2007-12-20

Surface of *Yersinia pestis*: LCRV, F1 Production, Invasion and Oxygen: A Dissertation

Kimberly Lea Pouliot
University of Massachusetts Medical School

THE SURFACE OF YERSINIA PESTIS: LCRV, F1 PRODUCTION, INVASION AND OXYGEN

A Dissertation Presented

By

Kimberly Lea Pouliot

Submitted to the Faculty of the
University of Massachusetts Graduate School of Biomedical Sciences, Worcester
In partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 20, 2007

Program of Molecular Genetics and Microbiology

December 20, 2007

THE SURFACE OF YERSINIA PESTIS:
LCRV, F1 PRODUCTION, INVASION AND OXYGEN

A Dissertation Presented
By

KIMBERLY LEA POULIOT

The signatures of the Dissertation Defense Committee signifies completion and approval as to style and content of the Dissertation

Jon D. Goguen, Thesis Advisor

Joan Mecsas, Member of Committee

Egil Lien, Member of Committee

Madelyn Schmidt, Member of Committee

Neal Silverman, Member of Committee

The signature of the Chair of the Committee signifies that the written dissertation meets the requirements of the Dissertation Committee

John M. Leong, Chair of Committee

The signature of the Dean of the Graduate School of Biomedical Sciences signifies that the student has met all graduation requirements of the school

Anthony Carruthers PhD.,
Dean of the Graduate School of Biomedical Sciences

Program in Molecular Genetics and Microbiology

December 20, 2007

COPYRIGHTS

Chapter II. Evaluation of the role of LcrV/TLR2-mediated immunomodulation in the virulence of *Yersinia pestis.* Kimberly Pouliot, Ning Pan, Shixia Wang, Shan Lu, Egil Lien, and Jon D, Goguen. 2007. Infection and Immunity 75: 3571-3580.

ACKNOWLEDGEMENTS

I must first thank my mentor, Jon Goguen, for his unwavering patience, support and wisdom. He truly fostered my love of the biological sciences.

I thank my thesis committee, Egil Lien, John Leong, Madelyn Schmidt and Neal Silverman, for all their support and advice. Thanks also go out to my outside committee member, Joan Mecsas, for agreeing to participate in my defense.

I thank my collaborators: Ning Pan, Shan Lu, Shixia Wang, Eicke Latz, Leslie Shaw and Brian Akerley from UMMS; Ralph Isberg and Vicki Auerbach from Tufts.

I give special thanks to past lab members: Chrono Lee, Nancy Deitemeyer, and Karen Gingris for their technical assistance.

I would like to thank my family and friends for their continuing support and encouragement. Special thanks go to my parents and grandmother, my brother, Matthew Copeland, Roman Berman, Vanessa Melanson and Thomas Pfeiffer.

Abstract

Of the eleven species of bacteria that comprise the genus *Yersinia* of the family *Enterobacteriaceae*, three species are pathogenic for humans. *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* usually cause a mild, self-limiting mesenteric lymphadenitis or ileitis. *Yersinia pestis* causes a highly invasive often fatal disease known as plague. All three elaborate a type three secretion system that is essential for virulence and encoded on closely related plasmids. In *Y. pestis*, all the effectors, structural components and chaperones are encoded on the 70kb plasmid, pCD1.

Of these, LcrV from *Y. enterocolitica* has been implicated in playing an immunosuppressive role through its interaction with host Toll-like receptor 2 (TLR2) and induction of IL-10. Through expression and purification of recombinant LcrV from *Escherichia coli* we show that only high molecular weight species of rLcrV are able to stimulate TLR2. In a highly sensitive subcutaneous mouse infection model we demonstrate no difference in the time to death between TLR2-sufficient or deficient mice. Analysis of cytokine levels between these two genotypes also shows no significant difference between splenic IL-10 and IL-6 or levels of bacteria. We conclusively show that this interaction, if it does occur, plays no significant role in vivo.

In a separate set of experiments, we also determined that the expression of F1, a peptide shown to be responsible for 37°C-dependent inhibition of invasion by *Y. pestis in vitro*, was significantly decreased under high oxygen conditions. This led us to re-examine the invasion phenotype both *in vitro* and *in vivo*. These results give new insights into virulence gene expression in *Y. pestis* by environmental cues other than temperature

Table of Contents

List of Figures

Chapter Two.

Chapter Four.

Chapter Five.

Chapter One.

Introduction

Plague and the biology of *Yersinia pestis*

Of the eleven species of bacteria that comprise the genus *Yersinia* of the family *Enterobacteriaceae*, three species are pathogenic for humans. *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* usually cause a mild, self-limiting mesenteric lymphadenitis or ileitis. In contrast, *Yersinia pestis* causes a highly invasive often fatal disease known as plague. Plague has been responsible for three major pandemics, each involving millions of deaths. The second of these, often known as the "Black Death" (1347-1351), killed close to one third of the European population. There are currently 1,000 to 3,000 cases of plague a year, mostly in Africa, Asia and South America, with 10-15 cases occurring in rural areas of the Southwestern United States. It is clear that *Y. pestis* is derived from a single clone of *Y. pseudotuberculosis*, and estimates based on a "mutational clock" theory indicate that this divergence occurred only 1500 to 20,000 years ago [1]. While the enteric yersiniae are transmitted to humans via the oral-fecal route, transmission of *Y. pestis* mainly occurs through the bite of infected fleas, an adaptation facilitated by the acquisition of two plasmids pMT1, pPCP1. pMT1 encodes genes necessary for colonization of the flea gut, while pPCP1 enhances dissemination from flea bite to bloodstream [2, 3].

Yersinia pestis is endemic in rodent populations in the southwestern United States, Africa, South America and Asia. The rat flea, *Xenopsylla cheopis,* feeds on infected rodents and other small mammals, engulfing bacteria-laden blood meals. Within

the flea gut, the bacteria produce two products of particular importance. A phospholipase D (Ymt) that is essential for protection from flea antibacterial defenses, and a surface polysaccharide that promotes aggregation of the bacteria and their adherence to structures in the flea proventriculus [4, 5]. This adhesion and aggregation occludes the proventriculus preventing ingestion of blood, causing the starving fleas to bite repeatedly, regurgitating *Y. pestis* into the bite wounds at each attempt. Fleas can infect a variety of rodent species, including many species of ground squirrels. These serve as important reservoirs in nature, as do rats, prairie dogs, and marmots. Human infection usually results from fleas infected by these natural hosts. *In vivo* experiments have demonstrated that following the bite of an infected flea, the pPCP1-encoded outer membrane protease, Pla, greatly enhances dissemination from the intradermal site [6]. Bacteria that lack this protease cause only a mild inflammation at the wound site and are completely unable to spread into the neighboring lymphatic system.

Spread into the lymphatics results in massive proliferation of the bacteria within draining lymph nodes, resulting in gross swelling and necrosis. Inguinal, cervical or axillary nodes are frequently involved. These engorged and painful nodes are termed “buboes”, and the resulting disease is known as bubonic plague. In classical bubonic plague, bacteria escape the nodes and enter the blood leading to fatal systemic infection. However, entry into the blood following fleabite may occur without bubo formation, resulting in primary septicemic plague. Dissemination via blood results in infection of many organs, including liver, spleen, lungs, and meninges, accompanied by extremely high levels of bacteremia. When untreated, bubonic and septicemic plague cause about

50% mortality. Lung involvement, or secondary pneumonic plague, can result in person-to-person transmission via contaminated droplets aerosolized through coughing. Transmission by aerosol results in primary pneumonic plague, which is universally fatal if untreated. Effective treatment requires institution of appropriate therapy within 20 hours of the onset of symptoms, a point at which the disease is difficult to recognize unless the index of suspicion is very high. The high mortality, ease of dissemination, and potential for secondary transmission of pneumonic plague make *Y. pestis* a significant cause for concern with respect to biological terrorism and warfare.

Evasion of Innate Immunity through thermally inducible virulence factors

During the course of infection, bacteria are able to reach extraordinary densities in the blood, up to 10^8 CFU per milliliter. This high density insures infection of feeding fleas. The ability of *Y. pestis* to attain this high concentration in the blood poses two interesting questions. How do the bacteria escape the antibacterial defenses that usually keep superficial infections localized? And how do the bacteria prevent induction of fatal septic shock before these high densities are achieved? The answers to both these questions lie in the ability of *Y. pestis* to manipulate the mammalian innate immune system. Multiple bacterial systems are involved, and often share a common property: activation by temperatures above 34°C. This allows expression within the mammalian host, but not the flea.

LPS

Toll-like receptors are central components of the mammalian innate immune system. So far, ten distinct human receptors have been identified, each able to recognize general motifs from bacterial, viral or fungal pathogens. Stimulation of these receptors frequently induces the expression of pro-inflammatory cytokines like TNF and IFN-γ. For example, lipopolysaccharide (LPS) is an immunodominant molecule expressed on the surface of Gram-negative bacteria that is specifically recognized by host Toll-like receptor 4 (TLR4) in complex with MD-2 [7]. LPS is typically composed of a lipid A portion anchored in the outer membrane, a polysaccharide core structure and an O-antigen, composed of repeating oligosaccharides. *Y. pestis* completely lacks the O-antigen structure and modifies the lipid A portion as to evade immune surveillance [8-10].

Rebeil et al. and Knirel et al. demonstrated that the lipid A portion of the *Yersinia pestis* LPS structure is thermally-regulated [11, 12]. At 26ºC, the lipid A portion of the LPS molecule is hexa-acylated, similarly to the LPS of *E. coli* [13]. A "missing" late acetyltransferase (*lpxL*) within the *Y. pestis* genome renders the lipid A portion predominantly tetra-acylated at 37ºC [14]. In vitro experiments demonstrate the importance of a modified LPS structure in virulence: the hexa-acylated form is able to stimulate TLR4, whereas the tetra-acylated form cannot [14]. These experiments also show that expression of the late acetyltransferase, *lpxl*, in wildtype *Y. pestis* leads to the addition of two additional acyl chains, resulting in a hexa-acylated lipid A at 37ºC. Infection experiments in wildtype mice demonstrate that the expression of a hexa-

acylated lipid A at 37ºC renders *Y. pestis* avirulent. However, in TLR4$^{-/-}$ mice, expression of hexa-acylated lipid A has no effect on virulence [14]. The contribution of a non-stimulatory LPS is an adaptation of *Y. pestis* to survive in the host by evading innate immunity, and may also prevent early onset of septic shock.

Type Three Secretion System

Many Gram-negative bacterial pathogens utilize a contact-dependent type three secretion system (TTSS) to inject their bacterial products directly into host cell cytosol. Upon host cell contact, proteins are translocated from the bacterial cytoplasm through a needle-like structure, or injectisome, that spans the inner and outer membrane. The tip of the needle connects to the host cell membrane and allows the bacterial proteins, or effectors, to enter the cell cytoplasm and interact with their intracellular targets. Typically, the function of these effectors is to modulate signaling pathways and functions based on cytoskeletal activity such as phagocytosis.

All three pathogenic Yersinia species elaborate a TTSS that is essential for virulence and encoded on closely related plasmids. In *Y. pestis*, all the effectors, structural components and chaperones are encoded on the 70kb plasmid, pCD1. Strains deficient in pCD1 are highly attenuated *in vivo* [15-17]. The assembly of the injectisomes and synthesis of effectors occurs only at 37°C. Upon host-cell contact, the injectisome is activated and the effectors are delivered directly into host cell cytosol. The collection of intracellular effectors, termed Yops (**Y**ersinia **o**uter **p**roteins), inhibit signaling cascades controlling phagocytosis (YopH, YopE, YopM), induce apoptosis and

inhibit cytokine production (YopJ) [18]. Three translocator proteins, YopB, YopD and LcrV (or V antigen) are required for efficient delivery of the Yops. Many studies suggest that YopB/D insert directly into the host cell membrane [19-21]. LcrV is necessary for both high-level expression of the Yops and as a structural component of the needle, and recently it has been suggested that it may also have an immunomodulatory role [22-24]. Studies using recombinant *Y. enterocolitica* LcrV expressed and purified from *E. coli* have shown an ability to induce the anti-inflammatory cytokine, IL-10, in a TLR2-dependent manner [25]. This ability, though not exhaustively tested for *Y. pestis* LcrV, has also been suggested for *Y. pestis* LcrV.

Pla

The only recognized virulence factor encoded on the 9.5 kb plasmid, pPCP1, is the integral, outer membrane protein, Pla, a multifunctional, 34.6 kD protein with plasminogen activator activity [2]. Unlike many of the virulence factors discussed here, Pla is constitutively expressed and active at both 30°C and 37°C. Sequencing of the *pla* gene reveals homology to *ompT* of *Escherichia coli* and *PgtE* of *Salmonella typhimurium*, proteases with weak plasminogen activator activity in comparison to Pla [26]. Though it possesses no significant homology to any known eukaryotic plasminogen activators, the activity of Pla is similar to that of human tissue or urokinase plasminogen activator, cleaving plasminogen at the same R_{560}-V_{561} site, releasing the active plasmin portion of the molecule [6]. The protease activity of Pla also degrades the C3b component of complement [6] and α_2-antiplasmin, an inhibitor of soluble plasmin. Collectively, these activities promote the degradation of fibrin. However, the most

prominent effect of Pla at the level of individual lesions is the suppression of neutrophil accumulation. In the liver, Pla^- *Y. pestis* are rapidly surrounded by neutrophils to form a microabcess. In contrast, Pla^+ bacteria grow freely packed within liver sinusoids with no sign of local inflammation [6]. The mechanism underlying this effect is not understood.

Several groups speculate that the central role of Pla during a subcutaneous infection is degradation of the fibrin matrices, via its plasminogen activator activity, that might otherwise encapsulate the bacteria at intradermal wound sites. It has been shown by several groups that *in vivo*, Pla enhances the progression to systemic disease after intradermal inoculation, but not for intravenous inoculation. Pla mutants are fully virulent after intravenous challenge compared to wildtype, but the LD_{50} increases a million-fold after subcutaneous infection of Pla-deficient strains compared to wildtype. Experiments conducted with plasminogen-deficient mice reveal that they are less susceptible to infection after subcutaneous challenge with *Y. pestis* [27].

In addition to its protease activity, Pla has been shown to have both adhesive and invasive properties *in vitro*. Lähteenmäki et al. observed *E. coli*–expressing Pla, but not *E. coli* alone, was able to bind to the extracellular matrix components, vitronectin and laminin [28]. In addition they observed that laminin, but not vitronectin, was degraded by Pla in the presence of exogenous plasminogen. In a subsequent study, they observed that *E. coli*-expressing Pla, but not *E. coli* alone, was able to invade endothelial cells in vitro [29]. A role for Pla-mediated adhesive and/or invasive functions has not been

demonstrated *in vivo*, nor has it been demonstrated that protease activity is absolutely required for adhesion and/or invasion.

The activities of Pla required for the progression of primary pneumonic plague are largely not known. Lathem et al. demonstrated that Pla is required for *Y. pestis* to cause fulminant pneumonia in intranasally infected mice; only 50% of mice infected with 1 x 10^4 CFU Pla-deficient *Y. pestis* succumbed to plague after 7 days, compared to 100% of mice infected with the wildtype strain, CO92 [30]. Infection of mice with Pla-deficient strains resulted in a significant reduction in bacterial load in the spleens and lungs after 48 hours compared to wildtype strain, CO92 (10^5 higher CFU/gram of lung in wildtype infected mice). However, they find that Pla is not essential for dissemination from the lung, unlike results from experiments where mice were infected subcutaneously. In contrast, studies by Welkos et al. demonstrate that the requirement for Pla is not substantial. They report only a 4-fold increase in the LD_{50} when mice are infected by aerosol with a $pPCP1^-$ strain compared to wildtype CO92 [31]. These differences could be due to growth conditions of the bacteria. For aerosol infection, Welkos et al. consistently grow the bacteria in liquid culture at 30°C, whereas Lathem et al. grew their bacteria in flasks at 37°C**,** conditions which the bacteria will be primed with many virulence determinants such as TTSS, F1 and non-stimulatory LPS [15, 30, 32 {Welkos, 2002 #52]}.

Interestingly, the plasminogen activator activity of Pla is largely dependent upon the structure of the bacterial lipopolysaccharide [33]. As previously mentioned, *Y. pestis* is unique in that its LPS structure contains only the outer membrane-bound lipid A

portion and a polysaccharide core; it completely lacks an O-antigen. "Smooth-type" LPS has O-antigen, composed of repeating oligosaccharides, whereas rough-type LPS lacks a long chain O-antigen. Studies by Kukkonen et al. observed that purified His_6-Pla reconstituted with rough (Re) type, but not smooth, lipopolysaccharide (LPS) from *Salmonella enterica* was able to effectively activate plasminogen [33]. It is likely that this effect is merely the result of steric hindrance, smooth LPS preventing access of the 94 kD plasminogen molecule to the active site of the protease located at the outer membrane surface. The structure of the fatty acid component of LPS does not appear crucial to Pla activity. A strain modified to express hexa-acylated LPS has similar Pla activity as to wild type, tetra-acyl LPS expressing strain [34]. Moreover, Pla activity does not change with temperature, although LPS structure is modified.

F1

Inhibition of phagocytosis is also accomplished, but to a lesser extent, by the production of a peptide "capsule" at 37°C [35]. The existence of this structure has been known for many years, and has been historically referred to as the Fraction 1 or F1 antigen. It is in fact not a true capsule, but comprises a highly expressed chaperone/usher type pilus that is readily shed by the bacteria. Encoded on the largest plasmid, pMT1, *caf1* is located within an operon that also encodes the regulator (*caf1R*), the chaperone (*caf1M*) and usher protein (*caf1A*) required for pilus biogenesis [36-38]. The 28.7 kD Caf1M protein shares homology with PapD, a chaperone protein of P pili in uropathogenic *Escherichia coli* [36] and Caf1A shares homology with PapC, the usher protein for P pili assembly [38]. Sequence analysis reveals homology between *caf1R* and

proteins of the AraC family, known to be DNA-binding transcription regulators. Though the mechanism whereby *caf1R* regulates *caf1* is not well understood, Karlyshev et al. speculate that caf1R is a positive regulator, as a C-terminal deletion mutant of caf1R within the caf1 operon expressed on a plasmid in *E. coli* retains the ability to generate F1 monomers [39].

F1 subunits of 15.5 kDa spontaneously self-assemble into pili that are readily shed from the bacteria during in vitro growth. Early work by Cavanaugh and Randall demonstrated that bacteria expressing F1 are resistant to phagocytosis by monocytes, but that phagocytosed F1-negative bacteria can replicate within the phagocyte and upon release from the cell were subsequently resistant to phagocytosis [40]. More recently, it was shown that production of F1 inhibits phagocytosis by the macrophage-like cell line, J774.1, as well as epithelial cell uptake by A549 cells [35, 41]. However, the role of F1 *in vivo* seems minor in comparison to TTSS, as studies have shown that F1 deletion mutants or strains lacking the entire pMT1 plasmid are fully virulent in non-human primates challenged by aerosol [42].

In addition, F1-deficient strains have been isolated from plague-infected individuals and from mice initially infected with a F1-sufficient strain of *Y. pestis* [43{{Burrows, 1958 #231] Friedlander, 1995 #230} [44]. However, the role of F1 in pathogenesis may be organism-specific, as a moderate loss of virulence is observed in rats and guinea pigs infected with F1-deficient strains [45]. Thus, the true function of F1 during human infection is unclear. To date, the capsule peptide, F1, and the

multifunctional TTSS protein, LcrV, are the only recognized vaccine antigens providing strong protection against *Y. pestis* infection [46-50].

Collectively, the expression of the type three secretion system (TTSS), F1 capsule peptide, the Pla protease and a non-stimulatory LPS allow *Yersinia pestis* to disseminate and replicate virtually unnoticed by the mammalian innate immune system, allowing bacteria to reach extraordinarily high densities in the blood. The thermal regulation on TTSS, F1 and LPS demonstrate the importance of bacterial growth temperature during infection studies; the state of the bacteria with respect to these structures at the time of infection may influence colonization and the subsequent course of disease.

Other Recognized Virulence Factors of *Yersinia pestis*

The acquisition of iron and the *pgm* locus

For bacterial pathogens, the ability to acquire iron *in vivo* is crucial for full virulence. Iron is tightly sequestered by the mammalian proteins, transferrin and lactoferrin. Encoded on the chromosome of *Y. pestis* is a 102 kb segment of DNA called the *pgm* locus, initially identified for ability of bacteria to bind large amounts of hemin or Congo Red (**p**i**gm**entation) from solid media at 26ºC, but not 37ºC [51, 52]. Within this locus is the *hmsHFRS* operon, encoding genes that produce enzymes required for the synthesis of the surface polysaccharide responsible for hemin and Congo red binding, and the *yersiniabactin* genes (*ybt* genes and *psn*) that synthesize *Y. pestis* siderophore [5, 53]. Due to recombination between flanking insertion sequences (IS100) with the same

orientation, the 102 kb *pgm* locus is spontaneously lost at a rate of 10^{-5} [54-56]. Loss of the entire locus results in a significant decrease in virulence; the LD_{50} for s.c.-infected mice increases from 2 to 10^7 CFU and LD_{50} for aerosol-infected mice increases from 2.3 x 10^4 to 10^6 CFU [31]. Loss of the *hms* operon has little effect on virulence when mice are infected subcutaneously [5]. Genes involved in the acquisition of iron are generally considered the most important virulence determinants for mammalian infection encoded within the *pgm* locus, although other genes may have important as yet unidentified effects. The polysaccharide produced by the *hms* system appears to play an important role in adherence of the bacteria to each other and to structures in the flea proventriculus, allowing the bacteria to prevent intake of blood to the gut, and as a result enhance transmission by causing increased biting and regurgitation by the infected flea.

pH 6 Antigen

pH6 antigen, or PsaA, is a chromosomally-encoded, thermally-regulated protein that is expressed only at temperatures between 35ºC and 41ºC and between pH 5 and pH 6.7. pH 6 antigen forms fimbriae on the surface of the bacteria and, similarly to F1, is exported by an usher-chaperone system. pH 6 antigen has been shown to interact with phosphatidycholine on the surface of alveolar epithelial cells and within pulmonary surfactant [57]. One study indicates that a pH 6 antigen mutant may be slightly attenuated after retro-orbital infection [58]. In a *pgm*-deficient background, they observed a 200-fold increase in the LD_{50}. The function of pH 6 antigen during infection is not known, though its regulation suggests it may be expressed within a bubo or within a vacuole.

In summary, the coordinate function and regulation, either by temperature or pH, of surface-associated virulence factors of *Yersinia pestis* is important for the establishment of disease (Table 1). Regulation of gene expression through environmental cues is crucial for the pathogenesis of plague. The interaction of these surface-associated factors with host cells *in vitro* may give insight into their putative function *in vivo*.

The central theme of this thesis is the analysis of interactions between surface-expressed molecules of *Y. pestis* and host cells. Despite the volume of work in this area, the functions of many of the key molecules described above remain uncertain, and we find some of the functions claimed to be unconvincing and worth revisiting.

In vivo studies in both rodents and non-human primates indicate the ability of *Y. pestis* to rapidly traverse epithelial barriers and into the bloodstream following aerosol or oral challenge [42, 59]. Several groups have shown that *Y. pestis* can mediate its internalization into cultured epithelial cells *in vitro* [41, 60]. We first attempted to identify *Y. pestis* factors involved in bacterial-mediated invasion of epithelial cells *in vitro* as a potential mechanism to cross these host barriers. However, others and we demonstrated that the expression of F1 at 37°C significantly inhibited the invasion phenotype [41]. This 37°C-dependent inhibition hindered further examination of this phenomenon, as it seemed an unlikely mechanism within a mammalian host. However, during the course of these experiments we found novel and biologically interesting cues for expression of F1 that led us to re-examine the invasion phenotype. These results give

new insights into virulence gene expression in *Y. pestis* by environmental cues other than temperature.

In addition to studying the interaction of *Y. pestis* with epithelial cells *in vitro*, we were also interested in testing the LcrV/TLR2 hypothesis in the context of *Y. pestis*. Multiple groups have demonstrated that recombinant LcrV from *Yersinia enterocolitica* has immunosuppressive effects through in induction of IL-10 and suppression of TNF through a TLR2/CD-14 dependent mechanism. As mentioned previously, LcrV is only one of two antigens shown to be immuno-protective against plague. The claim of its immunosuppressive effects has implications for the development of LcrV-based plague vaccines. One study attempted to design a modified *Yersinia pestis* LcrV molecule that lacked TLR2 stimulating ability [61]. After thorough examination, we show that stimulation of TLR2 by *Y. pestis* rLcrV, though it occurs in vitro, has no significant effect on the outcome of disease after subcutaneous or intravenous challenge with fully virulent *Y. pestis*.

Virulence Factor	Gene Location	Main Function in Mammals	Regulated by	Effect of Loss on LD_{50}*
pgm locus	Chromosome	Iron acquisition	constitutive	$>10^7$ (s.c.)
LPS	Chromosome	Innate Immune Evasion	temperature, 37°C	$>10^6$ **(s.c.)
pH 6 antigen	Chromosome	Formation of pili	temperature, 37°C and pH ~ 6.2-6.4	200-fold (r.o.)
F1	pMT1	Formation of pili	temperature, 37°C	None*** (aerosol)
Type three secretion	pCD1	Delivery of Yop effectors, inhibition of phagocytosis	temperature, 37°C	$>10^7$ (s.c., i.v.)
Pla protease	pPCP1	Plasminogen activation Inhibition of neutrophil accumulation	constitutive	10^8 (s.c.)

Table 1. ***Summary of Yersinia pestis Surface-Expressed Virulence Factors***. **Compiled from sources referred to in the text. **Infection of mice with stimulatory hexa-acylated lipid A form with bacteria grown at 37°C. ***As discussed in the text, the role of F1 in vivo is not well understood. Aerosol infection of non-human primates with $F1^+$ and $F1^-$ bacteria suggest difference in virulence, however this group almost exclusively uses 30°C-liquid grown strains for aerosol infection.*

Chapter Two.

