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Interactions of Francisella Tularensis With Components of the Host Fibrinolytic System

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Interactions of Francisella Tularensis With Components of the Host Fibrinolytic System

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**INTERACTIONS OF *FRANCISELLA TULARENSIS* WITH COMPONENTS OF THE
HOST FIBRINOLYTIC SYSTEM**

A Dissertation
Presented for
The Graduate Studies Council
The University of Tennessee
Health Science Center

In Partial Fulfillment
Of the Requirements for the Degree
Doctor of Philosophy
From The University of Tennessee

By
Shawn Russell Clinton
December 2010

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DEDICATION

I dedicate this dissertation to my mother,
Denese Clinton
and my father,
Kenneth Clinton
for encouraging and supporting my childhood dream of becoming a scientist.

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I thank my mentor, Dr. Mark A. Miller, for his guidance, encouragement, and patience over the last six years. I would like to thank my other committee members, Dr. James Bina, Dr. Richard Lee, Dr. Tony Marion, and Dr. Michael Whitt for their guidance and encouragement throughout the process of completing my dissertation work. I would like to thank past and present members of the laboratory, Dr. Christy Lavine, Dr. Irena Angelova-Fischer, Dr. Himangi Jayakar, and Jyothi Parvathareddy for their encouragement and support, but also for being great friends to me. I also thank Dr. Renee Bina and Dr. Thomas Hatch for answering my many questions about my work. I owe a special thanks to Cory Blackwell for all of the interesting scientific as well as non-scientific conversations that I have enjoyed over the years.

ABSTRACT

Francisella tularensis (FT) is a Gram-negative coccobacillus and causative agent of a life-threatening disease commonly referred to as tularemia. Due to the highly infectious nature of the organism, its previous development as a biowarfare agent and its potential use in acts of bioterrorism, this bacterium is listed as a Category A select agent by the Centers for Disease Control and Prevention (CDC). Efforts to understand the pathogenic mechanisms of FT within the host environment are vital for the development of safe and effective vaccines, as well as treatments, against tularemia. Though considered an intracellular pathogen, FT research of late has shown an abundance of extracellular bacteria in the plasma fraction of host's blood during infection. The goal of these studies was to identify and characterize the interactions of *Francisella tularensis* with components of the host fibrinolytic system.

Because many bacterial pathogens utilize proteins found in the host plasma fraction as a means to augment dissemination as well as to defend themselves against components of the host immune system, I first sought to determine if FT was able to bind these on its surface *in vitro* using fresh frozen human plasma. By using an ELISA assay I was able to detect significant levels of plasminogen and fibronectin binding to the bacterial cell surface. Based on this observation, further studies were designed to dissect possible *in vitro* and *in vivo* functions of these FT-bound host proteins.

Plasminogen (PLG) is a zymogen that upon activation is converted into the serine protease plasmin, which plays a key role in the proteolytic degradation of fibrin blood clots. Many pathogenic organisms have taken advantage of both the broad-specificity and abundance of this circulating host protein by binding it to their surface, which not only gives the pathogen an ability to degrade extracellular matrix components for dissemination but also aids in the defense against host immune responses. The ability of FT to acquire surface bound PLG that can be activated may be an important virulence mechanism that results in an increase in initial infectivity, survival, and/or dissemination of this bacterium *in vivo*.

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LIST OF ABBREVIATIONS

εACA	Epsilon Aminocaproic Acid
ANOVA	Analysis of Variation
BHI	Brain-heart Infusion
BSA	Bovine Serum Albumin
CDC	Centers for Disease Control
CF	Culture Filtrate
CFP	Culture Filtrate Precipitate
CFU	Colony Forming Units
CR3	Complement Receptor 3
CR4	Complement Receptor 4
ECM	Extracellular Matrix
ELISA	Enzyme-linked Immunosorbant Assay
FDA	Food and Drug Administration
FFP	Fresh Frozen Plasma
FH	Factor H
FPI	<i>Francisella</i> Pathogenicity Island
FT	<i>Francisella tularensis</i>
HRP	Horseradish Peroxidase
IgG	Immunoglobulin Gamma
i.n.	Intranasal
i.p.	Intraperitoneal
IP	Immunoprecipitation
kb	Kilobase
kDa	Kilodalton

LD ₅₀	Lethal Dose 50%
LPS	Lipopolysaccharide
LVS	Live Vaccine Strain
MAC	Membrane Attack Complex
MALDI-LTQ	Matrix-assisted Laser Desorption/Ionization - Linear Trap Quadrupole
MMH	Modified Mueller-Hinton
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Salin
PLG	Plasminogen
pNA	P-nitroaniline
PRMM	Pyrogallol Red Molybdate Methanol
PVDF	Polyvinylidene Fluoride
rIL-12	Recombinant Interleukin-12
RPM	Revolutions Per Minute
SDS	Sodium Dodecyl Sulfate
TBS	Tris-buffered Saline + 0.5% Tween-20
TMB	Trimethyl Benzamine
tPA	Tissue-type Plasminogen Activator
uPA	Urokinase-type Plasminogen Activator
2-ME	2-Mercaptoethanol

CHAPTER 1. INTRODUCTION

Francisella tularensis

Francisella tularensis (FT) is a Gram-negative intracellular coccobacilli and the causative agent of a life-threatening zoonotic disease known as tularemia. Being one of the most infectious bacteria known to man (LD50 ~10 organisms in humans for type A strains), *F. tularensis* is listed as a Centers for Disease Control and Prevention Category A select agent because of its potential use as a bioweapon. Named after Tulare County, California, where in 1911 a plague-like disease in ground squirrels was described by McCoy and Chapin (1), *F. tularensis* has been found throughout the Northern Hemisphere and infects a variety of vertebrate and invertebrate hosts (1-2). *Francisella* is the only member of the family *Francisellaceae* and is composed of the two species *F. tularensis* and *F. philomiragia*. Of the two *Francisella* species, *F. tularensis* is made of four subspecies: *tularensis* (type A), *holarctica* (type B), *mediasiatica*, and *novicida*.

F. tularensis subsp. *holarctica*, from which the attenuated live vaccine strain (FT LVS) was derived, is less virulent in humans than type A FT subspecies. This subspecies of FT is mostly found in Europe and Asia, but also found in North America. type B FT is biochemically differentiated from most other *Francisella* species by its lack of citrulline ureidase activity and an inability to produce acid from glycerol. Based on biochemical phenotyping, pathogenicity, and geographic distribution, three biovars of *F. tularensis* subsp. *holarctica* have been suggested but not yet recognized by international taxonomic committees: biovar I ery^S (erythromycin sensitive), biovar I ery^R (erythromycin sensitive), and *japonica* (3).

The most severe cases of human tularemia are often attributed to *F. tularensis* subsp. *tularensis* which make up approximately two-thirds of reported cases and found almost exclusively in North American infections (4). Type A FT can be biochemically differentiated from other species of *Francisella* by its demonstration of citrulline ureidase activity and by its ability to produce acid from glycerol fermentation. Recently, genomic analyses have further divided *F. tularensis* subsp. *tularensis* into two distinct subclades, with the A.I clade being found mostly in the central and eastern United States and the A.II clade mostly in the western United States (5). Hotspots of tularemia, especially those in and around Arkansas, are geographically associated with areas containing the A.I clade of FT (5).

Environmental Sources of *Francisella*

Though commonly associated with infections in lagomorphs, *F. tularensis* has been shown to infect a broad range of hosts (over 200), including mammals, birds, amphibians, and invertebrates. Because of this extended host diversity, there has been some difficulty in identifying a primary reservoir and clear routes of infection for FT in the environment. Sources of infection with *F. tularensis* in man and mammals are often associated with blood sucking insects such as the deer fly (*Chrysops discalis*) (6-7), mosquitoes (8-9) and ticks (*Dermacentor* and *Ixodes*) (6-7, 10) that feed on the blood of infected animals. *Francisella* has often been associated with waterways and is known to persist for long periods of time in moist environments. Additionally, *Francisella* has also been shown to infect protozoans such as *Acanthamoeba castellanii* (11) and has also

been shown to reside in cysts of this amoeba for up to 3 weeks post-infection (12). Recent work has also shown the ability of *F. tularensis* subsp *novicida* to form biofilms on chitin (13), a component of many arthropods and invertebrates found in aqueous habitats, making this a possible method of both survival under nutrient poor conditions and transmission to mammalian hosts. Overall, these data suggest that the life cycle of *Francisella* in the environment is quite complex and that this pathogen is well-suited for survival in a variety of conditions, whether inside or outside its chosen host.

Natural Infection and Symptoms of Tularemia

Cases of human infection with *F. tularensis* have often been noted in hunters of wild game, landscape laborers, and laboratory personnel (6, 14). Following exposure to this bacterium, symptoms of tularemia may become apparent in the range of 3 to 14 days, which may depend on the route of infection, number of bacteria, and immune status of the host. A fever is a general symptom in all forms of tularemia that in some cases could reach as high as 104°F. Other symptoms of tularemic infection may include chills, aches, and progressive weakness. Infected blood-sucking insects (6-10) and open-wound contact with infected animal tissue (6, 15-16) are common causes of ulceroglandular or glandular (without ulcerous lesions) forms of tularemia. It is believed that when bacteria enter the host through the broken skin they are carried by the lymph ducts to draining lymph nodes, causing a visible and painful enlargement of this tissue, followed by the dissemination of FT to other areas of the body.

Two less common, but potentially serious forms of tularemia are the oropharyngeal and oculoglandular manifestations of the disease. Ingestion of FT-contaminated materials or water is associated with oropharyngeal tularemia, typically displaying a pronounced cervical lymphadenopathy (17-19). Another less common, but significant, FT infection is known as oculoglandular tularemia, which may be caused by touching the eye with a contaminated hand (7, 20-21).

The pneumonic form of tularemia has the highest mortality (30-60% of untreated cases) and often is the result of inhalation of infectious particulate matter and aerosols. This form of the tularemia has been reported as the result of landscape workers inadvertently aerosolizing infected carcasses with lawn mowing equipment (18, 22-23). Farmers that have been involved with work such as hay-making have also been infected with pneumonic tularemia (24-25).

***Francisella* as a Potential Bioweapon**

Due to the high virulence and low inoculum needed (approximately 10 organisms) to cause a potentially fatal infection (26-27), this organism was studied in bioweapon programs in Japan, the United States, and the former Soviet Union as an offensive weapon (28). Being that FT is easily isolated from the environment and can be grown to large quantities without sophisticated equipment, it is of considerable concern that this organism could be used by various terrorist groups worldwide to infect human populations on a large scale. It has been estimated that 50kg of FT dispersed over a city of 5 million people would result in the infection of 250,000 individuals and approximately 19,000 deaths, with prolonged illness and relapses of infection lasting several weeks (29). The financial impact of such an intentional release of FT was

estimated by the Centers for Disease Control (CDC) to cost \$5.4 billion per 100,000 people infected (30) and would most likely overwhelm public health services. The financial and life-threatening potential of FT has led this organism to be designated a Category A select agent by the CDC, a group which also includes other dangerous organisms such as those causing anthrax and smallpox.

Vaccination against Tularemia

It is believed that survival of tularemia results in life-long immunity to reinfection with this disease in humans, suggesting that a protective vaccine is possible. Attempts to develop a safe and effective vaccine against tularemia have been met with various degrees of success in the past. Some of the first vaccination methods attempted were killed bacterial vaccines tested by Foshay during the 1930's and 1940's which consisted of formalized, heat killed, and oxidized preparations of virulent FT strains, all of which offered very limited protection against human tularemia although eliciting significant anti-FT serum antibody titers (31). Protection against infection with *F. tularensis* is currently limited to scarification with an attenuated live vaccine strain (FT LVS) of *F. tularensis* subsp *holarctica*, but is currently not licensed and does not illicit complete immunity against infection with type A strains of FT (26-27, 32). An attenuated Ft Schu S4 strain was reported by Twine *et al* to protect mice against wild-type homologous challenge after sublethal infection, though currently untested in humans (33). Murine models of infection have shown that anti-FT antibodies alone are able to protect against infection with type B strains of *Francisella* (34), but in studies using the more virulent type A strains interferon-gamma as well as CD4+ and CD8+ T-cells are needed to survive a lethal bacterial challenge (35-36). Immunization of mice with LPS extracted from FT bacteria offers protection against lethal challenge infections with FT LVS, but fails to protect against challenge with the highly virulent FT Schu S4 strain (36-37). Furthermore, infection of mice using a mutant strain of FT LVS with a transposon-inactivated *wbtA* gene results in an LPS O-antigen-deficient phenotype and further attenuation that offers protection in wt FT LVS challenge experiments (38-39) and suggests a dispensable role for anti-LPS antibodies in adaptive immune protection against lethal FT infection. Work by Huntley *et al* have shown that the immunization of mice with isolated native outer-membranes from FT LVS were reported to increase survival as well as decrease bacterial dissemination to the spleen and liver after an intranasal challenge with type A FT Schu S4 (40). All together, these studies seem to suggest that both humoral and cell-mediated immunity directed towards components of the bacterial envelope may provide our best hope in developing a safe and effective vaccine against lethal FT infection.

Survival of *Francisella tularensis* in the Host Environment

In the extracellular environment of the host, FT is resistant to complement-mediated lysis (41). Of particular interest is a requirement of host complement C3 deposition on the bacterial cell surface for opsonophagocytosis of FT by activating CR3 and CR4-mediated phagocytosis in macrophages and dendritic cells (42-43). Upon entry into a susceptible vertebrate host, *F. tularensis* is readily phagocytized by resident macrophages and dendritic cells via spacious, asymmetrical pseudopod loops (43). By a mechanism that is currently unknown, *Francisella* escapes from the phagosome beginning approximately one hour after being taken up by the phagocyte, prior to

lysosomal fusion and complete acidification of the vesicle and enters the cytoplasm (44). It then multiplies in the host cell cytoplasm (45-46) before an induction of ASC/caspase1 and apoptosis with subsequent release of bacteria to initiate another round of infection (47-49).

Genetic screening methodologies have identified many virulence genes associated with the ability of *Francisella* to infect host cells *in vivo* and *in vitro*, as reviewed by Meiborn and Charbit (50). Following the completed sequencing of several *Francisella* genomes, a group of highly conserved genes were identified in all FT genomes to date and are known as the *Francisella* pathogenicity island (FPI) (51). The FPI is approximately 30 kb in length and has been found in duplicate copies (51) within all known *Francisella* species except for *F. novicida* and *F. philomiragia* which have only a single copy (52). Within this operon, six genes (*pdpB*, *pdpC*, *iglD*, *iglC*, *iglB*, and *iglA*) have been identified as virulence factors that are necessary for host cell infection (53-58), though their functions are currently unresolved. Mutational studies of other genes outside of the FPI, such as those thought to be involved in capsule production (*capA*, *capB*, and *capC*), pili (*pilT*, *pilA*, and *pilF*), LPS O-antigen synthesis, and various other uncharacterized membrane/envelope proteins have also been shown to play important roles in the ability of FT to survive in the intracellular and extracellular host environment (55-56, 58-59).

Recent studies have shown that significant numbers of these released FT bacteria are found in the acellular plasma fraction of mice infected intradermally or intranasally with either FT live vaccine strain (LVS) (type B) or *F. tularensis* Schu S4 (type A) (60), and intranasally with *F. novicida* (61). These findings suggest that, in addition to utilizing the intracellular cytoplasmic niche for replication and protection from humoral immunity, FT may also have a significant extracellular phase. When taking into account both the high concentration and sheer multitude of proteins found in host plasma, there lies an opportunity for those freely circulating bacteria to interact with host molecules in ways to aid its survival and/or invasiveness within the infected host. This poorly studied phase of FT infection deserves attention and may unveil potentially valuable molecular targets for the treatment and/or prevention of tularemia.

One very interesting example of how *Francisella* interacts with a host plasma/serum component can be found within work with factor H. Several studies have shown that deposition of host complement component C3 on the surface of FT is required for opsonophagocytosis by activating complement receptor (CR3 and CR4)-mediated phagocytosis by macrophages and dendritic cells (42-43, 62). It is also known that FT is relatively resistant to complement-mediated lysis (41). A recent report suggested that resistance of FT to membrane attack complex-mediated lysis may be due (at least in part) to its ability to bind to factor H, a glycoprotein found in host plasma with complement regulatory activity (63). The normal function of factor H on the surface of host cells is to bind and inactivate C3 convertase (C3bBb) and ultimately prevent formation of the cytolytic membrane attack complex (MAC). By binding soluble factor H from host blood, FT is able to prevent MAC formation on the bacterial envelope, yet at the same time, acquire a necessary protein (iC3b) for gaining entry into phagocytic cells via complement receptor-mediated phagocytosis (63). It is possible that besides this ability of *Francisella* to bind to factor H, there lies the potential for other host plasma components to also play a significant role in its pathogenesis.

Interactions of Bacterial Pathogens with Host Plasminogen/Plasmin

It has long been established that a broad spectrum of both Gram-positive and Gram-negative bacterial pathogens gain a survival advantage by interacting with plasma proteins, and in particular, those components of the host coagulation/fibrinolytic system in humans (64-67). For instance, the ability to acquire surface-associated plasmin has been documented as an important virulence mechanism in Group A streptococci (68), *Borrelia burgdorferi* (69), and *Yersinia pestis* (70) by aiding in the organism's ability to penetrate the extracellular matrix and to disseminate to distal sites in the host. *Borrelia burgdorferi* has even been shown to utilize plasmin derived from the blood meal of ticks to aid in its passage from the gut to the salivary glands of its arthropod host, thus allowing the bacteria to be passed to a subsequent mammalian host (69). Plasminogen (PLG) is a 92-kDa glycoprotein zymogen that is involved in fibrinolysis. This precursor protein is converted to an active serine protease (plasmin) via cleavage of the peptide bond between residues R⁵⁶⁰ and V⁵⁶¹ *in vivo* via urokinase-type (uPA) and/or tissue-type (tPA) PLG activators. Plasmin has an important role in blood clot resolution because of its role in the degradation of fibrin polymers. Because plasmin has other substrates that include pro-collagenases, pro-metalloproteinases, and extracellular matrix proteins, such as fibronectin, laminin, and vitronectin, the ability of a bacterium to acquire surface associated plasmin can result in an enhanced ability of the pathogen to penetrate the extracellular matrix and disseminate to distal sites in the host (64, 66, 68).

Summary and Purpose

Francisella tularensis is a highly contagious bacterial pathogen with the potential to cause widespread illness and subsequently strain emergency response services in the event of a deliberate dispersal of the organism on human populations. Despite the best efforts of *Francisella* researchers that have been attempting to unravel the mechanisms behind this stealthy and effective pathogen for nearly a century, our work is far from complete. Though much work has been published on the intracellular aspect of its infectious cycle within the host, little has been shown on its extracellular phase which may provide valuable insight into *Francisella* pathogenesis. The overall goals of the studies described in this work sought to focus on the following: 1) To characterize the possible interactions of *Francisella* with human plasma proteins that have been shown to be exploited by other bacterial pathogens, in particular, plasminogen, 2) To identify receptors for any plasma proteins on the bacterial outer envelope by proteomic methodologies, and 3) To characterize any possible functions for these plasma proteins that may aid in the pathogenesis of *Francisella*. It is our hope that the studies described herein will contribute to the understanding of *Francisella* pathogenicity and to aid in efforts to develop treatments and vaccines against lethal tularemia.

CHAPTER 2. MATERIALS AND METHODS

Bacterial Strains and Culture

F. tularensis Live Vaccine Strain (FTLVS) was a kind gift of Dr. Karen Elkins (FDA, Bethesda, MD). FT LVS-pLux was obtained from James Bina (UTHSC, Memphis, TN) and contains a constitutively-expressed lux operon from *Photobacterium luminescens* with a FT promoter (pXB178). FT Schu S4 was obtained from the CDC. All bacterial cultures were grown overnight frozen stocks in either Brain-Heart Infusion broth (37g/L, pH 6.8) or modified Mueller-Hinton (MMH) broth supplemented with 2.5% ferric pyrophosphate, 10% glucose, and 10% cysteine at 37°C with shaking to mid-log phase ($OD_{600} = \sim 0.7$) before use. Bacterial stocks were made by pelleting an overnight culture and resuspending in MMH or BHI broth only. Stocks were stored at -80°C until use.

