Characterization of a *Francisella* pathogenicity island-encoded secretion system

by

Olle Maarten de Bruin
B.Sc., Vancouver Island University, 2003

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in the Department of Biochemistry and Microbiology

Supervisory Committee

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ABSTRACT

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ABSTRACT

Secretion is a fundamental process of bacterial microorganisms. It is responsible for diverse functions such as cell-to-cell communication, nutritional up-take, environmental adaptation, physiological responses, and evasion of the immune system of a host. To accomplish the task of secretion, bacteria have evolved multi-protein complexes, known as secretion apparatuses, which span the bacterial membranes serving as a conduit between the interior of bacteria and the extracellular milieu. *Francisella tularensis* is a Gram negative bacterium capable of growth inside macrophages. *Francisella tularensis* causes a rare but severe disease known as tularemia. The *Francisella* pathogenicity island (FPI) is a circa 30-kb genetic region that harbours genes of unknown function implicated in virulence of this organism. Although many of the FPI-encoded protein

products do not appear to have any known homologues, some of the FPI proteins show similarity to proteins involved in type VI secretion (T6S) of other bacteria. T6S systems are newly described bacterial virulence factors evolutionarily related to bacteriophages. We have tested the hypothesis the FPI encodes a secretion system. The FPI-encoded secretion system secretes a novel protein, IglC, into the extracellular milieu during broth growth. Systematic deletion mutagenesis determined the contribution of individual FPI genes to intramacrophage growth and secretion. We further characterized the secretion system by determining the subcellular localization of each FPI protein in the bacterial cell. An interaction between two inner membrane proteins, PdpB and DotU, was observed by co-immunoprecipitation, and the stability of PdpB requires DotU. Similarly, an interaction of IglA and IglB was demonstrated. Biochemical and fluorescence microscopy evidence suggest IglC is secreted into macrophages during intracellular localization of bacteria. Finally, a model of the FPI-encoded secretion system is presented. Our experiments provide biochemical, genetic and microscopy evidence that the FPI encodes a secretion system. The analysis of FPI-encoded secretion provides novel insights that may help us understand the role of FPI-encoded secretion in *Francisella* intracellular growth and virulence.

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have two amazing parents who I cannot speak highly enough of. I like to give many hugs and kisses forever to my children Jefferson and Svea. Finally, to the rest of my family, tack.

List of Abbreviations

aa	Amino acids
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
BLAST	Basic Local Alignment and Search Tool
BLASTP	Basic Local Alignment and Search Tool Protein
BMDM	bone marrow derived macrophages
bp	Base pair
cfu	Colony forming units
CDC	Center for disease control
COG	Conserved orthologous group
CR	Complement receptor
CU	Chaperone usher
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's Phosphate Buffered Saline
DR	Direct repeats
DUF	Domains of unknown function
EAEC	Enteroaggregative *Escherichia coli*
EEA1	Early endosomal antigen 1
Em	Erythromycin
Em^R	Erythromycin resistance
ER	Endoplasmic reticulum
ESAT-6	6 kDa early secretory antigenic target
ESX	early secretory antigenic target 6 system
FPI	*Francisella* pathogenicity island
GAP	GTPase activating protein
GC	Guanine + Cytosine
GEF	GTPase exchange factor
Hcp	Haemolysin co-regulated protein
HMM	Hidden Markov model
hr	Hour
Igl	Intracellular growth locus
IL-β	Interleukin-beta
iNOS	Inducible nitric oxide synthase
IAHPs	IcmF-associated homologous proteins
kb	Kilobase
kDa	Kilodalton
kg	Kilogram
Km	Kanamycin
Km^R	Kanamycin resistance
LAMP	Lysosome-associated membrane protein

LLO	Listeriolysin
LPS	Lipopolysaccharide
LVS	Live vaccine strain
M6PR	Mannose-6-phosphate receptor
MALDI-TOF	Matrix assisted laser desorption ionization-time of flight
Mb	Megabase
MDC	Monodansylcadaverine
µg	Microgram
µl	Microlitre
ml	Millilitre
mM	Millimolar
MOI	Multiplicity of infection
MW	Molecular weight
NADPH	nicotinamide adenine dinucleotide phosphate
NK	Natural killer
nm	Nanometre
NO	Nitric oxode
PI3K	Phosphoinositide 3-kinase
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
Pdp	Pathogenicity determinant protein
PI	Pathogenicity Island
PI(3)P	Phosphatidylinositol-3-phosphate
ROS	Reactive oxygen species
RNA	Ribonucleic acid
RNAP	RNA polymerase
RNS	Reactive nitrogen species
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SS	Secretion system
T1SS	Type I secretion system
T2SS	Type II secretion system
T3SS	Type III secretion system
T4P	Type VI pilin
T4SS	Type IV secretion system
T5SS	Type V secretion system
T6SS	Type VI secretion system
T7SS	Type VII secretion system
T8SS	Type VIII secretion system
T9SS	Type IX secretion system
TAT	Twin-arginine translocation
TSAC	Trypticase soy agar supplemented with 0.1% cysteine
TSBC	Trypticase soy broth supplemented with 0.1% cysteine
U112	Utah 112
WII	World War 2
WT	Wild type
Å	Ångstroms

Chapter 1. Introduction

1.1 *Francisella tularensis* - an intracellular pathogen causing tularemia

In 1911 an outbreak of a plague-like disease mainly affecting ground squirrels occurred in Tulare County, California, USA, prompting investigators to name the disease tularemia (Keim, Johansson et al. 2007; Sjostedt 2007). This zoonotic disease rarely affects humans, but when it does it is severely debilitating even causing death. The bacterium causing tularemia was isolated and named *Bacterium tularensis,* now *Francisella tularensis* in honor of the pioneering bacteriologist Dr. Edward Francis, who dedicated much of his time to study this pathogen (Keim, Johansson et al. 2007; Sjostedt 2007). *F. tularensis* is a Gram negative facultative intracellular gamma proteobacterium capable of infection and multiplication in immune cells, in particular macrophages, neutrophils, and dendritic cells (Anthony, Burke et al. 1991).

Taxonomically, three subspecies of *F. tularensis* are currently recognized, namely *tularensis, holarctica*, and *mediasiatica* (Keim, Johansson et al. 2007). Additionally, *F. philamoragia* and *F. novicida* are very closely related species (Oyston 2008). There are no human pathogens closely related to *F. tularensis*; however, genome sequencing and phenotypic studies suggest *Coxiella burnetii* and *Legionella pneumpohila* are the closest yet distantly related human pathogenic relatives of *F. tularensis* (Titball, Johansson et al. 2003; Keim, Johansson et al. 2007). Recent studies have identified a number of soil bacteria, fish pathogens and tick endosymbionts as closely related to *F. tularensis* (Ostland, Stannard et al. 2006; Keim, Johansson et al. 2007). It is likely these organisms will be included in *Francisellae* in the near future (Sjostedt 2007).

1.1.1 Disease and treatment

The subspecies *tularensis* and *holarctica* are capable of clinically important infection of varying severity depending on route and dose of infection with *tularensis* able to manifest fulminating tularemia potentially causing fatalities, whereas *holarctica* infection results in similar but less severe disease representations (Tarnvik and Chu 2007). Human infection by subspecies *mediasiatica* is not currently documented, but it is known this bacterium can infect and cause death of wild life; additionally, laboratory studies indicate the disease caused by this bacterium is comparable to subspecies *holarctica* in infectivity (Sjostedt, 2007). Both *F. novicida* and *F. philamoragia* have been known to cause tularemia in immunocomprimsed individuals, and *F. philamoragia* is associated with death of near-drowning victims (Keim, Johansson et al. 2007; Sjostedt, 2007).

Tularemia can be difficult to diagnose given its general flu-like symptoms, malaise, fever and chills, parallel those of several other bacterial infections (Oyston, Sjostedt et al. 2004; Matyas, Nieder et al. 2007). This represents a problem not only because the disease could become protracted and relapsing, but also because tularemia can be fatal (Ellis, Oyston et al. 2002). The route of infection influences distinct disease manifestations (Oyston, Sjostedt et al. 2004; Matyas, Nieder et al. 2007). Infection through the skin by mosquito bites or entry of the bacterium into wounds results in ulceroglandular tularemia, which is the most common form of the disease, whereas inhalation of the bacterium causes respiratory tularemia, the most life-threatening form of the disease (Oyston 2008). Other forms of tularemia associated with infection through

the eye or by consumption of contaminated water have been reported to occur, but are rare (Helvaci, Gedikoglu et al. 2000; Tarnvik and Chu 2007).

Ulceroglandular is the most common form of tularemia, and is associated with arthropod bites and contact with infected animals (Ohara, Sato et al. 1991; Oyston, Sjostedt et al. 2004). In this disease presentation, a lesion occurs at the site of infection, which develops into an ulcer or papule surrounded by a zone of inflammation (Anda, Segura del Pozo et al. 2001; Oyston, Sjostedt et al. 2004). Although the ulcer heals leaving a light red scar, fever and enlargement of a draining lymph node can occur within a few days (Evans, Gregory et al. 1985; Tarnvik and Chu 2007). If left untreated for over two weeks, suppuration of the lymph node is a distinct possibility resulting in serious complications (Helvaci, Gedikoglu et al. 2000). Oculoglandular tularemia occurs after infection of the eye, and is a rare but unpleasant form of tularemia resulting in eye lid swelling (Evans, Gregory et al. 1985). Gastrointestinal tularemia has been reported to affect individuals after incidences of consumption of contaminated water. Depending on the size of the infectious dose, the disease ranges from persistent diarrhea to development of bowel ulcers, which can be fatal (Luotonen, Syrjala et al. 1986). The most serious form of tularemia is called respiratory or pneumonic tularemia, which normally occurs after inhalation of the bacterium. Pneumonic tularemia can also develop after spread of the bacterium from an initial site of infection to the lungs (Tarnvik and Chu, 2007). Clinical presentations include high fever, delirium, vomiting and nausea, but are variable and, therefore, the disease is difficult to diagnose (Oyston, Sjostedt et al. 2004). This is problematic given inhalation of as few as 10 bacteria of subspecies *tularensis* is often fatal if untreated. In contrast, although inhalation of subspecies *holarctica* results in

severe disease, it is seldom fatal (Dahlstrand, Ringertz et al. 1971; Dennis, Inglesby et al. 2001). Person-to-person transmission of the disease has never been reported (Sjostedt, 2007).

Tularemia can be resolved by antibiotic treatment. The bacterium is resistant to penicillin and its derivatives, and wide-spread natural erythromycin resistance is documented (Ikaheimo, Syrjala et al. 2000). The drug of choice for treatment is currently the aminoglycoside gentamicin (Tarnvik and Chu, 2007). Recent data indicate the tetracycline doxycycline and the quinolone ciprofloxacin are useful as alternative antibiotics especially for treatment of milder forms of the disease (Ikaheimo, Syrjala et al. 2000).

A live-vaccine strain (LVS) derived from subspecies *holarctica* affording good protection against infection with *F. tularensis* has been developed (Saslaw and Carlisle 1961). However, LVS protection against respiratory infection with *F. tularensis* is incomplete (McCrumb 1961). Additionally, the nature of the attenuation of this strain is undefined, and there have been questions regarding the safety of using the vaccine; therefore, it is currently not in use (Oyston, Sjostedt et al. 2004). Albeit not as virulent as its parental strain, LVS is still capable of causing disease in mice, and has been used extensively as a model organism in various research programs. Recently, comparative genomics have helped identify genes mutated in LVS (Barabote, Xie et al. 2009). Re-introduction of two of the deleted genes, one a type IV pilin gene, the other an outer membrane protein, generated a strain, which is as virulent in mice as subspecies *holarctica* (Salomonsson, Kuoppa et al. 2009). Definition of the virulence defect of LVS

may allow its use as a *Francisella* vaccine. Attempts to generate epitope-based subunit and killed vaccines have been unsatisfactory to date (Gregory, Mott et al. 2009).

1.1.2 Bioweapons threat

F. tularensis is one of the most infectious bacterial species known to man (Saslaw and Carlisle 1961). The low dose of infectivity and severity of respiratory tularemia has lead to *F. tularensis* being classified a category A agent by the United States Center for Disease Control and Prevention meaning the bacterium is thought to be one of the most likely to be used in a bioweapon attack (Dennis, Inglesby et al. 2001). It is well-documented *F. tularensis* has been part of biowarefare programs of a number of countries (Dennis, Inglesby et al. 2001). There have been claims *F. tularensis* was used as a bioweapon during World War II (WWII), but these have not been confirmed (Oyston, Sjostedt et al. 2004; Alibek 1999). Indeed, tularemia is a disease of war times; however, there are several alternative explanations to this. For example, poor sanitary conditions and a documented increase in rodent populations carrying the disease during the 1990s war in former Yugoslavia and during WWII years in Russia are likely contributing factors to tularemia outbreaks (Sjostedt, 2007).

1.1.3 Epidemiology

Tularemia is considered a disease of the Northern hemisphere, affecting Asia, North American and Northern and Central parts of Europe (Keim, Johansson et al. 2007; Oyston, Sjostedt et al. 2004). Outbreaks of tularemia in humans appear to coincide with fatal *F. tularensis* disease in animal populations (Oyston, 2008). Although the true

reservoir and infectious cycle of this bacterium is unknown, it is becoming apparent *F. tularensis* is associated with aquatic environments (Sjostedt, 2007). For example, semi-aquatic animals such as beavers and muskrats are known to become infected; and human infection after consumption of contaminated water has been document on several occasions (Sjostedt, 2007; Anda, Segura del Pozo et al. 2001). Additionally, *F. tularensis* bacteria are capable of infection of amoeba offering a rational explanation for the association with water (Abd, Johansson et al. 2003; Titball, Johansson et al. 2003).

A risk factor of contracting tularemia is being fed upon by mosquitoes (Keim, Johansson et al. 2007). Given mosquitoes are likely to disseminate the disease, it has been suggested mosquito larvae hatched in watersheds become infected thereby turning mosquitoes into vectors of the disease. Additional factors may be involved in the *Francisella* cycle of parasitism. For example, ticks are important vectors of the disease, and animals important to the spread of Tularemia also include non-aquatic mammals such as hares, rodents and deer (Keim, Johansson et al. 2007). Mosquitoes and ticks harboring the bacterium could infect these animals by feeding upon them. Alternatively, it is possible animals become carriers after consuming contaminated water. Mosquitoes and ticks could then spread the disease after feeding on infected animals (Sjosted, 2007; Keim, Johansson et al. 2007; Oyston, Sjostedt et al. 2004).

To date, the bacterium has been isolated from over 200 different animal species (Oyston, Sjostedt et al. 2004). In spite of this apparent ubiquity, outbreaks of the disease occur in localized foci. The reason for this phenomenon is currently unknown, but appears linked to changes in poorly defined environmental factors (Sjostedt, 2007). It is documented import of infected animals are causes of localized outbreak foci. Two well-

known examples of such events occurred on Martha's Vineyard, an island outside the coast of Massachusetts, USA, and in the Czech Republic, Europe (Sjostedt, 2007). Import of hares from Western USA for hunting purposes to the elite community of Martha's Vineyard are likely factors giving rise to this isolated focus of tularemia in Eastern USA. In modern times, landscapers, often illegal immigrants, are the victim of tularemia in this location, especially since they lack adequate health insurance (Matyas, Nieder et al. 2007). Similarly, import of hares to Spain has contributed to the spread of the disease in Southwestern Europe (Perez-Castrillon, Bachiller-Luque et al. 2001). Additionally, in the Czech Republic, unexpected isolates of *F. tularensis* have been identified. It is probable import of prairie dogs from Western USA carrying the disease have contributed to the emergence of tularemia in this isolated locale, which may then have spread to other parts of Central Europe (Oyston, Sjostedt et al. 2004). An interesting locus of tularemia outbreaks is found in Central Sweden, in the town of Ljusdal (Tarnvik, Sandstrom et al. 1996). The reason for the re-occurring incidences of the disease here is not known; however, certain ecological and environmental conditions may be contributing factors. In the same country, recent outbreaks of tularemia have been unexpectedly reported in the city of Örebro, where a swamp, i.e. the breading ground of mosquitoes, was converted into a park area attracting immunocompromised elderly and children, who subsequently contracted the disease (Eliasson, Broman et al. 2006).

Changes in human behavior appear to have influenced not only the emergence but also the decline of tularemia in geographical areas. Traditionally, farmers and hunters were at risk of contracting the disease (Syrjala, Kujala et al. 1985). For example,

tularemia has been endemic in the former Soviet Union, but since the 1990s reported cases have decreased significantly likely due to changes in farming practices (Sjostedt, 2007). Decrease in hunting in the Rocky mountain region of Western USA may have contributed to the decrease in incidence of tularemia in this area, where it was once considered endemic (Sjostedt, 2007). Although *F. tularensis* causes a rare disease, the severity of its manifestations combined with the observation the intracellular life style of *Francisella* appears unique among microbes, much is to be learned about host-pathogen interactions from studying this intracellular bacterium.

1. 2 Host defenses and survival mechanisms of intracellular bacteria

Successful colonization of a host by a pathogen requires evasion of the immune system. The constant interaction of microbes with the immune system constitutes an evolutionary tug-of-war shaping bacterial virulence traits and contributing to defense mechanisms of immune cells (Bhavsar, Guttman et al. 2007). Professional phagocytotic leukocytes, such as neutrophils and macrophages, are well-equipped to deal with invading microbes (Haas 2007). Not surprisingly, bacterial pathogens have developed sophisticated means to subvert the killing action of leukocytes. Some bacteria are able to avoid phagocytosis by impairing the phagocytic machinery or scavenging opsonizing antibodies required for uptake; others have evolved means of surviving inside the host cell and are considered intracellular bacteria (Marques, Kasper et al. 1992; Forsberg, Rosqvist et al. 1994). Some intracellular pathogens force entry into non-phagocytotic cells by inducing cytoskeletal rearrangements (Cossart and Sansonetti 2004). Yet others specialize in surviving inside phagocytotic cells; these bacteria are sometimes referred to

as professional intracellular bacteria (Ray, Marteyn et al. 2009). In the following section, strategies of immune system avoidance by intracellular bacteria, which survive and prosper inside phagocytotic cells are reviewed. To better understand these strategies, the anti-microbial capacities of macrophages and neutrophils are first introduced.

1.2.1 Phagosomal maturation and microbicidal mechanisms

The innate immune system is a cornerstone of pathogen eradication. Central players of the innate immune system include macrophages, dendritic cells and neutrophils, which are capable of engulfing microbial organism by process of phagocytosis (Steinman 1991). Microbes trapped in a phagosome are subjected to a harsh treatment of microbicidal features, which normally leads to destruction of the internalized invader (Haas, 2007). Degradation of microbes in phagocytotic cells leads to antigen presentation and activation of an adaptive immune response, highlighting the interaction of the innate and adaptive immune response in clearance of infection (Yu and Finlay 2008). To develop into the ultimate microbicidal organelle, the phagosome undergoes a process of maturation gradually gaining and losing membrane proteins controlling fusion and fission events (Desjardins, Huber et al. 1994). These membrane proteins are effectors of phagosomal biogenesis ensuring controlled and highly orchestrated delivery of vesicles containing anti-microbial enzymes to the phagosome.

