incubation temperature. Bacterial loads of *Y.*

pestis-infected fleas were estimated by serially diluting individual flea lysates and plating them on SBA media in duplicate. Per flea bacterial load estimates were used in calculating the median bacterial burden of fleas at each time point and temperature within groups of fleas which fed on naïve mice at each time point and temperature tested.

Measurements of Midgut Peroxide Levels:

O. montana fleas were artificially fed either an infectious CO96-3188 blood meal (1×10^8 *Y. pestis* CFU/ml), or uninfected rat blood as previously described. Fed fleas were collected and stored at three respective temperatures. A total of 10 fleas from each temperature and group (infected and uninfected fleas) were individually collected on Days 1, 4. 7, and 10 post infection. Each flea was homogenized in 100 µl of phosphate buffered saline (PBS). Peroxide levels in each sample was determined using the Pierce Quantitative Peroxide Assay Kit (ThermoFisher, Scientific, Wilmington, DE), which is based on oxidation of ferrous to ferric ion in the presence of xylenol orange (Jiang et al. 1991). Samples were read at 595 nm using a plate reader to determine the optical densities. The data were expressed as the average of ten fleas per temperature and time point.

Statistical Analyses:

Maximum likelihood estimates for percent per flea transmission rates were calculated by using the Microsoft Excel Add-In PooledInfRate v. 4.0 (Ransom and Krueger 1954) for each time point at each temperature. Estimates were based on the number of infected fleas that were also determined to have fed on an individual mouse and whether subsequent transmission occurred in that mouse. Pooled percent per flea infection rates were also used to analyze the effect of day p.i. as well as temperature on transmission by using a pooled binomial regression

algorithm in R (binGroup package, the Comprehensive R Archive Network (CRAN), http://cran.r-project.org/web/packages/binGroup/index.html).

Pooled percent per flea transmission rates for each temperature, total mouse transmission rates for each temperature, and bacterial counts were compared using one-way ANOVA. The average bacterial CFU of infected, fed fleas at each time point were compared between temperatures using one-way ANOVA. CFU data are comprised of the bacterial loads of all infected fleas that fed on an individual naïve mouse for a particular time point and temperature. CFU calculations were determined for each group of fleas which fed on mice within a cohort (7 mice) for each time point at a particular treatment temperature. The bacterial CFU values to which each individual mouse within a cohort was exposed, were then averaged to obtain a single CFU value at each time point for all four temperatures. These averaged CFU values were then used in further statistical analyses. Differences among or between groups were considered statistically significant at $P < 0.05$. Contingency table analysis was also performed to determine differences in transmission efficiencies for each temperature and day p.i. Wilcoxon/Kruskal-Wallis tests (rank sums) were used to establish the significance of incubation temperature on i) in vitro bacterial viability, ii) average bacterial CFU data, iii) pooled percent per flea transmission rate, iv) mouse infection rate, and v) median flea bacterial loads. All statistical comparisons were performed using JMP statistical software (SAS Institute, Cary, NC).

3.3. Results

Low temperature effects on flea transmission efficiencies:

Transmission efficiencies of *Y. pestis* infected fleas held in soil and maintained at different experimental temperatures (6°C, 10°C, 15°C, and 23°C) showed no significant differences

between the control temperature, 23°C, and the three low temperatures tested for common post-infection time points among all temperatures, nor for common p.i. time points among the sub-ambient temperatures (Table 3.1). Fleas held at 6°C were able to survive in soil longer than all other temperatures tested and successfully transmitted to naïve mice at each time point tested out to day 42 p.i.. Pooled fleas held at 6°C had the greatest number of successful transmission events by infected fleas to naïve mice on each study day p.i., with all seven mice exposed on day's 7, 14, and 35 p.i., expiring from *Y. pestis* infection (Table 1). Pooled fleas maintained at 10°C and 15°C successfully survived and transmitted out to day 28 p.i., with an overall flea survival rate slightly higher at 10°C than 15ºC (data not shown). Pooled fleas maintained at 10°C, attained a 100% transmission outcome on day's 7, 14, and 21 p.i., as well as for 6 of the 7 mice exposed on day 28 p.i.. Pooled fleas held at 15°C also had comparable success in transmission to naïve mice from fleas surviving out to day 28 p.i., with 100% successful transmission to all 7 mice exposed on day's 7 and 14 p.i., and 6 out of 7 mice successfully obtaining plague infection on days 21 and 28 p.i. (Table 3.1). Interestingly, infected fleas held at the control temperature (23°C), were only able to survive out to day 11 p.i. when stored in soil with no maintenance feeds; however, these fleas had a 100% success rate of transmitting to naïve mice on Day 7 p.i.. To estimate the number of infected fleas which fed on a naïve mouse and resulted in a successful transmission event, percent per flea transmission efficiencies were calculated for each temperature and time point. Percent per flea transmission efficiencies were unable to be calculated for 6°C day's 7, 14, 35 p.i., 10°C day's 7, 14, and 21 p.i., 15°C day's 7 and 14 p.i. and 23°C day 7 p.i., because all seven mice tested succumb to infection; therefore likelihood calculations are not applicable. The highest percent per flea transmission efficiency (10.85%) for all temperatures, occurred in fleas held at 10°C on day 28 p.i.. Fleas maintained at

10°C also transmitted to 27 of the 28 mice tested, suggesting that this temperature falls within the optimal range for *Y. pestis* to survive and successfully transmit to naïve mice among the temperatures tested. The next highest percent per flea transmission efficiency transpired with fleas maintained at 6°C, which were able to survive and transmit out to day 42 p.i., resulting in 100% transmission occurring on days 7, 14, and 35 p.i.. Transmission efficiencies for pooled fleas held at 6°C ranged from 2.16-10.35% (Table. 3.1). Fleas held at 15°C survived out to day 28 p.i., and 6 out of the 7 mice on day's 21 p.i. and 28 p.i., successfully acquired *Y. pestis* infection with the highest percent per flea transmission efficiency on day 21 p.i. (9.82%) (Table 3.1). *Y. pestis* transmission was successfully observed for each challenge day post infection at each of the three low temperatures tested, whereas, fleas held at the control temperature (23°C) were unable to survive beyond 11 days p.i.. Poor survival at this temperature is likely the result of increased activity which in the absence of a maintenance blood meal resulted in rapid desiccation of these fleas when compared to fleas maintained in soil at the three lower temperatures tested. Overall flea infection prevalence for each of the four temperatures tested and on each day p.i. ranged from 85.7-100%. The average number of fleas feeding on each of seven mice per time point and temperature yielded a range of 12.6-15.0 fed fleas per naïve mouse (Table 3.1). No significant differences in the number of infected fleas that successfully took a blood meal from naïve mice were found on any of the challenge days or between temperatures (Table 3.1). Bacterial loads of each of the infected, fed fleas were quantified; however, a comparison among all four temperatures could only be performed on day 7 p.i., in which the average CFU's per temperature was significantly lower in the fleas stored at 23°C, when compared to the three lower maintenance temperatures tested (6°C, 10°C, and 15°C) (χ^2=0.0112). When quantifying bacterial loads for each infected, fed flea on all other day's p.i.

across the three low temperatures, the loads remained consistently high with no significant differences found between the average CFUs per flea for each time point p.i. at each of the sub-ambient temperatures. Our results indicate that temperature is an influential factor on flea survival and bacterial loads in the midgut of the flea when comparing ambient and sub-ambient temperatures. The data reported here on flea survival and bacterial loads relative to temperature are consistent with results previously reported by Williams et al. (2013).

Low temperature effects on reactive oxygen species in flea:

The reactive oxygen species assay identified significantly increased ROS levels in *Y. pestis*-infected fleas on day 10 post infection (p.i.), in fleas maintained at 23°C (P=0.0014), when compared to blood fed only fleas on the same day p.i. and temperature (Figure 3.3). Across the three low temperatures and time points, the *Y. pestis*-infected *O. montana* fleas maintained at 6°C, 10°C and 15°C, had no differences in the ROS levels, as well as, no differences were found among *Y. pestis*-infected *O. montana* fleas when compared to uninfected blood fed *O. montana* fleas on each temperature and time point p.i. (Figure 3.3). Bacterial loads of the *Y. pestis*-infected fleas, showed no significant difference between temperatures or time points (Figure 3.4). Our results indicate that ROS may limit *Y. pestis* colonization of *O. montana* fleas at 23°C after 10 days post infection, and that bacterial strategies to overcome ROS may enhance transmission.

3.4. Discussion

Our study suggests that fleas have a potential role as reservoirs of *Y. pestis* and the maintenance of plague foci or central areas in nature. Our findings also suggest that the temperatures encountered by fleas in their environments can significantly affect this reservoir

potential and the ability of infected fleas to survive in off-host environments and then transmit *Y. pestis* to susceptible hosts many days to weeks after they become infected and their original host has died of plague. This current study established the ability of *Y. pestis*-infected *O. montana* fleas to successfully survive in soil and transmit the plague bacterium to naïve mice up to day 42 p.i. when maintained at 6ºC without maintenance blood meals. *O. montana* is a temperate zone flea whose primary host is the California ground squirrel or rock squirrel. These fleas are commonly exposed to ground squirrel burrow temperatures reportedly ranging from 10-15ºC during winter months (Belovezhets and Nikol'skii, 2012). The capability of *Y. pestis* to survive and effectively grow at the lower temperatures tested in our study (6 ºC, 10 ºC, 15 ºC), and the pathogen's ability to persist within the midgut of the *O. montana* flea, suggests this flea and perhaps other flea species, could maintain a *Y. pestis* infection during the colder months and emerge as a viable transmitter during the subsequent transmission season. Indeed, Golov (1928) reported that three species of fleas (*Ctenophthalmus breviatus*, *Ceratophyllus tesquorum* and *Neopsylla setosa*) could survive for up to 220 days when held at 14-27°C and another species (*Ct. breviatus*) survived for 396 days at 0-15°C. Even more significantly, over half of *C. tesquorum altaicus* fed on *Y. pestis*-infected long-tailed susliks (*Spermophilus undulatus*) became infected and maintained their *Y. pestis* infections from mid-September to mid-June, a period of time sufficient to allow overwintering of the bacterium in its flea vectors and transmission by these fleas to susceptible susliks during the next transmission season (Bazanova and Maevskii 1996). Other studies have demonstrated successful transmission of *Y. pestis* by *X. cheopis* fleas at temperatures up to 30ºC during the early phase (days 1-4 p.i.) and late phase periods (days 7-28 p.i.), however, transmission of *Y. pestis* by *X. cheopis* at lower temperatures (e.g. 10°C) was unsuccessful perhaps because this semi-tropical and tropical flea species

survives poorly at such low temperatures or because of the overwhelming growth of *Y. pestis* observed in these fleas when they are incubated at low temperatures (Schotthoefer, Bearden et al. 2011).

Despite the many studies that have examined potential *Y. pestis* reservoirs to explain where this organism resides during inter-epizootic periods, the primary mechanism of survival and persistence remains uncertain (Gage and Kosoy 2005, Drancourt, Houhamdi et al. 2006). Nonetheless, different hypotheses for *Y. pestis* survival and maintenance have been proposed by numerous researchers, including low-level transmission of the pathogen between enzootic hosts and their associated fleas, *Y. pestis* survival in flea feces, prolonged survival of the pathogen, in the soil, persistence in infected and partially resistant rodent hosts, encystment in protozoan hosts, or within biofilms associated with soil nematodes (Pollitzer and Meyer 1961, Baltazard, Bahmanyar et al. 1963, Mollaret 1963, Darby, Hsu et al. 2002, Pushkareva 2003, Gage and Kosoy 2005, Drancourt, Houhamdi et al. 2006, Bizanov and Dobrokhotova 2007, Ayyadurai, Houhamdi et al. 2008, Eisen and Gage 2008, Boegler, Graham et al. 2012, Jones, Vetter et al. 2013). In studies performed by Mollaret et al. (1963) and Ayyadurai et al. (2008), viable *Y. pestis* was reported to be isolated after surviving months in both non-autoclaved and autoclaved soil (Mollaret 1963, Ayyadurai, Houhamdi et al. 2008). In a fatal human case study in 2008, Eisen et al, showed the ability of *Y. pestis* to remain viable in blood-contaminated soil for at least 3 weeks (Eisen, Petersen et al. 2008). In another study in which plague infected animals died, *Y. pestis* was reportedly isolated from carcasses after 11 months in rodent burrows (Karimi, Baltazard et al. 1963). Finally, a study which examined the ability of naïve scarified animals to successfully obtain plague infection from *Y. pestis*-infected soil, showed limited success in animals becoming infected after exposure to plague infected soil (Boegler, Graham et al. 2012).

In our study, we looked at whether the flea midgut can harbor *Y. pestis* for extended periods and act as a reservoir for this pathogen, particularly at low temperatures. It has been suggested that most fleas spend the majority of their time in the host nest or rodent burrow, rather than on the host itself (Benton 1980). The temperatures used in our study are similar to those previously reported for rodent burrows in temperate plague foci as well as foci located at higher elevation in tropical regions. Fleas residing in rodent burrows reside in distinctive and stable environments protecting the *Y. pestis* infected flea from extreme temperature fluctuations, swings in relative humidity and other conditions that might negatively affect flea survival through desiccation or other factors, thus allowing the pathogen to take advantage of these stable conditions to flourish within the flea's midgut and perhaps persist until the flea takes its next blood meal on a susceptible host, thus providing the opportunity to initiate new cycles of flea to rodent to flea transmission. Fleas usually are short lived, however several studies have been found them to survive off hosts for several months to more than a year in rodent burrows (Kartman, Prince et al. 1958, Bazanova and Maevskii 1996, Bazanova, Voronova et al. 2000)(Golov and Ioff, 1928)(Sharets et al. 1958). In our study, we found evidence that temperature plays a role in the ability of fleas to survive for extended periods. Our *Y. pestis*-infected *O. montana* fleas housed at 6ºC were found to harbor higher bacterial loads and remain capable of successful transmission to naïve mice out to day 42 p.i.. In infected fleas maintained at 10ºC and 15ºC, the growth, survival and transmission of *Y. pestis* was still higher when compared to fleas incubated at our control temperature (23 ºC) (Table 3.1, Figure 3.1 & 3.2). Many different variables are capable of playing a role in the survival of off-host fleas such as the age and sex of the flea, the flea species, infection with *Y. pestis,* or the availability of hosts to obtain a blood meal. In our study, flea survival may have been extended if maintenance feeds were provided to fleas during the

study, as was done in Williams et al. (2013), however, we still observed a trend in which our lower temperatures, mimicking burrow temperatures, showed an increase in plague growth, as well as, increased flea survival and transmission when compared to fleas maintained in soil at 23ºC. *Y. pestis* survival and growth in fleas maintained at 23ºC was significantly lower when compared to the infected fleas maintained at our three lower temperatures; indicating temperature contributed significantly to *Y. pestis* survival and growth within fleas, and may play a role in the focality of plague in temperate and tropical locations. The ability of temperature to influence the growth and transmission of *Y. pestis* in fleas is particularly interesting with respect to the focality of plague in the tropics where the disease typically does not occur or only briefly invades hot lowland areas and is found primarily in high, relatively cool upland areas.

In this study, we were able to demonstrate the ability of *Y. pestis*-infected *O. montana* fleas to successfully maintain infection and transmit *Y. pestis* to naïve rodent hosts out to day 42 p.i. when maintained at 6 ºC. Temperature seems to play a huge role in the growth of the bacterium within the flea, as well as the transmission efficiencies to mice. Flea survival at the lower temperatures is also significantly higher when compared to fleas held at 23 ºC. A previous study looking at lower temperatures and the effect on flea transmission found a significant increase in transmission efficiencies and growth of *Y. pestis* when infected fleas were maintained at temperatures lower than 23ºC (Williams, Schotthoefer et al. 2013). In order to determine why *Y. pestis* is better able to survive and be transmitted at these lower temperatures inside the flea gut, it is likely that further studies must be performed to examine transcriptional changes that are occurring *in vivo* at these lower temperatures but not at 23°C. Studies examining the lipopolysaccharide (LPS) of *Y. pestis* grown at 6ºC found temperature induced changes in this molecule which resulted in a rough LPS structure and a lack of expression, of genes which

encode the synthesis of the O-antigen, further allowing *Y. pestis* to avoid the innate immune responses once transmitted from the flea to the naïve rodent host (Suomalainen, Lobo et al. 2010). This change in the LPS structure may also play a role in the ability of *Y. pestis* to survive at the lower temperatures within the flea gut, a hypothesis that needs to be further investigated. With this current study it is known that temperature plays a pertinent role in *Y. pestis* survival and transmission from the flea to rodent host. To further elucidate how the *Y. pestis* transcriptional regulation is being altered at sub-ambient temperatures inside the flea, other studies must be performed to investigate temperature-related differences in gene expression, as well as, several global transcriptional regulators which could play major roles in regulating virulence and pathogenesis in *Y. pestis*.

CHAPTER III REFERENCES

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Table 3.1. Transmission efficiency of infected fleas held in soil at different temperatures during late phase (days p.i. 7 up to 42*)

Temp (°C)	Days p.i.	Flea infection prevalence (%)	Average no. fed, infected fleas per mouse (total in treatment group)	No. of mice infected (exposed)	Percent per flea transmission efficiency (95% CI)
6*	7	99.0	14.4 (102)	7 (7)	(N/A)[a]
	14	96.0	13.6 (99)	7 (7)	(N/A)[a]
	21	98.1	14.4 (101)	6 (7)	10.35 (4.55-28.72)
	28	98.1	14.4 (101)	5 (7)	8.94 (3.48-22.45)
	35	99.0	14.6 (102)	7 (7)	(N/A)[a]
	42	97.8	13.7 (41)	2 (3)	2.16 (0.14-11.83)
10	7	92.3	13.7 (104)	7 (7)	(N/A)[a]
	14	92.9	13.0 (98)	7 (7)	(N/A)[a]
	21	100	14.9 (104)	7 (7)	(N/A)[a]
	28	100	15.0 (105)	6 (7)	10.85 (4.77-30.29)
15	7	85.7	12.9 (105)	7 (7)	(N/A)[a]
	14	100	12.9 (104)	7 (7)	(N/A)[a]
	21	100	15.0 (105)	6 (7)	9.82 (4.36-26.35)
	28	99.0	13.4 (94)	6 (7)	7.47 (2.91-18.91)
23**	7	88.2	12.6 (97)	7 (7)	(N/A)[a]

***For 6°C fleas only. Fleas held at this particular temperature survived out to day 42 p.i..**
****Fleas held at 23°C in soil with no maintenance feeds were unable to survive out passed day 11 p.i.; therefore, transmission feeds were able to be performed at 7 days p.i..**
[a]When all pools are positive likelihood methods are not applicable. Likelihood estimates therefore do not exist in this case, indicated as N/A for these quantities.