Evaluation of the role of LcrV/TLR2-mediated immunomodulation in the virulence of *Yersinia pestis*

Introduction

The three Yersinia species elaborate a type III contact-dependent secretion system (T3SS) that is essential for virulence, and encoded on closely related plasmids. This apparatus allows delivery of effector molecules directly into host cell cytosol. These intracellular effectors, termed Yops, inhibit phagocytosis, induce apoptosis, and inhibit cytokine induction [18, 62]. Three translocator proteins, YopB, YopD and LcrV are required for efficient intracellular delivery of the Yops [62]. LcrV (V-antigen) is a multi-function protein essential for virulence. It comprises the tip of the injection needle of the T3SS [19, 63, 64], and along with LcrG has a regulatory role in Yop secretion [22]. In addition, LcrV has been shown to be one of only two proteins to serve as highly effective vaccine antigens against *Yersinia pestis* [46, 65, 66].

An immunomodulatory role for LcrV has also been proposed [67]. *In vivo* studies have shown that a recombinant *Y. pseudotuberculosis* LcrV/Protein A fusion, produced in *E. coli*, suppressed induction of TNF and IFNγ induced by injection of lipopolysaccharide (LPS) into Swiss Webster mice, and also enhanced the severity of disease and the bacterial burden following infection of treated mice with attenuated *Y. pestis*, *Salmonella typhimurium*, and *Listeria monocytogenes* [68]. Further studies utilizing recombinant *Y. enterocolitica* LcrV (rLcrV), also produced in *E. coli*, suppressed zymosan-induced TNF production in C57BL/6 macrophages [69]. It has been proposed that rLcrV interacts with both Toll-like receptor 2 (TLR2) and CD14 to induce interleukin-10 (IL-10) in transfected HEK293 cells [70]. Candidate residues

responsible for this interaction with TLR2 were identified in the N-terminal region of *Y. enterocolitica* LcrV based on the activity of cognate synthetic peptides [70, 71]. Lower activity was detected in similar peptides based on *Y. pestis* and *Y. pseudotuberculosis* LcrV sequence [71]. More recently, Overheim et al.. [72] showed that recombinant His-tagged LcrV derived from *Y. pestis* and purified from *E. coli*, induced IL-10 from murine macrophages and human monocytes. It also suppressed induction of TNF from murine macrophages. This group also showed that deletion of either of two domains within LcrV eliminated stimulation of IL-10 secretion, but that deletion of only one of these, near the C terminus of the protein, eliminated both induction of IL-10 secretion and suppression of TNF. Neither of these domains corresponds with--or overlaps the sequence of--the active peptide identified by Sing et al. in the LcrV of *Y. enterocolitica*. Overheim et al.. also presented evidence that a fragment of LcrV lacking the C terminus--which includes the domain responsible for suppression of TNF--was an effective vaccine against *Y. pestis* at lower doses than unmodified recombinant LcrV.

Toll-like receptors participate in many aspects of host defense against infections [73]. Stimulation of TLR2 results in the induction of pro-inflammatory cyokines such as TNF and IL-6. Like TLR4, the receptor for lipopolysaccharide (LPS), TLR2 is also responsible for the release of anti-inflammatory cytokines such as IL-10 and IL-4, although these are usually detected at a later time than pro-inflammatory mediators induced simultaneously via the same pathway [74, 75]. Curiously, TLR2 has been associated with immunosuppression in microorganisms in addition to *Y. enterocolitica* [76, 77], suggesting that exploitation of this pathway as a means to evade innate

immunity may be a common strategy among pathogens. However, no clear mechanistic basis for a predominantly immunosuppressive effect of TLR2 stimulation has been established.

In this study, we report that *Yersinia pestis*-derived rLcrV, purified from *E. coli* through Ni-NTA chromatography activates TLR2, as has been reported for *Yersinia enterocolitica* LcrV. However, further purification through gel filtration indicates that only a very high molecular weight multimer or aggregate, which comprises a small proportion of total rLcrV, has stimulatory activity. Stimulation with *Yersinia pestis* LcrV-derived peptides corresponding to stimulatory peptides derived from *Yersinia enterocolitica* LcrV failed to activate TLR2. In infection experiments, TLR2-deficiency in mice had no significant influence on the course of disease, levels of IL-10, or degree of inflammation in infected tissue. Taken together, these results strongly suggest that TLR2-mediated induction of IL-10 does not contribute significantly to the virulence of *Yersinia pestis*.

Materials and Methods

Bacterial Strains, Plasmids, Cell Lines, and Reagents. Virulent *Y. pestis* strain KIM1001[6], biotype mediavalis, was grown and quantified on solid medium (TB) containing 10 g Bacto-tryptose, 5 g NaCl, 3 g Beef Extract (paste form, Difco Cat # 212610) and 15 g agar per liter supplemented with 2.5 mM $CaCl_2$. Although this composition is identical to that given for pre-compounded Tryptose Blood Agar Base, we

have on occasion encountered significant problems with plating efficiency on the latter preparation, and therefore chose to work from the individual components. Unless otherwise indicated, plates were incubated at 25°C for 48 hours. prLcrV is described in Overheim at. al., and was the kind gift of Olaf Schneewind. Construction of $pLcrV_{Yp}$ is described below. HEK293 cells stably expressing human TLR2-YFP, or empty vector pcDNA3 were as described [78]. HEK293-TLR2-YFP/human CD14 cells were generated by stably expressing human CD14 in the vector pCEP4, selection in hygromycin, followed by FACSorting. 293 cells were maintained in DMEM supplemented with 10% FCS and 10μg/ml Ciprofloxacin (Biowhittaker), with addition of G418 (0.5 mg/ml, 293-pCDNA3 and 293-TLR2-YFP) or G418 plus hygromycin (400 U/ml, 293-TLR2-YFP/CD14). Cells were seeded at 3 x 10^4 per well in 96-well tissue culture plates (Costar) and stimulated for 16-18 hours before harvesting supernatant for cytokine analysis. LPS, from the *E. coli* strain 0111:B4, was purchased from Sigma and was subjected to two rounds of phenol re-extraction to remove contaminating TLR2 stimulating lipoproteins [79]. A synthetic triacylated lipohexapeptide Pam 3-$CysSerLys_4$ (P3C) was purchased from EMC Microcollections (Tuebingen, Germany). Synthetic LcrV peptides (HPLC purified to > 98%) were purchased from Genemed Synthesis, Inc.

Recombinant plasmids expressing LcrV from *Yersinia pestis*. *lcrV* from *Yersinia pestis* strain KIM [3] amplified by PCR was cloned into vector pBAD/gIIIB (Invitrogen) at NcoI and SalI sites resulting in $pLcrV_{Yp}$. This vector incorporates a signal sequence to promote export of the cloned protein to the periplasm. The signal sequence was included both to allow extraction by osmotic shock, and to reduce inclusion body formation.

Because osmotic shock proved to have little advantage, more convenient methods of extraction were used during preparation of recombinant protein (see below). The coding sequence of the rLcrV in this construct is as follows (cleaved domain of signal sequence italicized, non-native residues of mature protein lower case, native residues uppercase):

mkkllfaiplvvpfyshs tmvMIRAYEQNP...DTSGKvdhhhhhh

Note the added C-terminal hexahistidyl domain used in purification.

A second recombinant LcrV-producing plasmid, prLcrV, was the kind gift of Olaf Schneewind, and is described by Overheim et. al. [72]. This plasmid produces full-length rLcrV with the addition an amino-terminal decahistidyl domain.

Expression and Purification of rLcrV. *From plasmid pLcrV$_{Yp}$*: *Escherichia coli* LMG194 (Invitrogen) carrying pLcrV$_{Yp}$ was grown overnight at 37°C in 2xYT broth supplemented with 100 μg/ml ampicillin. Bacteria were then diluted into 4 L of fresh medium to an optical density at 600nm (OD600) of 0.1 and grown at 37°C in a New Brunswick Bio Flo 2000 fermenter (5 l/min aeration, 300 rpm agitation, antifoam as needed) to an OD600 of 0.5. Arabinose (0.002%) was then added to induce production of LcrV, and incubation continued for an additional 3 h. Cells were harvested by centrifugation at 12,000 x g for 10 min, resuspended in 50 ml of 50mM sodium phosphate buffer pH 8.0 containing 300 mM NaCl, and sonicated on ice (Branson Sonifier 450 with 0.75" solid stepped horn, output power setting 6, run for 4 minutes at 50% duty cycle). Alternatively, cells were disrupted via rapid decompression with a pre-chilled French pressure cell operated at 20,000 psi. The extract was centrifuged at 10,000

x g for 15 min, and the soluble fraction applied to nickel nitrilo-triacetic acid (Ni-NTA) column (1 ml bed volume) pre-equilibrated with 30 ml column buffer containing 50mM NaH_2PO_4, 300mM NaCl and 10mM imidazole, pH 8 (Qiagen). The column was washed with 15ml wash buffer containing 50mM NaH_2PO_4, 300mM NaCl, and 20 mM imidazole, pH 8. Bound protein was eluted in buffer containing 50mM NaH_2PO_4, 300mM NaCl, and 250mM imidazole pH 8. Proteins were subjected to extensive dialysis against endotoxin-free PBS (Cambrex), and immediately frozen at -80°C. Protein concentrations were determined by absorbance at 280 nm using an extinction coefficient of 0.515 calculated from the rLcrV sequence by the method of Gill and von Hippel [80] and confirmed by SDS-PAGE. All buffers were prepared using endotoxin-free reagents.

Analysis of purified rLcrV treated with 5 mM dithiothreitol (DTT) by mass spectroscopy (LC-MS) yielded a mass consistent with removal of the signal sequence: no material corresponding to the full-length precursor form was detected.

ii. Expression and purification from plasmid prLcrV: This plasmid was transformed into BL21DE3 and protein production induced and the rLcrV extracted and purified as described by Overheim et al. with the following modifications: (1) A French pressure cell was used to disrupt the bacteria as described above rather than sonication, (2) Triton X-114 phase separation and the following size-exclusion chromatography on G25 sephadex to remove Triton was omitted. This material was analyzed identically to rLcrV from $pLcrV_{yp}$ via size exclusion chromatography on Superose 12 resin as described below.

Size Exclusion Chromotography. One milligram samples of rLcrV in a 0.3 ml volume of PBS were loaded on a PBS-equilibrated Superose 12 column (Amersham Biosciences cat # 17-5173-01) and eluted with PBS at a flow rate of 0.54 ml/min. Endotoxin-free PBS (Cambrex) was used for column equilibration and elution. Absorbance of the eluate at 280 nm was monitored. Fractions of 330 ml were collected in the wells of microtiter plates and each tested for ability to stimulate TLR2 via induction of IL-8 as described above. Following each use, the column was washed with 1 M NaOH, followed by endotoxin-free water, 0.1 M HCl, and stored in 20% ethanol.

Mice. Female C57BL/6 (6-8 weeks of age) were purchased from Jackson Laboratory (Bar Harbor, Maine). $TLR2^{-/-}$ mice were originally a generous gift of S. Akira [81], and have now been back-bred to C57BL/6 for 11 generations. All mice were bred under specific-pathogen-free conditions. All animal studies were approved by the Univ. of Massachusetts Medical School Institutional Animal Care and Use Committee and all relevant policies regarding animal care, biosafety, and security were followed.

Experimental Infection of Mice. To prepare inocula for experimental infection, *Y. pestis* KIM1001 was inoculated heavily onto TB plates from suspensions stored at –70°C in TB (no agar) plus 5% glycerol, and incubated at 37°C for 24 hours. Bacteria were harvested from the plate with a loop and resuspended in sterile distilled water with a vortex mixer to match a turbidity standard of OD_{600} 0.3. It was important to use a low ionic strength medium for this initial suspension step to achieve good dispersion of the cells. This initial high density suspension was subsequently diluted as required using endotoxin free

injection grade PBS. For survival experiments, 10 age and sex-matched mice per treatment group were infected with *Yersinia pestis* KIM1001 by s.c. injection of 50 μl on the nape of the neck. For i.v. infection experiments, five mice per group were injected with 500 μl of inoculum containing the indicated doses in the tail vein. Survival was monitored every 12 hours for up to 21 days. All $TLR2^{-/-}$ were individually genotyped and bore uniquely numbered ear tags. For collection of organs, mice were sacrificed 48 hrs following i.v. infection by pentobarbital overdose followed by cervical dislocation. To determine bacterial load, spleens were homogenized in 1 ml of PBS and bacteria in the resulting suspension titered by serial dilution and plating. Differences in survival were analyzed by Kaplan-Meyer survival analysis and the log rank test.

Histopathology. Livers were fixed for in neutral buffered 4% formalin, and sections stained with hematoxylin/eosin.

Cytokine Determination. *From spleens*: Spleens were homogenized in 1 ml of PBS. Following removal of a sample for determination of bacterial titer, Ciprofloxacin (100μg/ml final concentration) and protease inhibitor cocktail (Roche catalog # 11-873-580, 1 x final concentration) were added. The suspension was centrifuged at 10,000 x g for 1 min. The resulting supernatant was filtered to remove bacteria (0.2 μm pore, polyvinylidene difluoride, Pall Acrodisc). Cytokines in the resulting supernatant were measured using ELISA kits from BD Pharmingen (moIL-10) or R&D systems (moTNFa, moIL-6) according to the manufacturer's directions. IL-10 levels were determined twice, each time in triplicate from the same set of tissue samples.

ii. Cytokine determination from tissue culture: Culture medium was removed from the wells and diluted as required with fresh medium for use in the assays. IL-8 was determined with a kit from R&D Systems (huIL-8). All cytokine assays were performed in triplicate. Significance of observed differences in median cytokine concentrations were analyzed by the non-parametric Pittman exact test.

NF-κB luciferase reporter assays. Cells were transfected with the NF-κB luciferase reporter plasmid (a gift of Katherine Fitzgerald, University of Massachusetts Medical School) using Genejuice (Promega) as described by the manufacturer. Following stimulation and incubation as indicated, cells were lysed with passive lysis buffer (Promega), and luciferase activity was measured by luminometry following addition of luciferase substrate.

Results

Preparation of recombinant *Y. pestis* LcrV. The *lcrV* gene from *Yersinia pestis* strain KIM1001 was cloned into the expression vector pBADgIIIB (Invitrogen) resulting in $pLcrV_{Yp}$. (See Materials and Methods for a detailed description of the protein produced by this construct.) rLcrV was expressed and purified by nickel chelation chromatography as described in Material and Methods. This method of purification yields primarily dimeric and tetrameric rLcrV as shown by native PAGE, SDS-PAGE, Western blot, and gel filtration chromatography (see below.)

***Yersinia pestis* rLcrV and stimulation of TLR2.** Recombinant his-tagged LcrV derived from *Y. enterocolitica* ($rLcrV_{Ye}$) has been reported to activate TLR2 in a CD14-dependent manner [70]. In these reports, LcrV from *Y. enterocolitica* was cloned and expressed in *E. coli* with N-terminal 6x His tag in the vector pQE30 (Qiagen), and purified by Ni^{++}-chelation chromatography. To determine if similar rLcrV preparations derived the *Yersinia pestis lcrV* gene are also able to stimulate TLR2, we transfected HEK293 cells with TLR2 either in the presence or absence of co-transfected CD14 and stimulated them for 18 hours with rLcrV purified as described above. As a measure of TLR2-stimulating activity, cell supernatants were assayed for interleukin-8 (IL-8) by capture ELISA. As shown in Figure 1A, TLR2 is required for induction of IL-8 by rLcrV, and this induction is enhanced by, but not dependent upon, the co-expression of CD14. These observations are similar to the results reported by Sing et al. for $rLcrV_{Ye}$, except that we find enhancement, rather than a strict requirement, for CD14.

Because Sing et al. relied primarily on an NF-kB reporter construct to indicate TLR2 activation in the HEK293/TLR2/CD14 system, we also examined activation with a similar reporter construct. The results (Fig. 1C) were consistent with the observations made using IL-8 release.

***Yersinia pestis* LcrV synthetic peptides fail to stimulate TLR2.** Peptides identical to specific domains of *Y. enterocolitica* LcrV and capable of stimulating TLR2 have been previously described [70, 71]. These are derived from the N-terminal globular portion preceding alpha helix 1 in the LcrV structure [63]. A peptide comprising residues 31 to 49, designated V7, was most active. We tested the ability of the synthetic cognate

peptide based on the *Y. pestis* sequence, which differs at single residue ($K^{43}\rightarrow N$) from the homologous region in *Y enterocolitica*, to activate TLR2. This peptide has identical residues at all of the positions established by Sing et al. to be required for TLR2 activation [82]. As shown in Figure 1B, this peptide is unable to stimulate HEK293 cells stably transfected with both TLR2 and CD14. Sing et al. [82] observed only weak activity with a longer synthetic peptide comprising residues 31 to 66 of *Y. pestis* LcrV [71]. A peptide comprising residues 27 through 43, corresponding to the inactive peptide designated V5 by Sing et al. [70], was also found to be inactive.

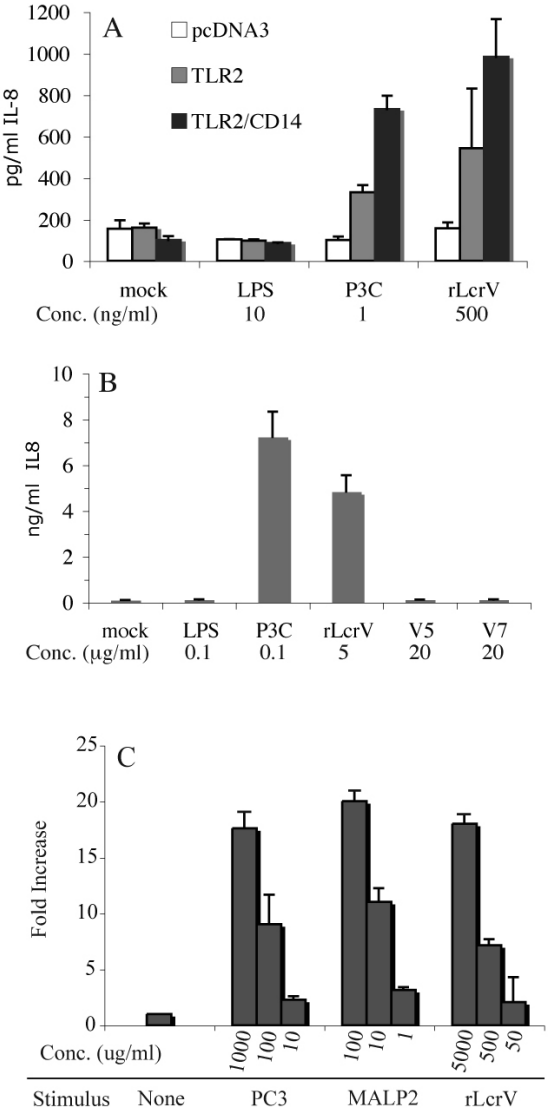

Figure 1. Activation of TLR2 by rLcrV. (A) HEK 293 cells stably transfected with vector alone (pcDNA3), TLR2 alone, or both TLR2 and CD14 were stimulated with 10 ng/ml LPS, 1 ng/ml P3C or 500 ng/ml rLcrV as indicated for 18 h. Supernatants were assayed by capture ELISA for the presence of IL-8 as an indicator of TLR2 activation. (B) HEK 293 cells stably transfected with both TLR2 and CD14 were stimulated with 100 ng/ml LPS, 100 ng/ml P3C, 5 μg/ml rLcrV or 20 μg/ml of the indicated synthetic peptide for 18 h. Supernatants were assayed by capture ELISA for the presence of IL-8 as an indicator of TLR2 activation. (C) The same cells as for panel B were transfected with an NF-κB luciferase reporter and stimulated with P3C, MALP2, rLcrV at the indicated concentrations for 18 h. Relative luciferase activity is shown. Data shown are means from triplicate assays with error bars indicating ranges and are representative of at least three experiments.

Multimers of rLcrV in purified preparations. It has been previously observed that in solution, rLcrV is not present in significant amounts in monomer form, but instead exists primarily as a mixture of dimers and tetramers [83]. Dimers can form through disulfide bonds between the single cysteine residue (C273) in each rLcrV molecule [63]. To determine the form(s) present in our rLcrV preparation, we analyzed samples by electrophoresis, gel filtration, and immunoblotting.

As shown in Figure 2, the major band of rLcrV seen by PAGE under native conditions migrates at a rate expected of rLcrV tetramer, and also contains higher molecular weight forms. The addition of reducing agent results in migration consistent with dimer. Under combined reducing and denaturing conditions, migration is consistent with monomer. Analysis by gel filtration on a calibrated Superose 12 column yields similar results (Figure 3). In the absence of reducing agent, elution time of the two primary peaks is consistent with tetramer and dimer. In the presence of glutathione, dimer forms the primary peak and the tetramer peak is dramatically reduced (Figure 3A, Inset). In some preparations, a small peak consistent with monomer was observed under reducing conditions, but in no case did we detect monomer without addition of glutathione to the elution buffer (data not shown). We conclude that disulfide bond formation in necessary for the existence of stable tetramers, but not for stable dimers.

Both native PAGE and gel filtration also revealed high molecular weight species (>200 kDal). These eluted near the void volume of the column during gel filtration. The integrated absorbance of this early peak was reduced only slightly in the presence of glutathione, indicating that its integrity is not dependent on disulfide bonds (Figure 3A, Inset). The presence of rLcrV in these high molecular weight fractions was confirmed by

immunoblotting (Figure 3B, Inset). Silver-stained SDS-PAGE gels showed only a single band consistent with rLcrV in these fractions (data not shown), indicating that rLcrV is the major protein component of this material.

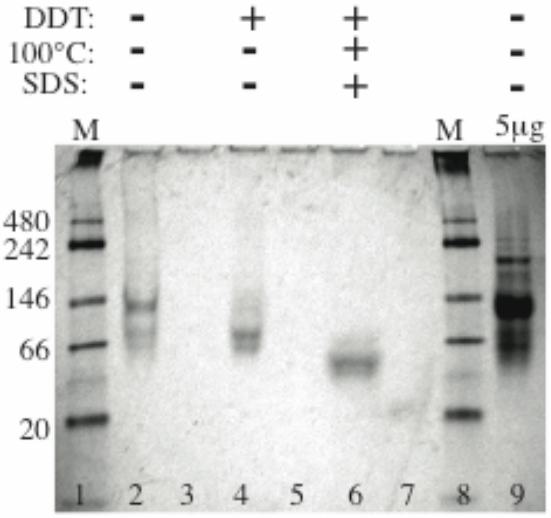

Figure 2. Analysis of rLcrV by PAGE. Lanes 1 and 8 contain molecular weight markers formulated for use in native gels, with their sizes in kDal indicate at left. In the absence of SDS, DTT, and thermal denaturation, rLcrV migrates at a rate consistent with tetramer, with some dimer also present (lane 2). Addition of DTT results in migration consistent with dimer (lane 4). The combination of DTT and denaturing conditions results in migration consistent with monomer (lane 6). Lanes containing rLcrV were separated by an empty lane, as diffusion of DTT into adjacent lanes affected migration. High molecular weight species greater than 200 kD are visible under native conditions when the gel is loaded with 5µg rLcrV (lane 9). The bands are less distinct than usual for SDS-PAGE because the buffers do not contain SDS, allowing some loss of detergent from the protein as it migrates.

The high molecular weight species containing rLcrV is responsible for TLR2-stimulating activity. To determine which form(s) of rLcrV contain TLR2stimulating activity, fractions from rLcrV preparations separated on Superose 12 were assayed for TLR2 stimulating activity (Figure 3). HEK293 cells stably transfected with TLR2 and CD14 were treated with a sample of each fraction, and IL-8 was measured as an indicator of TLR2 activation. Surprisingly, neither the dimer nor tetramer forms were able to stimulate TLR2. Virtually all of the TLR2-stimulating activity was associated with the higher molecular weight fractions, which contained less than 10% of the total rLcrV protein. The pattern of activity among fractions was remarkably consistent when

independent preparations were compared, even when different cell disruption techniques (sonication versus French pressure cell) that yielded different proportion of dimer and tetramer, but a consistent proportion of high molecular weight material, were used (compare Figures 3A and B).

Taken together, these results indicate that the TLR2 stimulating activity of our rLcrV resides in stable multimers or aggregates substantially larger than tetramer. rLcrV is the major, and perhaps the only significant, protein component of this material. However, the presence of non-protein components, or highly active protein components in low abundance, cannot be excluded. It is also possible that the tetramer and lower-molecular weight forms have some stimulatory activity obscured by the tailing peaks of the higher-activity fractions, but if so, this activity must be very weak.