The protein content of the phagosome and its membrane can be used to distinguish between three general stages of phagosomal maturation: early phagosomal, late phagosomal and the phagolysosomal stage (Desjardins, Huber et al. 1994). The early phagosomal membrane is endowed with the small GTPase Rab5, which controls initial

vesicular fusion events to the phagosome (Bucci, Parton et al. 1992). Rab5 recruits a PI(3)P producing complex known as p150-hVPS34 to the phagosomal membrane. Subsequently, PI3P anchors another vesicle-controlling protein EEA1 to the cytosolic membrane of the phagosome (Gaullier, Simonsen et al. 1998). Meanwhile, membrane-spanning ATPases translocate H^+ into the phagosome to create a milieu impeding microbial growth by favoring activities of hydrolytic enzymes and generating reactive oxygen species. At this early stage, the pH of the phagosome is mildly acidic (6.1-6.5) owing to membrane-integration of only a limited number of ATPase pumps (Beyenbach and Wieczorek 2006).

As the phagosome matures, the number of ATPases associated with its membrane has been observed to increase (Desjardins, Huber et al. 1994). Consequently, at the late phagosomal stage, the interior of the phagosome is more acidic (pH 5.5-6.0) (Desjardins, Huber et al. 1994). At this stage, vesicular fusion events have lead to the accumulation of proteases and LAMPs to the phagosome (Desjardins, Huber et al. 1994). The trafficking of the late phagosome is controlled by the GTPase Rab7, which is a distinguishing marker of this stage (Bucci, Parton et al. 1992). Rab7 ensures fusion with lysosomes resulting in complete maturation of this cellular compartment to a phagolysosome. Distinguishing features of this degradative organelle include an increased concentration of the protease cathepsin and a lack of the mannose-6-phosphate receptor present at an earlier stage (Griffiths, Hoflack et al. 1988). The phagolysosome is highly acidic (pH 4.5), which contributes to generation of reactive oxygen species (ROS). Furthermore, ROS are created as a result of transport of electrons to oxygen by the membrane-associated NADPH oxidase complex into the phagosome (Haas, 2007). Simultaneously,

microbicidal nitric oxide diffuses across the membrane into the phagosome, where it is converted to a range of reactive nitrogen species (RNS), which target bacterial components. For example, RNS and ROS target thiols, nucleic acids and lipids resulting in bacterial protein inactivation, lipid and DNA damage (Kehrer 2000). In addition, phagosomes contain antimicrobial enzymes such as proteases and hydrolases, which interfere with microbial functions and destroy bacterial structures (Pillay, Elliott et al. 2002). To further inhibit bacterial proliferation, lactoferrins present in the phagosome sequester iron required for growth of several bacteria (Masson, Heremans et al. 1969). Altogether, the arsenal of antimicrobial factors of the phagosome is able to eliminate the majority of bacteria encountered.

1.2.2 Microbes refractory to phagosomal elimination

Despite the formidable regiment of microbial killing mechanisms of the phagosome, some bacteria are able to survive and even prosper after phagocytosis by an immune cell. The survival mechanisms of intracellular bacteria can be divided into three major strategies: i) manipulation of phagosomal maturation and survival within a modified vacuole, ii) resistance to the harsh environment in the phagosome, and iii) escape from the phagosome into the cytosol of the host cell. Bacteria capable of manipulating phagosomal maturation include *Legionella pneumophila, Salmonella enterica* and *Mycobacterium tuberculosis*, whereas *Coxiella burnetii* is capable of survival in a phagolysosome (Horwitz and Silverstein 1980; Baca, Li et al. 1994; Ochman, Soncini et al. 1996; Pethe, Swenson et al. 2004). Pathogens able to avoid phagosomal killing by entering the host cytosol include *Listeria monocytogenes,*

Francisella tularensis and *Shigella flexneri* (Sansonetti, Ryter et al. 1986; Cossart 1998; Golovliov, Baranov et al. 2003). In this section, examples of intracellular survival strategies and bacterial modulation of the default endosomal maturation process are briefly introduced. A schematic overview of different phagosomal evasion strategies of intracellular bacteria is shown in figure 1.

1.2.3 *Mycobacterium tuberculosis*

Mycobacterium tuberculosis is one of the world's most prevalent pathogens infecting one third of humans and causing 2 million deaths per year (Dye, Bassili et al. 2008). After phagocytosis, the bacterium arrests phagosomal maturation at an early endosome-like stage to reside in a *Mycobacterium*-containing vacuole lacking Rab5 effectors required for further maturation (Fratti, Backer et al. 2001; Deretic, Singh et al. 2006). The arrest at this early stage is in part mediated by bacterial imitators of phosphatidyl inositol, which inhibit PI3K activity (Philips 2008). The *Mycobacterial* phosphatase SapM also prevents further maturation by hydrolizing PI(3)P of early phagosomes (Vergne, Chua et al. 2005). Additionally, a *Mycobacterial* lipid, LIM, is shed from the cell wall and dispersed throughout the endocytic network thereby preventing Ca^{2+} influx required for hVPS34 activation by calmodulin (Beatty and Russell 2000). Despite arrest in phagosomal maturation, recent studies have shown this pathogen is able to replicate in the cytosol of macrophages and dendritic cells (van der Wel, Hava et al. 2007). Both arrest of phagosomal maturation and escape into the host cytosol are dependent on a secretion system called ESX (MacGurn and Cox 2007 (MacGurn and Cox 2007; Smith, Manoranjan et al. 2008). Evidence exists one substrate of this

secretion system, ESAT-6, can form pores in host cell membranes suggesting a possible mechanism for phagosomal exit (Smith, Manoranjan et al. 2008).

1.2.4 *Legionella pneumophila*

Legionella pneumophila is a Gram negative, facultative intracellular bacterium that is capable of growth in phagocytic cells, including amoebae and alveolar macrophages. This bacterium is the causative agent of Legionnaires' disease, a pneumonic disease, which can be contracted after inhalation of contaminated water-droplets (Horwitz and Silverstein 1980). Legionnaires' disease generally only affects immunocompromised individuals, and failure to initiate treatment can be fatal. The combination of ageing water pipes in living quarters infested with bacteria and an elderly population has lead to an increase in number of reported cases world-wide (Patterson, Hay et al. 1997).

The intracellular survival strategy of *L. pneumophila* relies on a type VIB secretion system (see section 1.4.4), which translocates a large number of protein effector molecules altering host cell responses, including phagosomal maturation, vesicular trafficking and cell death (Banga, Gao et al. 2007; Pan, Luhrmann et al. 2008). Soon after phagocytosis, *L. pneumophila* avoids fusion with early- and late endosomal compartments to part from the default phagosomal maturation pathway (Swanson and Isberg, 1995). Instead, within 15 min post uptake, the bacterium-containing vacuole has acquired markers of the ER (Kagan and Roy 2002). The mechanism and identity of type IV secretion system effectors controlling host cell GTPases responsible for vesicular traffic between the ER and Golgi have been determined. For example, the GTPase Rab1

is modulated by *Legionella* proteins SidM (also known as DrrA) and LepB, which function as Rab1 effectors possessing GEF and GAP activity, respectively (Ingmundson, Delprato et al. 2007). The result of this control of vesicular trafficking is an ER-derived vacuole supporting bacterial replication.

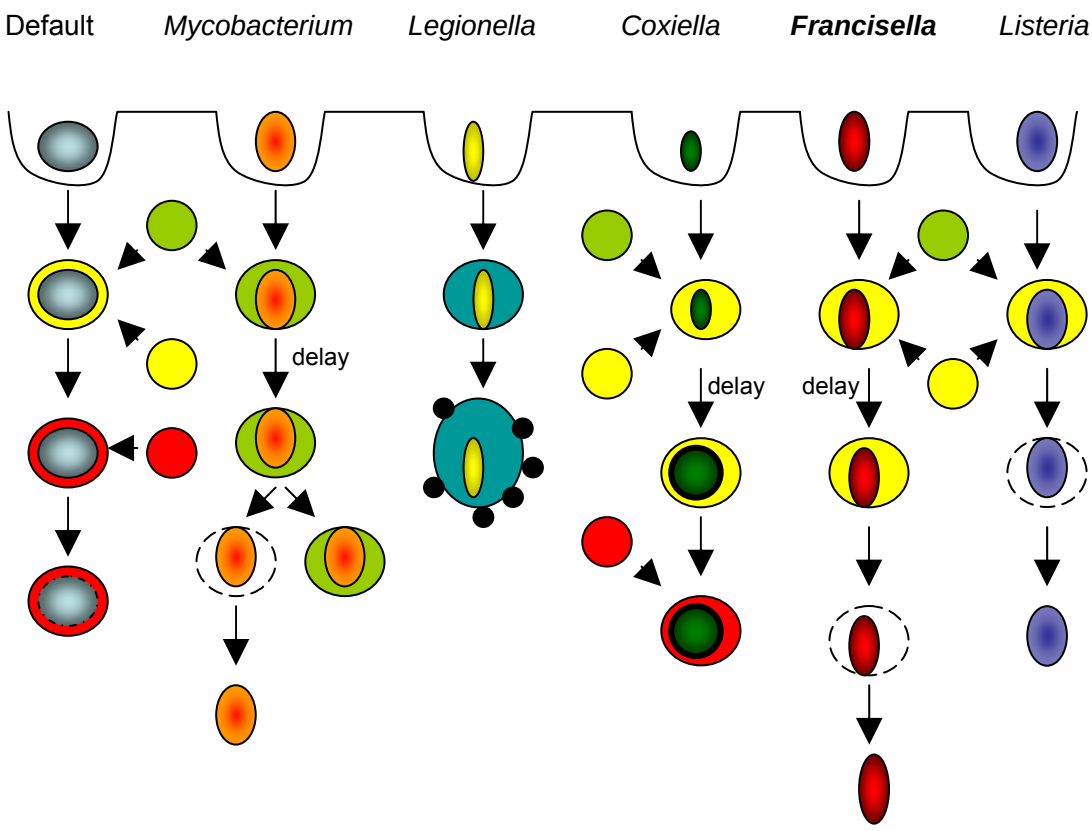

Figure 1. Evasion of phagosomal killing by intracellular bacteria. After entery into host cells via the phagocytic pathway, intracellular pathogenic bacteria are able to avoid killing by altering the default phagosomal pathway normally leading to phago-lysosomal fusion (default, far left) using a variety of strategies. See text for details. Green, indicates an early phagosomal stage; yellow, a late phagosomal stage; red, indicates a fully mature phagosome fused with lysosomes. Black circles indicate ribosomes. Delay, compared to default maturation these bacteria are able to delay phagosomal maturation at the indicated stage. Figure adopated from several sources including Santic et al. 2006, and Kumar et al. 2009.

1.2.5 *Coxiella burnetii*

Coxiella burnetii, the causative agent of Q fever, is a Gram negative obligate intracellular bacterium with a biphasic life cycle and is one of the most infectious organisms known to mankind (Voth and Heinzen 2007). After uptake by a phagocytic cell, the bacterium alters default endosomal maturation by recruiting the autophagic marker LC3 to the *Coxiella* containing vacuole thereby delaying phagosome-lysosome fusion (Gutierrez, Vazquez et al. 2005). The delay is thought to allow the bacterium to transit to a large-cell variant resistant to the killing action of the phagolysosome (Voth and Heinzen 2007). The large-cell variant resides in an acidified vacuole endowed with lysosomal proteins and ATPase pumps, where the bacterium replicates (Heinzen, Scidmore et al. 1996). Although it is clear *Coxiella* is well-adapted to life in a phagolysosome-like compartment, many of the bacterial mechanisms involved in survival in this destructive compartment are yet to be determined since the bacterium is refractory to genetic manipulation; however, a type VIB secretion system of *Coxiella* was recently shown to translocate eukaryotic-like effector proteins into host cells, likely manipulating cellular responses (Voth, Howe et al. 2009).

1.2.6 *Listeria monocytogenes*

Listeria monocytogenes is the causative agent of listeriosis, an acute intestinal tract infection normally contracted after ingestion of contaminated foods (Hamon, Bierne et al. 2006). This Gram positive bacterium rapidly escapes from the phagosome into the cytosol by degrading the membrane of the nascent phagosome. Escape is mediated by LLO, a secreted enzyme which inserts into the phagosomal membrane by binding

cholesterol (Bielecki, Youngman et al. 1990; Beauregard, Lee et al. 1997). A second bacterial enzyme PI-PLC is also needed for phagosomal escape (Camilli, Tilney et al. 1993). LLO insertion causes pores leading to vacuolar disruption and escape within 20 min of phagocytosis (Beauregard, Lee et al. 1997). Pore-formation also serves to delay phagosomal maturation due to loss of ion gradient across the membrane, thereby preventing phagosomal maturation and fusion with lysosomes (Shaughnessy, Hoppe et al. 2006). LLO activity is controlled at several levels; for example, acidification of the phagosome (pH 5.5) is required for escape as LLO is activated by acidic conditions (Beauregard, Lee et al. 1997). Additionally, a host factor found in the phagosome, GILT, is essential for activation of LLO (Singh, Jamieson et al. 2008). After escape, *Listeria* uses actin-based motility to dodge cytosolic defense factors, and to spread to neighboring cells (Lambrechts, Gevaert et al. 2008). Late during intracellular infection, this bacterium has been observed to trigger an autophagic response, which normally results in degradation of invading bacteria. However, through the action of LLO, *Listeria* is able to inhibit the maturation of the autophagosome to reside in a non-degradative double membranous vacuoles (Birmingham, Canadien et al. 2007).

1.2.7 Cytosolic immune responses of immune cells

Given the phagolysosome is highly bacteriocidal it is easy to assume it is in the best interest of intracellular bacteria to escape from the inhospitable milieu of the phagosome to the cytosol. However, bacteria in the cytosol are not guaranteed safety inside a host cell. Indeed, macrophages have evolved a variety of defense mechanisms against phagosomal escapees. One cytosolic antimicrobial defense mechanism employed

by macrophages is assembly and activation of a protein complex known as the inflammasome. Activation of this multi-protein complex leads to rapid host cell death, thus eliminating the intracellular niche of the parasitizing bacteria (Fink and Cookson 2006).

To activate the inflammasome, sensory molecules of the Nalp family of proteins or Ipaf proteins must first recognize bacterial factors such as components of the cell wall, flagella or DNA. Nalp or Ipaf subsequently interact with caspase-1 to form an active inflammasome complex, which causes caspase-1-mediated pore-formation in the macrophage plasma membrane leading to osmotic cell death (Fink and Cookson 2006). An additional consequence of inflammasome activation is caspase-1-triggered processing of pre-IL-β in to its mature form resulting in IL-β release and pro-inflammatory cellular responses (Henry and Monack 2007). Some bacteria are able to inhibit inflammasome function by targeting IL-β pre-cursor processing. For example, *Mycobacterium tuberculosis* is capable of inflammasome inhibition through the action of ZmpA, a predicted metalloprotease, which prevents IL-β maturation (Master, Rampini et al. 2008). Description of the inflammasome is in its infancy and the full extent of its contribution to cytosolic defenses has not been deciphered. It is clear additional defense mechanisms act in concert with the inflammasome to prevent intracellular proliferation of bacteria.

Poly-ubiquitinated cytosolic proteins are targeted for proteasome-dependent degradation (Voges, Zwickl et al. 1999). The eukaryotic ubiquitin system has therefore been implicated as a host defense mechanism against cytosolic pathogens. Indeed, cytosolically located *Salmonella* become poly-ubiquitinated leading to proteasome-mediated bacterial killing in macrophages (Perrin, Jiang et al. 2004) . In contrast,

Shigella is able to avoid poly-ubiquitination likely through actin–based motility (Perrin, Jiang et al. 2004). Ubiquitination is also important to the immune response known as autophagy: the formation of a double membranous vacuole, which like phagosomes matures into a degradative organelle by lysosomal fusion eliminating the enclosed bacteria (Nakagawa, Amano et al. 2004). Cytosolic bacteria, including *Shigella, Burkholdeira* and *Listeria,* are able to inhibit the autophagic response through secretion of certain effector molecules (see section 1.4.8 for effector definition) (Birmingham, Canadien et al. 2007). For example, *Shigella flexneri* secretes IcsB, which binds the autophagic protein ATG5 to inhibit autophagy (Ogawa, Yoshimori et al. 2005). Given several secreted bacterial effectors target the ubiquitin system, roles of bacterial effectors in autophagy avoidance by inhibiting the ubiquitin system is a possibility.

An autophagy-independent mechanism involving ubiquitination of cytosolic *Mycobacterium marinum* leading to LAMP-1 positive vacuolar encapsulation of bacteria has been documented; however, the fate of *M. marinum* in these vacuoles is yet to be determined. Apparently, *M. marinum* is capable of shedding its ubiquitinated cell wall, which may allow the bacteria to evade this putative host response (Collins, De Maziere et al. 2009). The ubiquitination of cytosolic bacteria has been proposed as an important host defense mechanism against other pathogens, but further research is required to substantiate this claim. Similarly, antimicrobial peptides of the macrophage cytosol are also suspected to contribute to bacterial killing; however, additional studies are required to clarify their involvement in human immune responses towards bacteria (Hiemstra, van den Barselaar et al. 1999).

Although much remains to be elucidated regarding the interplay of pathogens and cytosolic defense mechanisms, these defenses may contribute to the "opportunistic" vacuolar life style employed by some cytosolic pathogens, such as *Listeria* and *Francisella,* described below, and may explain why the majority of intracellular bacteria reside in modified endosomes rather than freely in the cytosol.

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1.2.8 Intracellular proliferation of *Francisella tularensis*

F. tularensis is a facultative intracellular bacterium that escapes from phagosomes of immune cells into the cytosol, where it is capable of massive replication (Golovliov, Baranov et al. 2003; Checroun, Wehrly et al. 2006). *Francisella* degrades the membrane of the trapping phagosome and escapes into the cytosol within 90 min of uptake (Golovliov, Baranov et al. 2003; Checroun, Wehrly et al. 2006). Prior to escape, the *Francisella* containing phagosome transiently acquires the early endosomal marker EEA-1 before becoming endowed with markers LAMP-1, LAMP-2 and CD-63, which are indicative of a late endosomal stage of maturation (Clemens, Lee et al. 2004; Santic, Molmeret et al. 2005). Notably, the phagosome is not enriched with degradative enzymes such as cathepsin D, thus current research suggest *Francisella* causes disruption of the phagosomal membrane before phagolysosomal fusion can occur (Clemens, Lee et al. 2004; Santic, Molmeret et al. 2005). Futhermore, when observed after 1h the *Francisella*-laden phagosome does not co-localize with the lysosomal tracer Trov, whereas phagosomes containing formalin-killed *Francisella* undergoing default maturation acquired this marker, thus suggesting *Francisella* is able to delay maturation of the phagosome (Santic, Molmeret et al. 2005). The stalling of maturation may be a

consequence of bacterial degradation of the phagosomal membrane, thereby allowing escape into the cytosol and protection from phagolysosomal components (Santic, Molmeret et al. 2005).

At a late endosomal stage, phagosomes are expected to have an acidic pH, yet controversy exists whether the *Francisella* containing phagosome is acidified before bacterial escape. Contradictory data regarding the acidity of the *Francisella* phagosome has been reported (Bonquist, Lindgren et al. 2008; Chong, Wehrly et al. 2008; Clemens, Lee et al. 2009). The most comprehensive study showed transient acidification occurring 20 min p.i. with gradual decrease in acidification at 40 and 60 min p.i . (Chong, Wehrly et al. 2008). In support of this notion, the *Francisella* containing phagosome acquires ATPase pumps; therefore, it seems likely transient acidification occurs prior to phagosomal escape (Chong, Wehrly et al. 2008; Clemens, Lee et al. 2009). Interestingly, inhibition of ATPase activity delays, but does not completely prevent escape of the bacteria (Chong, Wehrly et al. 2008; Clemens, Lee et al. 2009). Therefore, acidification may be required for full expression or activity of virulence factors enabling bacterial escape (Chong, Wehrly et al. 2008). *Francisella* does not appear to inhibit acquisition of vacuolar ATPases, rather, the observed decrease in acidity of the *Francisella* containing phagosome may be a consequence of membrane disruption leading to diffusion and equilibration of the acidic contents with the cell cytosol (Clemens, Lee et al. 2009).

