Characterization of PdpD, a *Francisella* Pathogenicity Island Protein.

by

Jagjit Singh Ludu
B.Sc., University of Victoria, 2004

A Thesis Submitted in Partial Fulfillment
of the Requirements for the Degree of

DOCTOR OF PHILOSOPHY

in the Department of Biochemistry and Microbiology

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Supervisory Committee

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Abstract

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Although its highly infectious nature has led to its classification as a potential bio-terror threat, very little is known about the pathogenesis of *Francisella*. A complete understanding of the mechanisms employed by *Francisella* to gain residence and replicate within macrophages will provide valuable insight into the means by which *F. tularensis*, and other intracellular pathogens such as *M. tuberculosis* and *L. pneumophila*, invade host cells, secrete effectors, alter phagosome biogenesis and disrupt vesicle traficking.

The overall theme of this dissertation is the analysis of genes encoded within a recently identified *Francisella* pathogenicity island (FPI). In particular, the chapters will focus on the identification, mutagenesis, and phenotypic analysis of Pathogenicity determinant protein D (*pdpD*), a ~135 kDa protein encoded within the FPI. Chapter 2 addresses the identification of the *Francisella* pathogenicity island, and the intramacrophage growth of several mutants found within this loci.

One of the greatest strengths in determining the roles of putative virulence genes is the ability of researchers to alter and amplify nucleic acids in a highly developed model platform and subsequently introduce the altered genetic

material into a pathogen. Although genetic transformation has been well developed and optimized in *E. coli*, where it is regularly used in cloning experiments, the introduction of DNA into *Francisella* has been a major deterrent in the mutagenesis of putative virulence factors. Chapter 3 focuses on engineered genetic elements and methods for transformation, antibiotic selection, deletion mutagenesis, and complementation in *Francisella* strains.

The chromosomes of *F. tularensis* strains carry two identical copies of the *Francisella* pathogenicity island, and the FPI of North American-specific biotypes contain two genes, *anmK* and *pdpD*, that are not found in biotypes distributed over the entire Northern Hemisphere. Furthermore, unlike other known intracellular pathogens, *F. tularensis* lacks a functional type III or type IV secretion system, which are necessary for other bacterium to arrest maturation of their respective phagosomes. Chapter 4 focuses on the virulence contribution of *anmK* and *pdpD* using *F. novicida*, which is very closely related to *F. tularensis* but carries only one copy of the FPI. In addition, the outer membrane localization of PdpD is examined in deletions of FPI genes encoding proteins that are homologues of known components of Type VI secretion systems.

Although each chapter is a continuum of research related to the *Francisella* pathogenicity island, each will be treated as a distinct work consisting of an introduction, materials and methods, results, and a discussion. Chapter 5 of this dissertation will consist of an overall conclusion section which will tie the 3 research chapters together as well as focus on future studies.

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Acknowledgments

As I come to the end of my graduate degree it is hard not to be nostalgic. When I first started my studies, a friend of mine told me that his Ph.D. degree was like a battle, and he was glad to have survived with his sanity intact. While I understand the symbolism of my friends' statement, I will probably never associate my degree with any major war. I personally would define this degree as a struggle. The struggle to carry on. The struggle to comprehend. The struggle to meet expectations. The struggle to keep pace. The struggle to not quit. And sometimes even the struggle to just stay awake. But this entire struggle is worth it. In the end if you can get through this struggle, you are left standing with something that you`ll carry for the remainder of your existence. That`s a really long time! Throughout my studies, one of the greatest things I`ve learned is the importance of having an amazing support system to overcome any obstacle. Completing a graduate degree without any support, would be like navigating the oceans without any wind in your sails. While it may be possible, I know for myself I could have never completed this degree without the support of a lot of great people that I am honoured to have in my life.

I would like to thank my supervisor, Dr. Francis Nano, for his unwavering support and commitment to obtain the best of my abilities. I have never been considered a great student. I never really did well in high school, and for most of my early undergraduate studies I was simply happy with getting by. Almost every teacher I had said that I had the "potential" to excel but for some reason,

whether that be for lack of effort or interest, I could never translate that into any success in an academic setting. I met Francis Nano during the spring semester of 2002, a time when I was at best an average student with little passion for science. Nonetheless, Francis opened his lab to me providing a new perspective on science and an opportunity to learn just for the sake of learning. He gave every discovery, no matter how big or small, the same excitement as landing on the moon for the first time. I was instantly hooked. Most importantly though, Fran believed in me and I think this simple gesture was what drove me to exceed even my wildest thoughts. It is the rare few that are lucky enough to have a teacher that changes their lives in such a profound manner, and I am forever grateful that I had the chance to meet Francis Nano; he is an extraordinary mentor and an amazing friend.

To the members of my supervisory committee, Drs. Perry Howard, Rob Ingham, and Terry Pearson, thank you for your support and guidance over the last four years. It was a privilege to have some of the brightest people I have ever met provide insight for my project and I appreciate the time and effort you put in to my graduate degree.

To my entire family, I am grateful for your support and could not imagine doing this without you. I have always considered myself lucky to have such an incredible family, and no words that I could write in this Acknowledgement could serve you justice.

To my girl Marianne, thank you for your unconditional love and support. You are my strength and provide me with the courage to do the things I normally could not even fathom. I am truly blessed to have you in my life.

Dedication

To my Mother and Father

Thank you

List of Abbreviations

% GC	Percent Guanine + Cytosine
Å	Angstroms
aa	Amino acid
ABC	ATP-binding cassette
Amp^R	Ampicillin resistance
AnmK	Anhydro-N-acetylmuramic acid kinase
Apaf-1	Apoptotic protease-activating factor 1
ATP	Adenosine-5'-triphosphate
BCG	Bacille Calmette-Guérin vaccine
BLAST	Basic Local Alignment and Search Tool
bp	Base pair
cfu	Colony forming units
CO_2	Carbon dioxide
COG	Conserved orthologous group
CR	Complement receptor
DMEM	Dulbecco’s Modified Eagle Medium
DNA	Deoxyribonucleic acid
Dot	Defect in organelle trafficking
DR	Direct repeats
DUF	Domains of unknown function
EEA1	Early endosomal antigen 1

Em	Erythromycin
Em^R	Erythromycin resistance
FHA	Filamentous hemagglutinin
FMDV	Foot and mouth disease virus
FPI	*Francisella* pathogenicity island
GAG	Glycosaminoglycan
GSP	General secretory pathway
GTP	Guanosine-5'-triphosphate
Hcp	Haemolysin co-regulated protein
hr	Hour
Hyg	Hygromycin
Icm	Intracellular multiplication
IFN	Interferon
Igl	Intracellular growth locus
iNOS	Inducible nitric oxide synthase
kb	Kilobase
kDa	Kilodalton
kg	Kilogram
Km	Kanamycin
Km^R	Kanamycin resistance
Lamp	Lysosome-associated membrane protein
LPS	Lipopolysaccharide
LVS	Live vaccine strain

M6PR	Mannose-6-phosphate receptor
Mb	Megabase
MHA	Mueller Hinton agar
µg	Microgram
µl	Microlitre
ml	Millilitre
mM	Millimolar
MOI	Multiplicity of infection
muBMDM	Murine bone marrow derived macrophages
MW	Molecular weight
NK	Natural killer
nm	Nanometre
NO	Nitric oxode
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
Pdp	Pathogenicity determinant protein
P_{FT}	Strong *Francisella* promoter
PI	Pathogenicity Island
Pro	Proline
RGD	Arginine-Glycine-Aspartate
RNA	Ribonucleic acid
RTX	Repeat in toxin
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

Ser	Serine
T1SS	Type I secretion system
T2SS	Type II secretion system
T3SS	Type III secretion system
T4SS	Type IV secretion system
T5SS	Type V secretion system
T6SS	Type VI secretion system
TAT	Twin-arginine translocation
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TSAC	Trypticase soy agar supplemented with 0.1% cysteine
TSBC	Trypticase soy broth supplemented with 0.1% cysteine
Vgr	Valine glycine repeats protein
WT	Wild type

Chapter 1 Introduction

1.1 Intracellular Bacterial Pathogens

Pathogenic bacteria have evolved various mechanisms to evade the host defences of an array of species ranging from complex mammals to simple single celled organisms. Bacteria can adopt an extracellular lifestyle in the blood or extracellular fluid, where they are free-living either as planktonic organisms or as biofilms attached to the surfaces of the body. *Clostridium botulinum* and *Staphylococcus aureus*, two potent food poisoning agents, rarely come into contact with a cell and exert their effects on a host via the secretion of an exotoxin. Bacteria can also colonize the surfaces of a host, as seen with *Vibrio cholerae*, which adheres to the mucosal surface of the small intestine without causing any contact mediated host cell response. Conversely, some pathogens, such as enteropathogenic *Escherichia coli*, adhere directly to the host cell surface without being internalized and direct rearrangements of the host cell cytoskeleton.

Lastly, some bacteria, such as *Legionella*, *Salmonella*, *Mycobacterium*, and *Francisella*, evade host cell killing by surviving and replicating inside phagocytic or non-phagocytic cells and are considered intracellular parasites. These pathogens are internalized by a host cell, where they ultimately reside free in the cytoplasm or within membrane bound compartments. To avoid killing by phagocytic host cells, intracellular pathogens have evolved three main strategies: (i) managing phagosome biogenesis at distinct phases in the endocytic

degradation pathway; (ii) physically adaptating to the harsh acidic environment found within phagolysosomes; and (iii) rapidly exiting the phagosome into the cytoplasm after degradation of the phagosomal membrane.

1.1.1 *Legionella*

Legionella pneumophila, the causative agent of Legionnaires' disease is a facultative intracellular pathogen capable of replicating within specialized vacuoles of phagocytic host cells such as macrophages and protozoa (Vinzing *et al*., 2008, Molmeret *et al*., 2004). Approximately 15,000 individuals are infected with *Legionella* each year in the United States, with symptoms including fever, chills, nausea, and occasionally diarrhea. Mortality results in 5% of cases when left untreated, however treatment with tetracycline leads to full recovery within 5 days (Kakeya *et al*., 2008).

Critical to the intracellular lifestyle of *Legionella* are the 23 *dot* (defect in organelle trafficking) and *icm* (intracellular multiplication) genes encoded at two separate regions of the *L. pneumophila* genome (De Buck and Lammertyn, 2007, Segal *et al*., 2005). Uptake has been shown to occur by conventional and coiling phagocytosis (See Figure 2C), with the *Legionella* containing phagosome surrounded by host organelles such as rough endoplasmic reticulum and mitochondria within five minutes following entry into monocyte-derived macrophages (See Figure 1) (De Buck and Lammertyn, 2007, Bitar *et al*., 2004). The *dot*/*icm* loci encode a functional Type IV secretion system which directs the formation of a specialized vacuole that escapes delivery to the default endosomal pathway (Segal *et al*., 2005). *Legionella* multiply to large numbers within the

modified vacuole until nutrient limiting factors trigger the release of the *rib* (release of intracellular bacteria) toxins that ultimately result in cytolysis of the host cell (Bruggemann *et al*., 2006, Salcedo et al., 2005).

1.1.2 *Salmonella*

Salmonella enterica, the causative agent of typhoid fever and various food borne illnesses, is an intestinal pathogen which infects approximately 40,000 individuals every year in the United States (Lavigne and Blanc-Potard, 2008, Miller *et al*., 2008). Infected individuals generally develop diarrhea, fever, nausea, and abdominal cramps 12 to 24 hours post-infection, with the illness lasting 4 to 7 days (James *et al*., 2008). In some cases, severe diarrhea requires hospitalization and the bacterium may spread from the intestines to the blood and other body sites unless an antibiotic regiment is initiated (James *et al*., 2008, Miller *et al*., 2008).

Salmonella spp. invade intestinal epithelial cells by adhering to microvilli and inducing membrane ruffles (See Figure 2D) that engulf the bacterium in a process similar to macropinocytosis (Patel and Galan, 2006). Critical to the internalization of *Salmonella* is a “syringe shaped” Type III secretion system which is involved in the injection of various effector molecules that induce cytoskeletal rearrangements and subsequent uptake of the bacterium (Panthel *et al*., 2008, Galan and Wolf-Watz, 2006). While *Salmonella* spp. have been shown to infect a broad range of host cells, macrophages serve as their primary replicative environment. Upon internalization, *Salmonella* resides inside a spacious phagosome which reduces to form an adherent membrane around one

or more bacteria (Patel and Galan, 2006). The *Salmonella* containing vacuole interacts briefly with the early endocytic pathway and quickly obtains and loses early endocytic markers such as EEA1 (early endosomal antigen 1) and the Rab5 GTPase (See Figure 1) (Madan *et al*., 2008, Bhattacharya *et al*., 2006). Several late endosomal markers are commonly associated with the vacuole as well, including Rab7, the LAMP's, and the vacuolar ATPase however there does not appear to be direct fusion with late endosomes (Madan *et al*., 2008, Bhattacharya *et al*., 2006). Acidification of the *Salmonella* containing vacuole results in the induction of a range of regulatory systems that encourage intracellular survival and confer resistance to antimicrobial peptides and oxidative stress (Lee *et al*., 2008, Prost *et al*., 2007). A decrease in pH and antimicrobial peptides are hallmarks of the phagosomal environment and such conditions activate many of the regulators that are involved in the pathogenesis of *Salmonella* (Lee *et al*., 2008, Prost *et al*., 2007). Sensory systems react to the phagosomal environment and synchronously coordinate the complex cascade of events that are required to modify the bacterial surface and promote intracellular replication (Lee *et al*., 2008, Prost *et al*., 2007). Environmental signals from the phagosome results in the induction, expression, and assembly of one of the *Salmonella* Type III secretion systems (Galan and Wolf-Watz, 2006). The *Salmonella* Type III secretion system promotes intracellular replication by translocating effector molecules across the phagosomal membrane to modify host vesicle trafficking, so that valuable metabolites, including amino acids and fatty acids, are directed to the vacuole (Panthel *et al*., 2008, Galan and Wolf-

Watz, 2006). Over 20 *Salmonella* proteins have been identified as translocated effectors which cross the phagosomal membrane into the host cell cytoplasm and are ultimately involved in the expansion of the vesicular compartment membrane (Panthel *et al.*, 2008).

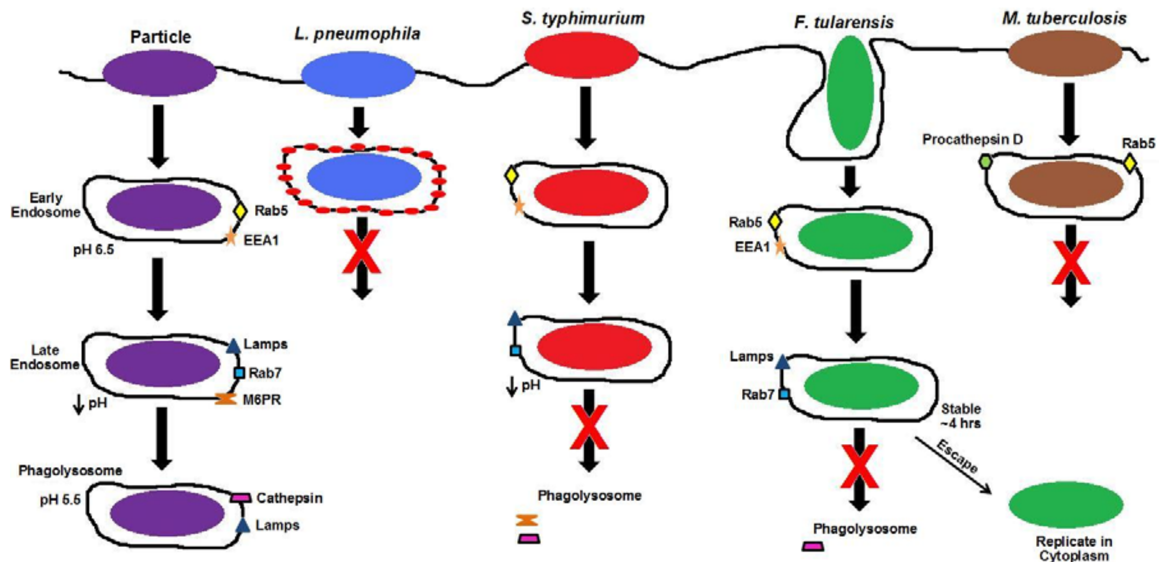

Figure 1: Intracellular pathogen evasion and trafficking within the endocytic pathway. A particle (leftmost) matures to an early endosome with a Rab5-rich domain containing EEA1 and other effectors of Rab5 that define the site of vesicle tethering during fusion. Maturation of the early endosome to a late endosome results in acquisition of Rab7, and the mannose-6-phosphate receptor (M6PR). Acquisition of the ATPase proton pump results in acidification of the vesicle, followed by fusion to lysosomes. The *Francisella* containing phagosome does not acidify or fuse to lysosomes, and is stable for 2-4 hours, upon which gradual disruption leads to bacterial release into the cytoplasm and subsequent replication. Activation by IFN-γ however, results in phagolysosomal fusion, as is the case with inert particles. (Borrowed from Santic et al., 2006)

1.1.2 *Mycobacterium*

Mycobacterium tuberculosis and *Mycobacterium leprae*, the causative agents of tuberculosis and leprosy respectively, are intracellular pathogens that parasitize and replicate within host macrophages through an arsenal of defence mechanisms against antimicrobial responses as well as by manipulating macrophage signalling (Hett and Rubin, 2008, Dye *et al.*, 2008). The World Health Organization estimates one-third of the world's population is asymptomatically infected with *Mycobacterium tuberculosis*, providing a reservoir for more than 8 million cases of active disease and 2 million deaths annually from tuberculosis (Dye *et al.*, 2008). Initially tuberculosis is characterized by weight loss, fever, night sweats, and loss of appetite, however, in latter stages can be more debilitating with cough, chest pain, and bloody sputum (Tabbara, 2007). Generally, tuberculosis can be cured with a 6 to 12 month antibiotic treatment which combines the antimicrobial agents isoniazid, rifampin, ethambutol, and pyrazinamide (Dye *et al.*, 2008, Tabbara, 2007).

The uptake of *M. tuberculosis* by a host cell occurs via conventional phagocytosis (See Figure 2B), and is facilitated by the binding lipoarabinomannan, a predominant *Mycobacterium* surface molecule, to macrophage mannose receptors (Rohde *et al.*, 2007). Similarly, three fibronectin binding proteins have been implicated in the uptake of *Mycobacteria* by macrophages via complement-mediated phagocytosis. Once internalized, *M. tuberculosis* resides in a membrane-bound phagosomal compartment that avoids fusion with lysosomes and is only mildly acidified (See Figure 1) (Deretic *et al.*,

2007, Rohde *et al*., 2007). The pH of a *Mycobacterium* containing phagosomal vacuole is ~6.5, while the pH of phagolysosomes containing inert particles is generally around 5 (Rohde *et al*., 2007). Ultimately, *M. tuberculosis* hinders the development of its phagosome, preventing lysosomal fusion, and residing in a compartment that has not fully matured to that of a phagolysosome (Deretic *et al*., 2006, Rohde *et al*., 2007). A hallmark of mycobacterial containing phagosomes is a block in Rab conversion (Deretic *et al*., 2006). In the normal endocytic pathway there is an abrupt replacement of Rab5 with Rab7 without vesicular trafficking. While Rab5 is present initially in mycobacterial containing phagosomes, there is a critical block in phagosomal Rab conversion and a complete absence of Rab7 (Katti *et al*., 2008, Deretic *et al*., 2006).

1.2 *Francisella tularensis*

1.2.1 History

Francisella tularensis, the causative agent of the zoonotic disease tularemia, is a highly infectious, gram-negative, non-motile, non-sporulating, facultative intracellular pathogen (Nano *et al.,* 2004). Originally isolated in 1912 when several ground squirrels exhibited “plague-like” symptoms in Tulare County, California, classic experiments by renowned American bacteriologist Edward Francis led to the discovery that *Francisella* was also the common agent of several human illnesses including rabbit fever, tick fever, lemming fever, Ohara’s disease, and deer fly fever (Elkins *et al.,* 2004). While the rate of reported human infections has steadily declined since the mid 20th century, tularemia still remains the single greatest zoonotic disease, with *F. tularensis*

infections outnumbering all other animal pathogens (Johansson *et al*., 2004, Elkins *et al*., 2003). Furthermore, *Francisella* infections have been documented in more than 200 species of mammals, as well as reported cases in the reptilia (reptiles), aves (bird), and actinopterygii (fish) classes of the chordate phylum (Farlow *et al*., 2005).

Recognized as a human pathogen since the onset of the 20th century, the first documented case of human tularemia occurred in 1914 in Ohio (Wherry and Lamb, 1914). Furthermore, prior to its isolation, there were reported cases of tularemia-like disease as early as 1818 in Japan and 1653 in Norway (Ohara, 1954, Scheel *et al.,* 1992). *Francisella* was initially designated as *Pasteurella tularensis* due to serological analysis, however, in 1966 DNA hybridization experiments demonstrated that the bacterium was not closely related to *Pasteurella* (Ritter and Gerloff, 1966). Ultimately, 16S rDNA sequencing would reveal that taxonomically *Francisella* belongs to the γ-subdivision of *Proteobacteria*, however shows no relationship to other organisms found within the sub-group (Forsman *et al*., 1994). To date, *Francisella* is the lone member of the *Francisellaceae* order, and the distinct taxonomical classification of the bacterium is confirmed by its greater cell wall lipid content and unique cellular fatty acid composition (Hood, 1977).

1.2.2 Bioterrorism

With less than ten organisms needed to cause fatality in humans, *Francisella tularensis* represents one of the most infectious pathogenic organisms. The highly infectious and fatal nature of *F. tularensis* has led to its

classification by the United States Center for Disease Control and Prevention as one of only six Category A agents (Nano *et al*., 2004, Dennis *et al*., 2001). Category A agents exhibit high morbidity or mortality rates, and are potential bioterrorism agents because of the ease with which they can be produced, stored, and dispersed. As early as 1932, Japanese, American, and Soviet germ-warfare laboratories had reportedly examined the feasibility of intentionally exposing humans to *F. tularensis* (Larsson *et al*., 2005). Furthermore, the American biological warfare agents program recruited volunteers who were infected with *F. tularensis* by exposure in an aerosilization chamber (Dennis *et al*., 2001). While there are no verified cases of deliberate usage of *Francisella* as a bio-weapon, Soviet scientists have claimed that *Francisella* was used in battles on the eastern front during World War II (Oyston *et al*., 2004). In addition, by the late 1950's *Francisella tularensis* became the primary focus of the United States biological warfare program, based on its ease of aerosolization, high infectivity, severity of disease, and ease of decontamination (unlike anthrax) (Oyston *et al*., 2004).

The World Health Organization estimates that aerosol dispersion of 50 kg of a virulent *Francisella* strain in a metropolitan area with a population of 5 million would lead to more than 19,000 fatalities and at least another 250,000 incapacitated individuals (Macintyre *et al*., 2000). Furthermore, illness would persist amongst the population for several weeks and disease reoccurrence would occur in the ensuing months. Economically, the cost of a *Francisella*

bioterrorist attack would reach approximately $5.4 billion per 100,000 affected individuals and cripple the economy of any modern city (Macintyre *et al*., 2000).

1.2.3 *Francisella tularensis* Subspecies

Four subspecies of *F. tularensis* have been identified. These include subsp. *tularensis* (type A), subsp. *holarctica* (type B), subsp. *novicida*, and subsp. *mediasiatica*. Although each subspecies exhibits its own distinct biochemical and pathogenic profile, they all have greater than 95% sequence identity at the genomic level (Nano *et al*., 2004). In terms of human illness, only the type A and type B strains, subspecies *tularensis* and *holarctica*, respectively, are associated with disease. Infection with type A strains results in significant mortality in humans, while infection with type B strains yield a mild flu-like disease (Johansson *et al*., 2004). *F. tularensis* subsp. *tularensis* has only been isolated in North America, and accounts for more than 70% of all *Francisella* infections in the United States (Staples *et al*., 2006). *F. tularensis* subsp. *holarctica* is spread throughout the Northern hemisphere, and is generally less virulent for humans due to a slower rate of dissemination and milder symptoms of disease (Farlow *et al*., 2005, Nano *et al*., 2004). Left untreated, upwards of 60% of *F. tularensis* subsp. *tularensis* infections can result in fatality. However, a simple 2-4 week treatment regime with common antibiotics results in a cure rate greater than 90% if applied immediately (Nano and Elkins, 2003). Transmission of *Francisella* can occur through the handling of infected animals, contamination of cuts or abrasions, vector-borne routes such as mosquito, fly, or tick bites, as well as by inhalation, with the path of entry playing a significant role in the

manifestation of disease level (Nano *et al*., 2004, Ellis *et al*., 2002). To date there have been no reported cases of transmission through human to human contact. As a result, isolation of infected individuals is not required (Staples *et al*., 2006, Farlow *et al*., 2005).

The *F. novicida* subspecies shows no known pathogenic effect towards humans despite exhibiting a high degree of virulence for mice. This attribute, as well as its high competency for transformation and allelic replacement, make it a very useful strain for studying virulence factors, and allows for analysis under less stringent Level II containment. Furthermore, the *F. novicida* subspecies allows for easier manipulation of genetic material associated with pathogenicity because it only contains a single copy of each virulence gene. This gives *F. novicida* a significant advantage over *F. tularensis* subsp. *holarctica* (e.g. Live Vaccine Strain) and *F. tularensis* subsp. *tularensis* strain, which contain duplicate copies of virulence genes, and require a substantially greater incubation period.

Francisella tularensis subsp. *mediasiatica* is geographically restricted to pockets of Southern Russia and Central Asia, and is found to infect rabbits, gerbils, and ticks (Sjostedt, 2007, Nubel *et al*., 2006). Phylogenetically, the *mediasiatica* subspecies is similar to *F. tularensis* subsp. *tularensis*, however exhibits a virulence profile similar to that of *F. tularensis* subsp. *holarctica* (Keim *et al*., 2007, Rohmer *et al*., 2007).

1.2.4 Disease and Treatment

Tularemia generally has an incubation period of 3-5 days, with initial onset of symptoms including fever, chills, headache, muscles aches, and malaise

(Dennis *et al*., 2001). Infection through breaks in the skin or the mucous membranes results in engorged and tender lymph nodes, while entry via the respiratory route leads to enlargement of lymph nodes in the hilum of the lungs (Tarnvik and Berglund, 2003). Clinical manifestations of tularemia can be classified by their mode of entry as either ulceroglandular and glandular, oculoglandular, oropharyngeal, respiratory/pneumonic, and typhoidal (Tarnvik and Chu, 2007).

Ulceroglandular and glandular tularemia are spread via vector-borne routes such as mosquito, fly, and tick bites, or through the handling of infected animals, and contamination of cuts or abrasions (Nano *et al*., 2004, Markowitz *et al*., 1985). Glandular tularemia is similar to the ulceroglandular form, but is differentiated from the latter by the absence of a characteristic skin lesion to indicate an entry point for the bacterium (Collison and Adams, 2003). The organism likely enters via an unapparent compromise in the skin and then spreads lymphatically or via the circulatory system (Collison and Adams, 2003). The ulceroglandular form of the disease accounts for ~80% of all tularemia cases and generally begins with the formation of a primary lesion at the site of bacterial entry (Collison and Adams, 2003, Anda *et al*., 2001). Once infected, the *Francisella* will spread lymphatically, usually causing painful localized swelling of the lymph nodes (lymphadenopathy) and ulceration of the skin at the point of entry (Guffey *et al*., 2007). The enlarged lymph nodes are characterized by excessive accumulation of fluid, tenderness, and a contour that is visible to inspection (Guffey *et al*., 2007, Markowitz *et al*., 1985). Inflammation normally

resolves rapidly if treatment is initiated within 5-7 days of the onset of symptoms (Tarnvik and Chu, 2007).

Oculoglandular tularemia accounts for only 1-2% of all clinical cases, with the conjunctiva serving as the point of entry for the bacterium (Evans *et al*., 1985). Transmission usually occurs through touch by a contaminated finger, or splashing of blood from infected tissue (Thompson *et al*., 2001). In addition to the general symptoms associated with *Francisella* infection, oculoglandular tularemia is identified by an intense red conjunctiva, fluid filled swelling which can be severe enough to protrude between the eyelids, and small ulcerative lesions on the inner membrane that coats the inside of the eyelids (Kantardjiev *et al*., 2007, Thompson *et al*., 2001).

Oropharyngeal tularemia is a rare disease acquired through the consumption of contaminated water and foods such as poorly cooked meat of an infected rabbit (Helvaci *et al*., 2000). Infected individuals will usually report a sore throat, inflammation or ulcers of the mouth area, abdominal pain, nausea, vomiting, diarrhea, and excessive swelling of the lymph nodes surrounding the neck (Helvaci *et al*., 2000).

Respiratory/pneumonic tularemia is acquired through inhalation of aerosolized *Francisella*, and exhibits symptoms characteristic of pneumonia such as chest pain, an increased pulmonary ventilation rate, and dry cough (Tarnvik and Chu, 2007). In many cases, the pneumonic form of the disease will appear as a complication of another form of tularemia (Roth *et al*., 2008). Approximately 10-15% of patients with ulceroglandular tularemia and 30-80% of those with

typhoidal tularemia will develop the pneumonic form of disease following spread via the circulatory system (Roth *et al*., 2008, Kirimanjeswara *et al*., 2007). Pathogenic differences between type A and type B *Francisella* strains are observed in respiratory infections (Tarnvik, 2003). Respiratory infection with type A *Francisella* results in the rapid onset of symptoms including chills, high fever, chest pain, painful breathing, shortness of breath, and profuse sweating (Tarnvik and Chu, 2007, Evans *et al*., 1985). Infections with type B *Francisella* strains however, rarely exhibit symptoms characteristic of pneumonia (Tarnvik and Chu, 2007).

The typhoidal form of disease accounts for 10-15% of tularemia cases, and is used to indicate severe disease in the absence of an indicated route of infection (Tarnvik and Chu, 2007). Diagnosis and subsequent treatment is very difficult due to the absence of ulcers and any observable infection or enlargement of the lymph nodes. The severity of the disease is likely due to the presence of *Francisella* in the circulatory system with patients presenting symptoms such as fever, chills, muscle pain, malaise, and weight loss (Tarnvik and Chu, 2007).