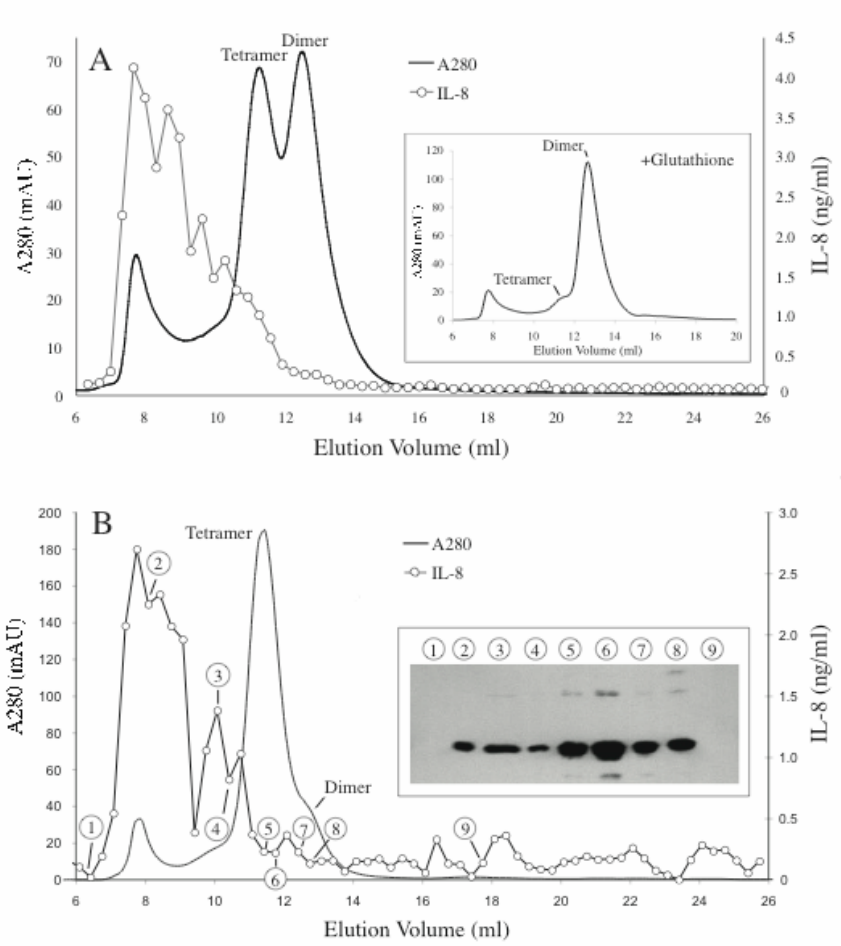

Figure 3. Analysis of rLcrV by gel filtration. (A). rLcrV purified from sonic extracts was separated on a calibrated Superose 12 column. Elution profile ($A_{280,}$ solid line) of the primary peaks indicates that rLcrV dimer and tetramer are the major species present. A high molecular peak, eluting near the void volume of the column, is also present. The profile obtained when 1 mM glutathione is included in the column buffer is shown in the inset. Note conversion of the tetramer peak to dimer, but retention of the high molecular weight fraction. Samples of fractions were also assayed for TLR2 stimulating activity (○) as in Figure 1. Note that this activity is associated almost exclusively with material eluting prior to rLcrV tetramer. (B). An independent preparation of rLcrV purified from extracts made via a French pressure cell was analyzed as in panel A. This preparation contained a much higher proportion of tetramer, and a similar proportion of high molecular weight material. Assay of fractions for TLR2 activating activity (○) again show the material eluting before tetramer to be most active. The inset shows an immunoblot of selected fractions prepared from a standard SDS-PAGE gel and probed with an LcrV-specific monoclonal antibody. The selected fractions are indicated by circled numerals. Note that the high molecular weight fractions contain rLcrV.

Analysis of an alternative rLcrV construct. Virtually all studies of the immunosuppressive properties of LcrV are based on recombinant constructs, with somewhat different structures. For example, the parent rLcrV fusion protein utilized by Overheim et al. [72] differs from ours in two significant ways: it has an amino terminal decahistidyl domain as opposed to the hexahistidyl carboxy-terminal domain in our construct, and includes the native *Y. pestis* LcrV sequence with no other additions. In contrast, our mature construct has three additional amino-terminal resides (*tmv*) that precede the native LcrV domain, and two additional carboxy-terminal residues (*vd*) which precede the added polyhistidine sequence. To determine if our finding that TLR2 stimulation is due to high molecular weight forms was the result of such differences, we purified and analyzed rLcrV produced by the construct of Overheim et. al. [72]. The results of these experiments are shown in Figure 4. As was found with our construct, all of the TLR2 stimulating activity of this rLcrV form was associated with fractions having much shorter elution times than dimer and tetramer. Also, in agreement with results obtained with our construct, almost all of the rLcrV is in the form of dimers and tetramers. No monomer is present. In fact, the preparation analyzed in Figure 4 contained less high molecular weight material than we had observed with our construct, and its TLR2-stimulating activity was correspondingly lower (Compare Figure 4 and Figure 3, noting the differences in scaling on the IL-8 concentration axis.) Thus, it appears that our basic observations are not sensitive to minor differences in rLcrV structure.

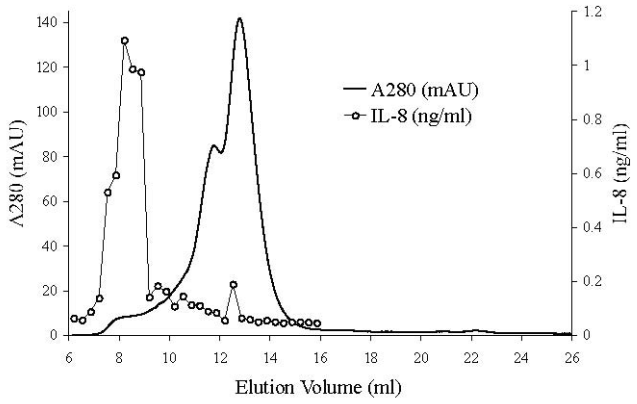

Figure 4. Analysis of an alternative rLcrV construct by gel filtration. rLcrV produced by the prLcrV construct of Overheim et al. purified as described (see Materials and Methods) was analyzed in experiments parallel to those of Figure. Note that the results are very similar, although the content of high molecular weight material is somewhat lower, as is the level of TLR2 stimulating activity of the corresponding fractions. Analysis via immunoblotting as in Figure 3 confirmed the presence of rLcrV in the active fractions (data not shown).

Infection of TLR2$^{-/-}$ mice. The interaction between *Yersinia enterocolitica* rLcrV and TLR2 is reported to result in an anti-inflammatory effect, including suppression of TNF and induction of interleukin-10 (IL-10) [69-71], leading to decreased macrophage activation. During *Y. enterocolitica* infection, this mechanism is proposed to contribute to evasion of innate immunity. To address the potential relevance of this mechanism to plague, we compared the disease produced in wild type and TLR2$^{-/-}$ mice following subcutaneous (s.c.) infection with virulent *Y. pestis*. A dose of 1000 CFU of *Y. pestis* strain KIM1001 was uniformly lethal for both C57BL/6 mice and C57BL/6 TLR2$^{-/-}$ mice, and no significant difference in mean time of survival was observed (Figure 5A). Subtle differences in resistance are more readily observed at doses that do not cause complete mortality. Accordingly, we conducted a second comparison using a dose of 100 CFU, again delivered s.c. (Figure 5B). This dose yielded 80% mortality in both wild type and

TLR2$^{-/-}$ animals. Again, no significant difference in mean time of survival between mouse genotypes was observed.

Cytokine levels and bacterial load in mice infected with *Y. pestis*. To determine if cytokine induction during infection was influenced by the TLR2 status of infected mice, we measured the levels of IL-10, TNF, and IL-6 in the spleens of five mice of each genotype two days following i.v. infection with 1000 CFU of KIM1001. The number of bacteria present in the spleen at this time was also measured.

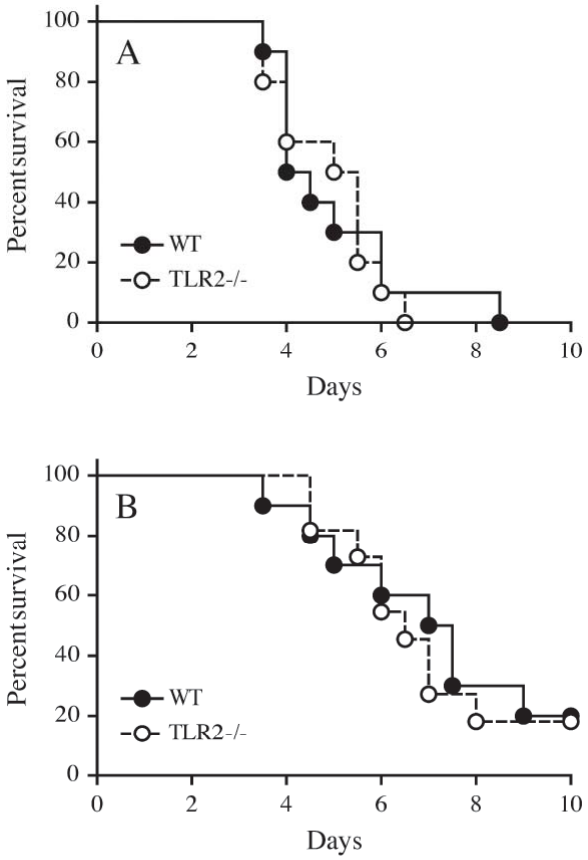

Figure 5. TLR2 deficiency and survival of *Y. pestis*-infected mice. WT (●) and TLR2$^{-/-}$ (○) mice, 10 animals per group, were infected s.c. with KIM1001. Survival was monitored for 14 days, and no deaths were observed beyond day 9. (A). Dose 1000 CFU. Note that survival time is very similar (mean: WT = 4.25, TLR2$^{-/-}$ = 5.25, p = 0.85). The results shown are representative of three experiments. (B) Dose 100 CFU. At this lower dose, survival time is also very similar (mean: WT = 7.25, TLR2$^{-/-}$ = 6.5, p = 0.75). The experiment was performed once at this dose. (Note: The TLR$^{-/-}$ group in this experiment contained 11 mice.)
f

Intravenous infection was used in this experiment because it results in essentially simultaneous infection of internal organs: with s.c. infection, the time of dissemination of the bacteria sometimes varies among the animals. We observed no significant difference in levels of either IL-10 or the pro-inflammatory cytokines TNF or IL-6 (Figure 6, panels A, B, and C). Although the difference in the level of TNF was not statistically significant, it was suggestive of a higher level in the TLR2 deficient mice. Note that this is inverse to the result predicted by the immunosuppression hypothesis. There was also no significant difference in bacterial titers within the spleens between the two genotypes (Figure 6, Panel D).

Inflammation at Foci of Infection: If TLR2-dependent stimulation of IL-10 production makes an important contribution to inhibition of local inflammation, we would expect enhanced inflammation at foci of infection in TLR2$^{-/-}$ mice. This should be readily observable in the livers of infected mice, because there is little inflammatory response to wild type *Y. pestis* in this tissue [6, 68] and any enhancement of the inflammation can be readily detected. Accordingly, we harvested livers from the mice used in the cytokine experiments described above and examined sections to determine the state of inflammation at foci of infection. As shown in Panels E and F of Figure 6, sections from both mouse genotypes show focal bacterial masses largely devoid of inflammatory cells. No evidence of enhanced inflammation in TLR2-deficient animals was observed.

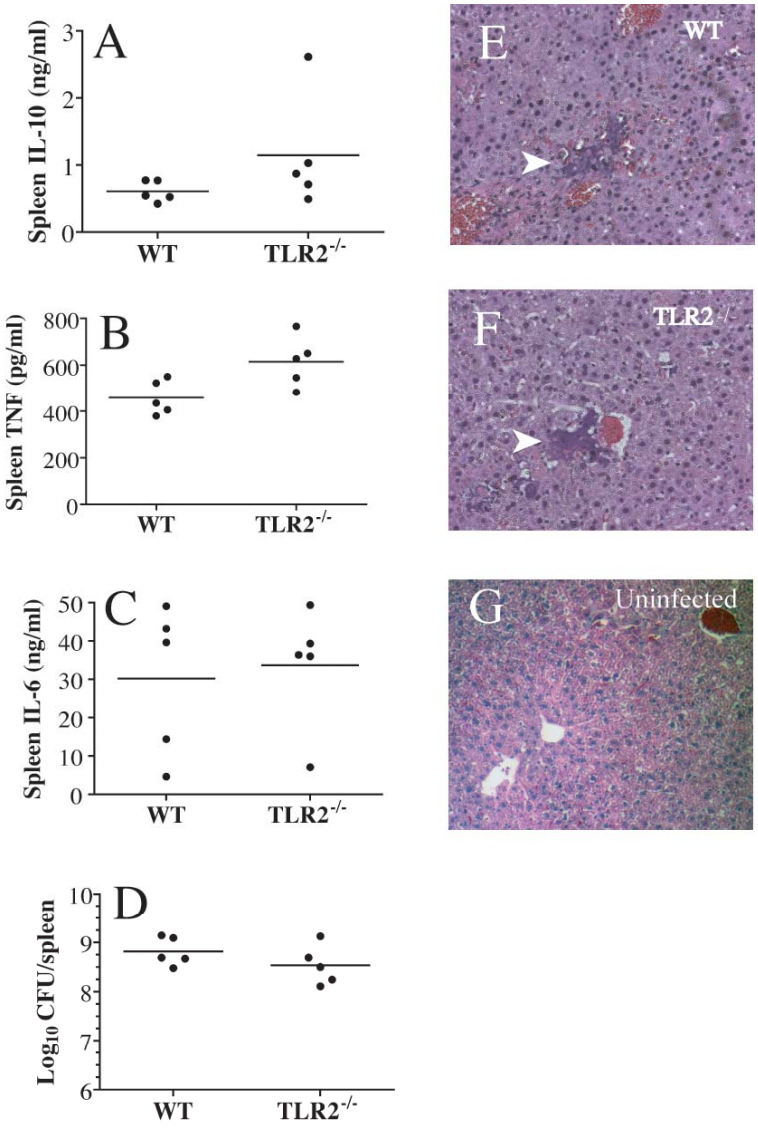

Figure 6. TLR2 deficiency has little effect during infection with *Yersinia pestis*. WT and TLR2$^{-/-}$ mice were infected i.v. with 1000 CFU KIM1001. Spleens and livers were harvested two days post-infection. Spleens were homogenized and cytokine levels were quantified by capture ELISA of IL-10 (A) TNF (B) and IL-6 (C). No significant differences were detected for any cytokine between genotypes, although the higher TNF levels in TLR2$^{-/-}$ mice were suggestive ($p = 0.056$). Bacterial loads in the spleens of the two genotypes (D) were also similar ($p = 0.2$). Liver sections from both genotypes stained with hematoxylin and eosin (E, WT; F, TLR2$^{-/-}$) showed a pattern typical of *Y. pestis* infection in wild type mice. Masses of bacteria occupy liver sinusoids with little sign of local inflammation (white arrows). For reference, (G) shows a liver section from an uninfected WT control.

Discussion

The major goal of this work was to examine the hypothesis that the interaction of LcrV and TLR2 contributes significantly to *Y. pestis* virulence, whether via the induction of IL-10 or by any other means. Our finding that TLR2-deficient mice showed no difference in their response to fully virulent *Y. pestis* as compared with wild type mice strongly suggests that this interaction plays little if any role in plague. It should be noted that the virulent *Y. pestis*/mouse infection model is a very sensitive one, in that specific genetic modifications of bacteria or mice often have large effects on virulence. For example, otherwise virulent mutants lacking the Pla protease [6, 32] and mutants with defects in iron acquisition [32] show an increase in LD_{50} of several orders of magnitude, as do strains modified to produce highly stimulatory LPS [84]. TLR4 deficient mice are highly susceptible to a strain producing stimulatory LPS, while wild type mice are highly resistant [84]. In both of these instances, reduced virulence (or enhanced resistance) was also associated with marked enhancement of inflammation at foci of infection. Thus it is clear that experimental manipulations in this system do indeed have very large effects when they are related to functional differences in the interaction between the bacteria and host defenses. Our failure to observe any indication of enhanced resistance, improved inflammatory response, or altered cytokine levels in TLR2 deficient mice must therefore be regarded as strong evidence that this receptor does not play a significant role in interactions contributing to virulence of *Y. pestis* during infection.

The literature regarding the induction of IL-10 by the LcrV of the yersiniae presents a somewhat confusing picture. Sing et al. report that specific residues are

required for induction IL-10 by LcrV of *Yersinia enterocolitica*, and that peptides containing these residues are effective inducers [70, 71]. However, they also find that a peptide from the cognate region of *Y. pestis* and *Y. pseudotuberculosis* LcrV has little activity [71]. On the other hand, Overheim et al. report that regions of LcrV entirely distinct from that defined by Sing et al. are required for IL-10 induction by *Y. pestis* LcrV. While Sing et al., provide evidence from a well-established in vivo model supporting a role for the TLR2/LcrV interaction during *Y. enterocolitica* infection [70, 71], these results are unfortunately dependent on the mouse strain employed [85]. No similar evidence has been published previously regarding *Y. pestis.* For this species, currently available in vivo data is indirect in that all the relevant experiments involve injection of mice with various forms of recombinant LcrV, followed by measurement of cytokine levels and/or challenge with LPS, attenuated *Y. pestis*, or other unrelated pathogens [68, 86].

A variety of fusion proteins have been used to demonstrate the immunosuppressive properties of LcrV. For example, Overheim et al. utilize a N-terminal decahistidyl tag [72]. Motin et al. fuse a 34 kilodalton fragment of Protein A to the N-terminus of a truncated LcrV lacking the first 67 residues [87]. The three-dimensional structure of LcrV was determined from a fusion protein containing five residues fused to an LcrV N-terminus beginning at residue 28 and a C-terminal addition of four residues plus a hexahistidyl tag [63]. This structure shows that both the N- and C-termini are very flexible and are located near each other, external to one of the LcrV globular domains. The flexibility and location of these termini is consistent with

tolerance for additions and deletions. Thus, it is unlikely that the three-residue N-terminal and the eight residue C-terminal additions -- the latter including a six residue His-tag -- present in our LcrV construct is less reflective of the properties of native LcrV than those employed by others. Moreover, we have shown directly that the rLcrV protein of Overheim et al. [72] behaves similarly to our own. It should be noted that the cytokine-inducing properties of native LcrV purified from *Y. pestis* or any other *Yersinia* species has not been studied.

Our results with recombinant His-tagged *Y. pestis* LcrV are consistent with those of others in that we do observe stimulation of TLR2 in vitro. However, we also show that the ability of this material to stimulate TLR2 is unexpectedly complex at the biochemical level. The major forms of rLcrV in our preparations, dimer and tetramer, have no TLR2 stimulating activity. Such activity is detected only in high molecular weight multimers or aggregates. Although the data from experimental infections discussed above argues strongly against a role for this activity in *Y. pestis* virulence, we consider three alternative hypotheses regarding TLR2 stimulation by LcrV, one of which implies physiological significance.

First, the stimulatory activity may result from the presence of a potent TLR2-activating contaminant (e.g. a lipoprotein, lipopeptide, peptidoglycan, etc.) constituting a small proportion of the aggregate, and not from rLcrV per se. Such contamination is both common in material purified from whole cell extracts and notoriously difficult to exclude. TLR2 stimulating activity initially ascribed to what were thought to be highly

purified materials has later been shown to result from such contamination (for example, see [79, 88, 89]. There is no general method to ensure freedom from such contaminants in protein preparations. Note that vulnerability to such contaminants is greatly increased when cells expressing a variety of Toll-like receptors, such as mouse macrophages are the targets of stimulation.

A second possibility is that the stimulatory activity is indeed due to rLcrV in the aggregates/multimers, but that this material is non-physiological and an artifact of over-expression and purification techniques. The tendency of recombinant proteins expressed at high levels in *E. coli* to form high molecular aggregates is well established. Exposure of mature rLcrV to cell extracts as occurs during purification could also be a critical to formation of the stimulatory material. In *Y. pestis* the level of LcrV expression is much lower, and exposure of mature LcrV to concentrated cell extracts does not occur. In this view, the stimulatory aggregates/multimers are purely an in vitro artifact and have no physiologic relevance.

A third possibility is that the stimulatory fractions contain structures that are, or resemble, physiological multimeric species that are normally detected by TLR2 as an indicator of pathogens with T3SS machinery. A study by Broz et al. demonstrated by scanning transmission electron microscopy (STEM) and quantitative immunoblotting that three to five monomers of LcrV form each tip complex of a needle {Broz, 2007 #240}. Perhaps such structures are occasionally released from bacteria in vivo and elicit pro-inflammatory responses via TLR2. However, there is no evidence to support this idea

from infection experiments with yersiniae. Also, PcrV, a related protein from *Pseudomonas aeruginosa*, does not stimulate TLR2 [70].

The association of TLR2 stimulation with aggregates/mutimers of rLcrV suggests that individual rLcrV molecules may interact weakly with TLR2 and that activation results from clustering of the receptor {Triantafilou, 2004 #241}. This would explain the failure of low molecular weight forms to cause activation. Other groups have not reported the molecular size of the active fraction in their recombinant LcrV preparations under non-denaturing conditions. Consequently, the presence of a similar stimulatory high molecular weight aggregate in their experiments cannot be excluded. The specific activity of our preparations is similar to that reported by others: if the activity they observe is not due to aggregates, which we find to constitute a small fraction of total protein, then the activity of rLcrV in their preparations on a per molecule basis must be correspondingly low. The clustering hypothesis suggests that rather than disrupting a specific TLR2/LcrV interaction, mutations which give rise to inactive recombinant LcrV preparations may interfere with formation of aggregates capable of activating TLR2.

Overheim et al. (22) have presented data suggesting that LcrV deletion mutants failing to stimulate IL-10 production are more effective immunogens, presumably due to the elimination of immunosuppressive activity. Our results show that highly purified LcrV preparations containing only dimer and/or tetramer lack TLR2 stimulating activity and hence may also have improved performance in vaccine applications. However, we have previously shown that, with DNA vaccines, production of LcrV multimers in vivo

was critical to providing an effective protective response, and also biased the response toward T_H1 as compared with the response produce by constructs producing only rLcrV monomer [46]. While we do not know the extent of multimerization occurring in vivo, the association of multimerization with TLR2 stimulation suggests the possibility that large multimers form in vivo and provide adjuvant activity in the context of the live vaccine through stimulation of TLR2, rather than immunosuppression. This adjuvant effect is more consistent with current understanding of TLR2, function than is an immunosuppressive effect.

In summary, our investigation provides no support for the hypothesis that activation of TLR2 by LcrV contributes to the virulence of *Y. pestis* via immunomodulation. In a sensitive infection model using virulent *Y. pestis*, elimination of TLR2 has no effect on the course of disease, or on cytokine levels observed in vivo. The bulk of recombinant LcrV protein has no TLR2 stimulating activity in vitro, and such activity is restricted to high molecular weight aggregates/multimers that contain LcrV but are of undetermined composition. Given the well-established sensitivity of the *Y. pestis* mouse infection model, its lack of response to TLR2 deficiency must be regarded as strong evidence that TLR2-induced immunomodulation does not have a significant role in plague. The early observations suggesting a direct immunosuppressive role for LcrV were based on direct injection of recombinant LcrV preparations into mice resulting in immunosuppression and elevated levels of IL-10 [86]. A TLR2-independent mechanism of IL-10 induction would be consistent with these early observations. However, the levels of IL-10 we observed in infected mice are modest (~1 ng/g of spleen, 1/10 the

level associated with induction by Sing et al.. [71] and hence not consistent with this hypothesis.

Detailed infection experiments have also been conducted using strains of *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* by Victoria Auerbuch et al. They also observed no differences in the course or pattern of disease, or in cytokine levels, between TLR2-sufficient and deficient mice. The conflicting results of Sing et al.. imply that LcrV/TLR2-mediated immunosuppression may operate under certain limited circumstances (i.e. with specific combinations of *Y. enterocolitica* strains and mouse strains), but, given the present weight of evidence, it is unlikely to be a phenomenon of general importance to virulence in the yersiniae.

Acknowledgements

This chapter was adapted from *Pouliot K., Pan N., Wang S., Lu S., Lien E., and Goguen J.D. (2007) Evaluation of the role of LcrV/TLR2-mediated immunomodulation in the virulence of Yersinia pestis. Infect Immun* ***75*** *3571-3580.* I would like to thank Nancy Deitemeyer and Chrono Lee for general technical assistance, and Neal Silverman and Eicke Latz for advice regarding purification of protein with minimal contamination by TLR-activating substances, as well as Olaf Schneewind for use of his rLcrV construct.

Addendum

Subsequent to the acceptance of this manuscript, additional data related to this work has been published. A study by Reithmeier-Rost et al. demonstrate that preparation of an untagged rLcrV from the *Y. enterocolitica* strain O:8 was able to stimulate mouse bone marrow derived macrophages (BMDM) significantly better than an untagged rLcrV from *Yersinia pestis* [90]. They observed approximately five-fold better induction of both TNF and IL-10 by *Y. enterocolitica* rLcrV compared to *Y. pestis* rLcrV. In vivo experiments show no significant difference in the survival of $TLR2^{-/-}$ mice compared to wildtype after subcutaneous challenge with the virulent *Y. pestis* strain, GB. They conclude that the low stimulatory activity of *Y. pestis* rLcrV compared to *Y. enterocolitica* they observe explains the survival of TLR2-/- mice, results consistent with our own. Binding studies by Abramov et al. show that LcrV peptides derived from *Y. pestis* are not only able to interact with human TLR2 but also with IFN-γ receptor [91]. Their data indicate that LcrV peptides comprising $LEEL_{32\text{-}35}$ and $DEEI_{203\text{-}205}$ are able to bind free TLR2 and human IFN-γ receptor. Neither of these peptides contains the required KDKN sequence recognized by Sing et al. to bind TLR2. We conclude that this interaction, if it occurs in vivo, does not seem to be biologically relevant as two independent groups, using strains from two *Y. pestis* biotypes, have shown no difference in survival between wildtype and TLR2-/- mice.

Chapter Three.

A Brief Review of Natural and Experimental Transmission of *Y. pestis*

Bubonic and septicemic plague, resulting from transmission of *Yersinia pestis* by fleas, are the most common forms of *Y. pestis* infection. Bubonic plague is characterized by the presence of grossly enlarged lymph nodes in conjunction with systemic infection, while septicemic plague is characterized by septicemia in the absence of buboes. This difference reflects more immediate access of the bacteria to the bloodstream in the case of septicemic plague, resulting in the appearance of symptoms prior to the appearance of buboes. Of thirteen cases of plague in the United States in 2006, five patients had primary septicemic plague and the remaining eight had bubonic plague [92].