Microscopic evidence has demonstrated degradation of the phagosomal membrane eventually leads to escape of *Francisella* into the host cytosol within 3 hours of infection. The exact timing is dependent up on the bacterial strain and cell type studied (Golovliov, Baranov et al. 2003; Clemens, Lee et al. 2004; Chong, Wehrly et al.

2008). Bacterial entry into the cytosol of macrophages can cause inflammasome-mediated cell death as described above in chapter 1.2.7 (Navarre and Zychlinsky 2000). Crucially, *Francisella* is able to delay activation of the inflammasome after phagosomal escape (Mariathasan, Weiss et al. 2005; Henry and Monack 2007). Although clearly important to *Francisella* intracellular survival, the molecular mechanism of inflammasome inhibition remains to be deciphered. After entry into the host cytosol, *Francisella* replication occurs approximately between 5 and 20 h p.i. resulting in a large number of bacteria grossly encompassing the cytosol of cells (Golovliov, Baranov et al. 2003; Checroun, Wehrly et al. 2006). The transcriptome of intracellular cytosolic *Francisella* has been reported. During this cytosolic stage it is evident the bacteria up-regulate genes required for nutrient acquisition and several novel genes of unknown function, some which appear to encode intracellular survival factors (Wehrly, Chong et al. 2009).

Eventually after 20 to 24 h p.i., *Francisella* re-enter double membranous vacuoles endowed with the autophagic probe MDC (monodansylcadaverine) and the autophagic protein LC3 (Checroun, Wehrly et al. 2006). Double membranous vacuoles containing bacteria have also been observed during late stages of macrophage infection with three different *Francisella* subspecies (*F. novicida, F. tularensis* and *F. holarctica)* and by different investigators (Mohapatra, Soni et al. 2008). The bacterium is able to survive in these acidified compartments, which have lysosomal features such as presence of LAMP-1 and cathepsin D (Checroun, Wehrly et al. 2006). Currently, the significance of autophagosome formation to *Francisella* is unknown; however, given autophagy as a macrophage defense mechanism is triggered rapidly after bacterial phagosomal egress, it

is speculated the autophagic response is important to *Francisella* rather than the macrophage (Nakagawa, Amano et al. 2004). Consistent with this notion, *Francisella* is apparently able to inhibit an early autophagic response as genes involved in autophagy are down-regulated during cytosolic replication of *Francisella* (Butchar, Cremer et al. 2008; Cremer, Amer et al. 2009). Although further research regarding the role of autophagy during *Francisella* infection is needed a suggested function of autophagy to the biology of *Francisella* is to provide environmental cues required for egress or re-infection of macrophages (Checroun, Wehrly et al. 2006; Wehrly, Chong et al. 2009).

Progress in the field of *Francisella* cell biology has been rapid in the last few years, yet many aspects of *Francisella* intracellular life remain poorly understood. Present evidence suggests the intracellular life style of *Francisella* is unique, combining intracellular survival strategies of other pathogens such as stalling phagosomal escape (*Mycobacterium, Legionella, Listeria*), phagosomal escape (*Listeria, Shigella*), and resistance to lysosomal degradation (*Coxiella*). Currently, there is a paucity of information regarding the virulence factors contributing to the successful intracellular life style of *Francisella.* Further work describing *Francisella* intracellular survival mechanisms will undoubtedly facilitate the identification of novel *Francisella* virulence factors. In the following section the current knowledge regarding bacterial factors important to *Francisella* pathogenicity are summarized.

1.3 Pathogenicity factors of *Francisella tularensis*

F. tularensis readily replicates in macrophages (Anthony, Burke et al. 1991). Sequencing of the *Francisella* genome have revealed few hints explaining its exceptional

ability to parasitize immune cells (Larsson, Oyston et al. 2005). Indeed, *Francisella* appear to lack classical virulence genes such as T3SSs and toxins. Gene disruption by mutagenesis has identified a number of genes, which appear to be important to intracellular survival of *Francisella* (Gray, Cowley et al. 2002; Maier, Casey et al. 2007). Mutants defective for intracellular growth are unable to cause disease underscoring the importance of intracellular replication to this pathogen (Lauriano, Barker et al. 2004; Nano, Zhang et al. 2004). Notably, most genes identified by random mutagenesis have not been complemented, and therefore, their role in intracellular growth cannot be unequivocally confirmed. Furthermore, the exact functions of the proteins encoded by a large number of these genes have not been determined. In some cases, roles of homologous proteins in other organisms have been investigated. A number of genes required for intracellular growth are located in the *Francisella* pathogenicity island, which is described in section 1.3.11 below.

Until recently, the vast majority of mutagenesis experiments were performed in low virulence strains of *F. holarctica* and *F. novicida*. Although strain-to-strain extrapolations should be conducted with caution, it is reasonable to assume genes required for intracellular growth of less virulent strains, such as *F. novicida* and *F. holarctica* LVS, serve a similar function in more virulent strains since the intracellular life styles of these bacteria are largely identical (Checroun, Wehrly et al. 2006; Qin, Scott et al. 2009; Wehrly, Chong et al. 2009).

1.3.1 Genes required for intracellular growth

A number of mutagenesis schemes as well as transcriptional profiling have identified genes required for intracellular growth of *Francisella* (Tempel, Lai et al. 2006; Fuller, Craven et al. 2008; Wehrly, Chong et al. 2009; Gray, Cowley et al. 2002; Maier, Casey et al. 2007). Only a limited number of genes required for intracellular growth have been identified and few have been characterized. For example, inactivated genes rendering *Francisella* unable to multiply intracellularly include *FTT0742,* encoding a putative lipoprotein (Tempel, Lai et al. 2006), *FTN1472,* encoding a phosphate kinase (Richards, Michell et al. 2008), *met* genes encoding a putative ABC transporter (Maier, Casey et al. 2007), and *FTT0989* (Brotcke and Monack 2008). Of these, FTT0989 may be particularly interesting since it has a putative Sec-dependent secretion signal and shows similarity to bacterial transglutaminases, which active Rho GTPases of the host (Maier, Casey et al. 2007).

Mutagenesis of regulators of virulence genes, genes of the FPI, genes required for LPS biosynthesis and genes encoding heat shock proteins also result in strains with growth-defects in macrophages (Baron and Nano 1998; Gray, Cowley et al. 2002). The factors encoded by these genes are discussed in more detail in other sections below. The molecular explanation for the intracellular growth defects of a small number of mutated genes has been investigated. It appears some genes required for intracellular growth are defective for *Francisella* phagosomal escape, whereas others are necessary for replication in the host cytosol.

1.3.2 Genes required for phagosomal escape

Phagosomal escape is essential to *Francisella* pathogenesis (Lindgren, Golovliov et al. 2004; Santic, Molmeret et al. 2005). The FPI genes *iglC, iglD* and *pdpA* are required for phagosomal escape and appear to be important virulence factors of *Francisella* (Bonquist, Lindgren et al. 2008; Chong, Wehrly et al. 2008; Schmerk, Duplantis et al. 2009). Phagosomal maturation and escape kinetics of *iglC* and *iglD* mutants have been investigated in both LVS and *F. novicida,* whereas a *pdpA* mutant has only been studied in *F. novicida.* Current data suggests there is a subtle difference in intracellular trafficking of *iglC* and *iglD* mutants between LVS and *F. novicida*. LVS lacking *iglC* and *iglD* are able to stall phagosome maturation similarly to wild type bacteria, but are unable to escape from this noxious, confined compartment (Lindgren, Golovliov et al. 2004; Bonquist, Lindgren et al. 2008; Schmerk, Duplantis et al. 2009). Contrastingly, whereas about 1/3 of *F. novicida iglC* mutants are able to escape, the 2/3 failing to exit phagosomes become trapped in mature phagolysosomes (Santic, Molmeret et al. 2005; Chong, Wehrly et al. 2008; Schmerk, Duplantis et al. 2009). Consistent with these observations, only about 30% of LVS *iglC* and *iglD* mutant bacteria are found in acidified phagosomes, which is significantly less than the amount reported for *F. novicida* mutants (about 75%) (Bonquist, Lindgren et al. 2008; Santic, Molmeret et al. 2005).

Mutants of both LVS and *F. novicida* are capable of some limited replication in macrophages, about 1 log, which is 100 logs less than replicating wild type bacteria, highlighting the importance of these genes to the intracellular life of *Francisella* (Bonquist, Lindgren et al. 2008; Santic, Molmeret et al. 2005; Lindgren, Golovliov et al.

2004). Interestingly, replication of LVS *iglC* and *iglD* strains occurs in LAMP-1 positive vacuoles (Bonquist, Lindgren et al. 2008); similarly, a *pdpA* mutant of *F. novicida* is capable of equally limited replication in LAMP-1 positive vacuoles (Schmerk, Duplantis et al. 2009).

An additional *F. tularensis* gene required for phagosomal escape is *FTT1103,* which encodes a predicted lipoprotein with similarity to the periplasmic oxidoreductase enzyme DsbA (Qin, Scott et al. 2009). In several bacterial species DsbA is associated with protein secretion by catalyzing disulfide bond formation in exported proteins (Ha, Wang et al. 2003). The *FTT1103* mutant remains LAMP-1 associated and is inaccessibility to cytoplasmically delivered *Francisella* antibody indicative of a phagosomal escape defect (Qin, Scott et al. 2009). Given *dsbA* is required for phagosomal escape, a function of the DsbA protein in a secretion system important to the intracellular growth of *Francisella* has been suggested (Qin, Scott et al. 2009).

Enzymes and pore-forming proteins are involved in phagosomal escape of a number of pathogens. Acid phosphatases catalyze hydrolysis of phosphomonoesters at acidic conditions and are involved in intracellular survival of several bacterial species (Saha, Dowling et al. 1985; Baca, Roman et al. 1993). The *F. tularensis* genome contains several acid phosphatases and their requirement for phagosomal escape of *F. novicida* in macrophages has been investigated by electron microscopy (Mohapatra, Balagopal et al. 2007; Mohapatra, Soni et al. 2008). An *acpA* mutant displays delayed phagosomal egress, whereas a quadruple mutant of *acpABC* and *hap* phosphatases is completely defective for phagosomal escape up to 24 h p.i. suggesting a role of acid phosphatases in phagosomal escape of *Francisella* (Mohapatra, Soni et al. 2008). Rather

than acting directly on the phagosomal membrane, AcpA could alternatively act intrabacterially inducing the activity of other proteins required for phagosomal escape (Mohapatra, Balagopal et al. 2007).

1.3.3 Genes required for replication in the cytosol

A small number of engineered mutants, which escape the phagosome into the cytosol of host cells with wild type kinetics, but are defective for intracellular growth have been identified. A deletion of *ripA*, encoding an inner membrane protein of unknown function results in such a phenotype (Fuller, Craven et al. 2008). Similarly, deletion strains of either *FTT0369* or *FTT1676* reach the cytosol, but fail to replicate in macrophages (Wehrly, Chong et al. 2009). These mutants are not auxotrophs, and the molecular mechanism for their cytoplasmic growth defect is currently unknown. A role of RipA in avoiding antimicrobial factors, possibly autophagy, has been speculated; however, *ripA* re-entered LAMP-1 positive double membranous autophagosome-like vacuoles at the same rate as wild type (Fuller, Craven et al. 2008). All three genes are required for virulence, and all mutants were fully complemented *in trans* (Fuller, Craven et al. 2008; Wehrly, Chong et al. 2009). FTT1676 is a predicted outer membrane protein, whereas FTT0369 is a novel protein of unknown function. Consistent of a role of these proteins during cytosolic localization of the bacterium, transcriptional profiling revealed *FTT1676* and *FTT0369* are up-regulated during the cytosolic phase of the *Francisella* intracellular infectious cycle (Wehrly, Chong et al. 2009).

1.3.4 Inhibition of the inflammasome

After initially inhibiting activation of the inflammasome, replication of *F. novicida* in the macrophage cytosol induces inflammasome-mediated host cell death called pyroptosis (see section 1.2.7 for inflammasome activation) (Mariathasan, Weiss et al. 2005). *F. novicida* mutants replicating faster than wild type are hypercytotoxic, whereas mutants that fail to replicate do not activate the inflammasome suggesting replication kinetics are linked to inflammasome activation (Mariathasan, Weiss et al. 2005; Weiss, Brotcke et al. 2007). Two hypercytotoxic mutants, *FTT0748* and *FTT0584*, replicating at wild type levels in macrophages have been identified (Weiss, Brotcke et al. 2007). The rapid cell death of these mutants is dependent upon caspase-1 suggesting FTT0748 and FTT0584 directly or indirectly inhibit inflammasome activation (Mariathasan, Weiss et al. 2005; Weiss, Brotcke et al. 2007). Thus, these virulence factors suppress both macrophage cell death and pro-inflammatory signaling by inhibiting inflammasome activation. The amino acid sequence of FTT0748 suggests this protein encodes an IclR family of transcriptional activators, whereas FTT0584 shows similarity to a protein of unknown function of *Legionella pneumophila*. As FTT0748 may be a regulator of transcription, this protein could be indirectly involved in inflammasome inhibition possibly through activation of unidentified virulence factors. Both mutants are attenuated *in vivo* suggesting a delay in inflammasome activation is important to the virulence of *F. novicida* and possibly other *Francisella* subspecies (Weiss, Brotcke et al. 2007).

1.3.5 Respiratory burst inhibition

The respiratory burst of phagocytotic cells, especially of neutrophils, is a potent antimicrobial defense mechanism (Nauseef 2004). During bacterial infection, the respiratory burst generating NADPH oxidase complex fails to assemble on the *Francisella*-containing phagosome resulting in respiratory burst inhibition and disruption of superoxide production (McCaffrey and Allen 2006). A mutagenesis screen has identified three mutants, which fail to inhibit the respiratory burst of neutrophils (Schulert, McCaffrey et al. 2009). The mutations were located in *carA, carB,* and *pyrB,* which encode the small and large subunits of carbamoylphosphate synthase and aspartate carbamoyl transferase, respectively. These mutants are uracil auxotrophs and supplementation of uracil restored wild type intracellular growth (Schulert, McCaffrey et al. 2009).

Another protein implicated in respiratory burst inhibition of *Francisella* is the broad substrate acid phosphatase AcpA. Purified *Francisella* AcpA inhibits neutrophil respiratory burst *in vitro* (Reilly, Baron et al. 1996). The respiratory burst-inhibiting ability of *acpA* mutants is yet to be investigated, but *acpA* is defective in escape from the phagosomal compartment of macrophages (Mohapatra, Balagopal et al. 2007).

1.3.6 Nitric oxide production, lipopolysaccharide and phase variation

Whereas the respiratory burst is inhibited by *Francisella*, nitric oxide (iNOS) is important to *Francisella* killing in activated macrophages (Anthony, Morrissey et al. 1992). *F. holarctica* LVS undergo phase variation resulting in antigenically distinct LPS (Cowley, Myltseva et al. 1996). The phase variation affects the ability of LVS to inhibit

nitric oxide production of rat macrophages. In wild type LPS confirmation negligible levels are produced, whereas antigenic shift results in production of large amounts of nitric oxide thereby suppressing intracellular growth. Interestingly, the LPS present after antigenic shift is similar to the LPS of *F. novicida* (Cowley, Myltseva et al. 1996). Therefore, the LPS of *F. novicida* appears locked in a nitric oxide-inducible confirmation offering an explanation to the attenuation of *F. novicida* in rats and possibly also in humans. LPS of many Gram negative bacteria are potent proinflammatory inducers, however, the LPS of *F. tularensis* is a poor proinflammatory stimulant. The finding *F. tularensis* LPS is not recognized by LPS-Binding-Proteins of the host may account for its low toxicicity.

Resistance of *F. tularensis* to killing by complement also appears to be mediated by LPS as mutants of genes involved in LPS biosynthesis are sensitive to complement-containing serum (Cowley, Gray et al. 2000; Raynaud, Meibom et al. 2007). Not surprisingly, a number of the LPS mutants are defective for intracellular growth (Cowley, Gray et al. 2000). In particular, mutants of the LPS O-anitgen locus *wbt* are required for intracellular growth (Maier, Casey et al. 2007; Raynaud, Meibom et al. 2007).

1.3.7 Capsule

Capsules are protective barriers of bacteria found outside the cell wall, usually difficult to visualize by conventional staining or microscopy methods. A putative capsule is important to *Francisella* virulence, but very little is known regarding its composition or function (Sandstrom, Lofgren et al. 1988). Mutations in genes homologous to capsule biosynthesis genes of other bacteria, such as *cpsK,* negatively affect the virulence of

Francisella lending weight to the hypothesis a capsule is required for *Francisella* virulence (Su, Yang et al. 2007; Weiss, Brotcke et al. 2007).

1.3.8 Iron acquisition

Intracellular bacteria rely on a variety of biosynthesis pathways to utilize limited nutrients available in the cytosol of host cells. Genes required for iron acquisition and siderophore production called *fsl* have been identified in *F. novicida* and LVS (Ramakrishnan, 2008). A mutant of *F. novicida fslA* is required intracellularly in *F. novicida* and *fsl* strains are avirulent in mice (Deng, Blick et al. 2006; Su, Yang et al. 2007; Weiss, Brotcke et al. 2007). The iron acquisition genes were found to be induced during the cytosolic stage of intracellular growth of *F. tularensis* (Wehrly, Chong et al. 2009). *F. tularensis* may employ additional strategies to utilize iron intracellularly as a mutant of *FTT0918,* encoding a 58 kDa protein required for efficient utilization of iron not involved in siderophore production is defective for intracellular growth and virulence (Twine, Bystrom et al. 2005; Lindgren, Honn et al. 2009).

1.3.9 Stress response

Intracellular pathogens initiate a vigorous stress response to acclimatize to the intracellular milieu (Raivio 2005). Transcriptional profiling identified a large list of putative stress responsive genes induced during macrophage infection including ClpB and HtpG (Wehrly, Chong et al. 2009). Indeed, the ClpB protein is up-regulated during intracellular growth and mutants of *clpB* and *htpG* fail to replicate in macrophages (Gray, Cowley et al. 2002; Wehrly, Chong et al. 2009). Furthermore, ClpB of LVS has been

shown to be important for resistance to cellular stress and virulence in mice (Meibom, Dubail et al. 2008). Analysis of the bacterial membrane of *clpB* suggested the amounts of five unidentified proteins are reduced in the membrane of this strain (Meibom, Dubail et al. 2008).

The intracellularly-induced transcriptional regulator MglA positively controls expression of a number of genes involved in oxidative and general stress responses (Baron and Nano 1998; Guina, Radulovic et al. 2007). Since *mglA* is required for intracellular growth and is induced during intramacrophage growth, it is possible MglA controls expression of factors required for a *Francisella* stress response in the macrophage; however, this has not been demonstrated to date (Baron and Nano 1998; Baron and Nano 1999).