Francisella tularensis is naturally resistant to first-generation cephalosporins as well as penicillins such as ampicillin, methicillin, and amoxicillin (Ikaheimo et al., 2000, Baker *et al*., 1985). Aminoglycosides, such as gentamicin, kanamycin and streptomycin are active against *Francisella*, and their bactericidal properties ensure minimal chance of relapse following therapy (Urich and Petersen, 2008). During the 1940's streptomycin was introduced as an effective means for treating tularemia, resulting in a dramatic decline in mortality,

from ~30% to 3% (Urich and Petersen, 2008, Ikaheimo *et al*., 2000). Due to toxicity issues and hypersensitivity reactions among drug handlers, streptomycin has been replaced with a gentamicin therapy which lasts 7-14 days (Tarnvik and Chu, 2007, Ikaheimo *et al*., 2000). Gentamicin is the drug of choice for treatment of severe cases of *Francisella* infection, and once daily regiments for the treatment of glandular tularemia have proven highly successful (Tarnvik and Chu, 2007). To date, there have been no reported cases of naturally occurring *Francisella* resistance to aminoglycosides, tetracyclines, chloramphenicol, or quinolones (Urich and Petersen, 2008). Furthermore, *Francisella* is not a member of the normal human microflora and the risk of antibiotic resistance in clinical therapy is minimal (Tarnvik and Chu, 2007).

1.2.5 *Francisella* Vaccines

A successful *Francisella* vaccine requires a cell-mediated immune response to provide protection against tularemia and overcome any successive exposure to the pathogen. To date, the Bacille Calmette-Guérin (BCG) vaccine against *Mycobacterium tuberculosis* and the Ty21a vaccine against *Salmonella typhi* are the only widely used vaccines against intracellular bacterial pathogens (Conlan and Oyston, 2007). Both the BCG and Ty21a vaccines are live attenuated strains that have an effectiveness of 80% and 70%, respectively (Manissero et al., 2008, Zhang et al., 2008).

The earliest *Francisella* vaccines consisted of whole killed bacteria that generated specific antibodies but lacked a cell mediated immune response (Conlan and Oyston, 2007). Many recipients of whole killed vaccinations still

developed severe infections, and immunization and challenge studies in mice, guinea pigs, monkeys, and humans demonstrated an inability to elicit a robust cellular immune response (Conlan and Oyston, 2007).

Immunization with lipopolysaccharide (LPS) isolated *from F. tularensis* affords good protection against systemic challenge with an attenuated or virulent type B strain. However, it does not protect against similar challenge with a highly virulent type A strain (Thomas et al., 2007, Conlan et al., 2002). Consequently, an LPS based vaccine may only be beneficial for combating natural type B infections arising from tick bites and contact with contaminated objects (Conlan and Oyston, 2007).

Several *Francisella* proteins, such as FopA and TUL4, have been shown to be highly immunogenic but are unable to elicit any protective immunity (Conlan and Oyston, 2007). The lack of success in finding a suitable candidate for a subunit protein vaccine can be attributed to the fact that only a few *Francisella* loci may be capable of providing protection as well as the need for the protein to be formulated with an appropriate adjuvant (Conlan and Oyston, 2007, Robinson and Amara, 2005).

The Live Vaccine Strain (LVS) of *Francisella* is the only vaccine provisionally accepted for therapeutic use in North America (Conlan and Oyston, 2007). Generated by successive passage on laboratory media and in mice, the LVS strain is a superior alternative to a whole cell killed vaccine (Conlan and Oyston, 2007). Vaccination studies with human volunteers demonstrated a 60% occurrence of disease in individuals vaccinated with a whole cell killed vaccine,

while only 17% of LVS vaccinees developed disease when challenged with a 10-50 cfu aerosolized dose of a highly virulent type A strain (Conlan and Oyston, 2007). Furthermore, the LVS strain substantially decreases the occurrence of laboratory acquired *Francisella* infections from 5.7 to 0.27 cases per 1000 at risk employees (Conlan and Oyston, 2007). To date, vaccinations with LVS or other live attenuated strains have proven to be the only effective means for preventing *Francisella* infections (Conlan and Oyston, 2007). Intuitively, a live attenuated strain containing even a few mutations will undeniably express many more protective antigens than could be integrated into a subunit vaccine (Conlan and Oyston, 2007). Several regulatory issues have prevented the widespread use of the LVS strain including the presence of numerous genetic mutations as well as safety issues concerning immuno-compromised individuals (Conlan and Oyston, 2007). The mutations identified within the LVS strain however can serve as a foundation for the attenuation of a well characterized *Francisella* isolate and generate a better defined live vaccine.

1.2.6 Immune Response

1.2.6.1 The *Francisella* LPS

The *F. tularensis* lipopolysaccharide (LPS) does not exhibit properties classically associated with endotoxins (e.g. induce B-cell proliferation, stimulate production of inflammatory cytokines by macrophages) and is biologically inert relative to the LPS of other gram-negative bacteria (Rahhal *et al.*, 2007). The failure of the *F. tularensis* LPS molecule to initiate any significant form of endotoxin-induced cellular response indicates that the *F. tularensis* LPS does not

interact with common LPS receptors (Ellis *et al*., 2002). It has been shown that *F. tularensis* strains undergo a phase variation in the LPS that affects the toxicity of the endotoxin (Kieffer *et al*., 2003, Cowley *et al*., 1996). The ability of *Francisella* to proliferate within the macrophages of an animal is dramatically dependent on the form of endotoxin variant (Kieffer *et al*., 2003, Cowley *et al*., 1996). It is commonly accepted within the *Francisella* research community that the limited endotoxicity of the LPS serves as a virulence factor that enables the bacterium to circumvent macrophage stimulation and subsequent cytokine release (Sjostedt, 2006, Kieffer *et al*., 2003). The LPS of *F. tularensis* subsp. *novicida* however, is “locked” into the more biologically active form of the LPS variant due to the lack of appropriate genetic information, and consequently elicits an innate immune response that ultimately leads to elimination of *F. novicida* (Elkins *et al*., 2003). In addition, *F. tularensis* subsp. *tularensis* that has undergone a phase shift to express the *F. novicida* form of the LPS are less virulent than their wild type counterparts (Vinogradov et al., 2004). Studies with rat macrophages demonstrate that the immunostimulatory effect of the *F. novicida* LPS is far greater than LPS derived from *F. tularensis*, ultimately resulting in the production of nitric oxide (NO) (Elkins *et al*., 2007, Cowley *et al*., 1996). Consequently, NO production limits the replication of *F. novicida* in rat macrophages, and co-infection of *F. tularensis* with the *novicida* subspecies greatly reduces intracellular growth (Elkins *et al*., 2007, Cowley *et al*., 1997). Similarly, *F. novicida* infection of human monocytic cells produces significantly

greater amounts of IL-1β and IL-8 than an equal dose of *F. tularensis* (Gavrilin *et al.*, 2006).

1.2.6.2 Innate Immune Response

Control of bacterial burden and subsequent survival of a host is initially dependent on the cytokines interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α) (Elkins *et al.*, 2003). Sub-lethal intradermal infections are readily converted to lethal doses when TNF-α is depleted prior to *Francisella tularensis* exposure (Elkins *et al.*, 2003). Furthermore, there is a significant decrease in the survival time of mice treated with IFN-γ neutralizing antibodies, with a time of death reduced from a month to approximately six days upon *Francisella* exposure (Elkins *et al.*, 2003). While the exact source of the initial onset of cytokines remains to be determined, natural killer (NK) cells and a specialized subset of NK/T cells have been shown to play an important role in innate immune responses to other intracellular pathogens (Fuller *et al.*, 2006, Elkins *et al.*, 2003). By virtue of its ability to enhance IFN-γ synthesis, interleukin 12 (IL-12) could also be involved in the initial control of *Francisella* infection. However, IL-12 knockout mice resolve primary sub-lethal and secondary lethal doses of *F. tularensis* equally well, in comparison to their wild type counterparts (Sjostedt, 2006, Fuller *et al.*, 2006).

Activation of macrophages by IFN-γ results in the synthesis of nitric oxide (NO) as well as other reactive nitrogen species, via inducible nitric oxide synthase (iNOS) (Fuller *et al.*, 2006 Lindgren *et al.*, 2005). IFN-γ stimulated control of intracellular *F. tularensis* replication is readily reversed by inhibitors of

NO *in vitro*, and mice deficient for iNOS do not survive sub-lethal intradermal infections (Lindgren *et al*., 2004). Although defense mechanisms of macrophage derived NO with respect to *Francisella* infection remain unknown, NO has been found to cause cell filamentation, induction of the SOS response, and DNA replication arrest in other intracellular gram-negative pathogens (Elkins *et al*., 2003, Lindgren *et al*., 2005, Schapiro *et al*., 2003). Ultimately with *Francisella*, NO controls the initial onset of infection, enabling the activation and expansion of a predominantly T-cell mediated immune response (Fuller *et al*., 2006 Lindgren *et al*., 2005).

Toll-like receptors (TLRs) represent an ancient family of proteins that are central to the innate immune system. TLRs have been implicated in the recognition of an array of microbial products such as double stranded RNA (TLR3), LPS (TLR4), flagellin (TLR5), and bacterial DNA (TLR9) (Katz *et al*., 2006). Found as transmembrane receptors on the surfaces of antigen presenting cells such as dendritic cells and macrophages, TLRs lead to the activation of a broad range of signal transduction pathways that facilitate the resulting specific immune response (Katz *et al*., 2006, Malik *et al*., 2006). The capacity to influence the synthesis of immunoregulatory cytokines and modulate expression of co-stimulatory molecule is a hallmark of TLRs, and demonstrates their pivotal role in bridging the innate and adaptive immune systems (Katz *et al*., 2006). The *Francisella* tularensis LPS does not interact with TLR4, a common receptor for the lipopolysaccharide of Gram-negative bacteria (Hajjar *et el*., 2006, Barker *et al*., 2006). Furthermore, TLR4 knockout mice infected with a highly virulent strain

of *Francisella* do not differ from their wild-type counterparts with respect to the course of infection (Collazo *et al*., 2006, Duenas *et al*., 2006). *Francisella* recognition via TLR2 and the subsequent production of TNF-α is essential for bacterial control and clearance, as TLR2 knockout mice are incapable of eliminating a *F. tularensis* infection (Katz *et al*., 2006, Malik *et al*., 2006). Dendritic cells derived from TLR6 knockout mice indicate that this recognition is the result of the TLR2/TLR6 heterodimer, and is an essential component for a proinflammatory host response and the upregulation of co-stimulatory molecules (Katz *et al*., 2006). Recently a link has been established between TLR2 activation via bacterial lipoproteins and the subsequent activation of Caspase-1 (formerly known as Interleukin-1 converting Enzyme) (Mariathasan *et al*., 2005). Depending on expression levels and isoforms, Caspase-1 can induce apoptosis, a defense mechanism of the innate immune system to microbial pathogens, which ultimately leads to cell death and cytokine production (Mariathasan *et al*., 2005).

1.2.6.3 Adaptive Immune Response

In general, antibodies offer minimal protective advantage in intracellular infections simply on the basis that the pathogen is largely sheltered from antibody activity by virtue of its localization (Elkins *et al*., 2003). In certain situations *Francisella* specific antibodies may provide some benefit, however this is thought to occur only with low dose systemic exposure (Bosio and Elkins, 2001, Elkins *et al*. 1999). Primary intradermal *Francisella* infections of B-cell knockout mice have a very similar course of infection relative to their wild-type

counterparts, and exhibit only a slight delay in clearance of the bacterium (Bosio and Elkins, 2001, Elkins *et al*. 1999). In addition, B-cell knockout mice vaccinated with LVS survive an intraperitoneal secondary challenge of up to 10^4 cfu (Bosio and Elkins, 2001). Wild type mice however are able to survive a similar secondary challenge of up to 10^6 cfu (Bosio and Elkins, 2001). Besides antibody production, B-cells have a variety of other functions including antigen presentation, as well as the secretion of chemokines and cytokines (Elkins *et al*., 2003, Elkins *et al*. 1999). During *Francisella* infections, B-cells have a noted involvement in the secretion and regulation of cytokines and chemokines, which ultimately regulates effector cells vital for the control of *F. tularensis* infection (Mariathasan *et al*., 2005, Lindgren *et al*., 2004).

Like other similar pathogens, *Francisella* infection is cleared primarily by T-cells which provide protective immunity (Elkins *et al*., 2003). While the activity of an immune response during the initial stages of a *Francisella* infection is almost entirely independent of T-cells, the final resolution and ultimate clearance of the pathogen is completely dependent on an α/β^+ T-cell line (Cowley *et al*., 2005, Elkins *et al*., 2003). When knockout mice lacking expression of α/β T-cell receptors are administered a primary intradermal infection, they display a T-cell independent response which controls bacterial growth initially; however ultimately succumb to overwhelming bacterial organ burdens 4-5 weeks following infection (Elkins *et al*., 2007, Yee *et al*., 1996). Naive T-cells will expand into a large effector cell population during an initial antigen encounter (Cowley *et al*., 2005). Of this effector cell population, some cells possess cytotoxic activities and

produce cytokines, such as IFN-γ and TNF-α, that are essential in macrophage activation (Cowley *et al*., 2005, Elkins *et al*., 2003). These effector T-cells work to limit the growth of the pathogen within its host cell. Deficiencies in either $CD4^+$ or $CD8^+$ T-cells does not inhibit the clearance of the pathogen, and both forms of depleted mice are capable of resolving primary and secondary *F. tularensis* infections (Elkins *et al*., 2003). However, a rare double negative (DN) population of $CD4^-CD8^-CD3^+\alpha\beta^+\gamma\delta^-NK1.1^-$ T-cells has been shown to be essential in effectively and specifically hampering the growth of *F. tularensis* in macrophages, and adoptively transferring immunity to *Francisella* (Elkins *et al*., 2007, Cowley and Elkins, 2003). Following a peak in the T-cell response during an infection, these double negative T-cells acquire a memory phenotype and efficiently control intracellular growth of *F. tularensis* (Malik *et al*., 2006, Elkins *et al*., 2007). During subsequent encounters, these memory cells can respond rapidly and effectively and are essential for successful vaccination (Malik *et al*., 2006, Elkins *et al*., 2007).

It is of importance to note that much of what is currently known about *Francisella* interactions with host immune mechanisms has been derived from studies using the attenuated type B *Francisella tularensis* subsp. *holarctica* live vaccine strain (LVS). Several differences in pathology, biochemistry, genomics, and physiology are noted between the LVS and more virulent type A *Francisella*, and while these studies provide reasonable insight into *Francisella* interactions with the immune system, they should not be assumed to apply directly to fully virulent *F. tularensis* subsp. *tularensis*. Two recent independent studies have

found that the LVS is 10000x less virulent than *F. tularensis* subsp. *novicida* in a avian embryo infection model, and that there is a far greater association of human macrophages with *F. novicida* compared to the type B vaccine candidate (Balagopal *et al*., 2006, Nix *et al*., 2006)

1.2.7 Intracellular Localization

1.2.7.1 Uptake of *Francisella*

While the majority of virulence studies have been conducted in monocytic cells of human or mouse origin, *Francisella* has also been found to infect non-phagocytic cell lines such as hepatic cells, fibroblasts, tick epithelial cells, endothelial cells, and HeLa cells (Sjostedt, 2006). Conventional phagocytosis, coiling phagocytosis, and ruffling/triggered macropinocytosis are the major strategies employed by intracellular bacterial pathogens to invade a eukaryotic host cell (Sjostedt, 2006). *Francisella tularensis*, however, enters macrophages via engulfment within spacious, asymmetric pseudopod loops (See Figure 2A), a mechanism that is entirely different from other known pathogens (Clemens *et al*., 2005). Enclosure of *Francisella* occurs when the pseudopod loop fuses with the plasma membrane, resulting in a spacious vacuole at the surface of the infected host cell (Clemens *et al*., 2005). Very quickly the *Francisella* containing vacuole undergoes a dramatic reduction in size and migrates toward the center of the macrophage (Clemens *et al*., 2005). Internalization of formalin killed *F. tularensis* occurs in a similar phagocytic process to that observed with live bacteria, indicating the need for preexisting *Francisella* surface molecules to trigger host cytoskeletal rearrangements (Clemens and Horowitz, 2007). Furthermore, heat

and protease treatment prior to infection does not abolish looping phagocytosis, whereas oxidation of surface carbohydrates by periodate treatment leads to internalization of the bacterium almost exclusively by conventional phagocytosis (Clemens and Horowitz, 2007, Clemens *et al*., 2005).

Complement and/or complement receptors have been found to play a critical role in the internalization of several important intracellular pathogens including *Mycobacterium tuberculosis* and *Legionella pneumophila*, the causative agents of TB and Legionnaire's disease, respectively (Clemens *et al*., 2005). Bacterial internalization by way of complement and/or its receptors represents a focal point in pathogenesis, since uptake via this process does not trigger an oxidative burst by macrophages (Clemens and Horowitz, 2007). Serum with intact complement activity, in particular complement component C3, is essential for efficient uptake of *Francisella* by macrophages (Clemens and Horowitz, 2007). Heat inactivation of serum significantly hinders ingestion of *Francisella* by human macrophages, suggesting sufficient levels of C3 complement are essential for uptake of the bacterium (Clemens *et al*., 2005). Furthermore, there is minimal uptake of *F. tularensis* by human macrophages in C3 depleted serum, while antibodies to complement receptors CR3 and CR4 significantly hinder internalization in a dose dependent manner (Clemens and Horowitz, 2007).

1.2.7.2 Membrane Trafficking

Although the initial *Francisella* containing phagosome exists as a spacious asymmetric pseudopod loop, it is quickly modified to a tight fitting vacuole within minutes of cell entry (Santic *et al*., 2006). A *Francisella* laden phagosome will

mature to an early endosome (See Figure 1) and acquire the early endosomal antigen 1 (EEA1) marker, a 162 kDa protein essential for fusion between early endocytic vesicles, followed by the subsequent acquisition of the late endosomal markers Lamp1 and Lamp2 (Santic *et al*., 2006). However, the relative amounts of Lamp1 and Lamp2 associating with the *Francisella* containing phagosome are consistently lower than vacuoles containing dead *F. tularensis* or latex beads (Clemens *et al*., 2005).

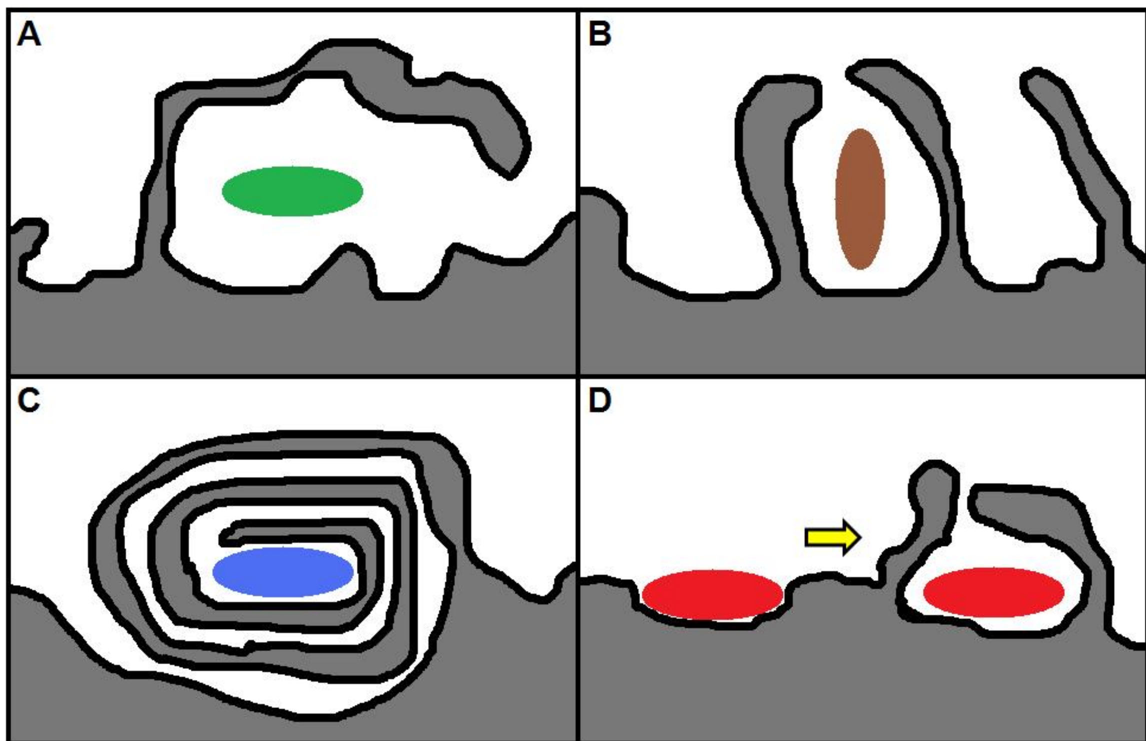

Figure 2: Morphology of invasion of *Francisella* and various other intracellular pathogens. Uptake of *Francisella tularensis* occurs via engulfment within spacious pseudopod loops **(A)**. Also shown is the uptake of *Mycobacterium tuberculosis* via conventional phagocytosis **(B)**, *Legionella pneumophila* via coiling phagocytosis **(C)**, and *Salmonella typhimurium* via triggered macropinocytosis **(D)**. (Borrowed from Clemens and Horowitz, 2007)

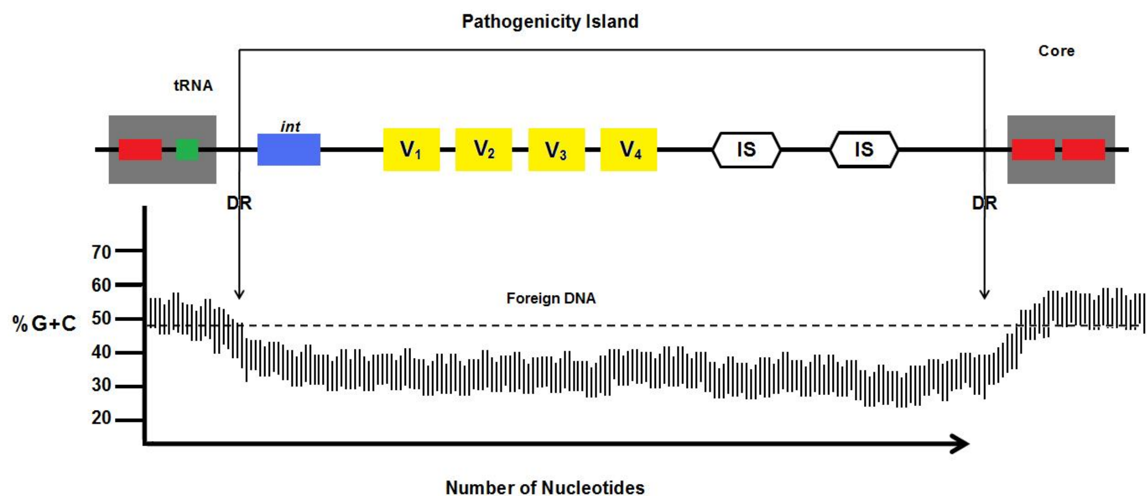

Figure 3: General structure of bacterial Pathogenicity Islands (PI's). PI's are unique DNA regions generally present in the genome of pathogenic bacteria but absent in non-pathogenic strains. PI's are usually inserted in the genome of host strains (thick grey bars) at specific sites that are frequently tRNA or tRNa-like genes (green bar). Mobility genes (*int*), are usually located at the beginning of the island, close to the tRNA locus orthe respective attachment site. PI's consist of genes related to bacterial virulence (V_1 to V_4), and are frequently interspersed with other mobility elements, such as insertion sequences (IS). The PI boundaries are frequently determined by direct repeats (DR), which are used for integration and excision of DNA elements. PI's generally have a unique %G+C content which differs significantly from the host genome. (Borrowed from Schmidt and Hensel, 2004)

Following a replicative stage in the host cytosol, *F. tularensis* induces cell death by apoptosis, releasing bacteria from the cell and enabling further infection of other macrophages (Santic *et al.*, 2006). *Francisella* mediated apoptosis serves a vital role in the pathogen life cycle as it enables the bacterium to escape a nutritionally depleted host cell without generating an inflammatory response and subsequent activation of neighboring monocytes (Santic *et al.*, 2006,

Sjostedt, 2006). *Francisella* induced apoptosis of macrophages resembles the intrinsic apoptotic pathway, with mitochondrial release of cytochrome *c* and subsequent formation of the apoptosome, which consists of cytochrome *c*, apoptotic protease-activating factor 1 (Apaf-1), and procaspase-9 (Clemens *et al*., 2005). Ultimately, the assembled complex leads to the auto-activation of procaspase-9, which in turn activates caspase-3, an effector caspase and important mediator of apoptosis of mammalian cells (Santic *et al*., 2006, Clemens *et al*., 2005).

1.2.8 Pathogenicity Islands

The concept of Pathogenicity Islands (PI's) was originally proposed in 1987 by Jorg Hacker at the University of Wurzburg while studying virulence characteristics of uropathogenic strains of *Escherichia coli* (Hacker *et al*., 1990, Knapp et., 1986). Hacker and his colleagues observed defined DNA segments with more than one linked gene encoding virulence factors such as the P-fimbirae adhesin and pore forming haemolysins (Hacker *et al*., 1990). Furthermore, Hacker was able to demonstrate that deletion of a PI led to an avirulent strain of *E. coli* (Hacker *et al*., 1990). The onset of the post-genomic era would reveal that these genetic elements are widespread throughout other virulent strains of *E. coli* as well as numerous other human pathogens including *Salmonella*, *Legionella* and *Mycobacterium* (Main-Hester *et al*., 2008, Danelishvili *et al*., 2007, Brassinga *et al*., 2003).

The genetic content of bacterial species is in a state of continuous change, with these dynamic elements exhibiting tremendous variation even

within strains of the same species (Welch *et al*., 2002). The evolution of a genome involves four distinct forms of modification including point mutations, rearrangements such as inversions or translocations, deletions, and insertions of foreign DNA (Schmidt and Hensel, 2004). The acquisition or loss of whole genetic loci can suddenly and drastically alter the virulence phenotype of a bacterium, with the insertion of foreign DNA serving as the primary force by which bacteria adapt to unique environments (Schmidt and Hensel, 2004). Conjugative plasmids, transposons, bacteriophages, insertion elements, genomic islands, and homologous recombination of foreign DNA are mechanisms of horizontal gene transfer employed by bacteria for the acquisition of new genetic material (Gal-Mor and Finlay, 2006, Hacker and Carniel, 2001).

1.2.8.1 Common Features of Pathogenicity Islands

Pathogenicity Islands (PI's) are defined as large chromosomal elements (See Figure 3) encoding virulence factors that have been acquired by horizontal gene transfer (Schmidt and Hensel, 2004). Several distinguishing features are associated with pathogenicity islands including:

i. PI's contain at least one genetic loci implicated in bacterial virulence.
ii. PI's are found in the chromosomes of a pathogenic bacterium but are absent from a benign member of the same species.
iii. PI's are large distinct chromosomal elements ranging from 10kb to 200 kb.
iv. PI's have a characteristic %GC composition that varies from the core genome and differs in its codon usage.

v. PI's are often situated next to tRNA genes which may serve as anchor points for the introduction of foreign genetic material due to the high degree of conservation among tRNA loci of various bacterial species.

vi. PI's are often associated with mobile genetic elements such as transposases or integrases.

vii. PI's are dynamic elements and exhibit deletions at a greater frequency than the typical rate of mutation.

viii. PI's are often an amalgamation of several genetic acquisitions as opposed to the integration of a single segment of DNA (Gal-Mor and Finlay, 2006, Schmidt and Hensel, 2004).

The transfer of genetic material between various bacterial strains and species is thought to occur through natural transformation, transduction, or via conjugative plasmids (Gal-Mor and Finlay, 2006, Schmidt and Hensel, 2004). Many bacteria are naturally capable of transformation due to the expression of transport systems which enable sampling of free DNA from the environment (Gal-Mor and Finlay, 2006). Although restriction modification systems generally degrade foreign DNA, some genetic material will be incorporated into the host chromosome due to selective pressure (Schmidt and Hensel, 2004). Virulence genes can also be transferred between bacteria via conjugative plasmids which replicate autonomously from the bacterial genome but in some cases can integrate into the host chromosome (Schmidt and Hensel, 2004). Lastly, while most PI's are too large to be carried in the genome of bacteriophages, transfer of virulence genes can occur by bacteriophages which enable recipient bacteria to colonize new environments (Bakhshi *et al.*, 2008). This phenomenon has been

observed with the infection of *Vibrio* with the CTXΦ phage, which carries a toxin that results in the emergence of new pathogenic strains of *V. cholerae* (Bakhshi *et al*., 2008, Stonehouse *et al*., 2008).

Pathogenicity island genes are tightly regulated and are expressed in response to environmental stimuli (Kage *et al*., 2008). Regulators of PI's can be encoded within the PI, within another separate PI, or by global regulators encoded elsewhere on the bacterial chromosome (Kage *et al*., 2008, Schmidt and Hensel, 2004). Furthermore, regulators are not exclusive to the PI and in many cases are responsible for the controlled expression of loci unrelated to virulence (Schmidt and Hensel, 2004, Boddicker *et al*, 2003). Generally, control of PI's is mediated by members of the AraC family of positive transcriptional regulators or by members of the two component response regulator family (Schmidt and Hensel, 2004, Boddicker et al, 2003). In some cases however, alternative sigma factors and histone like proteins have also been implicated in the regulated expression of PI encoded virulence genes (Laaberki *et al*., 2006).

1.2.9 The *Francisella* Pathogenicity Island

The *Francisella* pathogenicity island (FPI) was first identified by sequence analysis of the region surrounding Intracellular growth locus A and C (IglA and IglC), two closely linked loci found within the *iglABCD* operon that are essential for intracellular growth (See Figure 4) (Nano and Schmerk, 2007, Gray *et al*., 2002). Sequencing of a 17kb region downstream of the IglABCD genes and a 5kb region upstream of the operon revealed a %GC composition that differed from the core genome by 6.6% and 2.2%, respectively (Nano et al., 2004).