The most severe form of the disease, with a mortality rate of 100% when untreated, is *Y. pestis* pneumonia, known as pneumonic plague. Pneumonic plague may occur secondarily to bubonic or septicemic plague. When patients present with severe *Y. pestis* pneumonia and no indication of bubonic plague, the disease is classified as primary pneumonic plague. In 2005, the World Health Organization (WHO) reported 57 deaths resulting from primary pneumonic plague in a group of diamond miners in the Democratic Republic of the Congo (DRC) [93]. One of the largest recorded outbreaks of pneumonic plague occurred in Manchuria during 1910-1911 with an estimated 50,000 fatalities [94].

Coughing generates both small and large aerosolized respiratory droplets that can have a diameter of up to 100 microns. Evaporation of these small droplets results in droplet nuclei, having a diameter of only 2 to 20 microns {Wells, 1946 #238}. While inhalation of aerosols containing *Y. pestis* into the lungs is generally assumed to be the

cause of primary pneumonic plague and can certainly cause the disease, several observations suggest that such direct infection of the lungs may not be the usual mode of human-to-human transmission. First, transmission of pneumonic plague appears to occur via droplets rather than droplet nuclei, suggesting that direct penetration to the deep lung is unlikely. The infected droplets appear to be relatively large, as they are only detected within close proximity (1m) of infected patients {Wu, 1926 #107}. Secondly, *Y. pestis* readily colonizes the nasopharynx and upper airways, where large droplets are more likely to be deposited {Tang, 2006 #239}. These observations suggest to us that penetration to the lung is very likely secondary to asymptomatic or mildly symptomatic nasopharyngeal colonization.

Failure of the bacteria to reach the lung during the early stages of nasopharyngeal disease would result in progression to frank plague pharyngitis with the potential for systemic spread and the formation of cervical buboes, a rare but established form of disease. In a sense, this relationship is parallel to that of septicemic and bubonic plague to transmission via flea bite: in both cases, colonization via one route of transmission gives rise to two distinct forms of disease depending on dissemination to secondary sites. The rarity of nasopharyngeal plague suggests that penetration to the lungs following colonization is quite efficient**.**

It is also possible that nasopharyngeal colonization plays an important role in plague transmission among mammalian hosts in nature. In an attempt to mimic transmission between rodents in nature, studies by Rust et al. were able to sustain

transmission for an extended period among mice allowed to eat the carcasses of cage mates having died of plague. Rust et al. also found that all animals either fed infected carcasses or that consumed food infected with Y. pestis died within five days of consumption [59]. However, none of the ten rats challenged with infectious material by direct intubation of the stomach succumbed to plague. They conclude that all animals succumbed to terminal bacteremia and observed significant pathology in either the bronchial or mediastinal lymph nodes. In addition, they suggest that bacteria gained entrance through the pharyngeal mucosa [59].

The majority of work on progression and kinetics of plague has focused on the bubonic and septicemic forms of the disease. Intradermal inoculation of small numbers of wildtype *Y. pestis* results in a rapidly spreading systemic infection. Studies demonstrate that the absence of the *pgm* locus, absence of the type three secretion system in toto or many of its individual components, or the absence of the Pla protease results in a significant decrease in virulence [15]. For example, the loss of Pla causes up to a million-fold increase in the LD_{50} for subcutaneous infection, but no difference in virulence is observed when mice are infected intravenously [6]. *Y. pestis* also causes systemic disease when infection occurs by other routes. Hematogenous dissemination of *Y. pestis* from the lungs in pneumonic plague is well established. Less attention has been given to septicemia following oropharyngeal infection, although the literature contains sufficient data that this does occur. Although many researchers recognize the ability of *Y. pestis* to disseminate from the oral mucosa to the bloodstream, the mechanism by which

this occurs is not understood [59, 95]. Most in vivo studies of pneumonic plague have focused on protective vaccines or antibiotic therapies [47-49, 96-98].

In a study by Davis et al., all twenty-one African Green monkeys infected with *Y. pestis* via aerosol succumbed to primary pneumonic plague within ten days [42]. In all cases, examination of the lungs revealed multilobar pneumonia, pleural effusion, and fluid in the airways, similar to that observed in human pneumonic plague. Interestingly, bacteria and inflammation observed in the larynx and tracheal submucosa was associated with the mucosa-associated lymphoid tissue (MALT). The authors speculate that this may be the site where bacteria enter the bloodstream, and argue that further investigation of the mucosa-associated lymphoid tissue as a site for bacterial entry is warranted.

Aerosol or intranasal infection of mice results in a similar pathology; intranasal infection of BALB/c mice with 5×10^3 wildtype organisms resulted in death of all animals between days 3 and 5. Monitoring of peripheral organs during the experiment reveals the bacterial burden reaching 10^6 in the spleen and liver by day two, and 10^8 organisms in the lung. Little inflammation was noted in the lung until after bacteria had disseminated to peripheral sites. The authors note that the presence of bacilli in macrophages may indicate a vehicle by which they travel [99].

Butler et al. observed that six hours after ingestion, bacteria were recovered from the spleens of rodents, but not from the Peyer's patches or mesenteric lymph nodes (MLN) [95]. No pathology was observed in the lungs of any rodents, suggesting that

bacteria were not aspirated during oral challenge causing primary pneumonic plague. Previous studies have shown that reducing the normal intestinal flora with antibiotic treatment or reducing stomach acidity with bicarbonate increases the susceptibility of animals to enteric infection by other pathogens [100 {Knop, 1975 #228, 101]. In this study, there was no difference in *Y. pestis* infectivity observed between control animals and animals either intragastrically-administered streptomycin or sodium bicarbonate, suggesting to the authors that bacteria are rapidly absorbed in the gut or that *Y. pestis* cannot survive in an environment changed by the reduced number of normal intestinal flora. Interestingly, unlike the conclusions of Rust et al., the authors never suggest any involvement of the naso-pharyngeal area as a portal of entry for bacteria. An alternative and perhaps simpler interpretation of these results is that bacteria responsible for systemic infection never reach the stomach; instead they penetrate mucosal barriers in the upper alimentary tract.

These studies as well as cases of pharyngeal plague in humans suggest *Y. pestis* is able to traverse epithelial barriers within the lung and/or upper airways, however, a mechanism whereby bacteria cross these barriers has not been conclusively demonstrated *in vivo*. There are several possible mechanisms where traversal of the epithelial barrier could occur: direct destruction of the epithelia by type three secreted effectors, damage caused by inflammation or the direct invasion of epithelial cells followed by penetration across the epithelial layer. In Chapter Four of this work, we investigated the biological relevance of invasion during pneumonic transmission; and in Chapter Five we addressed virulence factors and their regulation involved in this process.

Chapter Four.

Enhanced Invasion by *Yersinia pestis* at Atmospheric Oxygen Levels

Introduction

Colonization of epithelium by bacterial pathogens often involves adhesion to or invasion of epithelial cells, and it has been known for some time that *Y. pestis* has both these abilities [41, 60]. However, adhesion and invasion have never been convincingly connected with *Y. pestis* virulence, and in vitro studies have suggested that such a role is unlikely because an abundant surface protein, known as F1 (or Caf1), interferes with these functions through inhibition of cell-cell contact [41].

F1 has been historically described as a capsular antigen because it forms a substantial layer around the bacteria, but its structure and properties differ greatly from classical polysaccharide capsules. The F1 protein is a typical chaperone-usher pilin which self-assembles to form pili-like structures of varying length only loosely associated with the bacteria surface. The caf operon, which encodes the pilin, usher, and chaperone, is strongly regulated by temperature, although the mechanism is not known. It is not expressed at 30°C, but strongly expressed at 37°C. Because expression of F1 has been shown to block both adhesion and invasion *in vitro*, only caf mutants of *Y. pestis* have been shown to be invasive to mammalian cells in culture when grown at 37°C in liquid medium [41]

Growth conditions for *Y. pestis* used in mouse infection experiments vary widely among investigators. To avoid the risk of aerosol generation when preparing inocula for infection studies, we typically grow the bacteria on solid media under ambient oxygen

conditions. We generally incubate at 26°C, similar to the temperature within fleas, for subcutaneous infection studies, and at 37°C for pulmonary infection studies. Infection experiments conducted by investigators at United States Army Research Institute for Infectious Disease (USAMRIID) use fully virulent *Y. pestis* strains grown on agar slants for s.c. experiments, but bacteria grown in shaking liquid culture for aerosol infection, always grown at 30°C [42]. Moreover, almost all *in vitro* studies of the interaction *Y. pestis* with mammalian cells, including our own, have been conducted with attenuated *Y. pestis* strains grown in shaking liquid cultures [41, 60] To determine if these differences in growth conditions might affect behavior of the bacteria, we conducted parallel in vitro interaction experiments with mammalian cells and *Y. pestis* grown on both solid media and in aerated liquid cultures. Surprisingly, bacteria grown on solid medium in a standard air incubator at 37°C proved to be much more invasive than those grown in shaking liquid cultures when incubated at 37°C.

Because this observation has potential implications for both *in vitro* studies of *Y. pestis*/mammalian cell interactions and for behavior of the bacteria in the upper airways where oxygen concentrations at the epithelial surface are high, we investigated the basis of this effect and found that atmospheric oxygen levels suppress F1 production.

We also examined the influence of growth at atmospheric oxygen levels on disease following upper respiratory inoculation of mice. Contrary to our hypothesis based on these in vitro observations, preliminary results of in vivo experiments clearly indicate that bacteria grown under microaerophilic conditions, which induce F1

production, are more virulent via low-volume intranasal infection than bacteria grown at atmospheric oxygen levels. Furthermore, we show that bacteria are unable to survive for an extended period of time in an intracellular environment *in vitro*.

Material and Methods

Bacterial strains and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 2. For liquid-grown *Yersinia pestis*, strains were grown in TB medium containing 10 g Bacto-tryptose, 5 g NaCl, 3 g Beef Extract (paste form, Difco Cat # 212610) per liter supplemented with 2.5 mM CaCl overnight at 30°C or 37°C where indicated. For growth of solid-grown *Yersinia pestis*, 5 x 10^6 CFU were inoculated as a thin film and grown on TB medium supplemented with 2.5 mM CaCl and 15 g per liter agar for 16 hours at 28°C or 37°C where indicated. For growth of bacteria under a reduced oxygen environment, bacteria were inoculated onto TB agar as described above and incubated in an unvented polycarbonate jar (BBL) with a CampyPak™ Plus Microaerophillic System envelope (BBL). *Escherichia coli* strains were grown in Luria-Bertani broth or on Luria-Bertani agar at 37°C. MC4100pJL272 expressing *invasin* (*inv*$^+$) and MC4100pRI203::Tn*pho*A (*inv*$^-$) were grown in LB media supplemented with 100μg/ml ampicillin (kind gift of John Leong, University of Massachusetts). β2155 was grown in LB media supplemented with 50μg/ml diaminopimelic acid, DAP (Sigma).

Cell Lines and Tissue Culture Reagents. . The type I human alveolar epithelial cell line, WI26 (American Type Tissue Collection, MD) was maintained in Minimum

Essential Medium (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen) and 10μg/ml Penicillin/Streptomycin (Invitrogen). HeLa human epithelioid cells were maintained in Dulbecco's modified Eagle's Medium (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum and 10μg/ml Penicillin/Streptomycin. All cell lines were maintained at 37°C with 5% CO_2.

Cell Invasion. Cells were seeded at 5 x 10^4 per well in 24-well tissue culture dishes (Costar) and grown overnight at 37°C with 5% CO_2. The day of the experiment, cells were washed twice with warm, serum-free RPMI 1640 (Invitrogen). For liquid grown bacteria, cell were pelleted and washed once with phosphate-buffered saline (PBS, 135 mM NaCl, 2.68 mM KCl, 10 mM $NaHPO_4$, 1.76 mM KH_2PO_4), and resuspended in PBS. For solid-grown *Y. pestis*, cells were washed off plates with a small volume of sterile water and resuspended in PBS. Bacteria were diluted into warm serum-free RPMI 1640 at a concentration of 10^6 CFU/ml. Warm medium was removed from the cells and one milliliter of the bacterial suspension was added to give a multiplicity of infection (MOI) of 10. Tissue culture dishes were then centrifuged for 10 minutes at 182 x *g* at room temperature and incubated for one hour at 37°C with 5% CO_2. Cells were washed as above and extracellular bacteria were killed by the addition of 50 μg/mL gentamycin in serum-free RPMI 1640 for one hour at 37°C with 5% CO_2. Cells were washed with RPMI and one milliliter of ice cold water was added to lyse cells. Dishes were placed on ice for 10 minutes and numbers of intracellular bacteria were determined by serial dilutions of the lysate. All experiments were done in triplicate wells in at least three independent experiments. The final inoculum was verified by CFU determination. For

cell invasion experiments extending over an hour, cells were infected and treated with gentamycin as above, were washed extensively and then incubated with serum-free RPMI for the duration of experiment to exclude the possibility of gentamycin leaking into the cell and killing intracellular bacteria. Intracellular bacteria were quantified as above.

Microscopy. Duplicate 24-well tissue culture plates (Costar) were seeded with 5 x 10^4 cells per well. To assess invasion efficiency, one set of samples was subjected to a standard gentamycin protection. For electron microscopy, WI26 cells were infected as described above, washed three times with serum-free RPMI, and then fixed by incubating overnight in 2.5% glutaldehyde in 0.05% sodium phosphate buffer pH 7.2 at 4°C. Monolayers were washed twice with 0.1M sodium phosphate buffer at 4°C and then treated with 1% osmium tetroxide in sodium phosphate buffer for one hour at room temperature. Osmium was washed from the cells with two washes of sterile water for 20 minutes at 4°C. Cells were dehydrated with a series of increasing ethanol concentrations from 10% to 100%. Propylene oxide (100%) was added and then cells were infiltrated with Spurr's resin mixture overnight at room temperature. The next day, fresh resin was added three times for one hour. Samples were placed in an oven and allowed to polymerize for 48 hrs at 60°C.

Construction of *Yersinia pestis* mutants. The Δcaf1 mutant was constructed by allelic exchange as described in Edwards et al. [102]. Two DNA fragments of ca. 1-kb flanking the *caf1* locus were amplified from KIM6 by colony PCR using the primer pair 5" ATC GAT GGT ACC CTT CCA GTA TCA GTG GGT TC 3' and 5' TGC TGC TGC TGC

TGC GTA ACC GTA TCT AAC CAA TAA 3' for the upstream fragment and the primer pair 5' GCA GCA GCA GCA GCA TGG CGA TAA CGG AAC TGA T 3' and 5' ATC GAT GAG CTC CGT GAC AGT AGG AGC ACA ACT T 3' for the downstream fragment. These two fragments were joined by PCR to produce a 2-kb amplicon with a central unique restriction site, MluI. The flanking and restriction sites were used to clone the resulting amplicon into the corresponding sites of pRE107 (generous gift of D. Schifferli, University of Pennsylvania) to generate pK101. To delete *caf1* from *Y. pestis*, pK101 was electroporated into β2155 (50μl, 10^9 CFU) cells with 2000 volts using an *E. coli* TransPorator (BTX). Cells were allowed to recover for 2 hours in LB broth supplemented with 50μg/ml DAP at 37°C. Bacteria were plated onto LB agar supplemented with 50μg/ml DAP and 50μg/ml ampicillin to select for transformants. For matings, β2155 pK101 and *Y. pestis* cells were mixed at a high density and were grown on TB agar supplemented with 50μg/ml DAP overnight at 30°C. To select for *Y. pestis* clones with integrated plasmids, dilutions of the mating mixture were plated on TB with 50μg/ml ampicillin and grown overnight at 30°C. Isolates were selected for sucrose resistance on TB supplemented with 5% sucrose and screened for ampicillin sensitivity to confirm plasmid loss. The caf1 deletion mutant was confirmed by PCR and F1-specific immunoblotting.

Two additional strains were also constructed for potential use as readily distinguishable competitors in infection experiments. KIM1001ΔxylB was created by allelic exchange using plasmid, pGK01ΔxylB (generous gift of B. Akerley), using methods described above. This mutation was confirmed by growth on MacConkey agar (Difco)

supplemented with 1% xylose. For creation of nalidixic acid resistant *Y. pestis*, a high density of KIM1001 were grown on ten TB agar plates containing 50 μg/ml nalidixic acid. Resistant clones were confirmed by streaking single colonies onto TB supplemented with containing 50 μg/ml nalidixic acid.

Antibodies. Mouse anti-F1 monoclonal antibodies (clone VA18) were purchased from Meridian Life Sciences (formally Biodesign). Polyclonal anti-Pla antibodies were produced by injecting rabbits with *E. coli* over-expressing Pla and purified by ammonium sulfate precipitation followed by adsorption to a Pla-deficient *Y. pestis* strain. Alkaline phosphatase conjugated anti-mouse and anti-rabbit IgG were purchased from Sigma.

SDS-PAGE and Immunolotting. Total protein extracts from 5 x 10^6 *Y. pestis* cells were run on SDS-PAGE (10% acrylamide) (Biorad) at 100V for 1 hour. For immunoblotting, proteins were transferred to a PVDF membrane (Millipore) for 1 hour at 100V. Membranes were blocked overnight in 5% nonfat milk in PBS (PBSM) at 4°C. Membranes were incubated with primary antibody diluted in 5% PBSM at room temperature with agitation for two hours. After three washes for 5 minutes each in PBS/0.1% Tween, membranes were incubated with secondary antibody diluted in 5% PBSM at room temperature with agitation for two hours. After three washes for 5 minutes each in PBS/0.1% Tween, membranes were washed for 20 minutes with high pH Tris buffer (100 mM Tris Buffer, 100 mM NaCl, 5 mM $MgCl_2$ pH 9.5) and developed with 0.005% 5-Bromo-4-Chloro-3-indolyl phosphate (BCIP) (Sigma) and 0.005% nitro blue tetrazolium resuspended in high pH Tris Buffer.

Mice. Female C57BL/6 (6-8 weeks of age) were purchased from Jackson Laboratory (Bar Harbor, Maine). All mice were bred under specific-pathogen-free conditions. All animal studies were approved by the Univ. of Massachusetts Medical School Institutional Animal Care and Use Committee and all relevant policies regarding animal care, biosafety, and security were followed.

Experimental Infection of Mice. To prepare inocula for experimental infection, the strains to be used were inoculated onto TB plates from suspensions stored at –70°C in TB (no agar) plus 5% glycerol, and incubated at 28°C for 24 hours. Suspensions from these plates were made in sterile water, and one hundred microliters of the suspension was spread evenly onto two agar plates of solid TB media using a sterile spreader. Bacteria were grown for 16 hours at 37°C under either atmospheric levels of oxygen or within a microaerophillic chamber as described above. Bacteria were harvested from the plate with a loop and resuspended in sterile distilled water with a vortex mixer to match a turbidity standard of OD_{600} 0.35. It was important to use low ionic strength medium for this initial suspension step to achieve good dispersion of the cells. This initial high density suspension was subsequently diluted as required using endotoxin free injection grade PBS (Cambrex). Anesthetized mice (ketamine 26.7 mg/ml and xylazine 6.7 mg/ml) 50 μl i.p. were infected intranasally as in Iyer et al. with the indicated dose of KIM1001 pBR322 Δtet in a total volume of 4 μl [103]. Two 1 μl drops were administered to each nostril. Mice were monitored for 7 days**.**

Results

Growth of bacteria in liquid at 37°C inhibits invasion

Several groups have shown that growth of *Yersinia pestis* at 37°C significantly inhibits invasion *in vitro* {Cowan, 2000 #42{Liu, 2006 #45}*}*. Mouse infection studies conducted by other groups typically use bacteria grown in liquid culture at 30°C or 37°C. We wanted to determine both quantitatively and qualitatively the invasion efficiency of *Y. pestis* grown at 30°C or 37°C. We conducted invasion experiments at both temperatures with both pPCP1-sufficient and deficient bacteria, as original observations of Cowan et al. indicated that the presence of pPCP1 enhances invasion. In order to visualize interactions between *Yersinia pestis* and cultured epithelial cells, electron microscopy images were prepared from infected HeLa and WI26 cells. Cell monolayers were inoculated with 10^6 CFU/well of each bacterial strain and incubated for one hour at 37°C. As shown in Figure 7, adherent bacteria and bacteria being engulfed by cell membrane were visualized in samples infected with either MC4100pJL272 (Panel A) or 30°C-grown KIM6 (Panel B). Virtually no bacteria were seen in MC4100pTn::*phoA* inv$^-$ infected monolayers, and significantly less adherent bacteria were visualized in 37°C-grown KIM6 (Panel C) or JG154 (Panel C, inset) infected monolayers. We were able to visualize both adherent and intracellular bacteria, in addition to interesting cell membrane projections and ruffling. In an experiment using HeLa cells, we also were able to visualize by electron microscopy intracellular and adherent *Y. pestis* as well as MC4100 pJL272 (data not shown). When we quantified numbers of bacteria from a duplicate

experiment, we found significantly less invasion by 37°C grown and pPCP1$^-$ *Y. pestis* when grown in liquid culture.

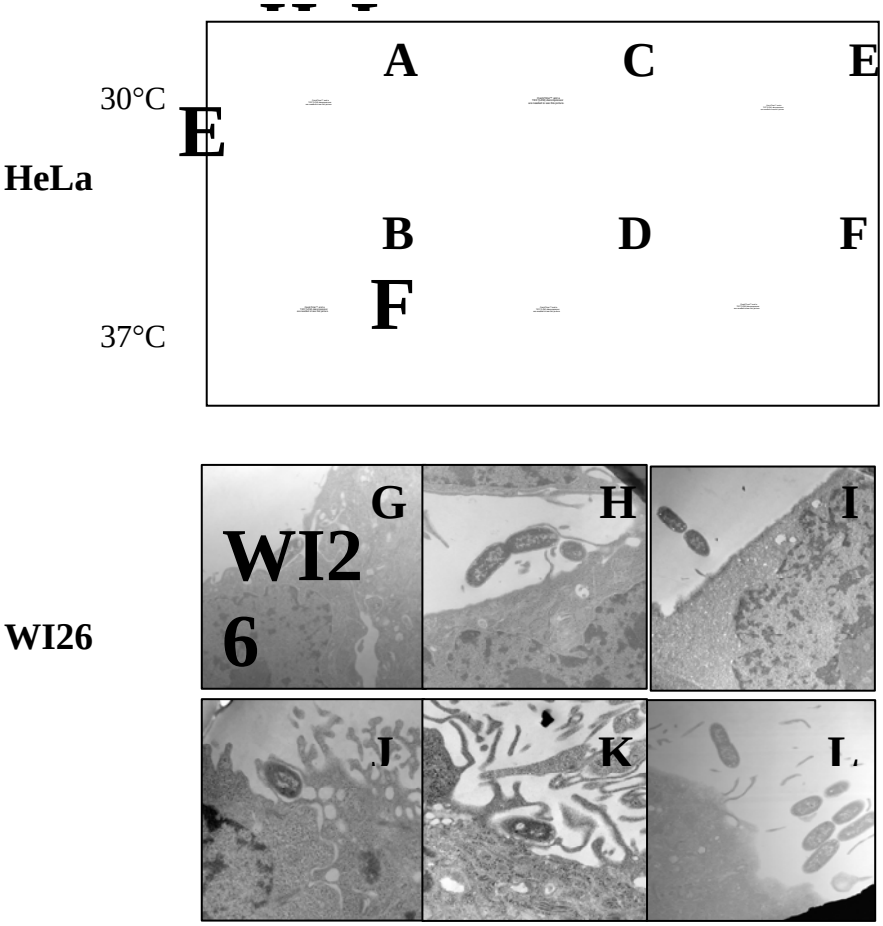

Figure 7. *Y. pestis* grown in liquid culture at 37°C inhibits invasion as shown by gentamycin protection assay and as visualized by electron microscopy (EM) . HeLa (top) and WI26 (bottom) cells were seeded in 24-well tissue culture plates at 5 x 10^4 cells per well. The next day, triplicate wells were infected for an hour with pPCP1$^+$ or with pPCP1$^-$ *Y. pestis* grown in liquid medium at 30°C (HeLa, WI26) or 37°C (WI26). To determine the assay efficiency, triplicate wells were infected with MC4100pJL272 (G, J) or with the vector control (not shown). After extensive washing, monolayers were incubated at 37°C with 50 μg/ml gentamycin for an hour. For visualization by EM, monolayers were prepared as described in the Materials and Methods. Adherent and internalized *Y. pestis* was observed in both HeLa (A-F) and WI26 cells (H, K). Significantly less adherent 37°C-grown bacteria were observed compared to 30°C-grown bacteria (compare H and K to I). Consistent with results by Cowan et al., the presence of pPCP1 enhanced invasion (compare H and K to L).

Enhanced Invasion of WI26 cells by *Yersinia pestis* grown under conditions of high O_2 availability

When preparing inocula for animal infection experiments, we typically grow the bacteria on solid TB medium in Petri dishes under ambient atmospheric conditions at 37°C. We asked whether bacteria grown in this way were similarly invasive compared with bacteria grown in aerated liquid cultures at 37°C. Growth rate experiments were first conducted to determine the time to log phase growth of solid-grown bacteria. Bacteria were grown as a thin film on solid TB media and 0.785 cm^2 samples were extracted at regular intervals and total bacteria was determined by dilution and plating. We established log phase growth of a inoculum of 5 x 10^6 colony forming units (CFU) on solid media to persist ca. 16 hours for both 30°C and 37°C grown *Y. pestis*.

We compared the invasive capacity of liquid- versus solid-grown *Y. pestis* at 37°C. We were surprised to discover that bacteria grown on solid media at 37°C exposed to an air interface retained significantly greater invasive capacity in the human lung-derived epithelial cell line, WI26, as quantified by gentamycin protection assay than those grown in liquid culture at the same temperature (Figure 8). We hypothesized that bacteria that were exposed to atmospheric levels of oxygen on solid media were responsible for the difference in invasive capacity that we observed. To address this question we utilized a microaerophillic chamber, lowering the available oxygen to approximately half atmospheric levels. Bacteria were grown on solid media at 37°C exposed to either an air interface or were incubated within the microaerophilic chamber.