1.3.10 Genes required for virulence

A number of genetic screens have identified genes needed for virulence in an animal model of infection. There is a large overlap between genes needed for intracellular growth and virulence indicating the importance of intracellular growth to *Francisella* (Su, Yang et al. 2007; Weiss, Brotcke et al. 2007; Kraemer, Mitchell et al. 2009). However, a number of genes required for virulence, which have not been identified as necessary for intracellular growth have been identified. Many of these virulence genes encode proteins of unknown function lacking known homologues. Their contribution to virulence and phagosomal escape is worthy of further investigation, particularly given effector proteins of secretion systems of many intracellular pathogens

affect virulence, but do not influence intracellular growth kinetics (Knodler, Vallance et al. 2003).

1.3.11 The *Francisella* pathogenicity island

Genomic islands acquired by bacteria through horizontal gene transfer are mobile genetic elements of linked genes conferring a selective advantage to harboring organisms in an ecological, pathogenic, fitness or resistance context (Dobrindt, Hochhut et al. 2004). The term pathogenicity island (PI) is reserved for genomic islands contributing pathogenicity traits to organisms (Schmidt and Hensel 2004). Hallmarks of pathogenicity islands include presence of virulence genes flanked by tRNA, a G+C content different from the core genome of the organism, and an association with additional mobile genetic elements including transposases. PIs are absent in non-pathogenic species, and often have a mosaic genetic structure indicating integration of virulence genes have occured during separate acquisition events (Schmidt and Hensel, 2004).

Recently, the genetic organization of a *Francisella* PI (FPI) was described (fig. 2). This region is approximately 30 kilo bases with an average G+C content significantly lower than the average of the core chromosome of *Francisella* of 33.2 percent (Nano, Zhang et al. 2004). The average G+C content between the genes *pdpA* and *pdpE* is remarkably low, 26.6 percent, suggesting the region may have been acquired from an unusual source such as a low G+C Gram positive bacterium or *Plasmodium* since their genomes have similar G+C contents to this region (Nano and Schmerk 2007).

Duplication of the FPI in *F. tularensis* subspecies *tularensis* and *holarctica* indicates the region once was capable of mobility (Nano, Zhang et al. 2004). The *F.*

novicida chromosome only harbors one copy of the FPI, and it is possible the additional copy contributes to rendering these subspecies more pathogenic than subspecies lacking a second copy (Nano and Schmerk 2007). The region is also flanked by transposon insertion elements, which may potentially have been important in mediating acquisition of foreign DNA. Another feature of the region is genes encoding 23S and 16S rRNA, which may have served as anchor points for insertion of the island (Nano, Zhang et al. 2004).

The FPI is organized in two putative operons, one consisting of 12 genes generally referred to as the *pdp* operon (spanning *pdpA* thru *pdpE*), and a second putative operon on the opposite coding strand commonly known as the *igl* operon, which includes the six genes *pmcA, pdpD* and *iglABCD*. Insertion mutagenesis in the *iglABCD* genes renders *F. novicida* unable to multiply in macrophages (Gray, Cowley et al. 2002). Similarly, a targeted deletion of both *iglC* genes of *F. holarctica* LVS causes a replication defect in macrophages (Lai, Golovliov et al. 2004). In contrast, only one study focusing of *pdpA* has investigated the role of the genes in macrophage-infection of the *pdp* operon. This study demonstrated, similarly to inactivation of *igl* operon genes, *pdpA* is required for intracellular growth (Schmerk et al, 2009).

Whereas some genetic evidence link the FPI to virulence, intracellular growth and phagosomal escape of the bacteria exists (see section 1.3.2), almost nothing is known regarding the protein products of the FPI. A proteomics study identified IglC as being highly up-regulated during intracellular growth, and unidentified proteins of molecular masses largely matching those predicted of IglA, IglB and IglD were also found to be induced intracellularly (Golovliov, Ericsson et al. 1997). Much of the work presented in

this thesis has focused on describing the protein products of the FPI, and investigating the contribution of individual FPI genes to *Francisella* intracellular growth. It should be noted the names of the genes of the larger *"pdp"* operon have been changed during the course of this study.

1.3.12 FPI regulation

Regulation of virulence factors, such as PIs, ensuring timely expression and coordination of their functions is an important aspect of bacterial pathogenesis. To accomplish this task, expression of PI genes can involve complex networks of several regulatory elements responsive to different stimuli (Schmidt and Hensel 2004). Indeed, a rather complex network of regulators appears to be involved in FPI gene expression. Recent studies have identified a number of proteins regulating FPI expression. One of these is MglA, which positively regulates FPI genes (Lauriano, Barker et al. 2004). The regulator FevR also controls expression of the FPI, and further, a *fevR* mutant is completely defective for intracellular growth (Brotcke and Monack 2008; Wehrly, Chong et al. 2009). MglA regulates the expression of FevR, but not vice versa (Brotcke and Monack 2008). In addition, PmrA, a so called orphan response regulator, positively affects FPI gene expression (Mohapatra, Soni et al. 2007). A regulator only controlling transcription of the *igl* operon has also been identified and named MirG (Buchan, McCaffrey et al. 2009). Hfq is the only known repressor protein of FPI genes identified, negatively affecting expression of genes of the putative *pdp* operon only (Meibom, Dubail et al. 2008).

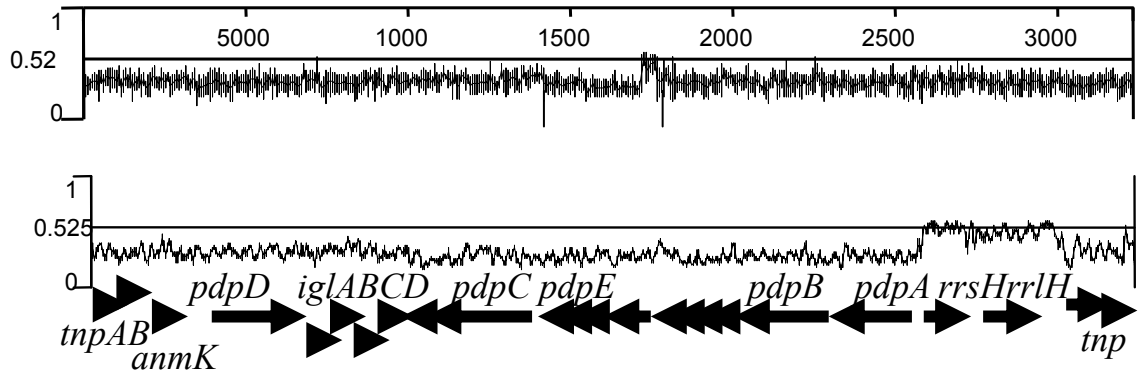

Figure 2. **Gene organization of the *F. novicida* form of the FPI.** Top graph shows G+C content of the 300 kb region of the *Francisella* chromosome encompassing the FPI. Two central vertical lines indicate extent of the FPI. Bottom graph shows G+C content of the FPI. The *anmKpdpD* genes are the first 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. However, the overall sequence of the FPI is otherwise highly conserved between *F. novicida* and *F. tularensis.* At the left end of the FPI are ORFs *tnpAB,* whereas on the far right end a *tnpA* gene is found; these ORFs are predicted to encode transposases. *rrsH* and *rrlH* encode 16S and 23S rRNA respectively. Genes are not drawn exactly to scale. Figure borrowed from Nano *et al*, 2004.

MglA is similar to the global regulator of the stringent starvation response, SspA (Baron and Nano 1998). Interestingly, in *Francisella,* a complex of MglA and SspA associates with RNAP to active transcription (Charity, Costante-Hamm et al. 2007). It is

notable *mglA* mutants are avirulent and are required for *Francisella* growth in macrophages. A mutant of the downstream located gene *mglB*, an SspB homologue, has an identical intracellular growth defect as *mglA* (Baron and Nano 1998); however, the involvement of MglB in FPI-regulation is yet to be addressed.

The induction of general stress response genes coincides with induction of FPI genes during macrophage infection (Wehrly, Chong et al. 2009). MglA apparently is responsive to stress and starvation conditions, and is induced during an early stage of infection when *Francisella* is located in a phagosome (Baron and Nano 1999; Guina, Radulovic et al. 2007). Despite several studies, no connection between FPI proteins and stress has been demonstrated (Lenco, Pavkova et al. 2005; Bonquist, Lindgren et al. 2008). Rather, it is likely MglA responds to conditions in the phagosome, which consequently leads to up-regulation of a number of genes with disparate functions important to the bacteria at this intracellular stage.

The involvement of multiple regulators of FPI expression suggests expression of FPI genes is highly controlled and orchestrated. Additionally, this may allow FPI expression to be controlled by multiple stimuli, such as different conditions within the macrophage. Indeed, it has been shown FPI transcripts are highly induced during different stages of the macrophage infection (Wehrly, Chong et al. 2009). FPI expression is highly up-regulated early on during macrophage infection coinciding with *Francisella* phagosomal escape. A second period of induction occurs between 16 and 20 h p.i., a period co-inciding with *Francisella* entery into an autophagy-like vacuolar stage (Wehrly, Chong et al. 2009). Given the FPI is up-regulated at multiple time points, the biological function mediated by the FPI may involve interaction with several disparate

host factors (Wehrly, Chong et al. 2009). Overall, the expression profiles of FPI-encoded genes are identical suggesting cooperation of these proteins in a shared function (Wehrly, Chong et al. 2009).

1.3.13 Signaling inhibition by FPI-encoded products

Intracellular LVS inhibits or otherwise delays proinflammatory signaling of infected immune cells (Telepnev, Golovliov et al. 2005). Mouse macrophagic cells infected with *F. holarctica* LVS showed a decrease in cell signaling compared to uninfected cells. LVS harboring a deletion of *iglC* are unable to inhibit the signaling response (Telepnev, Golovliov et al. 2005); thus, one possibility is signaling is uninterrupted as a consequence of the failure of *iglC* mutants to reach the cytosol. However, given down-regulation is observed as early as 15 min p.i., it is also possible IglC directly or indirectly is involved in cell signaling disruption (Telepnev, Golovliov et al. 2005). Similarly, macrophage infection with a *F. novicida pdpA* mutant failing to egress from the phagosome results in alterations of cell signaling pathways (Schmerk, Duplantis et al. 2009). Therefore, it is not clear whether down-regulation of signal transduction of immune cells is a consequence of *Francisella* phagosomal escape or involves a mechanism related to the function of IglC and PdpA.

1. 4 Secretion systems of Gram negative bacteria

Secretion is a fundamental process of bacterial microorganisms. It is responsible for diverse functions such as cell-to-cell communication, nutritional up-take, environmental adaptation, physiological responses, and evasion of the immune system of

a host. To accomplish the task of secretion bacteria have evolved multi-protein complexes known as secretion apparatuses, which span the bacterial membranes serving as a conduit between the interior of bacteria and the extracellular milieu. The Gram positive cell wall has only one membrane and, therefore, simple solutions regarding secretion are found in these organisms. Single membrane-spanning protein complexes, known as the Sec translocons and twine arginine translocons are examples of apparatuses sufficient for secretion in these organisms (Desvaux, Hebraud et al. 2009). Because of the added complexity of their cell wall, Gram negative bacteria are equipped with specialized secretion systems to interact and communicate with their surroundings.

Depending on structure and evolutionary origin, secretion systems are generally categorized into six different classes, type 1 thru type 6 (fig. 3). T1SS and T5SS are relatively simple systems involving only two or three proteins, whereas T2SS, T3SS, T4SS and T6SS are multi-component structures spanning the entire bacterial cell envelope. It has recently been proposed the chaperon-usher pilin biogenesis system be classified as a T7SS, and the extracellular nucleation-precipitation (ENP) pathway involved in assembly of curli surface-appendages be classified a T8SS (Desvaux, Hebraud et al. 2009). This new terminology takes into account the observation secretion of these pilin is accomplished by secretion apparatuses. Additionally, the surface-exposed structural components of T2SS and T4SS are structurally and evolutionarily similar to pilin indicting classification of pilin and protein secretion systems could be unified (Fronzes, Remaut et al. 2008).

Evolutionarily, T2SS are related to type 4 pilin, T3SS are structurally similar to flagella, whereas T4SS are related to conjugation systems (Desvaux, Parham et al. 2004).

Recently, it was also demonstrated T6SS and bacteriophages are evolutionary related (Leiman, Basler et al. 2009). Clearly bacteria have evolved multiple solutions to accomplish the essential mechanism of secretion. Given the recent unveilings of newly described secretion systems (for example, the *Mycobacterium* ESX system and T6SSs), it is probable additional systems exist that are yet to be identified.

In the context of bacterial secretion, the term **translocation** is used to describe the movement of a protein across a biological bilayer, such as a bacterial membrane, whereas **export** is used to describe transport of a protein across the inner membrane to the periplasmic space of Gram negative bacteria. The term **secretion** refers to transport of a protein from the inside to the outside of the cell (Desvaux, Parham et al. 2004). A secreted protein is found outside of the bacterial cell; as such, it can exist as a soluble protein, be surface-exposed, or be a subunit of a surface appendage (Pugsley 1993; Gerlach and Hensel 2007; Desvaux, Hebraud et al. 2009). Therefore, it is not sufficient for a protein to insert into the outer membrane of a bacterium to be considered secreted unless it is also surface exposed. Confusion regarding the term secretion likely stems from eukaryotic cell biology, where many signaling molecules are referred to as secreted if released freely into the extracellular space.

1.4.1 Sec export machinery

The Sec machinery translocates proteins across the inner membrane into the periplasmic space of Gram negative bacteria (Desvaux, Parham et al. 2004). The Sec machinery is an integral inner membrane pore-complex composed of SecYEG proteins and a peripheral ATPase known as SecA, which energizes export (Gerlach and Hensel

2007). Proteins targeted for Sec-dependent export contain an N-terminal signal peptide in their primary amino acid sequence, which is cleaved during export (Pugsley 1993). The Sec machinery is essential to biogenesis of several secretion systems and Sec-mediated export of proteins is important in translocation of several virulence factors; for example, the Sec machinery is involved in export of protein destined for secretion via T5SSs, and export of select substrates of T1SSs, T2SSs and T4SSs. Historically, Sec-dependent secretion was referred to as part of the general secretory pathway. However, since Sec-dependent translocation of Gram negative bacteria are involved in export rather than secretion, this terminology is out-dated (Desvaux, Parham et al. 2004). Furthermore, in addition to Sec, other export pathways of bacteria exist, such as the twin-arginine transport system, making the categorization as a general pathway obsolete (Voulhoux, Ball et al. 2001).

1.4.2 T1SS

The apparatus is the physical structure of secretion systems spanning the bacterial membranes. In terms of T1SSs, the apparatus is composed of an inner membrane ATP-binding cassette, a periplasmic-encompassing membrane fusion protein and a homotrimeric outer membrane channel exemplified by TolC of *E. coli* (Andersen, Koronakis et al. 2002). The ATP-binding cassette is responsible for substrate recognition and hydrolysis of ATP, which drives protein translocation (Binet and Wandersman 1995; Boardman and Satchell 2004). T1SSs are capable of secretion of large proteins up to 800kDa in an unfolded state (Hinsa SM, 2003). Conformational changes in the secretion complex allowing translocation are poorly defined; however, an uncleaved C-terminal

secretion signal of approximately 50 residues present in secreted proteins has been identified (Gerlach and Hensel 2007). The RTX toxin of *Vibrio cholerae* is a well-known example of a protein secreted via T1SSs (Boardman and Satchell 2004). Additional secreted substrates include many haemolysins important to the pathogenesis of several bacterial species (Schmidt and Hensel 2004).

1.4.2 T2SS and Type IV Pilin (T4P)

The foundation of the T2SS apparatus is a multi subunit inner membrane complex with a peripheral component, which catalyses secretion by ATP hydrolysis. A homometric channel-forming pore complex composed of 12-14 subunits, called the secreton, is inserted into the outer membrane allowing secretion by this system. The secreton is stabilized by a lipoprotein peripherally attached to the periplasmic side of the outer membrane (Nouwen, Ranson et al. 1999). The inner and outer membrane components are connected via a periplasmic linker protein (Desvaux, Parham et al. 2004; Gerlach and Hensel 2007). Important players of many bacterial pathogens, T2SS are responsible for secretion of a number of toxins and enzymes, including pore-forming molecules, such as aerolysin of *Aeromonas* spp., and enzymes such as lipase of *Burkholderia* spp. (Gerlach and Hensel 2007). All proteins secreted via this system are dependent on the Sec or Tat machinery for export.

Type IV pilin (T4P) are long, flexible appendages produced by a large number of Gram negative bacteria including the pathogens *Legionella pneumophila, Neisseria gonorrhoeae, Salmonella enterica* and *Pseudomonas aeurginosa*. In these pathogens T4P are essential to various virulence-related tasks such as adhesion, biofilm formation, invasion and twitching motility (Burrows 2005). Biogenesis of T4P requires a complex

ATPase-driven assembly apparatus spanning both bacterial membranes, which is structurally similar to T2SS apparatuses (Fernandez and Berenguer 2000; Peabody, Chung et al. 2003). Additionally, bioinformatics suggests an evolutionary relationship between the T2SS apparatus and the T4P biogenesis machinery (Fernandez and Berenguer 2000); therefore, it has been proposed T4P be classified as a specialized T2SS (Desvaux, Hebraud et al. 2009).

1.4.3 T3SS

T3SSs are found in a number of important bacterial pathogens including *Salmonella enterica, E. coli, Yersinia,* and *Shigella* spp (Kimbrough and Miller 2000; Sekiya, Ohishi et al. 2001). Central to the T3SS is a complex supramolecular structure known as the needle or injectisome designed to deliver effector molecules straight into eukaryotic host cells (Galan and Wolf-Watz 2006). T3SS apparatuses consist of about 20 different subunits. The base of this apparatus is a membrane-anchored, multi-ringed, heterocomplex evolutionary related to the bacterial flagellar assembly machinery (Saier 2004). On top of the base sits the aforementioned needle-like projection protruding from the bacterial surface (Kimbrough and Miller 2000). The base is required for the biogenesis and secretion of the needle complex, which is assembled into a hollow helical structure composed of polymerized needle subunits (Galan and Wolf-Watz 2006). Once assembly is completed, the base switches substrate specificities and becomes competent to secretion of effector molecules capable of hijacking eukaryotic cellular functions (Lavander, Sundberg et al. 2002). Small, acidic chaperones deliver effector molecules to the cytoplasmic-face of T3SS apparatus, where an inner membrane-associated ATPase

provides energy for effector-unfolding, chaperon-release, and contact-dependent translocation of effectors into host cells via the needle complex (Akeda and Galan 2005). Necessary for effector translocation, but not secretion, are secreted proteins forming a structure called the translocon. This structure inserts into the membrane of host cells forming a channel, which effectors can travel through (Hakansson, Schesser et al. 1996). Examples of T3SS effectors and their functions are given in section 1.4.8.