Figure 3.1. Average CFU's/temperature/day post infection (p.i.)

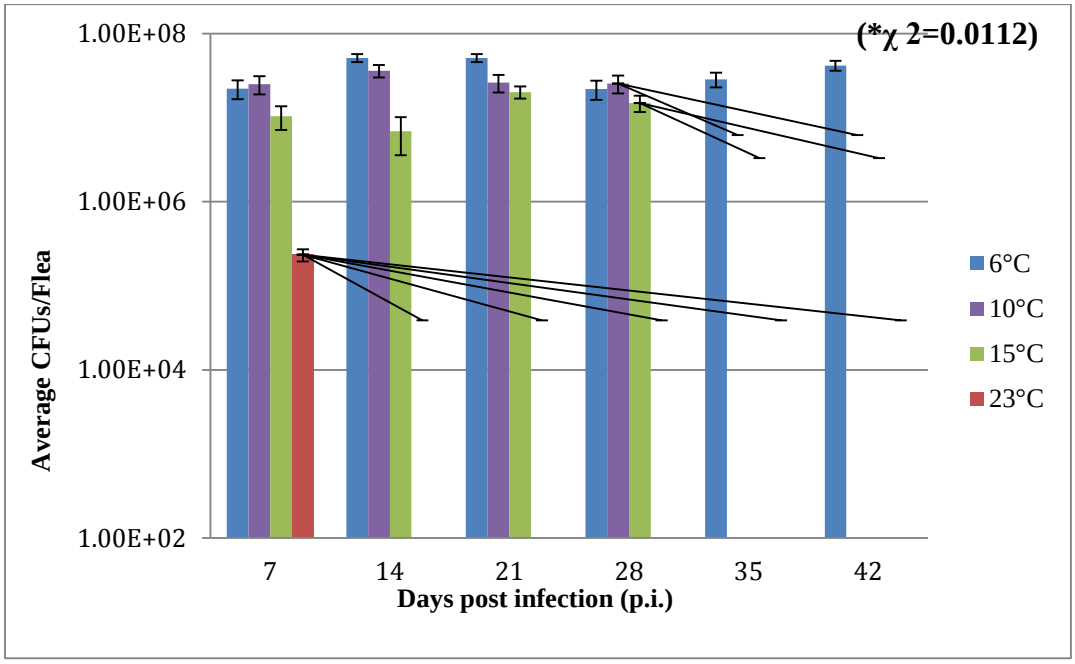

Figure 3.1. Histogram comparing average *Y. pestis* CFU per flea (Y axis) for temperatures 6°C, 10°C, 15°C, and 23°C and across all experimental time points denoted as days post infection (X axis). Sets of bars for each day post infection represent the average of the median CFU per infected, fed fleas for all temperatures at that time point. A significant difference in bacterial loads was found on day 7 p.i. between 6°C, 10°C and 15°C compared to 23 fleas. (X^2=0.0112)

Figure 3.2. Percent mouse mortality by temperature and time point

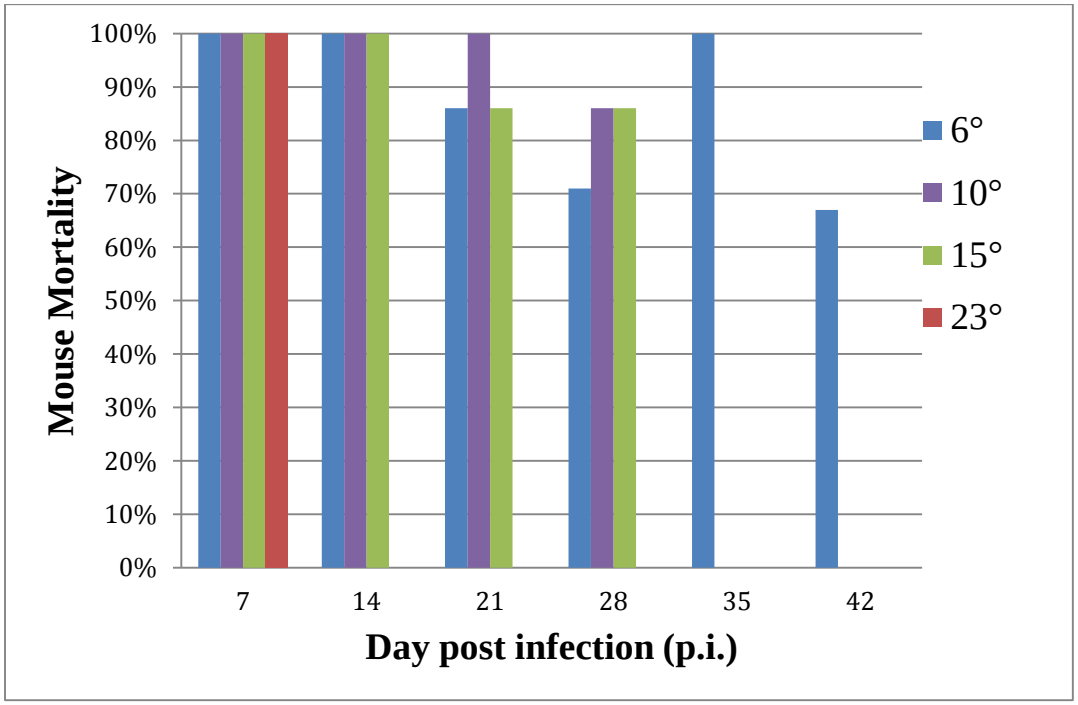

Figure 3.2. Histogram showing the number of mice that succumbed to *Y. pestis* infection, or were euthanized and confirmed to be infected by *Y. pestis* by bacteriophage lysis of culture isolates from mouse tissues, for each temperature and day post infection. The X axis denotes days on which naïve mice (7 mice were used for each time point and temperature) were exposed to infected fleas (infected fleas were obtained from three independent artificial infections for each temperature) and the Y axis indicates mouse mortality (dead or euthanized mice). Each temperature is denoted by a different block color (figure legend above). No significant difference was found in mouse mortality between temperatures on Days 7, 14, 21 and 28. Each temperature is in Celsius (°C).

Figure 3.3. ROS Assay Average OD in *Y. pestis*-Infected vs. Uninfected *O. montana* Fleas

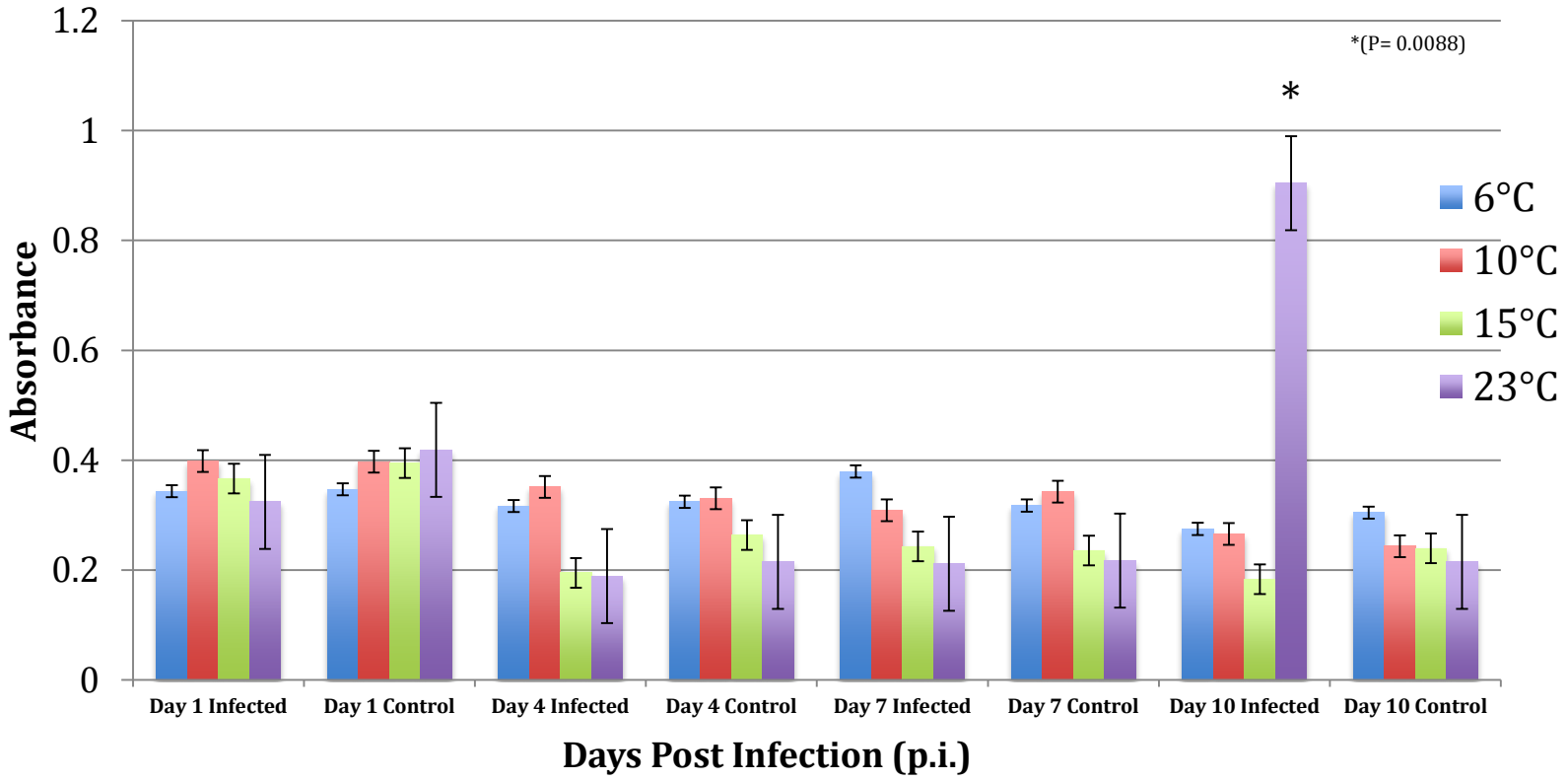

Figure 3.3. Histogram comparing the reactive oxygen species (ROS) assay of the average *Y. pestis* OD of fleas (Y axis) for temperatures 6°C, 10°C, 15°C, and 23°C and across all experimental time points denoted as days post infection (X axis). Sets of bars for each day post infection represent the average of the median OD per ten Yp-infected fleas, compared to ten blood fed fleas, for all temperatures at that time point. Temperatures are divided up by color, blue-6°C, purple-10°C, green-15°C, and red - 23°C. Solid bars are the *Y. pestis* infected fleas, and the patterned bars are the unifected-rat blood fed fleas.

Figure 3.4. ***Y. pestis*-infected ROS fleas average CFU's/temperature/day post infection (p.i.)**

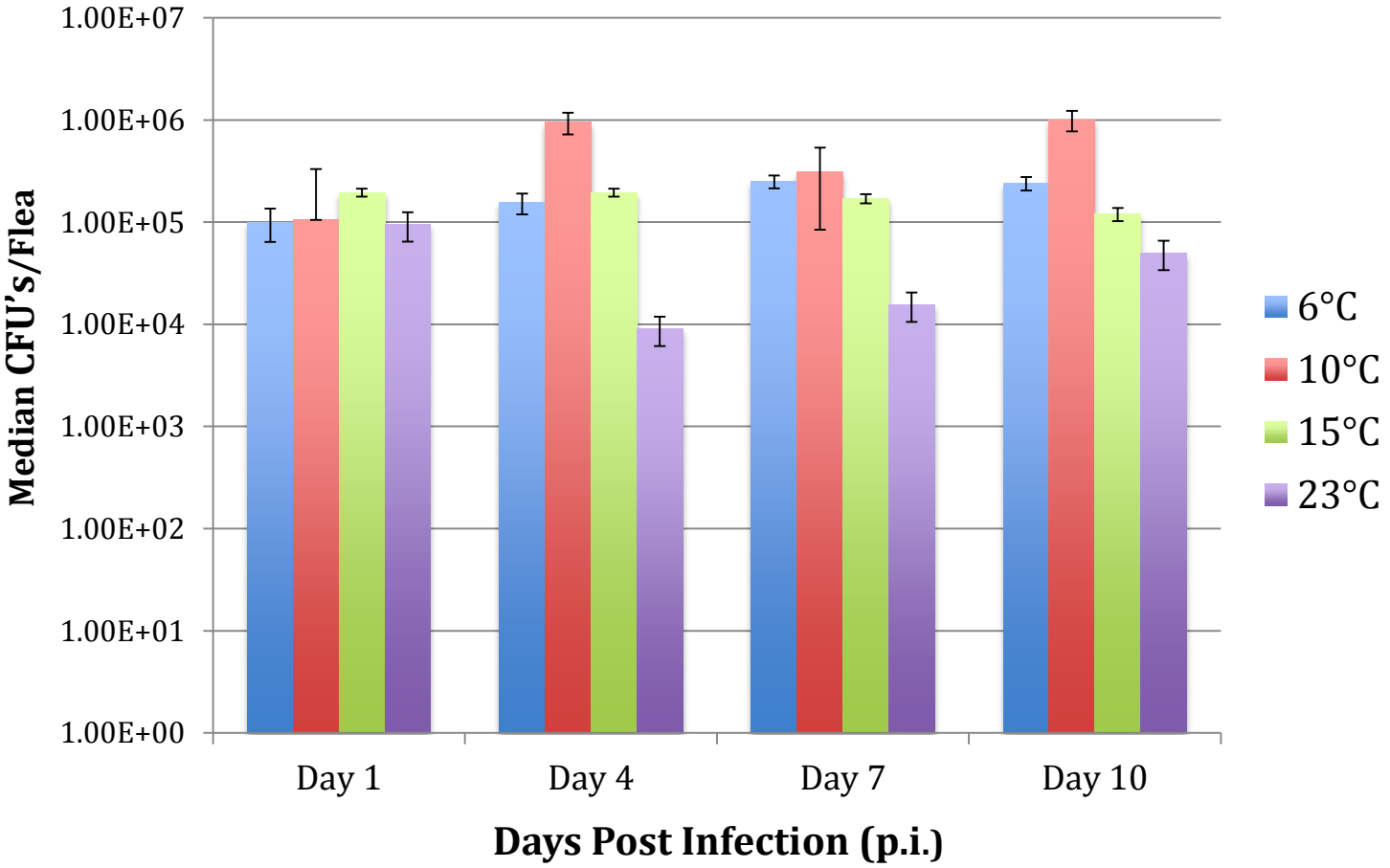

Figure 3.4. Histogram comparing average *Y. pestis* CFU per flea (Y axis) for temperatures 6°C, 10°C, 15°C, and 23°C and across all experimental time points denoted as days post infection (X axis). Sets of bars for each day post infection represent the average of the CFU per infected, fed fleas for all temperatures at that time point. Temperatures are divided up by color, blue-6°C, purple-10°C, green-15°C, and red- 23°C.

CHAPTER IV

Comparative Transcriptomics of *Yersinia pestis*: A Global View of Low Temperature Modulation of Gene Expression

Overview

Yersinia pestis, the causative agent of plague, is transmitted to mammals by infected fleas. *Y. pestis* is maintained within a distinct, closed life cycle encountering many changes in temperature and environmental conditions. Flea vectors are thought to optimally persist at temperatures between 21°C and 26°C however, survival of fleas at temperatures as low as 0°C, as might be encountered in the burrow of a hibernating rodent, have been reported. After transmission of *Y. pestis* to a mammalian host, the plague bacillus encounters a temperature shift to 37°C, inducing the expression of many well described *Y. pestis* virulence factors that confer resistance to host innate immunity. Gene expression in *Y. pestis* at a range of temperatures equivalent to those encountered in seasonally differentiated rodent burrows has never been examined. Identifying genes and factors which may be differentially expressed by *Y. pestis* at sub-ambient temperatures, may lead to new insights into vector-pathogen interaction and the virulence and pathogenesis of the plague pathogen. In this study, we used RNA-seq to compare the transcriptomes of *Y. pestis* grown at three sub-ambient temperatures (6°C, 10°C and 15°C), and found that the transcriptional profiles of *Y. pestis* at lower temperatures (6º, 10º and 15ºC), showed distinct gene expression patterns that varied significantly from those encountered at 23ºC. The number of differentially expressed genes (DEGs) for *Y. pestis* at each of the three lower temperatures tended to decrease as temperature increased (6ºC>10ºC>15ºC) relative to the ambient control temperature, 23ºC. When *Y. pestis* is grown at sub-ambient temperatures (6 °C, 10°C and 15°C) the largest proportion of the differentially expressed genes (DEGs) were

metabolic genes involved in the uptake and catabolism of amino acids and carbohydrates, resulting in up-regulation of the phosphotransferase system (*manXYZ*). The cyclic AMP receptor protein (Crp), was up-regulated at 6 °C, possibly regulating the differential expression of 81 genes at this growth temperature. The maltose operons, which include *malQPT, malEFG, malK, malM* and *malS*, was found to be expressed the highest in *Y. pestis* grown at 6°C, resulting in the mal operon genes being up-regulated by more than 2-fold, a statistically significant difference based on log2 fold changes ($p < 0.05$). At 6°C and 10°C, the LPS core biosynthesis protein CoaD was up-regulated. By contrast, the genes involved in the inner core biosynthesis, waaE, waaA and kbl were down-regulated potentially leading to an alternate LPS-type from that produced by *Y. pestis* at 23°C. Low temperature also played a role in the differential expression of *Y. pestis* outer membrane proteins (OMPs), as well as differential expression of an important adhesion and virulence gene, *yadB*, which was up-regulated at 6ºC and 10ºC, and is required for its role in bubonic plague pathogenesis. Our results suggest that *Y. pestis* growth at sub-ambient temperatures may play an important role in survivability and an expanded role in preparing the bacterium for pathogenesis within the mammalian host as has been suggested for *Y. pestis* during its transit through the flea.