As shown by Figure 9, invasive capacity was subsequently lost when bacteria were grown in the microaerophillic chamber.

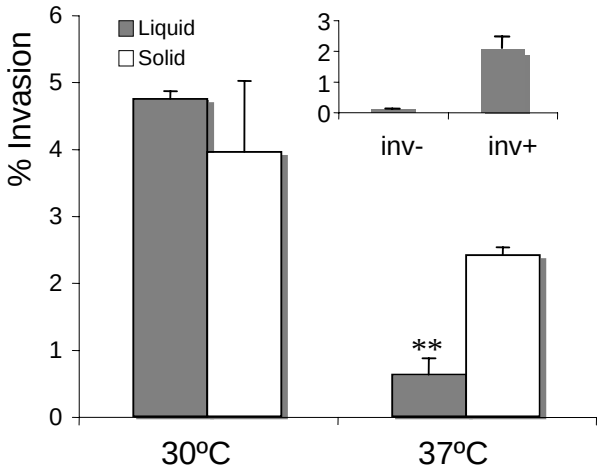

Figure 8. Enhanced invasion of WI26 cells by *Y. pestis* grown at an air interface. KIM6 (*pgm*$^-$ pCD1$^-$) was grown at 30°C or at 37°C in TB in a culture tube that was incubated for 16 h on a standard roller drum (liquid); or as a thin film on tryptic blood agar under atmospheric oxygen conditions (solid). Bacteria were washed with PBS and resuspended in serum-free RPMI at an MOI of 10 and were centrifuged onto a monolayer of WI26 cells and incubated for an hour at 37°C with 5% CO_2. After three washes with serum free RPMI, gentamycin (50μl/ml) was added for an hour at 37°C with 5% CO_2. WI26 cells were lysed with 1 ml ice cold water and intracellular bacteria were quantified by dilution plating on TB. Assays were conducted in triplicate wells in at least three separate experiments. MC4100 pJL272 (inv$^+$) and MC4100 pRI203::Tn*ph*oA (inv$^-$) are included as controls and are used in subsequent gentamycin protection assays to determine assay efficiency (inset). ** $p < 0.005$ (solid vs. liquid at 37°C).

To confirm that retention of invasive capacity during growth at 37°C was dependent on oxygen availability, not on an increase in carbon dioxide production, we also conducted gentamycin protection assays with bacteria grown in liquid cultures under a gradient of aeration regimes achieved by various volumes of medium in shaken culture flasks of identical size. This method has previously been utilized to show differential expression of virulence factors in *Haemophilus influenzae*.

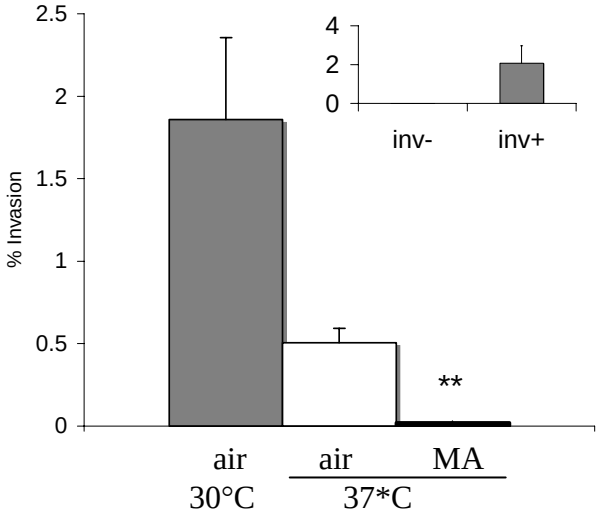

Figure 9. Low oxygen conditions inhibit high efficiency invasion. KIM6 (*pgm*$^-$ pCD1$^-$) was grown at 30°C or at 37°C as a thin film on TB agar under atmospheric oxygen conditions (air) or within a microaerophillic (MA) chamber. A standard gentamycin protection assay was conducted using WI26 cells as in Figure 8 with samples of *Y. pestis* grown under conditions described above. Assays were conducted in triplicate wells in at least three separate experiments. MC4100 pJL272 (inv$^+$) and MC4100 pRI203:: Tn*pho*A (inv$^-$) (inset). **p < 0.005.

As shown in Figure 10, high invasive capacity was attained only under the most vigorous aeration condition, in which the medium occupied 2% of the culture flask capacity. This result was independent on the presence of pPCP1 and is consistent with a requirement for very high oxygen levels for development of invasive capacity during 37°C growth.

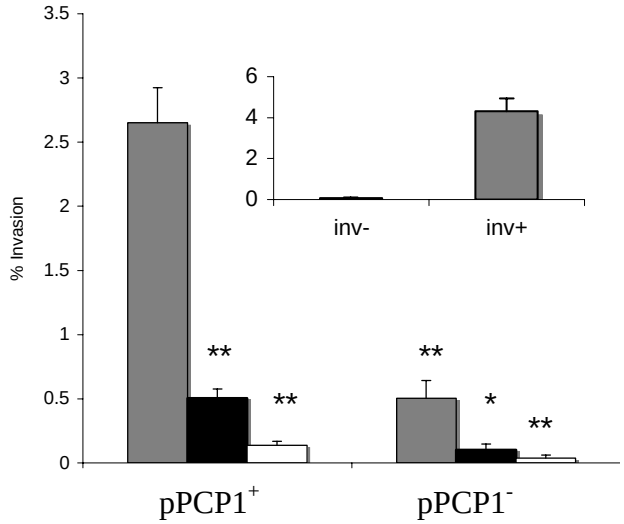

Figure 10. Requirement for high oxygen levels for efficient invasion during 37°C growth in liquid culture. KIM5 (*pgm*$^-$) and JG153 ((*pgm*$^-$ pPCP1$^-$) were incubated in 500 mL shaking flasks (250 rpm) in 10 mL, 60 mL or 200 mL volumes of trypic blood agar base without agar to log phase. Samples of bacteria were used for a standard gentamycin protection assay as described in Figure 7. Similar results were obtained with pCD1$^-$ bacteria. Assays were conducted in triplicate wells in at least three separate experiments. MC4100 pJL272 (inv$^+$) and MC4100 pRI203:: Tn*pho*A (inv$^-$) (inset). p < 0.005.

Because expression of F1 is known to interfere with invasion, we speculated that the difference we observed may be due to repression of F1 production under high-oxygen-availability conditions. Samples of bacteria from each growth condition were run on a denaturing SDS-PAGE and probed with monoclonal F1-specific antibody, or with polyclonal Pla-specific antibody as a loading control. As shown in Figure 11, F1 expression was significantly decreased when bacteria were grown on solid media in the presence of oxygen compared to bacteria grown on solid media within the microaerophlllic chamber (compare lanes 5 and 6).

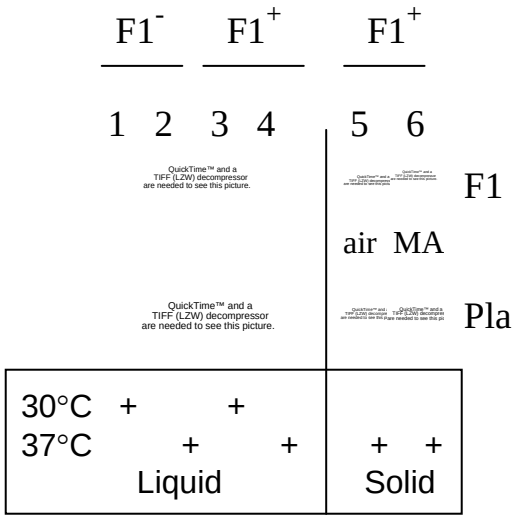

Figure 11. **Detection of F1.** Samples of bacteria from each growth condition as in Figure 8 were separated on SDS-PAGE PAGE and then transferred to a PDVF membrane and probed with F1-specific monoclonal antibody (Meridian Life Sciences). As a loading control, a duplicate membrane was probed with anti-Pla polyclonal antibody.

An F1 deletion mutant of KIM6 retains invasive ability at 37°C

If reduced F1 expression is responsible for the enhanced invasive activity of bacteria grown with high oxygen availability at 37°C, the invasive capacity of an F1-deletion mutant should be independent of oxygen availability or temperature during growth. We constructed an unmarked F1-deficient strain of *Y. pestis* by allelic exchange

and tested its invasive ability compared to the parental strain, KIM6. KIM6 and KIM6 (Δcaf1) were grown under relevant temperature and oxygen regimes at 37°C, and samples of bacteria were assayed for invasive ability with WI26 cells. As shown in Figure 12, neither oxygen availability nor temperature significantly affected the invasive ability of KIM6 (Δcaf1), but has significant effects on KIM6.

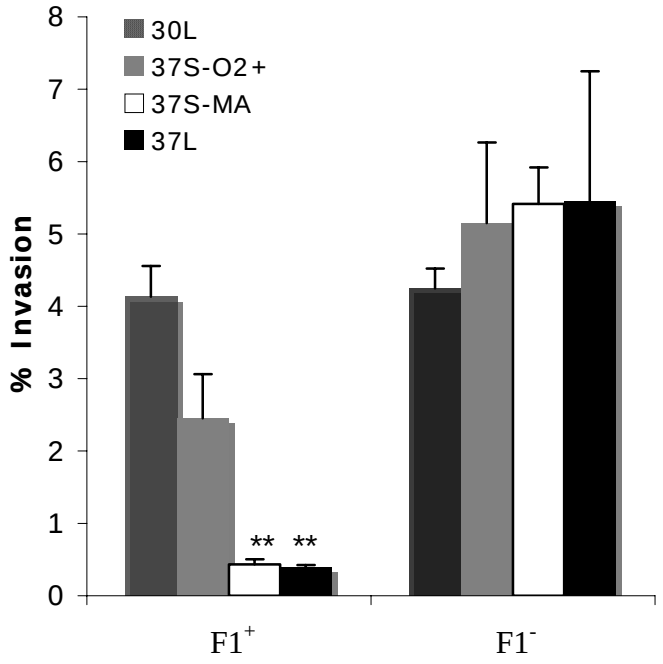

Figure 12. An F1 deletion mutant retains invasive ability at 37°C independent of oxygen availability. KIM6 F1$^+$ (*pgm*$^-$ pCD1$^-$) and KIM6 F1$^-$ (*pgm*$^-$ Δ *caf1* pCD1$^-$) were grown to log phase at 30°C or 37°C in liquid (L) or on solid media (S) under atmospheric oxygen levels (O_2^+) or in a microaerophillic chamber (MA). A standard gentamycin protection assay was conducted using WI26 cells as in Figure 8 with samples of *Y. pestis* grown under conditions described above. Assays were conducted in triplicate wells in at least three separate experiments. **$p < 0.005$ (invasion efficiency as compared to 37°C-grown on solid media)..

In vivo Experiments

These results led us to speculate that *Y. pestis* may have specific adaptations to growth in the upper airways where oxygen levels are high. Experiments by Davis et al. demonstrate that infection of African Green monkeys with the fully virulent Y. pestis strain CO92 is not dependent on the presence of F1 [42]. However, these studies were conducted with bacteria grown in liquid culture at 30°C. Thus, F1 was not present at the time of infection, and *in vitro* studies show that bacteria need multiple generations to make sufficient F1 (data not shown). Conclusive data on the role of F1 during intranasal challenge is not available.

To test the hypothesis that high-oxygen grown *Y. pestis* has enhanced ability to produce disease, perhaps via invasion/colonization of the nasopharynx, we conducted survival experiments comparing the virulence of bacteria grown under these two conditions. For these experiments, we followed methods similar to those as described by Iyer et al. to establish nasopharyngeal colonization with *Streptoccous pneumoniae* [103]**.** We infected mice with a small volume (4ul) of *Y. pestis* KIM1001 pBR322Δtet instilled in the nose. We compared the survival of mice infected with bacteria grown at atmospheric levels of oxygen to bacteria grown under microaerophillic conditions. Surprisingly we found the difference in oxygen availability had the opposite effect than we hypothesized. By day four, 4 of 5 of the mice infected with the highest dose (2×10^4 CFU) of microaerobically grown *Y. pestis* had died, while 5 of 5 mice infected with bacteria grown under atmospheric levels of oxygen were still alive (Figure 13). By day

12, all mice infected with the highest dose of microaerobically grown *Y. pestis* had died, while mice infected with bacteria grown under atmospheric levels of oxygen died more slowly, with 2 of 5 still alive at the conclusion of the study. At a lower dose (4×10^3 CFU), 4 of 5 mice infected with microaerophillically grown bacteria died between days five and nine, while we observed no deaths when mice were infected with bacteria grown under atmospheric levels of oxygen.

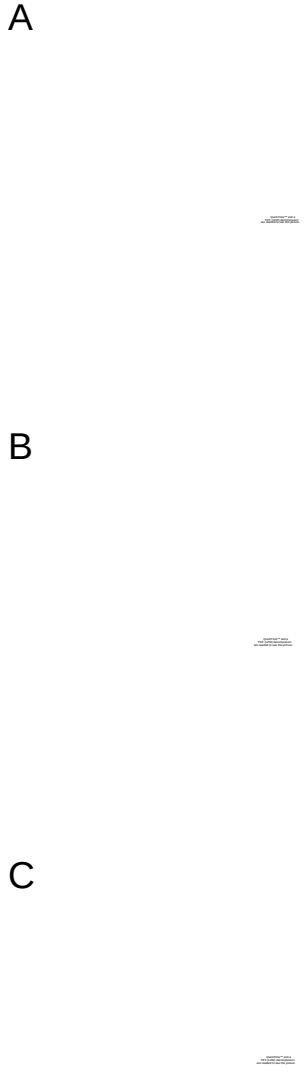

Figure 13. *Y pestis* grown under microaerophillic conditions are significantly more virulent than aerobically-grown bacteria in mice following low volume intranasal instillation. KIM1001 pBR322 Δtet was grown under atmospheric levels of oxygen (Δ) or in a microaerophillic chamber (○) for 24 hours on solid TB agar at 37°C. Bacteria were harvested from plates and resuspended in sterile water. Using a turbidity standard of 0.35, bacteria were diluted to the appropriate concentration in endotoxin-free, injection grade PBS (Cambrex) and confirmed by plating on TB. Five animals per group of C57Bl/6 mice were anesthetized with ketamine/ xylazine and were infected by intranasal instillation with 4μl of the inocula.. Survival was monitored for 7 days. Dose: 2×10^4 CFU (Panel A), 4×10^3 CFU (Panel B), 800 CFU (Panel C). Mice in both groups given the lowest dose (160 CFU) survived. The experiment was performed once at each dose. Mice were monitored for survival for 12 days. Survival curves were compared by log-rank test and p values were determined.

***Y. *pestis* bacteria do not survive inside cultured epithelial cells**

To determine if bacteria are able to replicate after entering the intracellular environment of cultured WI26 cells, internalized bacteria were quantified by gentamycin protection assay over a 12-hour period after infection. We infected WI26 monolayers with D1 (*pgm* ˉ) or KIM6 (*pgm* ˉ pCD1ˉ) grown at 30°C or 37°C on solid media and conducted a standard gentamycin protection assay. As shown in Figure 14, once inside the cell, bacterial numbers steadily decline in number, until virtually no bacteria can be recovered from the interior of the cell. This result, in combination with the infection experiments detailed above, argue against invasion occurring *in vivo*.

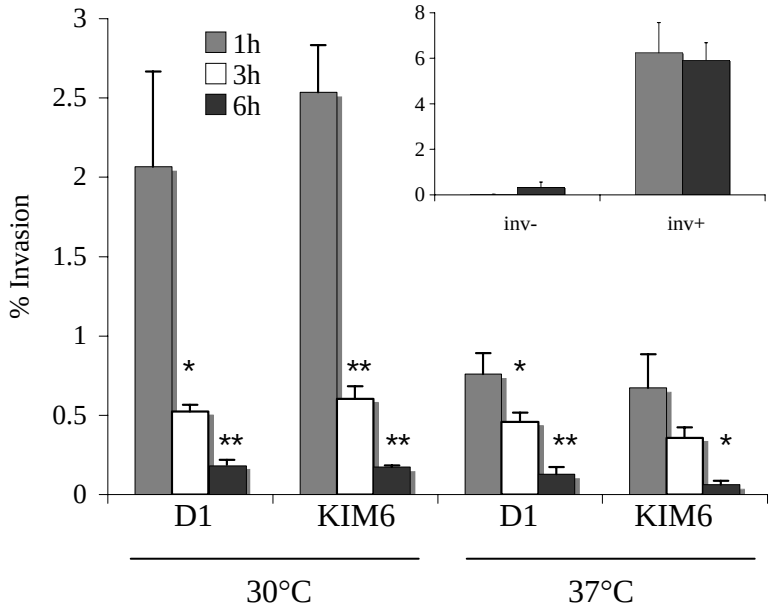

Figure 14. Intracellular survival following infection of WI26 cells. WI26 cell monolayers were infected with D1 (*pgm* ˉ), KIM6 (*pgm* ˉ pCD1ˉ) grown at 30°C or 37°C on solid media or with invasin-expressing or vector control MC4100 (inset). Standard gentamycin protection assays were performed as in Figure 8. After gentamycin treatment, cells were extensively washed with serum-free RPMI and allowed to incubate for 3 or 6 hours in the absence of gentamycin. Intracellular bacteria was quantified as in Figure 8. *$p < 0.05$ **$p<0.005$

Table 2. Bacterial strains and plasmids

Strain/Plasmid	Properties	Source or Origin
Yersinia pestis		
KIM1001	*pgm*$^{+}$; pMT^{+}, pCD1^{+} pPCP1^{+}	[6]
KIM1001pBR322Δtet	*pgm*$^{+}$; pMT^{+}, pCD1^{+} pPCP1^{+} A^{R}	[14]
D1	*pgm*$^{-}$; pMT^{+}, pCD1^{+} pPCP1^{+}	KIM derived, our laboratory
KIM5	*pgm*$^{-}$; pMT^{+}, pCD1^{+} pPCP1^{+}	KIM derived, our laboratory
KIM6	*pgm*$^{-}$; pMT^{+}, pCD1^{-} pPCP1^{+}	KIM derived, our laboratory
JG153	*pgm*$^{-}$; pMT^{+}, pCD1^{+} pPCP1^{-}	KIM derived, our laboratory
JG154	*pgm*$^{-}$; pMT^{+} , pCD1^{-}; pPCP1^{-}	KIM derived, our laboratory
Escherichia coli		
MC4100pR!203::Tn*pho*A	*Inv*$^{-}$; A^{R}	Gift of J. Leong
MC4100pJL272	*Inv*$^{+}$; A^{R}	Gift of J. Leong
β2155	Provides RP4 conjugation functions, pi protein for pi dependent suicide vectors; DAP auxotroph; K^{R}	Gift of B. Akerley
Plasmids		
pRE107	pi dependent suicide vector; Amp^{R}; *sacB*	Gift of D. Schifferli
pK101	pRE107; caf1 amplicon insertion	This study
pBR322 Δtet	pBR322 (Invitrogen), T^{S}	[14]
pGK01ΔxylB	pGK01; xylB amplicon insertion	Gift of B. Akerley

Discussion

Although fleas play an important role in the transmission of *Yersinia pestis* between mammalian hosts in nature, transmission can also occur by other routes. In epidemics within human populations, airborne transmission, primarily via droplets rather than fine aerosols, is thought to be important. Studies conducted during Manchurian pneumonic plague epidemics of the early 1900's revealed that droplet transmission, not nuclei, were more likely to be responsible for bacterial spread via coughing. During this epidemic, physicians treating plague-infected patients wore simple gauze masks that were sufficient to prevent transmission [94]. The poor efficiency of droplet penetration to the deep lung suggests the hypothesis that droplet transmission involves preliminary colonization of the upper airways and/or nasopharynx.

A key finding of this work is that the ability of *Y. pestis* to invade cultured epithelial cells is enhanced by growth during direct exposure to air, and that this enhancement is due to the repression of F1 production. This observation suggested to us that *Y. pestis* grown in air might have enhanced ability to colonize the nasopharynx. This ability could in turn result in enhanced vector-independent transmission, particularly if such colonization ultimately progresses to pneumonic infection.

One approach to testing this hypothesis is to establish a model of nasopharyngeal infection in mice and determine if the initial infection progresses to pneumonic disease and if growth conditions, particularly growth in air, affect the outcome of infection. In

and initial attempt to establish such a model, we adapted the low-volume intranasal instillation model of Iyer et al. used to establish nasopharyngeal colonization of *Streptococcus pneumoniae* to *Yersinia pestis*. [103]. Initial experiments with this model have yielded interesting, but unexpected, results.

For bacteria grown with exposure to air, forty percent (2 of 5) of mice receiving an inoculum of $2x\ 10^4$ CFU died as did 1 of 15 animals in lower dose groups. These deaths occurred between 4.5 and 5 days post-infection. Animals infected with bacteria grown under microaerophillic conditions behave differently in that none of the animals in the high dose group survived and 4 of 5 deaths occurred on day three. Based on these results, it is unlikely that the difference in LD_{50} between the two growth conditions is greater than 10-fold.

The rapid deaths of mice infected with microaerophillically grown bacteria is similar to that observed in models of primary pneumonic plague and suggests that pulmonary infection was established very early and progressed quickly. Two possible explanations for this result are that a larger number of bacteria reach the lung immediately after inoculation or that these bacteria were more resistant to host defenses during the initial stages of infection than their air-grown counterparts. At present, we cannot distinguish between these hypotheses, but the possibility that F1 expression induced microaerophillia results in more efficient penetration to the lung is an intriguing one. Whatever the mechanism, these results demonstrate the influence of growth conditions on the outcome of infection experiments and indicate a need to establish what

conditions yield bacteria with greatest phenotypic virulence. It is these conditions that should be employed in preparing inocula for use in protection studies for evaluating vaccine efficacy.

We do not yet know the basis of this phenotypic difference in virulence. It is likely to involve the increased expression of F1 under microaerophillic conditions. This can be readily tested in parallel experiments with a specific F1 mutant. If a preliminary colonization stage following low-volume intranasal instillation is established and goes on to produce pneumonic or systemic infection, we would expect this to be reflected by extended post-infection survival.

While the delayed deaths observed in the high dose group infected with air-grown bacteria may be consistent with a requirement for upper airway colonization, increased susceptibility to anti-bacterial defenses or more limited penetration of the initial inoculum directly to the lung could also have this effect. This, in the absence of any direct data on naso-pharyngeal colonization, prevents us from drawing conclusions regarding colonization at present.

An additional series of experiments can readily address these issues. The relative ability of bacteria grown under the two conditions to reach the lung rapidly following inoculation can be assessed by direct measurements of bacteria in the lung tissue excised from the bronchial tree a short time (e.g. 1 h) after infection. Marked strains have been constructed to do these experiments with a mixed inoculum. Because the phenotypic

difference established by growth prior to infection is likely to be transient *in vivo*, the relative abilities of both wildtype and F1$^-$ bacteria grown under different oxygen regimes to colonize the naso-pharynx can also be compared by direct measurements of bacteria in the naso-pharynx. One interesting possible outcome of such experiment would be the finding that F1$^-$ bacteria colonize efficiently but are much less likely to cause pulmonary infection. Another interesting question this raises is whether there are regions of the naso-pharynx that have a sufficiently oxygen rich atmosphere to repress expression of F1. This would probably be true in the immediate vicinity of the nares.

Oxygen availability as an environmental cue for gene expression has not previously been demonstrated for *Y. pestis*. Temperature, host-cell contact and pH have been the only recognized environmental cues for expression of many virulence factors. As F1 expression was never observed after growth at 30°C in liquid medium, our results indicate that 37°C growth is the primary cue and oxygen concentration serves as a secondary cue.

In regards to the regulation of F1, we attempted to create a *caf1R* deficient strain. Using similar methods used to construct our F1-deficient *Y. pestis* KIM6, we were able to confirm the presence of the co-integrate form from over sixty individual isolates in at least three separate mating experiments. Upon counter-selection on TB supplemented with 5% sucrose, all independent co-integrates resolved back to wildtype 100% of the time. This result, as well as similar experiments by colleagues at UMMS, led us to speculate that the lack of *caf1R* may be a lethal mutation (Xiaoyu Hu UMMS, personal

communication). Though a partial deletion mutant was described by Karlyshev et al., there is no mention in all the Yersiniae literature of a caf1R deficient *Y. pestis* [38]. Further experiments will need to identify the sensor and how this signal is relayed either directly or indirectly to caf1R.

Chapter Five.

Invasion of Cultured Epithelial Cells

Introduction

Direct invasion of epithelial cells in vitro has been well-characterized by the enteric yersiniae. The expression of the chromosomally-encoded invasin (*inv*) protein of *Yersinia pseudotuberculosis* in non-invasive *Escherichia coli* is sufficient for internalization into cultured mammalian cells [104-107]. Further investigation demonstrated that the specific binding of invasin to host β1 integrins mediated bacterial uptake [106]. However, in *Yersinia pestis*, invasin is not produced due to an insertion sequence within the *inv* gene [108]. The two other adhesins/invasins of the enteric yersiniae that have been identified, *Ail* and *YadA*, are also missing or inactive in *Y. pestis*.

These genetic analyses, as well as experiments by Sikkema et al. and Rosqvist et al., indicated that, unlike the enteric Yersiniae, *Y. pestis* was unable to invade epithelial cells in culture [109]. Sikkema et al. observed that *Y. pestis* was completely unable to invade HeLa cells in vitro; however these studies were conducted in tissue culture media in the presence of 10% fetal bovine serum. Rosqvist et al. also supplemented their media with 10% serum and used a Pla$^-$ strain of *Y. pestis*. Subsequent studies would show the inhibitory effects of these conditions on invasion experiments with *Y. pestis*.