Furthermore, the %GC content of the entire *Francisella* genome is considered to be relatively low at 32.5%, with only a limited number of microbes having a similar G+C composition (Nano and Schmerk, 2007). The availability of *Francisella* genomes reveals a sequence similarity greater than 95% between various biotypes (See Table 1) and a duplication of the FPI in subsp. *tularensis* and sub *holarctica* strains (See Figure 4B) that is not observed in *F. novicida* (Nano *et al.*, 2004).

Table 1: Predicted number of amino acid residues of *Francisella* Pathogenicity Island proteins. Molecular weights (MW) are indicated for subsp. *tularensis*.

Deduced Protein	***Francisella tularensis***			**MW (kDa)**	**BLAST / Function**
	subsp. *holarctica*	**subsp. *novicida***	**subsp. *tularensis***		
PdpA	820	820	820	95.3	----
PdpB	1093	1093	1093	127.6	IcmF motif, Secretion
IglE	125	125	125	14.5	----
VgrG	164	164	164	17.5	Type VI Secretion
IglF	554	576	576	67.6	----
IglG	173	173	173	18.4	----
IglH	476	476	476	55.3	----
DotU	207	207	207	24.6	VasF, IcmH, Secretion
IglI	383	383	383	44.6	----
IglJ	257	257	257	30.9	----
PdpC	1328	1325	1328	156.2	----
PdpE	191	188	191	22.1	----
IglD	398	398	398	46.5	----
IglC	211	211	211	22.4	----
IglB	514	514	514	58.9	Type VI Secretion
IglA	184	184	184	20.9	Type VI Secretion
PdpD	21	1245	1195	135.4	----
AnmK	NA	371	189 134	20.4 15.2	Kinase, Cell wall recycling

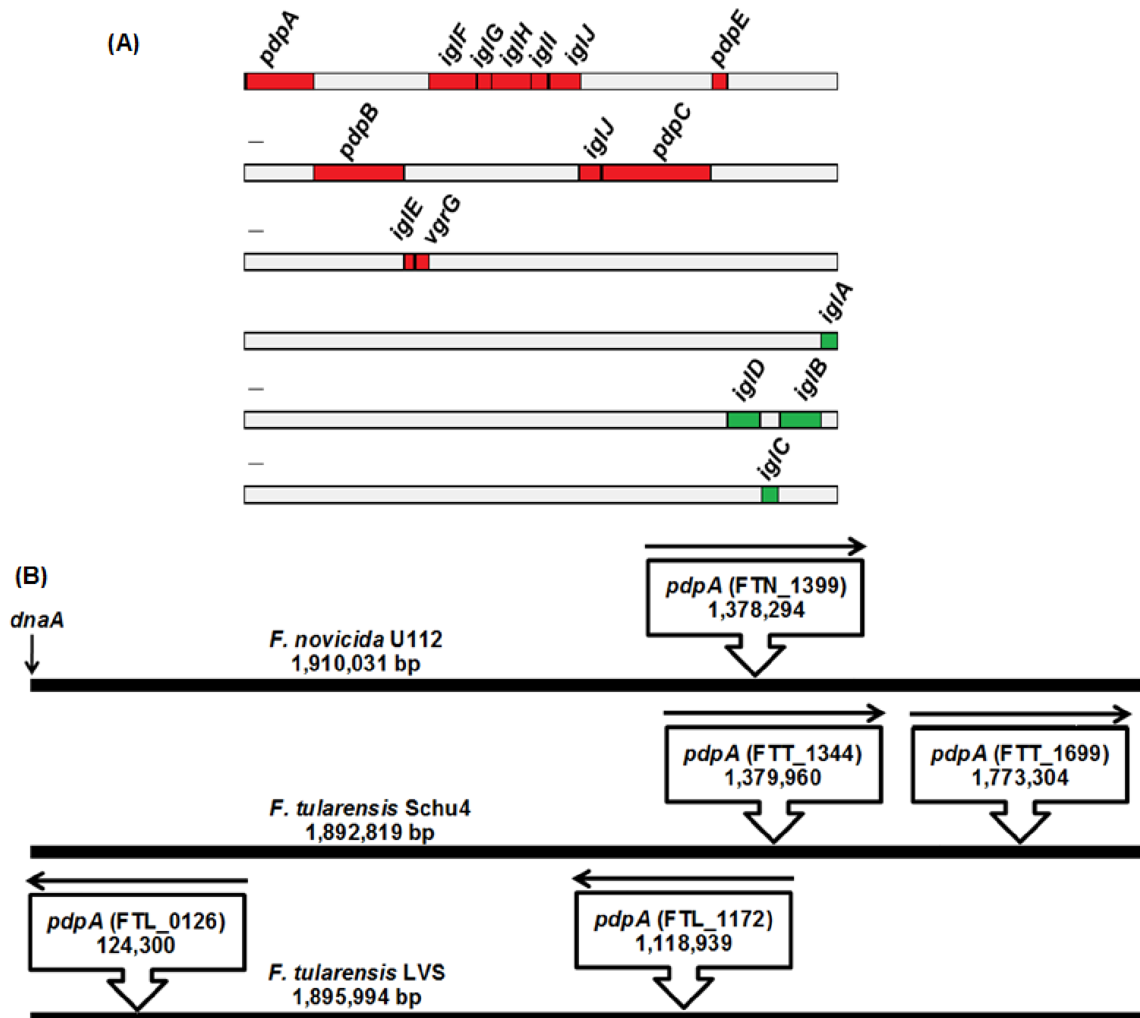

Figure 4: The *Francisella* Pathogenicity Island (FPI). Open reading frame organization of the FPI from *pdpA* to *iglA* in *F. tularensis* **(A)**. Distribution of the FPI in the genome of different *Francisella* species. Each respective genome is aligned with *dnaA* at the beginning. The position of the start codon for *pdpA* is used to indicate the start of the FPI **(B)**. (Borrowed from Nano and Schmerk, 2007)

Depending on the *Francisella* biotype, the FPI consists of 16 to 19 genes and contains four large open reading frames (ORF's) which range from 2.5 kb to 3.9 kb. While FPI genes show extensive conservation amongst *Francisella*

subspecies, *in silico* analysis has been unsuccessful in uncovering any significant homolog of many pathogenicity island proteins within the available database.

1.2.9.1 Regulation of the *Francisella* Pathogenicity Island

Expression of FPI encoded genes is mediated by the global regulatory proteins MglA and MglB (Brotcke *et al*., 2006, Santic *et al*., 2006, Lauriano *et al*., 2004). The MglA protein is the only recognized regulator of virulence factors in *Francisella tularensis*, and is responsible for the controlled expression of 102 genes (Brotcke *et al*., 2006). Furthermore, MglA regulates all the genes found within the FPI as well as a series of proteins implicated in response to oxidative and nitrosative stress (Guina *et al*., 2007). MglA and MglB form a bicistronic operon, and are homologues of the *Escherichia coli* stringent starvation transcription regulators, SspA and SspB (Hansen *et al*., 2005). Unlike SspA, whose expression peaks during stationary phase, MglA expression peaks during the exponential growth phase and is induced within 90 minutes of macrophage infection.

Iron limiting environmental conditions have been recognized as potent signals for the induction of virulence factors in a range of pathogenic microbes, including uropathogenic *Escherichia coli* (Lloyd *et al*., 2007). Under iron restrictive conditions, 80 genes were found to be upregulated under iron restrictive growth conditions, including 15 FPI genes with a minimum average increase of at least three-fold (Deng *et al*., 2006). While no direct link has been demonstrated between MglAB and its activation under iron restrictive conditions,

several homologues within other pathogenic bacterial species have shown activation under acid stress and nutrient starvation (Hansen *et al*., 2005).

Two-component signal transduction systems are commonly employed by bacteria to regulate the expression of specific genes (Mohaptra *et al*., 2007, Barker and Klose, 2007). In general, an environmental stimulus will trigger a membrane bound sensor to phosphorylate a cytoplasmic response regulator that ultimately mediates a change in gene expression (Mohaptra *et al*., 2007). Analysis of the available *Francisella* genomes indicates that there are no paired two-component signal transduction systems. However homologues of individual components of two component systems have been found and these have been designated orphaned response regulators (Mohaptra *et al*., 2007, Chamnongpol *et al*., 2002). The PmrA /B two-component signal transduction system is responsible for the expression of several genes in response to changes in environmental levels of iron and magnesium in *Salmonella* (Chamnongpol *et al*., 2002). An *F. tularensis* homologue of PmrA has been implicated in the regulation of 65 *Francisella* genes including 11 found within the MglA regulated FPI (Mohaptra *et al*., 2007). Furthermore, *Francisella* does not have a homologue of PmrB, the sensory molecule necessary for activation of PmrA, and the exact mechanism for transcriptional activation without a membrane bound kinase remains to be determined (Mohaptra *et al*., 2007, Chamnongpol *et al*., 2002).

1.2.9.2 PdpD and the *Francisella* Pathogenicity Island

While both Type A and Type B biotypes of *F. tularensis* are highly infectious and show extensive sequence similarity, the Type A strain shows a

marked capacity to cause mortality in infected humans (Beckstrom-Sternberg *et al.*, 2007). The absence of the gene encoding Pathogenicity determinant protein D (***pdpD***), a ~135 kDa protein encoded within the *Francisella* pathogenicity island, in *F. tularensis* Type B strains (See Figure 5A) may partially account for the variation in virulence observed in human infections between the type A and type B biotypes (Barker and Klose, 2007). Primary amino acid sequence analysis using the online bacterial protein subcellular localization predictor tool PSORTb v.2.0. (www.psort.org) suggests that PdpD localizes to the outer membrane of the bacterium (See Table 2), while the transmembrane predictive TMpred (www.ch.embnet.org/TMPRED), TopPred (www.bioweb.pasteur.fr/toppred), and DAS (www.sbc.su.se/~miklos/DAS/) programs weakly indicate the presence of two membrane spanning domains, one at the N- and C-termini, respectively. Furthermore, the availability of the complete genome sequence of the *F. tularensis* subsp. *tularensis* Schu4 (type A), *F. tularensis* subsp. *holarctica* LVS (type B) and *F. tularensis* subsp. *novicida* U112 strains have enabled comparisons of a range of *Francisella* species down to the nucleotide level. Although *in silico* analysis has been unsuccessful in uncovering any significant homolog within the available database, comparative genomics of the FPI reveals that the *pdpD* loci is found within the highly virulent Schu4 strain, is completely absent in the Live Vaccine strain (LVS), and contains a 144 base pair insert in the *F. novicida* subspecies (See Figure 5A). Furthermore, it must be noted that the remainder of the FPI remains intact and that the majority of genomic differences observed amongst *Francisella*

subspecies occur with genes that are not classically identified as bacterial virulence factors.

Table 2: Sequence analysis using the online bacterial protein subcellular localization predictor tool PSORTb v.2.0 (www.psort.org) of PdpD and several other known gram negative proteins. IglA is a recently identified cytoplasmic protein of *Francisella* presumably involved in protein secretion, while AcpA is an extracellular protein which inhibits the respiratory burst of neutrophils. FHA and OmpA are outer membrane proteins which serve as the central adhesion factors of *Bordetella* and *Neisseria*, respectively. The RtxA protein is a secreted toxin while TolC is a membrane localized protein essential in the secretion of RtxA. RecA is a common bacterial protein responsible for homologous recombination and resides in the bacterial cytosol.

	PSORTb v.2.0 Prediction	**Score**	**Experimental Observance**
PdpD subsp. *tularensis*	Outer Membrane	9.49	---
PdpD subsp. *holarctica*	Unknown	---	---
PdpD subsp. *novicida*	Outer Membrane	9.49	---
AnmK *Francisella*	Unknown	---	---
IglA *Francisella*	Cytoplasmic	9.88	Cytoplasmic
AcpA *Francisella*	Extracellular	9.72	Extracellular
FHA *Bordetella*	Outer Membrane	9.95	Outer Membrane
OpaA *Neisseria*	Outer Membrane	10.00	Outer Membrane
RtxA *Moraxella*	Extracellular	10.00	Extracellular
TolC Moraxella	Outer Membrane	10.00	Outer Membrane
RecA *Escherichia*	Unknown	---	Cytoplasmic

As previously mentioned, *pdpD* is encoded as a ~3.7 kb ORF within the pathogenicity island. Although iron restriction studies demonstrated that the majority of FPI genes are upregulated under iron limiting conditions, they were performed on the type B live vaccine strain, and consequently do not render any direct information pertaining to the upregulation of *pdpD* (Deng *et al*., 2006). Several clues provide indirect evidence however, mainly the point that both adjacent upstream and downstream genes are upregulated ~3.5 fold under iron restricted growth conditions (Deng *et al*., 2006).

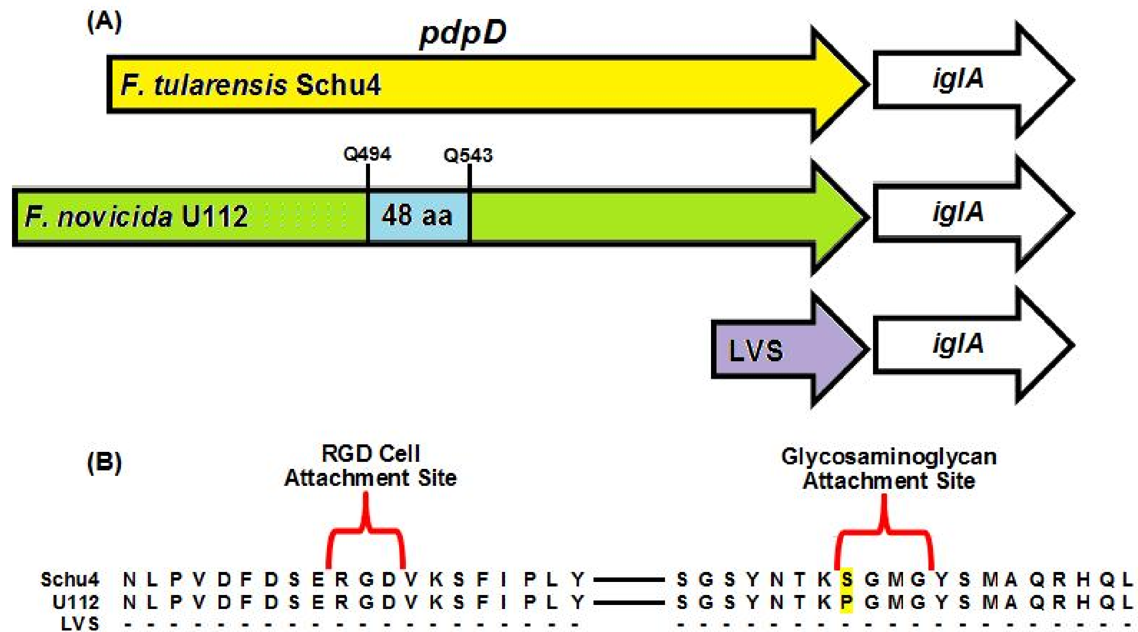

Figure 5: Pathogenicity determinant protein D (*pdpD*). The *pdpD* loci is found within the highly virulent subsp. tularensis (type A) strains, contains a 144 base pair insertion in the *F. novicida* subspecies, and is almost entirely absent in the *holarctica* biotype (type B) **(A)**. The *pdpD* loci of the *tularensis* and *novicida* subspecies both contain a RGD cell attachment sequence, however, the *F. novicida* subspecies contains a Ser→Pro mutation (highlighted in yelllow) in its predicted glycosaminoglycan attachment site **(B)**.

The *pdpD* gene exhibits several distinct variances within subspecies, which may account for the variability observed in virulence amongst various subspecies. As previously mentioned, basic bioinformatic analysis shows the complete absence of the gene in the LVS strain, while the Schu4 (type A) *pdpD* loci shows the presence of a glycosaminoglycan attachment site, as well as an Arginine-Glycine-Aspartate (RGD) cell attachment sequence (See Figure 5B). The *F. novicida pdpD* contains the RGD cell attachment sequences as well, however, contains a natural Ser→Pro mutation in its predicted

glycosaminoglycan attachment site. While various studies involving eukaryotic and prokaryotic genes have shown that proline substitutions can result in complete inactivation of proteins, effects seen with a deletion mutant suggest that this is likely not the case with PdpD (Wellner *et al.*, 1995).

Initially identified within fibronectin molecules, the Arginine-Glycine-Aspartate (RGD) tri-peptide motif was found to be responsible for mediating cell attachment (Senchou *et al.*, 2004). Integrins, a large group of heterodimeric molecules located within the plasma membrane of eukaryotic cells, are RGD receptors that mediate adhesion, movement, cell shape, and polarity upon binding of molecules such as fibronectin (Parkes and Hart, 2000). Integrins have also been exploited by various viral and bacterial pathogens that use surface exposed RGD motifs to attach to mammalian cells (Senchou *et al.*, 2004). Complement receptor 3 (CR3), a heterodimeric protein of the integrin superfamily, has been shown to be a essential component for efficient phagocytosis of *Francisella* (Clemens et al., 2005, Ernst, 1998)

Natural isolates of the highly contagious foot-and-mouth disease virus (FMDV), a viral disease of cattle, pigs, sheep, and other cloven-hoofed animals, binds to cells via a capsid surface exposed RGD motif (Zhao *et al.*, 2003). Genetically engineered derivatives of the virus in which the RGD sequence has been entirely deleted, or replaced with either a RGE, KFD, or a KGE sequence, results in a virus particle which is unable to bind host cells (Zhao *et al.*, 2003). Furthermore, wild-type virus infectivity decreases in a dose-dependent manner

when host cells are pre-incubated with a RGD containing peptide fragment (Zhao *et al.*, 2003).

In order to evade immune responses and obtain essential nutrients, many bacterial pathogens including *Yersinia pseudotuberculosis* adhere to and enter host cells during an infection (Parkes and Hart, 2000). Through attachment of host integrins via its membrane bound invasin protein, *Y. pseudotuberculosis* infects host cells through activation of cytoskeleton and the formation of pseudopodia (Parkes and Hart, 2000). As previously mentioned, *Francisella* uptake by macrophages also occurs through engulfment within spacious pseudopod loops (Santic *et al.*, 2006, Clemens *et al.*, 2005)

Exhaustive searches of available literature reveal few identified bacterial molecules that contain both a glycosaminoglycan attachment site as well as a RGD cell attachment motif. *Bordetella pertussis,* the causative agent of whooping cough, however, possesses a ~370 kDa surface protein, named filamentous hemagglutinin (FHA), which serves as the major adhesin of the intracellular pathogen (Alonso *et al.*, 2002, Ishibashi *et al.*, 2001). While FHA mutants of *Bordetella* are able to adhere and infect various cell lines *in vitro* via their fimbriae, complete colonization of cells of the respiratory tract requires an intact adhesin molecule (van den Berg *et al.*, 1999). Through its glycosaminoglycan attachment sites, FHA mediates attachment of *Bordetella* to GAG's abundantly found as part of proteoglycan complexes on cell surfaces in the bronchial airways, while a RGD motif enables attachment of the bacterium to host phagocytic cells via integrin receptors (Schmid-Hempel and Frank, 2007,

Menozzi *et al.*, 2002). Immunization of mice with purified FHA leads to long-term protection against lethal doses of wild-type *B. pertussis*, and has led to its inclusion in several commercially available acellular vaccines (Schmid-Hempel and Frank, 2007, Alonso *et al.*, 2002). Furthermore, pre-incubation of *Bordetella* or human host cells, with glycosaminoglycans or synthetic RGD containing peptides, significantly decreases adherence and invasion of the bacterium (Manning *et al.*, 2008, Ishibashi and Nishikawa, 2002).

1.3 Bacterial Secretion Systems

Protein secretion is a hallmark of all bacteria, and it has been estimated that ~20% of all peptides synthesized in the cytosol are destined for the outer surface and beyond (Thanassi and Hultgren, 2000). Bacteria obtain nutrients from their environment by secreting degradative enzymes such as proteases, nucleases, polysaccharidases, and lipases that break down macromolecules into their smaller molecular weight constituents which are then taken up by active transport (Lefevre *et al.*, 2008). Furthermore, pathogenic bacteria rely on secreted proteins to interact with and defend against eukaryotic host cells during an infection.

In Gram-positive bacteria, the general secretory pathway (GSP) is responsible for the secretion of extracellular and surface exposed proteins (Shelburne *et al.*, 2008). The GSP consists of a Sec translocase, comprised of at least five integral membrane proteins, as well as one or more cytoplasmic molecular chaperones (Wang *et al.*, 2008, Rosch and Caparon, 2004). SecA, a

homodimer which binds the integral membrane protein complex, ultimately facilitates translocation via ATP hydrolysis (Wang *et al.*, 2008).

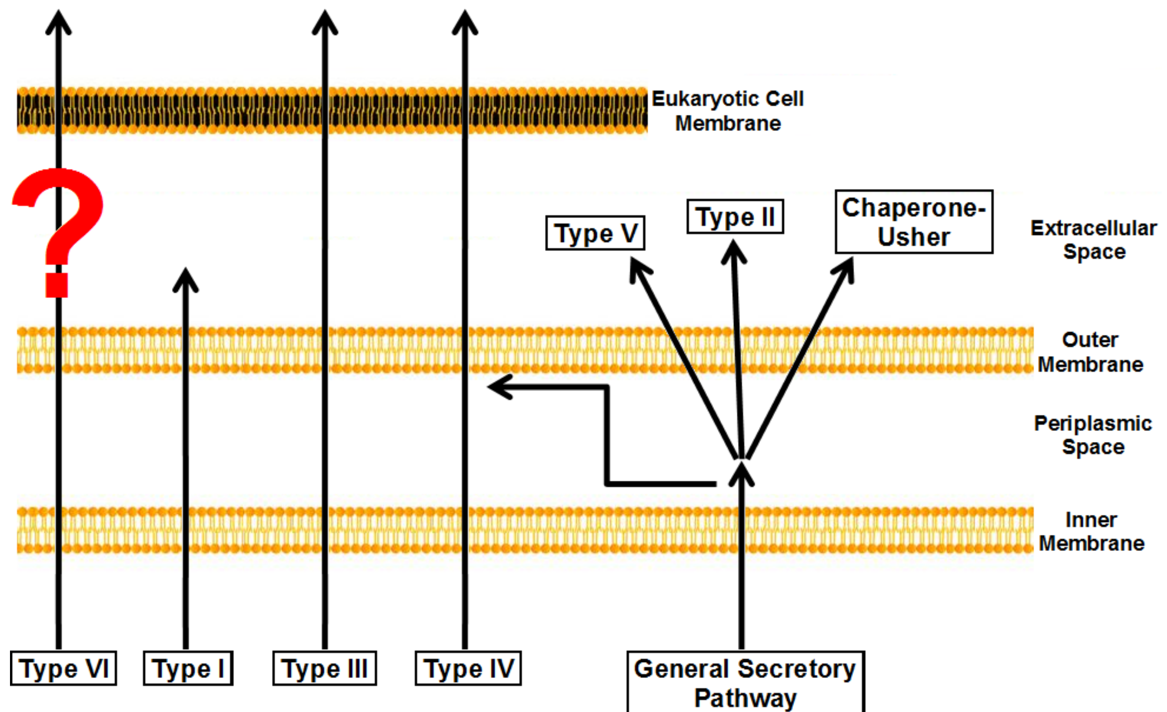

Figure 6: The protein translocation pathways of Gram-negative bacteria. The Type III and IV secretion systems are able to translocate proteins directly into a eukaryotic target cell. The exact mechanism of protein transport for the Type VI secretion system still remains to be determined.

In Gram-negative bacteria, successful secretion requires transport of the protein across an additional membrane to reach the cell surface and beyond. Similar to the Gram-positives, Gram-negative bacteria may rely on the multi-subunit GSP translocon to transport unfolded proteins into the cell periplasm (Wang *et al.*, 2008). However, in some cases the GSP is completely bypassed and a dedicated system that spans the inner and outer membrane is able to

export proteins in a single energy coupled event (Gerlach and Hensel, 2007). To date, six functionally and structurally distinct protein secretion systems (See Figure 6) have been identified in Gram-negative bacteria, as well as a chaperone-usher pathway which is responsible for the synthesis of adhesive pili or fimbriae (Nuccio and Baumler, 2007).

The Type I secretion system (T1SS) or ATP-binding cassette (ABC) transporters are hetrotrimeric complexes consisting of an ABC exporter found embedded within the inner membrane, a periplasmic protein that fuses the two membranes, and a pore forming outer membrane protein characterized by its β-barrel conformation (Gerlach and Hensel, 2007). The T1SS is responsible for the secretion of proteins as well as nonproteinaceous macromolecules into the extracellular medium in a single ATP hydrolysis coupled step (Boardman et al., 2007, Gerlach and Hensel, 2007). The best known T1SS substrate is the RTX (repeat in toxin) toxin, a pore forming haemolysin produced by a broad range of pathogenic Gram-negative bacteria (Boardman et al., 2007). In uropathogenic strains of *E. coli*, the RTX toxin is secreted by a dedicated T1SS and is encoded by a pathogenicity island which is responsible for expression, activation, and export of the pore forming haemolysin (Morova *et al*., 2008).

The Type II secretion system (T2SS) is dependent on the Sec machinery of the GSP to transport proteins from the cytoplasm to the periplasm (Korotkov and Hol, 2008). Found throughout Gram-negative bacteria, the T2SS consists of 12 to 14 components and is responsible for the secretions of enzymes and toxins across the outer membrane (Gerlach and Hensel, 2007). The ~9 nm

homomultimeric outer membrane pore consists of at least 10 to 12 sub-units that are rich in β-sheet structure, and is large enough to facilitate the transport of folded proteins and protein complexes. The best studied substrate of the T2SS is the pullulanase (PulA) of the opportunistic pathogen *Klebsiella oxytoca*, a cell surface anchored lipoprotein necessary for adhesion to the extracellular matrix of infected host tissue (van Bueren *et al.*, 2007). The GSP and the T2SS is generally encoded in the core genome of a bacterium, and has not been found within pathogenicity islands. However, several important protein substrates of the T2SS are encoded within pathogenicity islands, including the cholera toxin of *Vibrio cholerae* (Stathopoulos *et al.*, 2000).

The Type III secretion system (T3SS) is a syringe-like structure consisting of ~25 different subunits that mediates the injection of proteins directly into a eukaryotic host cell (Blocker *et al.*, 2008). Consequently, T3SS's must span the inner membrane, the periplasmic space, the outer membrane, the extracellular space and a host cellular membrane. Translocation of effector proteins by the T3SS can occur by extracellular pathogens via the cytoplasmic membrane or by intracellular pathogens via the phagosomal membrane (Field *et al.*, 2008). The T3SS is found encoded within two pathogenicity islands of *Salmonella enteric* as well as the locus of enterocyte effacement (LEE) in enteropathogenic *E. coli* (Field *et al.*, 2008). Furthermore, the injected effector molecules of the T3SS have been found to play a critical role in mediating adhesion to a host cell, interfering with host cell signalling and trafficking, evoking cytoskeletal

rearrangements, inducing apoptosis, and regulating cytokine production (Stavrinides *et al.*, 2008).

Similar to the T3SS's of *Salmonella* and *E. coli*, the Type IV secretion system (T4SS) is also capable of secreting macromolecules directly into a eukaryotic cell (Pei *et al.*, 2008). The T4SS consists of at least 10 subunits and is thought to have evolved from bacterial conjugation machineries. The *Agrobacterium tumefaciens* T4SS mediates the translocation of a DNA-protein complex into plant cells, but is a distinct system from those found in pathogens of humans and animals which only secrete protein (Schmidt and Hensel, 2004). The Type IV secretion system has been found in a number of human pathogens including *Helicobacter* pylori, whose T4SS is encoded on a 40kb pathogenicity island essential for the induction of an inflammatory response and cytoskeletal rearrangements (Andrzejewska *et al.*, 2006).

The Type V secretion system (T5SS), or autotransporters, encode all the information needed for successful translocation within the precursor protein itself (Schmidt and Hensel, 2004). The transport system and the substrate protein are synthesized in the form of a single preprotein which consists of three distinct domains: (i) an N-terminal signal sequence that targets export of the protein into the bacterial cytoplasm via the GSP; (ii) a secreted mature protein; and (iii) a C-terminal pore forming domain that facilitates translocation across the outer membrane (Gerlach and Hensel, 2007, Schmidt and Hensel, 2004). T5SS's are found encoded within pathogenicity islands of *Shigella flexneri*, as well as enteropathogenic strains of *E. coli* whose EspC autotransporter proteolytically

degrades human haemoglobin in order to obtain iron for bacterial growth (Drago-Serrano *et al.*, 2006, Schmidt and Hensel, 2004).

1.4 The Type VI Secretion System

The Type VI secretion system (T6SS) is a newly described mechanism of protein translocation in Gram-negative bacteria. While not strictly confined to human pathogens, the highly conserved T6SS gene clusters have been found in many pathogenic bacteria including *Vibrio cholerae*, *Pseudomonas aeruginosa*, *Yersinia pestis*, *Salmonella enterica*, and *Burkholderia mallei* (Bingle *et al.*, 2008, Pukatzki *et al.*, 2007). Furthermore, inactivation of T6SS encoding genes in *Vibrio cholerae* results in a defect in extracellular protein secretion and loss of virulence towards amoebae and J774 macrophages (Aubert *et al.*, 2008, Pukatzki *et al.*, 2007). Phylogenetically, there are four major groups of Type VI secretion clusters confined mostly to the Proteobacteria, but also including members of the Planctomycetes and Acidobacteria phyla (Bingle *et al.*, 2008). In addition, a divergent fifth group exists comprised solely of *Francisella* whose pathogenicity island encodes at least seven T6SS homologues (Bingle *et al.*, 2008).