1.4.4 T4SS

T4SSs are related to the conjugation mating machinery of bacteria; as such, these systems contribute to horizontal gene transfer and evolution of pathogens. In addition to DNA transfer, T4SSs are capable of injecting effector proteins directly into eukaryotic cells similarly to T3SS (Sexton and Vogel 2002). These protein secretion systems are important virulence factors of a number of human pathogens including *Legionella pneumophila, Coxiella burnetii* and *Helicobacter pylori* (Censini, Lange et al. 1996; Vogel and Isberg 1999). Interestingly, T4SS of the plant pathogen *Agrobacterium tumifaciens* is capable of injecting DNA into cells to cause disease (Christie, Atmakuri et al. 2005). A large diversity between T4SSs exists, and depending on evolutionary origin the various systems are divided into three groups. This classification is based on evolutionary relationship of T4SS components to conjugative plasmids of different incompatibility groups: IncP, exemplified by the *Agrobacterium* T4SS; IncI, including the *Legionella* T4SS; the GI lineage of T4SSs; and the IncF plasmid T4SS (Juhas, Crook et al. 2008). F and P have been categorized as T4A, I as T4B, and GI as "other" T4SSs (Christie 2004). GI, or "other" T4SSs, are well-conserved and exist in numerous

bacteria, where they are involved in transfer of antibiotic resistance genes, whereas T4A and T4B secretion systems are important virulence factors of several pathogenic bacteria (Juhas, Crook et al. 2007). Notably, T4A and T4B share only three homologues (Juhas, 2008). However, despite the limited sequence homology between T4A and T4B systems, biochemical evidence suggests the supramolecular structure of T4BSS of *Legionella pneumophila* and the prototypical T4ASS apparatus of *Agrobacterium tumifaciens* are highly similar (Vincent, Friedman et al. 2006). All T4SSs are multi-subunit envelope-spanning structures equipped with a secretion channel and a surface-exposed pilus structure (Juhas, Crook et al. 2008). The most comprehensively studied T4SS is the system of *Agrobacterium tumifaciens*, therefore, a general description of the *Agrobacterium* apparatus follows.

The T4SS apparatus is composed of multiple subunits named VirB1-11. Several proteins, including VirB6 and 8, form an inner membrane complex, whereas VirB10 forms a periplasmic-spanning structure linking the outer and inner membrane components (Das and Xie 2000; Jakubowski, Krishnamoorthy et al. 2004). A secretin-like VirB9 forms an outer membrane pore stabilized by a VirB7 lipoprotein (Cao and Saier 2001). VirB2 is the major structural subunit forming a surface-exposed pilus, whereas VirB5 is a minor subunit of the pilus (Christie, Atmakuri et al. 2005). Secretion is accomplished through ATP hydrolysis mediated by the peripherally-associated inner membrane proteins VirB11 and VirB4 (Christie, Atmakuri et al. 2005). Although other T4SS systems have different genetic composition, all appear to form highly similar apparatus machineries (Kutter, Buhrdorf et al. 2008).

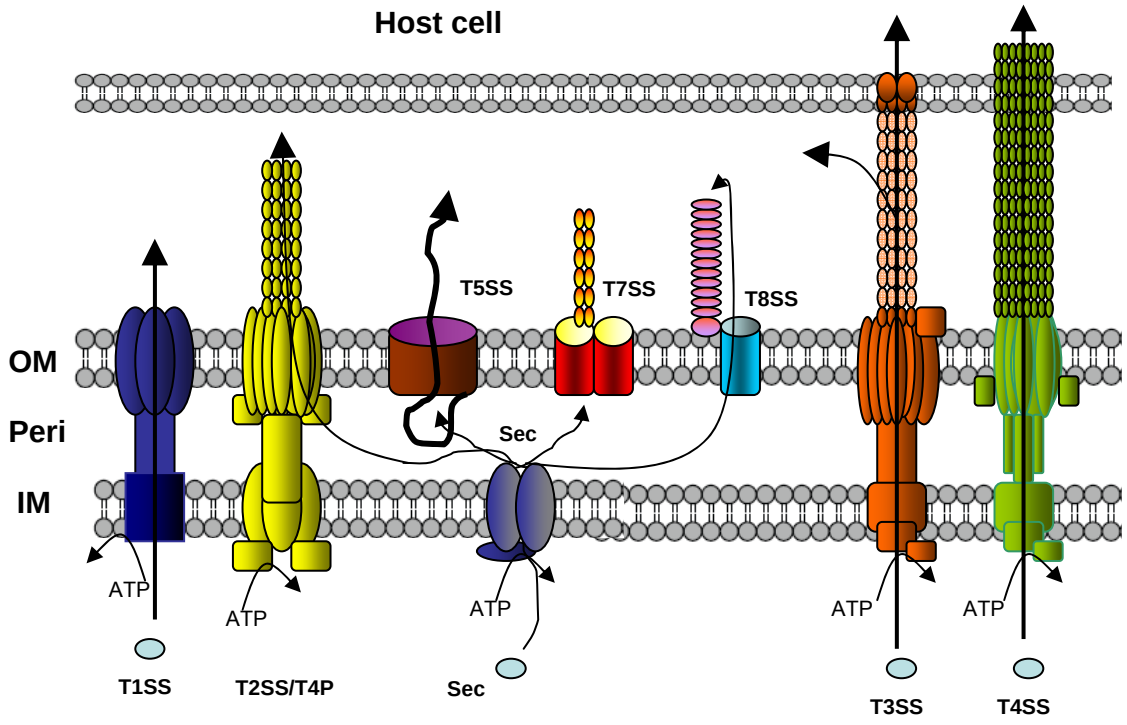

Figure 3. Schematic representation of secretion systems of Gram negative bacteria. IM, inner membrane of bacteria; peri, periplasm; OM, outer membrane. Large arrow-heads indicate routes of substrate secretion. T3SS and T4SS are able to translocate substrates directly into host cells. See text for detailed description of systems. Figure adapted from Gerlach and Hensel 2007, and Desvaux et al. 2009.

1.4.5 T5SS

Of all secretion systems of Gram negative bacteria, T5SSs are the most prevalent among species (Desvaux, Parham et al. 2004). Proteins secreted via the T5SS confer a wide variety of virulence traits including adhesion, autoaggregation, invasion, biofilm

formation and cytotoxicity (Henderson and Nataro 2001; Henderson, Navarro-Garcia et al. 2004). T5SSs offer a simple, yet elegant solution to secretion across the bacterial bilayer. After Sec-dependent export, components of T5SS insert and form a beta-barrel pore in the outer membrane (Henderson, Navarro-Garcia et al. 2004). A periplasmically-exposed passenger domain of the T5SS protein is then threaded through the T5SS pore resulting in its auto-proteolytic cleavage and secretion into the extracellular milieu (Brunder, Schmidt et al. 1997). After outer membrane insertion, some T5SSs are anchors retaining a secreted surface-protruding adhesion to the bacterial surface as exemplified by the *Yersinia* YadA adhesin (Hoiczyk, Roggenkamp et al. 2000). Because T5SS are capable of apparent self-secretion, T5SS are known as autotransporters (Henderson, Navarro-Garcia et al. 2004).

1.4.6 Chaperone-usher (CU) pili –T7SS

CU pili appear to be one of the most common types of bacterial surface structures, and are important virulence factors involved in adhesion to host cells and immune system evasion (Sauer, Remaut et al. 2004). CU pili are linear multisubunit surface appendages of a number of enteric bacteria, as well as of *Pseudomonas, Bordetella* and *Ralstonia* spp. To initiate pili assembly, individual subunits are first exported by the Sec machinery. In the periplasm, a specialized chaperone binds subunits to inhibit premature pilus assembly, and this pilin-chaperon complex is subsequently recruited to the outer membrane usher complex, which functions as a pilus assembly platform (Saulino, Bullitt et al. 2000; Vetsch, Puorger et al. 2004). The usher catalyses polymerization and translocation of pili to the surface through the usher channel consisting of a 24-stranded

beta-pore (Remaut, Tang et al. 2008). The outer membrane usher are homodimeric twin pore complexes (So and Thanassi 2006). The significance of the twinning of complexes is thought to allow proper and orderly pili assembly (Remaut, Tang et al. 2008). Polymerized pili have a complex quaternary structure consisting of multiple subunit types. For example, the CU pili of UPEC are composed of a homopolymer of over 1000 FimA subunits forming a helical structure and a distal end of multiple subunits, which serve different functions in adhesion and pili termination depending on the species (Fronzes, Remaut et al. 2008).

1.4.7 Curli –T8SS

In *E. coli* and *Salmonella* spp, curli form highly aggregative and flexible fibers known as thin aggregative fimbriae, which is the major protein-containing component of the extracellular matrix (Collinson, Emody et al. 1991). In addition to a role in biofilm formation and protection against environmental stresses, curli appear to be involved in bacterial pathogenesis functioning as adhesion and invasion causing factors (Kikuchi, Mizunoe et al. 2005). After Sec-dependent export, secretion of the major subunit of the *Salmonella* curli, AgfA, through a pore-forming outer membrane lipoprotein (AgfG) is facilitated by the periplasmic factors AgfE and AgfC (Robinson, Ashman et al. 2006; Gibson, White et al. 2007). Finally, biogenesis of curli structures is achieved by an extracellular nucleation-precipitation pathway that is not well-understood. Briefly, this involves aggregation of secreted AgfA into a fiber formed on top of membrane-associated nucleator subunit AgfB (Hammar, Bian et al. 1996).

1.4.8 Effector proteins of T3SS and T4SS

For certain pathogenic bacteria, the orchestrated hijacking of cellular responses of a host is of central importance to successful colonization and infection. The release of toxins into the surrounding environment introduces factors of error regarding reliable delivery to and contact with a cell of a mammalian host. To circumvent this problem, some bacteria come equipped with structures capable of injecting bacterial macromolecules, known as effectors, directly into the cytosol of cells. Whereas toxins are able to exert their function on cells after exogenous addition to living organisms, effector molecules require translocation by T3SS and T4SS straight into eukaryotic cells, where their mode of action modulates a diverse repertoire of cellular functions (Bhavsar, Guttman et al. 2007). An additional difference between toxins and effectors is toxins often have one solitary irreversible biochemical effect, whereas the action of effectors are often subtle, involving cooperation of several effector molecules to finely tune host responses to the benefit of the bacterium.

The unique arsenal of effector molecules of a bacterium is thought to largely contribute to differences in phenotypes and disease-causing abilities observed between bacteria harboring similar secretion systems. Several effector proteins have been shown to modulate immune responses by inhibiting signaling pathways. Yet other effectors of pathogens, including *Salmonella enterica* and *Legionella pneumophila,* target the cytoskeleton and vesicular transport system of the host to create conditions favorable for bacterial replication (Ensminger and Isberg 2009). For example, to maintain its intracellular niche, *Legionella pneumophila* translocates the effector SidF into host cells,

where SidF targets an apoptotic signaling pathway preventing cell death through interaction with Bcl2 family proteins (Banga, Gao et al. 2007). In contrast, the *Salmonella enterica* effector SseL is a deubiquitinase required for induction of delayed host cell death, which occurrs after bacterial replication (Rytkonen, Poh et al. 2007). It has been proposed apoptosis enables the bacteria to infect additional host cells leading to the spread of the infection. An interesting mechanism contributing to dispersal of *Salmonella* infection in hosts was recently reported. In gastrointestinal phagocytic cells the *Salmonella* effector SrfH interacts with the host cell protein TRIP6, which controls cell motility. SrfH is able to up-regulate the action of TRIP6 causing migration of infected cells to additional sites of the host (Worley, Nieman et al. 2006).

A recurring theme of bacterial secreted effectors is their mimicry of functions of eukaryotic proteins to alter the cellular responses of host cells (Galan 2009). Interestingly, effector molecules appear to have evolved convergently to eukaryotic proteins since there is an apparent lack of similarity at the primary amino acid level between mimicking effectors and eukaryotic proteins endowed with the same function (Galan and Wolf-Watz, 2006). Therefore, primary amino acid sequence predictions regarding the function of effectors have often proven fruitless (Galan and Wolf-Watz, 2006). However, *Legionella pneumophila* harbors a large number of proteins similar to eukaryotic proteins at the amino acid sequence level, some of which have been shown to be *bone fide* effector molecules of this pathogen (Pan, Luhrmann et al. 2008). Elucidating the crystal structure and biochemical activity of effectors have facilitated investigation of their *in vitro* function; however, to fully comprehend *in vivo* function knowledge of cellular biology of infection of a pathogen including the functions of co-

operative effector molecules appears necessary (Galan 2009). To exemplify this, the GAP activity of *S. typhimurium* SptP was originally thought to target and disrupt the cytoskeleton of the host cell; however, since this pathogen also injects effectors with Rho-GTPase activity acting in concert with SptP, the function of SptP was later shown to be to preserve cytoskeleton of the host cell (Galan and Wolf-Watz, 2006). Although much is still to be learned about effectors of secretion systems, a combination of molecular approaches has lead to insight into the functions of effectors of T3 and T4SS.

1.5 Type VI secretion systems

Recently, it was demonstrated semi-conserved gene clusters of Gram negative proteobacteria encode a novel secretion system, which has been coined a T6SS to distinguish it from secretion systems previously described (Mougous, Cuff et al. 2006; Pukatzki, Ma et al. 2006). Inactivation of genes in putative T6SS loci of a number of bacterial species suggests T6SS are required for a wide variety of biological functions ranging from survival in eukaryotic cells conferring pathogenesis ability to sensing environmental cues in bacterial ecology (Parsons and Heffron 2005; Bingle, Bailey et al. 2008; Suarez, Sierra et al. 2008; Weber, Hasic et al. 2009). Although experimentally proven to be functional only in select bacteria, T6SS clusters are present in about 25% of proteobacterial species sequenced to date, corresponding to 90 different bacteria. These clusters are composed of between 12 to 25 genes with some species harboring up to 6 different clusters with potential to encode a T6SS system (Bingle, Bailey et al. 2008; Filloux, Hachani et al. 2008; Boyer, Fichant et al. 2009). Bioinformatics analysis further suggests these clusters have been acquired by horizontal gene transfer during several acquisition events rather than by duplication events (Boyer, Fichant et al. 2009).

It should be noted research on T6SSs is in its infancy; therefore, only basic aspects of secretion have been described, whereas many details of these systems, such as secretion signals, substrate recognition, and discription of chaperones, are yet to be determined. Additionally, not much is known regarding the components of the T6 secretion apparatus, but some progress has been made describing certain aspects of its properties (Pukatzki, Ma et al. 2007; Zheng and Leung 2007; Aschtgen, Bernard et al. 2008; Leiman, Basler et al. 2009). The exact structure of the T6SS apparatus is yet to be visualized; however, purification and crystallization of conserved T6SS proteins suggest these proteins are capable of forming a structure similar to the tail of bacteriophages (Bonemann, Pietrosiuk et al. 2009; Leiman, Basler et al. 2009). In addition, bioinformatics analyses indicate several proteins of T6SSs are similar to bacteriophage proteins (Pukatzki, Ma et al. 2007; Leiman, Basler et al. 2009). Therefore, one possibility is part of the T6SS apparatus forms a structure similar to the bacteriophage tail.

Bacteriophages are parasitic entities thriving upon bacteria for their own propagation. Structurally, in addition to its genome-containing head, bacteriophages are composed of a tail consisting of tail fibers, a tail sheath, a tail tube and a baseplate. The tail sheath and tail tube form the majority of the tail structure with the hollow tail sheath surrounding the internal tail tube; whereas the base plate is connected at the bottom end of the tail (Leiman, Kanamaru et al. 2003).

To initiate infection of a bacterium, the phage uses its long extended tail fibers to attach to the bacterial cell wall, thereby forcing physical contact between the phage base plate and the bacterium. Upon attachment, a conformational change in the base plate

causes tail sheath contraction, much like a spring, thereby exposing and threading the static tail tube through the base plate (Leiman, Chipman et al. 2004; Kostyuchenko, Chipman et al. 2005). The bottom of the phage tail tube is equipped with a puncturing device, the tail-spike, harboring bacterial peptidoglycan-damaging lysozyme activity (Kanamaru, Leiman et al. 2002). Combined with the driving force of the tail sheath contraction, the lysozyme-activity of the tail spike creates a channel in the bacterial membrane allowing insertion of the tail tube and injection of phage genomic material into bacteria (Leiman, Kanamaru et al. 2003; Leiman, Shneider et al. 2003).

1.5.1 Secreted proteins

The genetic composition of T6SS clusters has been extensively analyzed; however, some the genomes of some bacteria contain apparently incomplete T6SS gene clusters (Das and Chaudhuri 2003; Bingle, Bailey et al. 2008; Boyer, Fichant et al. 2009). These bacterial genomes also encode multiple T6SS; therefore, it is difficult to gauge the exact minimum gene requirement needed to encode a T6SS (i.e. core components) as there may be functional overlap between clusters. Thus, to date no consensus minimum requirement of genes necessary to encode a T6SS exists (Filloux, 2008). Despite this, recent research has shed some insight on the importance of some bacterial genes encoding T6SS apparatus components and secreted proteins. Genetic and mutagenesis studies demonstrated genes located in T6SS gene clusters are required for secretion of two novel proteins belonging to the VgrG and Hcp family of proteins (Mougous, Cuff et al. 2006; Pukatzki, Ma et al. 2006; Pukatzki, Ma et al. 2007). Notably, in some bacterial

species the genes encoding the secreted Hcp and VgrG proteins are localized elsewhere in the genome outside of the T6SS cluster.

VgrG proteins have been demonstrated to be secreted into the growth supernatant by T6SS of *Vibrio cholerae* and *Edwardsiella tarda* (Pukatzki, Ma et al. 2006; Zheng and Leung 2007). VgrG proteins are interesting proteins for a number of reasons. For one, the first indication of an evolutionary relationship between T6SS and bacteriophages was based on the prediction VgrG is similar to the gp5-gp27 part of the T4 bacteriophage puncturing device (Pukatzki, Ma et al. 2007). Gp5-gp27 forms a trimeric complex, which constitutes the structure known as the tail-spike of bacteriophages. The crystal structure of amino acids 1 thru 483 out of 824 of an *E. coli* VgrG protein has been solved and shown to be similar to the gp27 portion of the puncturing device (Leiman, Basler et al. 2009). It is possible the remaining residues form a structure similar to gp5.

In *Vibrio cholerae,* three different VgrG proteins are expressed. Co-immunoprecipitation of these VgrG proteins suggests they may assemble into a heterotrimeric structure (Pukatzki, Ma et al. 2007). This structure could correspond to the trimeric gp5-gp27 complex, and could possibly constitute the tip of T6SS machineries. Supporting a structural role of secreted VgrG proteins, mutagenesis of *vgrG* results in failure of the bacterium to secrete other T6SS substrates (Pukatzki, Ma et al. 2006; Zheng and Leung 2007; Schell, Ulrich et al. 2007). However, there is also substantial evidence VgrG proteins have effector protein functions. Consistent with this hypothesis, VgrG-1 of *V. cholerae* has an actin cross-linking domain, which has been experimentally shown to be functional causing actin cross-linking of the cytoskeleton of the host cells (Sheahan, Cordero et al. 2004). Interestingly, the actin cross-linking

domain is dispensible for T6S by this pathogen (Ma, McAuley et al. 2009). The activity of VgrG-1 is dependent upon a functional secretion system suggesting VgrG-1 is delivered into the host cell by the T6SS. Further suggesting an effector function of VgrG proteins, several VgrG proteins have C-terminal domains with predicted similarity to proteins involved in virulence-associated functions (Pukatzki, Ma et al. 2007). These extensions show similarity to proteases, a *Mycobacterium* virulence factor of unknown function, and eukaryotic tropomyosin suggesting a possible role of the latter in cytoskeleton manipulation. In addition, several VgrG proteins have extensions with homology to bacterial proteins involved in adhesion suggesting a possible interaction with the membrane of host cells (Pukatzki, Ma et al. 2007).

How VgrG proteins exert their effector functions is not known, however, if VgrG proteins localize similarly to their bacteriophage homologues, these proteins would be located at the tip of the secretion apparatus. A mechanism has been proposed whereby the C-terminal effector domain of VgrG is released by proteolytic cleavage after insertion into the membrane of the host cell (Pukatzki, McAuley et al. 2009). Another suggested function of VgrG proteins is to penetrate the bacterial cell wall before secretion or serving as a cap controlling T6S (Pukatzki, McAuley et al. 2009). Finally, it is possible VgrG proteins are involved in puncturing of host cell membranes leading to pore-formation, which effector molecules could transit through (Pukatzki, Ma et al. 2007). Given multiple copies of v*grG* exist in several bacterial species, attributing a function to individual VgrG proteins by a mutagenesis approach could be a major challenge in some bacteria. In *V. cholerae,* deletion analysis of *vgrG* has proven successful in identifying

vgrG-1 and *vgrG-2* as required for virulence and secretion of Hcp, whereas *vgrG-3* appears dispensable (Pukatzki, Ma et al. 2007).