More recently, multiple groups have reported the ability of *Y. pestis* to invade epithelial cells in vitro [41, 60]. Utilizing a gentamycin protection assay, Cowan et al. demonstrated that the presence of serum during the experiment significantly inhibited invasion of HeLa cells by *Y. pestis*. Also, they reported that the presence of pPCP1 was

essential for high efficiency invasion ($pPCP1^+$ vs $pPCP1^-$ at 26°C was equal to an 18-fold increase). Their findings explain why data by previous groups led them to conclude *Y. pestis* to be non-invasive. In addition, their work defines some factors not required for invasion of HeLa cells by *Y. pestis*. The absence of pCD1 or the *pgm* locus did not affect the ability to invade cells and this result was independent of growth temperature. However, growth of bacteria at 37°C significantly decreased invasion efficiency. They speculated that Pla is the pPCP1-encoded factor that enhances invasion and the thermally-regulated F1 capsule peptide is responsible for the temperature-dependent inhibition that they observe.

Pla is the only recognized virulence factor encoded on pPCP1; *pst* and *pim* encode pesticin, the yersinia bacteriocin, and its cognate immunity protein, respectively [2]. The ability of Pla alone to mediate internalization by non-invasive strains of *E. coli* has been reported by several groups. Expression of Pla in the *E. coli* strains XL-1 or TB1 renders them invasive for the human endothelial cell line ECV304 or HeLa cells, respectively [29 Benedek, 2004 #44]. This invasive ability is substantially less than of *Y. pestis* as reported by Cowan et al.. Unfortunately, these studies did not conduct a direct comparison of invasion between any *Y. pestis* strain and Pla-expressing *E. coli*.

Benedek et al. attempted to ascertain the mechanism of Pla-mediated invasion. Not surprisingly, Pla-mediated invasion, but not adhesion, requires cytoskeletal arrangement within HeLa cells. Preincubation of HeLa cells with nanomolar concentrations of staurosporin, genistein, or the actin inhibitor, cytochalasin D, blocked

invasion up to 50% [110]. This study attempted to address invasion from the perspective of the target cell, but did not address which functions of Pla are involved in invasion. Lahteenmaki et al. constructed point mutants of Pla that abolished proteolytic ability and expressed them in *E. coli* [29]. They observed no difference in invasion between wildtype and the protease defective mutant.

Also using a gentamycin protection assay, a study by Liu et al. supported the hypothesis by Cowan et al. and demonstrated that F1 is the thermally regulated factor that inhibits invasion at 37°C [41]. They constructed a caf1 deletion mutant that restored invasion of A549 type II alveolar cells at 37°C.

Interestingly, contrary to the results of Cowan et al. and our own observations, they report that the presence of pPCP1 had no effect on invasion. We noted that in this study, bacteria were routinely grown in media different from our own to induce optimal expression of pH 6 antigen (brain heart infusion, BHI, versus TB). Bacterial metabolism of glucose present in BHI (2 mg/ml) causes acidification of the media due to the accumulation of acetic acid products [111-113]. This difference in growth condition may not only effect the regulation of pH 6, but also may alter the regulation of proteins associated with invasion, namely F1 and Pla. We asked whether this difference in growth condition had an effect on expression of either F1 or Pla. We tested the expression of these two proteins after growth in media that supports expression of pH 6 antigen.

The purpose of this study was to better characterize Pla-mediated invasion of human lung-derived epithelial cells in culture by *Yersinia pestis*. We addressed whether protease activity, adhesion, or plasminogen activation by Pla was involved in invasion. We demonstrate that expression of Pla from a vector utilizing the replication system from pPCP1 is unable to invade WI26 and HeLa cells when expressed in *E. coli*. However, when a high copy number plasmid is utilized we observe modest ability to invade cells in culture compared to *Y. pestis*.

Treatment of cells with a glycosylation inhibitor, previously shown to inhibit adhesion of *Y. pestis* (Thomas, 2006 #80}, resulted in a significant decrease in invasion. Interestingly, the difference observed was dependent on growth temperature of the bacteria. Invasion of bacteria grown at 37°C were unaffected by the loss of glycosylated host cell surface proteins. We were also able to exclude several integrins as potential receptors for the bacteria including the receptor for *invasin*, β1, and the predominant laminin-binding integrin, β4. Interestingly, even when we employed a $F1^-$ strain, *Y. pestis* grown at 37°C was not able to invade the cell line utilized in our 4 experiments. Collectively, these results led us to conclude that *Y. pestis* has at least two separate mechanisms capable of promoting invasion of cultured mammalian cells, and that utilization of these mechanisms is temperature dependent.

Materials and Methods

Bacterial strains, plasmids and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 3. For liquid-grown *Yersinia pestis,* strains were grown in TB medium containing 10 g Bacto-tryptose, 5 g NaCl, 3 g Beef Extract (paste form, Difco Cat # 212610) per liter supplemented with 2.5 mM CaCl overnight at 30°C or 37°C as indicated. For growth of solid-grown *Yersinia pestis*, 5 x 10^6 CFU were inoculated as a thin film and grown on TB medium supplemented with 2.5 mM CaCl and 15 g per liter agar for 16 hours at 30°C or 37°C where indicated. For growth of bacteria under a reduced oxygen environment, bacteria were inoculated onto TB agar as described above and incubated in an unvented polycarbonate jar (BBL) with a CampyPak™ Plus Microaerophillic System envelope (BBL). pH6 antigen was induced by the addition of 2 mg/ml glucose to the TB media. *Escherichia coli* strains were grown at 37°C in Luria-Bertani broth or on Luria-Bertani agar supplemented with 100μg/ml ampicillin. MC4100 pJL272 expressing *invasin* from *Yersinia pseudotuberculosis* and MC4100 pRI203 Tn::*phoA* (kind gift of John Leong, University of Massachusetts) were grown in LB media supplemented with 100μg/ml ampicillin. To buffer TB media supplemented with glucose, 1 x M9 salts (43 mM NaCl, 93 mM NH_4Cl, 110 mM KH_2PO_4, 450 mM Na_2HPO_4) were added.

Cell Lines and Reagents. The type I human alveolar epithelial cell line, WI26 (American Type Tissue Collection, MD) and HeLa human epithelioid cells were maintained as described above. MDA-435pcDNA3 vector control cells and MDA-435

pRc/CMV cells expressing the β4 subunit (generous gift of Leslie Shaw, University of Massachusetts Medical School, [114, 115] were maintained in Dulbecco's modified Eagle's Medium supplemented with 10% heat-inactivated fetal bovine serum and 10μg/ml penicillin/streptomycin supplemented with 500 μg/ml G418 (Clontech). F9 cells (DKO, as there are three copies of β1 in this cell line) and their β1-deficient counterpart (TKO) were maintained in Dulbecco's modified Eagle's Medium supplemented with 10% heat-inactivated fetal bovine serum and 10μg/ml penicillin/streptomycin in flasks pre-treated with 0.1% gelatin for 2 hours at 4°C or in 24-well tissue culture plates also treated with 0.1% gelatin for 2 hours 4°C for invasion assays {kind gift of John Leong, University of Massachusetts Medical School; {Stephens, 1993 #218}. All cell lines were maintained at 37°C with 5% CO_2. For glycosylation inhibition studies, cells were treated with 0.55μg/mL tunicamycin for 96 hours prior to infection (Sigma). For blocking experiments, cells were pretreated with 5-20 μg/ml polyclonal anti-human laminin or anti-human fibronectin antibodies for one hour prior to infection (MP Biomedicals). To determine the effect of plasminogen activation on invasion, bacterial cells were pretreated with 160nM human Glu- plasminogen at the indicated concentrations for one hour at 37°C prior to infection (Sigma).

Cell Invasion. Cells were seeded at 5 x 10^4 per well in 24-well tissue culture dishes (Costar) and grown overnight at 37°C with 5% CO_2. The day of the experiment, cells were washed twice with warm, serum-free RPMI 1640 (Invitrogen). Bacterial cells were pelleted and washed once with phosphate-buffered saline (PBS, 135 mM NaCl, 2.68 mM KCl, 10 mM $NaHPO_4$, 1.76 mM KH_2PO_4). Bacteria were diluted into warm serum-free

RPMI 1640 at a concentration of 10^6 CFU/ml. Warm medium was removed from the cells and one milliliter of the bacterial suspension was added to give a multiplicity of infection (MOI) of 10. Tissue culture dishes were then centrifuged for 10 minutes at 182 x *g* at room temperature and incubated for one hour at 37°C with 5% CO_2. Cells were washed as above and extracellular bacteria were killed by the addition of 50 μg/mL gentamycin in serum-free RPMI 1640 for one hour at 37°C with 5% CO_2. Cells were washed with RPMI and one ml of ice cold water was added to each well to lyse cells. Dishes were placed on ice for 10 minutes and intracellular bacteria were determined by serial dilutions of the lysate. All experiments were done in triplicate wells in at least three independent experiments. The final inoculum was verified by CFU determination.

Cytotoxicity assay. The Cytotoxicity Detection Kit (Roche) was used in accordance with the manufacturer's instructions. This kit measures release of lactate dehydrogenase.

SDS-PAGE and Immunolotting. . Total protein extracts from 5 x 10^6 *Y. pestis* cells were run on SDS-PAGE (10% acrylamide) (Biorad) at 100V for 1 hour. For immunoblotting, proteins were transferred to a PVDF membrane (Millipore) for 1 hour at 100V. Membranes were blocked overnight in 5% nonfat milk in PBS (PBSM) at 4°C. Membranes were incubated with primary antibody diluted in 5% PBSM at room temperature with agitation for two hours. After three washes for 5 minutes each in PBS/0.1% Tween, membranes were incubated with secondary antibody diluted in 5% PBSM at room temperature with agitation for two hours. After three washes for 5 minutes each in PBS/0.1% Tween, membranes were washed for 20 minutes with high pH

Tris buffer (100 mM Tris Buffer, 100 mM NaCl, 5 mM $MgCl_2$ pH 9.5) and developed with 0.005% 5-Bromo-4-Chloro-3-indolyl phosphate (BCIP) (Sigma) and 0.005% nitro blue tetrazolium resuspended in high pH Tris Buffer.

Antibodies. Mouse anti-F1 monoclonal antibodies (clone VA18) were purchased from Meridian Life Sciences (formerly Biodesign). Polyclonal anti-Pla antibodies were produced in rabbits injected with *E. coli* over-expressing Pla and purified by ammonium sulfate precipitation followed by adsorption to pPCP1-deficient *Y. pestis* strain, JG153. Poly-clonal anti-pH6 antisera was a generous gift of Dieter Schifferli (University of Pennsylvania). To remove non-specific antibodies, pH6 antiserum was adsorbed using whole cells of DSY23 resuspended in PBS and incubated for an hour at 4°C. Alkaline phosphatase conjugated anti-mouse and anti-rabbit IgG were purchased from Sigma.

Plasminogen activation assay. To test determine plasminogen activation activity, reactions were performed with whole bacterial cells washed and resuspended in assay buffer (0.1 M Tris, 0.1% Tween pH 7.4). Reactions were carried out in flat bottom 96-well plates (Costar) with c.a. 10^7 cells, 180 nM human Glu-plasminogen (Sigma), and 5nM of the chromogenic substrate Val-Leu-Lys-*p*-nitroanilide (Sigma, S2251) in 50μL final volume of assay buffer. Substrate hydrolysis by plasmin was followed by recording the absorbance at 405 nm over 60 minutes, and the rate of plasminogen activation calculated as the second derivative of the curve obtained.

Whole cell ELISA. Bacterial cells from growth conditions described above were washed in 1 ml PBS and diluted in 0.1 M NaH_2PO_4 / 0.1M Na_2HPO_4 buffer to 5 x 10^6 CFU/ml. Assay plates (ICN) were coated with 50µl/well of the bacterial suspension and allowed to incubate overnight at 4°C. Plates were blocked with 1% BSA diluted in PBS for one hour at room temperature. F1-specific antibodies were diluted in 1% BSA/PBS. Fifty microliters was added to each well of the assay plate and incubated for two hours at room temperature. Plates were washed three times with PBS and 0.1% Tween. Anti-mouse IgG was added at a concentration of 1:10,00 in 1% BSA/PBS and incubated for two hours at room temperature. Plates were washed three times with PBS/0.1% Tween. Plates were developed with p-nitrophenyl phosphate (Sigma) and absorbance read at an optical density (OD) of 405 nm.

Results

Part I: Pla-mediated invasion

Pla expressed in E. coli

Our observations, as well as observations by Cowan et al., indicate that expression of Pla significantly enhances invasion of cultured epithelial cells by *Y. pestis*. It has not been demonstrated which function(s) of Pla are involved in invasion, or if Pla acts alone or in conjunction with another factor. A study by Lahteenmaki et al. shows no requirement for the protease activity of Pla for the invasion of endothelial cells in culture [29]. Using *E. coli* strains that express Pla or Pla point mutants that disrupt protease

activity, they demonstrate that Pla point mutants without protease activity had no defect in invasion as compared to wildtype, though no comparison of invasion by any *Yersinia pestis* strain was conducted for comparison.

We transformed the *E. coli* strain XL-1 with the Pla-expressing plasmid, pPLAI, or the vector control, pOS50. As shown in Table 3, this vector is a pBR322 derivative but contains the origin of replication from pPCP1 resulting in a plasmid with a similar copy number to that of pPCP1. We confirmed the expression of Pla in XL-1 by Western blot and its protease activity as measured by plasminogen activation assay as indistinguishable from that of wildtype *Y. pestis* (data not shown). We tested each strain for its ability to invade WI26 and HeLa cells as quantified by gentamycin protection assay. As shown by Figure 15A, XL-1 expressing Pla was completely unable to invade either HeLa or WI26 cells.

These results are different from those obtained by Benedek et al. and Lahteenmaki et al., where expression of Pla in *E. coli* was sufficient for invasion of cultured epithelial cells. However, the constructs used in these studies utilized plasmid vectors from the pUC family, and thus have a much higher copy number than the pOS50 constructs used in the experiments described above.

To investigate this effect of this difference, Pla was cloned in a pBluescript vector (pBS*pla*), also pUC-derived, and was transformed into XL-1. The ability of XL-I expressing Pla to invade HeLa and WI26 cells was assessed by gentamycin protection

assay. As shown by Figure 15B, these strains are able to invade cultured cells, but to a much lesser extent that *Y. pestis.* Given these results, we hypothesize that in the context of *Y. pestis*, Pla does not mediate invasion alone, but rather in conjunction with other factors.

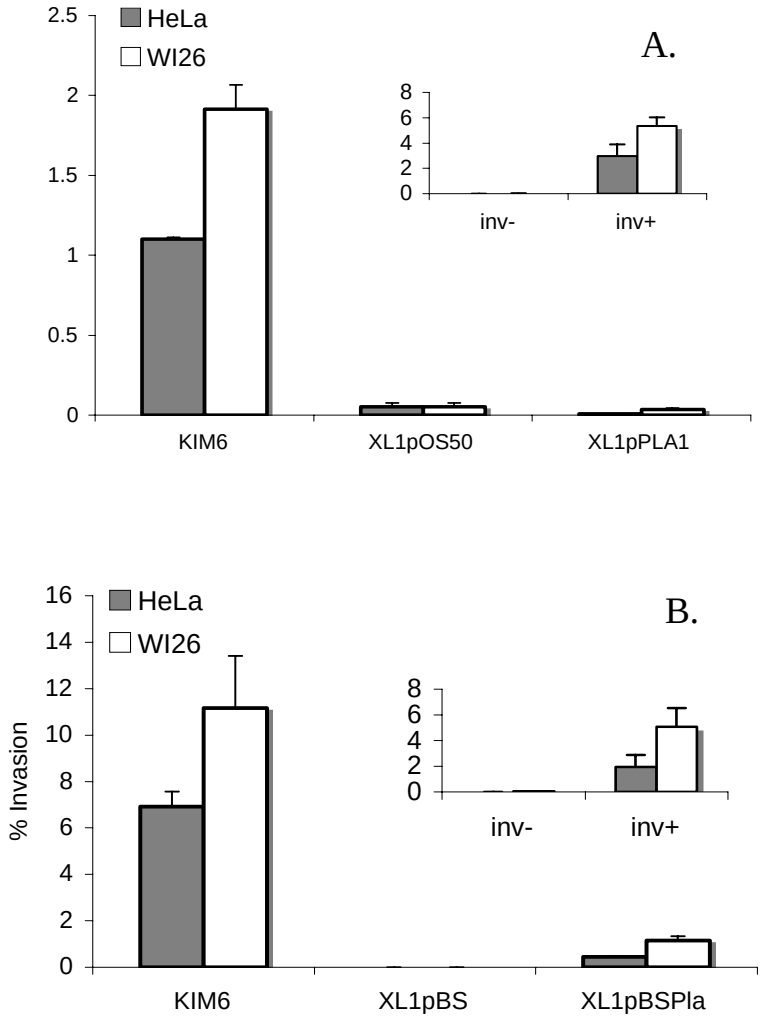

Figure 15. Pla-mediated invasion by *E. coli.* The *E. coli* strain, XL-1, was transformed with a vector control (pOS50) or pPLA1. XLI pPLA1 constitutively expresses a proteolytically active Pla (A). WI26 cells were seeded 5 x 10^4 cells per well in a 24-well tissue culture dish. Bacteria were washed once with PBS and resuspended in serum free RPMI at an MOI = 10. Cell monolayers were infected for an hour with the indicated strains. Intracellular bacteria were quantified by gentamycin protection assay (A). Same as in A, but XLI was transformed with the vector control, pBS, or with pBSPLA, which overexpresses Pla.

Protease Activity

We asked if the protease activity of Pla was required for invasion of cultured epithelial cells. We tested two point mutants of Pla for their ability to invade both HeLa and WI26 cells. As shown in Figure 16, the requirement for protease activity of Pla expressed in *Y. pestis* is dependent upon the cell line tested. For the human type II alveolar epithelial cell line, WI26, protease activity is required. However, protease activity of Pla is not required for the invasion of HeLa cells. This result is consistent with results obtained from other groups (S. Straley, personal communication).

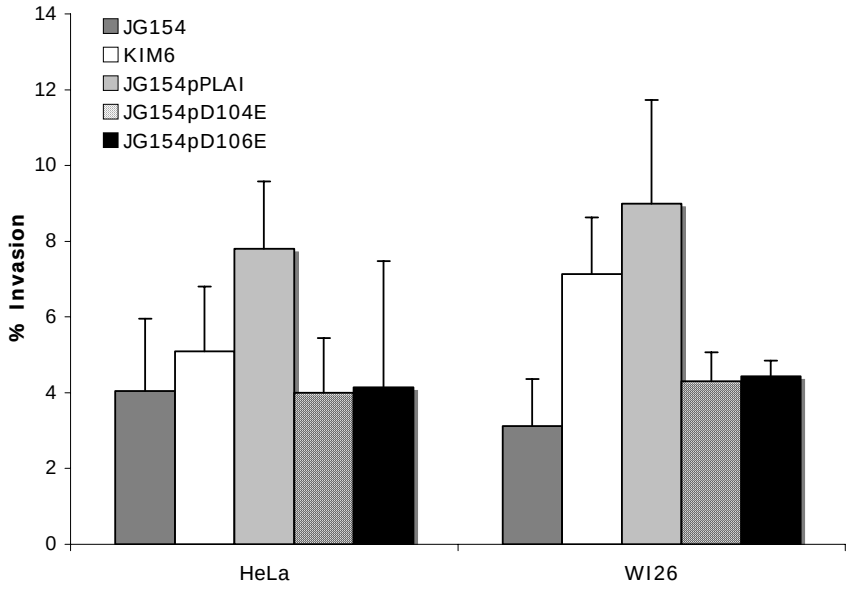

Figure 16. **The role of protease activity of Pla in invasion of cultured epithelial cells.** HeLa and WI26 cells were seeded 5 x 10^4 cells per well in 24-well tissue culture plates and allowed to incubate overnight. Monolayers were infected for one hour with bacteria grown in liquid media at 30°C. Intracellular bacteria was quantified by gentamycin assay as described above in Figure 8.. *$p < 0.05$ as compared to JG154 pPLAI.

Extracellular Matrix Binding

Binding extracellular matrix components and/or integrins is a common mechanism used by bacteria in the invasion of cultured mammalian cells. Enteric Yersiniae utilize β1 integrins to mediate internalization, and it has been shown that *Staphylococcus aureus* and *Streptococcus pyogenes* use extracellular matrix components to mediate internalization. The fibronectin-binding protein (FnBP) of *S. aureus* binds to fibronectin which acts as a bridge to α5β1 integrins to mediate uptake [116]. Similarly, the surface-expressed Sfbl protein of *S. pyogenes* also binds fibronectin and is internalized via interaction with α5β1 [117].

The ability to bind of Pla to bind laminin prompted us to determine if *Y. pestis* utilizes a similar mechanism for internalization. We asked if binding of laminin or a laminin-specific receptor was involved in invasion by Pla-expressing strains of *Y. pestis*. To address this question, we used two approaches. First, we attempted to block the Pla-laminin interaction using anti-laminin specific antibodies. Cell monolayers were incubated with either anti-laminin or anti-fibronectin antibodies or with RPMI alone. Reports by Sinha et al. indicate that incubation of 20 μg/ml anti-fibronectin antibodies with cultured epithelial cells was able to inhibit invasion by *S. aureus* [116]. Cells were infected with KIM6, JG154, or MC4100 (pJL272) expressing *invasin* or with the vector control. Intracellular bacteria were quantified by gentamycin protection assay. No difference in invasion efficiency of any strain was ever observed in the presence of anti-laminin antibodies.

Second, we tested whether the cell line MDA-435 transiently transfected with the integrin subunit, β4, were able to internalize Pla-expressing bacteria better compared to vector-transfected cells. Unlike HeLa and WI26 cells, which are epithelial cell derived, MDA-435 cells are derived from breast cell carcinoma. MDA-435 cell monolayers transfected with the β4 subunit or the pcDNA3 vector control expressing α6β1 were infected for one hour and intracellular bacteria was quantified by gentamycin protection. We observed that after infection with 30°C-grown *Y. pestis*, invasion is not significantly influenced by the presence of the β4 subunit. (Figure 17, Panel A). We speculate that if *Y. pestis* utilizes α6β4 to mediate bacterial entry, its contribution may be relatively small.

Interestingly, in all our invasion experiments with MDA-435 cells, infection of cells with 37°C-grown *Y. pestis* resulted in no observable invasion, with or without the presence of β4. Invasion was **never** observed in any *Y. pestis* strains tested, including an $F1^-$ strain (Figure 17 Panel B). This data, along with experiments outlined below lead us to hypothesize that their are least two mechanisms for invasion and that expression of at least one of these is strongly dependent on growth temperature.

Though invasion of cell line, MDA-435, was never observed by 37°C-grown *Y. pestis*, we asked if cell adhesion and/or cytotoxic effects of the type three secretion apparatus would also be non-functional. To date, there has not been a cell line identified that is not susceptible to the effects of TTSS. Infection of both WI26 and MDA-435 cells by KIM6 $F1^-$ grown at 30°C and 37°C resulted in significant adhesion (data not shown). In addition, MDA-435 was similarly effected by the cytotoxic effects of the type three

secretion system by 37°C-grown *Y. pestis*. Bacteria pre-grown to early log phase at 30°C were induced at 37°C for three hours. TTSS-sufficient and deficient bacteria were inoculated onto cell monolayers for 18 hours. We found that strains sufficient in TTSS were cytotoxic to both HeLa and MDA-435 cells after 4 hours of incubation at 37°C. These data demonstrate that the mechanism for adhesion is not sufficient for invasion.