4.1. Introduction

Yersinia pestis is the etiologic agent of plague. Plague is a rodent associated, flea-borne disease and responsible for emaciating human populations as a result of three large pandemics. Fleas are thought to acquire *Y. pestis* by feeding on plague infected mammals. The bacterium multiplies rapidly and forms masses within the flea gut at 26°C. Once the pathogen has been ingested by the flea, *Y. pestis* must adapt to the temperature shift (26°C), coming from a

mammalian host (37°C). To successfully adapt to changes in temperature, the bacterium must also adapt to changes such as nutrient requirements, enzyme activity, caspsule formation (37°C), and pigmentation production (Jackson and Burrows 1956, Tengerdy and Hillam 1973, Alonso, Hurtrel et al. 1982, Lindler, Klempner et al. 1990, Pendrak and Perry 1991). In addition, differential gene expression at these two temperatures may allow the bacterium to colonize its host efficiently, leading to commensalism or pathogenesis. More recently, studies have shown that *Y. pestis* transmits more efficiently to mammalian hosts and the bacterium replicates to higher levels in temperatures lower than 26°C (Williams, Schotthoefer et al. 2013). A study examining temperatures of rodent burrows during the winter months, found that *Y. pestis*-infected fleas had significantly higher bacterial loads then fleas maintained at 23°C. Furthermore, this study examined *Y. pestis*-infected fleas maintained at three sub-ambient temperatures (6°C, 10°C, and 15°C), and compared them to fleas maintained at 23°C. Surprisingly, fleas maintained at the three lower temperatures had higher transmission to rodent hosts and the lower the temperature, the better the flea survivability (Williams, Schotthoefer et al. 2013). Other studies in the past years have discovered many temperature-regulated virulence determinants (Straley and Perry 1995). With the completion of the annotated genome of *Y. pestis* CO92, studies comparing different *Y. pestis* strains, serotypes, and biovars, as well as its ancestor *Y. pseudotuberculosis* by DNA microarray were performed in order to compare different strains as well as determine the evolutionary genomic analysis (Parkhill, Wren et al. 2001). Subsequently, a study examining the transcriptional regulation of *Y. pestis* upon the increase of growth temperature from 26°C to 37°C in a chemically defined medium was performed and found that *Y. pestis* has the ability to extensively alter gene regulation in each environment (Han, Zhou et al. 2004). At the time, DNA microarray offered a tool for genome-

wide analysis of gene regulation at the transcriptional level (Kendall, Movahedzadeh et al. 2002). Currently, next-generation sequencing (NGS), is the state of the art technology in assessing the quantification of transcript levels as well as sequence information. Since microarray technology is limited to the amount of starting RNA, the quantification of transcript levels and the need for prior knowledge of sequence information, NGS has become the gold standard in transcriptome analysis. In this study, NGS was used to investigate whole transcriptomes of *Y. pestis* at lower temperatures (6°C, 10°C and 15°C compared to 23°C). This novel study will assess whether rodent burrow hibernation temperatures affect gene regulation, and determine the global transcriptional response to low temperature shifts; which could potentially lead to better survival or increased virulence. This data will provide a genome wide profile of gene transcription induced by low temperature shifts and will shed light on pathogenicity and host-microbe interaction of this deadly pathogen.

4.2. Materials and Methods

Bacterial strain, growth condition and preparation of bacterial pellet

A fully virulent *Yersinia pestis* CO96 strain was grown statically in Bacto Heart Infusion broth (Difco Laboratories) at 6°C, 10°C, 15°C, or 23°C for 3 days in duplicate, then concentrated by centrifugation at 12,000 x g for 10 minutes. Pellets were stored at -80°C after each culture was subjected to RNAlater® stabilizing reagent (Ambion) was added prior to freezing.

RNA extraction

Bacterial pellets were thawed on ice and homogenized in Lysis/Binding Solutions (Ambion). Each sample was further submitted to mechanical disruption using frozen pestles to lyse cells, followed by vortexing of each sample. The total RNA was isolated using the

RNAqueous Kit, according to the manufacturer's recommendations. RNA samples were eluted with RNase/DNase free water and yields were determined using the NanoDrop 2000 Spectrophotometer (ThermoFisher, Wilmington, DE). TURBO DNase was used to treat each sample to remove any contaminating genomic (Ambion). Additionally, the RNA integrity was checked on the Agilent Bioanalyzer using the Agilent RNA 6000 Kit (Agilent Technologies, Inc., Santa Clara, CA). Total extracted RNAs preparations were stored at -80°C until further use.

rRNA depletion

Ribosomal RNA depletion was performed on each Total RNA sample using the RiboMinus™ Transcriptome Isolation Kit (Invitrogen), 6 mg of total RNA were loaded and treated according to the manufacturer's recommendations. Each RiboMinus-depleted sample was eluted in RNase/DNase free water and further analyzed using the Agilent Bioanalyzer, Agilent RNA 6000 Kit (Agilent Technologies, Inc., Santa Clara, CA). All depleted RNA samples were stored at -80°C prior to further manipulations.

RNA-seq Library Construction

For RNA-sequencing, rRNA-depleted samples were used for cDNA library preparations using the Ion Total RNA-Seq Kit v2 (ThermoFisher, Wilmington, DE). Briefly, 10-500 ng of ribo-depleted RNA was fragmented using RNase III for 4 minutes. Each fragmented RNA sample was purified using the Magnetic Bead Clean-up Module (ThermoFisher Scientific) and yield and size distributions of each samples were assessed using the Agilent 2100 Bioanalyzer, Agilent RNA 6000 Kit (Agilent Technologies, Inc., Santa Clara, CA). Fragmented RNA was then used to further construct the whole transcriptome library by hybridization and ligation of specific primers to each sample (Ion adaptor mix, (ThermoFisher, Wilmington, DE). Next, reverse transcription was performed using 10X SuperScript III Enzyme (ThermFisher Scientific),

on each ligated RNA sample. The resulting cDNA samples were purified using the Magnetic Bead Clean-up Module (ThermoFisher Scientific) and yield and size distributions of each samples were assessed using the Agilent 2100 Bioanalyzer, Agilent High Sensitivity DNA Kit (Agilent Technologies, Inc.). The cDNA was further amplified and purified using the Magnetic Bead Clean-up Module (ThermoFisher, Wilmington, DE). Each amplified cDNA sample's yield and size distribution were assessed using the Agilent 2100 Bioanalyzer using the Agilent High Sensitivity DNA Kit (Agilent Technologies, Inc., Santa Clara, CA). Using the Agilent 2100 Bioanalzyer (Agilent Technologies, Inc.) smear analyses were performed to quantify the percentage of DNA that ranges in size from 50-160bp. Molar concentrations were then determined for each cDNA library using the size range from 50-1000 bp. After the smear analyses were performed and if the percentage of DNA in the 50-160 bp range is <50%; then determination of the library dilution factor could be completed which is required for template preparation. With less than 50% of the amplified DNA in the correct range, and for template preparation using the Ion PGM Template OT2 400 kit, or the Ion Hi-Q OT2 kit, library dilutions that give a concentration of 100 pM are required. (Ion Total RNA-Seq Kit v2 for Whole Transcriptome Libraries, (ThermoFisher, Wilmington, DE).

Ion Torrent Template Preparation and PGM sequencing

Diluted libraries were used as template for an emulsion PCR (emPCR) using reagent from either the Ion PGM OT2 400 Template Kit or Ion PGM Hi-Q Template kit (ThermoFisher Scientific). In brief, the DNA fragments were bonded onto spheres Ion Sphere™ particles (ISPs) and then amplified using the Ion OneTouch 2 Instrument (ThermoFisher, Wilmington, DE). Following the amplification, the emulsion was broken with successive washes using the Ion OneTouch ES instrument (ThermoFisher Scientific), and the spheres without template were

removed using the Dynabeads® MyOne™ Streptavidin C1 Magnetic Beads (ThermoFisher, Wilmington, DE). Next, the enriched ISP's which contain only template-positive ISP's were then mixed with Control Ion Sphere Particles (Ion PGM Hi-Q Sequencing Kit (ThermoFisher, Wilmington, DE). Next, sequencing primers were added to the ISP's mixture and placed in a thermocycler for 95°C for 2 minutes and then 37°C for 2 minutes to anneal the primers. Sequencing polymerase enzyme was added to the ISP mix and incubated for 5 minutes and room temperature. The resulting mixture was loaded on the Ion 318™ Chip for sequencing using the Ion Torrent Personal Genome Machine™ (PGM) (ThermoFisher, Wilmington, DE).

Bioinformatic analysis

All RNA-seq fastq data output files collected from the results of *Y. pestis* at 6°C, 10°C, 15°C and 23°C were aggregated and sent to the IDCR Bioinformatics Core for downstream bioinformatic analysis. Bioinformatic analyses were performed using Strand NGS software, Version 2.1, Build 163982 (Strand Scientific Intelligence, Inc., San Francisco, CA, USA). To begin, the quality control manager and preprocessing of each sample were assessed by removal of low quality bases (<Q20), rRNA sequences, adapter sequences, and short reads (<35bp). Next, RNA-seq bioinformatics analyses were applied to each sample by aligning the trimmed/filtered samples to the proper reference genome, *Y. pestis* CO92 (RefSeq genome NZ_CP009973.1) using the Strand NGS aligner, which uses a proprietary algorithm based on the Burrows Wheeler Transform. This aligner is significant because the aligner algorithm is capable of handling both short reads and long reads as well as allowing an arbitrary number of gaps and mismatches within the reads (Strand NGS Manual, Version 2.0, San Francisco, CA). Next, DESeq was integrated into the analysis tools for multi-conditional batch processing and used for normalization and differential analysis to produce classical statistical analysis (Z-scores and fold

changes) (Anders and Huber, 2010). Pathway and gene ontology analysis were performed and enabled comprehensive cross-comparisons of the genomic or proteomic data to identify key pathway/GO perturbations and underlying mechanistic regulatory points. A subset of the complete array of genes that map to the analyzed pathways and gene ontology groups are used in the analysis. Strand NGS provided the analyses and modeling results by a secure web-based analyses reporting system which will result in two excel files containing $\log_2$ fold change values and *p*-values that have been adjusted for multiple testing for each gene that was mapped.

Quantitative Real-Time PCR (qRT-PCR) Validation

A quantitative RT-PCR assay was designed to target genes that were both upregulated and downregulated in order to ensure the integrity of the PGM sequencing results. To validate our NGS transcriptome results, differentially expressed genes (DEGs) of *Y. pestis* were selected for qRT-PCR analysis from each temperature (6°C, 10°C and 15°C). Genes that are highly downregulated or highly upregulated at the different temperatures were tested and compared to NGS fold change values to verify gene expression patterns are credible before subsequent studies are conducted. Briefly, primers were designed for each selected differentially expressed gene (DEG). Primers were designed using Primer Express Software, v3.0 (Applied Biosystems, CA), and purchased from the Biotechnology Core Facility Integrated (DSR, Atlanta, GA). Real-time efficiency for each primer set were determined by amplification of a standardized cDNA dilution series, and specificity of the amplified PCR products were verified by melting curve analyses. Each real-time quantitative PCR reaction was performed using the CFX Connect™ Real-Time PCR Detection System (Bio-RAD). Briefly, RNA was isolated and prepared in duplicate from *Y. pestis* grown at 6°C, 10°C, and 15°C as described above. To avoid contamination with DNA, samples were treated with Turbo DNA-free DNase I (ThermoFisher Scientific, Wilmington,

DE). Next, each RNA sample subjected for qRT-PCR was reverse transcribed to synthesize cDNA in 20 µl reactions according to the manufacturer's protocols using the MultiScribe™ Reverse Transcriptase (Applied Biosystems, CA). A control reaction lacking reverse transcriptase was performed for each primer set using total RNA to ensure samples are not contaminated with DNA. Next, cDNAs were used as templates for qPCR using Power SYBR Green PCR master mix (PE Applied Biosysthems) and gene-specific primers (0.5 µM). Next, cDNA amplification was performed in 20 µl volumes for each reaction. Template and no-template controls were used for each primer set. All sample results were normalized against the expression level of the *proS* gene, which is constitutively expressed and not found to be differentially expressed at different growth temperatures used in this study. All reactions were assayed in triplicate, and the average Ct value were used to quantify the relative level of expression. Replicate raw data (threshold cycle number, or Ct) for each sample was averaged and then adjusted by comparing to the corresponding averaged Ct for the endogenous control gene to correct for any differences in the starting quantity of the material. Relative quantification using comparative Ct analyses were performed using the CFX Manager™ software (Bio-Rad), and relative expression levels across samples were performed using the comparative Ct method (ΔΔCt) (Livak and Schmittgen 2001). The qRT-PCR relative expression (fold change) ratios were compared to the RNA-seq DESeq fold changes to test for accuracy of the RNA-seq sequencing results.

4.3. Results

Transcriptional profile of Y. pestis at sub-ambient temperatures:

The transcriptional profiles of *Y. pestis* at low temperatures, mimicking the burrow temperatures (6º, 10º and 15ºC) of rodents including those in hibernation, showed very distinct transcriptional profiles. The number of differentially expressed genes (DEGs) for *Y. pestis* at each of the three lower temperatures were compared to the ambient control temperature, 23ºC (Figures 1 and 3.). Among 4,012 genes of *Y. pestis* CO92, of which 192 are pseudogenes (Parkhill, 2011), transcript abundances were the most differentially altered for *Y. pestis* grown at 6ºC, in which 1375 genes were either up- or down-regulated. Out of the 1375 DEGs for *Y. pestis* at 6ºC, 656 (48%) genes were up-regulated and 719 (52%) genes were down-regulated (Figure 4.1.). *Y. pestis* grown at 10º was found to have the second highest number of genes (1218) differentially expressed. Of these 1218 genes showing differential expression, 655 (54%) were up-regulated, and 563 (46%) were down-regulated (Figure 4.1). *Y. pestis* grown at 15ºC, resulted in the fewest number of differentially expressed genes compared to *Y. pestis* grown at ambient temperature. A total of 968 genes were differentially expressed at 15ºC, of which 442 (46%) genes were found to be up-regulated, and 526 (54%) were down-regulated (Figure 4.1.). Based on log2 fold changes ($p < 0.05$), transcript levels for 465 *Y. pestis* genes expressed at 6°C, changed more than 2-fold relative to those expressed at the control temperature of 23°C. Next, *Y. pestis* grown at 10°C yielded 409 genes whose transcript levels changed more than 2-fold based on log2 fold changes ($p < 0.05$), and for *Y. pestis* grown at 15°C, only 210 genes were found to have transcript levels changed more than 2-fold.

Transcriptional control by global regulators:

Crp:

In our study, *Y. pestis* grown at 6°C showed a 3.1-fold up-regulation of the cyclic AMP receptor protein (Crp). Crp is an important transcriptional regulator that plays a role in bacterial survival and virulence. In *Y. pestis*, Crp controls the expression of at least 214 known bacterial genes by a process known as catabolite repression, which typically occurs in response to environmental cues by sensing carbon availability (Busby and Ebright 1999, Zhan, Han et al. 2008). Of the 214 known *Y. pestis* genes that are regulated by Crp at mammalian temperatures, we identified 81 genes which exhibited up or down gene expression in response to growth at 6°C (Table 4.1). In addition, Hfq, which is a small RNA (sRNA) chaperone protein involved in posttranscriptional regulation, was found to be up-regulated (2.6-fold) at 6°C. Interestingly, neither *crp* or *hfq* were found to be up- or down-regulated at 10°C or 15°C relative to 23°C. Also regulated by Crp, is the maltose (*mal*) regulon, which was found to be up-regulated when *Y. pestis* was grown during 6°C but not at 10ºC or 15ºC. The *mal* operon, which includes *malQPT* and *malEFG* were up-regulated by more than 2-fold (range, 2.5-3.8), based on log2 fold changes ($p < 0.05$) in *Y. pestis* at 6°C (Table 4.1). The maltose operon encodes genes important for the uptake and metabolism of maltose, as well as enzymes that play a role in the conversion of maltose into glucose and glucose α-1- phosphate (Boos, Ferenci et al. 1981, Boos and Shuman 1998). The most highly up-regulated genes in *Y. pestis* grown at 6°C (ranging from 4.9-7.2 fold, based on log2 fold changes ($p < 0.05$), were the *gal* genes, comprising the galactose operon, which are known to be regulated by Crp, and recognized as playing a role in cell wall biogenesis, and commonly found in the LPS structure of *Y. pestis* at lower growth temperatures (Table 4.2) (Knirel and Anisimov 2012). As was determined for the *mal* operon genes, the *gal* operon was

not found to be up-regulated in *Y. pestis* at 10°C or 15°C. The *csrA* gene encoding a carbon storage regulator (CSR) homolog was found to be up-regulated at *Y. pestis* grown at 6°C and 10°C, and is part of the CSR system, necessary for glycogen metabolism and gluconeogenesis (Table 4.5). In addition, Crp-mediated expression of *csrA* is required for biofilm formation independent of glycogen metabolism (Willias, Chauhan et al. 2015). Other genes found to be up-regulated by Crp in response to growth temperatures at 6°C, included the *deoABCD* genes, which are important in amino acid metabolism and transport, and the glucose phosphotransferase (PTS) system which includes the mannose operon (*manXYZ*) and the *N*-acetylglucosamine operon (*nagABCE*) with fold change ranging from 3.1-15.6 ($p < 0.05$) (Table 4.1). Although inactive in *Y. pestis* epidemic strains, the Crp-regulated *zwf* gene, encoding glucose-6-phosphate dehydrogenase, showed increased expression at 6°C. Energy production genes of *Y. pestis* that are known to be regulated by Crp (*aceEF, atpI, adhE, epd, focA, pflA, ducA, lpdA, nirB*) also were significantly up-regulated (2.1-7.4 fold) at 6°C, suggesting an increased requirement in expression of genes involved in the ATP-proton motive force and oxidative phosphorylation pathways of *Y. pestis* (Table 4.1). Lipid biosynthesis (f*adDH*) and transport (*lipB*) genes, were found to be down-regulated by Crp in *Y. pestis* at 6°C. The universal stress response gene, *uspA*, altered by Crp was also down-regulated (3-fold) in *Y. pestis* at 6°C.

PhoP:

The transcriptional regulator gene *phoP* of the PhoPQ two-component regulatory system and the PhoP-regulated *mgtC* gene were expressed at levels >2-fold higher in *Y. pestis* grown at 6°C when compared to *Y. pestis* grown at 10°C, but no difference in expression of either gene was found between *Y. pestis* grown at 6°C and expression at bacterial growth during 15°C and

23°C (data not shown). It has been previously determined that PhoP and MgtC are known virulence factors necessary for survival of *Y. pestis* and other Gram-negative bacteria in macrophages, as well as for resistance to cationic antimicrobial peptides (CAMPs) of the mammalian innate immune response (Vadyvaloo, Jarrett et al. 2010). The expression of *phoQ* was found to be down-regulated in *Y. pestis* at sub-ambient temperatures when compared to pathogen growth at 23°C (data not shown).

The autoinducer-2 (AI-2) quorum-sensing system:

The autoinducer-2 (AI-2) quorum-sensing system has been linked to diverse phenotypes and regulatory changes in *Y. pestis* bacteria. Quorum sensing (QS) is a process of cell-to-cell communication that relies upon secretion and detection of chemical signals called autoinducers (AIs). *Yersinia pestis* possesses two conserved QS systems, the AI-1 pathway, which utilizes acyl homoserine lactones (AHLs) as signaling molecules, and the LuxS or AI-2 pathway, which uses a furanone autoinducer (Bobrov, Bearden et al. 2007). The AI-2 signaling pathway is commonly found in to many bacteria as homologues of the *luxS* gene. *LuxS* has two main functions, to generate the major methyl donor *S*-adenosylmethionine, and to detoxify *S*-adenosyl-l-homocysteine to homocysteine and 4,5-dihydroxy-2,3-pentandione (DPD), which yields AI-2 (Chen, Schauder et al. 2002). In our study, we found the *luxS* gene was up-regulated in *Y. pesti*s when grown at 6°C (2.5 fold) and 10°C (2.8 fold). No change was detected in the *luxS* gene in *Y. pestis* grown at 15°C compared to *Y. pestis* grown at 23°C. Neither the *ypsIR* nor the *ytbIR* gene pairs of the two AHL quorum sensing systems in *Y. pestis*, were found to be up- or down-regulated at any of the lower growth temperatures.