The major secreted protein of the vast majority of T6SSs investigated is Hcp. (Mougous, Cuff et al. 2006; Pukatzki, Ma et al. 2006; Schell, Ulrich et al. 2007; Wu, Chung et al. 2008). Hcp and Hcp-like proteins appear to have some interesting biochemical properties. Similarly to secreted VgrG proteins, Hcp lack a canonical signal peptide, suggesting secretion of these proteins occur in a one-step process independent of an export machinery such as Sec. Additionally, several isoforms of Hcp proteins exist in for example *V. cholerae* and Entero aggregative *E. coli*. Further, Hcp proteins have predicted molecular masses of 28kDa, but migrate unexpectedly at around 18 kDa in SDS-PAGE gels (Dudley, Thomson et al. 2006; Pukatzki, Ma et al. 2006).

The structure of Hcp has been solved demonstrating it forms a hexameric donut-shaped protein (Mougous, Cuff et al. 2006). Stacked Hcp proteins have been proposed to assemble into a membrane-spanning channel which other proteins are proposed to be transported through (Mougous, Cuff et al. 2006; Leiman, Basler et al. 2009). The fact that Hcp can polymerize to form tubes supports this notion (Ballister, Lai et al. 2008). These tubes are remarkably similar to the tail tube of bacteriophages (Kostyuchenko, Chipman et al. 2005) and, further, the crystal structure of Hcp is highly similar to a tail tube protein (Leiman, Basler et al. 2009). Therefore, current evidence suggest Hcp forms a structure related to bacteriophage tail tubes, which forms a secretion channel spanning the cell wall of the bacterium.

Adding complexity to the T6SS story, some bacteria lack an Hcp protein associated with T6S, and different proteins appear to be secreted by these systems. A

notable example is secretion of AaiC by a T6SS of Enteroaggregative *E. coli* (Dudley, Thomson et al. 2006). Although AaiC does not show similarity to any protein found in databases at the amino acid sequence level, its structure is predicted to be similar to Hcp according to our analysis using HMM (probability of 97). Interestingly, AaiC has a comparable migration pattern in SDS-PAGE gels to Hcp proteins (Dudley, Thomson et al. 2006). Although very little is known regarding AaiC it is possible AaiC forms a structure of the T6SS of EAEC similar to the proposed tube or channel formed by Hcp proteins in other T6SSs.

An additional protein identified as secreted by a T6SS is EvpP of *E. tarda***.** This protein interacts with Hcp inside the cell and extracellularly after secretion. However, EvpP is not required for secretion of VgrG or Hcp (Zheng and Leung 2007). Similarly to AaiC and Hcp proteins, EvpP runs aberrantly in SDS-PAGE gels, and has an apparent molecular weight closely matching Hcp. One could speculate EvpP interacts with Hcp to form the tip of a putative secretion channel of this pathogen.

Hcp proteins show high variability in amino acid content (Filloux, Hachani et al. 2008). Indeed, Hcp-like proteins of some organisms, including a *V. anguillorum* Hcp-like protein and AaiC of EAEC are not identified as similar to Hcp by BLAST analysis (Weber, Hasic et al. 2009). It is possible surface-localization and therefore exposure to immune cells has rapidly driven divergence of these proteins. Current data suggest Hcp forms a conduit together with VgrG, which other proteins may be transported through. Surface-exposure of Hcp and VgrG could account for the presence of these proteins in the supernatant of bacteria analogously to secretion of surface-exposed appendages of T3SS and T4P (Fernandez and Berenguer 2000; Filloux, Hachani et al. 2008). Consistent

with a role of Hcp as a structural protein, *hcp* is required for secretion of VgrG proteins of *V. cholerae* and of EvpP of *E. tarda* (Zheng and Leung 2007). Supporting a role of Hcp forming polymers, which create a membrane-spanning secretion channel, biochemical and microscopy evidence show Hcp is localized to the periplasmic space and to the membrane of the bacterium (Mougous, Gifford et al. 2007; Wu, Chung et al. 2008).

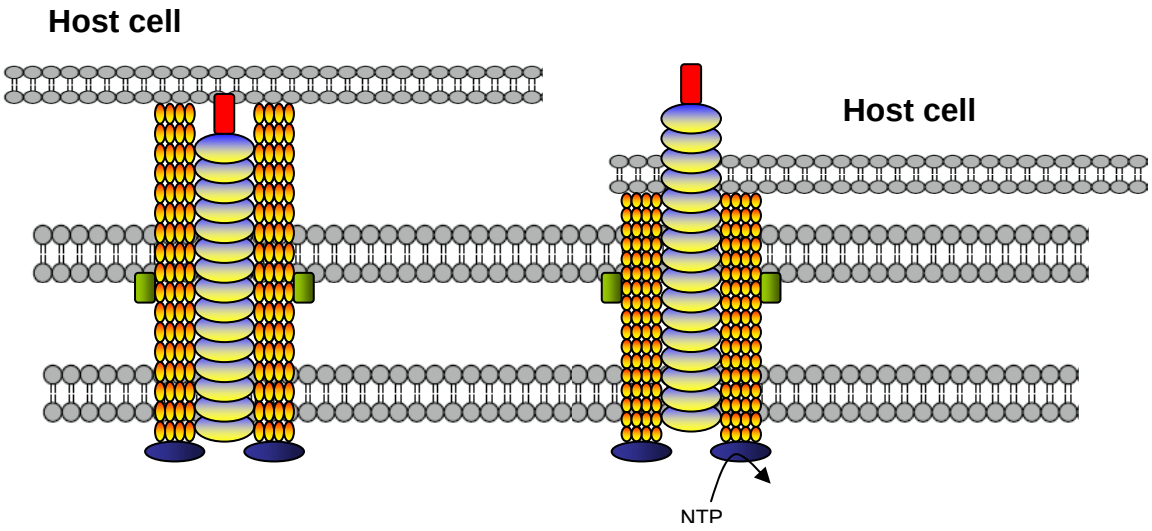

Figure 4. Model for T6S into host cells. Color-coded molecules are conserved T6SS components. Orange, IglA and IglB; blue/yellow, Hcp, dark blue, ClpV; green, lipoprotein; red, VgrG. After attachment to a host cell, ClpV-catalyzed contraction of IglA and IglB multimers together with the hole-puncturing ability of VgrG pierces the membrane of the host. Unidentified effector molecules could potentially be translocated through the hollow central Hcp secretion channel. Model is based upon published data introduced in this chapter.

1.5.2 Non-secreted core proteins

Most T6SS clusters contain the genes *icmF, dotU, iglA, iglB, sciB, sciC, clpV* and *sciN* encoding a predicted lipoprotein (Bingle, Bailey et al. 2008; Boyer, Fichant et al.

2009). None of these proteins have been found to be secreted into the growth supernatant of bacteria, but all of these proteins have been shown to be required for secretion of Hcp and/or VgrG in at least one study; therefore we will refer to theses proteins as non-secreted core proteins of T6SSs (Mougous, Cuff et al. 2006; Zheng and Leung 2007; Aschtgen, Bernard et al. 2008; Bonemann, Pietrosiuk et al. 2009). There is much division in nomenclature between T6SS homologues of different organism. For simplicity, we will use T6SS *Salmonella* nomenclature (*sci)* to identify genes and proteins when no consensus exists, with the exception of *iglAB* homologous proteins as they are so named in *Francisella.*

T6SS gene clusters were initially named IcmF-associated homologous proteins to signify these clusters contain homologues of the T4SS protein IcmF (and DotU), but no other T4SS homologues are encoded in theses clusters thereby distinguishing T6SSs from T4SSs (Das and Chaudhuri 2003; Pukatzki, Ma et al. 2006). IcmF and DotU proteins are accessory components of type IVB secretion systems (Sexton, Miller et al. 2004). Whereas *icmF* and *dotU* are only partially required for T4S, these two genes are absolutely required for T6S in all organisms examined (Zheng and Leung 2007; Wu, Chung et al. 2008). By definition, proteins required for secretion are deemed part of the secretion machinery (Cascales 2008). Therefore, it seems likely IcmF and DotU of T6SSs are important apparatus/machinery components. In several studies, additional machinery components of T6SSs have been identified, which include the aforementioned IglAB, SciBC, ClpV, and SciN proteins (Mougous, Cuff et al. 2006; Zheng and Leung 2007; Aschtgen, Bernard et al. 2008; Bonemann, Pietrosiuk et al. 2009). Although one could extend the findings regarding a T6S component studied in one organism to its

homologues, caution should prevail since there may be subtle differences of the roles of proteins between systems. Indeed, IglB has been shown to be required for T6S in several organisms, including *Edwardseilla tarda* and EAEC; however, an *iglB* mutant of *Burkholdeira* still secretes Hcp (Dudley, Thomson et al. 2006; Schell, Ulrich et al. 2007; Zheng and Leung 2007). However, since there are six copies of *iglB* in this organism, it is possible a homologue from a different T6SS cluster can complement the mutational defect. Such complementation or functional redundancy is not unprecedented in a T6SS context as mutagenesis of both *V. cholerae hcp* genes was required to render this organism avirulent (Pukatzki, Ma et al. 2006). Consistent with this observation, re-introducing a single copy of *hcp* on a plasmid was sufficient to restore secretion of VgrG proteins by this pathogen (Pukatzki, Ma et al. 2006).

A comprehensive mutagenesis study of *Edwardsiella tarda* demonstrated 13 out of 16 genes of the T6SS cluster of this organism are required for secretion (Zheng and Leung 2007). Among these are two core genes, *sciB* and *sciC*, which are found in nearly every T6SS cluster analysed (Boyer, Fichant et al. 2009). Curiously, other than being required for secretion in several organisms, no experimental evidence regarding the proteins encoded by these genes exists (Schell, Ulrich et al. 2007; Zheng and Leung 2007). Speculating a possible role of these proteins in T6S, it is notable consensual genomic organization analysis links *sciBC* together with *sciD* (Boyer, Fichant et al. 2009). Bioinformatics analyses suggest SciD is highly similar to the bacteriophage base plate protein gp25 (Leiman, Basler et al. 2009). The tail tube of bacteriophage T4 threads through six gp25 subunits, which form a ring surrounding the tube (Leiman, Chipman et al. 2004); therefore, SciD appears to be an apparatus protein possibly associated with one

of the bacterial membranes. Given gene synteny suggests a common function of encoded proteins it is possible SciBC form portion of the secretion apparatus together with SciD.

The majority of T6SS clusters encode a predicted lipoprotein called SciN (Cascales 2008; Boyer, Fichant et al. 2009). Recently, the EAEC homologue of this protein was experimentally shown to be a lipoprotein, which inserts into the outer membrane and is exposed in the periplasmic space (Aschtgen, Bernard et al. 2008). It is possible the lipoprotein forms an outer membrane pore allowing the secretion apparatus to encompass the lipid bilayer. Consistent with this hypothesis, the lipoprotein is required for Hcp secretion of EAEC (Aschtgen, Bernard et al. 2008). A yeast two-hybrid analysis of *E. tarda* suggests SciN, IglA, IcmF and DotU interact indicating these proteins may form a membrane-associated secretion apparatus complex (Zheng and Leung 2007). Although no further details regarding this putative secretion machinery was shed from this study, it is clear a ClpV protein plays an important role in T6S of several organisms (Mougous, Cuff et al. 2006; Zheng and Leung 2007; Bonemann, Pietrosiuk et al. 2009).

ClpV is similar to protein disaggregation chaperones and belong to the ring forming family of ATPases (Schlieker, Zentgraf et al. 2005). It has been hypothesized ATP hydrolysis by ClpV could drive substrate or effector translocation of T6SSs (Mougous, Cuff et al. 2006). Recently, a role of ClpV in context of IglAB assembly or interaction was demonstrated (Bonemann, Pietrosiuk et al. 2009). Purified IglA and IglB of *Vibrio cholerae* form large tubular structure with a central pore (Bonemann, Pietrosiuk et al. 2009). This structure is composed of 12-pronged cog-wheeled subunits with a large internal diameter. Interestingly, this structure is strikingly similar in shape and

dimensions to the tail sheath of bacteriophages (Kostyuchenko, Chipman et al. 2005). Additionally, IglB is predicted by the HMM to be similar to a tail sheath protein suggesting a possibility IglB is part of such a structure. The bacteriophage tail sheath forms a cylinder surrounding the tail tube (Kostyuchenko, Chipman et al. 2005). Upon attachment to a bacterium the tail sheath contracts and exposes the static tail tube (Kostyuchenko, Chipman et al. 2005). One possibility is a similar contraction mechanism of IglA and IglB polymers exposes Hcp, which is structurally similar to the tail tube, resulting in Hcp secretion into the growth supernatant during T6S of bacteria. Supporting this hypothesis, data reported indicate IglAB form a high molecular mass complex when purified *in vitro* in the absence of ATP (Bonemann, Pietrosiuk et al. 2009). In the presence of ATP and purified ClpV, the large tubular structure disintegrates and IglAB no longer migrate as a high molecular mass complex (Bonemann, Pietrosiuk et al. 2009). In view of this data, it is possible the threading of IglAB driven by ClpV and ATP observed in *V. cholerae* is analogous to the tail sheath contraction of bacteriophages (Aksyuk, Leiman et al. 2009). The ClpV protein evidently causes a conformational change in IglAB, which possibly could account for Hcp secretion. Altogether, it appears IglA, IglB and Hcp form a cell wall-spanning channel of T6SSs similar in structure to the tail of bacteriophages (Filloux, Hachani et al. 2008; Bonemann, Pietrosiuk et al. 2009; Leiman, Basler et al. 2009).

Current available data supports a model where T6SSs form a membrane-spanning complex with some general similarities to T3 and T4SSs (Fig.4). For example, since T6S requires contact between a bacterium and a target host cell to elicit a phenotype, there appear to be similarities between T6SSs and T3 and T4SSs in the mechanism of secretion

(Pukatzki, Ma et al. 2006). Furthermore, similarly to T3S and T4S current evidence supports the notion T6S occurs by a one-step process through a multi protein complex spanning both the inner and outer membranes. However, unlike other bacterial secretion systems, which have evolved from bacterial organelles, T6SS are evolutionary related to bacteriophages highlighting only general parallels should be drawn to other secretion systems. Given the many opportunities of gene shuffling of genetic elements one possibility is T6SS clusters represent chimeric fusions of genes encoding phage proteins and other bacterial factors. In support of this notion, not all T6SS proteins show similarity to those of bacteriophages; further, two essential T6SS proteins IcmF and DotU are homologous to proteins encoded by T4SSs, which are related to conjugation machineries.

1.5.3 Regulation of T6SS

Genetic manipulations, specifically over-expression of an activator has been necessary to identify secreted proteins of a number of T6 organisms suggesting T6S is tightly regulated and responsive to certain signals not present *in vitro* (Mougous, Cuff et al. 2006; Schell, Ulrich et al. 2007). Indeed, many T6SSs have been shown to be induced during *in vivo* conditions (Parsons and Heffron 2005; Pukatzki, Ma et al. 2006; Shalom, Shaw et al. 2007). Recently, it was demonstrated phagocytosis and bacterial internalization is required for VgrG-1 translocation by *V. cholerae* suggesting the bacterium responds to the intracellular environment to accomplish translocation (Ma, McAuley et al. 2009). Supporting tight regulation of secretion, a regulatory mechanism involving phosphorylation activating secretion of the *Pseudomonas* HIS-1 T6SS has been

identified (Mougous, Gifford et al. 2007). Given presence of homologous genes in other organisms, a similar regulatory mechanism appears to control secretion by a number of T6SSs, but is far from universal.

It has been shown T6SSs of *Salmonella* and *P. aeruginosa* are reciprocally regulated with T3SSs by two-component regulatory systems suggesting a functional relationship between systems (Parsons and Heffron 2005; Mougous, Cuff et al. 2006). For example, whereas T3SS are required for immediate infection, a T6SS of *P. aeruginosa* is involved in chronic infection of this pathogen (Mougous, Cuff et al. 2006). Additionally, other systems are induced by temperature (*Rhizobium leguminosarum*) or plant extracts (*Pectobacterium atrosepticum*) (Bladergroen, Badelt et al. 2003; Mattinen, Somervuo et al. 2008). Finally, it is noteworthy some clusters carry their own regulatory element, generally the sigma 54 alternative activator of RNA polymerase (Cascales 2008). The controlled regulation of T6SSs likely serves to coordinate secretion in a timely manner since inappropriate expression could potentially be harmful to the bacterium.

1.5.4 Phenotypic contributions of T6SSs

In many bacterial species, including *Pectobacterium* spp*., E. tarda, P. aeruginosa,* and *Vibrio cholerae,* mutagenesis analysis suggest T6SS clusters are involved in virulence although exactly how remains to be determined (Mougous, Cuff et al. 2006; Zheng and Leung 2007; Cascales 2008; Mattinen, Somervuo et al. 2008). Some studies have concluded specific T6SS-associated phenotypes. For example, in *Salmonella,* the T6SS limits intracellular spread of the bacterium. Inactivation of this

T6SS also causes hypervirulence in mice suggesting it is involved in mediating persistence in the host (Parsons and Heffron 2005). Additionally, a T6SS of *Aeromonas hydrophila* is required to prevent phagocytosis of this organism by immune cells (Suarez, Sierra et al. 2008). In the fish pathogen *Vibrio anguillurium,* T6S is not required for virulence, but instead is involved in stress responses, whereas in *Vibrio parahaemolyticus* this cluster appears to be necessary for biofilm formation (Weber, Hasic et al. 2009). The wide variety of functions mediated by T6SSs may be due to differences in effectors secreted by these systems. However, to date no evidence of effector secretion by T6SS outside of VgrG-1 of *V. cholerae* has been demonstrated. The T6SS of *Burkholderia mallei* is required for virulence and a gene encoding a protein with deubiquitinase-activity, TssM, is found within this cluster (Schell, Ulrich et al. 2007). It has been suggested this protein could interfere with the ubiquitin-proteasome degradation system of the cell thereby altering cell signaling (Schell, Ulrich et al. 2007). However, surprisingly, TssM secretion is not dependent upon the T6SS, therefore, its role as a T6SS effector must be regarded as controversial (Shanks, Burtnick et al. 2009).

In agreement with the notion T6SSs are mobile genetic elements it is clear T6SSs confer a selective advantage to the harboring bacterium. In turn, this provides a rational explanation for the high genetic variability between systems. Given the proven selective advantage of T6SSs there is reason to believe T6SS clusters are functional in many bacterial species. Overall, T6SS appear to be important players in the on going struggle of bacteria versus host and in bacterial adaptation to environmental conditions.