T6SS gene clusters were originally identified by their common possession of IcmF and DotU homologues, two proteins which only serve an accessory role in the T4SS of *Legionella pneumophila* (Bingle *et al.*, 2008, Suarez *et al.*, 2008). While not essential to the Type IV secretory apparatus, the IcmF and DotU homologues play a critical role in Type VI mediated secretion (Bingle *et al.*, 2008, Zheng and Leung, 2007). In addition, genes encoding domains of unknown

function DUF770 and DUF877 (*iglA* and *iglB*, respectively) always occur in T6SS gene clusters as a tandem pair, and have been shown to be essential for intracellular growth of *Francisella* (Bingle *et al*., 2008, de Bruin *et al*., 2007)

A hallmark of the T6SS is an apparent lack of a hydrophobic N-terminal signal sequence, with secreted proteins appearing in culture supernatants as unprocessed polypeptides (Pukatzki *et al*., 2007, Schell *et al*., 2007). The *Vibrio* T6SS is responsible for the secretion of haemolysin co-regulated protein (Hcp) and the valine glycine repeats (VgrG1-3) proteins, however Hcp and VgrG-2 are also part of the secretory machinery and inactivation of either results in the accumulation of substrates within the bacterium (Wu et al., 2008). In *P. aeruginosa*, the Hcp protein forms a 40 Å hexameric ring which can serve as a possible channel through which substrates are secreted (Mougous *et al*., 2006). The VgrG proteins are thought to assemble into a structure similar to the *E. coli* T4 bacteriophage phage tail-spike like complex, and may serve as a host cell membrane puncturing device and as a channel for the secretion of macromolecules (Wu et al., 2008, Pukatzki *et al*., 2007). Furthermore, VgrG-1 can covalently cross link host cell actin and it remains to be determined if its primary role is as a translocator or as an effector (Bingle *et al*., 2008, Pukatzki *et al*., 2007).

1.5 Dissertation Outline

Although its highly infectious nature has led to its classification as a potential bio-terror threat, very little is known about the pathogenesis of *Francisella*. A complete understanding of the mechanisms employed by

Francisella to gain residence and replicate within macrophages will provide valuable insight into the means by which *F. tularensis*, and other intracellular pathogens such as *M. tuberculosis* and *L. pneumophila*, invade host cells, secrete effectors, alter phagosome biogenesis and disrupt vesicle traficking.

The overall theme of this dissertation is the analysis of genes encoded within a recently identified *Francisella* pathogenicity island. In particular, the remaining chapters will focus on the identification, mutagenesis, and phenotypic analysis of Pathogenicity determinant protein D (*pdpD*), a ~135 kDa protein encoded within the FPI. Chapter 2 addresses the identification of the *Francisella* pathogenicity island, and the intramacrophage growth of several mutants found within this loci.

One of the greatest strengths in determining the roles of putative virulence genes is the ability of researchers to alter and amplify nucleic acids in a highly developed model platform and subsequently introduce the altered genetic material into a pathogen. Although genetic transformation has been well developed and optimized in *E. coli*, where it is regularly used in cloning experiments, the introduction of DNA into *Francisella* has been a major deterrent in the mutagenesis of putative virulence factors. Chapter 3 focuses on engineered genetic elements and methods for transformation, antibiotic selection, deletion mutagenesis, and complementation in *Francisella* strains.

The chromosomes of *F. tularensis* strains carry two identical copies of the *Francisella* pathogenicity island, and the FPI of North American-specific biotypes contain two genes, *anmK* and *pdpD*, that are not found in biotypes distributed

over the entire Northern Hemisphere. Furthermore, unlike other known intracellular pathogens, *F. tularensis* lacks a functional type III or type IV secretion system, which are necessary for other bacterium to arrest maturation of their respective phagosomes. Chapter 4 focuses on the virulence contribution of *anmK* and *pdpD* using *F. novicida*, which is very closely related to *F. tularensis* but carries only one copy of the FPI. In addition, the outer membrane localization of PdpD is examined in deletions of FPI genes encoding proteins that are homologues of known components of Type VI secretion systems.

While each chapter is a continuum of research related to the *Francisella* pathogenicity island, they will be treated as distinct works consisting of an introduction, materials and methods, results, and a discussion. Chapter 5 of this dissertation will consist of an overall conclusion section which will tie the three research chapters together as well as focus on future studies.

Chapter 2 A *Francisella tularensis* Pathogenicity Island Required for Intramacrophage Growth.

(Published in the Journal of Bacteriology in October 2004)

2.1 Introduction

Francisella tularensis is a highly infectious gram-negative coccobacillus that causes the zoonotic disease, tularemia (Ellis *et al*., 2002). This bacterial pathogen is known for its ability to cause a fulminating disease in humans after exposure to as few as 10 cells, and has raised considerable concern as a potential bioterrorist agent (Dennis *et al*., 2001). Because of its high infectivity and lethality, *F. tularensis* is one of six types of microbes classified by the U.S. Centers for Disease Control and Prevention as a Category A agent, one that poses the most serious threat as a vehicle of bioterror.

There are a variety of subspecies and biotypes of *F. tularensis*, but they all share greater than 95% DNA sequence identity. Although the type A and type B biotype strains are highly infectious, only type A strains, which are found exclusively in North America, cause significant mortality in infected humans. An attenuated variant of a type B biotype strain formed the basis of a live vaccine strain (LVS) of *F. tularensis*. Understanding the molecular basis of the differences in virulence levels of *F. tularensis* strains may help in the development of a rationally designed live vaccine strain.

F. tularensis is a facultative intracellular pathogen. The currently available evidence suggests that *F. tularensis* resides inside a membrane-bound phagosome during its initial growth in a macrophage and that it may be released

into the cytoplasm during a later phase of growth (Golovliov *et al*., 2003, Anothony *et al*., 1991). Very little is known about the bacterial virulence factors needed for infection, although it is clear that intracellular growth, especially in macrophages, is essential to the virulence of *F. tularensis*. A biochemical study of the live vaccine strain (LVS) of *F. tularensis* showed that four proteins are induced after *F. tularensis* entry into macrophages (Golovliov et al., 1997). The gene encoding the most prominently induced protein, the 23 kDa IglC protein, has been molecularly cloned and sequenced. Recently, Golovliov *et al*. deleted this gene and showed that the resulting mutant was unable to grow in macrophages or cause disease in mice (Golovliov et al., 2003).

Genetic approaches have also been used to discover other *F. tularensis* genes needed for optimal intracellular growth. The products of *mglA* and *mglB*, thought to be global regulators, are both required for intramacrophage growth and virulence in mice (Baron and Nano, 1998). Random insertional mutagenesis revealed that inactivation of *F. tularensis* genes encoding homologues of glutamine phosphoribosylpyrophosphate amidotransferase (purine biosynthesis), alanine racemase (peptidoglycan biosynthesis) and the heat-shock inducible ClpB protease reduce the ability of *F. tularensis* to grow in mouse macrophages (Gray et al., 2002). Perhaps most significantly, transposon-insertion into *iglB* and *iglC*, which are part of the pathogenicity island described in this work, profoundly affects intramacrophage growth (Gray et al., 2002). .

The strategy of parasitizing host cells is a common theme used by both bacterial and protozoan pathogens (Amer and Swanson, 2002). In many

bacterial intracellular pathogens, a specific gene, or set of genes, has been identified that promotes entry into host cells (Galan, 1996, Gaillard *et al*., 1991, Isberg et al., 1987). Two general types of cell entry mechanisms have been identified. One involves the tight binding of a bacterial surface protein to a host cell receptor, followed by engulfment of the bacterial cell by a zipper-like phagocytosis. A second type of uptake uses Type III secretion machinery to inject effector proteins into host cells, inducing membrane ruffling and macropintocytosis. The genetic loci in *Salmonella* and *Shigella* that encode the products needed for entry into mammalian cells are pathogenicity islands of common origin (Groisman and Ochman, 1993). The horizontal movement of this cluster of genes has enabled different species of bacteria to gain the ability to invade cells.

In this study we provide evidence for a *Francisella* pathogenicity island that is required for intramacrophage growth and virulence in mice. The presumed effector proteins encoded by the FPI genes show no definitive similarity to known prokaryotic virulence proteins, and thus represent novel factors required for virulence and intramacrophage growth. The gene encoding the PdpD protein appears to be absent in *F. tularensis* type B strain, and this absence may play a role for the wide difference in the virulence in human infections between the type A and type B strains.

2.2 Materials and Methods

2.2.1 Strains and molecular techniques

The following *F. tularensis* strains were used. *F. tularensis* B38 (ATCC 6223) is the type strain for the highly virulent subspecies *tularensis* (type A biotype). However, the B38 strain has lost virulence through laboratory passage. *F. tularensis* live vaccine strain (LVS, ATCC 29684) is the type strain for subsp. *holarctica* and represents the type B biotype. *F. tularensis* subsp. *novicida* (type strain U112, ATCC 15482) was used for all gene knockout and virulence work; the DNA sequence reported in this work is from the U112 genome. Unless otherwise stated, the *F. tularensis* subsp. *novicida* strains were grown in trypticase soy broth supplemented with 0.1% cysteine (TSBC). The generation time for subsp. *novicida* U112 and mutant strains grown in TSBC was in the range of 80 to 90 min. *F. tularensis* Schu4 is a fully virulent subsp. *tularensis* strain. Initial bioinformatics analysis was performed using the DNA sequence of the genome project of *F. tularensis*, strain Schu4 (http://artedi.ebc.uu.se/Projects/Francisella/); however, all of the analyses reported for the region covering *pdpD* through *pdpA* were done with the subsp. *novicida* strain U112 sequence (GenBank Accession No. AY293579). The sequence of the *pdpD* region of two type B strains were assigned GenBank numbers AY626806 and AY626807. Clinical isolates of *F. tularensis* were from the following locations in British Columbia, collected in the years indicated: B1, Mission (1993); B2 Vernon (1997); B6, Vernon (2002); and B7, Vandehoof (2003). These clinical isolates, and the Schu4 strain were manipulated using

BSL3 containment, and all other strains were handled under BSL2 conditions. Standard recombinant DNA and PCR techniques were used to manipulate or analyze DNA (Sambrook et al., 1989). Transposon mutagenesis and transformation of *F. tularensis* to create mutant 304-2 was performed as previously described (Cowley *et al*., 2000, Anthony et al., 1991). To construct the *pdpD* mutant, JL12, regions of the chromosome flanking *pdpD* were PCR-amplified, and ligated to an erythromycin resistance cassette (See Figure 10B). The region to the left of *pdpD* was apparently lethal in *E. coli* in high copy number vectors. Therefore it was amplified using the proof-reading *Pfx* polymerase (Invitrogen) and ligated to the low copy vector pWSK29 that had been digested with *Eco*R V which generates flush ends (Wang and Kushner, 1991). The primers used for this amplification were pdpDL-F, GGTACCTGGGTTATTTTTGCTGCTGA and pdpDL-R, CTCGAGGATCCATACTTACTACTCTTACAAGTAAACC. The resulting amplicon was 1,864 bp. The right side of *pdpD* was amplified by standard PCR techniques and cloned into pCR2.1 (Invitrogen) using primers pdpDR-F, CTCGAGCAATGATCTGGGTTTAAATTTAGC and pdpDR-R, GGTACCGCCATTTCTAAAGGGGTTGG. The resulting amplicon was 1,315 bp. The two recombinant clones were digested with *Xho* I and joined to an erythromycin resistance cassette that was engineered to contain flanking *Xho* I sites by PCR amplification using the primers EmXhoF, CTCGAGTGAATCGTTAATAAGCAAAATTC and EmXhoR, CTCGAGTTAAGGGATGCAGTTTATGC (Haas et al., 1993). The replacement of

pdpD by the erythromycin cassette was verified by using PCR with three *pdpD* primer sets to show that *pdpD* was absent, and combining primers for the erythromycin cassette with primers that hybridize to DNA flanking *pdpD* to generate amplicons.

Table 3: Primers used to amplify *Francisella* pathogenicity island genes.

Gene or Region	Primer	Sequence (5 '-)	Amplicon Size (bp)
	pdpD-1F pdpD-1R	5'-CAA GTG CTT GGT GGT GGT AA 5'-TGA TGT TTG ACC TGA ATT AGT GG	720
pdpD	pdpD-2F pdpD-2R	5'-TGG GTT ATT CAA TGG CTC AG 5'-TCT TGC ACA GCT CCA AGA GT	280 (U112) 136 (Schu4)
	pdpD-3F pdpD-3R	5'-TCC TGG CTT TGA TTT TGA GC 5'-AAA TCT TGT TCA TCA AAC GCA AT	678
iglA-iglB	pdpDR-F pdpDR-R	5'-CAA TGA TCT GGG TTT AAA TTT AGC 5'-GGT ACC GCC ATT TCT AAA GGG GTT GG	1315
pdpD-iglD	LA-F LA-R	5'-TAA AAT TGC ACA GCA GAT AAG AGC 5'-CGT ATA GCT GAT GGC TGG GCC	9932 (U112) 5665 (LVS)
iglA	iglA-F iglA-R	5'-GAG GGC GTT GTT AAG GTA ACT TGC 5'-GAG CAA CTT CTG TAG ATC CCC CAA A	567
iglB	iglB-F iglB-R	5'-GAG CTC TTG TGA TGC TGC TGA GTC T 5'-CTC GAG TCG CCA CTT GTT ACC TGT TG	1557
iglC	iglC-F iglC-R	5'-TTT GAA GGA ATG AAT ACT ACA ATG A 5'-GAG CCA TCT TCC CAA TAA ATC CTT	648
iglD	iglD-F iglD-R	5'-GAG GCG CAG CTA GCA CAG ATA AA 5'-CTC GAG GCT GGG CTA TCC CTC ATT	1209
iglC-D	iglCD-F iglCD-R	5'-TTG CGC AGC TAG CAC AGA TA 5'-TCT GCG AAC TTC AAT TCT CTT TC	704
Left of *pdpD*	pdpDL-F pdpDL-R	5'-GGT ACC TGG GTT ATT TTT GCT GCT GA 5'-TAC TTA CTA CTC TTA CAA GTA AAC C	1864
4kb Left of *pdpD*	O5-F O5-R	5'-AGT GTA ATG GAG CCC AAC CA 5'-GGT TTG CCA AAG CAG ATG AT	420

DNA sequencing of the FPI region was performed using custom primers. Sequence assembly and analysis was done using the LaserGene (DNAStar) suite of programs. Comparison of FPI deduced amino acid sequences to those in protein and nucleic acid data bases was done using on-line BLASTP with the default settings. TBLASTN and BLASTN were also used (Altschul et al., 1997). The location of transposon insertions was determined by amplifying the area of interest and sequencing parts of the amplicon by initiating DNA sequencing reactions from the Tn*Max2* transposon using the primers AAACATGCAGGAATTGACGA and TTCCTGAGCCGATTTCAAAG (Haas et al., 1993). Mutant 304-2 was genetically complemented by introducing DNA cloned into the kanamycin resistant, broad-host-range plasmid pDSK519 (Keen et al., 1997). Several primers were used to produce the amplicons shown in Figures 9 and 8, and they are listed in Table 3; the relative position of some of these primers is shown in Figure 10A.

Antibody was raised against IglA in a rabbit by injecting recombinant protein. The primers CTCGAGGGCGTTGTTAAGGTAACTTGC and CTCGAGCAACTTCTGTAGATCCCCCAAA were used to amplify the *iglA* gene as an *Xho* I-*Xho* I fragment. This amplicon was cloned into the *Sal* I site of pTZ18U, and transformed into BL21λDE3(pLysS) (Novagen). The hyper-expressed IglA was separated by 12% SDS-PAGE gel and purified using a Bio-Rad Model 491 Prep Cell. The acetone precipitated protein was emulsified in TiterMax adjuvant (Sigma), and injected subcutaneously three times at intervals of three weeks into a New Zealand White rabbit, following Canadian Council on

Animal Care protocols. Immune serum was used in immunoblots at a dilution of 1:2500. The antibody reactivity was visualized by reacting the blots with IRDye800-conjugated goat anti rabbit IgG (Rockland Immunochemicals, Gilbertsville, PA), and exposing the filters to excitation light in a LiCor Odyssey imaging system.

2.2.2 Infection of murine bone marrow-derived macrophages with bacteria

Bone marrow macrophages were infected with *F. tularensis* subspecies *novicida* strain U112 or mutants as previously described (Bosio and Elkins, 2001). Briefly, bone marrow cells obtained from femurs of healthy BALB/cByJ male mice were plated at $2x10^6$/well in 24-well plates (Costar, Corning, NY) for one week in complete tissue culture medium containing L929 supernatants (cDMEM). Macrophages were then infected with *F. tularensis* strains at a multiplicity of infection (MOI) of 1:20 (bacterium-to-macrophage ratio), monolayers were incubated for 2 h in cDMEM, washed, and incubated at 37˚C in 5% CO_2 for the remainder of the experiment. The common practice of adding gentamicin to kill extracellular bacteria cannot be used with the subspecies *novicida* strain in this assay as it is exquisitely sensitive to this treatment; it has been demonstrated that macrophages do not support *F. tularensis* extracellular growth in DMEM (Anthony et al., 1991). To determine bacterial uptake and replication, infected macrophages were lysed at the indicated time points with sterile distilled water for 3 minutes, diluted immediately in PBS, and plated on Mueller Hinton agar plates containing the appropriate antibiotics. The plates were incubated for 1-2 days at 37˚C in 5% CO_2, and colonies were counted.

2.2.3 Bacterial stocks

For macrophage and animal experiments, isolated colonies of the indicated bacteria were inoculated into modified Mueller-Hinton (MH) broth (Difco Laboratories, Detroit, MI) supplemented with ferric pyrophosphate and IsoVitalex ((Becton Dickinson, Cockeysville, MD) as previously described (Bosio and Elkins, 2001). Broth cultures were grown to mid-log phase as previously described, and one ml aliquots of bacteria were frozen in broth alone at -70˚C (Bosio and Elkins, 2001). These were periodically thawed for use, and viable bacteria quantified by plating serial dilutions on MHA plates. The number of CFU after thawing varied less than 10% over a 12 month period.

2.2.4 Animal and mouse infections

Six-to-eight week-old male specific-pathogen-free BALB/cByJ mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Animals were housed in sterile microisolator cages in barrier environment at the Center for Biologics Evaluation and Research. Mice were fed autoclaved food and water *ad libitum*, and all experiments were performed under Institutional Animal Care and Use Committee guidelines. Mice were given 0.1 ml of appropriately diluted bacteria intradermally at the base of the tail; actual doses of inoculated bacteria were simultaneously determined by plate count. All materials used in animals, including bacteria, were diluted in PBS (BioWhittaker, Walkersville, MD) containing <0.01 ng/ml endotoxin.

2.3 Results

2.3.1 The identification of a *F. tularensis* pathogenicity island

The sequencing of the *F. tularensis* genome, and the development of simple genetic tools in the *novicida* subspecies of *F. tularensis,* has facilitated analysis of virulence factors of *Francisella* (Prior *et al*., 2001, Karlsson *et al*., 2000). We previously isolated two mutations in the linked genes *iglB* and *iglC* that reduce the ability of *F. tularensis* to grow in macrophages (Gray *et al*., 2002). We performed bioinformatics analysis of the DNA in the region of the *F. tularensis* genome surrounding the location of these insertions, and discovered an apparent *Francisella* pathogenicity island (FPI) of approximately 30 kb (See Figure 7). In the left half of the FPI are eight open reading frames, four of which, *iglABCD*, appear to be organized into an operon. The deduced products of *iglA* and *iglB* have about 30% identity to hypothetical proteins found in several bacterial species, most of which are animal or plant pathogens, or plant symbionts. One set of similar genes, *impBC* of *Rhizobium leguminosarum* encode proteins thought to be involved in protein secretion (Bladergroen et al., 2003). Bladergroen and colleagues noted that presumed homologues of *impBC* are found organized in an identical fashion in operons in a number of gram-negative bacteria; this organization is maintained in the *F. tularensis iglABCD* operon (Bladergroen et al., 2003). The product of *iglC* has previously been shown to be very highly induced after entry of *F. tularensis* into mouse macrophages, and recently shown by us and others to be needed for growth in macrophages (Golovliov *et al*., 2003, Gray *et al*., 2002, Golovliov *et al*., 1997). However, the

deduced proteins IglC and IglD show no significant similarity to other known proteins. In the right half of the FPI are three large open reading frames (ORFs), named *pdpABC* (for pathogenicity determinant protein).

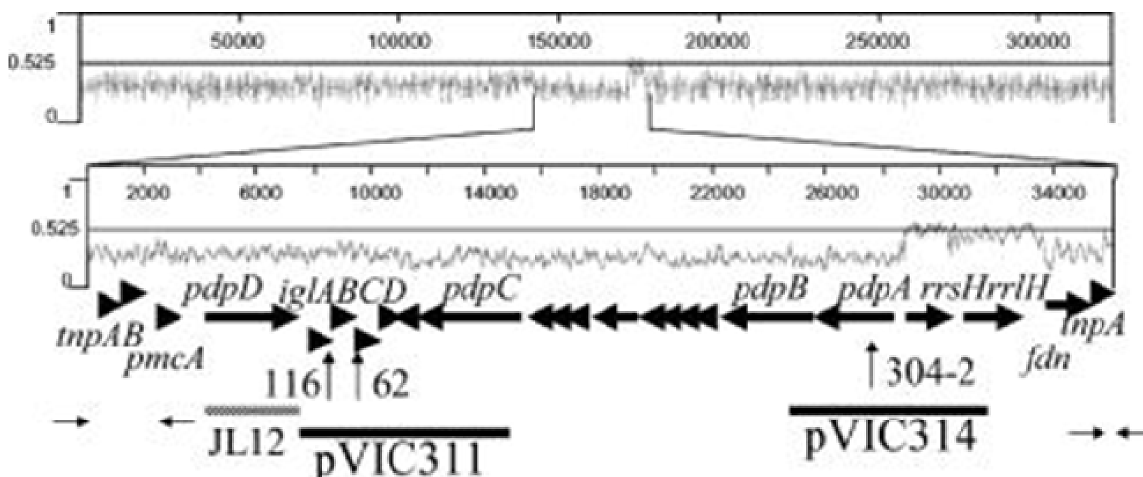

Figure 7: Gene organization and %G+C content of the FPI. Graph at the top shows the fractional G+C content of the 300 kb region of the *F. tularensis* subsp *tularensis* (strain Schu4) chromosome that encompasses the *Francisella* pathogenicity island (http://artedi.ebc.uu.se/Projects/Francisella/). The lower graph shows the G+C content of the FPI. The ORFs (arrows) from *pdpD* through *pdpA* are derived from the DNA sequence of *F. tularensis* subsp. *novicida* strain U112, determined in this work (GenBank Accession No. AY293579) and the remaining ORFs and sequence data is derived from the *F. tularensis* subsp. *tularensis* Schu4 sequence. The small opposing arrows indicate the approximate positions of 16 base pair inverted repeats that have previously been shown to be associated with *tnpA*. Vertical arrows labeled “62” and “116” indicate the location of the transposon insertions that originally indicated a cluster of virulence-associated genes. The region of the allelic replacement in the JL12 mutant is indicated by a shaded thick line. The 16S and 23S rRNA genes are indicated by *rrsH* and *rrlH* respectively; and the A-subunit of formate dehydrogenase by *fdn*. The smaller ORFs are not drawn to scale; the number of ORFs cited in the text are based on the *F. tularensis* subsp. *novicida* DNA sequence.

The region between *pdpB* and *pdpC* has eight relatively short ORFs, seven of which are below 800 bp and one of 1,431 bp. None of the deduced amino acid sequences of *pdpABC* or the smaller ORFs show substantial similarity to known proteins.

Pathogenicity islands are often recognized by the aberrant G+C content in their DNA, which differs from the rest of the resident genome. The *F. tularensis* genome has an overall G+C content of 33.2% (Prior *et al.*, 2001). The *F. tularensis* pathogenicity island has different regions that have variable G+C content. The region corresponding to *pdpD* through *iglD* has a G+C content of 31% (See Figure 7). The region from 1 kb to the left of *pdpC* to 204 bp to the right of the start codon of *pdpA* has a G+C content of 26.6% (bp 7,969-25,635 in GenBank AY293579). Immediately to the right of the presumed promoter region of *pdpA* lies a 5,050 bp region that is 51% G+C and encodes ribosomal RNA. The very different G+C content of this region is consistent with the need for conservation of the rRNA sequence, which permits changes in the G+C content primarily to adapt to the optimum growth temperature of the bacterium (Nakashima et al., 2003).

The recently released raw sequence data for the genome of *F. tularensis* LVS shows that there are two copies of the FPI in this genome (These sequence data were produced by the BBRP Sequencing Group at Lawrence Livermore National Laboratory and can be obtained from ftp://bbrp.llnl.gov/pub/cbnp/F-tularensis/F.tularensis.html.)

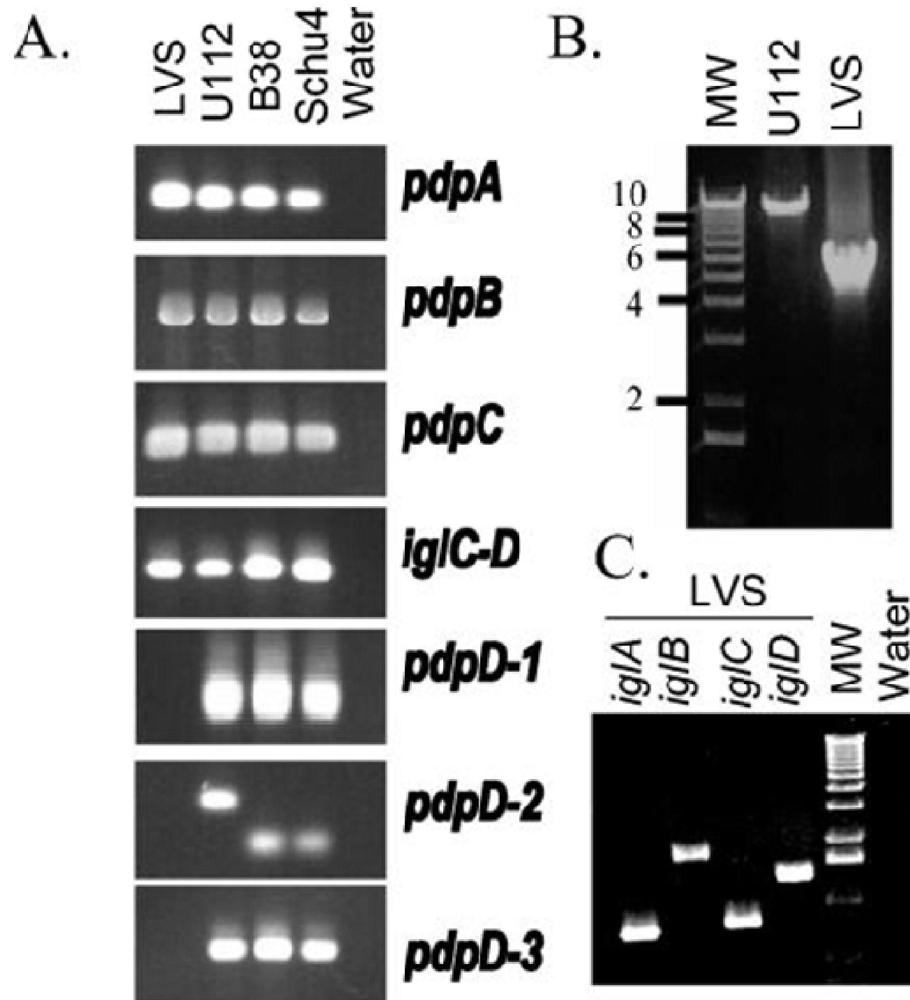

Figure 8: PCR amplification of FPI segments. (A) Results when primers were used to amplify internal fragments of *pdpA*, *pdpB*, *pdpC*, *pdpD*, and the junction between *iglC* and *iglD*. The resulting PCR products are shown, and provide evidence that the amplified regions in *pdpA*, *pdpB*, *pdpC* and *iglC-D* are very similar in the *F. tularensis* strains that were tested. **(B)** Long-range PCR encompassing *pdpD* to *iglD*. Primers were used to amplify a region of approximately 10 kb in strain U112. The product in the LVS reaction is approximately 5.5 kb. **(C)** Left to right, PCR products using primers for *iglA*, *iglB*, *iglC* and *iglD*. MW markers are as in Panel B. Lane labeled “water” is PCR reaction done with primers for *iglA* but with no template. Similar reactions were done with the three other sets of primers with identical results (data not shown). (Note: Primer design and PCR analysis performed by Na Zhang)

One of the LVS forms of the FPI is essentially identical to the Schu4 form (excluding the *pdpD* deletion, see below), from one set of inverted repeats on the left end to the other set on the right end. A second copy of the FPI in the LVS strain is identical from the inverted repeats on the left end through the ribosomal RNA genes. Thus, the presence of two copies of this region in LVS suggests that the FPI region was capable of movement at one time, and may still have the capacity to be mobile.

2.3.2 The *pdpD* gene is absent from Type B strains of *F. tularensis*

Upstream of *iglABCD* is a large ORF that we named *pdpD*, that shows no significant similarity at the deduced amino acid level to any known protein. The subspecies *novicida* form of PdpD protein is composed of 1,245 amino acids and is predicted to be 141 kDa with a pI of 6.84. Comparison of the deduced amino acid sequence of PdpD found by us in *F. tularensis* subsp. *novicida* (strain U112) to the PdpD found in *F. tularensis* subsp. *tularensis* (strain Schu4) showed that the subsp. *novicida* form had 50 additional amino acids. Forty-eight of these amino acids were found in a continuous stretch between Q494 and Q543. We examined the nature of this region of the *pdpD* gene employing PCR to amplify this region using DNA from the three widely available type strains of *F. tularensis* (B38, LVS, and subsp. *novicida* U112); Schu4 DNA, which represents the sequenced genome was also included (See Figure 8A). Several control PCR reactions, corresponding to two other regions of the *pdpD* gene, as well as regions of *iglC-D*, *pdpC*, *pdpB* and *pdpA* (see below) were performed.