Reagents

Human fresh frozen plasma (FFP) was purchased from Lifeblood Mid-South Regional Blood Center (Memphis, TN). Human Glu-PLG, human single-chain tissue PLG activator (tPA), and the plasmin colorimetric substrate (H-D-Val-Leu-Lys-pNA) were purchased from Molecular Innovations (Novi, MI). Bovine serum albumin (fraction V) was purchased from Thermo-Fisher Scientific (Pittsburgh, PA). Polyclonal sheep anti-human PLG and anti-human fibronectin antibodies were purchased from AbD Serotec (Raleigh, NC). Monoclonal anti-goat/sheep IgG-horseradish peroxidase conjugated secondary antibody (clone GT-34) and ϵ -aminocaproic acid (A7824) were purchased from Sigma-Aldrich (St. Louis, MO). Ninety-six well MAXISORP ELISA plates were purchased from Nunc (Rochester, NY).

Plasminogen Binding ELISA Assays

FTLVS was cultured overnight to mid-log phase in brain-heart infusion broth (pH 6.8), pelleted at 6,400 x g for 30 minutes, washed twice with phosphate-buffered saline (PBS), and resuspended in PBS with 0.1% Na azide to an $OD_{600} = 0.1$. The resulting bacterial suspension was added to microtiter plates (100 μ l/well) before being incubated overnight at 4°C to facilitate binding. The wells were then washed twice with 200 μ L of Tris-buffered saline (pH 7.45) containing 0.05% Tween-20 (TBST) to remove unbound bacteria and then pre-blocked with 200 μ l of TBST containing 1% bovine serum albumin (1% BSA-TBST) for 1 hour at RT° to prevent non-specific protein binding. After removal of the blocking solution, 90% citrated human plasma or 3 μ g/mL human Glu-PLG in 1% BSA-TBST was added to each well (100 μ l), with or without the indicated concentrations of ϵ ACA, and incubated for 1-2 hours at 37°C with gentle rocking. Wells were washed three times with TBST and then sheep anti-human PLG-specific antibody (1:2000 dilution in 1% BSA-TBST) was added (100 μ l/well) and allowed to incubate for 1 hour at 37°C. Unbound primary antibodies were removed by washing three times with TBST, followed by the addition of HRP-conjugated anti-sheep/goat IgG mAb (GT-34, 1:5000 dilution in 1% BSA-TBST; 100 μ l/well) and incubation for 1 hour at 37°C. Unbound secondary antibodies were removed by washing 4 times with TBST, and OptEIA TMB colorimetric substrate solution (Becton-Dickenson, Franklin Lakes, NJ) was added to

each well (100 μ l/well) and incubated at 37°C for 20 min. to allow color development. Absorbance at 450nm was determined using a SpectraMAX 340 plate reader (Molecular Devices, Sunnyvale, CA).

Indirect Immunofluorescence Assays

FTLVS was cultured and washed as previously described. After diluting the washed bacteria to $OD_{600} = 0.1$, 1 mL aliquots were incubated with a total of 40 μ g of PLG or PBS (negative control) for 30 minutes at 37°C with gentle rotation. Bacteria were then washed three times with PBS by centrifugation, resuspended in 100 μ l of PBS, followed by spotting 20 μ l of each sample onto glass coverslips. The samples were then air-dried overnight at 37°C. After methanol fixation, the coverslips were blocked with 1% BSA-PBS at room temperature before adding sheep anti-human PLG (1:100 diluted in 1% BSA-PBS) for 30 minutes at room temperature. The coverslips were gently washed with PBS before adding donkey anti-sheep/goat IgG:Dylight-488 (1:100 diluted in 1% BSA-PBS), followed by incubation for 30 minutes at room temperature. After washing again with PBS, coverslips were mounted onto glass slides using 100% glycerol containing 0.1M n-propyl gallate and images were collected on a Zeiss LSM 510 confocal microscope (Thornwood, NY) with an Axiovert 100M base with a 100X Plan Apochromat 1.4 NA oil DIC objective using the argon laser for 488 nm excitation and 505-530nm bandpass emission filter for imaging Dylight488 fluorescence and the HeNe1 543nm laser for illumination of the DIC images. Both images were collected using identical detector gain and amplifier offset settings, and the images shown are 1.0 mm optical slices. Digital images were visualized using Zeiss AxioVision LE software (Thornwood, NY).

Chromogenic Plasmin Activation Assay

FTLVS was cultured overnight to mid-log phase, washed twice with Tris-buffered saline (TBS) and then resuspended in TBS to an OD_{600} of 0.7. Aliquots of the bacterial suspension (50 μ l) was added to 50 μ l of TBS alone or TBS containing Glu-PLG (192 μ g/mL) and incubated for 1 hour at 37°C. The cells were washed 3x with TBST containing 0.1% BSA, and pellets were resuspended in 200 μ L of TBS and then split into two 100 μ l aliquots. 50 μ l of 50 mM Tris-HCl (pH 7.45) with or without 333 μ M of the chromogenic plasmin substrate (H-D-Val-Leu-Lys-pNA) and 50 μ l containing 1.2 μ g of tPA or TBS alone was added to each sample and incubated at 37°C for 3 h. Bacteria in each tube was pelleted via centrifugation and 150 μ l of each supernatant was pipetted into a 96-well plate (in triplicate) and absorbance at 405 nm was determined as a measure of PLG activity.

Membrane Protein Fractionation

Outer membrane enriched fractions were isolated by a procedure adapted from de Bruin, *et al* (71). FTLVS were grown in BHI broth (500 mL) to mid-log phase and then were pelleted via centrifugation at 6,400 x g for 30 minutes. Cells were resuspended in cold PBS and then lysed by sonication. Unlysed bacterial cells were separated from the whole-cell lysate by centrifugation at 10,000 x g for 20 minutes at

4°C. The insoluble membrane fraction was then isolated by ultracentrifugation for 1 hour at 100,000 x g at 4°C. After removal of the soluble protein fraction, the pelleted total membrane fraction was resuspended in 1% Sarkosyl with vortexing and subjected to a second round of ultracentrifugation for 1 hour at 100,000 x g at 4°C. The Sarkosyl-insoluble pellet was resuspended in 50mM Tris pH 8. The protein concentration of both the Sarkosyl-soluble and Sarkosyl-insoluble fractions was determined using the DC protein assay (Bio-Rad, Hercules, CA) according to manufacturer directions. Samples were stored at -20°C until use.

Far-Western Blotting Analysis

Approximately 100 µg of each protein fraction was precipitated using ice-cold acetone, pelleted via centrifugation at 18,900 x g at 4°C for 15 minutes, and air-dried at RT°. The samples were then solubilized by boiling in 1X SDS-PAGE sample buffer containing 2-mercaptoethanol. Duplicate 20 µl aliquots of each sample was subjected to 15% SDS-PAGE to separate the proteins based on their size. One set of the samples was then electrophoretically transferred to a PVDF membrane (Immobilon-Psq, Millipore). The PVDF membrane was pre-blocked with 1% BSA-TBST for 1 hour at RT° to minimize non-specific protein binding, and was then incubated in a solution of Glu-PLG (3 µg/mL in 1% BSA-TBST) for one hour with rocking at 37°C. Unbound PLG was removed by washing three times with TBST. Sheep anti-human PLG-specific antibody (diluted 1:2000 in 1% BSA-TBST) was added (100 µl/well) and allowed to incubate for 1 hour at RT° with rocking. The PVDF membrane was washed three times with TBST to remove unbound primary antibody. The membrane was then incubated in a solution of horseradish peroxidase-labeled anti-sheep/goat IgG monoclonal antibody (GT-34, diluted 1:5000 in 1% BSA-TBST) with rocking for 1 hr at RT°. The PVDF membranes were washed 3 times with TBST to remove unbound secondary antibody. The blot was developed using Pierce PicoWest chemiluminescence reagents and imaged using a Bio-Rad ChemiDoc XRS system (Bio-Rad, Hercules, CA).

Proteomic Identification of Plasminogen-binding FT Proteins

Protein bands were excised from Coomassie-stained SDS-PAGE gels, cut into small pieces, incubated in 50% acetonitrile/100 mM ammonium bicarbonate until colorless, and dried via vacuum centrifugation. The protein was digested by adding 20 µl of a 20 ng/µl trypsin solution and incubating overnight at 37°C. Peptides were extracted from the gel slices via sonication in 50 µl 60% acetonitrile/5%TFA, dried via vacuum centrifugation, and reconstituted in 15 µl 0.1% TFA. Tryptic peptides were desalted/enriched using a C18 ZipTip column (Millipore, Billerica, MA) according to manufacturer's instructions and the eluant was spotted on a MALDI plate and dried. Samples were analyzed using a MALDI-LTQ mass spectrometer (Thermo Finnigan, San Jose, CA). A full MS scan in high-mass range (m/z 600-4000, 5 microscans) was performed. The 50 most intense peaks in the full MS spectrum were selected, and MSMS scans were performed for those ions in high-mass range (m/z 50-4000, 5 microscans), the normalized collision energy for MSMS was 35. Xcalibur software (Thermo Fisher Scientific, Waltham, MA) was used process the mass spectrometric data, and the NCBIInr database and the Bioworks 3.2 search engine software (Thermo Finnigan, San Jose, CA) were used for database searching.

***In Vivo* Imaging of Mice Infected with Luciferase-expressing FT LVS**

Female Balb/c mice (5/group) were shaved and treated with a depilatory cream to carefully remove hair from the ventral area of the neck and torso to decrease light obstruction during image collection. Mice were lightly anaesthetized using Isoflurane before being intranasally infected with 1×10^6 CFU FT LVS-pLux (25 μ l/nare) or given sterile PBS alone. Six hours before infection, and every six hours for the duration of the experiment, mice were injected intraperitoneally with 200 μ L of ϵ ACA (150 mg/mL) or sterile PBS alone (72). Mice were granted access to chow and water *ab libitum* and also monitored daily for signs of disease. Each mouse was weighed and imaged daily using a Xenogen IVIS system (Caliper Life Sciences, Hopkinton, MA) to note disease severity and progress, with mice losing greater than twenty percent of their starting body weight or having impaired mobility being humanely euthanized.

CHAPTER 3. PLASMINOGEN/*FRANCISELLA* BINDING STUDIES AND THE IDENTIFICATION OF PUTATIVE BACTERIAL MEMBRANE-ASSOCIATED PROTEIN RECEPTORS

Introduction

The causative agent of tularemia, *Francisella tularensis*, is well-known among bacteriologists and physicians because of its highly infectious nature and potential to cause lethal infection in humans and other animals. Efforts to develop a safe and effective vaccine against tularemia have been unsuccessful to date and the only prophylaxis is by scarification with live vaccine strain of FT (FT LVS), though it is currently unlicensed and only available to military personnel and laboratory workers. Additionally, FT LVS was derived as an attenuated mutant of *F. tularensis* ssp. *holarctica*, a type B stain which is less virulent to humans than the type A *F. tularensis* ssp. *tularensis* (e.g. Schu S4) and has an unclear mechanism of attenuation. For these reasons, researchers in the field of FT vaccine research have been searching for alternative immunization strategies, such as using defined FT Schu S4 strains (33, 73-74) and subunit vaccine preparations (36-37, 40).

Francisella is not only one of the most infectious bacterial pathogens known to humans, but it also seems especially well prepared to survive in a wide array of other host environments as evidenced by its ability to infect over 200 species of vertebrates and invertebrates. Upon entering a mammalian host through breaks in the skin or by inhalation of infectious materials, FT bacteria are typically taken up by host phagocytic cells such as macrophages and dendritic cells. These bacteria are then believed to be carried by lymphatic ducts to the local draining lymph nodes. Later during the infection, FT spreads systemically and can be found in the liver, spleen, lungs, and freely circulating in the blood within or outside of phagocytic cells (60-61). Questions still remain as to how FT is able to spread so quickly (within days) to most areas of the host body and to the role that such high numbers of extracellular bacteria may play during its pathogenic cycle.

One common mechanism used by pathogenic organisms to disseminate within the host is to utilize components of the host fibrinolytic system. By taking advantage of both the broad specificity and the availability of relatively large quantities of the serine protease plasminogen (plasmin), many pathogens are able utilize the proteolytic activity of this host enzyme to promote penetration through the extracellular matrix as a means of dissemination as well as evasion of host innate immune responses (64-67). Bacterial pathogens have been shown to use a wide variety of both membrane and cytosolic proteins as plasminogen receptors, which suggests that the ability to bind plasminogen has proven itself beneficial as a means to allow safe passage within host environments.

This chapter illustrates work describing the interaction of plasminogen with the outer surface of FT LVS and FT Schu S4. I have clearly demonstrated that plasminogen binds to the outer envelope of FT. My work has also shown that plasminogen that has been pre-bound to the outer envelope of FT can be cleaved to its enzymatically active form (plasmin) by treatment with a plasminogen activator, as evidenced by its ability to cleave both a chromogenic plasmin substrate and an extracellular matrix component (fibronectin). Furthermore, I used a ligand blotting technique coupled with proteomic

methodologies to identify five putative plasminogen receptors from FT outer envelope preparations.

Binding of PLG from Fresh Human Plasma to the Surface of FT LVS

Until very recently, there were no published works describing the potential for FT to bind plasma proteins belonging to the host fibrinolytic system. Our first goal was to determine whether fibrinolytic plasma components could be recruited to the surface of FT. I used a simple enzyme-linked immunosorbent assay (ELISA)-based approach in which FT (grown to mid-log phase in modified Mueller-Hinton broth) was bound to microtiter plates and then incubated in the presence of fresh frozen plasma (FFP). I chose to use human fresh frozen plasma (FFP) as a source of ligands because it provides similar types and quantities of proteins that extracellular FT might encounter while circulating in the plasma fraction of whole blood. Binding of specific fibrinolytic plasma components was then detected using plasminogen-, fibrinogen-, and fibronectin-specific secondary antibody preparations. As a positive control I also measured binding of factor H, a plasma component that has been shown to bind to the surface of FT (63). In addition, BSA-coated well were used to establish levels of non-specific binding of plasma components. The results of this assay revealed that both plasminogen and fibronectin bind to FT, and it confirmed that factor H binds to FT (data not shown). However, I found no clear evidence that fibrinogen binds to the surface of FT. Though the binding of fibronectin to FT cannot be ruled out as a potential virulence factor at this time, I chose to focus my studies on the ability of FT to bind PLG because of its potential to enhance virulence and dissemination/invasiveness within the host.

In light of published findings that the proteome of FT grown *in vitro* in modified Mueller-Hinton broth differs significantly from that of FT cultured in cultured cells as opposed to FT cultured in brain-heart infusion broth, the latter of which closely resembles FT grown in cultured cells (75), we repeated the previously described study using FT cultured to mid-log phase in BHI broth and measured binding of plasminogen to the surface of FT. The results of this experiment indicated that the composition of FT culture media did not have a significant impact on the ability of FT to bind to plasminogen in FFP (**Fig. 3.1**). To confirm that binding of PLG to FT was a specific interaction involving exposed lysines, I also tested the ability of a lysine analogue and known inhibitor of PLG binding, ϵ -aminocaproic acid (ϵ ACA), to interfere with PLG binding to the bacteria. I found that ϵ ACA very effectively inhibited that ability of PLG to bind to FT.

Binding of Purified Human and Murine PLG to FT LVS and FT Schu S4

The plasma fraction of mammalian whole blood is a protein rich environment (60-80 mg/mL in humans) which contains dozens of proteins and other molecules that are necessary for homeostasis. To help eliminate the possibility of non-specific binding of PLG due to its high concentrations in human plasma (~200 μ g/mL) and also rule out any interference from other plasma proteins, I used purified human Glu-PLG (PLG) in ELISA experiments similar to those conducted with FFP. From these experiments with purified PLG I noted similar results to those observed when FFP was used (**Figs. 3.1** and **3.2**), which indicated that the binding of PLG required no other proteins from plasma.

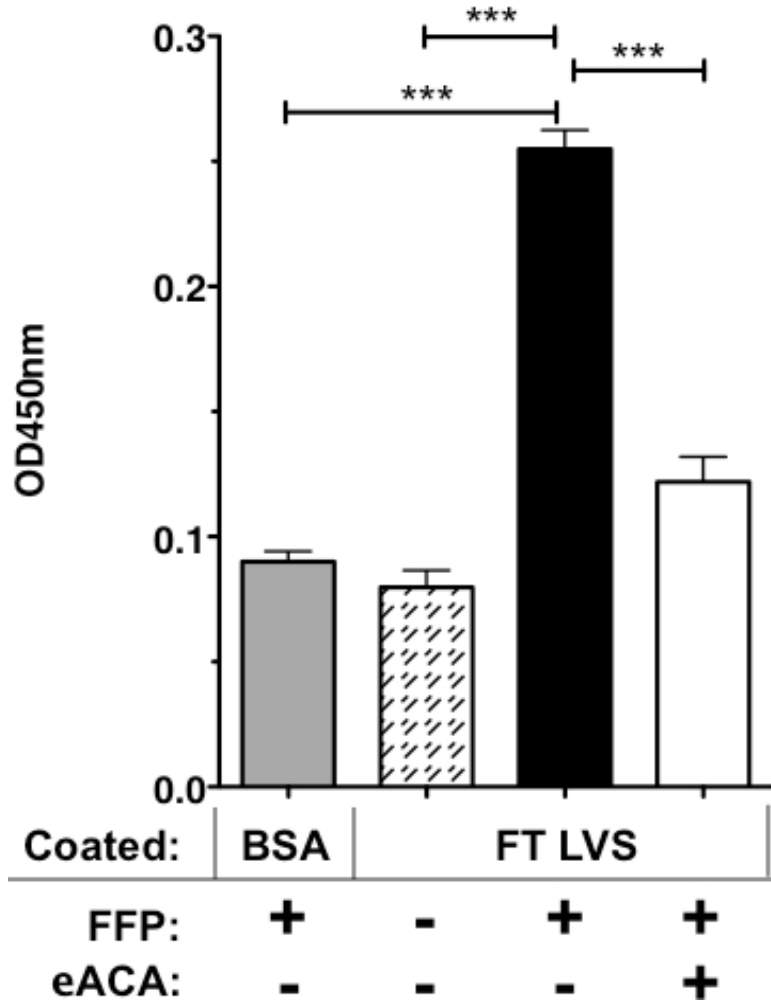


Figure 3.1. FT binds to PLG from human plasma. Microtiter plates coated with FT LVS cultured to mid-log phase in BHI broth were incubated for 1 hour with fresh frozen human plasma (FFP) in the presence or absence of 100 mM ϵ -amino caproic acid (ϵ ACA), a PLG-binding inhibitor. A modified ELISA was performed to measure FT LVS-bound PLG. The results shown are representative of 3 experiments of similar design. Bars indicate +/- SEM in triplicate. Statistical analysis was performed via one-way ANOVA using a Dunnett's Multiple Comparison post-test (***) P < .001).