Lipopolysaccharide Biosynthesis:

The lipopolysaccharide (LPS) of *Y. pestis* plays a significant role in resistance to host complement, antibiotics and and other host defense mechanisms and is considered an important virulence factor of *Y. pestis*. As in other *Enterobacteriacae*, LPS is a major component of the outer membrane of the *Y. pestis* cell wall and forms the outer layer of the LPS-phospholipid bilayer. In our study, we found that *coaD*, which encodes a LPS core biosynthesis protein, was up-regulated at 6°C and 10°C, and *waaE*, *waaA* and *kbl*, which are essential genes for the synthesis of the inner core were down-regulated at 6°C and 10°C, and *waaE* and *kbl* were down-regulated at 15°C. In addition, YPO0415 was found to be up-regulated in *Y. pestis* grown at 6°C, 10°C and 15°C and encodes a putative carbohydrate kinase necessary for LPS core synthesis, as well as the late stages of lipid A biosynthesis (Table 4.3). The LPS heptosyltransferase genes *waaC* and *waaQ* were up-regulated in *Y. pestis* grown at 15°C compared to *Y. pestis* expression at 6°C, 10°C and 23°C (Table 4.3).

Outer Membrane Proteins (OMPs):

Y. pestis is richly endowed with proteins in its outer membrane (OMPs). Many of the OMPs of *Y. pestis* have been associated with pathogenicity and evasion of the host immune response including resistance to complement and antibacterial peptides as well as invasion and survival within mammalian cells (Weiser and Gotschlich 1991, Weiser and Gotschlich 1991, Prasadarao, Wass et al. 1996, Fu, Belaaouaj et al. 2003, Llobet, March et al. 2009). In our study, we found that *Y. pestis* grown at 6°C had increased gene expression of the transcriptional regulators, *ompR* and *ompF*, in addition to up-regulation of the outer membrane protein genes *ompA* and *ompX* and the chaperone-encoding gene *ompH*. When the pathogen was grown at 10°C, increased gene expression of the two transcriptional regulatory proteins, *ompR*, *ompF*, and

ompH were observed. *Y. pestis* grown at 6°C and 10°C showed down-regulation of the outer membrane protein C gene, *ompC*. When *Y. pestis* was grown at 15°C, no changes in gene expression related to OMPs were found in comparison to *Y. pestis* grown at 23°C (Table 4.4).

Yersinia pestis metabolic adaption to growth at low temperatures:

The majority of the differentially expressed genes (DEGs) in *Y. pestis* at lower temperatures were those related to metabolism involved in the uptake and catabolism of amino acids and carbohydrates (Table 4.5). In particular, genes involved in transport and catabolism of the L-glutamate group of amino acids, arginine (*argC*), histidine (*hutIG*). The degradation of these amino acids gives rise to L-glutamate and the TCA cycle intermediates succinate, formate, and α-ketoglutarate. Additionally, the ABC-transport gene *metN*, involved in methionine uptake was up-regulated in *Y. pestis* at 6°C, 10°C and 15°C were upregulated in *Y. pestis* (Table 4.5). The hydroxyphenylacetate (HPA) transport gene (*hpaX*) of *Y. pestis*, was up-regulated at the three lower temperatures (6°C, 10°C and 15°C) with concomitant down-regulation of the HPA catabolic genes (*hpaBCI*) at sub-ambient temperatures when compared to *Y. pestis* at 23°C (Table 5.). Expression of the glucose phosphotransferase system (PTS) genes, *manXYZ*, was significantly increased in *Y. pestis* grown at the three sub-ambient temperatures relative to *Y. pestis* grown at 23°C. Significant down-regulation of the *actP* (6°C and 10°C), and *acs* (only at 6°C) genes, representing uptake of acetate and its conversion to acetyl-CoA, respectively, suggests that other mechanisms are being utilized at the lower temperatures of 6ºC and 10ºC to provide sufficient amounts of acetyl-CoA in order to potentiate the TCA cycle. The *uxaA* and *uxaC* genes encoding altronate hydrolase and glucouronate isomerase, respectively, involved in pentose and glucuronate interconversion, were up-regulated in *Y. pestis* grown at 6°C, 10°C and 15°C when compared to *Y. pestis* at our control temperature (Table 4.5). We also found that

genes necessary for respiration were significantly up-regulated (*hydN* and *dmsA*) in *Y. pestis* grown at 6°C, 10°C and 15°C as were those required for oxidative phosphorylation (*atpABDFHI*) in *Y. pestis* grown at 6°C (Table 4.5). Other genes that were up-regulated in *Y. pestis* at all three sub-ambient temperatures included lipid metabolism genes, *fadB* and *cdh*, though *accBD* and *acpD* only showed increased expression at 6°C (Table 4.5). Specific fatty acid degradation genes (*fadHDE*), also were up-regulated in *Y. pestis* at all three lower temperatures when compared to 23°C (Table 4.5).

Differential expression of Y. pestis pathogenesis-related genes:

Y. pestis contains many temperature-induced virulence factors, such as the iron acquisition systems, (*ybt*,*yfe* and *yfu* operons), and the oxidative and nitrosative stress response genes (*radC, hmp*), all of which contribute plague infection, and in this study, these genes were found to be down-regulated or exhibited no change in regulation when *Y. pestis* was grown at the sub-ambient temperatures. The *Y. pestis* F1 capsular antigen genes (*caf1M, caf1A*), were down-regulated at *Y. pestis* grown at 6°C, 10°C and 15°C, expected since the F1 antigen is only expressed at temperatures >33°C as would be encountered within the mammalian host and essential for virulence and evasion of the host immune system. Interestingly, the *Y. pestis* outer surface protein gene *yadB*, which is required for dissemination of *Y. pestis* from a subcutaneous inoculation site, specifically enhancing bubonic plague pathogenesis (Forman, 2008), was up-regulated in *Y. pestis* at 6°C (5.1-fold) and 10°C (3.5-fold) (Table 4.5). Other known virulence and transmission factors were not found to be differentially regulated in *Y. pestis* grown at the low temperatures when compared to pathogen growth at ambient temperatures, including the *hms* genes, necessary for biofilm formation within the flea, the *Y. pestis* plasminogen activator (*pla*), critical for dissemination from extravascular tissue at the fleabite site, and *ymt*, which is

necessary for bacterial survival in the flea vector. Up-regulation of the universal stress response gene B (*uspB*) was found in *Y. pestis* grown at 10°C (9.8-fold) and 15°C (6.4-fold), when compared to 23°C, and the universal stress response gene A (*uspA*) was up-regulated at 10°C (2.2-fold) and 15°C (2.1-fold), but was down-regulated at 6°C (-3-fold) (Table 4.5).

Validation of NGS sequencing results by qRT-PCR:

Quantitative reverse transcription-PCR confirmation was performed to further validate the NGS RNA-seq results. Target genes were selected by choosing genes which fell into one of the following criteria: most highly-upregulated, highly down-regulated, virulence gene, or involved in regulation of virulence genes. All sample results were normalized against the expression level of the *proS* gene which encodes a proline tRNA ligase. For the qRT-PCR samples, the average Ct values were used to quantify the relative level of expression ratios (fold change), and compared to the RNA-seq differentially expressed gene fold changes to test for accuracy of the RNA-seq sequencing results. Genes identified as being up-or down-regulated by NGS, were further validated by our qRT-PCR results (Figure 4.2). For each temperature, the Pearson correlation coefficient was used to measure the strength of a linear association between the qRT-PCR results and the NGS results. All temperatures showed a high association between the qRT-PCR fold changes, and the fold changes identified by NGS (all r values >0.900). *Y. pestis* primer sets can be found in Table 4.6.

4.4. Discussion

Y. pestis is a highly adaptive organism that inhabits different ecological niches as it cycles between arthropod vector and mammalian hosts and surviving under different, occasionally extreme, environmental conditions. The pathogenic lifestyle of *Y. pestis*, requires

strict control of virulence and general stress response genes. In the plague pathogen, the *crp* gene is found to be responsible for regulation of multiple virulence factors, including components of the type III secretion system (T3SS) and the plasminogen activator Pla (Lathem, Schroeder et al. 2014). It was previously demonstrated *Y. pestis crp* is positively regulated at the posttranscriptional level by the activity of the small-RNA (sRNA) chaperone, Hfq (Lathem, Price et al. 2007). The posttranscriptional activation of Crp was found to be necessary for expression of Pla in *Y. pestis*, and both Hfq and Crp are known to contribute immensely in the virulence of *Y. pestis* specifically during pneumonic plague infections (Lathem, Price et al. 2007, Lathem, Schroeder et al. 2014). Binding of Hfq to mRNA transcripts, results in either enhanced transcript stabilization or promotion of transcript degradation. In *Salmonella* Typhimurium, Hfq has been found to be responsible for controlling multiple metabolic pathways such as the purine and pyrimidine metabolism pathways, glycolysis/gluconeogenesis, pyruvate metabolism, the TCA cycle, and amino-acyl tRNA biosynthesis; whether Hfq plays the same role in the regulation of these metabolic pathways in *Y. pestis* is uncertain (Sittka, Pfeiffer et al. 2007, Sittka, Lucchini et al. 2008, Ansong, Yoon et al. 2009, Geng, Song et al. 2009, Sittka, Sharma et al. 2009, Bai, Golubov et al. 2010, Schiano, Bellows et al. 2010, Ansong, Deatherage et al. 2013, Kakoschke, Kakoschke et al. 2014). It is known however, that Hfq along with sRNAs, contribute to heightened virulence in *Y. pestis*, and have been implicated in the posttranscriptional regulation of the T3SS/Yop machinery in *Y. pestis* and *Y. pseudotuberculosis*. (Geng, Song et al. 2009, Schiano, Bellows et al. 2010, Schiano, Koo et al. 2014). Studies have found that the loss of both Crp and Hfq, resulted in attenuation of *Y. pestis*, further validating the importance of these two genes in virulence as well as the ability to adapt to different nutritional conditions (Zhan, Han et al. 2008). Since the *Y. pestis crp* gene is responsible for direct

transcriptional regulation of *pla*, loss of the Crp gene leads to reduced virulence of *Y. pestis* in the mammalian host and the inability of the bacterium to disseminate from the flea bite or inoculation site (Zhan, Han et al. 2008, Lathem, Schroeder et al. 2014). In the current study, there is ample evidence of Crp's regulatory function even at sub-ambient temperatures. *Y. pestis* grown at 6°C, resulted in the altered expression of 81 genes, known to be controlled by the *Y. pestis crp* gene product. In our previous studies, *Y. pestis*-infected-*Oropsylla montana* fleas maintained at sub-ambient temperatures (6°C, 10°C and 15°C), found that infected fleas maintained at 6°C, had higher bacterial loads and demonstrated increased transmission to naive mice when compared to the *Y. pestis*-infected *O. montana* fleas maintained at 23°C (Williams et al., 2013). From differential gene expression analysis performed in this study, it would be of interest to determine the role of Crp and its influence on the survival of the pathogen in the flea gut at 6°C and subsequent transmissibility to mammalian hosts. Other studies have already demonstrated that Hfq positively regulates *crp* in *Y. pestis* at the posttranscriptional level, and both of these genes were found to be up-regulated in this study when *Y. pestis* was grown at 6°C. Determining how the altered expression of the Crp-regulated genes at 6°C affects pathogen fitness and virulence would be of value. For example, *Y. pestis* grown at 6°C, resulted in up-regulation of the *crp* gene with concomitant up-regulation of the galactose operon (*galETKM*). Previous studies have found that when *Y. pestis* is grown at temperatures as low at 6°C, galactose can get incorporated into the LPS core oligosaccharide structure (Knirel and Anisimov 2012). *Y. pestis* galactose-containing LPS potentially could lead to increased virulence by further enhancing evasion of host innate immunity. Further studies could better elucidate the importance of the galactose operon at lower growth temperatures, and the significance of galactose incorporation into the LPS of *Y. pestis* at sub-ambient temperatures. Orthologous *gal*

genes have been identified in *Escherichia coli*, *Neisseria gonorrhoeae*, *Rhizobium meliloti*, *Erwinia amylovora* and other Gram-negative bacteria (Metzger, Sawyer et al. 1993). In a study examining the importance of the galactose operon in *E. amylovora*, the pathogen responsible for the necrotic disease, fire blight, in rosaceous plants, found that the *gal* operon is constitutively expressed independent of galactose concentration in the growth medium or environment. This study found that the *galE* gene is essential for virulence of *E. amylovora* pathogen, whereas *galT* and *galK* were not (Metzger, Sawyer et al. 1993). In addition, this study looked at the effects of a *gal* operon mutation on the incorporation of LPS O-antigen side chains and determined that this mutant elicited O-antigen side chain incorporation effects, but an intact O-antigen was not needed for successful virulence of the pathogen (Metzger, Sawyer et al. 1993). Similarly, Metzger et al, also demonstration that disruption of galactose metabolism affected capsule synthesis and virulence of *E. amylovora*, which were restored if galactose was added to the growth medium (Metzger, Sawyer et al. 1993). Galactose in *Y. pestis* is utilized by the LeLoir pathway, and the pathogen encodes the structural genes *galK*, *galT*, *galE*, which are organized in an operon induced by galactose. In *Y. pestis*, two gal promoters, which are modulated by the cyclic AMP (cAMP) receptor protein complex, and one gal repressor are encoded on the operon and essential for controlling regulation of the galactose operon (Cornwell, Adhya et al. 1987, Majumdar and Adhya 1987, Majumdar, Rudikoff et al. 1987). A more recent study examining the galactose network in Enterobacteria, found that galactose is highly important for survival and virulence of *Enterobacteriacae* (Csiszovszki, Krishna et al. 2011). This study determined that each pathogen has a unique galactose utilization system in order to optimize the system for different bacterial environments. Also of importance, this study found that the galactose mutarotase gene (*galM*) of *Y. pestis* is inactive due to a single base pair deletion; interestingly,

they found the ability of the *galM* gene to be restored by single base pair insertions. They hypothesized that during the evolution of *Y. pestis* from *Y. pseudotuberculosis,* in order to adapt to a vector-borne lifestyle, many genes were likely converted to pseudogenes. These workers suggest that *Y. pestis* pseudogenes are not deleted because meiotrophic reactivation of these genes may be necessary for survival in certain environments (Csiszovszki, Krishna et al. 2011). In the current study, we found that when *Y. pestis* was grown at 6°C, the galactose operon was the most highly up-regulated operon, including significant up-regulation (28.5 fold) of the *galM* pseudogene. Since *galM* was found to be significantly up-regulated in at 6°C, this could indicate possible restoration and reactivation of the gene, which may be necessary for bacterial adaptation and survival at colder temperatures. The incorporation of galactose into *Y. pestis* LPS at low temperatures, further implies that the galactose operon may have an important role in *Y. pestis* survival and virulence under these conditions.

The maltose operon of *Y. pestis*, which can also be influenced by the *crp* gene, was up-regulated when the pathogen was grown at 6°C. The maltose operon is well-characterized in other pathogens such as *E. coli*. This maltose operon includes *malQPT*, *malEFG*, *malK lamB malM*, and *malS* (Boos, Ferenci et al. 1981, Boos and Shuman 1998). Maltose genes are important for the uptake and metabolism of maltose, in addition to the conversion of maltose into glucose and glucose α-1- phosphate (Boos, Ferenci et al. 1981, Boos and Shuman 1998). The *mal* genes are regulated by the transcriptional activator, MalT, and responds to the presence of maltose and maltodextrins (Boos, Ferenci et al. 1981, Richet and Raibaud 1987, Richet and Raibaud 1989, Boos and Shuman 1998). It has also been demonstrated that some of the maltose operon genes can be controlled by catabolite repression (Chapon 1982, Chapon 1982, Chapon and Kolb 1983, Boos and Shuman 1998). MalT, is a member of the "STAND" (Signal

Transduction ATPases with Numerous Domains) family of transcription factors, and can receive input from multiple regulatory systems and subsequently transmit a new signal of its own (Marquenet and Richet 2007, Danot 2010). The maltose operon encodes structural genes, *malF* and *malG* for maltose metabolism, as well as components of an ATP-dependent transport complex for the uptake of maltose via the periplasmic space. In this system, *malK* hydrolyzes ATP to facilitate maltose transport and can also act as a regulatory protein. Increased levels of MalK lead to repression of the *mal* operon by inhibiting the binding of *malT* (Boos, Ferenci et al. 1981, Boos and Shuman 1998)(Reyes and Shuman, 1988; Panagiotidis et al., 1998; Joly et al., 2004; Richet et al., 2005). The maltose operon has been found to play a universal role in metabolism of maltose, and the genes have also been linked to virulence in *Y. pestis, Vibrio cholerae* and *Yersinia enterocolitica* (Lange, Kremer et al. 1994, Brzostek and Raczkowska 2001). In these studies, the addition of maltose to medium for *V. cholera* growth, resulted in the inhibition of cholera toxin secretion, as well as an increase in the production of the toxin-coregulated pilus. By comparison, *malF*-deficient *V. cholera* mutants were unable to synthesize or secrete the cholera toxin (Lange, Kremer et al. 1994). It has also been found that in *Y. pestis*, maltose can be utilized as a nutrition source, and the *malQ* pseudogene, encoding a maltodextrin phosphorylase, prevents glucose-1-phosphate from converting back to glucose to complete synthesis of the O-antigen unit. Thus, the synthesized core constituents *Y. pestis* LPS are devoid of extensive O-antigen (Perry and Fetherston 1997). In this study, at 6°C, the (*malQPT*) and (*malEFG*) genes were found to be up-regulated, but were not up-regulated when *Y. pestis* was grown at 10°C and 15°C. This up-regulation could have been related to increased need for energy production for bacterial survival at the lower temperature, and the potential effects that it plays on the virulence of the organism would need to be further assessed using *mal* mutant

strains of *Y. pestis*, and performing subsequent transmission studies.