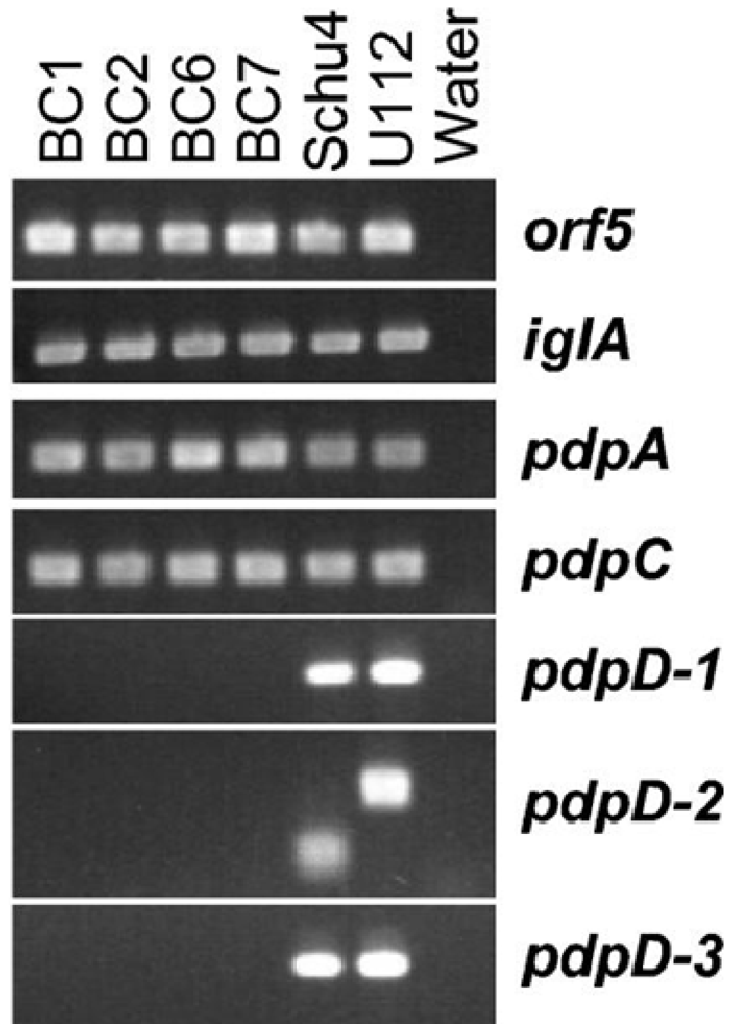

Figure 9: PCR analysis of clinical isolates of *F. tularensis*. The chromosomes of four type B isolates were amplified using primers corresponding to three regions of the *pdpD* gene as well as surrounding areas of the chromosome. The orf5 locus is 4 kb to the left of *pdpD* as shown in See Fig. 1. These results show that *pdpD* is missing or substantially different in type B clinical isolates as compared to the form found in type A strains or the *novicida* biotype. (Note: Primer design and PCR analysis performed by Na Zhang)

As expected, the PCR product that corresponds to codons 468 to 512 (for Schu4; codons 468-560 for U112) generated a product that was 136 bp when B38 or

Schu4 DNA was used as template and 280 bp when U112 DNA was used as template. Surprisingly, DNA from the live vaccine strain showed no PCR amplicon for three different PCR primer pairs for *pdpD*, while showing the expected amplicons for *pdpA*, *pdpB*, *pdpC*, and *iglC-D*. To corroborate that a substantial portion of *pdpD* was missing from the LVS strain we performed long-range PCR with primers that surrounded the *pdpD* gene (See Figure 8B). With Schu4 DNA as template a 9.9 kp product was generated; however LVS template generated a 5.5 kb product. To test if the full *iglABCD* operon was present in LVS we performed further PCR reactions. LVS template DNA generated products corresponding to the full lengths of *iglA* (555 bp), *iglB* (1,545 bp), *iglC* (636 bp) and *iglD* (1,197 bp). (See Figure 8C). DNA sequencing of this region in the LVS strain showed that a 4,249 bp region is deleted from this region (GenBank AY626806 and AY626807). The deletion extends from 107 bp upstream of *pmcA* to the first bp of codon 980 of *pdpD* (Schu4 form, or codon 1,030 of the U112 form of *pdpD*;) (See Figures 7 and 10). The genome sequence of the LVS strain confirms this deletion and shows that it occurs in both copies of the FPI in the LVS strain.

Since the LVS strain is an attenuated variant of a type B strain, the question arose as to whether the absence of *pdpD* represents a feature specific to the vaccine strain or is a feature of type B strains in general. To address this question we examined the DNA of four type B clinical isolates for the presence of *pdpD*. PCR amplification of three regions of *pdpD* indicates that this gene is missing from the clinical isolates or is significantly different in its nucleotide

sequence (See Figure 9). PCR amplification of other regions of the chromosome close to *pdpD* suggest that the clinical isolate forms are very similar to those found in the type strains, B38, LVS, and U112, of *F. tularensis*.

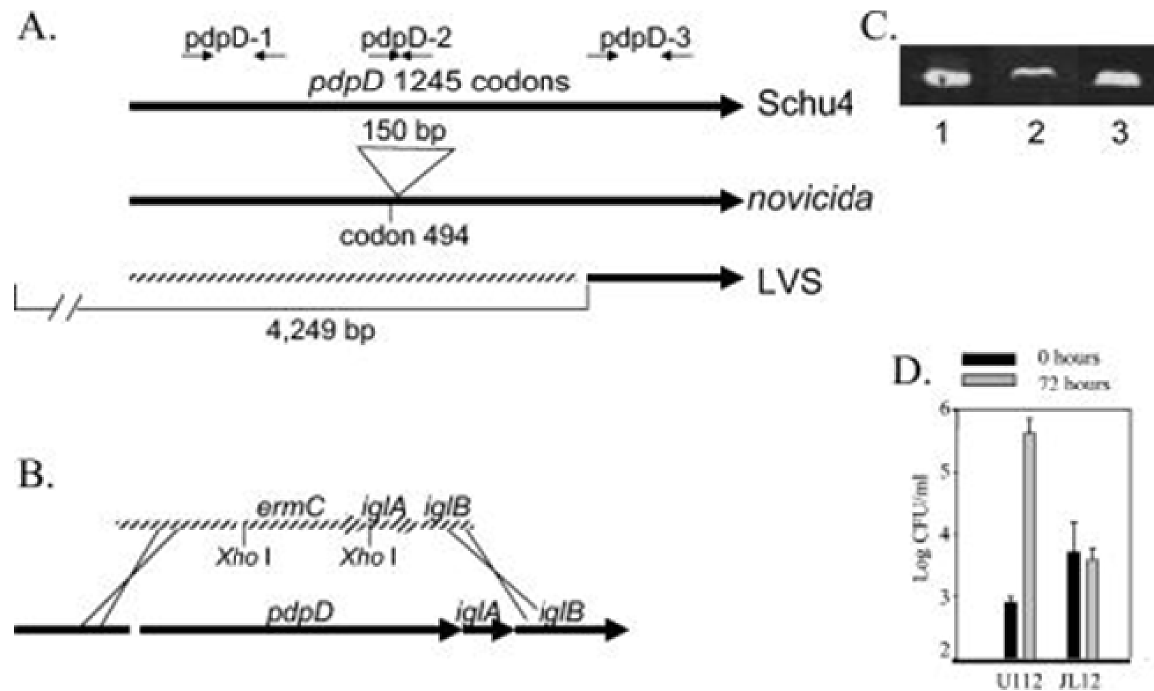

Figure 10: Properties of *pdpD* genomic region and phenotypes of a *pdpD* allelic exchange mutant. (A) Relative sizes of the *pdpD* genes in the *F. tularensis* strains Schu4, subspecies *novicida* and the live vaccine strain (LVS). **(B)** Recombinant constructs used to make the *pdpD*::Em^R allelic replacement mutant JL12 as described in the Materials and Methods. **(C)** Immunoblot of *E. coli*-produced, isolated recombinant IglA (Lane 1), and IglA expressed in the *pdpD* mutant, JL12 (Lane 2) and wild type U112 (Lane 3). When normalized to the amount of total protein loaded per lane, the intensity of the IglA band is six-fold higher in the U112 lane relative to the JL12 lane. **(D)** Relative growth of wild type subsp. *novicida* (U112) and JL12 in bone marrow derived macrophages after 72 hours. Representative data from one of three repetitions is shown. (Note: BMDM growth assays performed by Dr. Karen Elkins)

2.3.3 Disruption of *pdpD* results in a *F. tularensis* mutant defective for intramacrophage growth and virulence in mice.

The region corresponding to the *pdpD* gene was replaced with an erythromycin cassette using a recently described technique (See Figure 10B) (Lauriano *et al*., 2003). The resulting *pdpD* mutant, named JL12, still produced IglA, albeit at a lower level than wild type (See Figure 10C). This mutant failed to grow in mouse bone-marrow-derived macrophages (See Figure 10D). In two independent experiments, a total of 11/11 BALB/cByJ mice died within 7 days following intradermal infections with 10^5 cells of wild type *F. novicida* U112. In contrast, 12/12 mice survived intradermal infection with 10^5 cells of JL12. Multiple attempts were made to complement the *pdpD* mutation. However, we were unable to recover recombinant plasmids carrying *pdpD* in *E. coli* that were not lethal in *F. tularensis* subsp. *novicida*.

2.4 Discussion

There are multiple lines of evidence that the genomic region described in this work can be classified as a pathogenicity island. First, this work and others demonstrate that this region contains a cluster of genes encoding virulence factors (Golovliov *et al*., 2003, Gray *et al*., 2002). Second, much of the region has DNA with a G+C content that differs significantly from the rest of the *F. tularensis* chromosome. Third, this area is surrounded by transposable elements. Although transposable elements are common in *F. tularensis*, there is good evidence that this region is actually mobile (Johansson et al., 2001),. Golovliov *et al*. demonstrated that *iglC* is duplicated in the live vaccine strain of *F.*

tularensis, and the genome sequence data on the LVS strain shows that the entire FPI region is duplicated in that strain (Golovliov *et al*., 2003). This, in turn, suggests that this genomic area can move to and from replicons other than the *F. tularensis* genome. Very recent work shows that *iglA*, *iglC*, *pdpA* and *pdpD* are all regulated by MglA, suggesting that these FPI genes are coordinately regulated to produce a virulent phenotype in *F. tularensis* (Lauriano et al., 2004). Together, these features of this genomic area justify applying the term "pathogenicity island." Thus, this work represents the first description of a cluster of virulence genes, and the first description of a pathogenicity island, in *F. tularensis*.

There are numerous examples of large strain to strain differences in virulence levels among microbial pathogens. In the case of *F. tularensis*, type A strains are thought to have an approximate 10% mortality in untreated human cases, and infections with the type B strains are rarely fatal (Dennis et al., 2001). Until now, there was no known potential virulence factor that is present in the Type A strains that is not present in Type B strains. The presence or absence of *pdpD* may account, in part, for the strain to strain difference in virulence. Strangely, the *pdpD* mutant of *F. tularensis* described here has a lower virulence in mice than the LVS strain, which also lacks *pdpD*. Conceivably, the duplication of the FPI in the LVS strain, and the consequent increased gene dosage of the FPI-encoded genes may partially compensate for the absence of *pdpD*. Alternatively, the apparent polar effect on transcription of the erythromycin resistance cassette insertion may account for the lowered virulence phenotype in

the *pdpD* mutant, JL12. Interpreting the virulence role of the altered form of *pdpD* in the subspecies *novicida* is complicated by the different endotoxin and O-antigen that this strain possesses (Cowley et al.,1996). The endotoxin in particular clearly diminishes the ability of the *novicida* subspecies to grow in the macrophages of some animals (Cowley et al.,1996).

At present the most significant clue as to the function of IglA and IglB comes from the study of putative homologues in *Rhizobium leguminosarum* (Bladergroen *et al.*, 2003). In this species the IglAB homologues are thought to be needed for secretion of certain proteins. Hence, one possible hypothesis for the role of IglAB in *F. tularensis* is that they have a role in secretion of PdpABCD, and perhaps other proteins.

Chapter 3 Genetic elements for selection, deletion mutagenesis, and complementation in *Francisella* spp.

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3.1 Introduction

Francisella tularensis is a gram-negative cocco-bacillus that causes the acute febrile disease, tularemia (Ellis *et al.*, 2002). In North America, where both the *F. tularensis* subsp. *tularensis* (“type A”) strain and the *F. tularensis* subsp. *holarctica* (“type B”) strain can be found, tularemia ranges from a mild to a fatal disease. In Europe and Asia, where the *F. tularensis* subsp. *holarctica* and subspecies *mediasiatica* strains are found, tularemia is generally milder and almost never fatal. *F. novicida* is generally avirulent for humans, and has been used as a model for the biology of the more virulent strains of *Francisella*.

The biology of *F. tularensis* lends itself to genetic analysis, and some virulence factors have been identified using genetic approaches. For example, a variety of random transposon mutagenesis approaches have been applied to the analysis of *Francisella* strains (Maier *et al.*, 2006, Qin & Mann, 2006, Kawula *et al.*, 2004, Gray *et al.*, 2002) Recently, a comprehensive transposon mutant bank consisting of 16,508 unique insertions, has been made in *F. novicida*, allowing the identification of putative essential genes (Gallagher *et al.*, 2007). In addition insertional (Anthony *et al.*, 1994, Mdluli *et al.*, 1994) allelic replacement (Nano *et al.*, 2004, Lauriano *et al.*, 2003,) and deletion mutagenesis (Twine *et al.*, 2005, Golovliov *et al.*, 2003) have been used to identify or analyze genes needed for virulence.

Despite recent advances, there are still gaps in the molecular tools needed for the genetic analysis of *Francisella* species. In this study we describe engineered genetic elements and methods for antibiotic selection, deletion mutagenesis and complementation in *Francisella* strains.

3.2 Materials and Methods

Table 4: Bacterial strains and plasmids used in selection, deletion mutagenesis, and complementation of *Francisella* spp.

Strain or Plasmid	Genotype or Phenotype	Source or Reference
	Bacterial Strains	
E. coli DH5α	*E. coli* F$^-$ Φ80d*lac*ZΔM15 Δ (*lac*ZYA-*arg*F)U169 *rec*A1 *end*A1 *pho*A *sup*E44 *thi*-1 *gyr* A96 *rel*A1 λ$^-$	Invitrogen
F. novicida U112	Prototype *F. novicida* strain	Laboratory strain
JL0	*F. novicida* U112 with deletion of gene FTN1758.	This work
ODB2	JL0, Δ*iglA*	(de Bruin *et al.*, 2007)
	Plasmids	
pWSK29	Low copy cloning vector, Ap^R	(Wang, 1991)
pRK2013	Narrow-range plasmid carrying the transfer functions that operate *in trans* for broad-host plasmids. Km^R.	(Ditta *et al.*, 1980)
pMMB207	Broad-host-range plasmid, Cm^R	(Morales *et al.*, 1991)
pMP527	*Francisella* shuttle plasmid that is unstable in the absence of selection. Km^R	(LoVullo *et al.*, 2006)
pMP633	*Francisella* shuttle plasmid that is stable in the absence of selection. Hyg^R	(LoVullo *et al.*, 2006)
pJL-SKX	pWSK29 (with *Xho* I site removed) with integrating SKX cassette inserted at the *Bam*H I *Kpn* I sites. Ap^R Km^R	This work. See Figure 13
pJL-XKS	pWSK29 (with *Xho* I site removed) with XKS cassette inserted at the *Bam*H I *Kpn* I sites. Ap^R Km^R	This work. See Figure 13
pEN1	pMMB207 with Cm^R gene disrupted and Km-P inserted into *Pst* I site in the MCS region.	This work
pEN2	pMMB207 with Cm^R gene disrupted and Km-P inserted into the *Pst* I site in the MCS region.	This work

3.2.1 Strains and growth conditions

Strains and plasmids used in this study are listed in Table 4. *F. novicida* and *F. tularensis* LVS were grown using trypticase soy broth or agar supplemented with 0.1% cysteine (TSB-C, TSA-C). When needed, erythromycin (Em), or kanamycin (Km) was added to a final concentration of 15 μg/ml; hygromycin (Hyg) was used at 200 μg/ml, and filter-sterilized sucrose was added to media to a final concentration of 10%. *E. coli* strains were grown using LB broth or agar supplemented with Km (30 μg/ml), Em (100 μg/ml) or ampicillin (100 μg/ml) as needed.

3.2.2 Transformation and conjugation

DNA was introduced into *F. novicida* U112 and mutant strains exclusively by chemical transformation as described previously (Anthony *et al.*, 1991), with the following modification. Efficient chemical transformation of *F. novicida* normally requires the use of a complex synthetic medium, Chamberlain's broth (Chamberlain, 1965). In this work we found that the simple addition of 0.4% glucose rendered fresh trypticase soy broth equally suitable as a transformation medium. We also reduced the high experiment-to-experiment variation in transformation by using disposable plastic tubes and plate spreaders. DNA was introduced into *F. tularensis* LVS by electroporation as previously described (Maier *et al.*, 2004). Conjugation was performed essentially as describe by Golovliov and colleagues (Golovliov *et al.*, 2003) except that plasmid transfer functions were supplied by pRK2013 (Ditta *et al.*, 1980) via a tri-parental mating.

3.2.3 Cassette and plasmid construction

An *ermCsacB* cassette was generated through PCR amplification of *ermC* and *sacB* from Tn*Max2* (Haas *et al.*, 1993) and plasmid pUM24 (Ried & Collmer, 1987), respectively using the primers AACTCGAGAATTTTATAAGGAGGGAAAAATATGG (ermC-L), AAGCGGCCGCCGAAAAACAAGTTAAGGGATGC (ermC-R), AAGCGGCCGCGATCCTTTTTAACCCATCACATA (sacB-L)and AACTCGAGGGTTAGGAATACGGTTAGCCATT (sacB-R) as described below in the Results section. The previously identified *F. novicida* strong promoter (Gallagher *et al.*, 2007), that lies upstream of gene FTN_1451 (see www.francisella.org) was PCR-amplified using the primers AACTCGAGTTTGGGTTGTCACTCATCGT and AAGTCGACCAAGACGTTTCCCGTTGAAT, and the resulting amplicon was cloned into pWSK29 as a *Xho I-Sal I* fragment. The Em^R*sacB* cassette flanked by *Xho* I sites was ligated into the *Sal I* site adjacent to P_{FT}, thus destroying both the *Sal* I and the *Xho* I sites at the junction of the promoter and *ermC* gene (See Figure 11). The entire P_{FT}- Em^R-*sacB* cassette was PCR amplified using primers AACTCGAGTTTGGGTTGTCACTCATCGT and AACTCGAGGGTTAGGAATACGGTTAGCCATT in order to place *Xho* I sites at both ends of the cassette. An Em^R-*sacB* cassette with flanking *Bam*H I ends was created using essentially the same primer pair with *Bam*H I sites substituted for the *Xho* I sites at the ends of the primers. A similar series of steps was used to create a kanamycin resistance (Km^R) marker that lacked an internal *Xho* I site.

The Km^R marker was PCR-amplified from the P_{FT}-Km element created by Gallagher and co-workers (Gallagher *et al.*, 2007) using the primers TTTGGGTTGTCACTCATCGT and AACTCGAGTTTGGGTTGTCACTCATCGT. The *Xho* I fragment was ligated into the *Sal* I site downstream of the P_{FT} as described above in order to generate a Km^R element with flanking, but no internal, *Xho* I sites. Similarly the P_{FT}-Km cassette was amplified with primers containing the core sequences TTTGGGTTGTCACTCATCGT and TTAGAAAAACTCATCGAGCATCAA together with sequences containing the sites for the restriction enzymes *Eag* I, *Eco*R I, *Sal* I and *Pst* I in order to create cassettes with different restriction sites on their ends. The *iglA* gene was amplified for complementation experiments using the primers CCCCTCGAGAGCCGTTTTCAATATTG (iglA xho-L) and CCCCTCGAGCAACTTCTGTAGATCCC (iglA xho-R). The general PCR steps used for amplification consisted of a 3 min denaturation step at 92ºC, 33 cycles of denaturation for 45 s at 90ºC, annealing for 45 s at 55ºC, extension for 60 s at 73ºC followed by a final elongation of 10 min at 73ºC.

3.2.4 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western immunoblot

SDS-PAGE was performed according to standard techniques (Laemmli, 1970). To normalize the amount of protein added to each lane, the concentration of protein samples were determined by use of the BCA assay (Pierce). Separated proteins were transferred onto an Immobilon-FL (Millipore) membrane and blocked with 5% skim milk (Difco) in PBS. Rabbit anti-IglA, was used at

dilutions of 1:4,000. To detect bound antibody blots were incubated with IRDye800DX-conjugated goat anti-rabbit immunoglobulin G (Rockland, Gilbertsville, Pa.) and visualized in a LiCor Odyssey imaging system. Samples of the anti-IglA antibody have been deposited with the BEI program at the American Type Culture Collection.

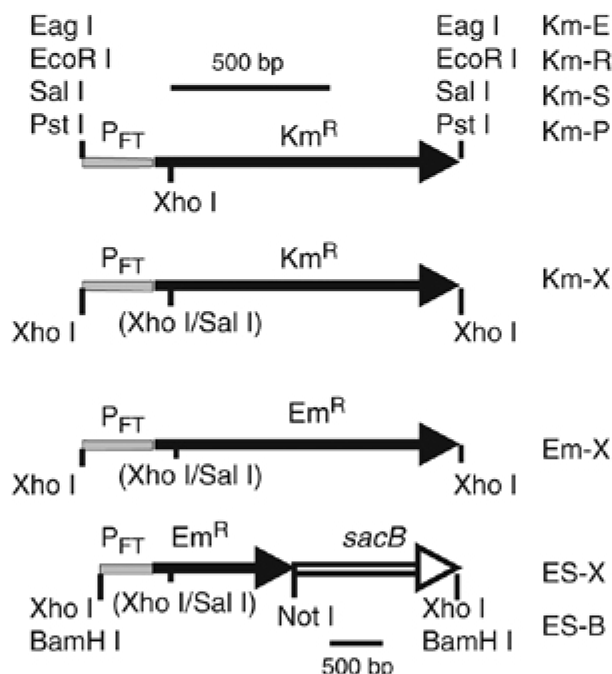

Figure 11: Cassettes for mutant construction in *Francisella* spp. Cassettes Km-E, Km-R, Km-S and Km-P are derived from Tn*903* Km^R gene and were generated by PCR to have *Eag* I, *Eco*R I, *Sal* I or *Pst* I sites respectively at each end. These cassettes have an *Xho* I site near the 5'-end of the Km^R gene. Cassettes Km-X and Em-X have had the internal *Xho* I site altered and contain flanking *Xho* I sites. The bar at the top indicating 500 bp applies to all of the cassettes except the Em^R-*sacB* cassette which has its own scale bar. Cassette ES-X consists of the same 5'-end from cassette Em-X linked to the *sacB* gene. A similar cassette, ES-B has *Bam*H I flanking sites. (Note: Km-E, Km-R, Km-S cassettes generated by Eli Nix)

3.2.5 Virulence tests

Infection of White Leghorn 7-day old chicken embryos was performed as previously described (Nix *et al.*, 2006). Two BALB/c mice were used for infection with JL0, via a subcutaneous infection, using guidelines from the Canadian Council on Animal Care.

3.2.6 Accession numbers

The GenBank Accession numbers for the Km^R cassettes KM-E, R, S, P (excluding the 6 bp at the ends for each restriction site) is EF526064; for the Em^R-*sacB* cassette with promoter, EF526066; for the SKX cassette, EF526067; for the XKS cassette, EF526068; for the promoter sequence, EF526069; and for the FTN1758 gene, EF526070.

3.3 Results

3.3.1 Use of *sacB* as a screening tool in gene deletion

We and others have extensively used the U112 strain of *F. novicida* to study the biology of growth in macrophages and virulence in animals. In order to make precisely defined mutants in this strain we attempted to use the *Bacillus subtilus sacB* gene as a counter-selectable marker. When we used *sacB* cassettes in *F. novicida*, we found that there was no detectable phenotype on sucrose-containing agar media. We reasoned that the lack of a sucrose-sensitive phenotype in *F. novicida* may be due to the well-substantiated finding that foreign genes express poorly in *Francisella* unless driven by a *Francisella*

promoter. Hence, we designed cassettes that incorporated a strong *F. novicida* promoter linked to antibiotic markers and to *sacB*. To create an appropriate *sacB*-containing cassette we first generated an *ermCsacB* cassette through PCR amplification of *ermC* and *sacB* from Tn*Max2* (Haas *et al.*, 1993) and plasmid pUM24 (Ried & Collmer, 1987), respectively. The primers ermC-L and ermC-R were used to amplify the erythromycin resistance (Em^R) cassette *ermC* as an *XhoI-NotI* fragment, while the sacB-L and sacB-R primer pair was used to generate a *NotI-XhoI sacB* amplicon. The respective amplicons were digested with *Xho* I and *Not* I, joined by ligation, and cloned into the low copy plasmid pWSK29 (Wang & Kushner, 1991). Colonies were replica plated, screened for erythromycin resistance and sucrose sensitivity in *E. coli*, and appropriate clones were analyzed by DNA sequencing. In order to create a cassette with a promoter that functioned well in *F. novicida*, we inserted a previously identified (Gallagher *et al.*, 2007) strong promoter, designated here as P_{FT}, that lies upstream of a putative outer membrane protein encoding gene, FTN_1451, in front of the Em^R marker in the Em^R-*sacB* cassette, ES-X, as described in the Materials and Methods. The introduction of this promoter improved the efficiency of obtaining integration of the cassette for some loci in *F. novicida* by at least 1,000-fold. Using this same approach we also introduced this promoter into a number of cassettes with selectable markers (See Figure 11) as described in the Materials and Methods section.

When the ES-X cassette was introduced into *F. novicida* it generated strains that became very large and mucoidal within 48 hours on agar media

containing 10% sucrose. However, the relative plating efficiency of ES-X-containing strains on agar with and without sucrose was the same. Despite the failure of *sacB* to kill *F. novicida* on sucrose-containing agar it allowed the easy detection of the loss of *sacB* by visual inspection of colonies.

We have used Em^R-*sacB* cassettes in two different ways to create deletion mutants in *F. novicida*. In one example we introduced an Em^R-*sacB* cassette into locus FTN_1758 (designated *1758*) that is found in *F. novicida* but not other *F. tularensis* strains.

This strain was then transformed with a recombinant molecule that contained the flanking genomic regions of the *1758* gene (See Figure 12). Following transformation and plating on sucrose-containing agar one colony out of 1,000 non-mucoidal colonies was found to have lacked the *1758* gene and the Em^R-*sacB* cassette (See Figure 12B), leaving a deletion of the *1758* gene. The resulting strain was designated JL0, and shown to be identical to wild type *F. novicida* in its growth phenotype in macrophages (data not shown) and in infection of 7-day old chicken embryos (data not shown, but See Figure 14).

In order to increase the efficiency of generating deletion mutants we developed a methodology that uses integration of a Em^R-*sacB* cassette followed by its excision. In this approach *F. novicida* is transformed with ES-X ligated to a construct of *F. novicida* DNA containing a gene deletion. Several Em^R-transformatants are then grown, with serial dilutions for 2-3 days to enrich for strains that have lost the integrated cassette, followed by plating on sucrose-containing agar.

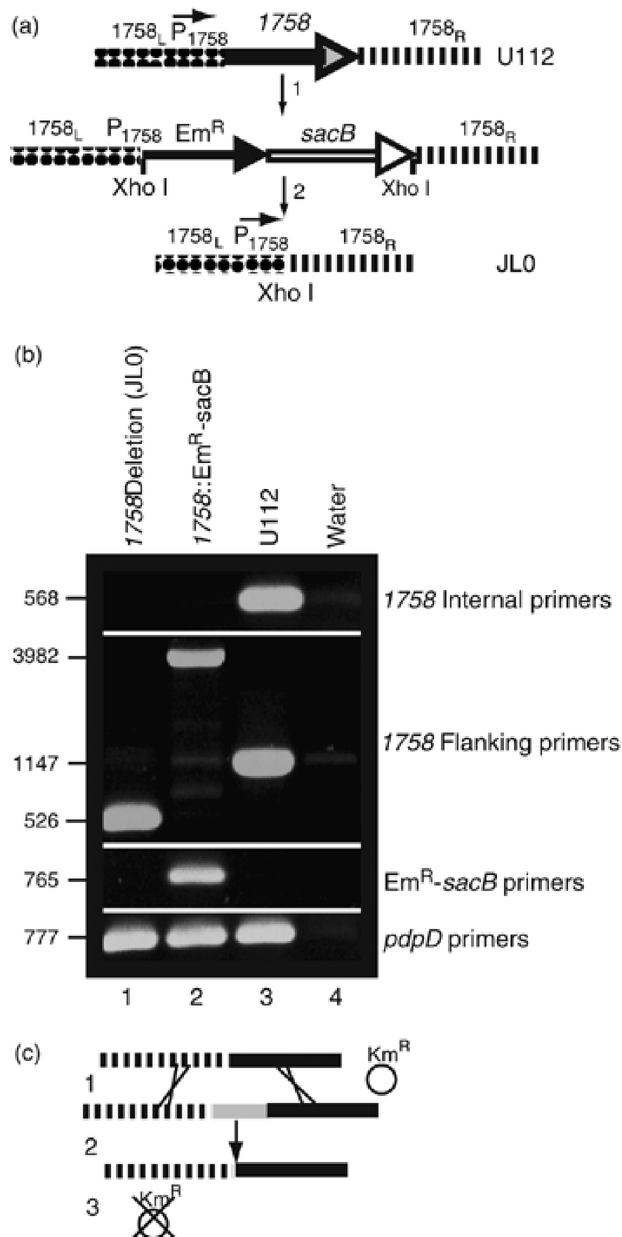

Figure 12: Approaches to mutagenesis in Francisella novicida. (A) Deletion mutagenesis by transformation with linear DNA to replace a cassette. Step 1. A recombinant clone was created that contained the flanking regions of the 621bp *1758* gene, separated by an *Xho* I site; 1758_L was 1528 bp and 1758_R was 1551bp. An Em^R-*sacB* cassette (2838 bp) was introduced into the *Xho* I site and this ligation product was used to transform *F. novicida* U112. Step 2. The linearlized recombinant clone containing the *1758* flanking regions was used to transform the *1758*::Em^R-*sacB* cassette-containing strain. The genetic elements are not drawn to scale. **(B)** PCR analysis of strains used in the steps to create a *1758* deletion. The Em^R-*sacB* cassette is 2,838 bp and the *1758* gene is 621 bp in length and this accounts for the different sized products in lanes 2 and 3 when *1758* flanking primers are used. The size of each PCR amplicon is shown at the left of the figure. **(C)** Deletion mutagenesis using co-transformation of a plasmid. Step 1. A linear recombinant deletion clone is transformed along with a plasmid. Step 2. The recombinant deletion integrates into 1-5% of the Km^R transformants. Step 3. The unstable plasmid is allowed to be lost by growth of the strain in non-selective medium.