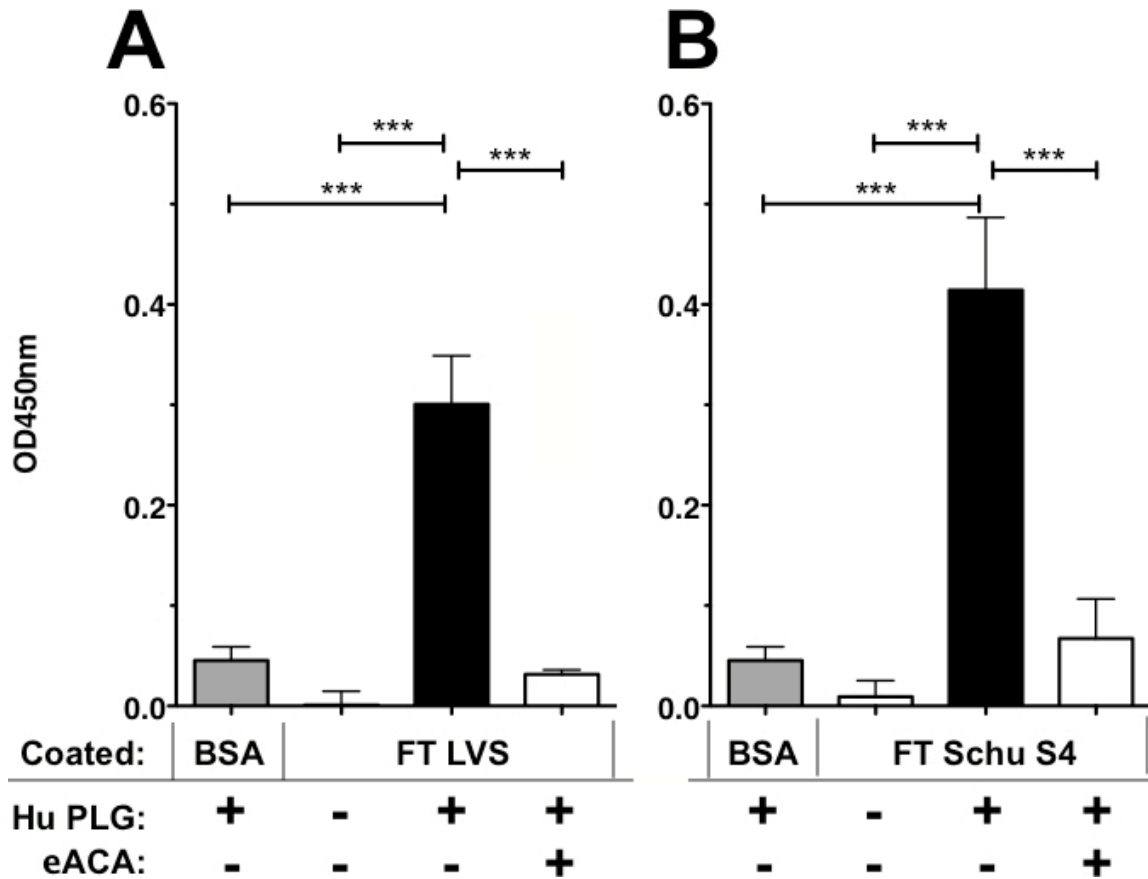


Figure 3.2. Purified human plasminogen binds to FT. FTLVS (**Panel A**) and FTSchuS4 (**Panel B**) were bound to microtiter wells and incubated for 2 hours with purified huPLG (3 $\mu\text{g}/\text{mL}$) in the presence or absence of 10 mM ϵACA . A modified ELISA was performed to measure FTLVS-bound huPLG. The results shown are representative of four (**Panel A**) and one (**Panel B**) experiments, respectively, of similar design. Bars indicate \pm SEM in triplicate. Statistical analysis was performed via one-way ANOVA using a Dunnett's Multiple Comparison post-test (***) $P < .001$.

Although the attenuated FT LVS and highly virulent FT Schu S4 strains are genetically very similar, their abilities' to cause disease are known to be quite different.

Experiments comparing the PLG-binding ability of these FT strains were conducted to note any strain-dependant differences within our modified ELISA model. From the results of these experiments I determined that human PLG binds to the highly virulent Schu S4 strain of FT at moderately higher levels than observed with FT LVS (**Fig. 3.2**). Additional experiments using purified murine PLG confirmed similar binding to FT LVS and FT Schu S4 also, which suggests that this is property is not confined to only human PLG (**Fig. 3.3**). The binding of PLG/plasmin typically involves interaction with the exposed lysine residues of a ligand. I confirmed that binding of PLG to FT is a lysine-dependent interaction by showing that increasing concentrations of epsilon aminocaproic acid (ϵ ACA) can inhibit binding of PLG to FT LVS in a dose-dependent fashion (**Fig. 3.4**). Together, these data clearly indicate a typical plasminogen-binding reaction with FT in which lysine residues of the bacterial receptor(s) play a necessary role in the said interaction.

Plasminogen Binds to the Outer Envelope of FT

In order for FT-bound PLG to both be activated to plasmin and also to be accessible to potential substrates, it would be important that the proenzyme be localized to the bacterial outer envelope of the bacterium. Therefore, I performed a series of experiments designed to determine whether PLG binds to the surface of FT. My first attempts to visualize PLG binding to the surface of FT involved the use of flow cytometric analysis. For these studies, FTLVS were pre-incubated in a PLG-containing solution, washed, and then labeled via sequential incubations with PLG-specific and a secondary antibody-fluorochrome conjugate. Unfortunately, I was unable to detect any PLG associated with FT using this technique. I posit that the failure of this technique was due to uncoupling of PLG:FT complexes due to shear forces associated with the fluidics system of the flow cytometer and/or the vortexing and centrifugation steps required for the washing steps during staining. I determined that a different technique that used much gentler washing and/or a formaldehyde fixation step would have a higher potential for success.

As an alternative method for visualization of PLG binding to the surface of FT, I performed an immunohistochemical analysis of FT following incubation with PLG. For this study, FTLVS was incubated in a PBS solution containing 40 μ g/mL human PLG and washed to remove unbound PLG, and then bacteria were immobilized onto a glass microscope slides via methanol fixation. PLG bound to FT was then labeled via sequential incubations with goat anti-PLG IgG and anti-goat IgG conjugated to DyLight-488. I then visualized the localization of PLG on the surface of FT via confocal microscopy. The images with PLG-coated FT in relation to uncoated bacterial controls clearly indicate a fluorescent signature which suggests that plasminogen binds to the outer envelope of FT (**Fig. 3.5**). I believe that this procedure proved successful because the washing steps performed on immobilized FT:PLG complexes were much gentler than those required for the flow cytometry-based approach. I also recognize that the methanol fixation step used in the protocol could have altered the integrity of the cell envelope, making it impossible for us to say with certainty that PLG binds to the true outer surface of FT; I can only say that PLG binds to the outer envelope of the

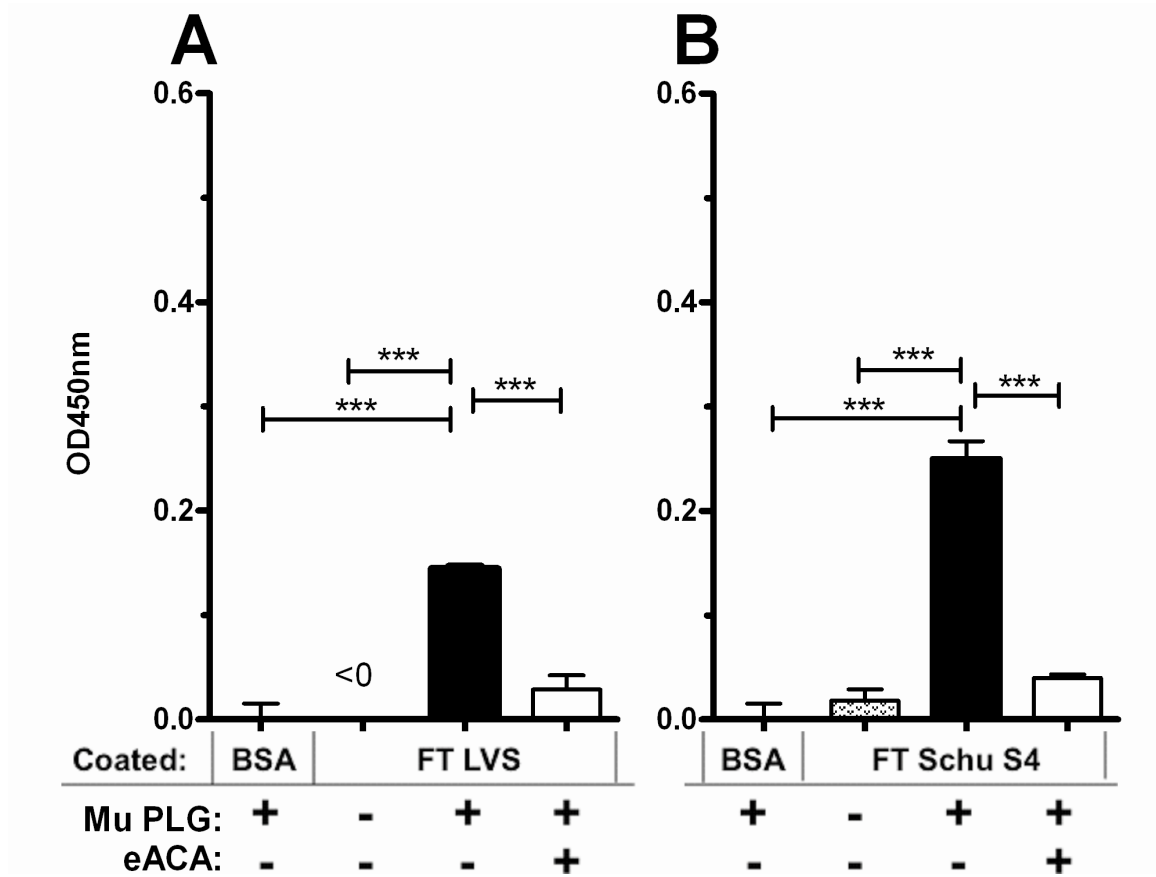


Figure 3.3. Purified murine plasminogen binds to FT. FTLVS (Panel A) and FT Schu S4 (Panel B) were bound to microtiter wells and incubated for 2 hours with purified muPLG (3 $\mu\text{g}/\text{mL}$) in the presence or absence of 10 mM ϵACA . A modified ELISA was performed to measure FTLVS-bound muPLG. The results shown are representative of four (Panel A) and one (Panel B) experiments, respectively, of similar design. Bars indicate \pm SEM in triplicate. Statistical analysis was performed via one-way ANOVA using a Dunnett's Multiple Comparison post-test (*) $P < .001$.**

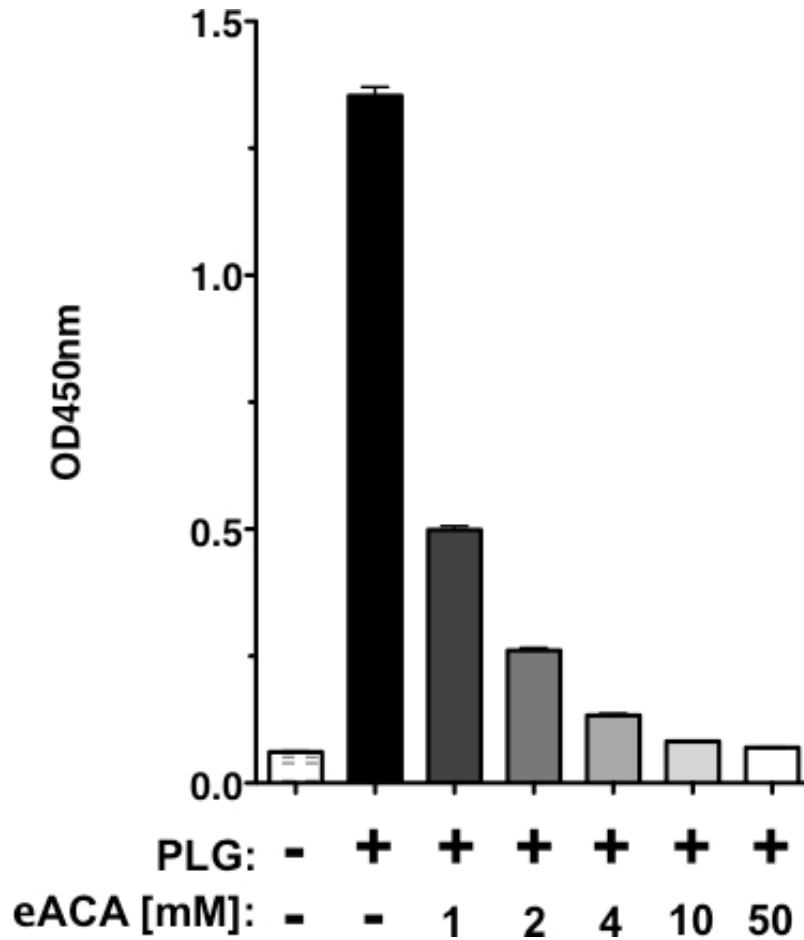


Figure 3.4. Binding of human PLG to FT is inhibited in the presence of ϵ ACA. FT LVS was coated onto microtiter plate wells and incubated for 2 hours with purified human Glu-plasminogen (PLG) (3 μ g/mL) in the presence or absence of titrated concentrations of ϵ ACA. The results shown are representative of 3 experiments of similar design. Bars indicate +/- SEM in triplicate. Statistical analysis performed via one-way ANOVA using a Kruskal-Wallis test determined a p-value of < 0.0001 relative to PLG incubated without inhibitor.

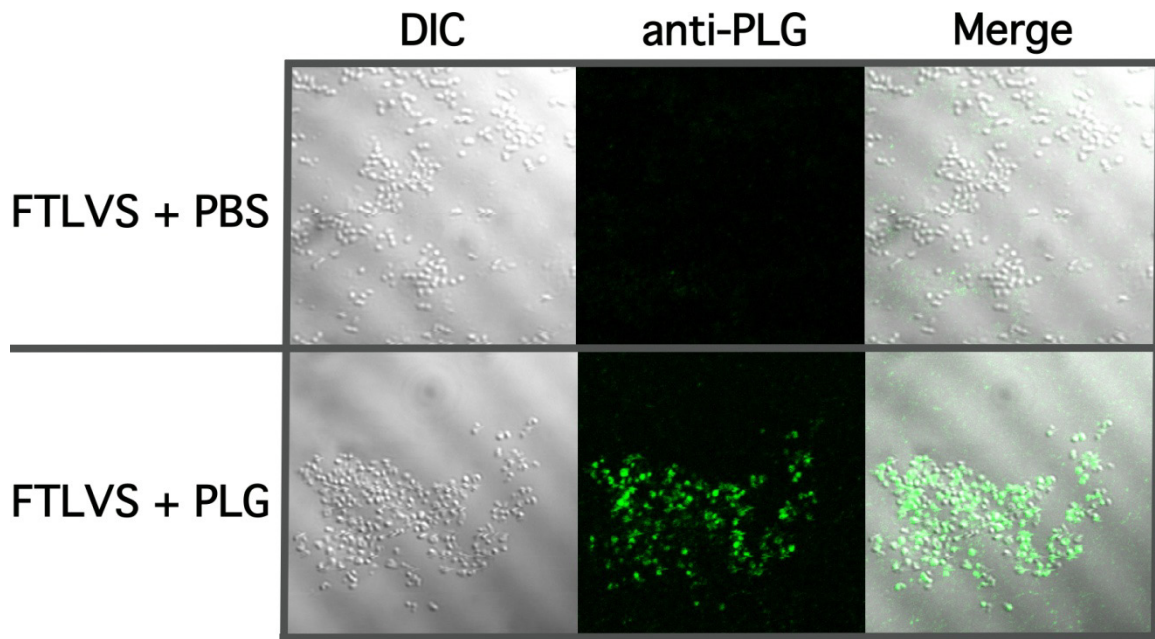


Figure 3.5. Demonstration of bacterial surface localization of human PLG.

Indirect immunofluorescence microscopy of PLG-associated FT LVS was performed as described in “Materials and Methods.” Bound PLG ligand was detected using sheep anti-human primary antibody followed by incubation with Dylight-488 conjugated anti-sheep/goat IgG secondary antibody. Samples were visualized using a Zeiss LSM510 confocal microscope on a Axiovert 100M base.

bacterium.

Identification of Putative *Francisella* Plasminogen-binding Membrane Proteins

Because our ultimate goals are to define potential virulence factors and to identify any potential therapeutic or vaccine targets within the FT epitome, I sought to identify potential PLG receptors within the bacterial membrane. The identification of putative PLG receptors on FT could potentially provide valuable targets for monoclonal antibody therapy or for immunization protocols to block these receptors from gaining access to this host protease and possibly limit any effects on its virulence. I wished to identify these PLG-binding FT proteins from the outer envelope protein fraction where they would most likely have the most effect on virulence in the host. To accomplish this, I utilized Sarkosyl soluble and insoluble FTLVS membrane fractions together with a Far-Western blot method and mass spectrometry (**Fig. 3.6**). Sarkosyl is a weak anionic detergent in which many outer membrane proteins of Gram-negative bacteria are insoluble (76), rendering them amenable to isolation by sedimentation via ultracentrifugation. After separation of these fractions by SDS-PAGE, the Sarkosyl-treated proteins were then transferred to a PVDF membrane and then incubated this membrane with diluted PLG and identified bound PLG by reaction with anti-PLG (**Fig. 3.6A**). I used the relative migration rates of the reactive bands to identify the reactive proteins on a duplicate Coomassie-stained polyacrylamide gel (**Fig. 3.6B**), which were then excised for proteomic analysis by mass spectrometry. Several prominent PLG-binding proteins were noted in the total membrane fraction of FTLVS, all but one of which was found in the Sarkosyl insoluble fraction (**Fig. 3.6B**). The identity of the prominent proteins from this assay (**Fig. 3.6C**) are the products of the following genes: FTL_1328 (outer membrane associated protein, fopA1), FTL_1042 (FKBP-type peptidyl-prolyl cis-trans isomerase family protein), FTL_0336 (peptidoglycan-associated lipoprotein), FTL_0421 (hypothetical lipoprotein, lpn-A), and FTL_0645 (hypothetical lipoprotein). In summary, through my experiments I was able to show that plasminogen is able to bind proteins found in the *Francisella* bacterial membrane fraction and putatively identify some of these receptors through Far-Western blotting methods and mass spectrometry.

Discussion

Within this chapter, assays were performed to evaluate the ability of *Francisella tularensis* to bind several host plasma proteins on its surface using fresh frozen human plasma. To confirm our initial findings of PLG binding to the FT bacterial membrane as detected by a modified ELISA assay using FFP as a ligand source, an additional assay with purified PLG was performed which demonstrated similar results and confirmed those findings. By including the plasminogen-specific binding inhibitor ϵ ACA with PLG in these experiments, this binding to FT was strongly inhibited. This data suggests that the ability of FT to bind PLG was dependant on exposed lysine residues found within the protein receptors on both FT LVS and FT Schu S4. Furthermore, I was able to identify several putative plasminogen receptors within a membrane fraction of FT.

Until recently, FT has been considered an intracellular pathogen whose dissemination to tissues distal to the site of initial infection was highly dependent on its

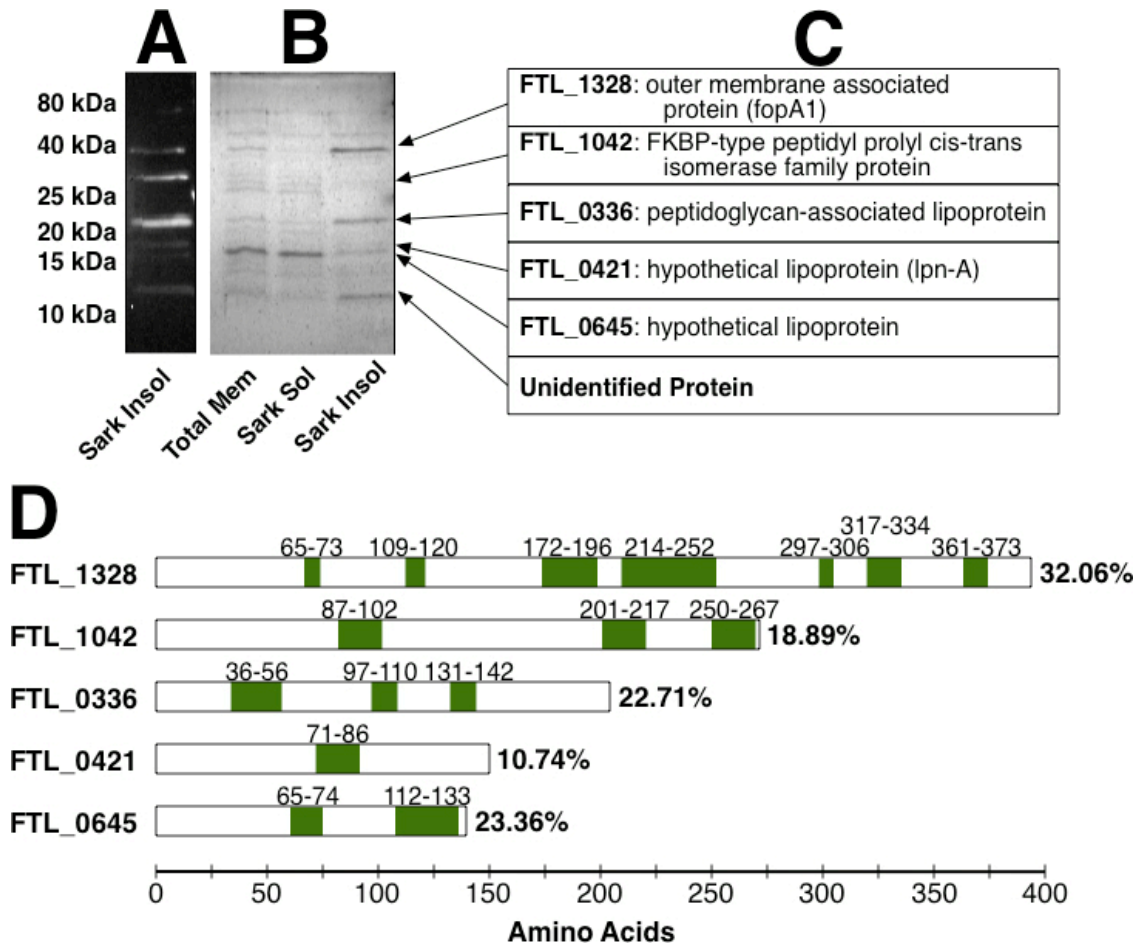


Figure 3.6. Identification of putative plasminogen-binding proteins of FT. Sarkosyl-soluble and insoluble protein fractions of FTLVS were separated by SDS-PAGE and transferred to PVDF membrane. Membranes were then blotted with Glu-plasminogen (3 µg/mL) followed by anti-plasminogen antibody and HRP-conjugated secondary antibody to detect plasminogen-binding proteins (**Panel A**). Protein bands on an identical Coomassie Blue-stained SDS-PAGE gel corresponding to those identified via blotting were excised (**Panel B**) and identified using proteomic methodologies (**Panel C**). The tryptic peptides identified via mass spectrometry that were used to identify the putative PLG ligands of FT are indicated in the peptide coverage maps (**Panel D**).

ability survive within host macrophages. The observation that FT can be found in high numbers in the acellular plasma fraction of its mammalian host (60-61) suggested that FT may have a significant extracellular component to its life cycle and that interactions between FT and one or more plasma proteins could contribute to its ability to survive and cause disease in the host. In fact, there are a number of examples of bacterial pathogens that utilize interactions with host plasma components to enhance their ability to gain entry to host cells and to penetrate the extracellular matrices of host cells/tissues. Some examples of these bacterial pathogens and interactions with host plasma components will be discussed below in further detail.