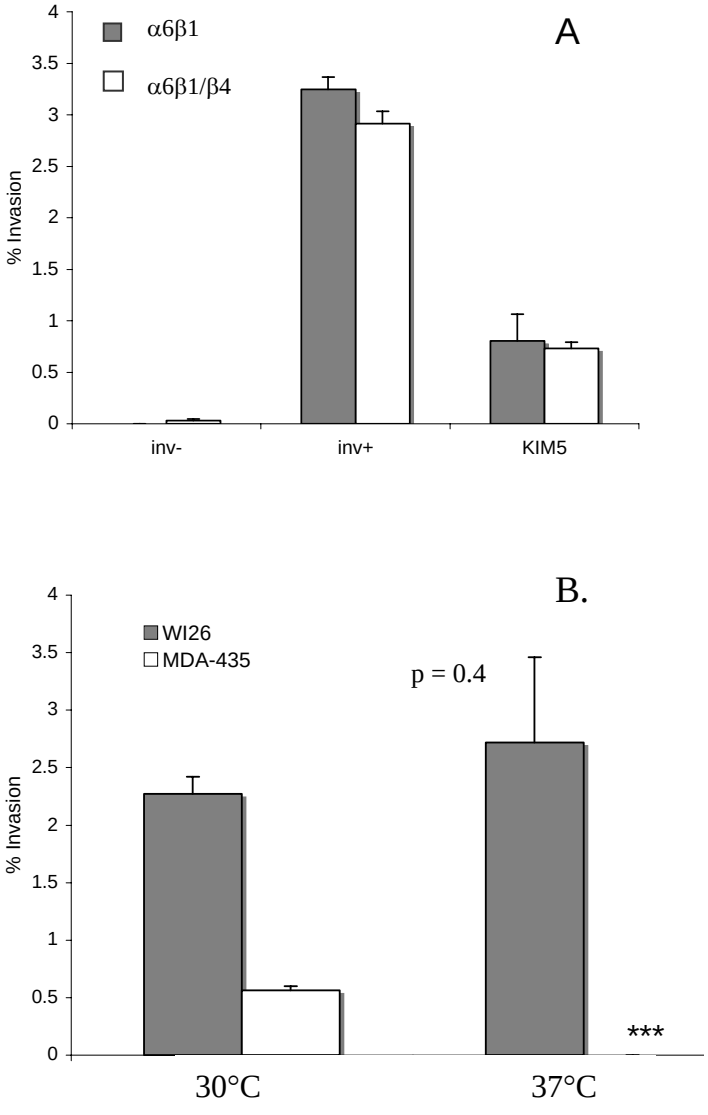

Figure 17. Invasion of 30°C-grown *Y. pestis* is not influenced by the presence of the β4 subunit of the laminin receptor. (A) MDA-435 cells were stably transfected with vector control (α6β1) or pRc/CMV expressing the β4 integrin subunit (α6β1/β4). Cells were seeded in 24-well tissue culture plates. The next day monolayers were infected for one hour with *Y. pestis* KIM6 F1$^-$ grown at 30°C (MOI = 10). MC411pJL272 (inv$^+$) is included as a control. Intracellular bacteria were quantified by standard gentamycin protection assay as in Figure 8. (B) WI26 cells and MDA-435 cells were seeded in 24-well tissue culture plates. The next day, monolayers were infected for one hour with either 30°C- or 37°C-solid grown *Y. pestis* KIM6 F1$^-$. (MOI = 10). Intracellular bacteria were quantified by standard gentamycin protection assay as in Figure 8. ***p < 001, 30°C-grown v.s. 37°C-grown KIM6 F1$^-$

Plasminogen Activation

One of the main functions of Pla is its ability to activate plasminogen. Activation of plasminogen by Pla has also been observed to degrade laminin [28]. We asked if plasminogen activation of Pla would enhance invasion of cultured epithelial cells. Cell monolayers were either pre-treated with human plasminogen for one hour prior to infection by *Y. pestis* or cells were treated at the time of bacterial inoculation. Intracellular bacteria were quantified by gentamycin protection assay. Pre-incubation of cells with plasminogen or concurrent plasminogen treatment and infection did not result in any decrease in invasion efficiency by pPCP1^{+} *Y. pestis* (incubation with 1μM Glu-plasminogen resulted in less than a 1.35-fold difference in invasion). The presence of plasmin and possible degradation of laminin had no effect on invasion efficiency by *Y. pestis.* Nor did the binding of Pla to plasminogen have any inhibitory effect on invasion.

Pla expression is repressed by pH

Results by Liu et al. indicate that the presence of pPCP1 had no effect on invasion. Studies by Cowan et al. and our own work have shown that pPCP1 and Pla, respectively, enhance invasion five to eighteen fold (Cowan et al, see figure 17 of this work). We asked if growth conditions utilized by Liu et al. that induced expression of pH 6 antigen had an effect on proteins we know to be important in invasion *in vitro*. As a standard practice for optimal induction of pH 6 antigen, bacteria were grown in TB supplemented with 2mg/ml glucose, the concentration found in BHI, the medium used by Liu et al. KIM6 was grown overnight as a thin film on TB agar supplemented with

2.5mM CaCl with or without 2mg/mL glucose at 30°C or 37°C under atmospheric levels of oxygen or within the microaerophillic chamber. Western blotting of whole cell lysates

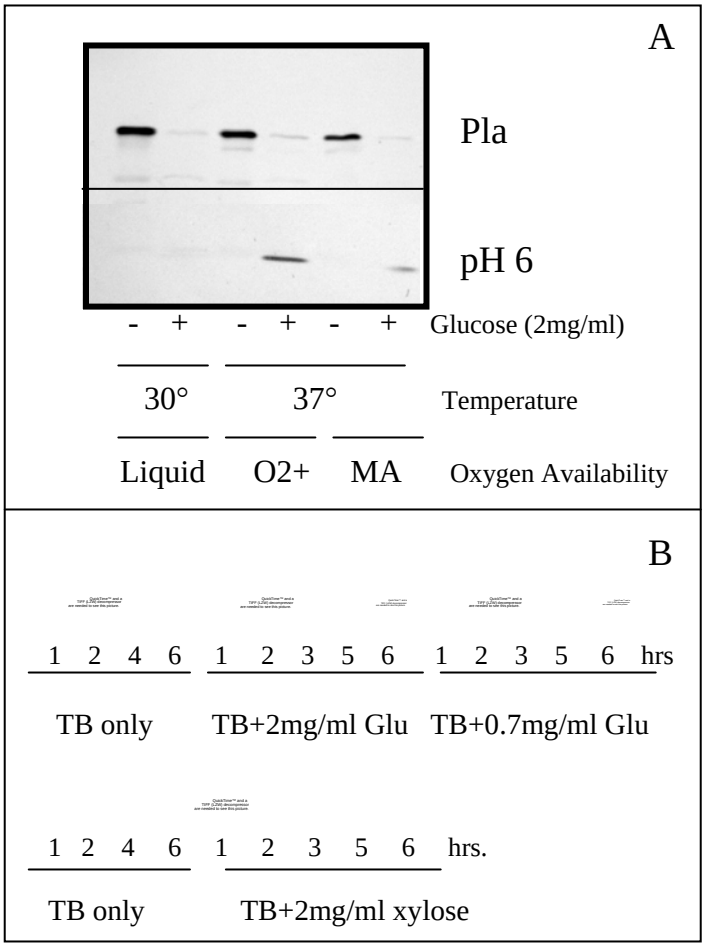

Figure 18. Pla expression is repressed by pH. (A) Bacteria were grown to log phase at 30°C or 37°C in liquid or on solid media grown under atmospheric levels of oxygen (air) or in a microaerophillic chamber (MA) in the presence or absence of 2 mg/ml glucose. Whole cell lysates from each condition were run on duplicate 10% SDS-PAGE, transferred to a PVDF membrane and then probed for the presence of Pla with polyclonal Pla-specific antibody and the presence of pH6 antigen with ploYclonal anti-pH 6 antibody. Note that under *any* growth condition containing glucose, Pla expression is significantly reduced, compared to pH6 expression, which is induced under low (c.a. 6.2-6.4) pH. Bacteria were grown in liquid culture at 30°C in shaking culture flasks in the presence or absence of glucose (2 or 0.7mg/ml) or xylose (2mg/ml) where indicated. Samples of bacteria were taken at different intervals and Pla expression was assessed as in panel A. Number s below indicate hours of incubation. For example, at 3 hours, the pH of TB alone was 7.12, while that of TB + 2 or 0.7 mg/ml glucose was 6.58. The pH of cultures containing xylose was 7.06 (B). at different intervals and Pla expression was assessed as in panel A. Number s below indicate hours of incubation. For example, at 3 hours, the pH of TB alone was 7.12, while that of TB + 2 or 0.7 mg/ml glucose was 6.58. The pH of cultures containing xylose was 7.06 (B).

demonstrated minimal differences in pH6 antigen expression in response to oxygen availability but more interestingly, a significant difference in the expression of Pla was detected when bacteria were grown on media containing glucose (Figure 18 A).

Furthermore, this result was independent of growth temperature and oxygen availability, as Pla was repressed at both 30°C and 37°C in the presence of glucose. Growth of bacteria in TB supplemented with M9 buffer did not yield any repression of Pla (data not shown), confirming that the change in pH was responsible for the difference in Pla expression observed.

To address this finding further, we conducted an experiment to observe when during growth and at what pH Pla was being repressed. A significant decrease in Pla was noted early in growth at a moderately lower pH (6.5) (Figure 18 B). These data indicate that Pla and pH 6 antigen are not actively expressed together. They also may explain the conclusions of invasion experiments of Liu et al.. They state that the presence of pPCP1 had no effect on invasion, though the intent of their study was to show the role of pH6 antigen in invasion, which is illustrated in their growth condition; brain heart infusion media supplemented with glucose.

We asked if F1 was similarly affected by pH conditions of the growth media. We similarly tested the effect of lower pH on the expression of F1. Surprisingly, we were able to demonstrate by whole cell ELISA and by western blot that bacteria grown in TB supplemented with 2 mg/ml glucose decreased F1 expression (Figure 19).

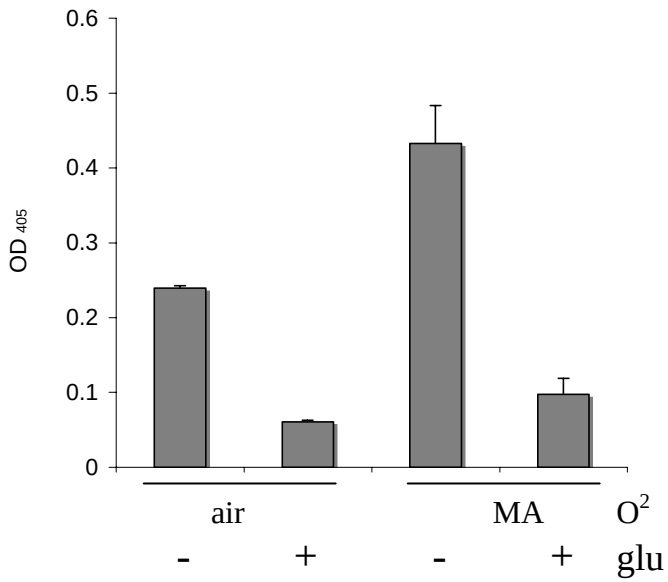

Figure 19. F1 is repressed by low pH. KIM5 was grown at 37°C on solid TB agar with or without 2mg/ml glucose under atmospheric (air) or microaerophillic conditions (MA). Whole cells from each condition were assayed for the expression of F1 with monoclonal anti-F1 anti-body by whole cell ELISA (as described in the Material and Methods).

Part II: Adherence and Invasion

Tunicamycin treatment inhibits the biosynthesis of high-mannose and complex glycoproteins in mammalian cells. Reducing the surface expression of oligosaccharides by tunicamycin treatment significantly decreased the ability of *Streptococcus pneumoniae* and *Legionella pneumophila* to adhere to the alveolar epithelial cell line, A549 [118]. Studies by Thomas et al. tested the hypothesis that adhesion of the fully virulent *Y. pestis* strain, GB, would similarly be decreased in response to a reduction in host cell glycoproteins. They demonstrate adhesion was decreased 55-65% after tunicamycin treatment of alveolar epithelial cell line, A549, and the bronchial epithelial cell line, BEAS2-B [119].

These observations by Thomas et al. indicate that *Y. pestis* uses an oligosaccharide moiety on the surface of mammalian cells for. Adhesion is likely an

important step in invasion by *Y. pestis*, though it is unclear what receptor *Y. pestis* binds to mediate internalization. We tested whether decreased glycosylation on the cell surface of would effect invasion of WI26 cells. Monolayers were cultured in the presence of 0.55μg/mL tunicamycin for 96 hours. At this concentration, tunicamycin was not cytotoxic to WI26 cells as demonstrated by a cell cytotoxicity assay (data not shown). After an hour infection, intracellular bacteria were quantified by gentamycin protection assay.

As shown in Figure 20, tunicamycin treatment decreased invasion by 30°C-grown *Y. pestis* by approximately 70%, indicating that adhesion to oligosaccharides mediates invasion. Tunicamycin treatment of WI26 cells had no effect on invasion by MC4100 pJL272. Observations that tunicamycin treatment decreased invasion by 30°C-grown, but not 37°C-grown *Y. pestis* led us to speculate that *Y. pestis* utilizes two separate mechanisms for invasion that are dependent on growth temperature. Protein(s) expressed at 30°C that are required for invasion are likely to bind these glycosylated molecules, whereas protein(s) expressed at 37°C do not require glycosylated surface expressed molecules. Also, this result indicates that the 30°C mechanism is not operative at 37°C. This result led us to speculate that adherence and invasion may be independent functions.

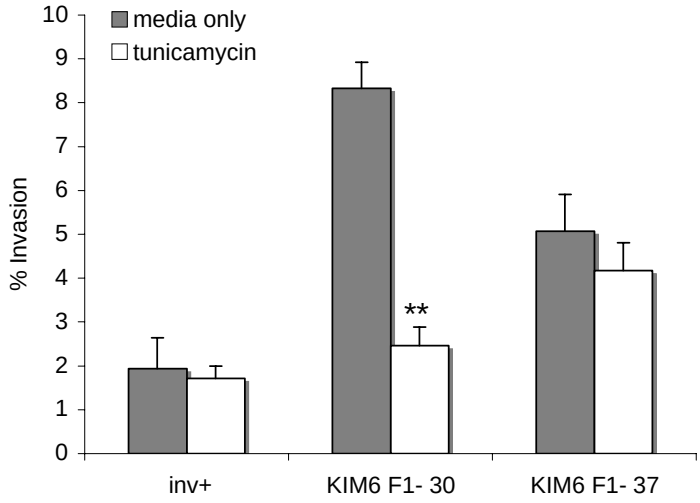

Figure 20. Tunicamycin treatment of WI26 cells specifically inhibits invasion by 30°C-grown, but not 37°C-grown *Y. pestis.* WI26 cells were seeded in 24 well tissue culture plates and were treated for 96 hours with 0.55 μg/ml tunicamycin diluted in tissue culture media. **.** F1-deficent *Y. pestis* was grown overnight in liquid medium at 30°C or 37°C. Cells were infected for an hour with bacteria at an MOI = 10. Intracellular bacteria were quantified by gentamycin protection assay as in Figure 8. **$p < 0.005$, treated v.s. untreated.

Part III. *Y. pestis* utilizes a separate invasion pathway from *Y. pseudotuberculosis*

In an attempt to determine the mechanism of *Y. pestis*-mediated invasion from the host cell perspective, we asked whether bacterial binding of β1 integrins, similar to *invasin* of *Y. pseudotuberculosis*, was involved in internalization. Binding of integrins, either directly or indirectly is a common mechanism of invasion by both Gram-positive and Gram-negative bacteria. Utilizing a standard gentamycin protection assay, F9 cells containing one intact copy of the β1 gene (DKO) and its β1- deficient counterpart (TKO) were infected with MC4100inv-, MC4100pJL272 or *Y. pestis* KIM6. As shown in Figure 21, the lack of β1 integrins had no effect on invasion by *Y. pestis*, but no invasion by

invasin-expressing E. coli was observed in the absence of β1 integrins. We conclude from these experiments that *Y. pestis* utilizes a separate mechanism from *Y. pseudotuberculosis* to mediate internalization.

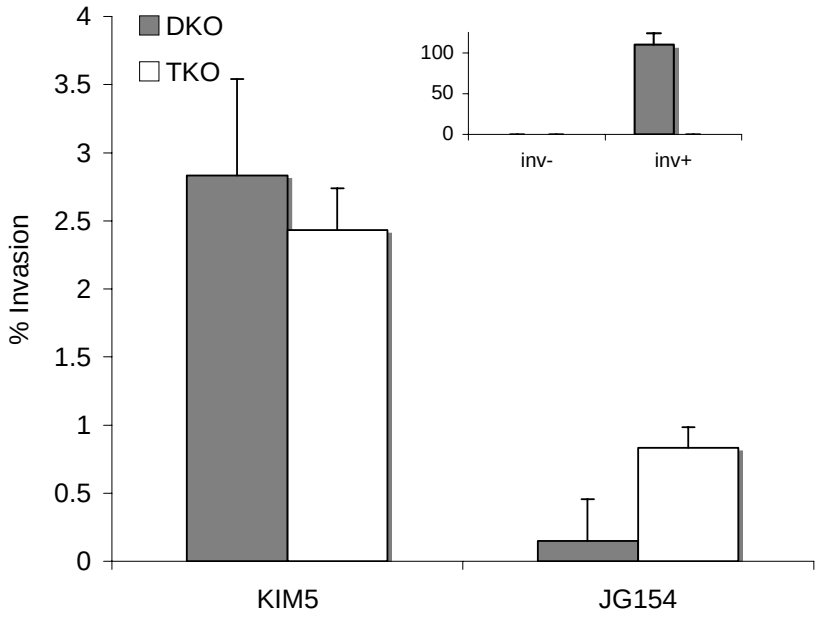

Figure 21. *Y. pestis* utilizes a separate invasion pathway from *Y. pseudotuberculosis*. β1-sufficient F9 cells (DKO) and β1-deficient cells (TKO) were seeded 5 x 10^4 in a 24-well tissue culture dish pretreated for 24 hours with 0.1% gelatin. Cell monolayers were infected with the indicated strains for one hour. Intracellular bacteria were quantified by gentamycin protection assay as in Figure 8. MC4100pJL272 and MC4100pR1203 are shown as controls for invasion efficiency (inset).

Table 3. Bacterial Strains and Plasmids

Strain/Plasmid	Properties	Source or Origin
Yersinia pestis		
KIM6	pgm$^-$; pMT$^+$, pCD1$^-$ pPCP1$^+$	KIM derived, this laboratory
KIM6 F1$^-$	pgm$^-$; pMT$^+$ Δcaf1 , pCD1$^-$ pPCP1$^+$	This study
]JG154	pgm$^-$; pMT$^+$, pCD1$^-$; pPCP1$^-$	KIM derived, this laboratory
Escherichia coli		
MC4100 pR!203	Inv$^-$; A^R	Gift of J. Leong
MC4100 pJL272	Inv$^+$; A^R	Gift of J. Leong
XL1		Stratagene
Plasmids		
pOS50	pBR322 derived, pPCP1 *ori*	[26]
pPLA1	pOS50; Pla $^+$; protease activity $^+$;3.2 kb *Hind*II frag of pPCP1;	[26]
pPLAD104E	pOS50; Pla $^+$; protease activity $^-$;3.2 kb *Hind*II frag of pPCP1;	our laboratory
pPLAD104E	pOS50; Pla $^+$; protease activity $^-$;3.2 kb *Hind*II frag of pPCP1;	our laboratory
pBS		Stratagene
pBSPla	Pla $^+$; protease activity $^+$	our laboratory

Discussion

This work demonstrates that unlike *invasin* of the enteric yersiniae, we have yet to identify a single protein in *Yersinia pestis* that can mediate efficient invasion of cultured epithelial cells. Though the presence of the Pla protease enhances invasion in *Y. pestis* as was first observed by Cowan et al.. In *E. coli* overexpression of Pla is required to promote even moderate levels of invasion. In contrast, invasion by *Y. pestis* seems to be a complex process involving multiple factors, some of which have temperature dependent expression. Moreover, the requirements for invasion differ significantly among cell lines. For example, the protease activity of Pla is required for invasion of WI26 cells but not for HeLa cells. At present, we have no clear indication of the receptor(s) or cell surface proteolytic targets are involved.

Our evidence indicates at least two separate pathways for invasion by *Y. pestis* that depend on growth temperature of the bacteria. For example, 30°C-grown *Y. pestis* will invade MDA-435 cells, but 37°C-grown will not. Nonetheless 37°C grown bacteria readily invade WI26, HeLa, and F9 cells. Results from tunicamycin treatment experiments also indicate multiple pathways. The invasion of WI26 cells by 30°C grown bacteria is reduced by tunicamycin, while invasion by 37°C grown bacteria is unaffected. This suggests the involvement of glycoproteins in invasion of 30°C grown bacteria, but not 37°C grown bacteria. Differences between invasion observed at different temperatures is highly unlikely to be related to Pla expression because Pla is expressed equivalently at both temperature regimes used. These pathways that are

differentiated by the temperature response almost certainly involve bacterial factors other than Pla.

The failure of 37°C grown bacteria to invade MDA-435 cells strongly suggests a missing a factor that is present in the other cell lines tested. It may be possible to exploit this difference to identify one important sole component contributing to invasion.

Induction of pH 6 antigen under different oxygen concentrations was accomplished by supplementing growth media with glucose and incubation of bacteria at 37°C. Acidic products are produced during the metabolism of glucose, causing a drop in pH and expression of pH6 antigen. Under these growth conditions, we observed significant differences in both Pla and F1 expression. Initially, we hypothesized that the added glucose may responsible for the differential expression levels of Pla and F1. To answer this question we grew bacteria in TB media in the presence or absence of buffer. We conclusively demonstrated that pH, not glucose, was responsible for the repression of Pla and F1 that we observe. Our results indicate that inducing conditions for pH 6 expression represses expression of Pla and F1.

These results have implications for many *in vitro* and *in vivo* experiments. Invasion studies by Liu et al. indicate, contrary to results by Cowan et al., that Pla has no effect on invasion. We speculate, in light of these new regulatory cues, that conditions conducive to the expression of pH 6 antigen repressed Pla expression. The take home message from these studies is to be aware of growth conditions.

Given the evidence we have for multiple invasion pathways and subtle differences between invasion in different cell lines (e.g. Pla protease activity required in WI26 but not HeLa) and the current lack of any definitive evidence indicating that invasion is important during *Y. pestis* infection, it is important to question the relevance of invasion observed *in vitro* to pathogen biology. It is perhaps likely that interactions between bacteria and cells *in vitro* that are essentially artifactual, in the sense that they do not represent significant interactions *in vivo*, more readily cause invasion phenomena than is generally thought. If this view is correct, it may be injudicious to devote effort to the study of invasion when the only data to support the phenomena in question was obtained *in vitro*.

Chapter Six.

Discussion

Discussion

This work presents two major findings; first, we conclusively show that the LcrV/TLR2 interaction that has gained wide acceptance in the Yersiniae field, plays no role during infection with fully virulent strains of plague and second, we discovered novel environmental cues for the expression of several virulence determinants, most importantly, F1.

Until recently, the hypothesis that interaction between Yersiniae LcrV and host TLR2 has an anti-inflammatory effect important in infection has been a widely accepted. With no clear data indicating that *Y. pestis* LcrV had similar activities or had any effect on the course of disease, Overheim et al. initiated work on the construction of a non-TLR2 stimulating *Y. pestis* LcrV construct for use in vaccine development. We tested the stimulatory activity of recombinant LcrV from *Yersinia pestis* and found that the majority of protein had no TLR2 stimulating activity *in vitro*, and such activity was restricted to high molecular weight aggregates/multimers that contain LcrV but are of undetermined composition.

Though we demonstrated an interaction between high molecular weight aggregates of *Yersinia pestis* rLcrV and host cell TLR2, this interaction had no significant consequence *in vivo*. In a sensitive mouse infection model, elimination of TLR2 had no effect on the course of disease, bacterial load, or on cytokine levels. Given the well-established sensitivity of the *Y. pestis* mouse infection model, its lack of response to TLR2 deficiency must be regarded as strong evidence that TLR2-induced

immunomodulation does not have a significant role in plague. This work, along with studies by Auerbuch and Isberg (2007) are show that this interaction is specific for some strains of *Y. enterocolitica* and certain strains of mice, but is not biologically relevant for *Yersinia pestis*. Since the publication of this work, Sing et al. have reported additional studies in agreement with our conclusions.

Vector-borne bacterial pathogens like *Yersinia pestis* that cycle between a vector and mammalian host encounter different environments and require strategies to control protein expression in a host-specific manner. This can be accomplished via species-specific environmental cues. Signals such as temperature, pH, iron, oxygen tension and osmolarity control the coordinate expression of virulence determinants in many bacteria [120]. For *Yersinia pestis*, induction of genes involved in growth and transmission by the flea an in rapid dissemination through evasion of innate immunity within the mammalian host need to be expressed in the appropriate environment. For example, expression of the type three secretion system (TTSS) and its many effectors within the flea is not beneficial, nor energy efficient, for the bacteria.

We previously demonstrated that increased oxygen availability significantly decreased expression of F1, which resulted in a greater ability to invade cultured epithelial cells. These novel observations of F1 regulation by oxygen availability led us to hypothesize that *Y. pestis* may utilize effective oxygen concentration to modulate other virulence genes. Regulation of virulence factors by oxygen availability has been observed in several species of bacteria. For example, in *Y. enterocolitica*, Schiel et al. demonstrate

that *yplA*, which encodes a phospholipase A, is repressed under increased aeration conditions [121]. In group B *Streptococci* (GBS) shows enhanced invasion of multiple cell lines under high aeration conditions, although the mechanism underlying this phenomenon has not been identified [122, 123]. Probably the best-characterized regulation system controlled by oxygen tension is in found in *Salmonella*species. *Salmonella* infects and penetrates intestinal epithelia, a microenvironment in which availability of oxygen is limited. High oxygen tension decreases invasion efficiency of non-typhoidal *Salmonella* by repression of *hilA,* a transcriptional regulator of genes encoded within the Salmonella Pathogenicity Island 1 (SPI-1). This is accomplished through the signal molecule guanosine tetraphosphate (ppGpp) whose regulation is required for both invasion and intracellular survival in the host [124].

Invasion of cultured epithelial cells has also been observed by many species of bacteria. The mechanism of invasion for the enteric *yersiniae* has been well characterized from both the perspective of the bacterial and host cell. *Y. pestis* is also invasive for cultured epithelial cells, though the mechanism is unknown. Studies showed that this invasive ability was significantly reduced when bacteria were grown at 37°C, independent of the presence of pCD1. This result led to disinterest in this process becuase it suggested the absence of invasion during infection. However, in a serendipitous experiment, we found F1 expression to be dramatically reduced under atmospheric levels of oxygen invasive ability concomitantly increased. We speculated that reduced F1 under high oxygen conditions may be an adaptation to efficient transmission by droplet infection. The results of preliminary infection experiments do

not provide support for this hypothesis. In contrast, they suggest that F1 either promotes transit to the lungs or provides significant resistance to against pulmonary defenses.

The role of F1 in vivo remains ambiguous. While some studies support the idea that F1 inhibits infection of rodents, other studies suggest that F1 plays no role. Additional studies utilizing the F1 mutants created in fully virulent KIM1001 will help elucidate the role of F1 in colonization and transit to the lungs. Additional work will also be neededto determine how F1 expression is regulated by oxygen, and if other virulence factors are also affected by oxygen concentration. Analysis of mutants with altered patterns of F1 expression and mutants defective in redox cycling will be important in revealing the mechanism of regulation.

.