The furanone-producing autoinducer-2 (AI-2) quorum-sensing system, which is a mechanism for interspecies cell-to-cell communication, relies primarily on secretion and detection of chemical signals and has been linked to many different bacterial phenotypes and regulatory changes in pathogenic bacteria. In a study by Jing and colleagues, the AI-2 quorum system was associated with metabolic activities and oxidative stress genes, which may contribute to *Y. pestis* survival (Dong, Wang et al. 2001, Dong, Gusti et al. 2002). This was confirmed by observing that a *luxS* mutant was more sensitive to killing by hydrogen peroxide, suggesting a potential requirement for AI-2 in evasion of oxidative damage. In addition, this study found that many membrane protein genes are controlled by LuxS, suggesting a role for quorum sensing in membrane modeling. It still remains unclear if LuxS plays a role in pathogenesis. One study using a *luxS* mutant strain of *Y. pestis* found that the lethal dose was the same for the mutant and wild-type strain of *Y. pestis*, indicating the *luxS* was not important for virulence (Bobrov, Bearden et al. 2007). In the current study, *luxS* was found to be up-regulated in *Y. pestis* when grown at 6°C and 10°C, and may be necessary for survival in colder temperatures to regulate metabolic activities. The *luxS* gene was not found to be differentially regulated in *Y. pestis* when grown at 15°C, relative to bacterial growth at 23°C. Quorum sensing in *Y. pestis* has also been shown to play a role in regulation of metabolism in the pathogen and altering the maltose and galactose operons. In our study, it is unclear whether quorum sensing, the *crp* gene or other factors are responsible for the increased expression of the galactose and maltose operons in *Y. pestis* at 6°C, since previous studies have linked each to successfully altering the operons (LaRock, Yu et al. 2013). In *Y. pestis*, quorum sensing has also been linked to controlling the glyoxylate bypass genes, *aceA* and *aceB*, which encode, isocitrate lyase and malate synthase, and

work together to bypass the carbon dioxide evolving steps of the TCA cycle. Metabolic variation in the TCA cycle is necessary for altering capability of pathogens to grow on fatty acid substrates. The *aceA* and *aceB* genes have been found to be significantly up-regulated by quorum sensing signals (LaRock, Yu et al. 2013). In this study, *aceA* was up-regulated in *Y. pestis* at all three of the lower growth temperatures, and *aceB* was up-regulated at 6°C and 10°C. Up-regulation of the glyoxylate bypass pathway would provide a selective advantage by allowing growth on the fatty acids of the blood meal inside of the flea vector and providing a way to scavenge acetate from the fatty acid breakdown and allowing earlier growth on the glucose in the blood. The glyoxylate bypass pathway has been implicated in the pathogenesis of several different bacterial species, *Salmonella*, *Mycobacterium tuberculosis* and *Pseudomonas aeruginosa* (McKinney, Honer zu Bentrup et al. 2000, Fang, Libby et al. 2005, Lindsey, Hagins et al. 2008). Quorum sensing mutants in *Salmonella* were unable to cause persistent infections, but had no effect on growth or acute infections (Fang, Libby et al, 2005). The direct effects of *luxS* up-regulation in this study will need to be further evaluated using *luxS* knock-outs in order to identify the possible role of quorum sensing in *Y. pestis* at these lower temperatures.

The LPS of *Y. pestis* characterized as a rough-type LPS lacking the O-antigenic polysaccharide (Chart and Rowe 1995). The LPS or endotoxin elicits a variety of inflammatory responses in mammalian hosts, and the hexa-acylated form of the LPS has been shown to efficiently activate the toll like receptor 4 (TLR4) in mammalian hosts, which results in the activation of the host innate immune system (Knirel and Anisimov 2012). A typical LPS consists of three main parts: the lipid A, core, and O-antigen. The lipid A portion is associated with toxicity in mammalian hosts, and the immunogenicity is associated with the polysaccharide components (Knirel and Anisimov 2012). In *Y. pestis*, different LPS types have been discovered,

and the different forms have been associated with differences in growth temperature. Growth of *Y. pestis* at ambient temperatures results in the synthesis of a hexa-acylated lipid A; whereas growth of the pathogen at 37°C results in a tetra-acylated LPS (Kawahara, Tsukano et al. 2002, Telepnev, Klimpel et al. 2009). The tetra-acylated form of the LPS in *Y. pestis* has been further evaluated and determined that the change in structure and number of acylations on the lipid A, results in an increased immune response or decrease in recognition in mammalian hosts. For example, the tetra-acylated form of the LPS was less immunogenic and resulted in immune evasion in a mouse model when compared to the hexa-acylated form of the LPS in which all virulence was ablated (Sun, Koumoutsi et al. 2011). The differences in LPS structures were studied in more detail, and found that *Y. pestis* growth at 6°C, which mimics burrow and rodent hibernation temperatures, resulted in biosynthesis of a completely different LPS type. Multiple differences were found in the core such as: a replacement of the terminal galactose with terminal d-*glycero*-d-*manno*-heptose; phosphorylation of the terminal oct-2-ulosonic acid with phosphoethanolamine, resulting in a lower content of GlcNAc, and; absence of glycine. Changes at this temperature in the lipid A were: a lack of 4-amino-4-deoxyarabinose and partial oxygenation of the fatty acids. This study suggested that cold temperature changes results in an alternative mechanism of control and synthesis of the *Y. pestis* LPS (Kawahara, Tsukano et al. 2002). Knirel and colleagues further identified the genes necessary for LPS biosynthesis (Figure 4). They found that three main gene clusters were responsible for the LPS core synthesis *waaI*, *waaII* and *wab* (Knirel and Anisimov 2012). Our study found that when *Y. pestis* was grown at 6°C and 10°C, the LPS core biosynthesis protein (*CoaD*) was up-regulated and genes involved in the inner core biosynthesis *waaE*, *waaA* and *kbl* were down-regulated, which are all found on the *waaI* gene cluster. *WaaE* and *kbl* were also down-regulated when *Y. pestis* was grown at 15°C.

On the *waaII* gene cluster, YPO0415 was found to be up-regulated in *Y. pestis* grown at 6°C, 10°C and 15°C (Figure 4). *WaaC*, part of the *waaI* gene cluster and *waaQ*, part of the of *waaII* gene cluster, both encode LPS heptosyltransferase enzymes, and were up-regulated only when *Y. pestis* was grown at 15°C. In the *waaI* gene cluster, four out of the seven LPS biosynthesis genes are down-regulated when *Y. pestis* is grown at 6°C and 10°C, which may contribute to the partial replacement of the Kdo (*3-deoxy-D-manno-oct-2-ulosonic acid*), residue in the side chain, to the close derivative, Ko (*D-glycero-D-mal-oct-2-ulosonic acid*) of the LPS core structure. In addition, a galacatose residue can be incorporated into the core, attaching to the heptose residue most distal from the lipid A, resulting in D-glycero- D-galactose-heptose at lower temperatures (Knirel and Anisimov 2012). It is unclear whether the highly expressed *gal* operon that we observed in *Y. pestis* at 6°C, could lead to this genotype where galactose residues become incorporated in the LPS core structure. Further studies evaluating the roles that different LPS-types have on virulence and pathogenesis of *Y. pestis* would need to be further elucidated. Next, structural variations of the lipid A component of *Y. pestis* have been found to occur when the pathogen is grown at different temperatures (Knirel and Anisimov 2012); (Kawahara, Tsukano et al. 2002). Changes in content and different acylated forms of lipid A have been discovered in *Y. pestis*. These changes have been observed when cultivation conditions of *Y. pesits* were altered, indicated temperature as a significant role leading to structural variations. When *Y. pestis* is grown at temperatures ranging from 20-28°C, it resulted in a mixture of tetra-acyl, penta-acyl and hexa-acyl forms of lipid A being produced (Knirel and Anisimov 2012) A rise in temperature to 37°C, resulted in a decrease in acylation of lipid A, and further hypothesized as playing a role in pathogenesis to better evade the host immune system, by decreased recognition. When *Y. pestis* was grown at 6°C, a mixture of tetra-acyl and hexa-acyl forms of lipid A were

discovered (Knirel and Anisimov 2012). Our study further demonstrated that genes necessary for LPS biogenesis were altered, which could explain the change in structure described by Knirel and colleagues. The temperature dependence of the catalytic activity of the enzymes could result in a change in the lipid A acylation patterns, which could lead to increased virulence in *Y. pestis* at lower temperatures. The LPS core is thought to contribute to serum resistance, and mediated by the effect of the LPS on the folding correctness and functional activity of the outer membrane protein Ail (OmpX), which plays known role in serum resistance (Bartra, Styer et al. 2008), and found to be up-regulated in our study when *Y. pestis* was grown at 6°C (6.7-fold). The temperature-dependent variations of the core and lipid A structures of the *Y. pestis* LPS may facilitate adaption to lower temperatures resulting in increased survival of *Y. pestis*, as well as, potentially making the pathogen more virulent, specifically when tetra-acylated forms of lipid A are formed, in addition to potential galactose incorporation into the LPS core.

In *Y. pestis*, outer membrane proteins (OMPs) have been linked to virulence and pathogenesis. Some outer membrane proteins (Yersinia outer proteins-Yops), are induced by growth in calcium-deficient medium at 37°C and partially secreted, and found to be directly involved in pathogenesis in the mammalian host (Bölin and Wolf-Watz 1984, Bölin, Portnoy et al. 1985, Bölin, Forsberg et al. 1988, Bölin and Wolf-Watz 1988). In *Y. pestis*, outer membrane protein R or OmpR, is found to regulate *ompCFX*, directly through OmpR-promoter DNA association, and a component of the OmpR-EnzV two component regulatory system (Reboul, Lemaitre et al. 2014). OmpR is required for both virulence and survival within macrophages. In addition, *Y. pestis* OmpA has also been found to be necessary for intracellular survival in macrophages and virulence in mice (Gao 1986, Bartra, Styer et al. 2008, Gao, Zhang et al. 2011). The osmotic regulator OmpR, regulates differentially the expression of major porin proteins

OmpF and OmpC. In *Y. enterocolitica* and *Y. pseudotuberculosis*, OmpR also has been found to be required for both virulence and survival within host macrophages. The role of OmpR in *Y. pestis* is thought to be involved in building resistance against phagocytosis by countering the toxic effectors secreted by polymorphonuclear leukocytes in the tissues, in addition to controlling adaptation to various stressful environments (Feng, Oropeza et al. 2003, Reboul, Lemaitre et al. 2014). This study found an increase in expression of the transcriptional regulators, OmpR and OmpF, in addition to up-regulation of the outer membrane proteins A and X, (OmpA and OmpX), as well as up-regulation of the chaperone protein OmpH when grown at 6°C. At 10°C, *ompR*, *ompF* and *ompH* were all up-regulated; whereas the outer membrane protein C gene, *ompC, was* down-regulated at 6°C and 10°C (Table 4.). Increased expression of *ompR*, a component of the OmpR-EnvZ two component regulatory system of *Y.pestis*, at 6°C and 10°C, may play a role in bacterial adaption and survival at the lower temperatures. *Y. pestis* encodes 24-two component regulatory systems, and only two of the systems (PhoP-PhoQ; OmpR-EnvZ) have been found to be important for pathogen virulence and immune evasion (Reboul, Lemaitre et al. 2014). The main role of two component regulatory systems is thought to be the enhancement of bacterial adaptation to environmental changes; therefore, the increased expression of OmpR at 6°C and 10°C, may play a role in allowing the pathogen to successfully survive at these colder temperatures. Previous studies have demonstrated that OmpF and OmpC are reciprocally regulated by medium osmolarity, and OmpC is predominantly expressed in high osmolarity environments, while the OmpF expression is repressed (Kawaji, Mizuno et al. 1979). This was demonstrated in this study, when *ompF* was found to be upregulated at 6°C and 10°C, and *ompC* was downregulated; further indicating a growth environment in which low osmolarity conditions were encountered by *Y. pestis*. OmpF is found to be predominantly expressed in

aqueous environments as well as adapting to increases in medium osmolarity (Nikaido 2003, Ayyadurai, Houhamdi et al. 2008). Expression of OmpC is increased in the flea midgut, while OmpF is predominately expressed in the aqueous habitats (as demonstrated in this study). The pore size of OmpC is smaller and has a slower flux; whereas, OmpF has a larger pore size and may play an additional role in scavenging for bacterial nutrients (Nikaido 2003). Lastly, OmpX in *Y. pestis* is required for efficient bacterial adherence, as well as conferring resistance to human serum (Kolodziejek, Schnider et al. 2010, Kolodziejek, Schnider et al. 2010). *Y. pestis ompX* was up-regulated in our study when grown at 6°C, and may play a role in increased virulence in mammalian hosts at lower temperatures. Much is still unknown about the porin regulation in *Y. pestis* and a comparison between porin regulation in *Y. pestis* and *Y. pseudotuberculosis* may provide new insights into possible evolutionary forces selecting for altered gene regulation.

Y. pestis is a facultative anaerobe and possesses a constitutive glyoxylate bypass and unregulated L-serine deaminase expression, but lacks detectable adenine deaminase, aspartase, glucose 6-phosphate dehydrogenase, ornithine decarboxylase, urease, and α-ketoglutarate dehydrogenase activities (Brubaker 1972, Brubaker 1991, Brubaker 1991, Holt, Krieg et al. 1994). At all temperatures, *Y. pestis* has nutritional requirements for L-isoleucine, L-valine, L-methionine, L-phenylalanine, and glycine (or L-threonine); these auxotrophies, some of which are capable of reversion, are due to cryptic genes. At 37°C, *Y. pestis* also requires biotin, thiamine, pantothenate, and glutamic acid (Perry and Fetherston 1997). In our study, the glyoxylate bypass genes were up-regulated in *Y. pestis* grown at 6°C and 10°C, as indicated by genes *aceA* (encoding an isocitrate lyase) and *aceB* (encoding a malate synthase). These genes permit the synthesis of tricarboxylic acid cycle intermediates from two carbon precursors. AceA catalyzes the reversible aldol cleavage of threo-isocitric acid to succinic and glyoxylic acids;

whereas AceB catalyzes the condensation of acetyl-coenzyme A (CoA) with glyoxylic acid to form malate. *AceAB* are up-regulated during growth in the presence of two carbon compounds such as ethanol or acetate (Kornberg 1966, Kornberg and Smith 1966, Hillier and Charnetzky 1981). In our study, when *Y. pestis* is grown at sub-ambient temperatures (6 °C, 10°C and 15°C) the largest proportion of the DEGs were those related to metabolism were involved in the uptake and catabolism of amino acids and carbohydrates, resulting in up-regulation of the phosphotransferase system (*manXYZ*). *Y. pestis* appears to use amino acids, arginine, histidine and methionine, as primary carbon, nitrogen, and energy sources, in which the carbon gets funneled into the TCA cycle. Fatty acid degradation (*fadHDE*) was up-regulated at the sub-ambient temperatures when compared to 23°C, and respiration was highly up-regulated at the lower temperatures (*hydN* and *dmsA*), while oxidative phosphorylation (*atpABDFHI*) was highly up-regulated only at 6°C.

Gene reduction of *Y. pestis* has shown that genes are inactivated or deleted under selective pressure, and that this process is likely related to the interaction of the bacterium with the nutrient rich environments in which the pathogen is primarily inhabiting, such as flea midguts or mammalian hosts. The genome of *Y. pestis* indicates that the pathogen possesses a full complement of the biosynthetic metabolic pathways, yet *Y. pestis* is dependent on satisfying its nutritional requirements by acquiring them from the environment in which it is residing. Characteristic of parasitic relationships, *Y. pestis* metabolism is very inefficient, and more than half of the carbon sources that are imported into the cell are excreted as waste material and respiratory metabolism is more carbon efficient than anoxic metabolism. Additionally, oxidative metabolism produces lower amounts of the acidic by-products produced by the organism, which are known to inhibit cellular growth. This aversion to acidity, results in *Y. pestis* using gluconate

as a carbon source (Englesberg, Chen et al. 1954, Chain, Carniel et al. 2004). *Y. pestis* must apply a considerable number of adaptive strategies in order to survive in both the mammalian host and the flea. Previous studies have shown the ability of the pathogen to live successfully in a flea gut for over a year, further implicating the flea as a potential long-term reservoir for *Y. pestis* (Gage and Kosoy 2005). Factors affecting *Y. pestis* growth and infection efficiency in the flea greatly depend on the mammalian host from which the flea obtains its blood meal. The closed environment of the flea gut will eventually lead to nutrient deprivation born out by long periods with only sporadic blood feedings; therefore, the metabolic pathways of *Y. pestis* may have adapted in such ways to allow for complete utilization of the blood meal under austere conditions. Future studies evaluating the transcriptome of *Y. pestis*-infected fleas, would lead to even more insight on the pathogen-vector interactions allowing for increased persistence of *Y. pestis* or changes in virulence at these lower temperatures.

Y. pestis has many temperature-regulated virulence and pathogenesis factors, facilitating immune evasion and dissemination, which are up-regulated upon entry into a mammalian host (37°C). At 37°C or mammalian temperature, the pathogen encounters low Ca^{2+} concentrations and a nutrient rich environment. The pCD1 plasmid, encodes the Yop virulon, a T3SS, and Ysc (Yersinia SeCretion). There are 29 different Ysc proteins which all play a role in inhibiting phagocytosis, inflammation, and inducing apoptosis of macrophages and other phagocytes. This plasmid also encodes the V antigen (LcrV), which functions in producing the type three secretion apparatus, as well as suppression of the host immune response. The outer membrane protein plasminogen activator gene, *pla*, is found on the pPCP1 plasmid and promulgates dissemination of the pathogen by interfering with blood coagulation and host complement activation pathways. The pMT1 plasmid of *Y. pestis* encodes the F1 caspular antigen (F1) expressed at 37°C,

enhances resistance to phagocytosis. Also encoded on this plasmid is the *Yersinia* murine toxin (Ymt), which is optimally expressed in the flea at 26°C, and encodes a phospholipase D required for flea gut colonization. *Y. pestis* transmission and infection could not be accomplished without the chromosomal *pgm* locus, which contains the *hmsHFRS* genes essential for biofilm formation within the flea vector; therefore expressed at 26°C. The *pgm* locus also harbors the *Yersinia* high-pathogenicity island (HPI), which encodes a siderophore-based iron acquisition system (Ybt) needed for infection in the mammalian host (Hinnebusch, Perry et al. 1996). Consistent with current knowledge regarding temperature regulation of *Y. pestis* virulence factors, our study found further down-regulation of the *Y. pestis* F1 capsular antigen at low temperature, in addition to down-regulation of *pla*. Interestingly, one potential virulence gene, *yadB*, was found to be up-regulated at 6°C and 10°C growth temperatures. YadB encodes the outer surface protein gene *yadB*, and is required for adhesion and dissemination of *Y. pestis* after a flea bite and is necessary for the progression of bubonic plague (Vadyvaloo, Jarrett et al. 2010). Thus, *yadB* up-regulation at burrow temperatures ranging between 6-10°C, could prepare the pathogen for the host environment and is implemented specifically when *Y. pestis*-infected fleas feed on a hibernating rodent host.