Complement and complement regulators are proteins found in plasma/serum that play an important role in host defense against pathogenic organisms. The activation of complement in the presence of bacteria enhances the ability of the host immune system to clear these organisms, primarily mediated by opsonophagocytosis and the **relatively** chemotactic recruitment of phagocytic cells to areas of infection. Factor H (FH) is the major complement regulatory protein which protects host cells from the actions of complement from the alternative, classical, and lectin pathways. Most specifically, FH accelerates the degradation of C3b to an inactive form (iC3b) on the host cell surface, ultimately resulting in diminished C5-convertase formation and safeguarding self-cells from destruction. A wide range of bacterial pathogens (including *Francisella*) subvert the destructive mechanisms of complement by acquiring surface-bound complement control proteins. Using ELISA and *in vitro* phagocytosis assays, Ben Nasr and Klimpel reported that both virulent and avirulent FT are able to bind C3-derived fragments for aiding in entry into monocytes and dendritic cells (63). They were also able to show that FT could bind factor H from human serum and plasma by both Western-blot and flow cytometric analyses. These authors suggested that acquisition of FH on the surface of FT may prevent the assembly of C5b-C9 membrane attack complex (MAC)(63). The finding that FT could bind FH from human plasma caught my attention and led me to want to explore the possibility that other plasma/serum proteins from the host could also bind FT in ways that may be beneficial in its survival. Besides factor H, a number of Gram-positive bacterial pathogens including *streptococcal* spp. (77-78), *staphylococcal* spp. (79-82), and *Bacillus anthracis* (83-84), as well as Gram-negative bacteria such as *Borrelia* spp. (85-87) have been shown the potential to augment their invasive capacity by interacting with such plasma proteins as fibrinogen, fibronectin, and/or PLG. *Yersinia pestis* is probably the most characterized example of a pathogen that exploits the host fibrinolytic system to penetrate host tissues. *Yersinia* expresses a surface serine protease (designated Pla) whose substrates include several complement components, PLG, and alpha2-antiplasmin (the primary circulating inhibitor of plasmin). Pla also has adhesin activity and binds to ECM preparations as well as purified laminin (a glycoprotein of mammalian basement membranes)(88). Because Pla upregulates plasmin activity, and because laminin is a substrate of plasmin, *Yersinia* can very efficiently penetrate basement membranes of host tissues (70, 89). Clearly, interaction with plasma components is a strategy that is used by many bacterial pathogens to gain a survival advantage within their hosts.

It is well known that the binding of plasminogen to its ligand is primarily through recognition of lysine residues. Though the inclusion of ϵ ACA in my *in vitro* experiments does not **definitively** prove that the lysine residues within the receptor proteins are necessary for binding of PLG to FT to occur, it is highly suggestive based on the

observed inhibition. This interaction could of course be further studied by mutational studies. However, to perform these studies it would be necessary to mutate each lysine residue in each putative PLG receptor protein. This would be a labor-intensive endeavor which could also result in clones with many deleterious growth defects, making the recovery of every possible mutant a difficult and possibly futile effort. Instead, a much easier approach to define the necessity of lysine residues on FT for binding PLG may be to simply modify them by using common biotinylation reagent kits and repeating the ELISA studies with the treated bacteria. If by modifying all of these residues the ability of FT to bind PLG is diminished greatly, then this may provide even more supporting evidence to the inhibition data that I have shown. These types of experiments could be included in further studies.

Using a ligand-blotting technique coupled with proteomic methodologies I identified five FT LVS proteins that were able to bind to PLG, each of which are highly conserved among the various FT type A and B strains. Three of these proteins are lipoproteins (gene products of FTL_0336, FTL_0421, and FTL_0645). Two of the lipoproteins (fopA1 and lpnA) are unique to FT while the third, peptidoglycan-associated lipoprotein (PAL), is highly conserved among Gram-negative bacteria. Interestingly, the specific use of surface-exposed lipoproteins as receptors for host PLG is not unusual and has been well documented in other human bacterial pathogens, such as some members of the genus *Borrelia* and *Treponema*. Several members of the genus *Borrelia* use complement regulator-acquiring surface proteins (CRASP) to bind both PLG and complement factor H to aid in the ability of the organism to both disseminate and to resist innate immunity (90-95). An additional example of a PLG-binding lipoprotein is OppA of *Treponema denticola*, which has been suggested to play a role in periodontal disease in humans (96). With this in mind, there lies the possibility that lipoproteins of *Francisella* species may have the capacity to bind multiple host-derived proteins in addition to PLG.

In summary, these results clearly show that PLG from host plasma has the ability to bind several FT membrane-associated proteins in a lysine-dependant manner. Further studies in this area of FT research may help better our understanding of its pathogenesis, especially as it pertains to the extracellular phase of its infection cycle. The next chapter focuses on both *in vitro* and *in vivo* studies aimed at characterizing the active protease, plasmin, on the surface of FT LVS.

CHAPTER 4. DEMONSTRATION OF PROTEOLYTIC ACTIVITY OF PLASMIN-BOUND FT LVS

Introduction

After internalization by host phagocytic cells, FT escapes into the cytoplasm before replication (45-46). Following bacterial replication in the cytoplasm, there is an induction of apoptosis (47-49) to enable its subsequent release into the extracellular environment for another cycle of infection. Though most work has focused on the intracellular aspects of FT infection, several reports have described the presence of many extracellular bacteria in mouse models of tularemia (60-61). The significance of the extracellular life cycle phase of FT infection may yield valuable clues toward our complete understanding of its infectious cycle and ultimately contribute to a safe and effective vaccine against lethal tularemia.

In the extracellular environment of the host, FT is known to interact with certain soluble protein components and uses these molecules to enable it to survive and replicate intracellularly, including complement proteins (41-43), factor H (63), and surfactant protein A (97). Most of all, these studies suggest that utilization of a variety of host-derived proteins may provide FT with a means to ensure its virulence capacity within a given host compartment, including plasma/serum.

In Chapter 3 I investigated the ability of FT to bind plasma proteins in an ELISA assay, and followed up on those findings by using purified plasminogen to confirm it as a putative ligand. The ability of PLG to bind FT was also confirmed visually by means of confocal microscopy of ligand-coated bacteria and specific antibodies. Furthermore, I used a ligand blotting technique coupled with proteomic methodologies to identify five putative plasminogen receptors from FT outer envelope preparations.

The use of host coagulation/fibrinolytic system components by many types of bacterial pathogens to gain a survival advantage has been well documented for some time now (64-67). For instance, the ability to acquire surface-associated plasmin has been studied as an important virulence mechanism in human pathogens such as Group A streptococci (68), *Borrelia burgdorferi* (69), *Borrelia hermsii* (86) and *Yersinia pestis* (70) by aiding in the organism's ability to penetrate the extracellular matrix and to disseminate to distal sites in the host and beyond. Work on *Borrelia burgdorferi* has even shown plasmin derived from the mammalian blood allows the pathogen to disseminate from the arthropod gut to the salivary glands, aiding in the transfer of these bacteria to another host at a subsequent feeding (69). Plasminogen (PLG) is a 92-kDa glycoprotein zymogen that is primarily involved in the degradation of clots (fibrin). Activation of this precursor protein by tissue-type (tPA) or urokinase-type (uPA) plasminogen activators converts it to an active serine protease (plasmin). Plasmin has an important role in blood clot resolution because of its role in the degradation of fibrin polymers. Plasmin has a broad-specificity, including many known substrates such as pro-collagenases, pro-metalloproteinases, and extracellular matrix proteins, such as fibronectin, laminin, and vitronectin. Likewise, the ability of a bacterium to acquire surface associated plasmin can result in an enhanced ability of the pathogen to penetrate the extracellular matrix and disseminate to distal sites in the host (64, 66, 68).

In order for plasminogen to be used as a virulence factor, the protein must be

activated by plasminogen activators found within the host or in some cases produced by the pathogen themselves. This chapter outlines a series of experiments with the purpose of describing possible functions for the binding of PLG to the outer envelope of both attenuated (FT LVS) and virulent (FT Schu S4) subspecies of *Francisella tularensis* as seen in Chapter 3 and my published work (98). In this chapter, I provide experimental evidence that plasminogen which has been pre-bound to the outer envelope of FT can be cleaved to its enzymatically active form (plasmin) by treatment with a plasminogen activator, as evidenced by its ability to cleave both a chromogenic plasmin substrate and an extracellular matrix component (fibronectin).

Plasmin Activation on the Surface of FT LVS *In Vitro* by a Plasminogen Activator

Francisella tularensis is well-known for its ability to disseminate to points distal to the route of entry and has recently been found extracellularly in high numbers in the plasma fraction of mice (60-61). In several other bacterial systems, surface-bound PLG can be converted to its proteolytically active plasmin form and has been shown to contribute to the organism's virulence by aiding in survival and enhancing tissue penetration in the host (64-67). To test whether PLG bound to FT LVS can be converted to plasmin, I first used a chromogenic plasmin substrate (H-D-Val-Leu-Lys-pNA) to detect proteolytic activity following the addition of a PLG activator (tPA) (**Fig. 4.1**). I found that there is a plasminogen activator-dependant conversion of the chromogenic substrate which is based on the observed increase in absorbance readings at 405nm after cleavage of the plasmin-specific peptide sequence from its chromophore (p-nitroaniline). This finding not only indicates this is a possible mechanism *in vivo*, but also that there appears to be no intrinsic plasminogen activator produced by FT based on a lack of substrate conversion in a control reaction that lacked tPA.

Some examples of potential substrates for plasmin-bound bacteria, including FT, are components of the extracellular matrix (ECM). To determine whether FT-bound plasmin could potentially participate in the breakdown of extracellular matrices, I allowed PLG to bind to the surface of FT, activated it via treatment with tPA, and then tested the ability of the FT surface-bound plasmin to use fibronectin, an ECM component, as a proteolytic substrate (**Fig. 4.2**). These results demonstrate plasminogen bound to the surface of FT can be converted to its active form, plasmin. This active serine protease can proteolytically degrade fibronectin, and potentially other protein components of extracellular matrices.

Murine Infection with FT LVS-pLux with the Inclusion of a Plasminogen Binding Inhibitor

The acquisition of plasmin on the surface of bacterial pathogens has been shown in numerous examples to increase their potential virulence and dissemination within mammalian hosts (64-67). Based on our findings of the ability of FT to bind and allow the activation of plasmin on its surface *in vitro*, I hypothesized that if plasminogen/plasmin was unable to bind to the outer envelope of FT LVS during the course of infection, then it may be possible to observe signs of reduced disease severity. To test this, I performed an *in vivo* dissemination study using the IVIS whole animal imaging system. This experiment was performed by employing FT that had been engineered to express light (FT LVS-pLux) and a treatment group that received a series

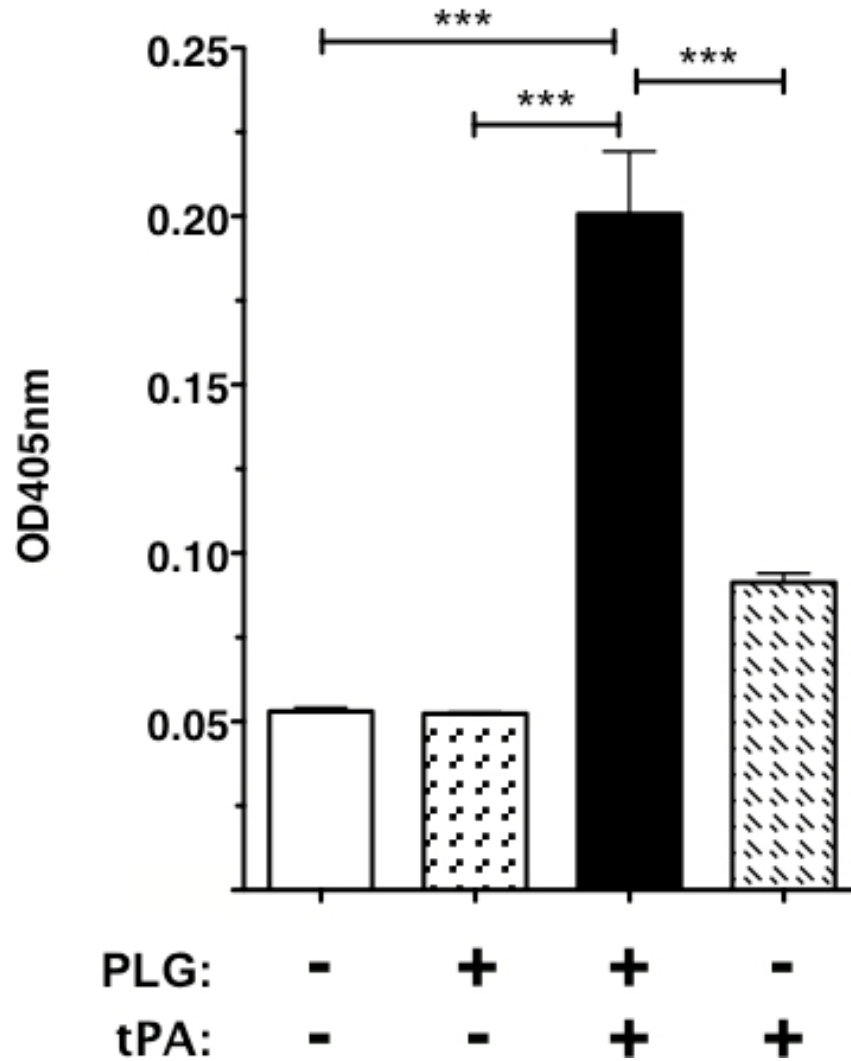
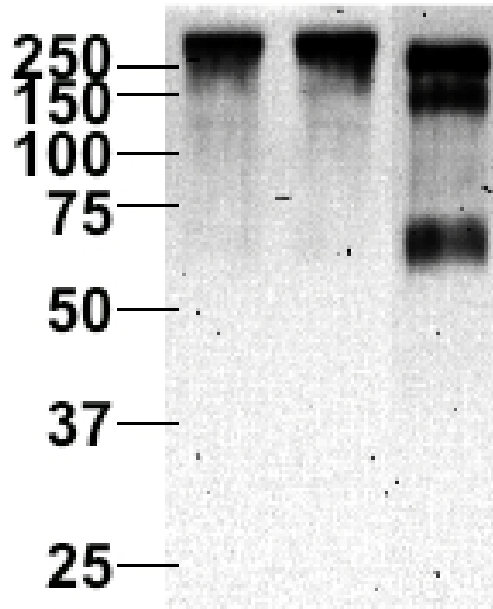


Figure 4.1. FT surface-bound plasminogen can be converted to plasmin. FT LVS was incubated with purified human plasminogen (PLG) at a concentration of 96 $\mu\text{g}/\text{mL}$. After removal of unbound plasminogen, a chromogenic plasmin substrate (D-VLK-pNA), tissue plasminogen activator (tPA), or both were then added to test the proteolytic ability of each sample preparation. Conversion of the chromogenic substrate was measured by comparison of $\Delta 405 \text{ nm}$. The results shown are representative of 3 experiments of similar design. Bars indicate \pm SEM in triplicate. Statistical analysis was performed via one-way ANOVA using a Dunnett's Multiple Comparison post-test (***) $P < .001$.



FT alone: + + -
FT/PLG: - - +
tPA: - + +

Figure 4.2. Fibronectin is a substrate for plasmin bound to FT. FTLVS (10^9 CFU) were incubated with 100 μ g of purified human PLG and 0.5 μ g tissue tPA for 1 hour at 37°C. After removal of unbound PLG and tPA, 3 μ g fibronectin was added and allowed to incubate for 24 hours at 37°C. Supernatant from each preparation were separated by SDS-PAGE and transferred to PVDF membrane. Degradation of fibronectin was detected by Western blot analysis.

of ϵ ACA infusions that could potentially prevent the binding of plasminogen to the surface of FT. I hoped that by using FT LVS-pLux it would enable us to use the luminescent signal as a possible means to visualize any changes in distribution of the bacteria in the mice over time that plasminogen inhibition may cause. Female Balb/c mice (5/group) were infected intranasally (i.n.) with 1×10^6 CFU FT LVS-pLux while under light anaesthesia. In addition, six hours before infection and every six hours for the duration of the experiment, mice were injected intraperitoneally with 200 μ L of ϵ ACA (150 mg/mL) or sterile PBS alone to evaluate the effects that a plasminogen binding inhibitor may have on disease progression. These mice were weighed prior to infection and every day for the course of the experiment to monitor the progressive weight loss that is caused by FT infection. As expected there was a dramatic loss of weight in the FT LVS-pLux-infected mice as compared to those that were mock-infected with PBS only (**Fig. 4.3**). Comparison of the FT LVS-pLux infected groups that received injections of ϵ ACA showed only an insignificant reduction in weight loss at day 6 as compared to mice that were given PBS only. To look for differences in the distribution of luminescent FT bacteria through the course of this experiment, images of lightly anaesthetized mice were collected using a Xenogen IVIS system (**Fig. 4.4**). Analysis of these images revealed no obvious differences in the distribution of FT in this murine infection model with or without i.p. injections of the plasminogen inhibitor. All but one mouse in the FT-infected group receiving ϵ ACA survived to the sixth day, at which all remaining mice were sacrificed due to the severity of infection.

Discussion

Infection with *Francisella tularensis* is often associated with bacterial dissemination to sites distal to the primary entry point. Most of what is known about FT pertains to its intracellular phase of infection, whose dissemination to tissues distal to the site of initial infection was highly dependent on its ability survive within host macrophages. Recently, the detection of high numbers of extracellular FT in the acellular plasma fraction of mice (60-61) suggest that FT may have a significant extracellular component to its life cycle and that interactions between FT and one or more plasma proteins could contribute to its ability to disseminate within the host. Results from our ELISA and confocal microscopy experiments detailed and discussed in Chapter 3 provided evidence of the ability of FT to bind PLG and the identity of several putative bacterial plasminogen receptors from a membrane protein fraction.