Non-mucoidal colonies are screened for Em^R, and antibiotic sensitive colonies are then screened by PCR to detect strains containing the desired deletion. Using the ES-X cassette and these approaches we have created a number of gene deletions, including a Δ*iglA* strain (de Bruin *et al.*, 2007).

3.3.2 Deletion mutagenesis via cotransformation with plasmid DNA

F. novicida is unusual in that it readily takes up and integrates linear DNA. This property of *F. novicida* makes it amenable to deletion mutagenesis by combining the transformation of unmarked DNA along with a selectable marker (See Figure 12C). To test this approach we transformed *F. novicida* with linear DNA containing a chromosomal deletion along with the stable, hygromycin resistant (Hyg^R) plasmid pMP633, or the unstable, kanamycin resistant (Km^R) plasmid pMP527 as selectable markers (LoVullo *et al.*, 2006). In all experiments we used an approximate 10-fold excess of the chromosomal fragment to the plasmid DNA. We first demonstrated that transformation and selection for pMP633 (Hyg^R) along with a chromosomal fragment carrying a Km^R cassette in the *1758* gene resulted in 3% (14/500) of the Hyg^R colonies also being Km^R, thus demonstrating co-transformation. When an unmarked deletion of the *1758* gene was co-transformed with pMP633 about 4% (2/48) of the Hyg^R colonies had a deletion of the *1758* gene as detected by PCR (See Figure 12 for expected patterns). We also performed co-transformation using small deletions (120 and 156 bp) in the *pdpC* gene. Using the stable pMP633 plasmid (Hyg^R) as the selectable marker in two experiments we found integration of the co-transformed DNA in 66% (8/12) or 5% (6/122) of the Hyg^R colonies. When the unstable

plasmid pMP527 (Km^R) was used as the selectable marker in three experiments, we found that the co-transformed DNA integrated in <0.4% (0/270), 1% (6/600) and 1% (2/200) of the Km^R colonies. In one of the deletion mutants created by co-transformation with pMP527, the plasmid was lost during the replica-plating of colonies, verifying its instability during this series of genetic manipulations.

3.3.3 An integrating element for complementation

Since disruption of the *1758* gene did not affect the virulence of *F. novicida*, we reasoned that this locus should be a good location to insert gene cassettes for genetic complementation. Hence, we constructed a plasmid that contains a Km^R cassette with the strong promoter, and the cassette was flanked by DNA that corresponds to the regions that flank the *1758* gene in the *F. novicida* chromosome. Two versions of this cassette were made so that one contains a unique *Xho* I site before the Km^R cassette (pJL-XKS) and the other has the *Xho* I site after the cassette (pJL-SKX, See Figure 13) and is thus influenced by the P_{FT} promoter. Sites for *Xho* I are rare in the *F. tularensis* chromosome, and thus most genes can be PCR-amplified as *Xho* I-*Xho* I fragments. Exogenous genes can be introduced into the *Xho* I sites in both orientations with respect to the P_{FT} promoter and both orientations with respect to the *1758* promoter. Once a recombinant gene is inserted into the *Xho* I site the entire plasmid can be digested with *Bam*H I, *Eco*R I, *Eag* I or Sac I and the resulting linear DNA used to transform *F. novicida*. Integration of the XKS/SKX vector readily occurs into the chromosome via a double cross-over event.

The utility of the SKX vector was demonstrated by introducing the *iglA* gene into a Δ*iglA* strain. A PCR reaction was used to amplify the region from 365 bp upstream of the *iglA* start codon to 81 bp downstream of the stop codon. The *iglA*-containing fragment included the *iglA* promoter, and was cloned into SKX downstream and in the opposite orientation to the Km^R cassette. As can be seen in Figure 14A the introduction of SKX::*iglA* into the Δ*iglA* strain resulted in the production of IglA at a level close to that observed in the wild type strain. The complemented strain regained much of its virulence as measured in a chicken embryo model of infection (See Figures 14B-D).

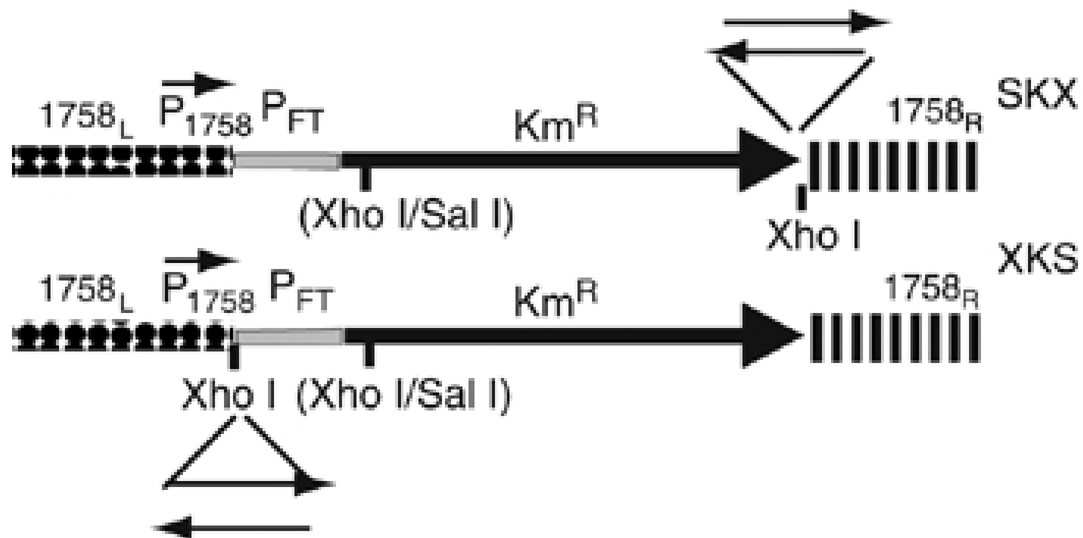

Figure 13: Cassettes for integration of recombinant genes into the chromosome of *Francisella novicida*. A Km^R gene with a strong *F. novicida* promoter P_{FT} was cloned between the chromosomal regions (1,528 bp upstream and 1551 bp downstream) flanking *1758*. A unique *Xho* I site follows the Km^R gene and promoter in pJL-SKX and precedes the Km^R gene in pJL-XKS. The entire constructs are incorporated into the *Bam*H I and *Kpn* I sites of the low copy plasmid pWSK29 that has been modified to remove its *Xho* I site. Recombinants can be linearlized with *Bam*H I, *Eco*R I, *Eag* I or *Sac* I if these sites are missing

from the cloned insert. The DNA sequences for the SKX and XKS cassettes can be found at GenBank under accession numbers EF526267 and EF526070 respectively.

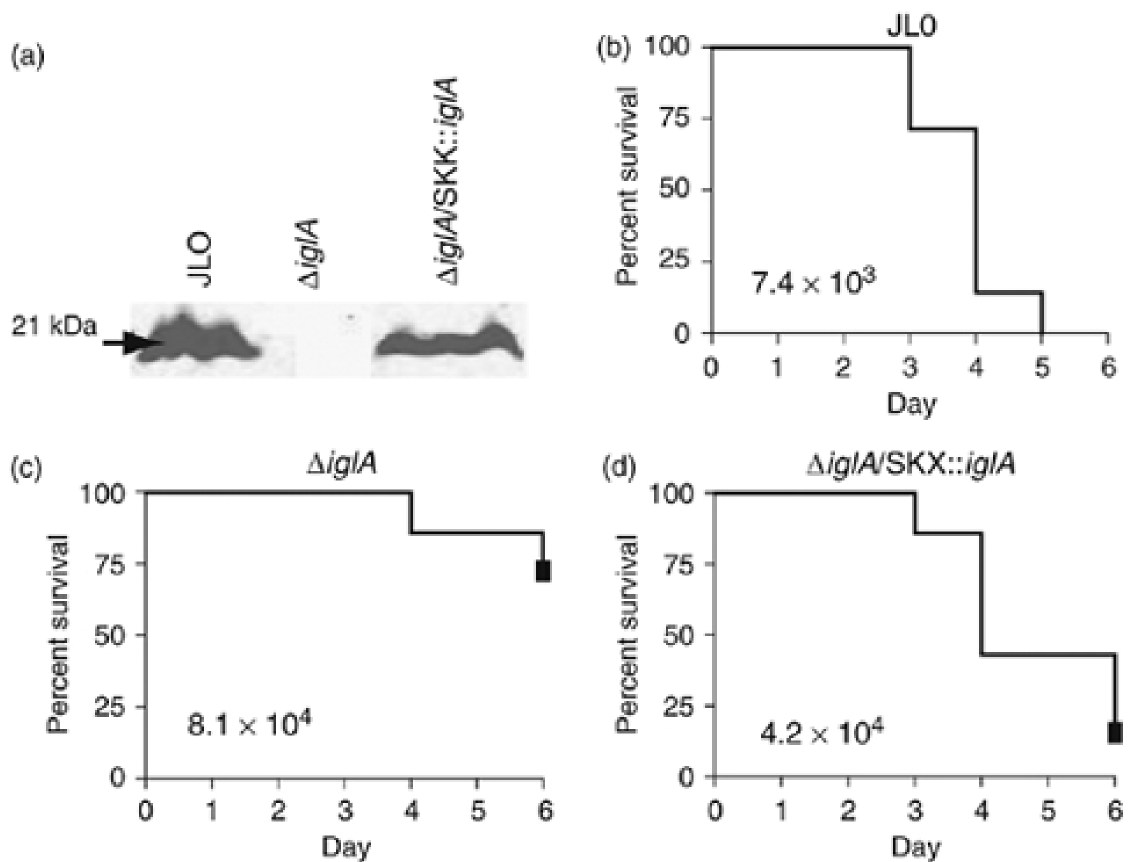

Figure 14: Genetic complementation using the integrating pJL-SKX vector in *Francisella spp*. (A) Immunoblot analysis of expression of IglA from pJL-SKX. The *iglA* structural gene and the upstream presumptive promoter region were cloned into the pJL-SKX vector in the opposite orientation as the Km^R cassette and introduced into the Δ*iglA* strain, ODB2. Quantification of the fluorescence signal from the Western immunoblot shows that expression of IglA in the complemented strain was about 0.5-fold that of the parent strain, JL0. **(B-D)** Survival of chicken embryos infected with Δ*iglA* strain and complemented strain. The numbers inside of each graph show the inoculating dose of *F.*

novicida. Each graph represents the infection of seven chicken embryos. While the Δ*iglA* mutant, ODB2, was avirulent the expression of IglA in ODB2 largely restored virulence. (Note: Immunoblot and complementation performed by Olle de Bruin)

3.3.4 Engineered broad-host-range plasmids

Previously we had introduced derivatives of the broad host range plasmid RSF1010 into *F. novicida* (Nano *et al.*, 2004). We had noted that while we were able to introduce a recombinant plasmid carrying *F. novicida* DNA we were never able to introduce the parent cloning vector. One possible reason for this phenomenon is that the parent vector lacked a promoter strong enough to drive sufficient expression of the selectable antibiotic cassette. To test this idea we inserted a Km^R cassette carrying the strong P_{FT} promoter into the broad host range, Cm^R plasmid, pMMB207 (Morales *et al.*, 1991) and used it to transform *F. tularensis* LVS. From one Km^R colony plasmid DNA was recovered and used to transform *E. coli* DH5α. The pMMB207::Km^R recombinant was isolated from *E. coli* and partially digested with *Dra* I and religated so as to destroy the Cm^R marker. Chloramphenicol can be used as a therapeutic agent to treat tularemia and hence Cm^R markers may be considered inappropriate to use with virulent *F. tularensis* strains. The resulting plasmid, designated, pEN1, (See Figure 15) and a derivative with the Km^R cassette oriented in the opposite direction, pEN2, were tested for their efficiency to transform *F. novicida* and electroporate into *F. tularensis* LVS. As expected the plasmids, when isolated from *E. coli* transformed more efficiently into the LVS (2-9 X 10^6 transformants per µg of

plasmid DNA) than into *F. novicida* since the former apparently lacks a restriction-modification system. Transformation of pEN1 into *F. novicida* was 3-4 orders of magnitude less efficient than electroporation into the LVS, and it was difficult to obtain even a single *F. novicida* transformant in some experiments. In contrast, conjugation via tri-parental matings of pEN1 to *F. novicida* and *F. tularensis* LVS generated approximately the same number of exconjugants (10^{-5} per recipient cell). However, in some experiments no exconjugants were obtained for the LVS strain, and we attribute this to its fragility in the co-culture conditions. Hence, with the appropriate use of electroporation or conjugation for a particular strain one can recover several hundred transformants or exconjugants in a typical experiment.

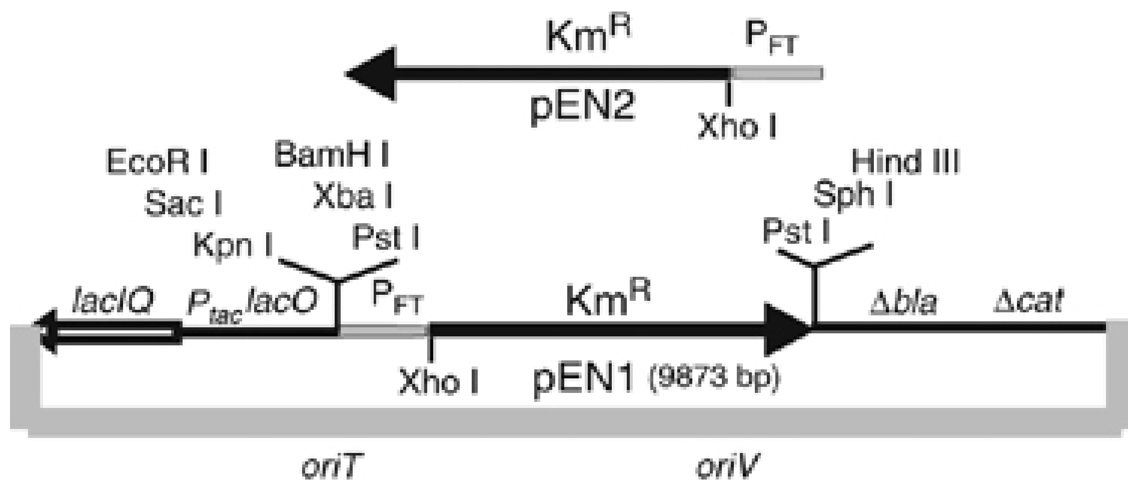

Figure 15: Organiztion of the pEN1 and pEN2 plasmids. Diagram shows relative position of unique cloning sites on pEN1 and pEN2. Ap^R and Cm^R are not encoded by these plasmids. The sites for *Bam*H I, *Kpn* I, *Sac* I, *Sph* I and *Eco*R I are appropriate for cloning *Francisella* genes since these sites are relatively rare in *Francisella* genomic DNA. The thick, gray line indicates the backbone of the plasmid and the relative positions of *oriT* and *oriV*. The

elements of the plasmid are not drawn to scale. (Note: Vector generated by Eli Nix)

Plasmid pEN1 was unstable in *F. novicida* when grown in the absence of antibiotic selection; after 20 h of broth cultivation of *F. novicida*(pEN1) up to 99.9% of the cells lacked the plasmid. When the pFNL10-based plasmid pMP633 (Hyg^R) was transformed into *F. novicida* harboring pEN1, the number of Km^R colonies approximately equaled the number of Km^R-Hyg^R transformants, indicating that the two plasmid are compatible. Isolation of whole chromosomal DNA from *F. novicida* (pEN1) followed by restriction digestion with *Hin*d III and agarose gel electrophoresis showed that the linearized plasmid band stains about 5-10 fold as intensely as chromosomal bands (data not shown), indicating that pEN1 is a low copy plasmid.

3.3 Discussion

In this work we described approaches for making unmarked deletion mutants in *F. novicida*, and tools to genetically complement mutations. In many cases it is essential to make deletion mutations rather than antibiotic resistant allelic replacements, since the latter often lead to disruption of expression of genes downstream of the inserted antibiotic resistance marker. Of course, the approaches for making deletion mutants can also be used for making gene substitutions, for example, with site-directed mutant gene alleles. Although the *sacB* gene cannot be used to select for the loss of a cassette, it still has proven useful in *F. novicida* as a tool to screen for the presence of a cassette.

The importance of inserting a *Francisella* promoter in front of the selective markers can only be understood in the context of the biology of exogenous gene expression in *Francisella* species. It is well established that exogenous gene expression in *Francisella* is dependent on a *Francisella* promoter or very high gene dosages of the exogenous gene (Gallagher *et al.*, 2007, LoVullo *et al.*, 2006, Qin & Mann, 2006,). Antibiotic markers that lack an appropriate promoter will preferentially generate strains with insertions downstream of endogenous *Francisella* promoters, thus creating a bias in many experiments

The Km^R-based genetic elements described in this work can be used in any *Francisella* species or subspecies and the Em^R-based elements can be used in *F. novicida*, *F. tularensis* type A, and many North American type B strains. The pEN1/pEN2 plasmids represent a different plasmid incompatibility group than the plasmids commonly used in *Francisella*, and thus allow an expansion of the types of complementation experiments that can be performed. In addition, the use of conjugation eliminates the need for electroporation equipment and the generation of aerosols, which facilitates gene transfer experiments to highly virulent *F. tularensis* strains in a BSL3 environment.

Finally, the success in using tri-parental matings to *F. novicida* and *F. tularensis* potentially enhances cloning experiments by allowing the use of any *E. coli* strain to serve as the cloning vehicle prior to matings to *Francisella*. The use of tri-parental matings obviates the need to construct clones in potentially unstable and poorly transformable *E. coli* strains carrying integrated broad host range plasmids.

Chapter 4 The *Francisella* Pathogenicity Island Protein PdpD is Required for Full Virulence and Associates with Homologues of the Type VI secretion System.

(Published in the Journal of Bacteriology)

4.1 Introduction

Francisella tularensis, the causative agent of the zoonotic disease tularemia, is a gram negative, facultative intracellular bacterial pathogen (Eigelsbach *et al*., 1961). *F. tularensis* is remarkable in that it is both highly infectious and capable of infecting a very broad array of animal species. During a short period of time in the early 20th Century, *F. tularensis* was independently found to be the cause of zoonotic diseases in Europe, Asia and North America. It was also observed that the clinical outcome of tularemia was most severe in North America, where fatalities occurred at a rate 10-100 fold higher than the rate found in Europe or Asia. Exchange of *F. tularensis* strains between Russian, Japanese and American scientists led to the discovery that two major biotypes existed, a pan-Northern Hemisphere "*holarctica*" (or "type B") biotype and a North American-specific biotype ("*tularensis*" or "type A"). Both American and Russian scientists used rabbit models of infection to discriminate the highly virulent from less virulent forms (Olsufjev and Emelyanova, 1963, Bell *et al*., 1955). Cumulatively the human clinical disease pattern and the experimental rabbit infection results led to the widely held belief that the *F. tularensis* type A strains were much more virulent in humans than their type B counterparts. However, a review of the literature shows that there is no solid experimental or clinical basis for this conventional belief. Indeed, the notion has been challenged recently by a

retrospective study by CDC scientists of fatal and non-fatal cases of tularemia in the United States (Staples *et al.*, 2006). Although this study is limited by the strong bias generated by the strains sent to the Center for Disease Control and Prevention, it does highlight the possibility that a subset of type A strains (A.II) (Johansson *et al.*, 2004), found mostly in the Western U.S. are less virulent than the A.I subset found mostly in the Eastern United States.

The recent availability of genomic information for *F. tularensis* has enabled comparison of type A and type B genomes, as well as European and North American type B strains, down to the nucleotide level (Beckstrom-Sternberg *et al.*, 2007, Larsson *et al.*, 2005, Rohmer *et al.*, 2007). While there are many genomic rearrangements and single nucleotide polymorphism differences among strains, there are very few cases of genes being absent from one biotype and present in another. Of those overt differences only one locus, *anmKpdpD*, is clearly associated with a cluster of known virulence genes, namely those found in the *Francisella* Pathogenicity Island (FPI) (Nano and Schmerk, 2007, Nano *et al.*, 2004).

The recently identified FPI is a ~30 kb genetic element with an average G+C content that differs from the core genome by 2.2% for the *anmK-iglD* operon and by 6.6% for the larger *pdpA-pdpE* region (Nano and Schmerk, 2007, Nano *et al.*, 2004) (See Figure 16). Aberrant G+C contents are an important signature of DNA introduced into a chromosome by ancient horizontal DNA transfer. All of the type A and type B biovars have two identical copies of the FPI, while the *F. novicida* biotype contains a single copy of the FPI. In every instance to date,

inactivation of FPI genes has led to decreased intramacrophage growth and decreased virulence (de Bruin *et al*., 2007, Santic *et al*., 2007, Weiss *et al*., 2007, Temple *et al*., 2006, Golovliov *et al*., 2003, Gray *et al*., 2002,). Although the function of the proteins encoded by the FPI have not been determined, at least four of the genes in the FPI encode proteins that are homologues of proteins that are part of a type six secretion system (T6SS) found in other pathogens. The T6SS is thought to mediate the export of virulence effector proteins in a *sec*-independent manner in a variety of animal (Zheng and Leung, 2007) and plant pathogens or symbionts, including *Vibrio cholerae*, and *Pseudomonas aeruginosa* (Mougous *et al*., 2006, Pukatzki *et al*., 2006, Bladergroen *et al*., 2003). There is substantial evidence that T6SSs are tightly controlled and up-regulated during an infection, and these properties may explain why proteins in *Francisella* that are secreted by its T6SS have not been identified in the past (de Bruin *et al*., 2007, Mougous *et al*., 20007, Schell *et al*., 2007). Recent evidence suggest that the *V. cholerae* T6SS produces a secreted structure that is predicted to have cell-puncturing properties, but the full picture of secretion by T6SS is yet to be elucidated (Pukatzki *et al*., 2007).

As a gram negative bacterial pathogen *Francisella* is expected to have mechanisms to secrete proteinaceous virulence factors to the surface of the bacterium or into the extracellular milieu. Gill and co-workers found a *tolC* and a *tolC*-like homologue in *Francisella*, and showed that these genes contribute to resistance to bactericidal small molecules (Gil *et al*., 2006). They also showed that the *tolC* homologue is required for virulence, and suggested that their

evidence indicated a role for TolC in a type I secretion system. Bina and colleagues showed that an AcrB RND efflux pump contributes to drug resistance and virulence in the *F. tularensis* LVS strain (Bina *et al*., 2008). Importantly, Hager *et al.* showed true secretion of seven proteins in *F. novicida* that is dependent on genes that are homologous to type IV pili genes (Hager *et al*., 2006). Surprisingly, inactivation of secretion components or one of the secreted proteins generated strains that had enhanced virulence, and enhanced dissemination of *F. novicida* when introduced via an intradermal injection.

Table 5: Bacterial strains used in the study of PdpD and its association with homologues of the Type VI secretion system.

Bacterial Strain	Relevant Characteristics	Source and/or Reference
U112	Wild type *Francisella novicida*	(Larson *et al*., 1955)
JLO	U112 with deletion in FTN_1390, which is the site where the integrating SKX vector inserts. This strain has growth and virulence phenotypes identical to U112.	(Ludu *et al*., 2008)
GB2	U112 with point mutation in the global virulence regulator, *mglA*	(Baron *et al*., 1998)
SC92	O-antigen mutant of U112	(Cowley *et al*., 2000)
JL12	An *ermC* allelic exchange mutant of *pdpD*	(Nano *et al*., 2004)
ODB2	JL0 with deletion of *iglA*	(de Bruin *et al*., 2007)
Δ*pdpD*-10b	An in-frame deletion mutant missing codons 513 to 789 of *pdpD*	This work
Δ*pdpD*-20d	An in-frame deletion mutant missing codons 215 to 1138 of *pdpD*	This work
Δ*anmK*	An in-frame deletion mutant missing codons 18 to 355 of *anmK*	This work
Δ*pdpD*/SKX::*pdpD*	Δ*pdpD*-20d complemented with the	This work

	integrating pJL-SKX::*pdpD* construct	
Δ*pdpD*/SKX:: *anmKpdpD*	Δ*pdpD*-20d complemented with the integrating pJL-SKX::*anmKpdpD* construct	This work
Δ*pdpD*/pMP633:: Km^R*anmKpdpD*	Δ*pdpD*-20d complemented with the pMP633::Km^R*anmKpdpD* construct	This work
Δ*anmK*/SKX:: *anmK*	Δ*anmK* complemented with the integrating pJL-SKX::*anmK* construct	This work
Δ*iglAB*/SKX:: *anmKpdpD*	A Δ*iglA* mutant complemented with the integrating pJL-SKX::*anmKpdpD* construct	This work
Δ*iglB*/SKX:: *anmKpdpD*	A Δ*iglB* mutant complemented with the integrating pJL-SKX::*anmKpdpD* construct	This work
Δ*pdpB*/SKX:: *anmKpdpD*	A Δ*pdpB* mutant complemented with the integrating pJL-SKX:: *anmKpdpD* construct	This work
Δ*dotU*/SKX:: *anmKpdpD*	A Δ*dotU* mutant complemented with the integrating pJL-SKX:: *anmKpdpD* construct	This work
SC92/SKX:: *anmKpdpD*	A LPS mutant complemented with the integrating pJL-SKX:: *anmKpdpD* construct	This work
F. tularensis subsp. *holarctica* LVS	Live Vaccine Strain. Type b biotype.	(Larson *et al*., 1955) ATCC
F. tularensis LVS (pMP633:: Km^R*anmKpdpD*)	The LVS strain complemented with the pMP633::Km^R*anmKpdpD* construct	This work
E. coli DH5α	*sup*E44 Δ(*lacIZYA-argF)*U169 (Φ80*lacZ*ΔM15) *hsd*R17 *rec*A1 *end*A1 *gyr*A96 *thi*-1 *rel*A1	(Hanahan, 1983) Invitrogen

Table 6: Plasmids used in the study of PdpD and its association with homologues of the Type VI secretion system.

Bacterial Strain	Relevant Characteristics	Source and/or Reference
pWSK29	Low copy cloning vector, Amp^R.	(Wang *et al*., 1991)
pJL-SKX	pWSK29 (with Xho I site removed) with integrating SKX cassette inserted at the *Bam*H I *Kpn* I sites, Km^R, Ap^R	(Ludu *et al*., 2008)
pMP633	*Francisella* shuttle plasmid, Hyg^R.	(LoVullo *et al*., 2006)

pJL-ES-X	An *ermCsacB* cassette with flanking Xho I restriction sites	(Ludu *et al.*, 2008)
pWSK29::*pdpD*	A *pdpD* clone with 2,100 bp of the up and downstream flanking regions	This work
pWSK29::Δ*pdpD*-10b	A construct containing a 277 codon in-frame *pdpD* deletion and flanking Xho I restriction sites	This work
pWSK29::Δ*pdpD*-20d	A construct containing a 924 codon in-frame *pdpD* deletion and flanking Xho I restriction sites	This work
pJL-SKX::*pdpD*	An integrating complementation vector carrying the *pdpD* locus	This work
pJL-SKX::*anmK*	An integrating complementation vector carrying the *anmK* locus and 397 bp of upstream sequence.	This work
pJL-SKX::*anmKpdpD*	An integrating complementation vector carrying the *anmKpdpD* loci and 397 bp upstream of *anmK*.	This work
pMP633::Km^R*anmKpdpD*	*Francisella* shuttle plasmid carrying the SKX::*anmKpdpD*. Km^R, Hyg^R. The insert in pMP633 contains the Km^R cassette through the end of *pdpD*, which was PCR amplified from pJL-SKX::*anmKpdpD* and cloned into the EcoR V site of pMP633.	This work

In this work we examined the role of *anmK* and *pdpD* in *Francisella* virulence, and in the course of these studies discovered an interaction of homologues of T6SS components with PdpD. Thus here we examine the virulence role of a protein found in the North American specific biotype of *F. tularensis* that is missing in the pan-Northern Hemisphere biotype, and provide new knowledge about a poorly understood secretion system.

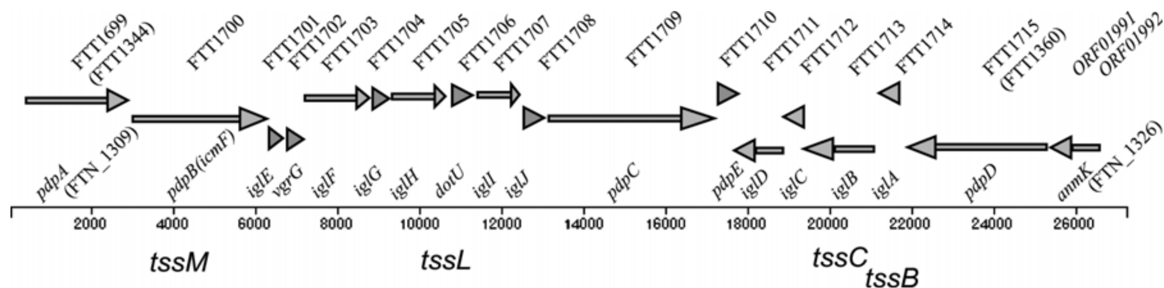

Figure 16: Diagrammatic representation of the *F. novicida* form of the FPI using a consensus nomenclature of the FPI genes. The *anmKpdpD* genes are the first and second ORFs in an apparent operon that runs from *anmK* through *iglD*. This operon has a G+C content of 30.6% in contrast to the 33% G+C average content for the *Francisella* chromosome. The *pdpA-pdpE* region has a G+C content of 26.6% which is the region of the lowest G+C content in the chromosome. The *F. novicida* form of the FPI differs from the *F. tularensis* type B form in having *anmK* and *pdpD*. *F. tularensis* type A biotypes have *pdpD* but have one of two different-sized *anmK* forms. The names of the FPI genes are indicated above the line that indicates the base pair numbers starting from the start of *pdpA*. Below the line are the consensus designations for T6SS genes. For reference, the *F. tularensis* Schu 4 genomic designations for one of the Schu 4 FPI copies are included above the arrows that indicate the direction of each gene. For the first and last genes in the FPI, the numbers in parentheses indicate alternative genomic designations for the Schu strain (FTT) or the *F. novicida* species (FTN).