In conclusion, transcriptome studies, as the one described here, demonstrated their value by presenting a global view for differential expression of genes from selected environments, tissues, or whole animals. Next generation sequencing has made it possible to examine the complex array of genes associated with selected biological functions in a single sample, and is extremely beneficial when an existing reference genome is available. In this novel study, we examined *Y. pestis* gene regulation at burrow temperatures of hibernating rodents, and provided new insights on virulence and survival strategies of this pathogen at sub-ambient temperatures.

Our study used a powerful approach that integrated gene expression measurements with large-scale genome-wide association data to identify genes whose expression were altered by changes in environmental growth temperatures.

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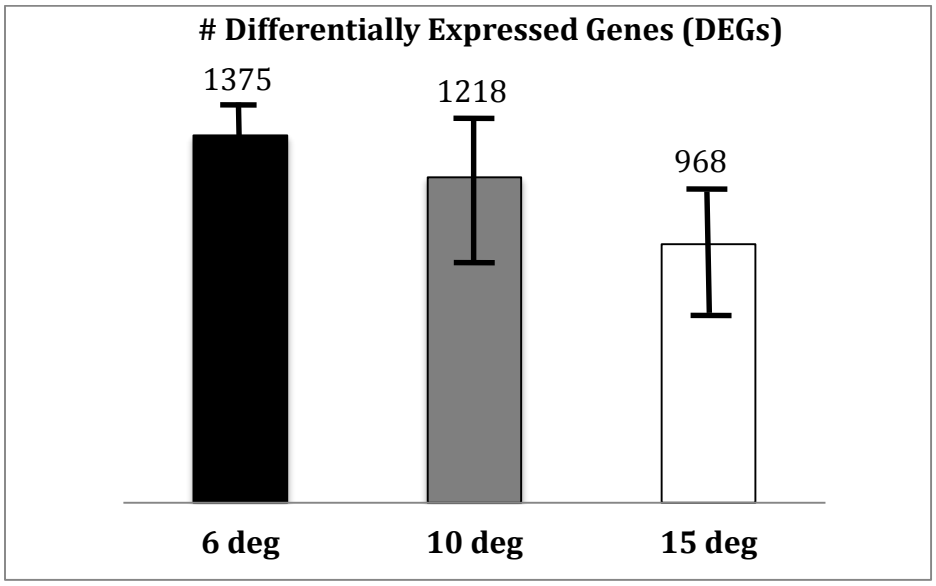
Figure 4.1. Overview of the differentially expressed genes (DEGs) of *Y. pestis* at low growth temperatures (6°C, 10°C, and 15°C compared to 23°C)

A.



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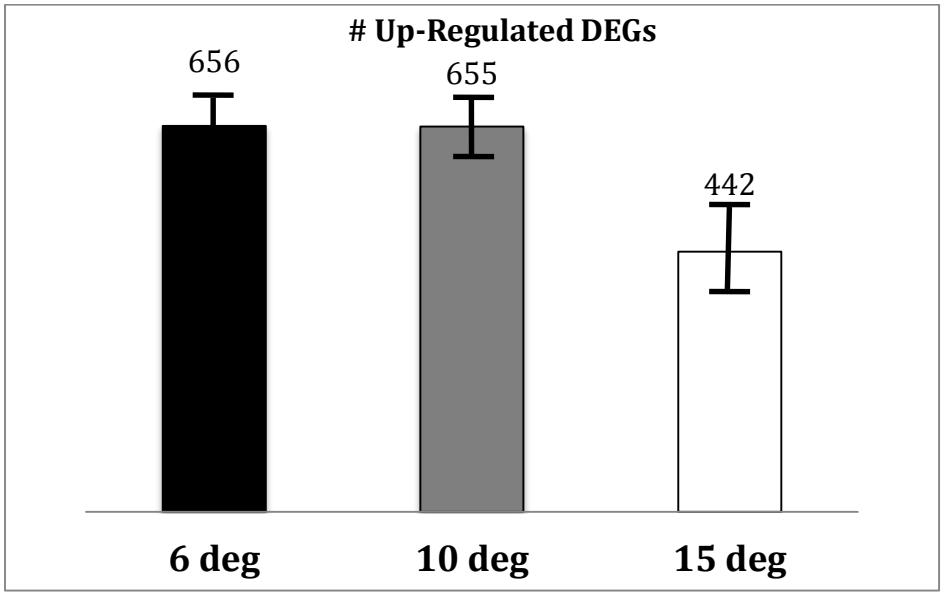


C.

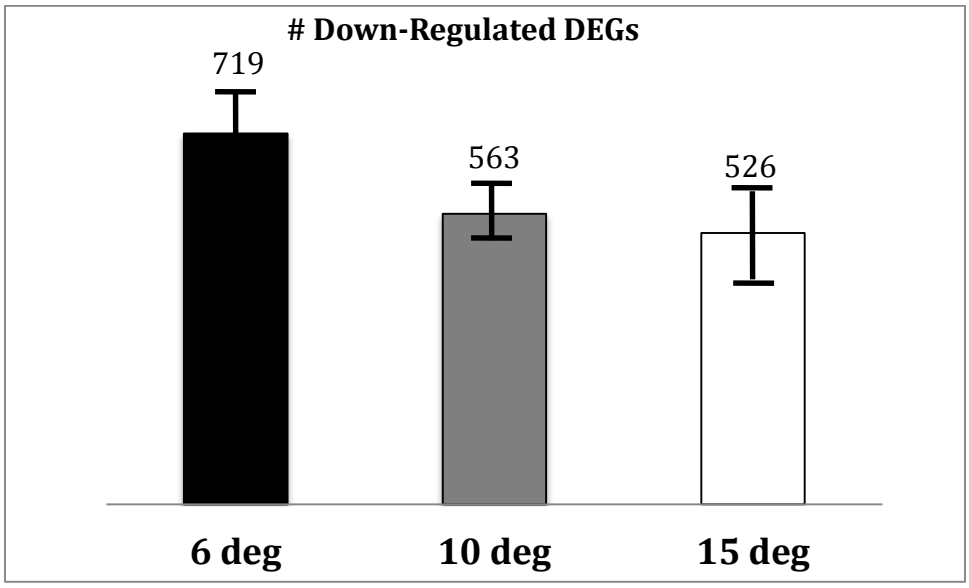


Figure 4.1. A. Histogram showing the differentially expressed genes (DEGs) from the NGS-RNA-seq transcriptome study examing *Y. pestis* gene expression at 6°C, 10°C, 15°C compared to 23°C. B. Histogram of the number of total up-regulated genes of *Y. pestis* at 6°C, 10°C, 15°C when compared to 23°C. C. Histogram of the number of total down-regulated genes of *Y. pestis* at 6°C, 10°C, 15°C when compared to 23°C. (Black bar-6°C, Grey bar-10°C, White bar-15°C). Y-axis depicts gene number, and X-axis depicts *Y. pestis* growth temperature.

Table 4.1. Table of genes up- or down-regulated ≥2-fold by the Crp gene of *Y. pestis* at 6°C

Gene	Function	Up/Down Regulation	Fold Change
crp	cyclic AMP receptor protein	Up	3.1
Amino Acid Transport and Metabolism			
gltJ	glutamate/aspartate ABC transporter, permease protein	Down	-6.6
serA	D-3-phosphoglycerate dehydrogenase	Up	10.2
serC	Phosphoserine aminotransferase	Up	3.4
glnH	glutamate and aspartate transporter subunit	Down	-4.4
hpaE	4-hydroxyphenylacetate catabolism	Down	-7.5
hpaR	4-hydroxyphenylacetate catabolism	Down	-2.8
deoC	2-deoxyribose-5-phosphate aldolase	Up	2.7
deoA	thymidine phosphorylase	Up	3.5
deoB	phosphopentomutase	Up	2.5
deoD	purine nucleoside phosphorylase	Up	2.8
tdk	Thymidine kinase	Down	-3
Carbohydrate Transport and Metabolism			
manX	PTS enzyme IIAB, mannose-specific	Up	3.1
manY	PTS system, mannose/fructose/sorbose family, IIC subunit	Up	5.3
manZ	PTS enzyme IID, mannose-specific	Up	7.5
nagB	Glucosamine-6-phosphate deaminase	Up	3
nagA	N-acetylglucosamine-6-phosphate deacetylase	Up	8.9
nagC	*N*-acetylglucosamine-6P-responsive transcriptional repressor NagC, ROK family	Up	3.4
nagD	Phosphatase NagD predicted to act in *N*-acetylglucosamine utilization subsystem	Down	-2.3

nagE	PTS system, *N*-acetylglucosamine-specific IIA component	Up	15.6
zwf	Glucose-6-phosphate 1-dehydrogenase	Up	8.7
maeB	malic enzyme	Up	2.3
malE	maltose ABC transporter periplasmic protein	Up	3.5
malF	ABC superfamily (membrane), maltose transport protein	Up	3.8
malP	maltodextrin phosphorylase	Up	2.8
malQ	4-alpha-glucanotransferase	Up	2.7
malT	transcriptional regulator MalT	Up	2.5
dppA	dipeptide ABC transporter, substrate-binding protein	Down	-2.8
dppB	dipeptide ABC transporter, permease protein	Down	-5
dppC	dipeptide transporter	Down	-3.1
dppD	dipeptide transporter ATP-binding subunit	Down	-4
dppF	dipeptide transporter ATP-binding subunit	Down	-4.1
gntT	gluconate transporter	Up	3.6
uxaC	Uronate isomerase	Up	2.1
pgk	phosphoglycerate kinase	Up	2.5
aroA	5-Enolpyruvylshikimate-3-phosphate synthase	Up	2.1
argH	Argininosuccinate lyase	Up	4.5
ugpC	SN-glycerol-3-phosphate transport ATP-binding protein UgpC	Down	-2.7
mglA	Galactose/methyl galactoside ABC transport system, ATP-binding protein MglA	Down	-2.9
mglC	Galactose/methyl galactoside ABC transport system, permease protein MglC	Down	-3.5
ugpC	SN-glycerol-3-phosphate transport ATP-binding protein UgpC	Down	-2.7
Cell Wall/Membrane Biogenesis			
galE	UDP-galactose-4-epimerase	Up	179.4
galT	galactose-1-phosphate uridylyltransferase	Up	185.3

galK	galactokinase	Up	53.1
galM	aldose 1-epimerase	Up	28.5
Energy Production and Conversion			
aceE	Pyruvate dehydrogenase E1 component	Up	4.4
aceF	Dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex	Up	6
atpI	ATP synthase subunit I	Up	2.1
adhE	Alcohol dehydrogenase/Acetaldehyde dehydrogenase	Up	2.5
epd	D-erythrose 4-phosphate dehydrogenase	Up	2.7
focA	formate transporter	Up	3.6
pflB	formate acetyltransferase	Up	7.4
dcuA	anaerobic C-4-dicarboxylate symporter	Up	2.5
lpdA	Dihydrolipoamide dehydrogenase of pyruvate dehydrogenase complex	Up	3.7
nirB	nitrite reductase (NAD(P)H) subunit	Up	3.8
aceA	isocitrate lyase	Down	-4.4
sdhC	Succinate dehydrogenase cytochrome b-556 subunit	Down	-2.2
sdhD	Succinate dehydrogenase hydrophobic membrane anchor protein	Down	-4.1
sdhA	Succinate dehydrogenase flavoprotein subunit	Down	-2.4
sdhB	Succinate dehydrogenase iron-sulfur protein	Down	-2.5
glk	glucokinase	Down	-2.3
glpA	sn-glycerol-3-phosphate dehydrogenase subunit A	Down	-4.4
glpB	Glycerol-3-phosphate dehydrogenase subunit B	Down	-3.8
glpC	anaerobic glycerol-3-phosphate dehydrogenase subunit C	Down	-3.3
glpD	glycerol-3-phosphate dehydrogenase	Down	-6.8
glpF	facilitator for glycerol uptake	Down	-14.1

Lipid Transport and Metabolism			
fadD	long-chain-fatty-acid--CoA ligase	Down	-3
fadH	2,4-dienoyl-CoA reductase [NADPH] (2,4-dienoyl coenzyme A reductase)	Down	-2.7
lipB	Octanoate-[acyl-carrier-protein]-protein-N-octanoyltransferase	Down	-5
Transcription and signal transduction mechanisms			
hexR	Phosphogluconate repressor HexR, RpiR family	Up	6
ctyR	transcriptional repressor	Down	-5.9
uspA	Universal stress protein A	Down	-3
pdhR	Transcriptional repressor for pyruvate dehydrogenase complex	Up	5.4
fxsA	suppressor of F plamsid exlusion of phage T7	Down	-4
Replication, recombination and repair			
deaD	ATP-independent RNA helicase	Down	-2.1
hupA	DNA-binding protein HU-alpha (HU-2)	Up	3.2
iciA	chromosome replication initiation inhibitor protein	Up	2.1
Coenzyme transport and metabolism			
cyoE	protoheme IX farnesyltransferase	Down	-2.6
General function and prediction unknown			
actP	acetate permease	Down	-13.6
acs	acetyl-coenzyme A synthetase	Down	-5.3
yohK	LrgB family protein	Down	-2.5
Translation			
ppiA	Peptidyl-prolyl cis-trans isomerase ppiA precursor	Up	4.9

Table 4.2. Table of the galactose operon genes up-regulated in *Y. pestis* at 6°C

Galactose operon regulated by crp in *Yersinia pestis* at 6°				
Gene	**Function**	**Up/Down Regulation**	**Fold Change**	**Log2 Fold Change**
galE	UDP-galactose-4-epimerase	Up	179.4	7.5
galT	galactose-1-phosphate uridylyltransferase	Up	185.3	7.5
galK	galactokinase	Up	53.1	5.7
galM	aldose 1-epimerase	Up	28.5	5.4
galR	galactose operon repressor galR	Up	2.6	1.0
mglB	beta-methylgalactoside ABC transporter, periplasmic binding protein	Up	2.1	1.0

Table 4.3. Table of the known genes necessary for the biosynthesis of the *Y. pestis* LPS that are up- or down-regulated ≥2-fold at 6°C, 10°C, and 15°C compared to 23°C

Lipopolysaccharide (LPS) Genes		Gene Function	Up/Down Regulation		
			6°	10°	15°
waaI	*coaD (kdtB)*	Lipopolysaccharide core biosynthesis protein	4.1	3.2	NC *
	waaE (kdtX)	Lipopolysaccharide core biosynthesis glycosyl transferase	4.3	3.1	2.6
	waaA (kdtA)	3-deoxy-D-manno-octulosonic-acid transferase	5.2	3.6	NC *
	waaC	Lipopolysaccharide heptosyltransferase I	NC *	NC *	3.3
	waaF	Lipopolysaccharide heptosyltransferase II	NC *	NC *	NC *
	hldD	ADP-L-glycero-D-manno-heptose-6-epimerase	NC *	NC *	NC *
	kbl	2-amino-3-ketobutyrate coenzyme A ligase	-2.4	-3.6	-5.3
waaII	*YPO0415*	putative carbohydrate kinase	4.5	10.2	3.4
	waaQ	LPS inner-core heptosyltransferase III	NC *	NC *	3.7
	waaL	LPS biosynthesis enzyme	NC *	NC *	NC *
	YPO0418		NC *	NC *	NC *

***Represents genes that were not up- or down-regulate when compared to 23°C**

Table 4.4. Table of the differentially expressed outer membrane protein (OMP) genes up- or down-regulated ≥2-fold of *Y. pestis* at 6°C, 10°C, and 15°C compared to 23°C

Outer membrane protein (OMP) genes		Up/Down Regulation		
Gene	**Gene function**	**6°**	**10°**	**15°**
ompR	Transcriptional regulatory protein ompR	3.8	2.3	NC *
ompF	Transcriptional regulatory protein ompF	3.4	2.1	NC *
ompC	Outer membrane protein C, porin	-3.4	-5.0	NC *
ompA	Outer membrane protein A	2.4	NC*	NC *
ompH	Chaperone protein	2.7	2.0	NC *
ompX	Outer membrane protein X	6.7	NC *	NC *

***Represents genes that were not up- or down-regulated when compared to 23°C**

Table 4.5. Differentially expressed genes (DEGs) up- or down-regulated ≥2-fold of *Y. pestis* grown at 6°C, 10°C, and 15°C compared to 23°C