One well-documented and relatively common method for bacteria to disseminate and avoid the host innate immunity is through the acquisition of host plasminogen/plasmin on its surface (64-67). In this chapter I showed that plasminogen bound to the surface of FT could cleave a plasmin-specific chromogenic substrate, thus demonstrating its proteolytic potential. I also showed by Western-blot analysis the degradative effect that plasmin-coated FT LVS had on an extracellular matrix component, fibronectin. This observation is not at all unique to FT and similar findings are in the literature for several other bacterial pathogens to date (69, 83, 99-100). These findings support our hypothesis that the ability of FT to bind to serum plasminogen may enhance its ability to penetrate extracellular matrices, however, more in-depth studies of this potential virulence factor needed to be performed *in vivo* to firmly establish that FT's ability to bind to plasminogen offers a survival advantage to this highly pathogenic bacterium.

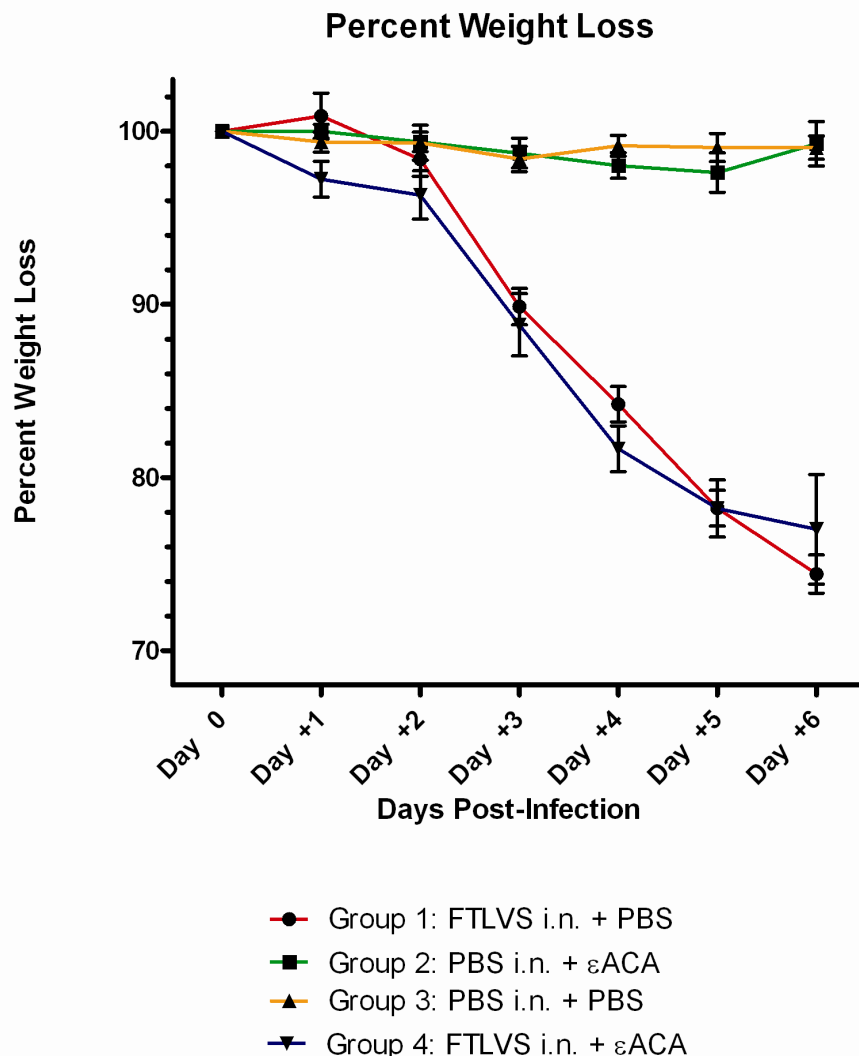
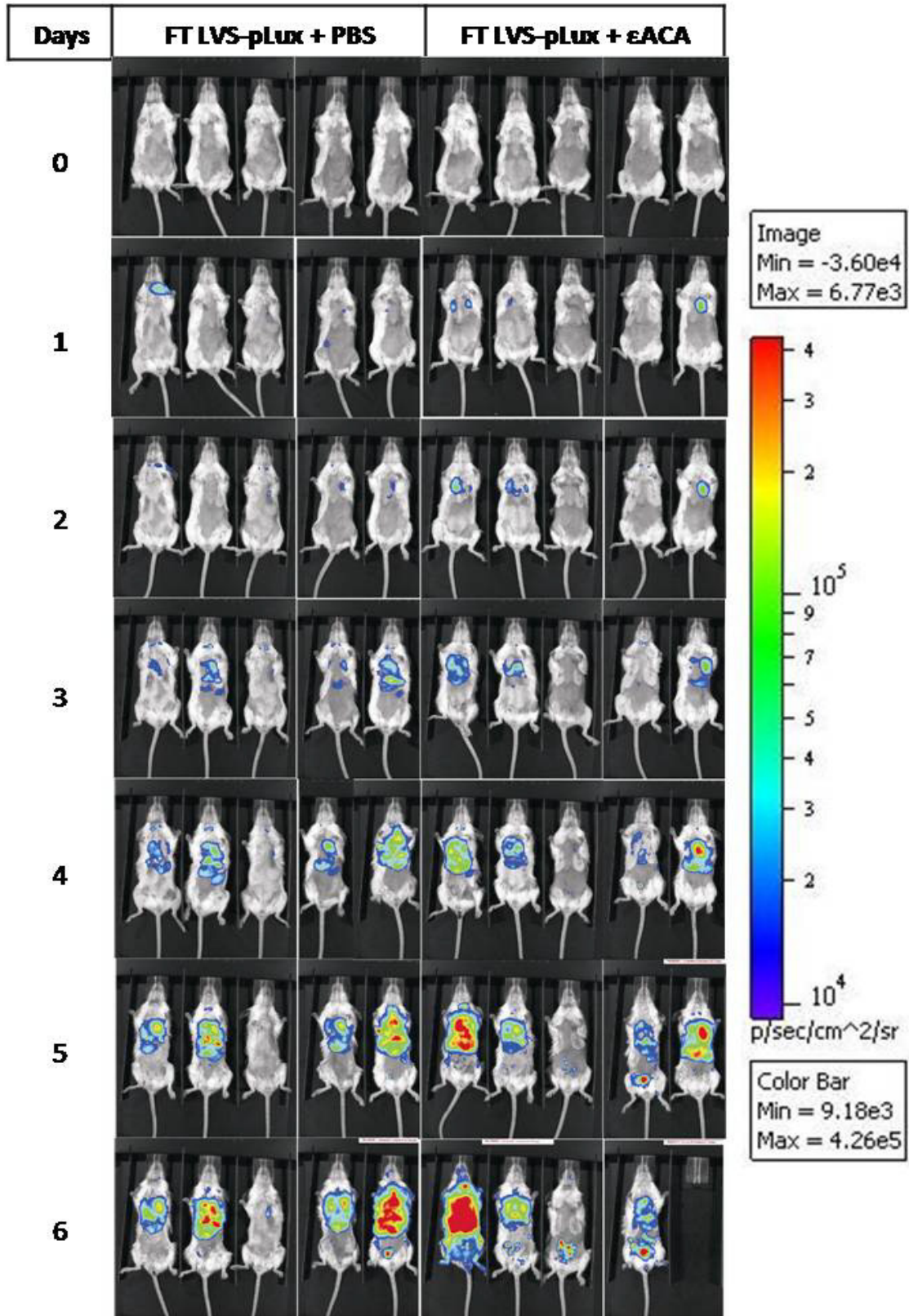


Figure 4.3. Daily weight measurements of Balb/C mice after intranasal infection with FT LVS-pLux. Female Balb/C mice were infected intranasally with 1×10^6 CFU of ($\sim 1,000 \times$ LD50) luciferase-expressing FT LVS (FT LVS-pLux). Before and during the course of infection, mice were given intraperitoneal injections of ϵ ACA or PBS alone every six hours. Mice were weighed daily as a measure of disease progression, and the results are reported as percentage of starting weight.

Figure 4.4. Murine infection with FT LVS-pLux with intraperitoneal ϵ ACA injections. Female Balb/C mice were infected intranasally with 1×10^6 CFU of ($\sim 1,000 \times$ LD50) luciferase-expressing FT LVS (FT LVS-pLux). Before and during the course of infection, mice were given intraperitoneal injections of ϵ ACA or PBS alone every six hours. Dissemination of FT LVS-pLux was monitored daily via whole animal live imaging using an IVIS Spectrum imaging system. Exposure times varied based on luminescent signal intensities in an effort to collect between 600 and 60,000 counts, and image scaling was normalized by converting total counts to photons/second.



If FT can bind plasmin on its surface, then this active protease could contribute to the distribution of this bacterium through the course of tularemia. In an effort to test this hypothesis *in vivo*, I administered a plasminogen-binding inhibitor (ϵ ACA) every six hours to mice that had been infected with a lethal dose of FTLVS-pLux (1,000 X LD₅₀) and monitored disease progression by monitoring outward clinical symptoms, weight, and actual bacterial distribution patterns via whole live animal imaging. Unfortunately, I observed no significant differences in disease progression relating to treatment with ϵ ACA. At the time of my *in vivo* experiment, I found only one published work which utilized ϵ ACA in an influenza virus infection model (72), and none featuring the numerous bacterial pathogens that are known to interact with plasminogen. However, during the writing of this manuscript, a paper was published that used ϵ ACA to inhibit plasminogen in a staphylococcal infection model found that this drug actually *increased* the severity of disease and *decreased* the time to death relative to mice receiving injections of PBS only. Based on these findings, one may speculate that the inhibition of plasminogen/plasmin, or that ϵ ACA itself, has an unknown detrimental effect upon the innate immunity of the host that may negate any impairment caused on the virulence of the pathogen(s). Neutrophils and monocytes are an important line of defense against bacterial infection, and it has been shown that the activation of these cell types increases the number of plasminogen binding sites on their surface (101-103). Though the function of plasminogen bound to the surface of neutrophils and monocytic cells is not very clear, it is possible that the presence of a plasminogen inhibitor such as ϵ ACA may uncouple some important function(s) needed to clear bacterial pathogens such as FT.

Here I have shown that FT LVS can bind to PLG and that surface-bound PLG can be activated by tPA to its proteolytic form (plasmin). The binding of PLG on the surface of FT could play a role in several phases of tularemia, including the initial entry into the host through insect bites and/or broken skin where active fibrinolytic processes would provide an early opportunity for FT to acquire proteolytic activity that might augment the establishment or dissemination of infection. During later phases of tularemia the acquisition of plasmin on the cell surface may contribute to its pathogenicity by degrading host innate effector molecules and extracellular matrix components. Based on the new report that FT-bound plasmin can degrade immunoglobulins (104) as well as the established ability of FT to acquire surface-bound factor H (63), it also appears likely that FT uses plasma components to interfere with host humoral immune mechanisms throughout the course of FT infection. Future studies to identify additional plasma components that can be surface acquired by FT may uncover novel virulence mechanisms used by this pathogen during its extracellular life cycle.

CHAPTER 5. GENERAL DISCUSSION

Summary

Our current understanding is that following entry through broken skin or mucus membranes, *Francisella tularensis* (FT) is taken up by resident phagocytic cells before disseminating throughout the host. These FT-infected cells are believed to travel by means of lymphatic vessels and finally replicating in the draining lymph nodes as well as major organs such as the lungs, spleen, and liver. Besides being found inside host cells, FT can also be found in surprising numbers as extracellular bacteria during later stages of tularemia (60-61). As might be expected, being in contact with host plasma proteins outside of the cell leaves FT exposed to components of innate immunity, such as complement proteins, antimicrobial peptides, and various opsonizing proteins. Despite all of these potential killing mechanisms of the mammalian host, FT appears not only resistant to these threats (41, 97, 105), but in some instances has been shown to use such host proteins to its advantage. In one such example, it has been suggested by Ben Nasr and Klimpel that FT circumvents the assembly of the complement membrane attack complex on its surface while enabling complement-mediated opsonophagocytosis by using host factor H (63). FH protects host cells from activation of complement via the alternative pathway by recognition of sialic acid and other polyanions present on the mammalian cell surface (106). An affinity for sialic acid/polyanions helps direct FH activities toward mammalian cells, which is absent on the surface of most bacteria, thus providing a way for FH to discriminate host from pathogen. The binding of FH to the host cell surface dissociates C3bBb and makes C3b susceptible to factor I-mediated cleavage. Similar complement aversion mechanisms are not only found in FT, but other bacterial pathogens such as *Streptococcus pyogenes* (107), *Haemophilus influenza* (108), *Neisseria gonorrhoeae* (109), *Neisseria meningitidis* (110), and *Borrelia* spp (93, 111). These bacteria have been shown to express receptor molecules that allow them to exploit the action of FH and other complement inhibitors thereby increasing their survivability in the host extracellular environment. Interestingly, a study utilizing monoclonal antibodies directed against a factor H binding protein (GNA1870) from *N. meningitidis* has demonstrated a bacteriocidal effect in the presence of human serum (112), underlining the necessity of this host protein for its survival in the extracellular environment.

Besides components related to the complement pathways, many bacterial pathogens have previously been shown to utilize host plasma proteins such as plasminogen/plasmin to increase dissemination and/or subvert innate immune responses in the host (64-67). Because there is a lack of published work on FT interactions with host plasma proteins, I first sought to determine whether certain proteins that are commonly used to enhance virulence (fibrinogen, fibronectin, and plasminogen) have a significant binding affinity for this bacterium. In Chapter 3 I described ELISA assays in which I was able to confirm the association of factor H, fibronectin, and plasminogen with FT-coated microtiter plates after incubation with human fresh frozen plasma (**Appendix A**). Because the binding of a protease to the bacterial outer envelope has the potential to increase pathogenicity within the host, I chose to focus our efforts on characterizing the plasminogen:FT interaction.

A wide variety of bacterial pathogens are known to utilize plasminogen/plasmin in ways thought to aid in their survival and dissemination within the host extracellular

environment. The type of plasminogen receptor proteins used by these bacteria exhibit tremendous diversity, ranging from the surface exposed lipoproteins (*B. burgdorferi*) to glycolytic enzymes such as glyceraldehydes-3-phosphate dehydrogenase and α -enolase (Group A streptococci) (64, 113-114). PLG is well-known for its affinity to lysine residues on the surface of protein receptors. Our work clearly showed that plasminogen binding to FT could be inhibited by the inclusion of the lysine analog, epsilon aminocaproic acid (ϵ ACA) in our ELISA experiments, indicating the involvement of lysine residues in binding to these bacterial receptors. Furthermore, I was able to identify several putative plasminogen receptors from a FT LVS membrane fraction through Far-Western blotting approach and proteomic identification.

In order for plasminogen to be converted to its active form (plasmin), it must be activated by plasminogen activators, such as tissue type plasminogen activator (tPA). Because this is also relevant in the case of PLG on the bacterial outer envelope, I decided to test the ability of FT to activate bound PLG in a set of experiments. By using a chromogenic plasmin substrate, I demonstrated the ability of plasminogen to be converted to an active protease on the bacterial outer envelope in the presence of tPA. The presence of plasmin on the surface of pathogenic bacteria such as *Borrelia* spp is known to enhance their ability to penetrate the extracellular matrix and augment dissemination/tissue penetration within the host (115-118). When plasmin-coated FT bacteria were incubated in the presence of fibronectin, a component of extracellular matrices (ECM), it clearly demonstrated proteolytic cleavage of this substrate in my experiments *in vitro*.

Finally, because it is unknown how the inhibition of PLG binding to FT *in vivo* might affect the observable aspects of lethal tularemia, I sought to try and answer this question in a murine model. I conducted an *in vivo* experiment by infecting ϵ ACA-injected mice with FT LVS-pLux, but was unable to prevent lethal infection or detect any significant changes in disease progress with this treatment. This result was not too surprising considering the multiple functions of plasmin within the context of overall homeostasis. In addition, since this type of treatment has yet to be shown as an effective means to inhibit the disease-causing potential of other known plasmin-binding pathogenic bacteria, it is possible that other infection models failed to respond positively to plasminogen inhibition.

Binding of Human Plasma Proteins to FT and the Identification of Putative Receptors

The first experiments presented in this work involved incubating human FFP with FT bound to ELISA plates. This was done primarily to screen for possible interactions with known and unknown plasma/serum proteins (fibrinogen, fibronectin, plasminogen) by detection with antibodies specific for each antigen. These particular plasma proteins were chosen because of their established roles in aiding survival and/or dissemination in other bacterial pathogen models. Since it was known that factor H is able to bind FT (63), this served as an appropriate positive control. Using a modified ELISA assay, human PLG was shown to bind significantly to the immobilized FT bacteria. Similar ELISA experiments using purified human and murine PLG instead of whole plasma revealed that binding to FT was independent of any other host protein and also not a host-specific interaction. In addition, the ELISA experiments with purified PLG (3 μ g/mL)

detected binding to FT at a concentration far lower than might be found in whole plasma (~200 µg/mL for humans).

Following up on my experiments with FT LVS, I then wanted to assay the ability of PLG to bind a more virulent subspecies of FT. My results revealed that the binding of PLG to FT was shown to occur in both the attenuated FT LVS and highly virulent FT Schu S4 subspecies. Interestingly, my ELISA data appears to show a slightly higher amount of both human and murine PLG binding to FT Schu S4 in relation to FT LVS. These results were in agreement with a report that was published very recently by Crane, *et al* (104). Within this report the authors noted an interesting effect on opsonization after treatment with PLG by which the active protease degraded anti-FT IgG molecules, resulting in decreased uptake of virulent, but not avirulent FT by macrophages. Their results indicated an ability of highly virulent FT to bind greater amounts of PLG/plasmin relative to the less virulent FT LVS. Additionally, their work also showed that only FT Schu S4, but not FT LVS, could bind the active form of the protease (plasmin). These experiments were conducted by adding a plasminogen activator (uPA) together with plasminogen for the detection of bound ligand as opposed to adding plasminogen alone. Together these data open the possibility that having plasmin on the outer envelope of FT could play a role in the ability of those subspecies to have increased survivability and/or invasiveness in the host by decreasing the number of these bacteria that are taken up by host phagocytes.

Because the localization of FT-bound PLG would likely require surface exposure to allow its activation to plasmin by host plasminogen activators and also for proteolytic activity to occur, I then performed confocal microscopy experiments with pre-coated bacteria. FT LVS that were allowed to incubate in the presence of human PLG, washed, and then fixed to glass coverslips were then used to detect this ligand on the bacterial outer envelope. By using a fluorescently labeled secondary antibody, confocal microscopic analyses suggest that PLG does indeed bind to the surface of FT. However it remains a possibility that some of the staining observed was the result of PLG penetration into the FT outer envelope via lipid mixing caused by the methanol fixation step. An attempt to get around this fixation step through the use of flow cytometry proved unsuccessful, possibly due to the effect of sheer forces displacing the fluorescently-labeled PLG from the bacterial outer envelope. Perhaps cross-linking of bacterial surface-bound PLG with formaldehyde or glutaraldehyde prior to antibody staining may resolve this issue for future studies by flow cytometry.

Many bacterial pathogens have previously been shown to utilize host plasminogen/plasmin to increase tissue invasion and subvert innate immune responses in the host. Within this broad range of bacterial species lies an equally diverse group of unique membrane and cytoplasm-associated plasminogen receptors that have been identified (64-67). Because the identification of PLG receptors in FT may provide us with potential therapeutic or vaccine targets, I set out to try and identify these proteins from membrane fractions. Our SDS-PAGE/Far-Western blotting experiments and the subsequent mass spectrometric identification of the most prominent FT LVS PLG-binding membrane proteins resulted in the identification of five putative PLG receptors: peptidoglycan-associated lipoprotein (FTL_0336), IpnA (FTL_0421), a hypothetical lipoprotein (FTL_0645), FKBP-type peptidyl prolyl cis-trans isomerase family protein (FTL_1042), and fopA1 (FTL_1328). I found it interesting that two of the identified PLG receptors, fopA1 and IpnA, are poorly characterized and both highly conserved lipoproteins that are unique to FT. One very recent study reported an increase in the

survival of mice that had been intradermally infected with FT LVS and later passively immunized with anti-fopA and anti-lpnA monoclonal antibody preparations (119). Whether or not the observed increase in mouse survival from tularemia in those experiments is related to the antibodies' ability to block PLG or other plasma proteins from binding to receptor sites cannot be determined at this time, but should be considered in future studies.