1.6 *Francisella* secretion systems

As a Gram negative bacterium, *F. tularensis* is expected to possess secretion systems enabling communication and protein translocation. The following section summarizes published data regarding secretion and *Francisella.*

1.6.1 T2SS

T2SSs mediate secretion of important virulence factors in a number of bacteria. A secretion system of *F. novicida* apparently similar to T2SS/T4P of other organisms has been characterized, which appears to be non-functional in other subspecies of *Francisella* (Hager, Bolton et al. 2006). This system is related to type IV pilin, and includes the components *pilF, pilG, pilQ,* and *pilA* encoding an ATPase, an inner membrane spanning component, an outer membrane secretin and a fiber formation protein, respectively, as inferred by homology to T2SS/Tfp genes of other organism (Hager, Bolton et al. 2006). Wild type *F. novicida* secretes high levels of chitinases, as well as proteins of unknown function (Fsps) and the protein PepO into the supernatant during broth growth, whereas mutagenesis of *pilF, pilG, pilQ,* or *pilA* disrupts this secretion (Hager, Bolton et al. 2006). This data supports the role of *pilFGQA* in an *F. novicida* T2SS rather than functioning as a T4P biogenesis machinery.

Chitinases of protozoans parasitizing the mosquito gut are important virulence factors; therefore, a similar role of the *F. novicida* secreted chitinases has been proposed, but not molecularly demonstrated (Hager, Bolton et al. 2006). In contrast, the secreted protein PepO was shown to have metalloprotease activity (Hager, Bolton et al. 2006). Several biological observations have lead to the hypothesis a loss of *pepO* contributes to

increased virulence of *F. tularensis* as compared to *F. novicida.* For one, *pepO* is a pseudogene in clinically relevant subspecies; additionally, an *F. novicida pepO* mutant is more virulent than wild type *F. novicida* and has increased organ burden loads compared to infection with wild type bacteria (Hager, Bolton et al. 2006). Furthermore, since PepO was shown to have metalloprotease activity, a role of PepO in vasoconstriction leading to increased inflammation and virulence attenuation of *F. novicida* has been postulated (Hager, Bolton et al. 2006). However, in studies performed by others, independently generated *pepO* mutants are avirulent (Brotcke, Weiss et al. 2006; Zogaj, Chakraborty et al. 2008). Different virulence assays were employed between studies, which may explain the discrepancy. Although proteomics analysis of supernatant proteins did not find evidence of T2SS by *F. holarctica* or *F. tularensis,* it should not be ruled out these systems are tightly regulated and functional in these clinically relevant subspecies as well.

1.6.2 Type IV pilin (T4P)

Pilin are secreted surface structures involved in motility, adhesion, and virulence of a number of bacterial species. Sequencing of several *Francisella* genomes revealed a number of genes potentially involved in T4P biogenesis. Some molecular aspects of the T4P system of *Francisella* have been unraveled; however, there is a lack of knowledge regarding the role and contribution of individual genes to pilin biogenesis and function in *Francisella*. Specifically, controversy exists regarding the gene encoding the major pilin subunit. PilA of *F. holarctica* is a surface-exposed protein proposed to be the major pilin subunit (Forslund, Kuoppa et al. 2006). Indeed, PilA proteins generally encode the major

pilin subunit of T4P of bacteria (Fernandez and Berenguer 2000). However, *F. holarctica* LVS is a natural *pilA* mutant, yet it produces T4P appendages on its surface (Chakraborty, Monfett et al. 2008).

Deletions of LVS *pilF,* homologous to ATPase assembly protein and, unexpectedly, deletion of *pilT,* encoding a putative ATPase pilin disassembly protein, are both required for pilin secretion to the surface. Consistent with an important role of T4P to *Francisella* biology, *pilA, pilT* and *pilF* of LVS are required for virulence of this bacterium. In addition, *pilF* and *pilT* are defective for adherence to macrophages suggesting a role of T4P in adhesion to host cells of this pathogen (Chakraborty, Monfett et al. 2008).

As introduced above, there is controversy regarding the molecular aspects of *Francisella* T4P formation. Studies on *F. novicida* T4P have revealed *pilT* and *pilF* are required for pilin appendage formation as described for *F. holarctica* LVS (Chakraborty, Monfett et al. 2008; Zogaj, Chakraborty et al. 2008); additionally, a *pilE4* mutant.but not a mutant of a *pilA* homologue, lacks surface appendages therefore suggesting *pilE4* encodes the major pilin subunit of *F. novicida* T4P (Zogaj, Chakraborty et al. 2008). Several putative *pilA* genes exist in *F. novicida,* but all differ significantly at the amino acid level from *F. holarctica* PilA, and anti-LVS-PilA does not react with *F. novicida* surface proteins suggesting a different role of PilA homologous proteins of *F. holarctica* and *F. novicida* (Forslund, Kuoppa et al. 2006). Indeed, the role of *F. novicida* PilA-like protein appears to be restricted to a T2SS described above (Hager, Bolton et al. 2006; Zogaj, Chakraborty et al. 2008). Although *F. holarctica* secretes PilA to its surface, it is presently not clear whether *F. holarctica* PilA constitutes the major pilin subunit in this

subspecies, therefore, its role in T4P and T2S deserves further investigation (Forslund, Kuoppa et al. 2006). Complicating matters, it was recently shown introduction of *pilA* of either *F. tularensis* or *F. novicida* complements T4P secretion of a *N. gonorrhoeae pilA* mutant suggesting PilA is an important surface-exposed protein of both *F. novicida* and *F. tularensis* (Salomonsson, Forsberg et al. 2009).

There are numerous examples of functional overlap between T4P and T2SSs, and such overlap could be expected between a *Francisella* T2SS and T4P given how closely these two systems appear to be related evolutionarily (Fernandez and Berenguer 2000). Currently, the only molecular evidence of cross-talk between the T2S and T4P systems of *Francisella* is the observation a *pilF* mutant of *F. novicida* defective for pilin fiber biogenesis is also incapable of T2S (Zogaj, Chakraborty et al. 2008). Therefore, there appear to be two separate systems with limited redundancy for T2 and T4P secretion of *Francisella.*

1.6.3 Efflux pumps, AcrAB, TolC and T1SS

RND efflux systems are tripartite transporters consisting of an integral cytoplasmic membrane RND pump protein, a periplasmic membrane fusion protein (AcrAB), and an outer membrane pore protein homologous to *Escherichia coli* TolC, which are physically linked on the chromosome (Fernandez-Recio et al., 2004). In addition to their role in efflux pump systems some TolC proteins can also form the outer membrane pore of T1SSs involved in transport of important virulence factors in a number of pathogens (Desvaux, Parham et al. 2004). The role of *Francisella* TolC homologues and AcrAB RDN efflux pumps in antimicrobial defenses, intramacrophage growth and

virulence has been investigated (Gil, Platz et al. 2006; Bina, Lavine et al. 2008; Qin, Scott et al. 2009). Neither component is required for intracellular replication. AcrAB, as well as the outer membrane protein TolC, and the TolC-like protein FtlC are required for resistance to anitimicrobial compounds and antibiotics indicating they encode multidrug resistance efflux pumps (Gil, Platz et al. 2006; Bina, Lavine et al. 2008; Qin, Scott et al. 2009). There is no biochemical evidence AcrAB, TolC and FtlC of *Francisella* participate in the same secretion system. Additionally, *Francisella* harbors several TolC homologues and none of these are physically linked with either AcrAB or each other as may be expected if performing a common function. Therefore, an additional role of TolC as an outer membrane component of a T1SS responsible for secretion of unidentified virulence factors has been proposed (Gil, Platz et al. 2006). However, if this were the case one would expect mutagenesis of *tolC* to result in avirulence of the bacterium. Indeed, mutagenesis of LVS *tolC* results in a strain less virulent than wild type bacteria in a mice infection study (Gil, Platz et al. 2006); however, a *tolC* mutant of *F. tularensis* is only marginally less virulent than wild type *F. tularensis* (Kadzhaev, Zingmark et al. 2009). Therefore, a role of TolC outside of multi drug resistance is under challenge. Given the present molecular and immunological data, a possible role of AcrAB and TolC working synergistically to expel a broad range of antibiotics and harmful compounds seems plausible.

Mutagenesis studies have also identified two putative T1SS components, which when inactivated rendered *F. novicida* defective for intracellular growth (Mdluli, Anthony et al. 1994). The mutated genes encode proteins similar to T1SS components of *E. coli,* ValAB. In *Francisella,* these two proteins indeed appear to encode a functional

secretion system evidently involved in transport of LPS to the outer membrane (McDonald, Cowley et al. 1997).

1.6.4 Type VI secretion and the FPI

Sequencing of the *F. tularensis* genome suggests an absence of many classical virulence factors such as toxins or type III and type IV secretion systems. As seen in the proceeding survey of *Francisella* secretion systems, it is clear novel factors are key players in *Francisella* secretion, which function differently from those of established model secretion systems (Chakraborty, Monfett et al. 2008). Previous studies have identified a number of genes, which when deleted or otherwise inactivated render *Francisella* unable to grow intracellularly in host cells (Gray, Cowley et al. 2002; Qin and Mann 2006; Tempel, Lai et al. 2006; Maier, Casey et al. 2007; Fuller, Craven et al. 2008). Many of these mutations occurs in genes located in the FPI, and a number of the genes of the FPI show similarity to those found in loci encoding T6SSs of other organisms, therefore suggesting a role of a putative secretion system in intracellular growth of *Francisella* (de Bruin, Ludu et al. 2007; Bingle, Bailey et al. 2008). Of the homologous genes, *iglAB, dotU* and *pdpB* (*icmF*-like) are found in every T6SS identified to date (Bingle, Bailey et al. 2008). However, several of the FPI genes do not show similarity to T6SS genes and *Fransicella* apparently lack genes encoding the important T6SS components Hcp and ClpV. Therefore, the relationship between the FPI and T6SS is worthy of investigation. Indeed, the assumption the FPI encodes a T6SS was recently challenged since there is only limited similarity between T6SS and FPI genes (Bingle, Bailey et al. 2008; Boyer, Fichant et al. 2009). Using genetic, biochemical and

microscopy approaches, the work presented in this thesis tested the hypothesis the FPI encodes a secretion system.

Chapter 2. The *Francisella* pathogenicity island protein IglA localizes to the bacterial cytoplasm and is needed for intracellular growth

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2.1 Introduction

Francisella tularensis is the etiological agent of the severe, febrile disease tularemia. Although there have been rare isolates of *F. tularensis* in Australia, tularemia is mainly a disease of the Northern hemisphere that is spread by blood-sucking mosquitoes, flies, and ticks or acquired from contact with infected animals such as rabbits, rodents, and beavers (Oyston, Sjostedt et al. 2004). Occasionally, local outbreaks of tularemia are associated with contact or consumption of contaminated natural water. In addition, *F. tularensis* is potentially a threat as a bioterrorist agent due to its high infectivity and lethality when inhaled. *F. novicida* is highly related at the DNA level to *F. tularensis*, and serves as a model organism since it is very virulent in mice while being avirulent in humans.

F. tularensis is a gram-negative, facultative intracellular bacterium capable of survival and replication in macrophages (Anthony, Burke et al. 1991). A common virulence strategy of intracellular pathogens is to favorably modulate the intracellular milieu of hosts for their own benefit. In *Legionella pneumophila,* a type IV secretion system (T4SS) delivers effectors that allow the pathogen to replicate in ribosome-studded phagosomes that fail to fuse with lysosomes (Segal, Purcell et al. 1998; Vogel, Andrews et al. 1998). *Salmonella enterica* relies on a pathogenicity island-encoded type III secretion system (T3SS) to modify phagosome biogenesis (Ochman, Soncini et al. 1996; Vazquez-Torres, Xu et al. 2000), including inhibition of phago-lysosomal fusion (Uchiya,

Barbieri et al. 1999) and the NADPH oxidase-mediated killing by host cells (Vazquez-Torres, Xu et al. 2000). Other intracellular pathogens, such as *Listeria monocytogenes,* degrade the phagosomal membrane and escape into the cytoplasm to replicate freely (de Chastellier and Berche 1994). *F. tularensis* initially resides in a phagosome which accumulates some late endosome markers. After about four hours most *F. tularensis* cells escape the phagosome and grow in the cytoplasm. (Anthony, Burke et al. 1991; Abd, Johansson et al. 2003; Clemens, Lee et al. 2004; Santic, Molmeret et al. 2005). Although an intact *iglC* gene is needed for *F. tularensis* to escape phagosomes, the role of IglC is unknown.

We recently described a *Francisella* pathogenicity island (FPI) harboring several genes necessary for intracellular growth. Four FPI genes, *iglABCD,* are organized in an apparent operon (Nano, Zhang et al. 2004). The production of IglC mRNA is in part dependent on MglA (Lauriano, Barker et al. 2004) which is thought to be a global regulator of virulence factors in *F. tularensis*. By analogy with its *Escherichia coli* homologue, SspA, MglA likely interacts with RNA polymerase to directly or indirectly alter transcription of several genes (Hansen, Qiu et al. 2005). Disruption of *mglA* or *mglB* results in mutants that are severely attenuated for virulence (Baron and Nano 1998). IglC has been shown to be induced about four-fold during intracellular growth relative to broth growth and necessary for virulence (Golovliov, Ericsson et al. 1997; Gray, Cowley et al. 2002; Cossart and Sansonetti 2004), and it was recently demonstrated that inactivation of *iglC* and *mglA* result in mutants that remain in phagosomes that fuse with lysosomes (Lindgren, Golovliov et al. 2004; Santic, Molmeret et al. 2005). Although an *iglA* transposon insertion mutant has been shown to be defective for intracellular growth,

it could not be ruled out that the observed phenotype was due to interruption of transcription of downstream genes, including *iglC* (Gray, Cowley et al. 2002).

In this study, we use *F. novicida* to investigate the properties of IglA and its role in *F. novicida* intracellular growth. *F. novicida* is particularly suited for these studies since, unlike *F. tularensis*, it contains only one copy of the FPI, and this simplifies the construction of mutants. Further, the biology of *F. novicida* growth in human macrophages is indistinguishable from that of *F. tularensis* strains and thus *F. novicida* serves as a valid surrogate for virulent strains when studying basic aspects of *Francisella* intracellular growth. In this work we supply evidence that IglA is a cytoplasmic protein that interacts with IglB, and is required for intramacrophage growth.

2.2 Materials and Methods.

All experiments conducted in this study were performed by Olle de Bruin.

2.2.1 Bacterial strains and culture conditions.

All strains used in this work are listed in Table 1. *F. novicida* strains were grown in trypticase soy broth supplemented with 0.1% cysteine (TSBC) or on trypticase soy agar supplemented with 0.1% cysteine (TSAC) unless stated otherwise. Kanamycin (45 μg/ml) or erythromycin (30 μg/ml) or 10 % sucrose were added as needed.

2.2.2 Subcellular fractionation.

1000 ml of overnight *F. novicida* U112 culture was harvested and resuspended in 50 ml of cold phosphate buffered saline (PBS). Cells were broken 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 x *g* at 4˚C, and a sample was taken as the total protein fraction. The lysate was subjected to ultracentrifugation (Beckman L8-70, rotor Type 45 Ti) for 1 hr at 100,000 x *g* at 4˚C to pellet the membranes. The supernatant (soluble protein fraction) was removed, whereas the membrane pellet was resuspended in 2.5 ml of 1% Sarkosyl (Sigma). The sarkosyl soluble (inner membrane) and the sarkosyl insoluble (outer membrane) were separated by ultracentrifugation for 1 hr at 100,000 x *g* at 4˚C in a Beckman TLA-100.3 ultramicrocentrifuge. The activity of the inner membrane-associated enzyme NADH oxidase was determined per mg of protein (McDonald, Cowley et al. 1997) for each of the fractions as a measure of the relative mixing of the different cell compartments. The soluble fraction contained 3%, the sarkosyl soluble membrane fraction 79% and the sarkosyl insoluble membrane fraction 18% of the NADH oxidase activity. In addition, we found that 90% of IglC was found in the soluble fraction (data not shown) and 10% was in the total membrane fraction. IglC could not be detected in the sarkosyl-soluble or sarkosyl-insoluble membrane fractions. As IglC has previously been shown to be a soluble protein (Golovliov, Ericsson et al. 1997), this served as another control of our fractionation experiment. Isolation of periplasmic proteins was performed as described by Nossal and Heppel (Nossal and Heppel 1966).

2.2.3 Co-immunoprecipitation.

500 µl of soluble fraction was pre-cleared by incubation with 20 µl protein-G/Agarose beads (40% slurry; EMB Bioscience, La Jolla, CA) and 10 µg nonspecific antibody for 1 h at room temperature (RT). Beads and bound proteins were removed by centrifugation and the soluble fraction was incubated with 10 µl rabbit anti-IglA serum or nonspecific antibody for 1 h at RT followed by addition of 75 µl protein-G/Agarose beads and incubation 1h at RT. Complexes were recovered by centrifugation, 6500 rpm, 3 min, and beads were washed three times with 150mM NaCl, 10mM Na_2H_3PO, pH 7.2. After the final wash, complexes were resuspended in 30 µl SDS-PAGE loading buffer and the sample was boiled for 5 min. Beads were removed by centrifugation and released proteins were separated on a 12% Sodium dodecyl sulphate-polyacrlamide electrophoresis (SDS-PAGE) gel. The immunoprecipitated material was examined by immunoblotting with anti-IglA to confirm that IglA was present (data not shown).

2.2.4 SDS-PAGE and Western blotting.

To normalize the amount of protein added to each lane, the concentration of protein samples were determined by use of the BCA assay (Pierce). SDS-PAGE was performed according to standard techniques. Separated proteins were transferred onto a Trans Blot® nitrocellulose (BioRad) or Immobilon-FL (Millipore) membrane and blocked with 5% skim milk (Difco) in PBS. Anti-IglA, and anti-IglC antibody were used at dilutions of 1:4,000 and 1:500 respectively. To detect bound antibody blots were incubated with IRDye800DX-conjugated goat anti-rabbit or IRDye700DX-conjugated

goat anti-rat immunoglobulin G (Rockland, Gilbertsville, Pa.) and visualized in a LiCor Odyssey imaging system.

2.2.5 MALDI-TOF.

Following SDS-PAGE separation of proteins in-gel digestion with trypsin was carried out, and peptides extracted. 10 µl of the peptide sample was loaded on to a C18 zip tip and washed three times in 10µl of 0.1% TFA and eluted with 2 µl of 50% ACN and 0.1% TFA containing 10 mg/ml 4-hydroxy alpha cyanocinnamic acid. MALDI-TOF MS analysis of the peptides was carried out using a Voyager-DE STR (Applied Biosystems, Foster City, CA). Mass fingerprint analysis was done using Mascot (Matrix Science, UK).

2.2.6 Construction of *iglA* deletion mutant.

IglA deletion mutant, ODB2, was constructed using a two-step integration-excision method. 1.5 kilobasepair (kbp) regions flanking *iglA* were amplified with primers iglA L-F 5' cgcggccgcagcaaaaatgctggaggtgt, iglA L-R 5' cctcgagcatcaaccttgaatttgggatt, for the left-hand flanking region, and with primers iglA R-F 5' cctcgagctcttgtgatgctgctgagtct, iglA R-R 5' cgcggccgcaataccagccaggcttaccc, for the right-hand flanking regions. These were cloned into plasmid pCR2.1 (Invitrogen) and verified by sequencing. The flanking regions were then joined by ligation. The flanking region construct was ligated to an erythromycin resistance-*sacB* cassette and the ligation mixture was used to chemically transform *F. novicida* JL0 to erythromycin resistance as previously described (Mdluli, Anthony et al. 1994). The JL0 strain (Ludu et al.,

unpublished data) is a derivative of the *F. novicida* U112 prototype strain that has a deletion in a sucrose hydrolase gene, and thus is sensitive to *sacB* expression in the presence of sucrose. An erythromycin resistant colony was grown and plated on TSAC containing 10% sucrose which acts as a counter selective marker for the *sacB* gene. Sucrose sensitive strains were examined for loss of *iglA* by PCR.