Chromosomal Genes:		Fold Change		
Amino Acid Transport and Metabolism		6°	10°	15°
ureA	urease gamma subunit	2.5	4.1	3.1
ureB	urease beta subunit	2	6.9	3.1
ureC	urease alpha subunit	2	7.2	3.5
ureD	urease gamma subunit	2	4.7	3.3
ureF	urease beta subunit	2	4.2	3
ureG	urease subunit alpha	2.5	2.4	2.4
gltJ	aspartate/glutamate ABC transport system	6.6	NC*	NC*
argC	*N*-acetyl-gamma-glutamyl-phosphate-arginine	4.5	4.7	3.4
argH	Argininosuccinate lyase	4.5	4.9	5.1
argD	Acetylornithine/succinyldiaminopimelate	6.6	10	3.1
metB	O-succinylhomoserine (Thiol)-lyase	NC*	2.3	NC*
metC	cystathionine beta-lyase	-6.6	-5.6	-3.3

metF	5,10-methylenetetrahydrofolate reducatase	3.6	7.9	NC*
metH	5-Methyltetrahydrofolate--homocysteine methyltransferase	NC*	NC*	-4.9
metN	Methionine import ATP-binding protein MetN 1	8.4	5.6	3.4
metQ	*D*-methionine-binding lipoprotein MetQ	3.2	NC*	NC*
ilvC	ketol-acid reductoisomerase	-11.2	5	2.7
livM	leucine/isoleucine/valine transporter permease subunit	NC*	NC*	3.4
lysC	amino acid metabolism	2.5	2.8	2.1
nanA	acetylneuraminate lyase	42.3	4.3	2.3
nanT	Putative sialic acid transporter	6.2	6.7	NC*
cysK	cysteine synthase A	-2.7	-2.3	-4.2
cysM	Cysteine synthase	3.3	2.1	NC*
cysE	Serine acetyltransferase	-2.6	-2.4	NC*
cysB	HTH-type transcriptional regulator CysB	2.9	2.5	2.3
cysJ	Sulfite reductase [NADPH] flavoprotein alpha-component	-5.8	NC*	NC*
cysQ	Inositol monophosphatase family protein	NC*	2.7	2.1
cysS	Cysteine--tRNA ligase	NC*	NC*	2.1
csyI	hypothetical protein	-4.7	-3	-4.3

ybtS	salicyclate synthase Irp9	-6.6	-2.6	-3.1
hisD	histidinol dehydrogenase	2.5	NC*	NC*
hisB	imidazole glycerol-phosphate dehydratse/histidinol phosphatase	NC*	-2.3	-3.1
aspC	aromatic amino acid aminotransferase	2.9	22.8	8.5
aspA	amino acid metabolism	2.9	2.5	2.3
serA	D-3-phophoglycerate dehydrogenase	10.2	6.3	3.4
serB	Phosphoserine phosphatase SerB	4.9	2.2	3.2
serC	Phosphoserine aminotransferase	3.4	NC*	NC*
proC	pyrroline-5-carboxylate reducatase	3.2	2.8	NC*
speC	ornithine decarboxylase isozyme	NC*	NC*	3.4
leuB	3-isopropylmalate isomerase large subunit	4.1	2.1	NC*
leuC	isopropylmalate isomerase large subunit	8	4.3	NC*
dapB	dihydrodipicolinate reducatase	-2.9	3	3.4
dapX	hypothetical protein	2	2.3	2.3
thrA	bifunctional aspartokinase I/homoserine dehydrogenase	2.3	2.5	3.2
glnH	glutamine ABC transporter periplasmic binding protein	-5.2	-2.4	NC*
glnQ	glutamine ABC transporter periplasmic binding component	-10.7	-10.3	NC*

glnP	glutamine ABC transporter periplasmic binding permease	-12.2	-2.9	-6
hutI	imidazolonepropionase-histidine	3.1	3	NC*
hutG	histidine degradation	3.3	3	2.3
hpaC	hypothetical protein	-2.3	-3.2	NC*
hpaB	component B of 4-hydroxyphenylacetate acid-hydroxylase	-4.6	-3.3	-3.9
hpaX	4-hydroxyphenylacetate permease	5.3	3.9	3.2
hpaI	2,4-dihydroxyhept-2ene-1,7-dioate hydratase	-3.5	-3.9	NC*
ansB	periplasmic L-asparaginase II	-9.7	-2.8	-5.4
artQ	arginine 3rd transport system permease	-5	-4.2	NC*
Carbohydrate Transport and Metabolism				
lamB	maltoprotein precursor	-3	-5.5	NC*
manX	PTS system, mannose, specific IIAB component	8.1	6.4	2.6
manZ	PTS system, mannose specific IID component	7.5	9.1	4.7
manY	PTS enzyme, IIC, mannose-specific	5.3	3.9	2.7
tpiA	triosephosphate isomerase	3.2	2.2	NC*
eno	phosphophyruvate hydratase-glycolysis/gluconeogenesis	2.2	NC*	NC*
sgbU	putative L-xylulose 5-phosphate-3-epimerase	-2.1	NC*	NC*

nagA	*N*-acetylglucosamine-6-phosphate deacetylase	8.9	11.6	10.3
nagB	glucosamine-6-phosphate deaminase	3	NC*	NC*
eda	keto-hydroxyglutarate-aldolase/keto-deoxy-phosphogluconate-Pentose Phoshate	4.2	NC*	NC*
zwf	glucose-6-phosphate dehydrogenase	8.7	2.3	2
malK	ATP-binding component of transport system for maltose	-6	-5.5	NC*
malP	Maltodextrin phosphorylase	2.8	NC*	NC*
malQ	4-alpha-glucanotransferase	2.7	NC*	NC*
malT	MTH-type transcriptional regulator malT	2.5	NC*	NC*
malZ	Maltodextrin glucosidase	2.1	-2.2	NC*
malE	Maltose-binding periplasmic protein	3.5	-2.2	NC*
malS	Alpha-amylase	3.3	-3.9	NC*
fruK	1-phosphofructokinase	-5	-5.6	NC*
bglA	6-phospho-beta-glucosidase A	-5	-6.1	-7
galK	galactokinase	53.1	NC*	NC*
malG	malG type permease	2	-2.7	-2.1
malF	malF-type permease	3.8	-4	-4.1
fba	fructose-bisphosphate aldolase	2	NC*	NC*

pgk	phosphogluycerate kinase	2.5	2.8	NC*
tktA	transketose-pentose phosphate	2.1	NC*	NC*
uxaA	altronate hydrolase	2.4	4.3	4.6
uxaB	altronate oxidoreductase	NC*	3	NC*
uxaC	glucouronate isomerase	2.1	3	3.2
deoB	phosphopentomutase	2.5	6.1	2.1
gntV	glucuronate isomerase	NC*	2	NC*
glgX	glycogen debranching enzyme	NC*	9.7	5
glgC	glucose-1-phosphate adenylytransferase	-2.9	4.5	5.1
glgA	glycogen synthase	6	5.3	2.3
glgP	glycogen phosphorylase	NC*	2.6	NC*
csrA	carbon storage regulator homolog	2.2	2.6	NC*
ompA	putative outer membrane protein porin A	2.4	NC*	NC*
lpp	major outer membrane protein	NC*	2	NC*
ail	attachment invasion locus protein	-3.4	-2.8	-2.5
Cell cycle control				
minD	cell division inhibitor	3.1	4	3.3

Cell motility				
flgK	cell distal portion of basal body rod	-5	-4.2	3.3
flgI	flaggellar basal body P-ring protein	-3.3	-2.8	-2.6
cheD2	methyl-accepting chemotaxis protein	2.2	5.4	NC*
Cell Wall/Membrane Biogenesis				
kdtX	Lipopolysaccharide core biosynthesis glycosyl transferase	-3.1	-4.5	-3.5
mscL	Large-conductance mechanosensitive channel	-2.2	-2.4	-2.6
acrA	accridine efflux pump	2.6	2.6	NC*
tonB	transport protein tonB	-9.7	NC*	NC*
ompC	outer membrane porin protein C	-3.4	-5	-4
galE	UDP-galactose-4-epimerase	179.4	3.2	4.5
galT	galactose-1-phosphate uridylytransferase	185.3	2.9	NC*
alr	alanine racemase	9.3	5.7	NC*
Coenzyme transport and metabolism				
panF	sodium/panthothenate symporter	NC*	2.1	NC*
btuB	vitamin B12/cobalamin outer membrane transporter	3.4	NC*	-2.3
btuC	vitamin B12/cobalamin outer membrane transport protein	2.3	2.3	NC*

thiC	thiamine biosynthesis protein	NC*	NC*	2.3
panC	pantoate-beta-alanine ligase	NC*	NC*	-2.5
metK	*S*-adenosylmetehionine synthetase	4	2.8	NC*
tbpA	thiamine transporter substrate binding subunit	2.1	NC*	NC*
menG	ribonuclease activity regulator protein	NC*	NC*	-2
cyoE	protoheme IX farnesyltransferase	-2.6	-3.1	-2.6
Defense Mechanisms				
pcp	outer membrane lipoprotein	3.5	5.1	2.9
Energy Production and Conversion				
ppc	phosphenolpyruvate carboxylase	5.5	NC*	NC*
qor	quinone oxidoreductase, NADPH-dependent	2.9	NC*	NC*
hydN	electron transport protein	6.5	10.4	12
aceE	pyruvatedehydrogenase subunit EI	4.4	NC*	NC*
aceF	dihydrolipoamide dehydrogenase	6	2.6	NC*
lpdA	dihydrolipoamide dehydrogenase	3.7	NC*	NC*
hmp	flavohemoglobin nitric oxide dioxygenase	-5	-4.2	-2.6
maeB	malic enzyme	2.3	8.7	2.6

adhE	CoA-linked acetaldehyde dehydrogenase	2.3	NC*	NC*
pntA	NAD(P) transhydrogenase subunit alpha	3.6	4.3	3.3
putA	trifunctional transcriptional regulator/proline	-5.2	-2.8	NC*
nirB	nitrate reductase NAD(P)(H) subunit	3.8	-3.2	3.4
atpF	F0F1 ATP synthase subunit B	2.5	NC*	NC*
sdhC	succinate dehydrogenase cytochrome large subunit	-2.2	NC*	NC*
sdhD	succinate dehydrogenase cytochrome small subunit	-4.1	-2.2	-2.6
sdhB	succinate dehydrogenase catalytic subunit	-2.5	NC*	NC*
atpB	ATP synthase subunit A	3.4	NC*	NC*
atpI	ATP synthase subunit I	2.6	NC*	NC*
frdD	fumarate reductase subunit	NC*	3	NC*
nqrE	Na+-translocating NADH_quinone	-2.7	-4.2	-3.6
uxaC	pentose and glucuronate interconversions	2.1	3	NC*
aceA	Isocitrate lyase	5.2	4.4	2.3
aceB	Malate synthase A	4.1	2.1	NC*
dmsA	respiration	5.1	4.3	2.9
ccmA	cytochrome c-type biogenesis	2.6	2.5	2.1

hcp	hydroxylamine reductase	NC*	NC*	2.3
Oxidative Phosphorylation				
atpD	ATP synthase subunit beta	3.7	NC*	NC*
atpA	ATP synthase subunit alpha	3.4	NC*	NC*
atpH	ATP synthase subunit delta	4.5	2.1	NC*
atpF	ATP synthase subunit b	2.5	NC*	NC*
Fatty Acid Degradation				
fadH	fatty acid oxidation complex subunit alpha	2.5	2.8	2.1
fadD	long-chain-fatty-acid--CoA ligase	6.8	4.2	2.1
fadE	acyl-CoA dehydrogenase	3	3	2
fadL	long-chain fatty acid transport protein	NC*	10.5	2.7
Inorganic Ion Transport and Metabolism				
sbp1	exported sulfate-binding protein	-2.6	-3	-2.6
zntA	zinc, lead, cadmium, and mercury transporting ATPase	-3.5	NC*	NC*
pstB	putative phosphate transport ATP-binding protein	6.2	5.7	4.5
cysP	thiosulfate-biding protein	-2.5	NC*	NC*
cysA	sulfate transport ATP-binding protein	-2.9	-4.5	-2.5

dps	DNA protection during starvation conditions	-3.2	4.4	4.6
wrbA	TrpR binding protein	NC*	2.1	2.3
bfr	bacterioferritin	8.3	6.2	2.2
hmuT	periplasmic heme binding protein	-5.9	-3.4	NC*
hmuS	heme uptake system component	-15	-5.1	-3.4
hmuR	TonB-dependent outer membrane receptor	-3.3	-3.5	NC*
terB	tellurium resistance protein	10.8	10.9	4.6
katY	catalse; hydroperoxidase HPI(I)	-36.4	-26.3	-41.1
kdpA	potassium-transporting ATPase subunit A	NC*	2.7	NC*
yfeB	ATP-binding protein for iron and manganese ABC transporter	-2.2	-2.6	-3.1
yfeA	periplasmic binding protein for iron and manganese ABC transporter	-2.4	NC*	NC*
metN	DL-methionine transporter ATP-binding subunit	8.4	5.6	3.3
metQ	DL-methionine transporter substrate binding subunit	3.2	NC*	NC*
nirC	nitrite reductase small subunit	NC*	2.1	NC*
secY	preprotein translocase	3.2	NC*	NC*
hmuU	hemin ABC transporter permease	-12.6	-9.9	-2.8
modB	molybdate transporter permease protein	NC*	3.6	NC*

Lipid Transport and Metabolism				
accB	acetyl-CoA carboxylase biotin carboxylase subunit	2.1	NC*	NC*
accC	acetyl-CoA carboxylase biotin carboxylase subunit	2.7	NC*	NC*
fadB	multifunctional fatty acid oxidation subunit complex	6.2	4.2	2.1
acpD	acyl carrier protein phosphodiesterase	2.2	2	NC*
cdh	CDP-diacylglycerol pyrophosphatase	6.8	4.3	3.5
acpP	acyl carrier protein	NC*	-2.4	NC*
lipB	lipoyltransferase	-5	-4.6	-4.7
Intracellular Trafficking and Secretion				
ftsY	cell division protein	3.1	3	2.4
cysQ	inositol monophosphatase family protein	NC*	2.7	NC*
tatE	twin arginate translocase system permease	-4.8	NC*	NC*
Nucleotide Transport and Metabolism				
amn	purine metabolism	2.5	NC*	NC*
pyrB	aspartate carbamoyltransferase catalytic subunit	NC*	2.1	NC*
udp	uridine phosphorylase	5.4	5.1	2.5
purH	bifunctional phosphoribosylaminoimidazolecarboxamide	-3.9	-3.1	NC*

cpdB	bifunctional 2',3'-cyclic nucleotide 2'-phosphodiesterase	NC*	2.9	NC*
nrdF	ribonucleotiode-diphosphate reductase subunit beta	-6.4	-5.4	-3.4
nrdE	ribonucleotiode-diphosphate reductase subunit alpha	-5	-4.2	-3
nrdH	glutaredoxin-like protein	-4.8	-4.1	-3.1
purL	phosphoribosylformylglycinamidine synthase	-3.6	-3.3	-2.1
upp	uracil phosphoribosyltransferase	-2.4	-2.1	NC*
deoC	cytosine deaminase	2.8	6	NC*
Posttranlational modification, protein turnover, chaperones				
bcp	bacterioferritin comigratory protein	4.3	2.3	4.2
hslJ	putative heat shock protein	-2.1	-2	-2.2
grxA	glutaredoxin 1	2.1	NC*	NC*
hslU	ATP-dependent protease ATP-binding subunit	-2.1	-2.2	-3.8
clpB	protein disaggregation chaperone	-2.9	-2.4	-3.4
tig	peroxidase	2.2	NC*	NC*
htpG	heat shock protein 90	-2.6	-2.6	-4
gst	glutathionine S-transferase	NC*	2.5	NC*
hfq	RNA-binding protein	2.6	NC*	NC*

ibpB	heat shock protein	-90.4	-19.8	-10.9
Insertion Elements				
YPO4050	transposase	-2.2	NC*	NC*
Replication, recombination and repair				
priC	primosomal replication protein n	-2.7	NC*	NC*
mutM	formamidopyrimidine-DNA glycosylase	NC*	-2.3	-5.4
dnaB	replicative DNA helicase	2.2	NC*	NC*
deaD	ATP-dependent RNA helicase DeaD	-2.1	-2.9	-2.3
xthA	exonuclease III	3	NC*	NC*
mutY	adenine DNA glycosylase	3.3	2.4	NC*
ihfB	integration host factor beta subunit	2.7	NC*	NC*
hupA	DNA-binding protein HU-alpha	3.2	NC*	NC*
tnp	transposase for the IS1541 insertion element	16.3	NC*	-2.8
Secondary metabolite biosynthesis, transport and catabolism				
trxC	thioredoxin 2	7.4	9.9	2.3
irp2	HMWP2 nonribosomal peptide synthetase (yersinabactin)	-4.4	-3.3	-3.1
irp1	HMWP1 nonribosomal peptide/polyketide synthase	-11	-4	-9.3

fabG	2,5-dichloro-2,5-cyclohexadiene-1,4-diol dehydrogenase	2.1	NC*	NC*
Transcription and signal transduction mechanisms				
slyA	transscriptional regulator SlyA	-2.7	NC*	NC*
slyD	transscriptional regulator SlyD	2.3	NC*	NC*
psaE	putative regulatory protein	-3.9	-4	-2.5
treR	trehalose repressor	-5.6	-7.8	-12.9
cytR	DNA-binding transcriptional regulator	-5.9	NC*	NC*
hexR	DNA-binding transcriptional regulator	6	4	3.1
ybtA	AraC-type transcriptional regulator for yersiniabactin uptake	-5.7	-4.8	-3
flgM	anti-sigma28 factor FlgM	2.4	NC*	NC*
hepA	ATP-dependent helicase HepA	2.8	2.4	2.1
cspE	cold shock protein E	-19.8	NC*	NC*
cspa1	major cold shock protein	-3	-7.2	-4
cspa2	major cold shock protein	-7.2	-6.8	-3.3
rpoS	RNA polymerase sigma factor	NC*	2.2	NC*
rseA	sigma E factor negative regulatory protein	NC*	2.2	**NC***
rseB	periplamsic negative regulator of sigmaE	-2.2	NC*	NC*

uspA	universal stress protein A	-3	2.2	2.1
csrA	carbon storage regulator	2.2	2.6	NC*
uspB	universal stress protein B	NC*	9.8	6.4
slmA	nucloetide occulusion protein	2.3	3.4	4.1
pspA	phase shock protein A	NC*	2.2	3
gcvA	positive regulator of gcv operon	NC*	NC*	-3.1
Translation				
infA	translation initiator facor IF-1	2.5	NC*	NC*
infB	translation initiator facor	2	NC*	NC*
rplA	50S subunit ribosomal protein	2.9	NC*	NC*
rplB	50S subunit ribosomal protein	3.8	NC*	NC*
rplD	50S subunit ribosomal protein	4	NC*	NC*
rplE	50S subunit ribosomal protein	3.3	NC*	NC*
rplF	50S subunit ribosomal protein	2.4	NC*	NC*
rplI	50S subunit ribosomal protein	3.9	NC*	NC*
rplJ	50S subunit ribosomal protein	2.3	NC*	NC*
rplK	50S subunit ribosomal protein	4.1	NC*	NC*

rplN	50S subunit ribosomal protein	5.4	NC*	NC*
rplO	50S subunit ribosomal protein	4.2	NC*	NC*
rplP	50S subunit ribosomal protein	4.3	NC*	NC*
rplQ	50S subunit ribosomal protein	2.3	NC*	NC*
rplT	50S subunit ribosomal protein	2.6	NC*	NC*
rplV	50S subunit ribosomal protein	5	NC*	NC*
rplW	50S subunit ribosomal protein	2.8	NC*	NC*
rplX	50S subunit ribosomal protein	3	NC*	NC*
rpmA	50S subunit ribosomal protein	2.2	-2.1	NC*
rpmD	50S subunit ribosomal protein	4.7	NC*	NC*
rpmF	50S subunit ribosomal protein	2.9	NC*	NC*
rpmG	50S subunit ribosomal protein	4	NC*	NC*
rpmH	50S subunit ribosomal protein	2	NC*	NC*
rpmI	50S subunit ribosomal protein	3.7	NC*	NC*
rpmJ	50S subunit ribosomal protein	3.6	NC*	NC*
rpsA	30S subunit ribosomal protein	2.7	NC*	NC*
rpsC	30S subunit ribosomal protein	4.3	NC*	NC*

rpsD	30S subunit ribosomal protein	6.7	NC*	NC*
rpsE	30S subunit ribosomal protein	3.6	NC*	NC*
rpsF	30S subunit ribosomal protein	2.2	NC*	NC*
rpsH	30S subunit ribosomal protein	2.6	NC*	NC*
rpsJ	30S subunit ribosomal protein	2.1	NC*	NC*
rpsK	30S subunit ribosomal protein	4.4	NC*	NC*
rpsL	30S subunit ribosomal protein	2.3	NC*	NC*
rpsM	30S subunit ribosomal protein	5	NC*	NC*
rpsN	30S subunit ribosomal protein	3.5	NC*	NC*
rpsQ	30S subunit ribosomal protein	4.3	NC*	NC*
rpsR	30S subunit ribosomal protein	2.1	NC*	NC*
rpsS	30S subunit ribosomal protein	3.1	NC*	-2.8
rpsT	30S subunit ribosomal protein	2.4	NC*	NC*
pheS	phenylalanine-tRNA synethase subunit alpha	2.4	NC*	NC*
infC	translation initiator facor IF-3	2.8	NC*	NC*
General function and prediction unknown				
hns	global DNA-binding transcriptional regulator	NC*	-2	NC*

dkgB	2,5-diketo-D-glyconate reducatase B	-3.1	-2.6	NC*
trmE	tRNA modification GTPase TrmE	-2.5	-2.3	-9.5
asr	acid shock protein	-6.4	-2.8	-3.4
tus	DNA replication terminus site-binding protein	NC*	-2.8	NC*
ilvB	ilvB operon leader peptide	NC*	2.9	NC*
actP	acetate permease	-13.6	-5.6	-2.9
acs	acetyl coenzyme A synthethase	-5.5	NC*	NC*
yddG	drug efflux pump	-2.8	NC*	NC*
Plasmid Genes				
caf1M	putative F1 chaperone protein	-3.3	-2.6	NC*
caf1A	F1 capsule antigen	-15.6	-10.9	-8.3
rop	putative replication regulatory protein	-10.1	-6.3	-2.6
pla	coagulase/fibrinolysin precursor	-12.7	-3.6	-2.3
parA	partitioning protein	3.3	-2.1	NC*
parB	partitioning protein	6.4	NC*	NC*
mgtC	modulator of P-type ATPase	-5.5	-9.4	-4.9
pspG	phage shock protein G	-3.6	NC*	NC*

yadB	putative outer membrane virulence factor	5.1	3.5	NC*
Quorum Sensing				
luxS	*S*-ribosylhomocysteine lyase, synthesis of autoinducer AI-2	2.6	5.4	NC*

***Represents genes that were not up- or down-regulated when compared to 23°C**

Table 4.6. Differentially expressed genes (DEGs) up- or down-regulated ≥2-fold of *Y. pestis* secretome and outer surface component genes at 6°C, 10°C, and 15°C compared to 23°C.