Plasminogen Bound to the Surface of FT LVS Demonstrates Proteolytic Abilities in the Presence of a Plasminogen Activator

Though the intracellular phase of FT infection has been well-documented, the recent reporting of high numbers of extracellular bacteria during tularemia brings to light the potential interaction of this pathogen with host plasma proteins. Our previously described experiments in Chapter 3 sought to characterize the ability of FT to bind any of several plasma proteins that are known to be used by pathogenic bacteria to increase their virulence and/or tissue invasion within the host. Following an ELISA-based screening experiment using human plasma, the detectable binding of plasminogen drew our attention because of the potential benefits to virulence in having this host protease zymogen bound to its outer envelope. Because any benefit of having the zymogen form of this protein bound to the outer envelope is limited by its ability to be converted to an active protease by a plasminogen activator, I sought to further characterize the proteolytic potential of PLG-coated FT bacteria.

The first set of experiments to measure plasmin activation involved incubating PLG-coated bacteria in the presence of tissue type plasminogen activator (tPA) and a plasmin-specific chromogenic substrate (D-VLK-pNA). When this plasmin substrate is cleaved of its peptide moiety by the active protease, it in turn causes the development of a yellow color that can be measured by an increase in the spectrophotometric absorbance of the attached chromophore, p-nitroaniline (pNA), at 405 nm relative to controls lacking one or more necessary components. Our results clearly indicated the generation of a significant amount of plasmin activity when PLG-coated FT LVS were incubated in the presence of both tPA and this plasmin-specific substrate, which showed a three-fold increase in absorbance at 405 nm relative in relation to PLG-coated bacteria that lacked tPA. Based on these findings, I concluded that not only is plasminogen able to bind the surface, but that it is also possible to generate the active form of this enzyme (plasmin) which may also degrade known host protein substrates.

Next, in order to test the ability of plasmin-coated FT to degrade a host protein found in the ECM, I conducted experiments to look at the ability of these proteolytically activated bacteria to degrade fibronectin. By incubating plasmin-coated FT LVS with purified fibronectin, I was able to show through Western-blot analysis that these treated bacteria clearly possessed a proteolytic affect upon this substrate. This opens the possibility that FT could use the ability of plasminogen/plasmin acquisition on its surface to aid in the ability to both penetrate the ECM to disseminate more easily in the host and to avoid the effect of active and passive immune responses. Direct evidence of the contribution of plasminogen/plasmin to tissue penetration in the host is difficult to prove *in vivo*, but some *in vitro* models have been used. These types of assays have been performed in other well-studied plasminogen-utilizing pathogens within the genus *Borrelia*. Coleman *et al* has shown in their work where plasmin-coated *B. burgdorferi* have the ability to penetrate endothelial cell layers *in vitro* (115). By using an *in vitro*

model of the blood-brain barrier, *B. burgdorferi* has demonstrated its ability to cross this cell layer in the presence of plasminogen (117). Though rare, cases of tularemia meningitis are present in the literature and this may represent an interesting example of how FT could cross ECM barriers to gain access to less hostile environments such as the central nervous system (120-126). However, as this is obviously an inappropriate experiment in humans, further studies on the possible contribution of plasmin to tularemic meningitis would require the development of an appropriate animal model of infection which is currently unavailable at this time. In all, these data suggest that the binding of plasminogen by FT has the potential to augment its ability to penetrate tissue barriers in the host.

The generation of plasmin on the surface of FT has recently been shown by Crane *et al* to decrease both the opsonization of FT-specific antibodies as well as the opsonophagocytosis of these bacteria by phagocytic cells (104). Most importantly, their findings also indicate that plasmin has a proteolytic effect on immunoglobulins, which they state may help explain the inability of humoral immunity alone to prevent lethal infection with virulent strains of FT (104). I feel that their report helps to support our findings in outlining some of the possible ways in which plasminogen/plasmin bound to the outer envelope of FT could augment the survivability and perhaps the invasiveness of this bacterial pathogen *in vivo*.

Murine Tularemia Studies with the Inclusion of a Plasmin Inhibitor

Our previous studies described experiments which provided evidence of plasminogen's ability to bind the surface of both a virulent and an attenuated strain of FT and provided the identification of putative receptors from its membrane fraction. Within those experiments I showed how this binding of plasminogen to FT could be inhibited in the presence of a lysine analog (ϵ ACA). I also provided experimental evidence of the activation of plasminogen to plasmin on the bacterial outer envelope and a demonstration of its proteolytic potential on an ECM component, fibronectin.

Because it is unknown how the inhibition of PLG binding to FT *in vivo* might affect any directly observable aspects of lethal tularemia such as weight loss, survival, and bacterial distribution, I sought to try and answer this question in a murine model. I approached this question by conducting an *in vivo* experiment by giving regular doses of the plasminogen inhibitor ϵ ACA to naïve Balb/c mice before and during a lethal challenge with FT LVS-pLux. I chose to use FT LVS-pLux for the infection because this method would allow us to track the bacteria by capturing luminescent images of live animals through the course of infection by using the Xenogen IVIS photodocumentation system.

Unfortunately I was unable to prevent lethal infection or detect any significant changes in disease progress with FT-infected mice that had received the plasminogen inhibitor or PBS alone. The weight-loss curves and times to death were essentially the same for both infection groups, thereby leaving the *in vivo* effects of plasminogen-bound FT indeterminable by our method. This was not too surprising considering the multiple functions of plasmin within the context of overall homeostasis and that this type of treatment has yet to be shown as an effective means to inhibit the disease-causing potential of other known plasmin-binding pathogenic bacteria. In fact, there has been a

report on a murine staphylococcal infection model that describes an increase in disease severity while administering regular doses of a closely related lysine analog, tranexamic acid. It is possible that these types of experiments have been tried with other plasmin-utilizing pathogenic bacteria and not reported because they also failed to yield significant results.

Perhaps the pleiotropic functions of plasmin in the host are too complex to simply inhibit them all completely. In considering this possibility, a more direct approach should be used in the context of studying its affect on the course of tularemia infection to avoid interfering with other processes in the host. In one example, plasmin-dependent metalloproteinase-9 activation has been found necessary for macrophage recruitment to sites of inflammation (127). It is possible that inhibiting plasmin activity in my *in vivo* experiments resulted in a decrease in the infiltration of phagocytes to sites of FT infection. Being that phagocytic uptake of FT is an important step in clearance of these organisms, the use of plasminogen inhibitors may not be the best choice for future studies. A more suitable method for preventing plasminogen/plasmin binding *specifically* to the outer envelope of FT may be found by the use of monoclonal antibodies against the identified plasminogen receptors that I have identified. This may be a consideration for future studies in the matter.

In conclusion, I hope that these studies have brought to light another aspect to consider in developing safe and effective treatments and vaccines against infection with *Francisella tularensis*. Published works describing the extracellular phase of FT infection are now coming to light and the possible contributions of plasminogen/plasmin during this phase should not be ignored (**Fig. 5.1**). Though not likely to be a major contributor towards its pathogenicity, further studies on the ability of FT to acquire plasmin may reveal that this process works in concert with other known or unknown virulence factors during tularemic infection.

Future Directions

Because of my work and that of others, we now know that FT is able to interact with at least two host plasma/serum components (factor H and plasminogen) and that these proteins may contribute in some degree to its virulence and survivability within the host. However, expanding upon the experiments presented in this work may be necessary to define clear roles, especially *in vivo*.

My *in vivo* experiments utilizing the lux-expressing strain of FT sought to determine changes in the distribution of bacteria following an intranasal challenge during treatment with a plasminogen inhibitor. Perhaps variations on this experiment could be designed that focus on *other routes of infection* which could be used to more accurately determine the effects of plasminogen binding to FT. One problem with my *in vivo* infection model was that by giving the bacteria intranasally it is extremely difficult to ensure that all FT remained in contact with the respiratory tract and are not swallowed. This brings to question whether the intended CFUs delivered *for this route* were indeed the same between any two mice.

A second problem with the original *in vivo* model lies in the diminishing signal from the bacteria at day 0. Being that such few bacteria are delivered and that they must travel from the nares to various parts of the respiratory tract, the dilution of

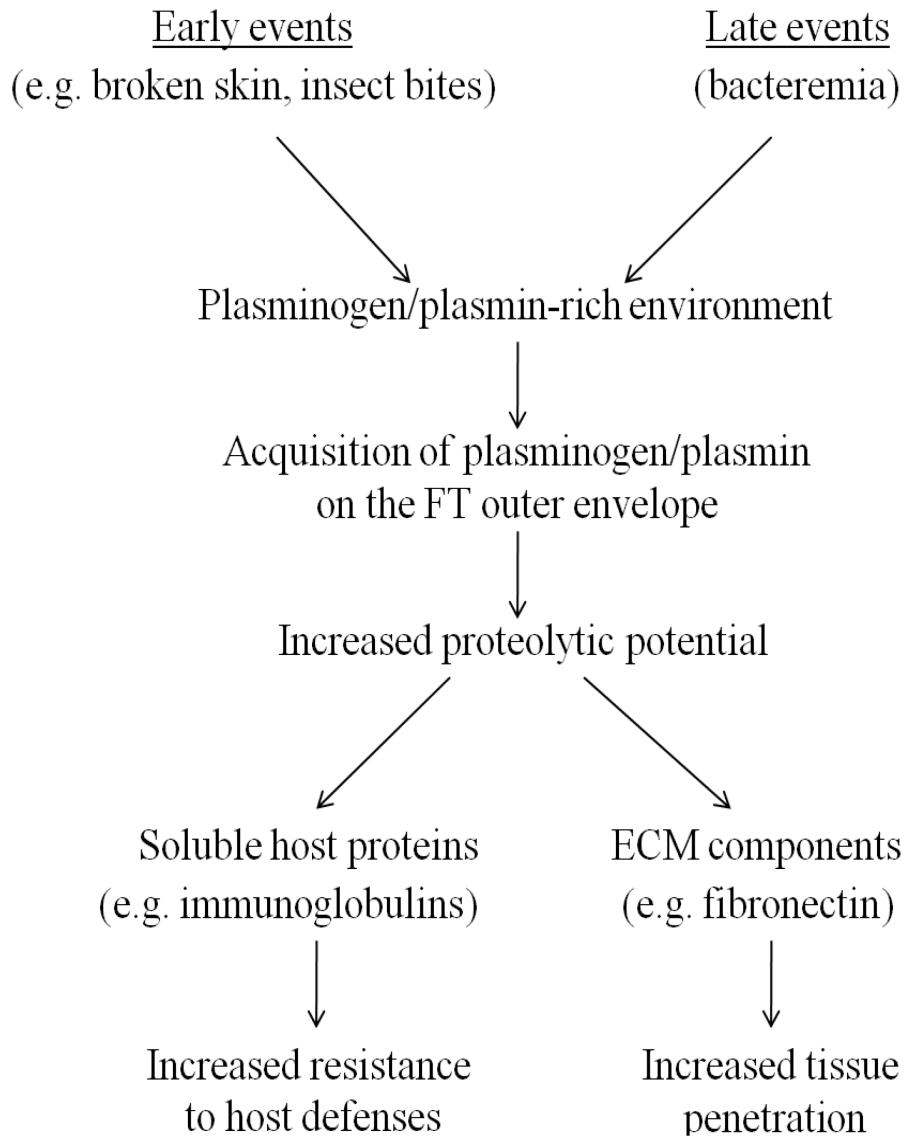


Figure 5.1. Diagram summarizing the potential roles that plasminogen may play during FT infection. Both examples of early and late stages of FT infection occur within plasminogen-rich environments. It is within damaged tissue and/or blood where FT would be likely to acquire plasmin on the outer surface. This protease-bound pathogen could then aid in the degradation of host proteins, as indicated in recently published work. Further work is needed to describe the *in vivo* effects of this process.

signal at this point removes a potentially important data point. Entry into the host could be the first opportunity for FT to encounter available plasminogen and changes in the distribution of bacteria at the initiation of infection would be difficult to discern with this methodology.

The third and most important variation on this line of experimentation lies in the use of appropriate routes of infection for measuring the effects of plasminogen on FT infection. To measure the effect(s) of FT with the ligand we may want to ensure contact with plasminogen throughout the initial point of infection. This goal may better be attained by placing the bacteria within the tissue as opposed to infecting mice by a mucosal surface as found in the respiratory tract. It is unclear how much plasminogen may be encountered via the respiratory route and this may be inviting error into the interpretation of results from the very start of the experiment.

One way to possibly resolve these issues could be to use an intradermal or subcutaneous infection model. This route may be advantageous to the goals of this work for several reasons. First, by giving the bacteria under the skin the lux signal will be more concentrated and have less tissue to absorb the light, thus improving the resolution of an initial infection image. Second, both the distribution and intensity of signal away from the injection point could more accurately be used to gauge changes induced by inhibitors or the pre-coating of bacteria with plasminogen. It is possible that a quantifiable change in the diameter of the luminescent might show differences between groups of mice. Finally, this route most closely resembles a more naturally occurring infection (arthropod vector or entry through broken skin) and may be more appropriate in a general manner. Establishing this method might then allow one to closely measure changes in tissue penetration/dissemination of FT while treated with plasminogen inhibitors. Alternatively, these experiments could be used to compare different mouse strains or PLG knock-out mice to look for changes in the distribution of FT by intradermal/subcutaneous routes.

Other experiments may include ways to address the importance of FT surface lysine residues in binding PLG on its surface. As discussed in Chapter 3, mutating each lysine on the bacterial surface would be a near impossibility if one wished to recover each and every mutant clone. Luckily, biotinylation kits are commercially available that allow the user to modify lysine residues on proteins by reacting them with biotin. This simple and effective method may allow us to essentially block the kringle domains of PLG from binding lysine on the bacterium. These lysine-blocked bacteria could then be coated onto ELISA plates and experiments could be performed as mentioned previously in this work to show its effect on PLG binding. A loss of signal in these experiments would provide further evidence for a dependence of lysine in the FT-PLG binding. An additional experiment with the same lysine-blocked FT could be used in a Far-Western blot format to show a loss of PLG binding to bacterial surface proteins. However, one would have to consider the increase in molecular weight and how this would affect the migration rate of the modified FT proteins during SDS-PAGE.

Aside from plasminogen, other host-derived proteins should also be considered. Given the multitude and quantities of available host proteins in blood and other fluids, it is unlikely that we have found all cases where FT may use host proteins and other molecules to its advantage. Though only briefly mentioned in this work, soluble fibronectin was also positive for binding to FT in our preliminary ELISA screening

method with a single putative receptor protein being identified (**Appendix B**). The ability of bacterial pathogens to bind fibronectin has been studied in such infection models as *Pseudomonas aeruginosa* (128), *Borrelia burgdorferi* (85, 129) , and has been well documented in Gram-positive cocci (130-131) as a means for adherence and uptake of these organisms into host cells. It is possible that FT uses a similar mechanism to enter non-phagocytic cells, which is another set of experiments that should be considered for future work. Additionally, the FT culture filtrate components that were identified in my previous work (**Appendix C**) might also interact with host molecules and should be taken into account in future studies as well.

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APPENDIX A. PRELIMINARY FT SCREENING ASSAYS USING HUMAN PLASMA

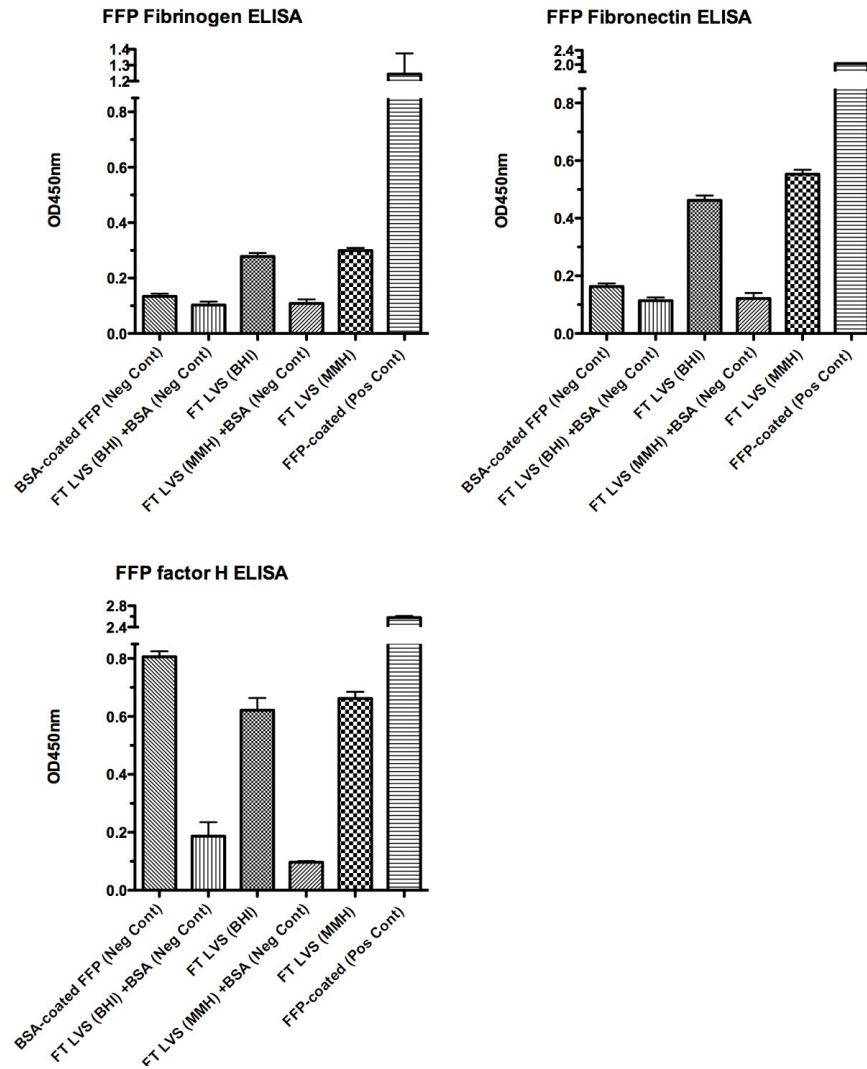


Figure A.1 Preliminary FT screening assays using human plasma. Microtiter plates coated with FT LVS cultured to mid-log phase in MMH or BHI broth were incubated for 1 hour with fresh frozen human plasma (FFP). A modified ELISA was performed to measure FT LVS-bound ligands as indicated. The results shown are representative of 2 experiments of similar design. Bars indicate +/- SEM in triplicate.

APPENDIX B. IDENTIFICATION OF A PUTATIVE FIBRONECTIN-BINDING PROTEIN FROM FT

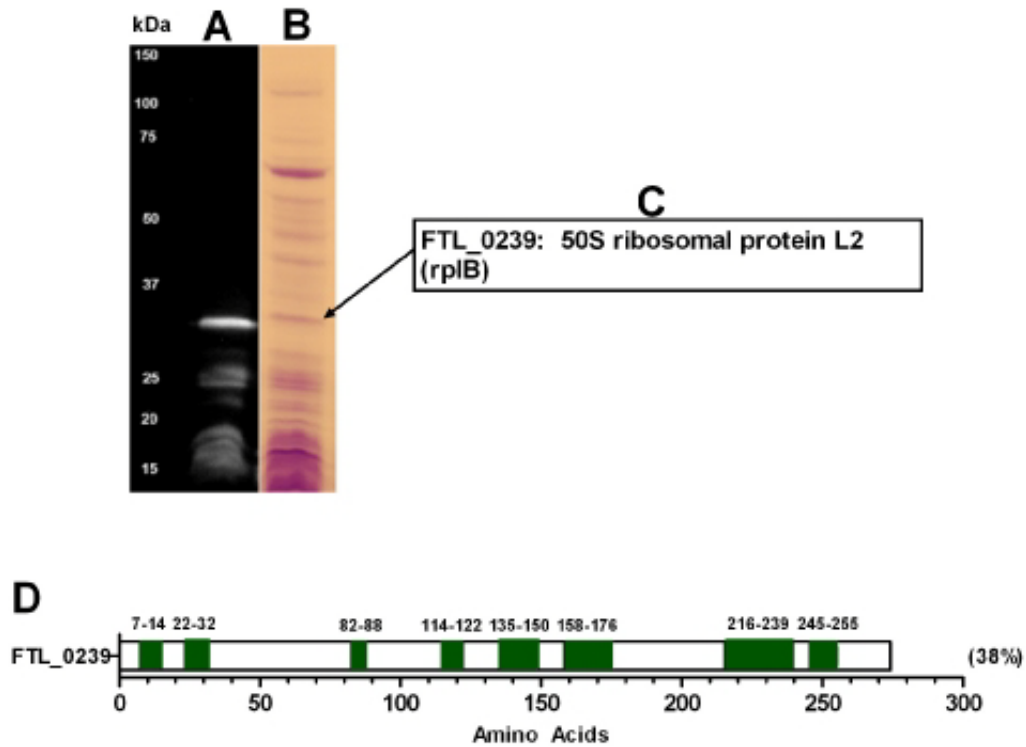


Figure B.1. Identification of a putative fibronectin-binding protein from FT. A Sarkosyl- insoluble protein fraction of FTLVS was separated by SDS-PAGE and transferred to PVDF membrane. Membranes were then blotted with human fibronectin (3 $\mu\text{g}/\text{mL}$) followed by sheep anti-fibronectin antibody and HRP-conjugated anti-sheep IgG secondary antibody to detect fibronectin-binding proteins (**Panel A**). Protein bands on an identical Coomassie Blue-stained SDS-PAGE gel corresponding to those identified via blotting were excised (**Panel B**) and identified using proteomic methodologies (**Panel C**) with the indicated peptide coverage map (**Panel D**).