References

1. Achtman, M., et al., *Yersinia pestis, the cause of plague, is a recently emerged clone of Yersinia pseudotuberculosis.* Proc Natl Acad Sci U S A, 1999. **96**(24): p. 14043-8.
2. Sodeinde, O.A. and J.D. Goguen, *Genetic analysis of the 9.5-kilobase virulence plasmid of Yersinia pestis.* Infect Immun, 1988. **56**(10): p. 2743-8.
3. Deng, W., et al., *Genome sequence of Yersinia pestis KIM.* J Bacteriol, 2002. **184**(16): p. 4601-11.
4. Hinnebusch, B.J., et al., *Role of Yersinia murine toxin in survival of Yersinia pestis in the midgut of the flea vector.* Science, 2002. **296**(5568): p. 733-5.
5. Hinnebusch, B.J., R.D. Perry, and T.G. Schwan, *Role of the Yersinia pestis hemin storage (hms) locus in the transmission of plague by fleas.* Science, 1996. **273**(5273): p. 367-70.
6. Sodeinde, O.A., et al., *A surface protease and the invasive character of plague.* Science, 1992. **258**(5084): p. 1004-7.
7. Akira, S. and K. Takeda, *Toll-like receptor signalling.* Nat Rev Immunol, 2004. **4**(7): p. 499-511.
8. Prior, J.L., et al., *Characterization of the lipopolysaccharide of Yersinia pestis.* Microb Pathog, 2001. **30**(2): p. 49-57.
9. Prior, J.L., et al., *The failure of different strains of Yersinia pestis to produce lipopolysaccharide O-antigen under different growth conditions is due to mutations in the O-antigen gene cluster.* FEMS Microbiol Lett, 2001. **197**(2): p. 229-33.
10. Kawahara, K., et al., *Modification of the structure and activity of lipid A in Yersinia pestis lipopolysaccharide by growth temperature.* Infect Immun, 2002. **70**(8): p. 4092-8.
11. Knirel, Y.A., et al., *Cold temperature-induced modifications to the composition and structure of the lipopolysaccharide of Yersinia pestis.* Carbohydr Res, 2005. **340**(9): p. 1625-30.
12. Rebeil, R., et al., *Characterization of late acyltransferase genes of Yersinia pestis and their role in temperature-dependent lipid A variation.* J Bacteriol, 2006. **188**(4): p. 1381-8.
13. Anisimov, A.P., et al., *Effect of deletion of the IpxM gene on virulence and vaccine potential of Yersinia pestis in mice.* J Med Microbiol, 2007. **56**(Pt 4): p. 443-53.
14. Montminy, S.W., et al., *Virulence factors of Yersinia pestis are overcome by a strong lipopolysaccharide response.* Nat Immunol, 2006. **7**(10): p. 1066-73.
15. Lathem, W.W., et al., *Progression of primary pneumonic plague: a mouse model of infection, pathology, and bacterial transcriptional activity.* Proc Natl Acad Sci U S A, 2005. **102**(49): p. 17786-91.
16. Une, T. and R.R. Brubaker, *In vivo comparison of avirulent Vwa- and Pgm- or Pstr phenotypes of yersiniae.* Infect Immun, 1984. **43**(3): p. 895-900.
17. Straley, S.C. and W.S. Bowmer, *Virulence genes regulated at the transcriptional level by Ca2+ in Yersinia pestis include structural genes for outer membrane proteins.* Infect Immun, 1986. **51**(2): p. 445-54.

18. Viboud, G.I. and J.B. Bliska, *Yersinia outer proteins: role in modulation of host cell signaling responses and pathogenesis.* Annu Rev Microbiol, 2005. **59**: p. 69-89.
19. Holmstrom, A., et al., *LcrV is a channel size-determining component of the Yop effector translocon of Yersinia.* Mol Microbiol, 2001. **39**(3): p. 620-32.
20. Neyt, C. and G.R. Cornelis, *Insertion of a Yop translocation pore into the macrophage plasma membrane by Yersinia enterocolitica: requirement for translocators YopB and YopD, but not LcrG.* Mol Microbiol, 1999. **33**(5): p. 971-81.
21. Neyt, C. and G.R. Cornelis, *Role of SycD, the chaperone of the Yersinia Yop translocators YopB and YopD.* Mol Microbiol, 1999. **31**(1): p. 143-56.
22. Matson, J.S. and M.L. Nilles, *LcrG-LcrV interaction is required for control of Yops secretion in Yersinia pestis.* J Bacteriol, 2001. **183**(17): p. 5082-91.
23. Mueller, C.A., et al., *The V-antigen of Yersinia forms a distinct structure at the tip of injectisome needles.* Science, 2005. **310**(5748): p. 674-6.
24. Nakajima, R., V.L. Motin, and R.R. Brubaker, *Suppression of cytokines in mice by protein A-V antigen fusion peptide and restoration of synthesis by active immunization.* Infect Immun, 1995. **63**(8): p. 3021-9.
25. Sing, A., et al., *Yersinia V-antigen exploits toll-like receptor 2 and CD14 for interleukin 10-mediated immunosuppression.* J Exp Med, 2002. **196**(8): p. 1017-24.
26. Sodeinde, O.A. and J.D. Goguen, *Nucleotide sequence of the plasminogen activator gene of Yersinia pestis: relationship to ompT of Escherichia coli and gene E of Salmonella typhimurium.* Infect Immun, 1989. **57**(5): p. 1517-23.
27. Goguen, J.D., T. Bugge, and J.L. Degen, *Role of the pleiotropic effects of plasminogen deficiency in infection experiments with plasminogen-deficient mice.* Methods, 2000. **21**(2): p. 179-83.
28. Lahteenmaki, K., et al., *Expression of plasminogen activator pla of Yersinia pestis enhances bacterial attachment to the mammalian extracellular matrix.* Infect Immun, 1998. **66**(12): p. 5755-62.
29. Lahteenmaki, K., M. Kukkonen, and T.K. Korhonen, *The Pla surface protease/adhesin of Yersinia pestis mediates bacterial invasion into human endothelial cells.* FEBS Lett, 2001. **504**(1-2): p. 69-72.
30. Lathem, W.W., et al., *A plasminogen-activating protease specifically controls the development of primary pneumonic plague.* Science, 2007. **315**(5811): p. 509-13.
31. Welkos, S., et al., *Determination of the virulence of the pigmentation-deficient and pigmentation-/plasminogen activator-deficient strains of Yersinia pestis in non-human primate and mouse models of pneumonic plague.* Vaccine, 2002. **20**(17-18): p. 2206-14.
32. Welkos, S.L., A.M. Friedlander, and K.J. Davis, *Studies on the role of plasminogen activator in systemic infection by virulent Yersinia pestis strain C092.* Microb Pathog, 1997. **23**(4): p. 211-23.
33. Kukkonen, M., et al., *Lack of O-antigen is essential for plasminogen activation by Yersinia pestis and Salmonella enterica.* Mol Microbiol, 2004. **51**(1): p. 215-25.

34. Pouillot, F., et al., *Evaluation of O-antigen inactivation on Pla activity and virulence of Yersinia pseudotuberculosis harbouring the pPla plasmid.* Microbiology, 2005. **151**(Pt 11): p. 3759-68.
35. Du, Y., R. Rosqvist, and A. Forsberg, *Role of fraction 1 antigen of Yersinia pestis in inhibition of phagocytosis.* Infect Immun, 2002. **70**(3): p. 1453-60.
36. Galyov, E.E., et al., *Expression of the envelope antigen F1 of Yersinia pestis is mediated by the product of caf1M gene having homology with the chaperone protein PapD of Escherichia coli.* FEBS Lett, 1991. **286**(1-2): p. 79-82.
37. Galyov, E.E., et al., *Nucleotide sequence of the Yersinia pestis gene encoding F1 antigen and the primary structure of the protein. Putative T and B cell epitopes.* FEBS Lett, 1990. **277**(1-2): p. 230-2.
38. Karlyshev, A.V., et al., *A new gene of the f1 operon of Y. pestis involved in the capsule biogenesis.* FEBS Lett, 1992. **297**(1-2): p. 77-80.
39. Karlyshev, A.V., et al., *Caf1R gene and its role in the regulation of capsule formation of Y. pestis.* FEBS Lett, 1992. **305**(1): p. 37-40.
40. Cavanaugh, D.C. and R. Randall, *The role of multiplication of Pasteurella pestis in mononuclear phagocytes in the pathogenesis of flea-borne plague.* J Immunol, 1959. **83**: p. 348-63.
41. Liu, F., et al., *Effects of Psa and F1 on the adhesive and invasive interactions of Yersinia pestis with human respiratory tract epithelial cells.* Infect Immun, 2006. **74**(10): p. 5636-44.
42. Davis, K.J., et al., *Pathology of experimental pneumonic plague produced by fraction 1-positive and fraction 1-negative Yersinia pestis in African green monkeys (Cercopithecus aethiops).* Arch Pathol Lab Med, 1996. **120**(2): p. 156-63.
43. Winter, C.C., W.B. Cherry, and M.D. Moody, *An unusual strain of Pasteurella pestis isolated from a fatal human case of plague.* Bull World Health Organ, 1960. **23**: p. 408-9.
44. Williams, J.E., D.N. Harrison, and D.C. Cavanaugh, *Letter: Cryptic infection of rats with non-encapsulated variants of Yersinia pestis.* Trans R Soc Trop Med Hyg, 1975. **69**(1): p. 171-2.
45. Meyer, K.F., et al., *Live, attenuated Yersinia pestis vaccine: virulent in nonhuman primates, harmless to guinea pigs.* J Infect Dis, 1974. **129**: p. Suppl:S85-12.
46. Wang, S., et al., *A DNA vaccine producing LcrV antigen in oligomers is effective in protecting mice from lethal mucosal challenge of plague.* Vaccine, 2004. **22**(25-26): p. 3348-57.
47. Andrews, G.P., et al., *Fraction 1 capsular antigen (F1) purification from Yersinia pestis CO92 and from an Escherichia coli recombinant strain and efficacy against lethal plague challenge.* Infect Immun, 1996. **64**(6): p. 2180-7.
48. Chiuchiolo, M.J., et al., *Protective immunity against respiratory tract challenge with Yersinia pestis in mice immunized with an adenovirus-based vaccine vector expressing V antigen.* J Infect Dis, 2006. **194**(9): p. 1249-57.
49. Heath, D.G., et al., *Protection against experimental bubonic and pneumonic plague by a recombinant capsular F1-V antigen fusion protein vaccine.* Vaccine, 1998. **16**(11-12): p. 1131-7.

50. Titball, R.W., et al., *Expression of the Yersinia pestis capsular antigen (F1 antigen) on the surface of an aroA mutant of Salmonella typhimurium induces high levels of protection against plague.* Infect Immun, 1997. **65**(5): p. 1926-30.
51. Pendrak, M.L. and R.D. Perry, *Characterization of a hemin-storage locus of Yersinia pestis.* Biol Met, 1991. **4**(1): p. 41-7.
52. Perry, R.D., M.L. Pendrak, and P. Schuetze, *Identification and cloning of a hemin storage locus involved in the pigmentation phenotype of Yersinia pestis.* J Bacteriol, 1990. **172**(10): p. 5929-37.
53. Bearden, S.W., J.D. Fetherston, and R.D. Perry, *Genetic organization of the yersiniabactin biosynthetic region and construction of avirulent mutants in Yersinia pestis.* Infect Immun, 1997. **65**(5): p. 1659-68.
54. Fetherston, J.D., P. Schuetze, and R.D. Perry, *Loss of the pigmentation phenotype in Yersinia pestis is due to the spontaneous deletion of 102 kb of chromosomal DNA which is flanked by a repetitive element.* Mol Microbiol, 1992. **6**(18): p. 2693-704.
55. Buchrieser, C., M. Prentice, and E. Carniel, *The 102-kilobase unstable region of Yersinia pestis comprises a high-pathogenicity island linked to a pigmentation segment which undergoes internal rearrangement.* J Bacteriol, 1998. **180**(9): p. 2321-9.
56. Buchrieser, C., et al., *The 102-kilobase pgm locus of Yersinia pestis: sequence analysis and comparison of selected regions among different Yersinia pestis and Yersinia pseudotuberculosis strains.* Infect Immun, 1999. **67**(9): p. 4851-61.
57. Galvan, E.M., H. Chen, and D.M. Schifferli, *The Psa fimbriae of Yersinia pestis interact with phosphatidylcholine on alveolar epithelial cells and pulmonary surfactant.* Infect Immun, 2007. **75**(3): p. 1272-9.
58. Lindler, L.E., M.S. Klempner, and S.C. Straley, *Yersinia pestis pH 6 antigen: genetic, biochemical, and virulence characterization of a protein involved in the pathogenesis of bubonic plague.* Infect Immun, 1990. **58**(8): p. 2569-77.
59. Rust, J.H., Jr., et al., *Susceptibility of rodents to oral plague infection: a mechanism for the persistence of plague in inter-epidemic periods.* J Wildl Dis, 1972. **8**(2): p. 127-33.
60. Cowan, C., et al., *Invasion of epithelial cells by Yersinia pestis: evidence for a Y. pestis-specific invasin.* Infect Immun, 2000. **68**(8): p. 4523-30.
61. Overheim, K.A., et al., *LcrV plague vaccine with altered immunomodulatory properties.* Infect Immun, 2005. **73**(8): p. 5152-9.
62. Cornelis, G.R., *The Yersinia Ysc-Yop virulence apparatus.* Int J Med Microbiol, 2002. **291**(6-7): p. 455-62.
63. Derewenda, U., et al., *The Structure of Yersinia pestis V-Antigen, an Essential Virulence Factor and Mediator of Immunity against Plague.* Structure, 2004. **12**: p. 301-306.
64. Mueller, C.A., et al., *The V-Antigen of Yersinia Forms a Distinct Structure at the Tip of Injectisome Needles.* Science, 2005. **310**: p. 674-676.
65. Hill, J., et al., *Regions of Yersinia pestis V Antigen that Contribute to Protection against Plague Identified by Passive and Active Immunization.* Infect Immun, 1997. **65**: p. 4476-4482.

66. Williamson, E.D., et al., *Human immune response to a plague vaccine comprising recombinant F1 and V antigens.* Infect Immun, 2005. **73**(6): p. 3598-608.
67. Brubaker, R.R., *Interleukin-10 and Inhibition of Innate Immunity to Yersiniae: Roles of Yops and LcrV (V Antigen).* Infect Immun, 2003. **71**: p. 3673-3681.
68. Nakajima, R., V.L. Motin, and R.R. Brubaker, *Suppression of Cytokines in Mice by Protein A-V Antigen Fusion Peptide and Restoration of Synthesis by Active Immunization.* Infect Immun, 1995. **63**: p. 3021-3029.
69. Sing, A., et al., *Yersinia enterocolitica Evasion fo the Host Innate Immune Response by V-Antigen-Induced IL-10 Production of Macrophages is Abrogated in IL-10 Deficient Mice.* J Immun, 2001: p. 1315-1321.
70. Sing, A., et al., *Yersinia V-Antigen Exploits Toll-like Receptor 2 and CD14 for Interleukin 10-Mediated Immunosuppression.* J Exp Med, 2002. **196**: p. 1017-1024.
71. Sing, A., et al., *A Hypervariable N-terminal region of Yersinia LcrV determines Toll-like receptor 2-mediated IL-10 induction and mouse virulence.* PNAS, 2005. **102**: p. 16049-16054.
72. Overheim, K.A., et al., *LcrV Plague Vaccine with Altered Immunomodulary Properties.* Infect Immun, 2005. **73**: p. 5152-5159.
73. Akira, S., S. Uematsu, and O. Takeuchi, *Pathogen recognition and innate immunity.* Cell, 2006. **124**(4): p. 783-801.
74. Akira, S. and K. Takeda, *Toll-like Receptor Signalling.* Nat Rev, 2004. **4**: p. 499-458.
75. Netea, M.G., J.W.M. Van der Meer, and B.J. Kullberg, *Toll-like Receptors as an escape mechanism from the host defense.* Trends Microbiol, 2004. **12**: p. 484-488.
76. Gehring, A.J., et al., *The Mycobacterium tuberculosis 19-kilodalton lipoprotein inhibits gamma interferon-regulated HLA-DR and Fc gamma R1 on human macrophages through Toll-like receptor 2.* Infect Immun, 2003. **71**(8): p. 4487-97.
77. Netea, M.G., et al., *Toll-like Receptor 2 Suppressed Immunity against Candida albicans through Induction of IL-10 and Regulatory T-cells.* J Immun, 2004: p. 3712-3718.
78. Latz, E., et al., *Lipopolysaccharide rapidly traffics to and from the Golgi apparatus with the toll-like receptor 4-MD-2-CD14 complex in a process that is distinct from the initiation of signal transduction.* J Biol Chem, 2002. **277**(49): p. 47834-43.
79. Hirschfeld, M., et al., *Cutting edge: repurification of lipopolysaccharide eliminates signaling through both human and murine toll-like receptor 2.* J Immunol, 2000. **165**(2): p. 618-22.
80. Gill, S.C. and P.H. von Hippel, *Calculation of protein extinction coefficients from amino acid sequence data.* Anal Biochem, 1989. **182**(2): p. 319-26.
81. Takeuchi, O., et al., *Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components.* Immunity, 1999. **11**: p. 443-451.
82. Sing, A., et al., *A hypervariable N-terminal region of Yersinia LcrV determines Toll-like receptor 2-mediated IL-10 induction and mouse virulence.* Proc Natl Acad Sci U S A, 2005. **102**(44): p. 16049-54.

83. Tito, M.A., et al., *Probing molecular interactions in intact antibody: antigen complexes, an electrospray time-of-flight mass spectrometry approach.* Biophys J, 2001. **81**(6): p. 3503-9.
84. Montminy SW, et al., *Virulence factors of Yersinia pestis are overcome by a strong lipopolysaccharide response.* Nat. Immunol., 2006. **7**(10): p. 1066-1073.
85. Sing, A., et al., *Contribution of toll-like receptors 2 and 4 in an oral Yersinia enterocolitica mouse infection model.* Int J Med Microbiol, 2003. **293**(5): p. 341-8.
86. Nedialkov, Y.A., V.L. Motin, and R.R. Brubaker, *Resistance to Lipopolysaccharide Mediated by the Yersinia pestis V Antigen-Polyhistidine Fusion Peptide: Amplification of Interleukin-10.* Infect Immun, 1997. **65**: p. 1196-1203.
87. Motin, V.L., et al., *Passive Immunity to Yersiniae Mediated by Anti-Recombinant V Antigen and Protein A-V Fusion Peptide.* Infect Immun, 1994. **62**: p. 4192-4201.
88. Kirschning, C.J., et al., *Human toll-like receptor 2 confers responsiveness to bacterial lipopolysaccharide.* J Exp Med, 1998. **188**(11): p. 2091-7.
89. Yang, R.B., et al., *Toll-like receptor-2 mediates lipopolysaccharide-induced cellular signalling.* Nature, 1998. **395**(6699): p. 284-8.
90. Reithmeier-Rost, D., et al., *The weak interaction of LcrV and TLR2 does not contribute to the virulence of Yersinia pestis.* Microbes Infect, 2007. **9**(8): p. 997-1002.
91. Abramov, V.M., et al., *Attachment of LcrV from Yersinia pestis at dual binding sites to human TLR-2 and human IFN-gamma receptor.* J Proteome Res, 2007. **6**(6): p. 2222-31.
92. www.cdc.gov/ncidod/dvbid/plague/. 2007.
93. www.who.int/csr/disease/plague/en/, 2007.
94. Wu, L.T., *A treatise on pneumonic plague*. Health. 1926: Geneva: League of Nations.
95. Butler, T., et al., *Experimental Yersinia pestis infection in rodents after intragastric inoculation and ingestion of bacteria.* Infect Immun, 1982. **36**(3): p. 1160-7.
96. Anderson, G.W., Jr., et al., *Recombinant V antigen protects mice against pneumonic and bubonic plague caused by F1-capsule-positive and -negative strains of Yersinia pestis.* Infect Immun, 1996. **64**(11): p. 4580-5.
97. Anderson, G.W., Jr., et al., *Protection of mice from fatal bubonic and pneumonic plague by passive immunization with monoclonal antibodies against the F1 protein of Yersinia pestis.* Am J Trop Med Hyg, 1997. **56**(4): p. 471-3.
98. Byrne, W.R., et al., *Antibiotic treatment of experimental pneumonic plague in mice.* Antimicrob Agents Chemother, 1998. **42**(3): p. 675-81.
99. Lawson, J.N., C.R. Lyons, and S.A. Johnston, *Expression profiling of Yersinia pestis during mouse pulmonary infection.* DNA Cell Biol, 2006. **25**(11): p. 608-16.
100. Bohnhoff, M., C.P. Miller, and W.R. Martin, *Resistance of the Mouse's Intestinal Tract to Experimental Salmonella Infection. Ii. Factors Responsible for Its Loss Following Streptomycin Treatment.* J Exp Med, 1964. **120**: p. 817-28.

101. Field, L.H., et al., *Persistence and spread of Candida albicans after intragastric inoculation of infant mice.* Infect Immun, 1981. **31**(2): p. 783-91.
102. Edwards, R.A. and D.M. Schifferli, *Differential regulation of fasA and fasH expression of Escherichia coli 987P fimbriae by environmental cues.* Mol Microbiol, 1997. **25**(4): p. 797-809.
103. Iyer, R., N.S. Baliga, and A. Camilli, *Catabolite control protein A (CcpA) contributes to virulence and regulation of sugar metabolism in Streptococcus pneumoniae.* J Bacteriol, 2005. **187**(24): p. 8340-9.
104. Leong, J.M., R.S. Fournier, and R.R. Isberg, *Identification of the integrin binding domain of the Yersinia pseudotuberculosis invasin protein.* Embo J, 1990. **9**(6): p. 1979-89.
105. Leong, J.M., R.S. Fournier, and R.R. Isberg, *Mapping and topographic localization of epitopes of the Yersinia pseudotuberculosis invasin protein.* Infect Immun, 1991. **59**(10): p. 3424-33.
106. Leong, J.M., P.E. Morrissey, and R.R. Isberg, *A 76-amino acid disulfide loop in the Yersinia pseudotuberculosis invasin protein is required for integrin receptor recognition.* J Biol Chem, 1993. **268**(27): p. 20524-32.
107. Leong, J.M., et al., *An aspartate residue of the Yersinia pseudotuberculosis invasin protein that is critical for integrin binding.* Embo J, 1995. **14**(3): p. 422-31.
108. Simonet, M., et al., *Invasin production by Yersinia pestis is abolished by insertion of an IS200-like element within the inv gene.* Infect Immun, 1996. **64**(1): p. 375-9.
109. Sikkema, D.J. and R.R. Brubaker, *Resistance to pesticin, storage of iron, and invasion of HeLa cells by Yersiniae.* Infect Immun, 1987. **55**(3): p. 572-8.
110. Benedek, O., G. Nagy, and L. Emody, *Intracellular signalling and cytoskeletal rearrangement involved in Yersinia pestis plasminogen activator (Pla) mediated HeLa cell invasion.* Microb Pathog, 2004. **37**(1): p. 47-54.
111. Santer, M. and S. Ajl, *Metabolic reactions of pasteurella pestis. I. Terminal oxidation.* J Bacteriol, 1954. **67**(4): p. 379-86.
112. Santer, M. and S. Ajl, *Metabolic reactions of Pasteurella pestis. III. The hexose monophosphate shunt in the growth of Pasteurella pestis.* J Bacteriol, 1955. **69**(6): p. 713-8.
113. Santer, M. and S. Ajl, *Metabolic reaction of Pasteurella pestis. II. The fermentation of glucose.* J Bacteriol, 1955. **69**(3): p. 298-302.
114. Shaw, L.M., et al., *Activation of phosphoinositide 3-OH kinase by the alpha6beta4 integrin promotes carcinoma invasion.* Cell, 1997. **91**(7): p. 949-60.
115. Shaw, L.M., C.E. Turner, and A.M. Mercurio, *The alpha 6A beta 1 and alpha 6B beta 1 integrin variants signal differences in the tyrosine phosphorylation of paxillin and other proteins.* J Biol Chem, 1995. **270**(40): p. 23648-52.
116. Sinha, B., et al., *Fibronectin-binding protein acts as Staphylococcus aureus invasin via fibronectin bridging to integrin alpha5beta1.* Cell Microbiol, 1999. **1**(2): p. 101-17.
117. Molinari, G., et al., *Two distinct pathways for the invasion of Streptococcus pyogenes in non-phagocytic cells.* Cell Microbiol, 2000. **2**(2): p. 145-54.

118. Thomas, R.J. and T.J. Brooks, *Oligosaccharide receptor mimics inhibit Legionella pneumophila attachment to human respiratory epithelial cells.* Microb Pathog, 2004. **36**(2): p. 83-92.
119. Thomas, R. and T. Brooks, *Attachment of Yersinia pestis to human respiratory cell lines is inhibited by certain oligosaccharides.* J Med Microbiol, 2006. **55**(Pt 3): p. 309-15.
120. Mekalanos, J.J., *Environmental signals controlling expression of virulence determinants in bacteria.* J Bacteriol, 1992. **174**(1): p. 1-7.
121. Schmiel, D.H., G.M. Young, and V.L. Miller, *The Yersinia enterocolitica phospholipase gene yplA is part of the flagellar regulon.* J Bacteriol, 2000. **182**(8): p. 2314-20.
122. Johri, A.K., et al., *Transcriptional and proteomic profiles of group B Streptococcus type V reveal potential adherence proteins associated with high-level invasion.* Infect Immun, 2007. **75**(3): p. 1473-83.
123. Johri, A.K., et al., *Oxygen regulates invasiveness and virulence of group B streptococcus.* Infect Immun, 2003. **71**(12): p. 6707-11.
124. Thompson, A., et al., *The bacterial signal molecule, ppGpp, mediates the environmental regulation of both the invasion and intracellular virulence gene programs of Salmonella.* J Biol Chem, 2006. **281**(40): p. 30112-21.