System	Up/Down Regulation		
Protein Secretion Systems	**6°**	**10°**	**15°**
Type 1 secretion			
secA	NC*	NC*	NC*
secE	NC*	NC*	NC*
Twin arginine translocation			
tatE	-4.8	NC*	NC*
Two-partner secretion			
YPO0600	NC*	NC*	NC*
YPO0599	NC*	3.2	2.7
YPO3721	NC*	NC*	NC*
Type 2 secretion			
yacC	NC*	NC*	NC*
YPO0033	2.7	2.4	2.1
Type 3 secretion			
yopB	-4.1	-3.8	-2.4
yopD	-3.6	-3.2	-2.8
yopE	-2.3	-3.3	-3.1
yopR	-2.1	-2.2	-2.6
yopT	-3.6	-5.0	-5.1
Type 5 secretion			
yapA	12.7	4.1	2.6
yapC	-4.1	-2.2	-2.1

yapH	-2.0	2.5	-2.1
Type 6 secretion			
YPO3704	3.2	2.3	NC*
YPO3705	NC*	NC*	NC*
YPO3706	NC*	-3.6	-2.6
YPO3707	-2.0	-2.5	-3.8
YPO2727	-2.3	NC*	NC*
YPO0506	NC*	NC*	NC*
YPO0514	-2.3	-3.6	-4.1
YPO0516	3.1	NC*	NC*
Putative secretome components			
Chaperone/Usher transport systems			
caf1R	NC*	NC*	NC*
caf1M	-3.6	-3.2	-2.8
caf1A	-2.3	-3.3	-3.1
caf1	NC*	NC*	NC*
psaA	NC*	NC*	NC*
psaE	-2.0	-2.5	-3.8
papD	NC*	NC*	NC*
Secreted/exported protein genes			
YPO1698	NC*	NC*	NC*
YPO1699	NC*	NC*	NC*
YPO1700	-2.4	-2.3	NC*
YPO3675	NC*	NC*	NC*
yeiB	NC*	NC*	NC*
sanA	NC*	NC*	NC*

YPO0337	NC*	NC*	NC*
YPO3699	-2.4	NC*	NC*
YPO4064	NC*	NC*	NC*
YPO0033	6.2	4.2	2.6
YPO1858	2.3	3.0	3.1
YPO0800	NC*	NC*	Up
YPO2857	NC*	NC*	NC*
YPO3069	NC*	NC*	NC*
yiaF	NC*	NC*	NC*
ybgF	NC*	NC*	NC*
Insecticidal-like toxin genes			
yitR	NC*	NC*	NC*
yitA	NC*	NC*	NC*
yitC	NC*	NC*	NC*
yipB	NC*	NC*	NC*
YPO2312	-3.2	NC*	NC*
Haemolysin-heamagglutinin-like genes			
YPO0900	-2.8	NC*	NC*
YPO3721	NC*	NC*	NC*
YPO0599	NC*	5.2	3.7
YPO4005	NC*	NC*	NC*
Outer membrane components			
Outer membrane protein genes			
ompH	2.7	2.0	NC*
ompA	2.4	NC*	NC*

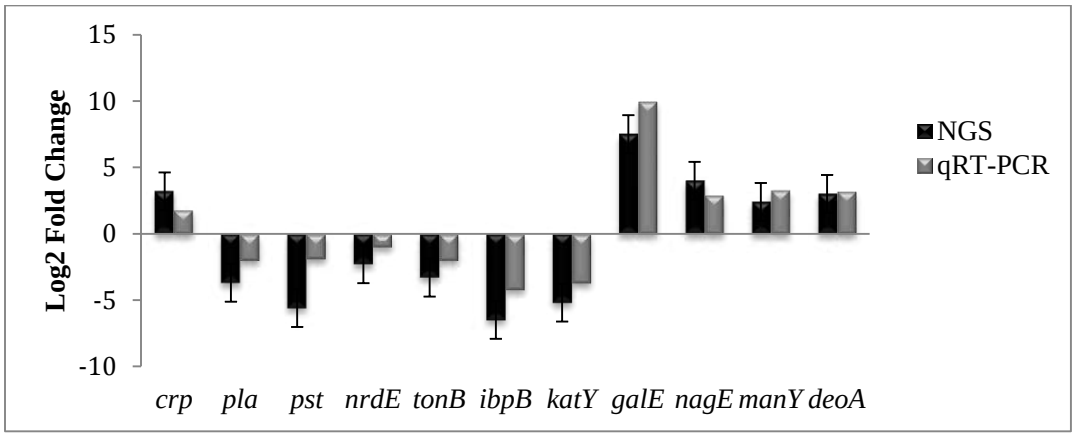
ompC	-2.2	-2.6	NC*
tolC	NC*	NC*	NC*
hmsH	NC*	NC*	NC*
hmsF	NC*	-3.1	NC*
yaeT	NC*	NC*	NC*
btuB	4.3	NC*	-2.3
ail	-3.2	-2.6	-2.1
YPO1011	-2.1	-3.1	-5.3
YPO1313	-4.3	-3.3	NC*
Lipoprotein genes			
spr	NC*	NC*	NC*
lgt	NC*	NC*	NC*
ybaY	-3.6	NC*	NC*
yiaD	NC*	NC*	NC*
YPO0420	NC*	NC*	NC*
YPO1635	NC*	NC*	NC*
YPO2202	NC*	NC*	NC*
YPO2292	4.8	3.4	2.3
pal	3.2	NC*	NC*
lolD	NC*	-3.6	-5.2
slyB	NC*	NC*	NC*
lpp	2.3	3.0	NC*
rlpA	-3.9	-3.6	NC*
dapX	5.8	3.9	2.6
yfiO	NC*	NC*	NC*

YMPT1.32	-2.4	-3.6	-5.2
YPO1354	NC*	NC*	NC*
YPO2073	2.3	3.0	3.1
LPS modification genes			
pmrL	NC*	NC*	NC*
pmrM	NC*	NC*	NC*
msbB	-3.2	-2.6	-2.1
eptB	NC*	NC*	NC*
wzxE	NC*	NC*	NC*
wecC	NC*	NC*	NC*

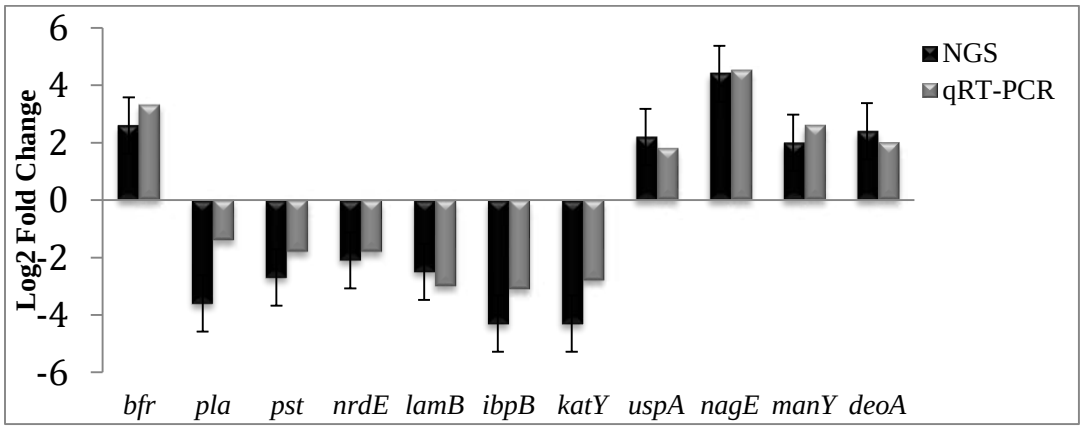
***Represents genes that were not up- or down-regulated when compared to 23°C.**

Figure 4.2. Quantitative reverse transcription qRT-PCR confirmation of RNA-seq (NGS) results

A.



B.



C.

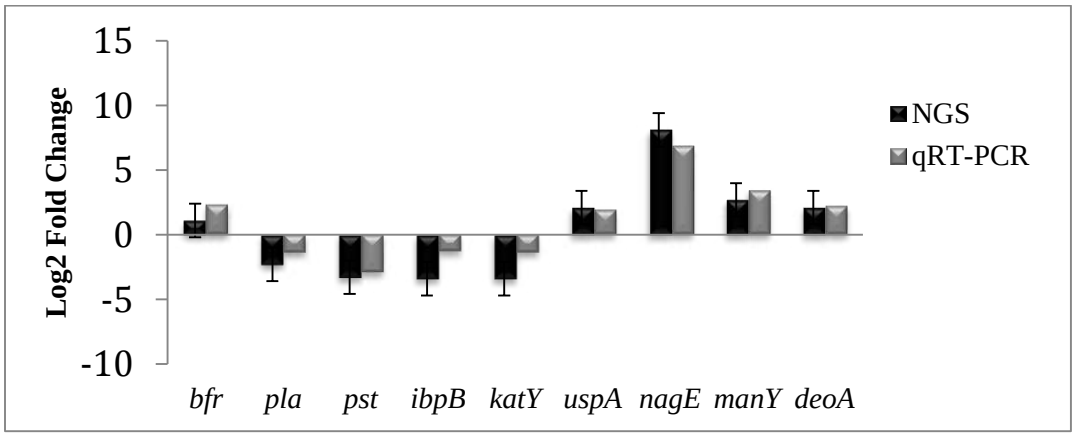


Figure 4.2. Histogram showing the quantitative reverse transcription (QRT) PCR confirmation results compared to the NGS results. The quantity of each mRNA was determined relative to that of the reference gene *proS.* Fold-differences in transcript levels of the *Y. pestis* genes at each growth temperature (6°C, 10°C, 15°C compared to 23°C) were compared to the NGS transcriptome fold changes (dark grey bars) and qRT-PCR (light grey bars). Figure A. Validation of the *Y. pestis* grown at 6°C, comparing qRT-PCR results compared to the 6°C NGS fold change results. Figure B. Validation of the *Y. pestis* grown at 10°C, comparing qRT-PCR results compared to the 10°C NGS fold change results. Figure C. Validation of the *Y. pestis* grown at 15°C, comparing qRT-PCR results compared to the 15°C NGS fold change results. For each temperature, the Pearson correlation coefficient was used to measure the strength of a linear association between the qRT-PCR results and the NGS results.

Figure 4.3. Schematic overview of the differentially expressed genes (DEGs) up- or down-regulated ≥2-fold of *Y. pestis* grown at 6°C, 10°C, and 15°C compared to 23°C

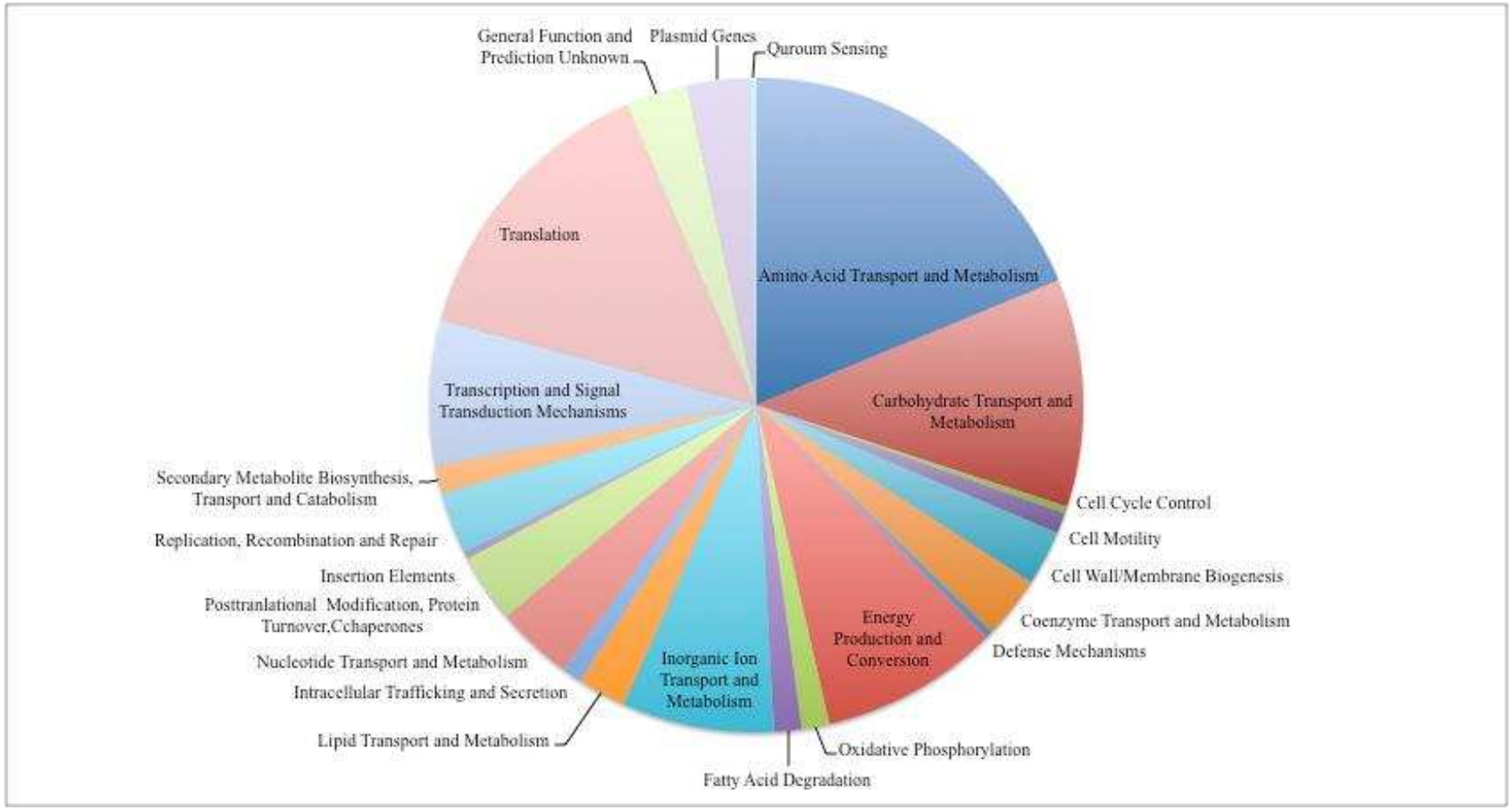


Figure 4.4. Organization of the LPS Biosynthesis gene clusters of *Y. pestis*

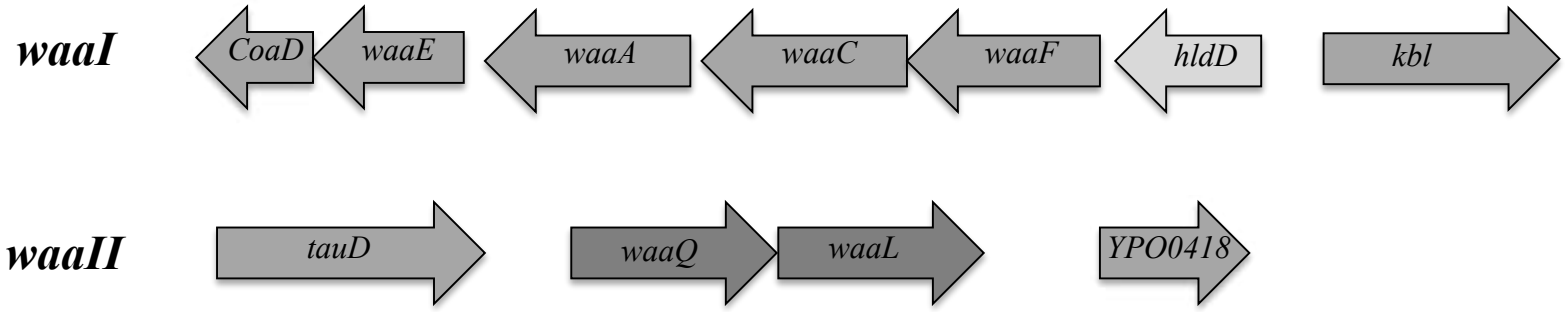


Figure 4.3. Figure of the known genes necessary for the biosynthesis of the *Y. pestis* LPS. The genes in the clusters *waaI* contain most of the genes necessary for the synthesis of the inner core, which comprises the genes of Kdo transferase waaA; heptosyltransferases, waaC and waaF to transfer LD-HepI and LD-HepII, and glucosyltransferase waaE. In addition, it includes the gene of heptose 6-epimerase HldD catalyzing the synthesis of ADP-LD-Hep from its biosynthetic precursor ADP-DD-Hep. WaaQ, located in the waaII cluster, encodes a heptosyltransferase gene. The enzyme encoded by this gene transfers LD-HepIII to LD-HepII; glucose must be present on LD-HepI in order to accomplish the transfer. Gene cluster *waaII* is a homologue of the gene of ligase waaL, which attaches the O-antigen to the core; however, with the absence of O-antigen in *Y. pestis*, waaL attaches the GlcNAc residue to the core; therefore, this residue is not a true component of the core. (Reconstructed from Knirel et al., 2012).