APPENDIX C. STUDIES USING FT LVS CULTURE FILTRATE PREPARATIONS AS AN IMMUNIZATION AGAINST TULAREMIA

Summary and Purpose

Francisella tularensis (FT) is the causative agent of a life-threatening disease known as tularemia. The high virulence and low inoculum needed to cause tularemia led several countries to pursue the development of *Francisella* as a biowarfare agent starting in the 1930's and is a high-priority organism in anti-bioterrorism research. Incomplete understanding into the details of its pathogenicity have complicated immunoprotection studies for countering an FT infection and have led many researchers over the years to examine the successful vaccination strategies used for other pathogenic organisms. Several of those approaches have been previously explored for FT vaccination, including live attenuated strains, killed bacteria, lipopolysaccharide preparations, and purified proteins with various degrees of protection seen in murine animal models. Preliminary results in our laboratory has found that intraperitoneal immunization using a FT LVS culture filtrate (CF) preparation offers protection against intraperitoneal and intranasal infection in a murine model. I hypothesized that immunization with recombinant forms of FT CF antigens would induce a long-lasting and effective immune response against a lethal FT challenge. The overall goals of the studies described herein sought to focus on the following: 1) to identify the protective antigens in FT CF and 2) to evaluate the ability of recombinant forms of these antigens to stimulate the protective immune responses necessary for developing a safe and effective vaccine against tularemia.

Introduction

Francisella tularensis (FT) is a highly infectious Gram-negative intracellular pathogen which is the causative agent of tularemia. *F. tularensis* has been found throughout the Northern Hemisphere and infects a variety of vertebrate and invertebrate hosts (2, 6). *Francisella* is the only member of the family *Francisellaceae* and is composed of the two species *F. tularensis* and *F. philomiragia*. Of the two *Francisella* species, *F. tularensis* is made of four subspecies: *tularensis* (type A), *holarctica* (type B), *mediasiatica*, and *novicida*. The most severe cases of human tularemia are often attributed to *F. tularensis* subsp *tularensis* (type A) which makes up approximately 2/3 of reported cases and found almost exclusively in North American cases of infection (4). Human cases of tularemia are often associated with wild game hunters, landscape laborers, and laboratory workers (6, 14) that are exposed to infectious materials such as animal tissue (6-7) or by arthropod vectors (6-10). Because FT is both easily obtainable from the environment and highly infectious, this organism is classified as a class A select agent by the CDC consequential to its former role as a biowarfare agent (28) and its potential use for bioterrorism purposes. It is for these reasons that a safe and effective vaccine against tularemia is needed.

While our current understanding is that surviving an infection with *Francisella* results in life-long immunity to tularemia in humans, it also opens the possibility that a protective vaccine against this disease is possible. Attempts to develop a safe and effective vaccine against tularemia have been met with various degrees of success in the past. Some of the first vaccination methods attempted were killed bacterial vaccines

tested by Foshay during the 1930's and 1940's which consisted of formalin treated, heat killed, and oxidized preparations of virulent FT strains, all of which offered very limited protection against human tularemia although eliciting significant anti-FT serum antibody titers (31). Protection against infection with *F. tularensis* is currently limited to scarification with an attenuated live vaccine strain (FT LVS) of *F. tularensis* subsp *holarctica*, but is currently not licensed and does not illicit complete immunity against infection with type A strains of FT (26-27, 32). An attenuated FT Schu S4 strain was reported by Twine *et al* to protect mice against wild-type homologous challenge after sublethal infection, though currently untested in humans (33). Murine models of infection have shown that anti-FT antibodies alone are able to protect against infection with type B strains of *Francisella* but in studies using the more virulent type A strains interferon, -gamma as well as CD4+ and CD8+ T-cells are needed to survive a lethal bacterial challenge (35-36). Immunization of mice with LPS extracted from FT bacteria offers protection against lethal challenge infections with FT LVS, but fails to protect against challenge with the highly virulent FT Schu S4 strain (36-37). Furthermore, infection of mice using a mutant strain of FT LVS with a transposon-inactivated gene encoding *wbtA* results in an LPS O-antigen-deficient phenotype and further attenuation that offers protection in wt FT LVS challenge experiments (38-39) and suggests a dispensable role for anti-LPS antibodies in adaptive immune protection against lethal FT infection. Therefore, even though there exists a high level of cross-reactivity between antisera of to the LPS O-antigen of both type A and B subspecies of FT, experimental evidence clearly indicates that bacterial antigens other than lipopolysaccharide may also play a role.

Various strains of *F. tularensis* possess homologous features of type II, type IV, and the newly described type VI secretion systems found in other bacterial pathogens such as *Klebsiella*, *Bartonella*, *Brucella*, *Legionella*, and *Vibrio* (132-135). Recent studies have shown that several immunogenic *Francisella* proteins are detected in culture filtrates from both high and low-pathogenicity strains. Surprisingly, a single report from almost 50 years ago describes a protective immune response against virulent *Francisella* strains following immunization of mice with culture filtrate preparations (136). Preliminary data from our laboratory has shown that immunizing mice with culture filtrate (CF) preparations from a *wbtA*-inactivated strain of FT LVS are able to consistently elicit protection in mice from wt FT LVS challenge, which has also been used to passively immunize naïve Balb/c mice using pooled sera. Following up on this study, I identified four immunodominant protein antigens (*g roL*, *sucB*, *aceE*, and *aceF*) from Western Blot studies using frozen sera taken from those mice before lethal FT LVS challenge. The work presented here also includes immunization experiments using recombinant versions of these and other proteins that are identified.

To date, only a few subunit vaccine formulations have been reported in the literature, including recombinant proteins *lpnA* (137-138) and *fopA* (139), despite several papers documenting at least 94 FT protein antigens that are recognized by immune sera from mice and/or humans (140-143). The lack of published FT subunit vaccine work together with a wealth of available immunoproteomic data clearly indicates an opportunity for FT vaccine researchers to explore these antigens in murine experiments.

Materials and Methods

Extraction of FT culture filtrate components

F. tularensis Live Vaccine Strain (FTLVS) was a kind gift of Dr. Karen Elkins (FDA, Bethesda, MD). A 10 μ L inoculum taken from frozen stocks of wild-type FT LVS or FT LVS 48B10 (LPS O-antigen deficient mutant) were used to start an overnight culture in modified Mueller-Hinton (MMH) broth supplemented 0.025% ferric pyrophosphate, 0.1% glucose and 0.1% cysteine, 0.625 mM CaCl_2 , 0.530 MgCl_2 , (plus 10 $\mu\text{g}/\text{mL}$ kanamycin for FT LVS 48B10 strain) and cultured at 37°C with 200 RPM rotation. This overnight culture was used to dilute ~500 mLs of pre-warmed MMH to a final OD^{600} reading of 0.1 on a spectrophotometer, using an aliquot of uninoculated MMH as a blank. Frequently, small culture samples were read spectrophotometrically until an OD^{600} reading of 0.4-0.5 was reached. After reaching this desired mid-log phase culture density, the entire culture was dispensed into 2 x 250 mL Cornell conical centrifugation bottles, and were spun at 4,000 RPM and 4°C for 30 minutes. Carefully, the culture supernatant was poured into two additional fresh conical centrifugation bottles and the previous step was repeated to pellet any bacteria that became dislodged from the bacterial cell pellet. After the second centrifugation step, the clarified supernatant was filtered through a 0.2 micron bottle-top filtration unit to remove any residual bacterial cells and particulates. To this clarified culture filtrate, an equal volume of pyrogallol-red molybdate methanol (PRMM) buffer solution (0.05 mM pyrogallol red, 0.16 mM sodium molybdate, 1.0 mM sodium oxalate, 50.0 mM succinic acid, 20% [vol/vol] methanol in H_2O , adjusted to pH 2.0 with HCl) were added into a sterile 1L glass bottle and incubated overnight at 4°C with magnetic stirring to enhance precipitation of the proteins, an adaptation of a previously described method. (144-145) The PRMM-buffered FT culture filtrate was then poured into 4 x 250 mL conical centrifuge bottles, and spun at 4,000 RPM and 4°C overnight. After removing the supernatant, all precipitate material was dislodged from the conical bottles using approximately 10 mLs of PBS w/ 2.5% glycerol and vigorous pipetting. The pooled collections of precipitate were then split into 2 x 30 mL round-bottom polypropylene centrifugation tubes with 100% acetone added to a final concentration of 80% (v/v), vortexed, and stored at -20°C for 30 minutes before further centrifugation at 20,000 RPM for 15 minutes at 4°C. After the final spin, the acetone-containing supernatant is removed and the precipitate is allowed to air-dry at room temperature. To the dry precipitate, approximately 5 mLs of PBS w/ 2.5% glycerol was added before vortexing to resuspend the pellet and then frozen at -20°C for further experiments. Total protein concentrations were spectrophotometrically estimated using pyrogallol-red-molybdate buffer and a bovine serum albumin protein standard solution and standard curves generated by a shift in absorbance from 470 nm to 590 nm (146).

SDS-PAGE analysis of FT LVS culture filtrate precipitate

Equal amounts of FT LVS whole bacterial lysate and culture filtrate (CF) were precipitated using ice cold acetone, incubation at -20°C for approximately 30 minutes, then centrifuged at 15,000 RPM for 20 minutes, removed supernatant, and air-dried at room temp. Each sample was resuspended in 1x Laemmli denaturing SDS-PAGE sample buffer with 2-mercaptoethanol, boiled for 15 minutes, and loaded on a 4-20% polyacrylamide gradient gel (Pierce) before running ~40 minutes at a constant 150V. At

completion, the gel was Coomassie stained and photographed using a ChemiDocXRS system (Bio-Rad Laboratories, Hercules, CA).

Murine immunization studies

Groups of 4 female Balb/c mice were immunized intraperitoneally (i.p.) with 50 μg of FT LVS CF and/or 0.5 μg rIL-12 (adjuvant), or sterile phosphate-buffered saline (PBS) alone in a total volume of 1 mL. A positive control group was immunized with a sublethal inoculum of 5×10^4 CFU of live FT LVS intradermally (i.d.). Immunizations were given at Day 0, 5, and 15. At +31d after the final immunization, mice were lightly anaesthetized with isoflurane and challenged by i.n. infection with 1×10^4 CFU of live FT LVS in a total volume of 50 μL (25 μL /nare). For passive immunization studies, pooled serum taken +25 days after mice were successfully immunized with FT LVS (48B10) CF and rIL-12 were give to groups of naïve Balb/c mice i.p. (100 μL sera + 400 μL sterile PBS) or immune mice (500 μL sterile PBS alone) and challenged 14 hours later with a lethal i.p. dose (1×10^6 CFU) of FT LVS. Weight and appearance were monitored daily and mice losing 20% of their starting weight were sacrificed.

Immunoproteomic analysis of sera from FT CF-immunized mice

Immunoprecipitation (IP) experiments were perform using pooled sera (+25d after final immunization) from a total of 10 Balb/c mice following immunization with PBS or FT LVS CF (48B10) and rIL-12 were mixed with whole bacterial lysate from wild-type FT LVS containing a protease inhibitor cocktail. After incubation at 4°C for 2 hours with rocking, this mixture was added to pre-swollen Protein A Sepharose CL-4B beads (GE) to bind antibody-antigen complexes overnight with rocking at 4°C. The following day, this slurry was gently micro-centrifuged at 1,000 RPM for 2 minutes to remove supernatant from the beads, followed by two additional washing steps to remove unbound material using PBS and micro-centrifugation at 1,000 RPM. After pipetting away as much of the remaining wash as possible, an equal volume of 2x Laemmli SDS-PAGE sample buffer containing 2-ME was added to the beads, which were then boiled for 10 minutes to completely solublize the Protein A-bound antibody-antigen complexes. The samples were removed from the heat and allowed to cool to room temperature before briefly micro-centrifuging at 15,000 RPM to pellet the beads. The supernatant was subjected to denaturing SDS-PAGE analysis using 4-20% acrylamide gradient mini-gels (Pierce) for ~40 minutes at a constant 150V. After electrophoresis was complete, gels were Coomassie stained and stored at 4°C in gel destaining solution (50% methanol + 10% acetic acid). To confirm IgG specificity from the IP experiments, gels containing separated FT proteins were electrophoretically transferred to Immobilon-P_{sq} PVDF membranes (Millipore), blocked overnight with 5% non-fat milk in PBS at 4°C, probed with pooled mouse antisera, and detected using a goat anti-mouse IgG-HRP 2° antibody. The blots were developed using SuperSignal West Pico Chemiluminescent Substrate and images were documented on a ChemiDocXRS system (Bio-Rad Laboratories, Hercules, CA). The corresponding immunoreactive bands as seen on Western blots were excised from Coomassie-stained gels using sterile scalpels and those proteins were identified by mass spectrometry. Four of the most intensely immunoreactive FT LVS bands from Western blot analysis were identified using vMALDI-LTQ methods similar to those described in Chapter 2.

Results

Precipitation of FT culture filtrate components using pyrogallol-red molybdate methanol buffer

Until recently, little was known about the extracellular proteins and potential secretion systems of *Francisella* (133-134, 147). Additionally, there have been no studies on the immunological response to protein antigens found in the media during culture of FT. I first sought to extract FT culture filtrate (CF) components by utilizing a pyrogallol-red molybdate methanol (PRMM) method which has been previously shown to enhance the recovery of bacterial proteins (144). I chose this method because PRMM has been shown to allow the precipitation of proteins in solutions that are too dilute to extract by other methods, such as those involving cold acetone or trichloroacetate (145). To accomplish this, FT LVS was cultured to the intended density in MMH broth, bacteria were then removed from the media by centrifugation and sterile filtration, and finally combined with PRMM solution and processed as described in the Materials and Methods. Separation of the extracted FT culture filtrate components by SDS-PAGE analysis revealed several prominent bands (**Fig. C.1**).

FT LVS culture filtrate as an immunogen against tularemia in a murine model of infection

To evaluate the ability of immunizations with FT LVS CF to protect mice from a lethal FT LVS intranasal (i.n.) challenge, I injected groups of 4 female Balb/c mice intraperitoneally (i.p.) with 50 µg of FT LVS CF and/or 0.5 µg rIL-12 (adjuvant), or sterile phosphate-buffered saline (PBS) alone in a total volume of 1 mL. A positive control group was immunized with a sublethal inoculum of 5×10^4 CFU of live FT LVS intradermally (i.d.). Immunizations and subsequent boosters were given at Day 0, 5, and 15. At +31d after the final immunization, mice were lightly anaesthetized with isoflurane and challenged by i.n. infection with 1×10^4 CFU of live FT LVS in a total volume of 50 µL (25 µL/nostril). Weight and appearance were monitored daily and mice losing 20% of their starting weight were sacrificed. Results from this experiment indicated a protective effect in mice receiving FT LVS CF+rIL-12 (100% survival) with an increased time to death and survival in the group receiving only FT LVS CF (1/4 surviving) (**Fig. C.2**). As expected, all mice in the PBS group were dead by day 4 and all mice receiving a sublethal FT LVS immunization survived through the duration of the experiment. Together, these results clearly indicate a protective component in FT LVS CF for further evaluation as a potential vaccine against tularemia. Because it is known that immunization with (lipopolysaccharide) LPS O-antigen from FT can elicit protective immunity against infection with low virulence strains of FT, I wished to determine if this component is responsible for the protective effect of FT LVS CF immunization. To evaluate the contribution of LPS O-antigen in the observed protection, a similar experiment was conducted using CF derived from an LPS O-antigen deficient mutant (FT LVS 48B10). Surprisingly, a similar protective effect was seen in the LPS O-antigen deficient CF as was observed in the experiment using CF from wt FT LVS with all mice surviving the study (**Fig. C.3**). Additional experiments with Balb/c mice showed that protection against infection with FT LVS could be maintained at least 72 days after the final immunization, which suggests that the protective effect seen using CF as in

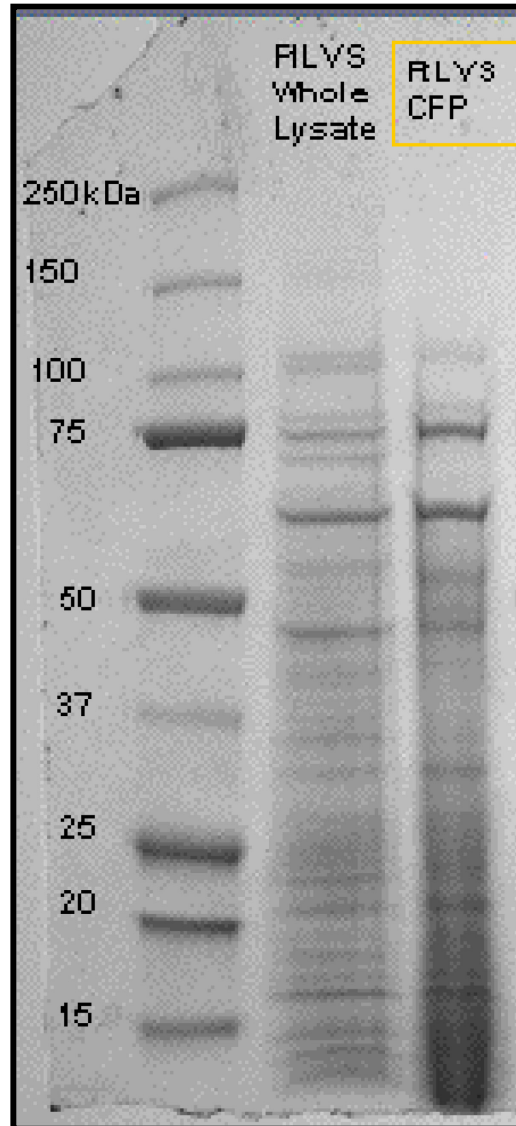


Figure C.1. Coomassie-stained 4-20% SDS-PAGE gel of FT LVS CF precipitate.