4.2 Materials and Methods

4.2.1 Strains and growth conditions

The bacterial strains and plasmids used in this study are listed in Tables 5 and 6, respectively. Detailed descriptions of Δ*iglB*, Δ*pdpB* and Δ*dotU* will be described elsewhere, but they were made essentially as described below (see Mutagenesis and Complementation). *F. novicida* and *F. tularensis* LVS were

grown using trypticase soy broth or agar supplemented with 0.1% cysteine (TSB-C, TSA-C). When needed, erythromycin (Em) or kanamycin (Km) was added to a final concentration of 25 μg/ml and 15 μg/ml, respectively. For deletion mutagenesis experiments, filter-sterilized sucrose was added to media to a final concentration of 10%. *E. coli* strains were grown using LB broth or agar supplemented with Km (30 μg/ml), Em (100 μg/ml) or ampicillin (100 μg/ml) as needed.

4.2.2 Transformation of *Francisella*

Genetic constructs were introduced into *F. novicida* by a previously described chemical transformation protocol with the modification that Chamberlain's broth was replaced with TSB-C supplemented with 0.4% glucose (Anthony *et al.*, 1991, Ludu *et al.*, 2008).

4.2.3 SDS-PAGE and Immunoblotting

SDS-PAGE was performed according to standard techniques (Laemmli *et al.*, 1970). To normalize the amount of protein added to each lane, the concentration of protein samples were determined by use of the BCA assay (Pierce). Separated proteins were transferred onto an Immobilon-FL (Millipore) membrane and blocked with 5% skim milk (Difco) in PBS. Rabbit anti-IglA, was used at dilutions of 1:4,000, while rabbit anti-PdpD, was used at dilutions of 1:1,000. To detect bound antibody, blots were incubated with IRDye800DX-conjugated goat anti-rabbit immunoglobulin G (Rockland, Gilbertsville, Pa.) and visualized in a LiCor Odyssey imaging system. In some blots monoclonal anti-

IglB, IglC, and PdpB were used, and these were detected using IRDye800-conjugated goat anti-mouse antibody. All of the rabbit antisera and the mouse hybridomas described above have been deposited with the American Type Culture Collection's BEI program. Anti-FopA rabbit serum was kindly supplied by Dr. Michael Norgard.

The three different anti-PdpD rabbit antisera used in this study were made by New England Peptide (Gardner, MA) by immunizing New Zealand White rabbits with injections of either a recombinant fragment of PdpD (amino acids 748-966) or KLH-conjugated peptides (amino acids 259-272, and 963-976). Rabbit anti-VgrG was made by injections with purified recombinant protein.

4.2.4 Fractionation of *Francisella*

Approximately $2x10^{11}$ CFU of two day old plate grown *F. novicida* were harvested and resuspended in 50 ml of cold phosphate buffered saline (PBS) supplemented with 35 µl of a bacterial protease inhibitor solution. Agar plate-grown *F. novicida* were used because they appeared to produce more PdpD than broth grown cells. Cells were lysed by repeated passage through a French Pressure cell (American Instruments Co, Silver Spring, MD) at ~1200 PSI. Unbroken cells were removed by 20 min of centrifugation at 10,000 × *g* at 4ºC, and a sample was taken as the total protein fraction. The lysate was subjected to ultracentrifugation (Beckman L8-70, rotor Type 45 Ti) for 2 hrs at 100,000 X *g* at 4°C to pellet the membranes. The supernatant (soluble protein fraction) was removed, while the membrane pellet was resuspended in 2.5 ml of 1% Sarkosyl (Sigma). The sarkosyl soluble (inner membrane) and the sarkosyl insoluble

fractions (outer membrane) were separated by a second ultracentrifugation for 2 h at 100,000 X *g* at 4°C in a Beckman TLA-100.3 ultramicrocentrifuge. The pelleted membrane fraction was resuspended in SDS-PAGE running buffer, and all samples were separated and blotted using standard techniques.

4.2.5 Biotinylation of *Francisella* outer membrane proteins

The biotinylation of potentially surface exposed proteins was carried out using the EZ-Link Sulfo-NHS-LC-LC-Biotin (Pierce) labeling agent. Plate grown *F. novicida* strains were resuspended in 10 ml of cold PBS, washed three times by pelleting at 10,000 X *g* and resuspended in cold PBS. Following the final wash, cells were resuspended in 5 ml of PBS, and a 500 µl aliquot was transferred to a 1.5 ml tube containing 250 µL of a 15 mg/ml solution of Sulfo-NHS-LC-LC-Biotin. Cells were incubated for 30 min at room temperature, pelleted at 8,000 X *g*, and washed in 1 ml of biotinylation salt solution (50 mM Tris, 300 mM NaCl, pH7.5), and two 1 ml washes of cold PBS. Following the final wash, bacteria were resuspended in 50 µL of PBS, lysed by adding 500 µL of B-PERII (Pierce), and centrifuged at 15,200 X *g* for 1 min. The supernatant was transferred to a new 1.5 ml tube and 200 µl of Ultralink immobilized NeutrAvidin beads (Pierce) was added. Tubes were incubated with gentle rocking for 30 min at room temperature, followed by five washes in which the mixture was pelleted at 1,000 X *g* for 1 min and resuspended in 1 ml of Tris-buffered saline (50 mM NaCl, 25 mM Tris, pH 7.5, 0.2% Tween-20). Protein was recovered by resuspending the pelleted NeutrAvidin beads in 40 µl of standard SDS-PAGE sample buffer, and boiling at 95ºC for 15 min. The heated mixture

was gently pelleted to remove NeutrAvidin beads, and 15 µl of supernatant was separated on a 4-10% NuSep gradient gel.

4.2.6 Mutagenesis and complementation of *anmK* and *pdpD*

To create deletion mutations, a ~8 kb clone which encompasses the entire *pdpD* gene as well as ~2 kb flanking each side of *pdpD* was cloned into pWSK29. This recombinant contained a unique *Pac* I site that lay near the middle of *pdpD*, at position 2021. The clone was linearized by *Pac* I digestion, and subjected to exonuclease BAL-31 digestion. Bal-31 reactions were stopped at 5 min intervals for 30 min by heat inactivation of Bal-31, and the resulting digested plasmids were ligated and electroporated into *E. coli* DH5α . From hundreds of transformants five clones each from the 10, 20 and 30 min Bal-31 reactions were sequenced and five were found to have in-frame *pdpD* deletions of varying sizes. These deletion constructs were used to create *F. novicida* deletion mutants as previously described (Ludu *et al*., 2007). Briefly, the in-frame *pdpD* deletion mutant clones were digested with *Xho* I, ligated to a *ermCsacB* cassette and transformed into *F. novicida* JLO to form Em^R co-integrates. Colonies that resulted from exision of the co-integrate were scored by the loss of a mucoidal phenotype in the presence of 10% sucrose and the loss of Em^R. Ultimately, these mutagenesis experiments led to the creation of five *pdpD* mutants (named 10b, 10f, 20d, 20e and 20g) whose deletions range in size from 462 to 968 amino acid residues, relative to the 1,245 residues observed in the wild-type form. The sequence of the two mutants described in this work, which represent the smallest and largest deletions, have been deposited with GenBank

and have been assigned the accession numbers EU341813 (*pdpD10b*), and EU341814 (*pdpD20d*).

Complementation of the *anmK* and *pdpD* mutations was done by inserting *anmK*, *anmKpdpD* or *pdpD* into the integrating vector, pJL-SKX and introducing linear recombinant DNA into *F. novicida* JL0. The pJL-SKX vector inserts into the *F. novicida* chromosome at the FTN_1758 locus which is located at bp 1,887,821 in the chromosome (*pdpD* is at bp 1,399,803) (Rohmer *et al*., 2007). The FTN_1758 ORF is deleted in *F. novicida* JL0. As the FTN_1758 locus is missing from *F. tularensis* LVS we were unable to integrate the vector into the LVS chromosome. Instead we PCR amplified the pJL-SKX::*anmKpdpD* recombinant from the promoter that lies in front of the Km^R cassette to the end of *pdpD*, and cloned the resulting amplicon into pMP633, and introduced this recombinant into *F. tularensis* LVS (LoVullo *et al*., 2006). The sequence of the primers used for this amplification and all others used in this work will be made available upon request.

4.2.7 Intracellular growth assays

Bone marrow cells were isolated from femurs of healthy BALB/c male mice and cultivated in 96-well cell culture plates at 4 X 10^5 cells/well (Costar) for one week in complete Dulbecco's Modified Eagle Medium (cDMEM) containing 10% fetal bovine serum (FBS), 1% L-glutamine, 1% MEM non-essential amino acids, 1% HEPES buffer solution, and 10% conditioned L929 supernatant. The resultant bone marrow-derived macrophages (BMDMs) were infected with *F. novicida* strains at a multiplicity of infection (MOI) of 20:1 (bacterium-to-

macrophage). Infected monolayers were incubated for 1 h in cDMEM to allow for phagocytosis to occur, washed five times in Dulbecco's Phosphate Buffered Saline (DPBS), and incubated at 37°C in 5% CO_2. To determine bacterial replication, infected macrophages were lysed in 0.1 % deoxycholate at 0, 24, and 48 h post infection. The lysates were serially diluted in DPBS containing 0.1 % gelatin and plated on TSAC. It has been previously demonstrated that *F. tularensis* extracellular growth in standard DMEM is not supported which makes the macrophage infection assay an appropriate determination of intracellular growth. As a negative control, the *F. novicida mglA* mutant GB2, which does not grow in macrophages, was incorporated into all macrophage growth experiments (Baron *et al*., 1998).

The various cell lines were grown in DMEM essentially as described for BMDMs. The cell lines that were used included J774A.1 mouse macrophage cell line, NIH/3T3 mouse embryonic fibroblast cell line, COS-7 monkey kidney fibroblast cell line, HeLa human cervical epithelial cell line, C2C12 mouse muscle fibroblast cell line, HEK-293 human kidney epithelial cell line, MDCK dog kidney epithelial cell line, 4T1 mouse mammary gland epithelial cell line, CMT-93 mouse rectum epithelial cell line, C6 rat brain fibroblast cell line, LLC-PK1 pig kidney epithelial cell line, and Caco-2 human colon epithelial cell line.

4.2.8 Chicken embryo and mouse infections

For the *in vivo* analysis of mutants, *F. tularensis* strains were grown to the late log phase (optical density at 600 nm, 1.0) and diluted in PBS for injection. The inoculating dose was calculated retrospectively by determining the CFU

following dilution and plating on TSA-C. Fertilized White Leghorn eggs were obtained from the University of Alberta Poultry Research Station, and chicken embryos were incubated at 37°C with high humidity for seven days prior to infection. Throughout the experiment the embryos were mechanically tilted to a 45° angle every 40 min. Following the seven day initial incubation, chicken embryos were injected with various doses of 100 µl of *F. novicida* diluted in PBS under the chorioallantoic membrane as described previously (Nix et al., 2006). Chicken embryos were then monitored daily for death for up to 6 days.

For in vivo infections six-to-eight week-old male specific-pathogen-free BALB/cByJ mice were purchased from the Jackson Laboratory. Animals were housed in sterile micro-isolator cages in barrier environment at the Center for Biologics Evaluation and Research. Mice were fed autoclaved food and water *ad libitum*, and all experiments were performed under Institutional Animal Care and Use Committee guidelines. Mice were given 0.1 ml of appropriately diluted bacteria intradermally at the base of the tail; actual doses of inoculated bacteria were simultaneously determined by plate count. All materials used in animals, including bacteria, were diluted in PBS (BioWhittaker) containing <0.01 ng/ml endotoxin. Graphing and statistical analyses (standard error of the mean, and the P-value of an unpaired t test) of experiments was done using Graph Pad Prism 4.03 software.

4.3 Results and Discussion

4.3.1 Variation of the *anmK-pdpD* region among *F. tularensis* biotypes

We previously reported the presence of *pdpD* in *F. novicida* and in *F. tularensis* type A strains and the absence of the *pdpD* gene in five *F. tularensis* type B strains (Nano *et al.*, 2004). The presence of *pdpD* in type A strains was interesting in that strains of this biotype are considered more virulent than strains of the type B biotype. In our original description of the FPI we identified the gene upstream of *pdpD, anmK,* as a putative molecular chaperone (originally called *pmcA*). Recently, the conserved orthologous group associated with *anmK* (*pmcA*), COG2377, has been reannotated, and members of this group are now recognized as being part of the anhydro-N-acetylmuramic acid kinase family. This enzyme is responsible for the utilization of exogenous or recycled 1,6-anhydro-N-acetylmuramic acid which is a component of the cell wall peptidoglycan. The *E. coli anmK* homologue (*ydhH*, or *b1640*) is known not to be essential for viability, and, since *anmK* is missing from many strains of *F. tularensis*, it is clear that it is not essential for *Francisella*, as well.

The release of several *F. tularensis* genomes and our analysis of the *anmK* region of the type A strain B38, revealed that, in addition to the difference in this region between type A and type B strains, there are also differences in the *anmK-pdpD* region between the recently identified clades, *F. tularensis* type A.I and A.II (See Figure 17). In the two representative strains of the type A.I clade, strains Schu4 and FSC033, the *anmK* region has two premature stop codons at positions 190 and 328 when compared to the *anmK* form found in *F. novicida,*

which has only one stop codon at position 372. In the A.I form of *anmK* the stop codon at position 190 is followed with a start codon at position 194. The deduced AnmK proteins found in most bacteria are about 380 amino acids (371 in *F. novicida*) in length, and hence, the stop codon at position 190 in the clade A.I form of *anmK* suggest that AnmK is not functional in this strain. In two representatives of the type A.II clade, WY96-3418 and B38, the *anmK* gene has a stop codon at position 328 in addition to the stop codon at position 372 found in all forms of *anmK*. Outside of the internal stop codon regions, the deduced amino acid sequences of the ORFs of the *anmK* genes show 96% identity between the clades A.I and A.II forms, and 98% identity between the *F. novicida* and the clade A.I forms.

Homologues of AnmK are very widely found in bacteria, and since the large majority of these are not pathogens it seems likely that *anmK* does not have a role in virulence that involves pathogen-host interactions. However, the presence of *anmK* could increase the overall fitness of a strain, and we provide evidence below that there is a small effect of the loss of *anmK* on *F. novicida* virulence. Nevertheless, we have reasoned that the *anmK* does not have a specific biological role in the virulence of *Francisella*, and we have included analysis of mutants in *anmK* only as a necessary component of our study of *pdpD*.

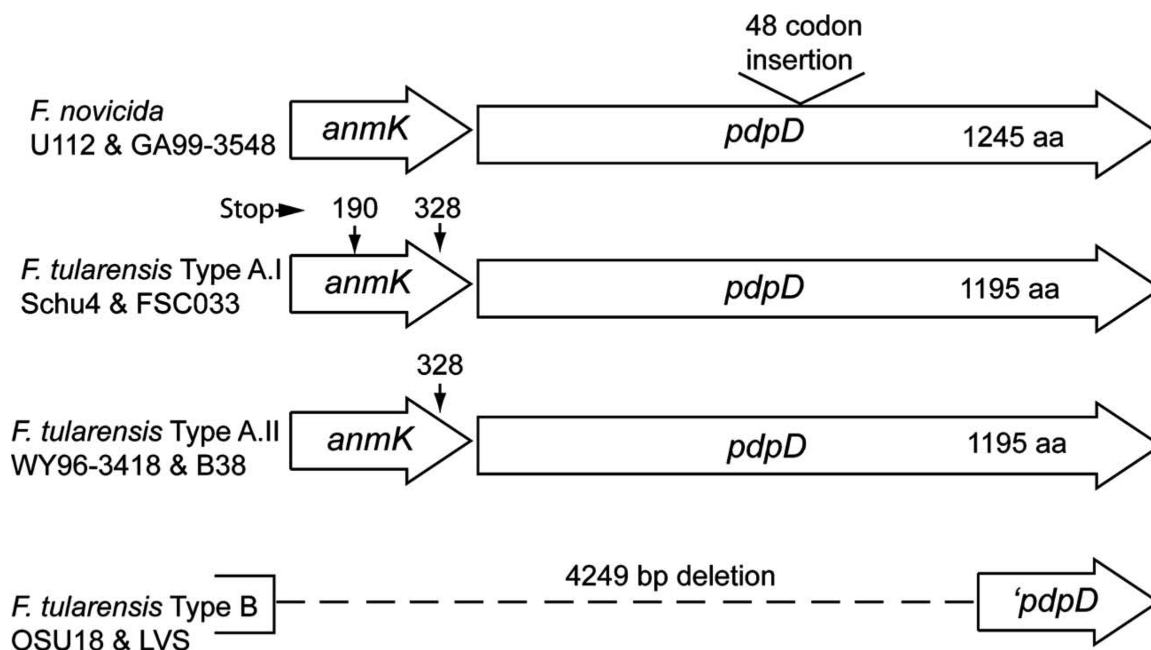

Figure 17: The *anmK* and *pdpD* loci vary in *F. tularensis* subspecies. The *F. novicida* form of the FPI differs from the type B biotype forms in having *anmK* and *pdpD*, while the type A biotypes have *pdpD* but two distinct, truncated forms of *anmK*. The *F. novicida* strains U112 and GA99-3548 encode an intact AnmK protein consisting of 371 amino acid residues. The *anmK* genes of *F. tularensis* Type A.I strains Schu4 and FSC033 contain two premature stop codons at positions 190 and 328, while the *anmK* genes of *F. tularensis* Type A.II strains WY96-3418 and B38 have a single premature stop codon at position 328. The *anmK* locus is absent from subsp. *holarctica* (type B) strains OSU18 and LVS. The sequence for the *F. tularensis* B38 form of *anmK* region has been deposited with GenBank and has been assigned the number EU341812.

The *F. novicida pdp*D gene encodes a 1,245 amino acid protein (140,663 MW), and the *pdpD* genes found in both type A.I and A.II clades of *F. tularensis* encode proteins of 1,195 amino acids (135,394 MW). The deduced amino acid sequence identity between *pdpD* genes found in the two clades is 100 percent. There is no significant identity of PdpD with other proteins as determined by a

BLASTP search of the non-redundant protein databases. The *F. novicida* form of PdpD has 50 more amino acids than PdpD from type A strains of *F. tularensis*, and 48 of these amino acids constitute a hydrophilic stretch of amino acids near the center of PdpD.

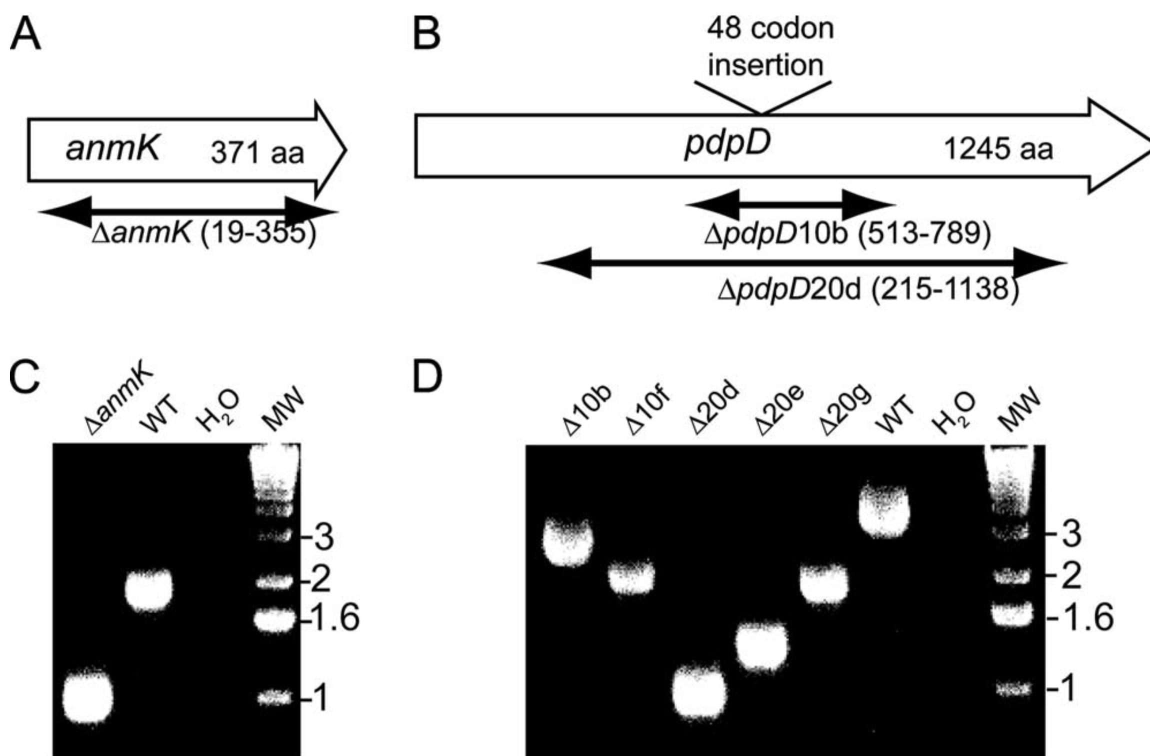

Figure 18: Mutagenesis of *anmK* and *pdpD*. The extent of the deletions in *anmK* **(A)** and *pdpD* **(B)** are diagrammed. Unless otherwise stated, all reference to a Δ*pdpD* mutant in this work refers to mutant Δ*pdpD20d*. Panels **(C)** and **(D)**. The *anmK* and five in-frame deletion mutants of *pdpD* were examined by PCR using primers which flank the *anmK* and *pdpD* loci. The DNA sequences of mutated loci for strains used in this work were submitted to GenBank.

4.3.2 Mutagenesis of the *anmKpdpD* region

The product of the *pdpD* gene had not been detected in previous studies including proteomic analysis of type A *F. tularensis* (Pavkova *et al.*, 2006, Twine *et al.*, 2006). Hence it was important to generate mutants in the *pdpD* gene in order to help identify the presumptive product of *pdpD*, in addition to determining whether PdpD plays a role in virulence. Since our previous gene replacement mutation of *pdpD* had affected the expression of the downstream gene, *iglA*, we thought it was important to construct deletion mutations, which usually have minimal polar effects on transcription/translation coupling. To make deletion mutations we took advantage of the unique *Pac* I site that lies near the center of *pdpD*. A recombinant clone containing *pdpD* and surrounding regions was digested with *Pac* I and deletion mutations were created by treatment with the processive exonuclease Bal-31 and the subsequent recovery of a number of deletion mutation clones (See Figure 18). Five in-frame deletion mutations were recovered and the analyses of two of them are shown in this work. A deletion of the complete *anmK* gene was made as well (See Figure 18). Genetic complementation of the Δ*anmK* or the Δ*pdpD* mutations was accomplished by introducing *anmK* or *anmKpdpD* into a chromosomal integrating vector pJL-SKX which directs insertions into the chromosome 0.48 Mb from *pdpD*. The region 397 bp upstream of *anmK*, which is the presumptive promoter region, was included in both complementation constructs.

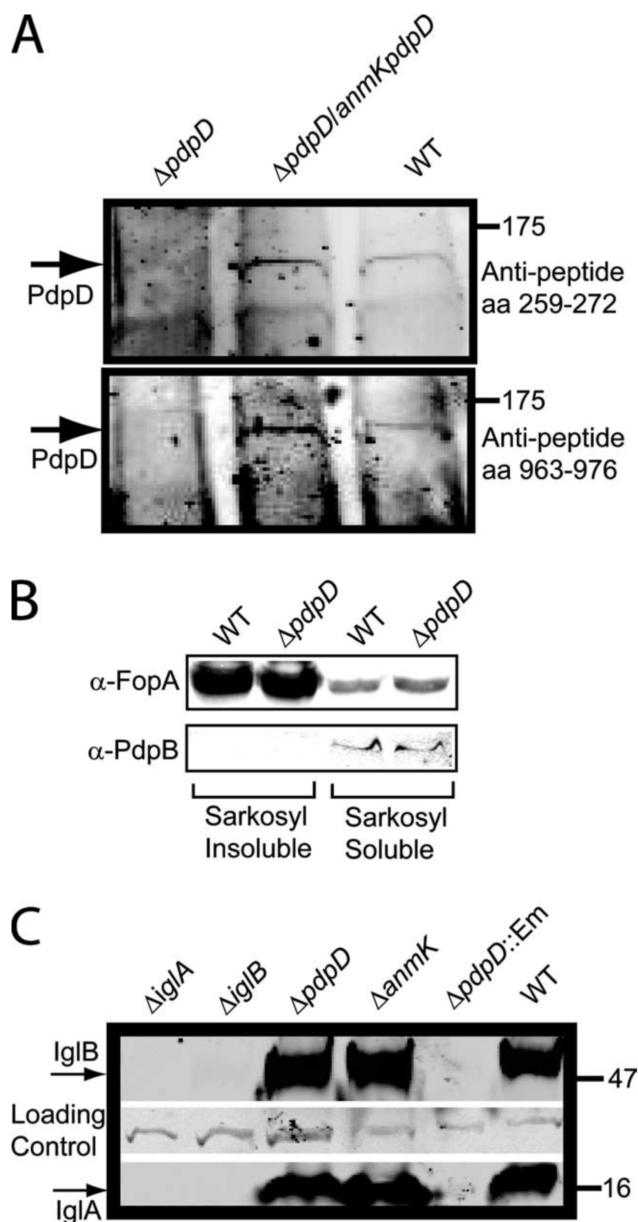

Figure 19: Immunoblot analysis of *pdpD* mutants. (A) Immunoblots developed with anti-PdpD peptide antibodies. Samples were normalized to 15 µg protein per lane prior to separation on a 4-12% gradient gel. **(B)** Sarkosyl solubilization separates markers for the inner and outer membrane. Membrane fractions were separated by sarkosyl solubilization and subjected to Western blotting. All lanes were loaded with 5µg of protein. **(C)** IglA and IglB expression profiles in *F. novicida* mutants. In all blots molecular weight markers are shown on the right. (Note: FopA and PdpB control blots performed by Olle de Bruin)

In order to detect PdpD by antibody reactivity, we generated three antisera. Two antisera were raised against peptide sequences found in PdpD at amino acid positions 259-272 and 963-976 which were predicted to be antigenic by on-line bioinformatic tools (e.g., http://bio.dfci.harvard.edu/Tools/antigenic.pl). A third antiserum was produced against a recombinant fragment (aa 748-996) of PdpD. Although the anti-recombinant and anti-peptide 963-976 sera reacted well with recombinant protein they did not reproducibly react with any protein band in whole cell extracts of wild type *F. novicida* that was absent from a *pdpD* mutant strain. However preparation of sarkosyl insoluble extracts of *F. novicida* apparently enriched PdpD sufficiently to allow its detection with all three antisera (See Figure 19A, and data not shown). As well, over-expression of PdpD occurred in strains carrying the complementation construct, pJL-SKX::*anmKpdpD*, and this complementation allowed enhanced detection of PdpD (See Figure 19A). Using sarkosyl insoluble fractions from a strain over-expressing PdpD allowed us to visualize a ca. 140,000 relative molecular mass protein using all three types of antisera that was absent from the *pdpD* mutant. The identical pattern of reactivity generated by the three antisera, and the absence of the band in the Δ*pdpD* mutant strains, provided confidence that the reactive bands corresponded to PdpD. This is especially important since detection of PdpD was very difficult, even when it was concentrated before Western blotting.

Sarkosyl was shown to separate inner and outer membrane fractions by testing for the presence of the outer membrane protein, FopA, in the sarkosyle

soluble and insoluble fractions (See Figure 19B). Measurement of the fluorescent intensity of the blots indicates that the outer membrane protein FopA is enriched about 15-fold in the sarkosyl insoluble fraction. The inner membrane protein, PdpB was detected only in the sarkosyl soluble fraction. These results indicate that the sarkosyl treatment was generating a valid separation of inner and outer membrane proteins.

The deletion mutations in *anmK* and *pdpD* did not have a detectable effect on the expression of the downstream genes *iglA* or *iglB* (See Figure 19C). The amounts of IglA and IglB expressed in the *pdpD* deletion strain contrasted sharply with the amounts expressed in the gene replacement mutant of *pdpD* (See Figure 19C). The lack of both IglA and IglB in both the Δ*iglA* and Δ*iglB* mutants (first two lanes of Figure 19C) highlights the previous finding that the lack of expression of one protein leads to the apparent degradation of the other (de Bruin *et al*., 2007).

4.3.3 Over-expression of PdpD affects the surface localization of IglA, IglB and IglC and the localization of IglC is dependent on T6SS component homologues

The enrichment of PdpD in the sarkosyl insoluble fraction suggested that it localized to the outer membrane. However, because the antibody reactive bands were so faint, probably due to the small amounts of PdpD that were made, it was difficult to determine the distribution of PdpD in the bacterial cell. To help ascertain if PdpD localized to the surface of *F. novicida* we reacted surface exposed proteins in the PdpD over-expressing strain (carrying pJL-

SKX::*anmKpdpD*) with biotin. After biotin labeling we separated the biotin-labeled proteins using strepavidin binding, and analyzed proteins eluted from the strepavidin by Western blotting. Probing the blots with anti-PdpD antisera failed to yield reactive bands of the appropriate relative molecular mass for PdpD. Since small amounts of IglA were previously shown to be exposed to surface biotinylation we probed our Western blots with anti-IglA; we also reacted the blots with monoclonal antibodies against IglB, IglC and PdpB, that we expected to serve as negative controls (Melillo *et al*., 2006). Although antibody against the inner membrane protein, PdpB, did not detect a protein band on the blot, antibody against IglA, IglB and IglC did react, and reacted more strongly in samples over-expressing PdpD (data not shown, but see below).

IglA and IglB are homologues of proteins that are part of type VI secretion systems (T6SS). There are at least two other FPI genes, *pdpB* and *dotU*, that encode proteins that are homologous to the IcmF and DotU families of proteins that are parts of both type IV and type VI secretion systems. To assess the role of T6SS proteins in the surface localization of IglA, IglB, and IglC, we introduced pJL-SKX::*anmKpdpD* into a number of strains and carried out surface biotinylation of these strains (See Figure 20). Our results showed that increased amounts of IglA and IglB were exposed to surface biotinylation when pJL-SKX::*anmKpdpD* was present regardless of the genetic background (See Figure 20A-B). However, the amount of IglC exposed to biotinylation was dramatically affected by the absence of *pdpB* and *dotU* (See Figure 20A).