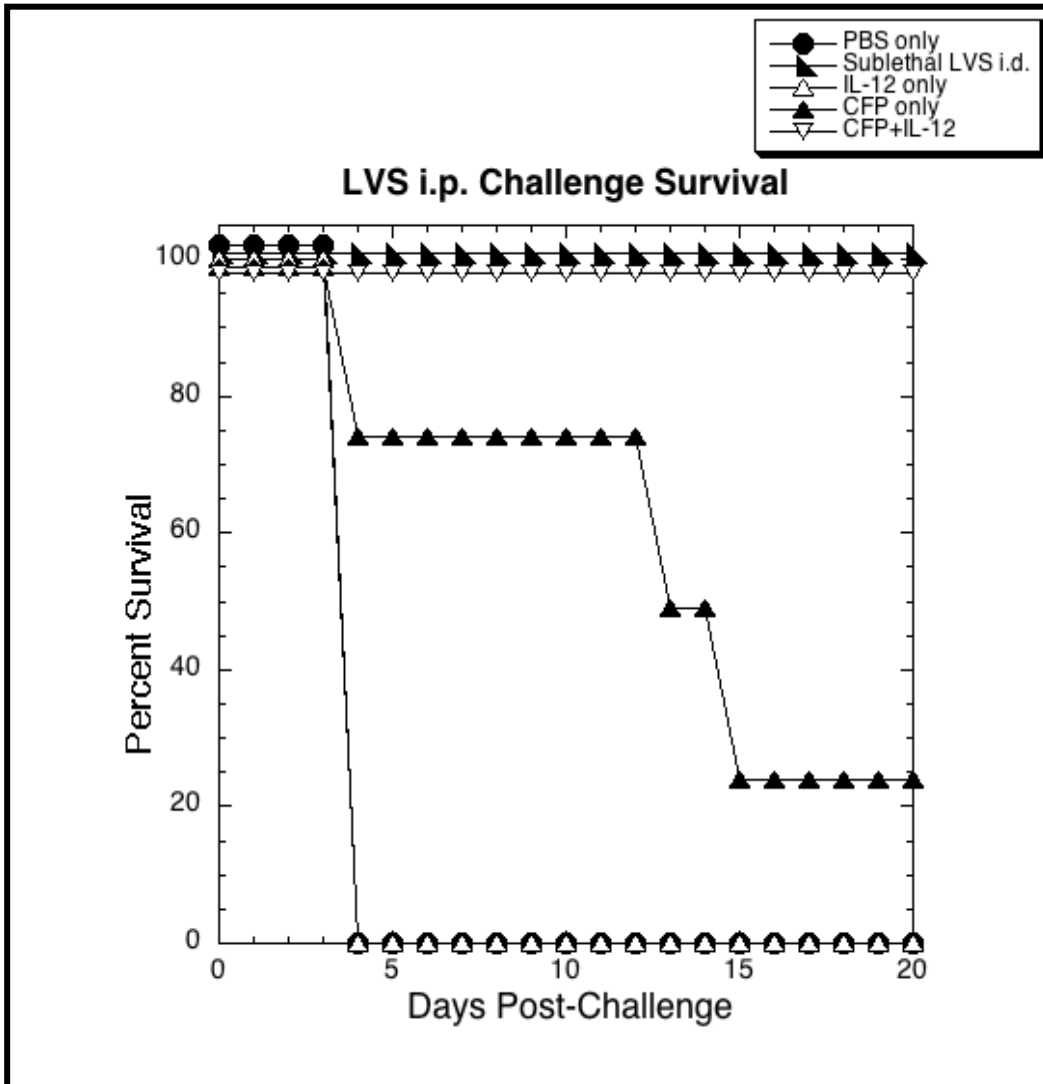


Figure C.2. Kaplan-Meier survival plot of Balb/c mice following i.p. immunization with FT LVS CF and rIL-12 adjuvant. Balb/c mice (4/group) were immunized with the vaccine formulations indicated on days 0, 5, and 15. On day 31, all mice were challenged i.n. with FT LVS. The health and survival of the mice were monitored daily for signs of tularemia. Results are reported as days surviving post-challenge with FT.

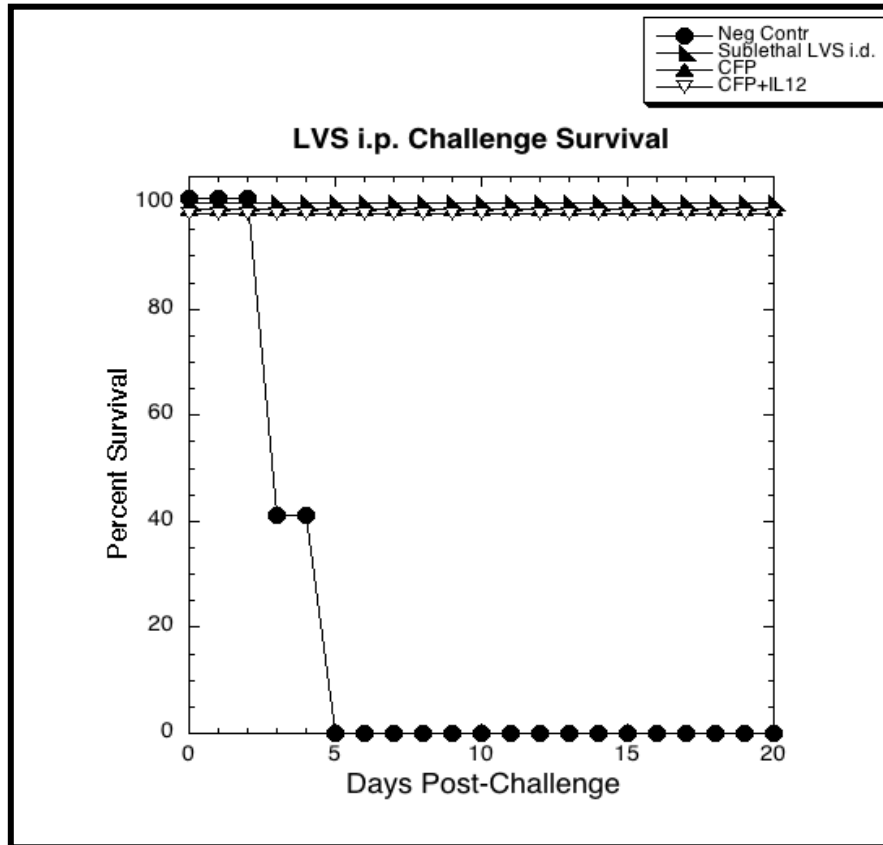


Figure C.3. Kaplan-Meier survival plot of Balb/c mice following i.p. immunization with FT LVS (48B10) CF and rIL-12 adjuvant. Balb/c mice (5/group) were immunized with the vaccine formulations indicated on days 0, 5, and 15. On day 31, all mice were challenged i.p. with FT LVS. The health and survival of the mice were monitored daily for signs of tularemia. Results are reported as days surviving post-challenge with FT.

immunogen is also long-lived (data not shown). In summary, these data indicate that the protective component found in FT LVS CF is not likely LPS, but possibly a protein antigen.

Sera from mice immunized with FT LVS (48B10) CF can be used to passively transfer humoral immunity to naïve mice

It is thought that protection against FT is mediated by both the humoral (immunoglobulins) and cell-mediated (T-cells) adaptive immune responses to these bacterial antigens during an active infection. To determine whether the protection seen in the previous experiments were dependant on cell-mediated adaptive immunity, pooled antisera taken +25 days after mice were successfully immunized (**Fig. C.3**) with FT LVS (48B10) CF and rIL-12 were give to groups of naïve Balb/c mice i.p. (100 uL sera + 400 uL sterile PBS) or immune mice (500 uL sterile PBS alone) and challenged 14 hours later with a lethal i.p. dose of FT LVS. As indicated, it was concluded that i.p. injection of anti-CF sera could protect mice from lethal FT challenge (**Fig. C.4**). Together, these data suggest that because immunity from FT LVS CF+rIL-12 –immunized mice could be passively transferred to naïve mice, it suggests that FT protein-specific antibodies play a role in this model. These findings also suggest that further investigation on the protective effect of FT LVS CF immunization for the possible discovery of novel vaccine components against tularemia.

Immunoproteomic analysis of passive transfer experiments involving FT LVS 48B10 CF

To try and elucidate the protective components of the previously successful immunization, immunoprecipitation (IP) experiments were performed using pooled sera (+25d after final immunization) from a total of 10 Balb/C mice following immunization with PBS either FT LVS CF (48B10)+ rIL-12 were mixed with whole bacterial lysate from wild-type FT LVS. This approach allows antibodies from the sera to bind their specific antigens for isolation and later identification. After incubation at 4°C for 2 hours with rocking, this mixture was added to pre-swollen Protein A Sepharose CL-4B beads to bind antibody-antigen complexes overnight with rocking at 4°C. The following day, this slurry was gently washed several times with PBS to remove supernatant containing unbound FT antigens. After pipetting away as much of the remaining final wash as possible, an equal volume of 2x Laemmli SDS-PAGE sample buffer containing 2-ME was added to the beads, which were then boiled for 10 minutes to completely solublize the Protein A-bound antibody-antigen complexes. After cooling, the supernatant was subjected to denaturing SDS-PAGE analysis using 4-20% acrylamide gradient mini-gels (Pierce) to allow separation of the proteins according to their molecular weight. After electrophoresis was complete, gels were then Coomassie stained (**Fig. C.5**) and stored at 4°C in gel destaining solution (50% methanol + 10% acetic acid). To confirm IgG specificity from the IP experiments, duplicate gels containing separated FT proteins were electrophoretically transferred to Immobilon-P_{sq} PVDF membranes (Millipore), blocked overnight with 5% non-fat milk in PBS at 4°C, probed with pooled mouse antisera, and detected using a goat anti-mouse IgG-HRP 2° antibody. The blots were developed using a chemiluminescent substrate and images were documented for analysis (**Fig. C.6**). The corresponding immunoreactive bands as seen on Western blots were excised from Coomassie-stained gels using sterile scalpels and those proteins were identified by

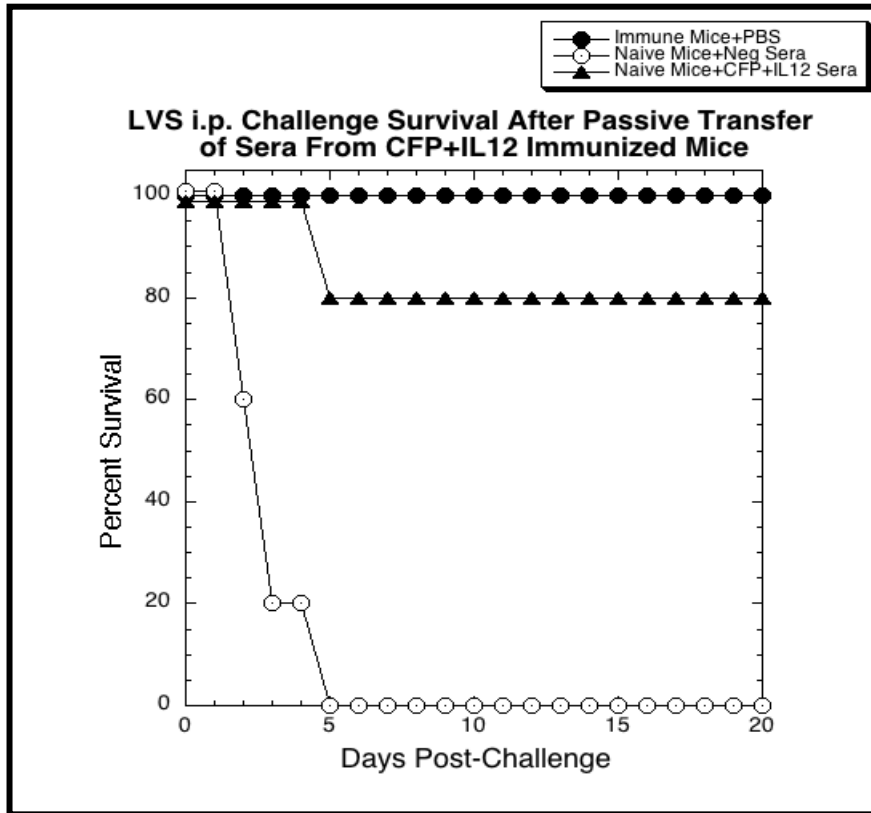


Figure C.4. Kaplan-Meier survival plot of naïve Balb/c mice following passive immunization with pooled serum from mice immunized with FT LVS CF and rIL-12 adjuvant. Balb/c mice (5/group) were passively immunized with the vaccine formulations as indicated. Fourteen hours after receiving injections, all mice were challenged i.p. with FT LVS. The health and survival of the mice were monitored daily for signs of tularemia. Results are reported as days surviving post-challenge with FT.

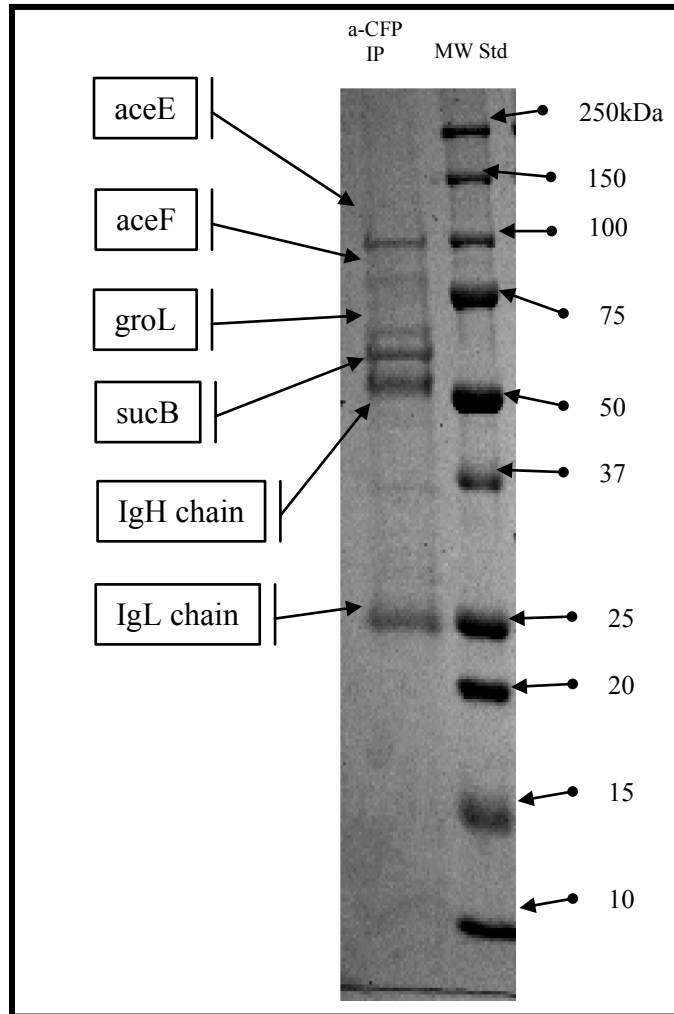


Figure C.5. SDS-PAGE and Coomassie-stained anti-CFP immunoprecipitated FT LVS proteins identified so far using anti-CFP immune sera. FT LVS protein antigens and mouse immunoglobulin chains are indicated.

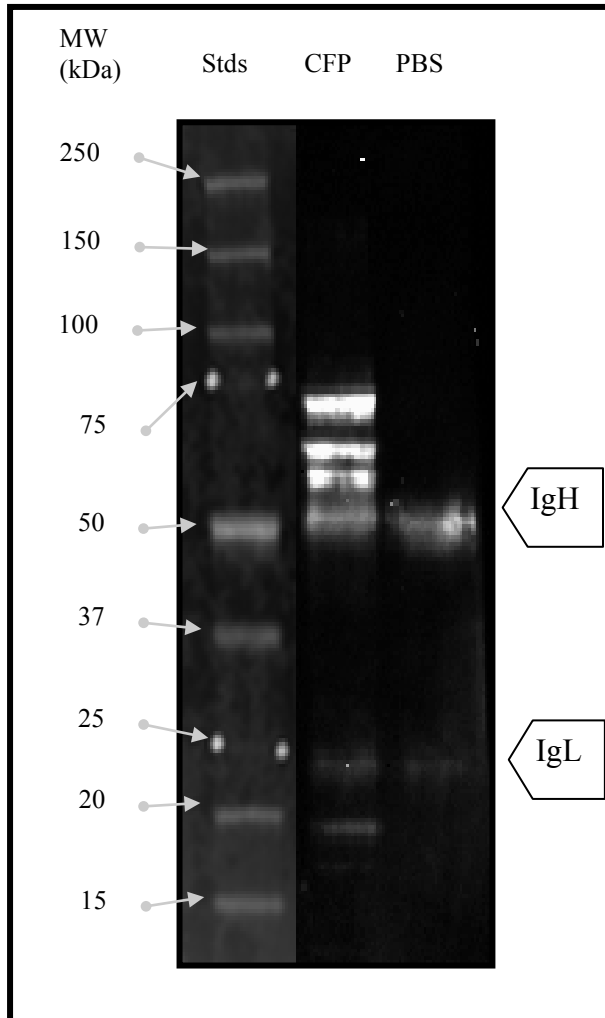


Figure C.6. Western blot analysis of immunoprecipitated FT LVS lysate using pooled negative control sera and anti-CFP sera (1°Ab) followed by detection with goat anti-mouse IgG:HRP (2°Ab).

mass spectrometry. Four of the most intensely immunoreactive FT LVS bands from Western blot analysis were identified by using vMALDI-LTQ as GroL, sucB, aceE, and aceF.

Unfortunately, later immunization experiments were conducted using recombinant forms of the four identified proteins expressed in *E coli* BL21(DE3) with rIL-12, but these preparations failed to elicit a protective immune response against FT infection. Based on these findings, we therefore concluded that the immunoreactive proteins that were identified from FT LVS CF preparations did not warrant further immunization experimentation in mice. Perhaps additional studies utilizing alternative methods will allow the protective antigens in FT LVS CF to be isolated and further characterized as potential vaccine components against tularemia.

Discussion

The effectiveness of *F. tularensis* as an intracellular pathogen, together with an incomplete understanding of the pathogenic mechanisms used by the organism to establish infection, have led vaccine researchers to look for unique approaches to elicit protective host responses. However, to date, the only available preventative measure against human tularemia is by immunization with the unlicensed FT Live Vaccine Strain (LVS), and this protection is thought to be incomplete for type A strains of *F. tularensis*, such as SCHU S4. The susceptibility of mice to FT LVS and the ability to elicit protective immunity following a sublethal infection with this organism have allowed researchers in the field an experimental model to tease apart the interaction between FT and the host adaptive immune responses outside of BSL-3 conditions. Recent studies have begun to shed light on the extracellular proteins and potential secretion mechanisms of various strains of *F. tularensis* (133-134, 147). Our studies have indicated that immunization of mice with precipitated FT LVS culture filtrate (CF) components can protect Balb/c mice against homologous bacterial challenge.

The LPS O-antigen is known to elicit a protective immune response against FT LVS in murine models. Because it is likely that co-precipitation of FT LPS along with proteins in our CF preparations by the PRMM buffer might have been a major immune determinate in our murine immunization models, an additional CF preparation was made using an LPS O-antigen deficient mutant of FT LVS. Additional studies found that CF prepared using an LPS O-antigen deficient strain of FT LVS (48B10) could also elicit protective immunity, thus ruling out a requirement for antibodies specific for LPS O-antigen in this model.

To further define the role of adaptive humoral immunity afforded by FT LVS 48B10 CF, passive transfer experiments were performed using pooled sera from immunized mice which transferred serum-mediated protection against wild-type FT LVS lethal challenge to naïve mice. Since there was protection afforded by the passive transfer of FT CF from bacteria lacking the complete O-antigen, this suggested to us that protein antigens within this prep were responsible for the successful immunization against FT LVS. Using pooled anti-CF sera from successfully immunized mice, I performed immunoprecipitation experiments, followed by the SDS-PAGE molecular weight separation and mass spectrometric identification of four highly immunoreactive protein antigens (aceE, aceF, sucB, and GroL) excised from polyacrylamide gels.

However, an experiment in which recombinant forms of these proteins were expressed and purified from *E. coli* failed to elicit protection against challenge with FT LVS.

It is not clear why immunization with recombinant FT CF proteins failed to protect the mice in our challenge experiment, despite the presence of antibodies to the given antigens. One possible explanation might be a difference in the way that these proteins are post-translationally modified in *E. coli* versus FT LVS which caused them to be less effective than naturally derived antigens from FT CF. Another critical issue with the CF immunization model was that there was a lack of protection seen in mice when challenged with the highly virulent FT SchuS4. Together, these data made it impractical to carry out additional experiments with any likelihood of positive results and the CF project was suspended indefinitely.

VITA

Shawn Russell Clinton was born in Hattiesburg, Mississippi in 1977. He graduated from the University of Southern Mississippi in June, 2001 with a Bachelor of Science in Medical Technology (*cum laude*). Following graduation, he immediately began work at the University of Mississippi Medical Center as a Clinical Laboratory Scientist/Clinical Histocompatibility Technologist in the Department of Pathology. In August of 2004, he entered the Integrated Program in Biomedical Sciences at the University of Tennessee Health Sciences Center. He completed a Doctor of Philosophy in Molecular Sciences in 2010.