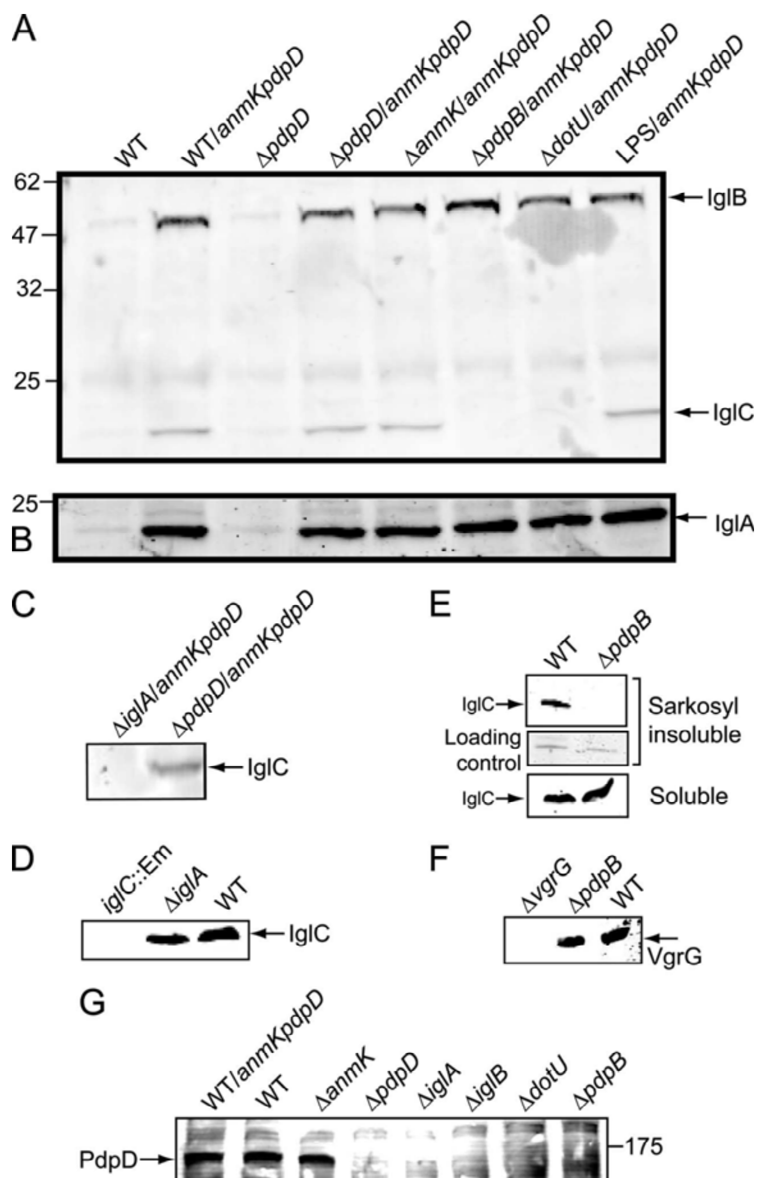

Figure 20: Effect of PdpD over-expression on surface biotinylation of IglA, IglB, and IglC. **(A)** Reactivity of surface-exposed biotinylated proteins with anti-IglB and anti-IglC monoclonal antibodies. **(B)** Reactivity with anti-IglA polyclonal serum. **(C)** Surface biotinylation of IglC in an Δ*iglA* strain. **(D)** Control for Panel C. IglC is found in the cytoplasm in the Δ*iglA* strain. **(E)** IglC fails to localize to the outer membrane in a Δ*pdpB* background. **(F)** The PdpB deletion does not affect expression of the downstream gene, *vgrG*. **(G)** Presence or absence of PdpD in the outer membrane in the *F. novicida* Δ*pdpD*, Δ*iglA*, Δ*iglB*, Δ*dotU*, and Δ*pdpB* strains.

The wild type *F. novicida* showed a small amount of IglC in a biotinylation accessible state, but even this small amount failed to be biotinylated in the *pdpB* and *dotU* backgrounds, even when PdpD was over-expressed (See Figure 20A). Similarly, in an Δ*iglA* or Δ*iglB* background IglC failed to be biotinylated, even when pJL-SKX::*anmKpdpD* was present (See Figure 20C and data not shown) even though IglC was shown to be in the soluble fraction of the wild type and Δ*iglA* strains (See Figure 20D). Biotinylation of IglA, IglB or IglC was identical to the wild type strain in an O-antigen mutant strain of *F. novicida* (See Figure 20A, far right).

Previous work had shown that IglC was primarily localized to the cytoplasm, with a small amount being found in the outer membrane (Golovliov *et al*., 1997). Any amount of this protein found outside of the cytoplasm might be attributed to cross-contamination among cell fractions. To discriminate between this possibility and a true physiological localization of IglC to a site accessible to biotinylation we prepared sarkosyl insoluble fractions from wild type *F. novicida* and from a Δ*pdpB* strain (See Figure 20E). We reasoned that a lack of outer membrane localization of IglC in a strain lacking a canonical component of both type IV and type VI secretion systems would support the interpretation that this experimental approach provided a meaningful localization profile. In the wild type strain but not in the Δ*pdpB* strain IglC was detected in the sarkosyl insoluble pellet, which represents an enrichment of outer membrane components (See Figure 20E). It should be noted that the amount of total protein loaded for the outer membrane fractions (top part of panel E) was about 10-fold higher than the

amount analyzed in the soluble portion of the bacterial extract (bottom part of panel E). Hence the results presented here are consistent with previous studies showing the IglC is localized predominantly to the cytoplasm (Golovliov *et al.*, 1997). To demonstrate that the Δ*pdpB* mutation did not disrupt expression of downstream genes which may encode unidentified components of the T6SS protein homologues we assessed the expression of VgrG which is encoded by a gene downstream of *pdpB*. This analysis (See Figure 20F) showed that VgrG was expressed at wild type levels.

The enhanced localization of IglC to the outer membrane by the over-expression of PdpD, and the dependence of this localization on T6SS homologues suggested that PdpD localization to the outer membrane would be dependent on the T6SS. To test this idea we extracted proteins from the sarkosyl-insoluble (outer membrane) fraction of wild type *F. novicida* and mutant strains with deletions in genes encoding T6SS homologue components and performed Western blots to detect PdpD (See Figure 20G). Since it was difficult to consistently obtain numerous samples of sarkosyl insoluble fractions with clear banding patterns, biotinylation of proteins followed by strepavidin extraction was used to prepare samples of outer membrane proteins. As with IglC, localization of PdpD to the outer membrane required IglA, IglB, DotU and PdpB, but not AnmK. These results are consistent with the growing consensus of the required components of a T6SS. PdpB which contains an IcmF motif together with IglAB may form the foundation of a T6SS, as these three proteins make up the “IcmF associated homologous protein” group which was the bioinformatic basis for

defining the T6SS. DotU homologues are often associated with IcmF-related proteins.

We interpret the localization data on IglA, IglB and IglC as indications that these proteins interact with PdpD. The enhanced exposure of IglA, IglB and IglC to surface biotinylation when PdpD is over-expressed, suggest that there is some form of co-localization of the four proteins. The fact that the enhanced localization of IglC is eliminated in strains with Δ*iglA*, Δ*dotU*, and Δ*pdpB* mutations argues that the altered surface exposure of IglC is not simply due to leakage of IglC through a membrane when PdpD is over-expressed. It is noteworthy that the amount of surface biotinylation of IglC in the Δ*iglA*, Δ*dotU*, and Δ*pdpB* genetic backgrounds appears to be below the level found in the wild type *F. novicida* strain, and, thus, the effect of these mutations is independent of the expression level of PdpD. Although our biotinylation studies suggest protein interactions or co-secretion they do not define the nature of the secretion or the final destination of the IglA, IglB, IglC or PdpD. The IglA, IglB, IglC and PdpD proteins all lack an N-terminal region that corresponds to a signal peptide used by either the Sec or the twin arginine transport (TAT) systems, and hence the transport of these FPI-encoded proteins is presumably not mediated by Sec or TAT processes.

We previously showed that immunoprecipitations of IglA co-precipitated one protein that was identified by MALDI-TOF as IglB (de Bruin *et al*., 2007). We also showed that the absence of IglB by mutation led to the loss of IglA, presumably by protease digestion. IglC was made in the absence of IglA or IglB.

Hence, there is evidence that IglA and IglB interact but there is no evidence that IglC interacts with IglA or IglB.

4.3.4 Intracellular growth of PdpD mutants

We had previously reported that an insertion mutation in *pdpD* reduced the virulence and intramacrophage growth ability of the resultant strain (Nano *et al.*, 2004). We noted that the insertion depressed the expression of the downstream-encoded IglA protein (See also, Figure 19C above) which was later shown to be needed for intracellular growth and virulence (de Bruin *et al.*, 2007). To more accurately assess the role of *pdpD* in intracellular growth we tested one Δ*pdpD* strain for growth in bone marrow derived macrophages (BMDM). We found that Δ*pdpD20d* grew in BMDM identically to the wild type strain (See Figure 21A). The Δ*anmK* strain also showed no defect in intramacrophage growth (data not shown). We also tested the Δ*pdpD* strain in 11 cell lines of different tissue and species origins (See Figure 21B) and found that in each case the Δ*pdpD* strain grew like wild type *F. novicida*. Although mouse macrophages, including the mouse macrophage cell line, J774, are the most commonly used cell types to study *Francisella* intracellular growth our results suggest that other cell lines, such as the human colon epithelial cell line Caco-2 might serve as an equally important host cell for in vitro studies of *Francisella* intracellular growth.

The contrasting intracellular growth phenotypes for the *pdpD* gene replacement mutant and the Δ*pdpD* mutant highlight a phenomenon that we have observed for many mutants with lesions in FPI genes. In several cases we have found that insertion mutations and some small partial in-frame deletion

mutations generate strains with phenotypes that are different from complete markerless deletion mutations. This suggest that small perturbations of FPI gene expression interferes with intracellular growth and virulence, and this is to be expected if all of the FPI genes encode virulence associated proteins.

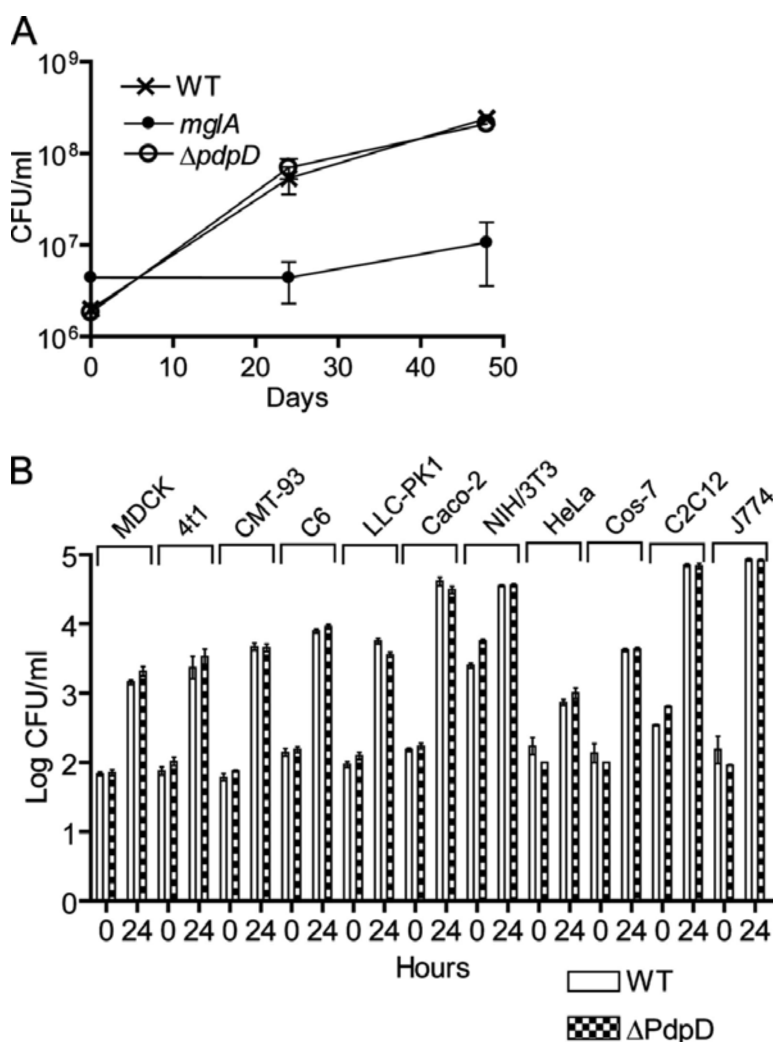

Figure 21: Intracellular growth of Δ*pdpD* mutant. (A) Murine bone marrow derived macrophages were infected at an MOI of 50:1 and viable *F. novicida* were counted at 0, 24 and 48 hours after infection. The *mglA* strain, which is defective for intracellular growth, served as a negative control. **(B)** The growth of a Δ*pdpD* mutant was examined in 11 different cell lines and found to grow as well as wild type *F. novicida*. Error bars represent the standard errors of the means.

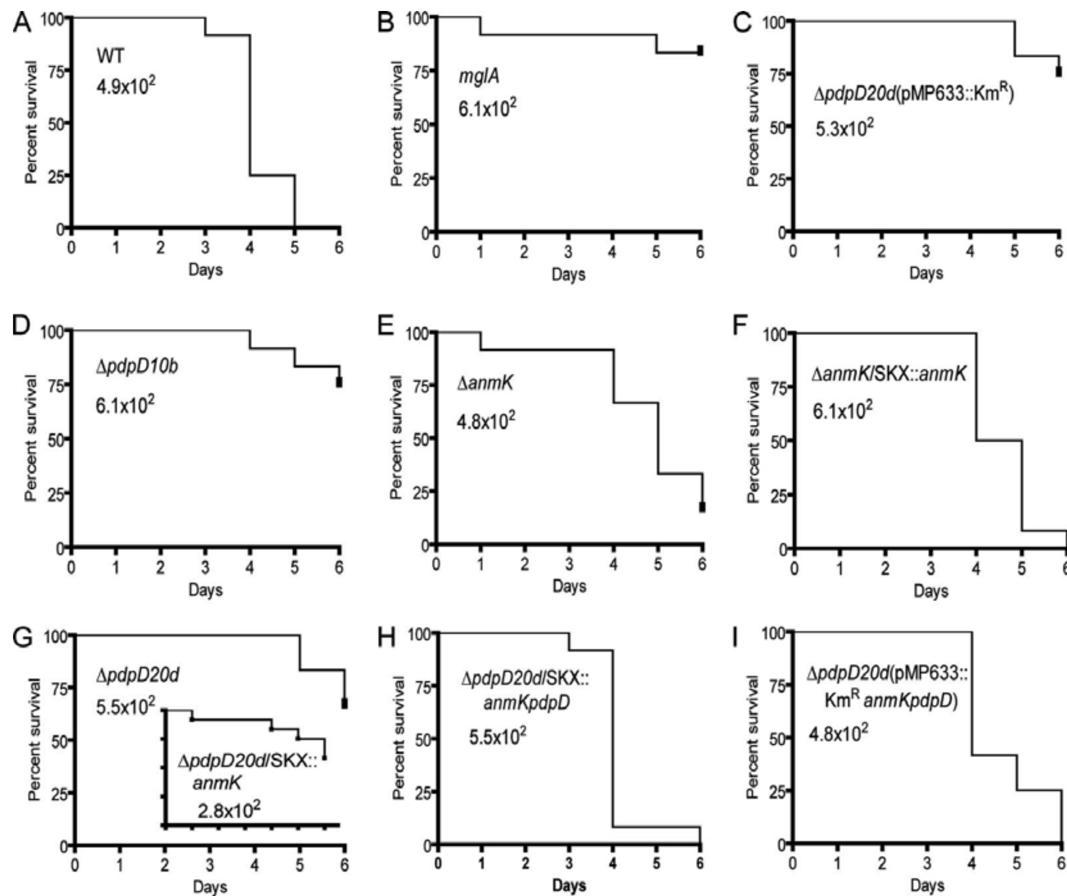

Figure 22: Virulence of Δ*anmK* and Δ*pdpD* mutants in chicken embryos. The time-to-death profile of seven day old chicken embryos is shown for the wild type strain **(A)** and a standard avirulent strain **(B)** of *F. novicida*. The mutant strains Δ*pdpD10b*, Δ*anmK*, and Δ*pdpD20d* are shown in Panels **(D)**, **(E)**, and **(G)** respectively. A mock-complemented strain is shown in **(C)** and genetically complemented strains are shown in Panels **(F)**, **(H)**, and **(I)**. The *F. novicida* strain and the inoculating dose are shown inside of each graph. The P-value for the differences between the wild type strain and all of the mutant strains was less than 0.001. The P-value for the differences between the wild type and all of the complemented strains was greater than 0.05, showing that they are not significantly different; however, in some of the repetitions the Δ*pdpD*/SKX::*anmKpdpD* complemented strain was more virulent at a statistically significant level. All of the *F. novicida* experiments were done with 12 chicken embryos and were repeated three times.

4.3.5 Virulence phenotype of *anmK* and *pdpD* deletion mutants in chicken embryos and in mice

To assess the effects of gene deletions on virulence we used a combination of infections of chicken embryos and mice. To reduce animal suffering we performed the bulk of the infections with chicken embryos and compared these results with smaller experiments using mouse infections. Both the large and small deletions in *pdpD* resulted in strains that were attenuated for virulence in chicken embryos (See Figure 22D and G). Complementation of the genetic defect using the integrating recombinant pJL-SKX::*anmKpdpD* completely restored virulence (See Figure 22H). Similarly, genetic complementation using a plasmid vector restored virulence (See Figure 22I) but a mock complementation with the plasmid without the *anmKpdpD* insert did not (See Figure 22C). Complementation of Δ*pdpD* strain with SKX::*anmK* alone did not restore virulence (insert in Figure 22G). Deletion of *anmK* had a small effect on virulence, and this defect could be reversed by genetic complementation with a wild type copy of *anmK* (See Figure 22E-F). A strain that we use for a universal negative control, GB2 (*mglA*) showed its usual low virulence (See Figure 22B). When *pdpD* was introduced into the *F. tularensis* type B strain LVS, the resulting strain always generated more rapid deaths than the parent strain (data not shown). However, this slight increase in apparent virulence was not statistically significant on a consistent basis.

The pattern of the virulence phenotypes for Δ*pdpD20d* in mouse experiments showed the same pattern as was observed for chicken embryos

(See Figure 23). The time-to-death for the Δ*pdpD*/SKX::*anmKpdpD* strain was slightly shorter than for the wild type *F. novicida* strain, but this difference was statistically significant at a low level of confidence (P=0.04) using an unpaired t test, and thus is of marginal biological significance.

The complementation of the Δ*pdpD* strain with pJL-SKX::*anmKpdpD* restored virulence but complementation with pJL-SKX::*pdpD* failed to restore virulence. In the Δ*pdpD*/SKX::*pdpD* strain both *anmK* and *pdpD* were present in wild type forms, but were separated on the chromosome, with a copy of *anmK* in the FPI and a copy of *pdpD* inserted into pJL-SKX. Presumably, *pdpD* requires the promoter region located in front of *anmK* to express properly.

The biochemical roles of FPI-encoded proteins are unknown, and the biochemical properties of only a few of them have been studied. IglC was the first of the FPI-encoded proteins to be discovered as a protein that is highly induced following *F. tularensis* infection of macrophages (Golovliov *et al*., 1997). Subsequent work has shown that mutants with a deleted or disrupted *iglC* gene fail to grow in macrophages, are deficient for escape from phagosomes, and fail to down-regulate pro-inflammatory response in macrophages (Telepnev *et al*., 2005, Lindgren *et al*., 2004, Golovliov *et al*., 2003). Although these studies provide insights into the cell biology events surrounding *F. tularensis* infection they do not ascribe a biochemical role for IglC. The role of IglC could be direct or it could be through its interactions with one or several other proteins.

There is some published evidence that IglC primarily localizes to the cytoplasm with a small proportion of IglC localizing to the outer membrane, and this is consistent with the data presented in this work (Golovliov *et al*., 2003).

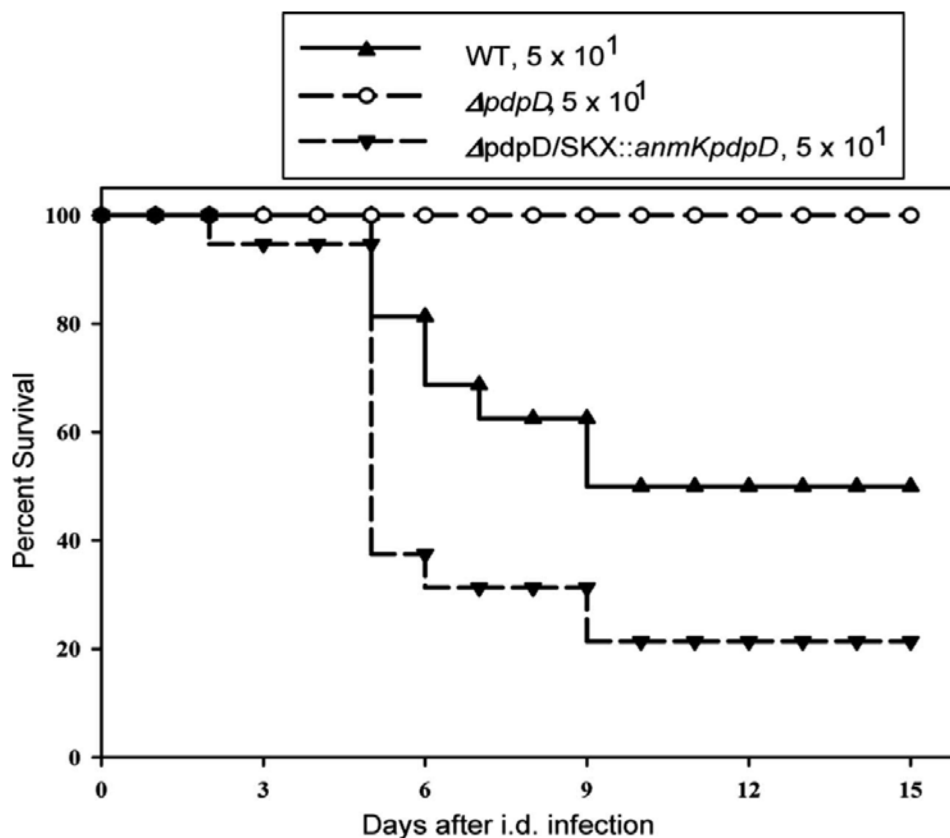

Figure 23: Survival patterns of mice infected intradermally with a low dose of *F. novicida* or *pdpD* mutants of *F. novicida*. BALB/cByJ male mice were infected with 5×10^1 of the indicated bacterial strain intradermally, and monitored for survival. Results are expressed as percentage of surviving mice of the total within each group. Results are shown through day 15 after infection; mice were further monitored through day 30, and no other deaths occurred. Total group sizes were for wild type *F. novicida*, 16 mice; for Δ*pdpD* mutant, 17 mice; and for Δ*pdpD*/SKX::*anmKpdpD*, 14 mice. The P value for the wild type infection survival curve compared to that for the Δ*pdpD* strain is 0.0001 and for the wild type infection survival curve compared to that for the complemented strain is 0.039. (Note: Murine infections performed by Dr. Karen Elkins)

Recently the structure of IglC was determined and found to have limited structural similarity to gp5, a component of the hole-poking device of bacteriophage T4 (Sun *et al*., 2007). Conceivably, IglC could play a role in a secretion channel in the outer membrane of *Francisella*, or, alternatively, it could play a role in forming a channel in a host cell membrane structure. Whatever the role of IglC it seems likely that it interacts with PdpD, and this interaction is linked to events carried out by T6SS homologues encoded by the FPI.

In this work we have shown that the *pdpD* gene is required for full virulence of *F. novicida* but not for intracellular growth. We have provided evidence that the PdpD protein localizes to the outer membrane in a fashion dependent on homologues of the T6SS. Over-expression of PdpD increased the amounts of IglA, IglB and IglC that are exposed to surface biotinylation. Our data suggest that the localization of IglC to a surface biotinylation susceptible site requires IglA, IglB, PdpB and DotU, which are all putative components of the FPI-encoded T6SS. These same proteins are required to localize PdpD to the outer membrane. Hence, there appears to be some form of interaction of PdpD with IglA, IglB and IglC, and the processes that affect the secretion of IglC to the outer membrane appear to be the same that affect the secretion of PdpD.

Chapter 5 Conclusions and Future Studies

In the short period since its discovery, there have been significant advancements in what is known about the *Francisella* pathogenicity island. Depending on the specific strain, the FPI consists of 16-19 genes and is duplicated in the *tularensis* and *holarctica* biotypes. While very little is known about its virulence factors, the FPI represents a new frontier in understanding the pathogenesis of *Francisella*. Furthermore, every FPI mutant has exhibited severe attenuation in murine and avian embryo infection models, demonstrating their importance to the pathogenesis of *Francisella*. One of the greatest challenges facing *Francisella* researchers will be decoding the exact roles of FPI encoded genes with respect to virulence.

Pathogenicity determinant protein D (*pdpD*), a ~135 kDa protein encoded within the *Francisella* pathogenicity island of the highly virulent Schu4 strain, is completely absent in the Live Vaccine strain (LVS), and contains a 144 base pair insert in the *F. novicida* subspecies. It must also be noted that the remainder of the FPI remains intact and that the majority of genomic differences observed amongst *Francisella* subspecies occur with genes that are not classically identified as bacterial virulence factors. PdpD may serve as a bridge between highly virulent Type A *Francisella* strains and their less virulent Type B counterparts. The absence of the PdpD gene in the less virulent Type B strains may partially account for the attenuation seen with the LVS, as well as its inability to successfully infect the respiratory tract and alveolar macrophage cell lines.

Furthermore, two recent independent studies have found that the LVS is ~10000x less virulent than *F. tularensis* subsp. *novicida* in a avian embryo infection model, and that there is a far greater association of human macrophages with *F. novicida* compared to the type B vaccine candidate. While the exact contribution of PdpD to the pathogenesis of Type A strains and *F. novicida* remains to be determined, its association with a highly conserved pathogenicity island implies an important role in *Francisella* virulence.

A mutant generated via allelic exchange of the *pdpD* gene with an *ermC* (erythromycin resistance) cassette renders an attenuated strain (named JL12) incapable of growth in murine bone marrow-derived macrophages (muBMDM). This *pdpD* mutant however, exhibited polarity effects on the neighbouring IglA gene, with the expression of the downstream protein six-fold less in the JL12 mutant relative to the wild type counterpart. In addition, it has been hypothesized that IglA and IglB are necessary components in the secretion of FPI encoded virulence factors, therefore an allelic exchange mutant does not unequivocally demonstrate that *pdpD* is the sole constituent responsible for the loss of pathogenicity. Consequently, to determine if the limited virulence of the allelic exchange mutant is in fact due to the loss of *pdpD* it was necessary to generate a mutant in which the expression of the neighbouring genes has been returned to wild type levels. Through western blot analysis it was shown that the in-frame *pdpD* deletion mutant (∆*pdpD*-20d) does not exhibit polarity effects on its neighboring IglA gene, and any subsequent characteristics of the mutant could reasonably be attributed to the functional loss of the gene.

While we have shown that PdpD localizes to the *Francisella* outer membrane and associates with several homologues of a Type VI secretion system, very little is known about its actual function. PdpD possesses a glycosaminoglycan attachment site as well as an Arginine-Glycine-Aspartate (RGD) cell attachment sequence which have been implicated in the internalization or adhesion of several infectious micro-organisms. Any study of PdpD as a potential adhesin would require extensive mutagenesis of these attachment sites. Furthermore, it will be important to determine the binding the sites of these attachment motifs since bacteria possess several adhesion factors which are utilized to bind various receptors found on an array of host cell lines, and with *Francisella* alone, the mannose receptor, complement receptor 3, Fcγ receptor, surfactant proteins A and D, as well as an assortment of integrins have all been implicated in phagocytic uptake of the bacterium.

The virulence mechanisms of *Francisella* and the exact function of FPI encoded proteins can only be accelerated by the development of consistent genetic protocols and the availability of molecular tools. The polarity effect observed with JL12 mutant reveals the need for clean markerless deletion mutants to examine the exact roles of FPI encoded proteins. In response to this problem, the Nano lab has developed genetic elements for the deletion mutagenesis and complementation of *F. novicida*.

An original *Francisella* transformation protocol developed by Tyeryar and Lawton in 1969 and subsequently modified by Anthony *et al.* was found to be inconsistent and impractical, requiring a complex synthetic medium

(Chamberlain's media) consisting upwards of thirty components. However, simply replacing complex media with trypticase soy broth supplemented with 0.1% cysteine and 0.4% glucose (TSB-CG) has resulted in a simplified and consistent means of transforming *Francisella novicida*. Transformation of *Francisella* with a standardized preparation of plasmid pLA6811, a positive control commonly used in *F.novicida* transformations, generates ~25-fold greater transformants per µg of DNA when using the simplified TSB-CG media compared to the traditional Chamberlain's media.

Complementation of genetically attenuated bacteria generally occurs through the introduction of a stable host plasmid carrying the wild type gene of interest. A recently developed *Francisella* shuttle plasmid named pFNLTP1 demonstrated a high efficiency of transformation and genetic stability without compromising the intracellular growth of *Francisella*, but did not serve well in the complementation of FPI encoded genes. The high copy number of this vector likely results in constitutive expression of a greater number of proteins, ultimately leading to toxicity for the *Francisella* host. Consequently, we developed two integrating complementation vectors (pJL-SKX and pJL-XKS) which result in the introduction of a single copy of a respective gene into the *Francisella* chromosome. Once inserted into the *Francisella* genome, the complementing element is highly stable and does not require selective pressure to ensure maintenance within the bacterium.

While the majority of tools developed so far have been for use in the *novicida* subspecies, there is a need for molecular tools that are transferable

between all *Francisella* strains, especially the virulent biotypes. Limited insight into the genetic basis for the attenuation of the LVS has been the central argument against commercial licensing and large scale usage, and any subsequent live vaccine will require defined mutations within a virulent *Francisella* strain. Using the LVS strain as a foundation for which to generate defined mutations in highly virulent Type A strains will ultimately require genetic elements that can be utilized in all *Francisella* biotypes. This will allow for quicker initial analysis in *F. novicida* under less stringent Level II containment, and the subsequent transfer of genetic elements to highly virulent strains under Level III biosafety containment.

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