The *iglA* and *iglB* allelic replacement mutants, ODB7 and ODB1, were constructed as previously described (Lauriano, Barker et al. 2004). Briefly, 1.5kbp regions flanking *iglB* were PCR amplified with primers iglB L-F 5' cgcggccgcgaagaagataattcttcttctgaaaccg, iglB L-R 5' cctcgag attgtcataacaaaatcctctctactt, iglB R-F 5' cctcgagtgactatagatactaggcttgaacca, iglB R-R 5' cgcggccgctcaaaggcttttggaaatcaa incorporating Xho I sites and ligated to an erythromycin resistance cassette with added Xho I sites. *F. novicida* U112 was transformed with the construct as previously described (Mdluli, Anthony et al. 1994). The same primers used for construction of ODB2 were used for ODB7.

2.2.7 *In cis* complementation.

IglA and its promoter region were amplified with primers IglA int-L 5' CCCCTCGAGAGCCGTTTTCAATATTGGTTT and IglA int-R 5' CCCCTCGAGCAACTTCTGTAGATCCCCCAAA incoporating added XhoI sites and ligated to a kanamycin resistance cassette carrying a *F. novicida* promoter (Ludu et al., unpublished data). The construct was used to transform ODB2 as previously described (Mdluli, Anthony et al. 1994).

Figure 5. IglA regulation by MglA and MglB. Western blot showing lack of IglA in *mglA* and *mglB* mutants but present in the wild type strain U112. All samples were normalized to 6 μg protein per lane.

2.2.8 Macrophage infection assay.

Macrophage infection assays were performed essentially as described previously (Anthony, Burke et al. 1991). Briefly, J774.1 mouse macrophage-like cells were infected with *F. novicida* strains at a multiplicity of infection of 50:1 (bacterium-to-macrophage), and monolayers were incubated for 2 h in Dulbecco's Modified Eagle Medium containing 10% fetal bovine serum (DMEM), washed five times in Dulbecco's Phosphate Buffered Saline (DPBS), and incubated at 37 ˚C in 5% CO_2. Macrophages were lysed in 0.1% deoxycholate at 0, 24, 48 and 72 h post infection. To determine bacterial growth, lysed macrophages and culture supernatants were serially diluted in DPBS and plated on TSAC. As *F. novicida* does not grow in DMEM, this allows for an adequate determination of intracellular growth (Anthony, Burke et al. 1991).

2.2.9 Chicken embryo infections.

Fertilized White Leghorn eggs were obtained from the University of Alberta Poultry Research Station. Seven-day old embryos were injected under the chorioallantoic membrane with various doses of 100 µl of *F. novicida* diluted in PBS as previously described (Nix, Cheung et al. 2006). The embryos were monitored for death for 6 days.

2.3 Results.

2.3.1 IglAB homologues in diverse bacteria are organized in a conserved gene cluster.

Homologues of *iglA* and *iglB* exist in several bacterial species that are either animal or plant pathogens or plant symbionts, but there are no known homologues of *iglC* or *iglD*. IglAB homologues in *Vibrio cholerae, Salmonella enterica, Rhizobium leguminosarum,* and other bacteria are found in a cluster of genes encoding proteins known as IcmF-associated homologous proteins (IAHPs) (Folkesson, Lofdahl et al. 2002; Das and Chaudhuri 2003; Sexton, Miller et al. 2004). Recently, it was demonstrated that this gene cluster encodes components of a T6SS in *Vibrio cholerae* (Pukatzki, Ma et al. 2006).

In light of the emerging role of IAHP/T6SS in the secretion of proteins we re-examined the ORFs in the FPI to determine if components of a type VI secretion system may be present. Three essential components of a T6SS are a protein with an IcmF-motif and two linked genes that correspond to *iglA* and *iglB*. A BLASTP search revealed that an IcmF region was found as part of the C-terminal third of PdpB which aligned with the

corresponding regions of proteins belonging to the IcmF conserved orthologous group (COG3523.2 with an E-value of 7 X 10^{-9}). The identification of IglA and IglB as members of COGs is much clearer. IglA has strong identity to members of COG3516 (E-value of 2 X 10^{-20}) and IglB has strong identity with COG3517 (E-value of 2 X 10^{-102}). Remarkably all of the relatives of *iglAB* are organized in the same order, and are always adjacent to each other on the chromosome. The *iglAB* genes together with an *icmF*-containing gene form the core set of genes that suggest the presence of a type VI secretion system. We also found through BLASTP analysis that the deduced product of an ORF 380 bp downstream of *pdpB* shows a weak similarity (E-value 0.15) to the family of *vgr*-encoded proteins, such as VgrG, which is secreted by a T6SS in *V. cholerae*. Vgr proteins are hydrophilic proteins that contain valine-glycine repeats, and are found in a number of gram negative pathogens. Another ORF, 4587 bp downstream of *pdpB* show similarity (E-value, 0.0005) to proteins in COG3455 that includes the IAHP-associated protein DotU. The clustering of *iglAB* and the *icmF*-containing *pdpB* gene, together with two other IAHP-associated genes strongly suggests that the FPI carries a type VI secretion system.

2.3.2 IglA expression in an *mglAB* background.

Previously, RT-PCR analysis of the level of *iglA*, *iglC* and *iglD* transcripts revealed a role of MglA in regulating expression of the *iglABCD* operon mRNA production (Lauriano, Barker et al. 2004). We wished to test if IglA protein expression levels are depressed in mutant *mglA* and *mglB* backgrounds. Western immunoblot

analysis of IglA in an *mglA* mutant and an *mglB* background revealed that IglA is not expressed at detectable levels in these strains (Fig. 5).

2.3.3 IglA expression during intramacrophage growth.

Previous studies provide evidence that MglA expression peaks at about 5 hours after infection of macrophages (Baron and Nano 1999), and that IglC expression is maximal at between 6 and 24 hours after infection (Golovliov, Ericsson et al. 1997). To access the pattern of IglA expression during *F. novicida* infection of macrophages, we lysed J774 macrophages at various time points after infection with the wild type strain U112 and examined the lysates for IglA using immunoblotting. In our assays IglA was first detectable at 8 hours post-infection, peaked at 10 hours, and showed a decline by 12 hours (Fig. 6). In broth grown cultures IglA appeared to be maximally expressed at the late logarithmic phase of growth.

2.3.4 IglA is cytoplasmically located.

Knowing the cellular localization of a protein can help lead to a hypothesis as to its biological role. To investigate the subcellular localization of IglA, we fractioned *F. novicida* U112 into soluble and membrane-associated fractions and determined the amount of IglA in each fraction by immunoblot analysis. The data from this experiment revealed that IglA is exclusively a soluble protein (Fig. 7). Although IglA lacks a signal peptide sequence, it could not be ruled out that IglA localizes to the periplasm by a novel mechanism. Therefore, we isolated the periplasmic contents from *F. novicida* and determined by immunoblotting that IglA does not localize to this compartment. We also

failed to detect IglA in culture supernatant (data not shown). The data from these experiments strongly suggest that IglA is a cytoplasmic protein. In agreement with this, the IglA homologue in *Salmonella enterica* has been predicted to be localized to the cytoplasm (Folkesson, Lofdahl et al. 2002).

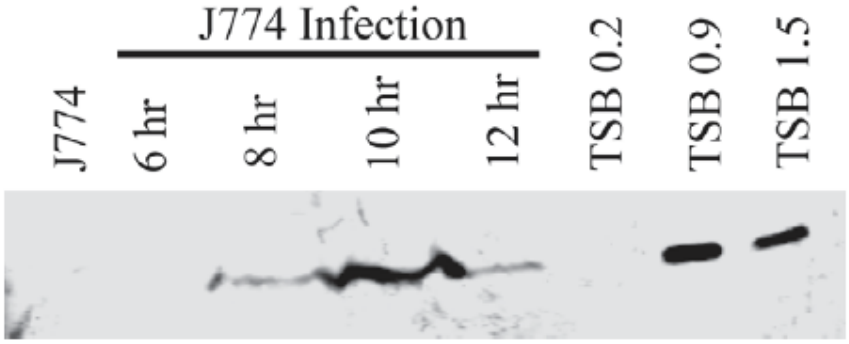


Figure 6. IglA expression in J774 macrophages. Western blot showing expression of IglA during infection of macrophages. J774 macrophages were infected with parent strain U112 (m.o.i 300:1) and lysed at the indicated time post infection. Loading was normalized according to the number of viable bacteria (CFU) in each sample as determined by plating on TSA-C plates. Lane J774, uninfected macrophages. TSB, broth grown U112 grown to indicated optical density (600 nm). All samples were normalized to 10^7 CFU by viable counts. The macrophage cell lysates altered the appearance of the IglA bands, but control experiments showed that the cell lysates did not mask IglA reactivity with antibody.

2.3.5 IglA interacts with IglB

To investigate interactions of IglA with other *F. novicida* proteins we performed immunoprecipitations with anti-IglA antibody on soluble proteins. A co-precipitating protein with a relative molecular mass of approximately 60 was detected (Fig 8A). This

protein band was excised and subjected to MALDI-TOF analysis, and the resulting peptide fragment masses were submitted to searches against predicted peptide fragments of prokaryotes in the MASCOT data bank. This analysis revealed that the only significant match was IglB from *F. novicida* (Fig. 8B). The relative molecular mass of the co-precipitated protein is consistent with this result as IglB is predicted to be 58 kDa. Immunoprecipitations performed with an *iglA* null strain did not result in the appearance of the 60 kDa band, nor did immunoprecipitations of U112 done with pre-immune serum. These results strongly suggest that IglA and IglB interact in the cytoplasm of *F. novicida.* Supporting the hypothesis that IglA interacts with IglB is the finding that IglB mutants but not IglC mutants lack detectable IglA (see below, Fig. 8). Presumably a lack of association of IglA with IglB makes the former susceptible to degradation.

2.3.6 Deletion mutagenesis of *iglA* and complementation of the mutant strain.

An *iglA* deletion mutant, ODB2, was constructed using a two-step integration-excision method (Fig. 9A). First, the PCR-amplified 1.5kbp regions flanking *iglA* were joined so as to leave *iglB* intact, including its ribosome binding region. This recombinant construct was ligated to an erythromycin resistance-*sacB* cassette and the ligation mixture was used to chemically transform *F. novicida* JL0 to erythromycin resistance. The JL0 strain is a derivative of U112 that has a deletion in one of its putative sucrose hydrolase genes, and is thus sensitive to sucrose when *sacB* is expressed. This strain behaves like wild type in our virulence assays (data not shown). An erythromycin resistant colony was grown and plated on agar media containing 10% sucrose which acts as a counter selective marker for the *sacB* gene. Sucrose sensitive strains were examined for loss of

iglA by PCR (Fig. 9B). Attempts to genetically complement the Δ*iglA* strain by incorporating *iglA* into a *F. tularensis* plasmid pFNLT1 (Maier, Havig et al. 2004) failed, presumably because the over-expression of IglA was lethal to *F. novicida*. Hence, an *in cis* complementation approach was devised, allowing *iglA* to be incorporated into the chromosome linked to a kanamycin resistance marker (Fig. 10A and B). The *iglA* deletion strain failed to produce IglA as determined by Western immunoblotting (Fig. 11). However, the Δ*iglA* strain retained expression of IglC at parental strain levels. *In cis* complementation of the Δ*iglA* strain resulted in a strain that regained partial expression of IglA. An insertion mutant of *iglB* gave a reduction in the amount of IglC that was made, and this is not surprising since many insertion mutations decrease the expression of downstream genes. Surprisingly, this same mutant lacked expression of IglA, suggesting that the co-expression of IglB is needed for expression of IglA or to prevent degradation of IglA. Disruption of *iglC* however, does not affect the amount of IglA detected (Fig. 11).

2.3.7 IglA is required for growth in the J774 macrophage cell line.

Previous work has suggested that IglA is required for *F. novicida* intramacrophage growth and virulence; however, its role has never been unequivocally demonstrated. In order to assess the requirement for IglA expression in intramacrophage growth we used our defined deletion and complemented strains to infect a culture of the J774 macrophage cell line. The data shown in figure 12 illustrates that the Δ*iglA* strain is incapable of intramacrophage growth, as is the *iglC* negative strain, CG62. The Δ*iglA* strain that was complemented for IglA production partially regained its ability to grow in

macrophages. The residual defect in intracellular growth is not unexpected since we showed that the expression of IglA was not at wild type levels. Δ*iglA* replicated as the parental strain in broth (data not shown).

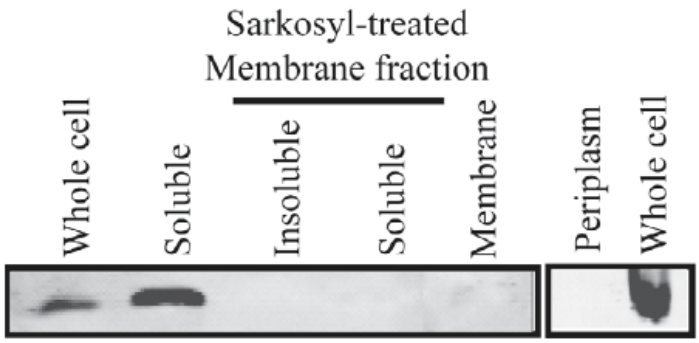
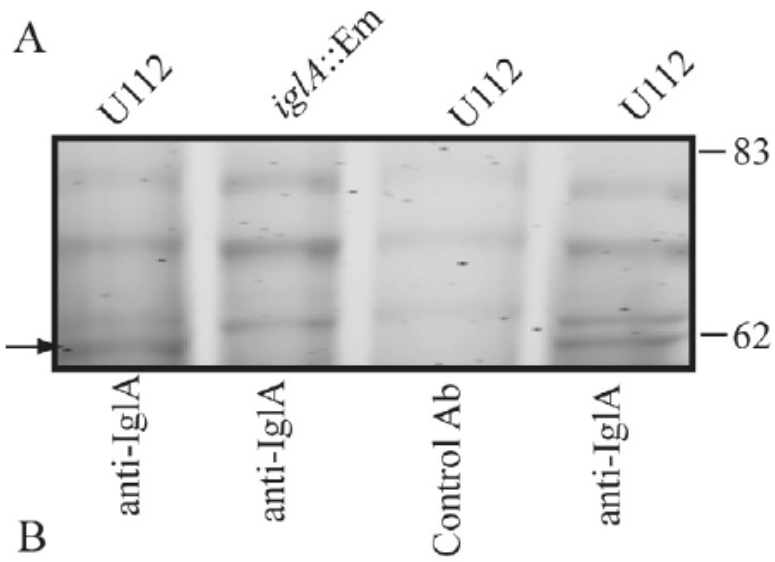


Figure 7. Subcellular localization of IglA. Anti-IglA was used to probe Western immunoblot of subcellular fractions of *F. novicida.* The sarkosyl insoluble fraction represents an enrichment of outer membrane protein and the sarkosyl soluble fraction contains largely inner membrane protein. Samples were prepared as outlined in Methods and normalized to 10 μg protein per lane before separation on a 12% SDS-PAGE gel. Results are representative of three independent experiments.

2.3.8 The Δ*iglA* strain has lowered virulence in chicken embryos.

When the Δ*iglA* strain was used to infect chicken embryos it caused low mortality when compared to wild type *F. novicida* (data not shown). The wild type strain of *F. novicida* caused 100% mortality at day 5 post infection at an infecting dose of 600 CFU, whereas the Δ*iglA* strain caused only 14% mortality at day 6 with an infecting dose of 4,500 CFU or 50% mortality at day 6 with an infecting dose of 45,000 CFU (data not shown).



MISREDFVMTINKLSLTDELLNNFGGSTEVDSVLKNIDFDVSDDASKVLS 50
LSTDYNARNLMALSLVLANNDNINNYNQKYIQKVITVIDKLIDLQVNSII 100
SNDEFRALEQEWLKVQEVCQEDYDNVEVSILDVKKEELQYDFERNLYDIS 150
SSDFFKKVYVSEFDQYGGEPYGAILGLYNFENTTNDIIWLTGMGMVAKNS 200
HAPFIASIDKSFFGVKDLSEITHIKSFEALLEHPRYKEWNDFRNLDVAAY 250
IGLTVGDFMLRQPYNPENNPVQYKLMEGFNEFVDYDKNESYLWGPASIHL 300
VKNMMRSYDKTRWFQYIRGVESGGYVKNLVACVYDNKGILETKSPLNVLF 350
ADYMELSLANIGLIPFVSEKGTSNACFFSVNSAKKVEEFVDGFDSANSRL 400
IANLSYTMCISRISHYIKCVIRDKIGSIVDVESIQKILSDWISEFVTTVY 450
QPTPLEMARYPFRNVSIEVKTIPGKPGWYSCKINVIPHIQFEGMNTTMTI 500
DTRLEPELFGTNNN 514

Figure 8. Co-immunoprecipitation of a 60kDa protein with IglA. Panel A. Anti-IglA serum co-immunoprecipitates a circa 60 kDa soluble protein (arrow, lanes 1 and 4). The band is absent in control reactions with non-specific antibody (lane 3) and in immunoprecipitations with an *iglA* mutant (lane 2). Numbers shown indicate molecular mass standards. Results are representative of those of three experiments. Panel B. MALDI-TOF identified the 60 kDa protein as IglB. Underlined sequences indicate peptides identified by MALDI-TOF.

2.4 Discussion.

There is growing evidence that the *iglABCD* operon is needed for *F. tularensis* intracellular growth and virulence and that the MglAB proteins are involved in regulating

the expression of *iglABCD*. However, there is very little genetic and corresponding biochemical data demonstrating the roles of MglAB and IglAB and their corresponding homologues in other bacteria. For example, while it is clear that MglA plays a role in regulating the amount of *iglABCD* transcript it is unclear if the role precisely corresponds to that of the *E. coli* SspA protein. The data that exists for the functioning of SspA suggest that much of the regulation of stationery phase proteins occurs indirectly via the repression of H-NS, and that some of the effect of SspA is post-transcriptional (Hansen, Qiu et al. 2005).

There is also growing evidence that proteins encoded by IAHP clusters, of which IglAB homologues are important components, are involved in secretion of proteins from gram-negative bacteria (Mougous, Cuff et al. 2006; Pukatzki, Ma et al. 2006). There are approximately 30 homologues of *iglAB* and in every case the two genes are adjacent to each other and arranged in the same gene order. In this work we provided biochemical evidence that the IglAB proteins physically associate with each other and are localized to the cytoplasm. The surprising finding that inactivation of the *iglB* gene results in the disappearance of the IglA protein suggest that the presence of IglB is required for IglA to be stable.

IglA was first identified as a locus that when inactivated by a transposon insertion rendered *F. novicida* defective for growth in macrophages (Gray, Cowley et al. 2002). However, it could not be ruled out that the effect was due to interruption of transcription of downstream genes. In this report, we provide strong evidence that IglA is necessary for intracellular growth as a non-polar *iglA* deletion mutant was defective for growth in a mouse macrophage-like cell line. *In cis* complementation of the Δ*iglA* strain restored

intramacrophage growth although the growth was slower than in the wild type strain. The *in cis* complementation strategy created two *iglA* promoter regions on the chromosome, one on either side of a kanamycin resistance cassette. It is conceivable that this results in aberrant regulation of *iglA* expression, which could explain why the growth of the complementation strain lags early during infection. We were unable to complement the *iglA* deletion mutant *in trans* with pFNLTP1::*iglA*, a high copy-derivative of an endogenous *Francisella* plasmid. Presumably, over-expression of IglA was lethal to *F. novicida.*

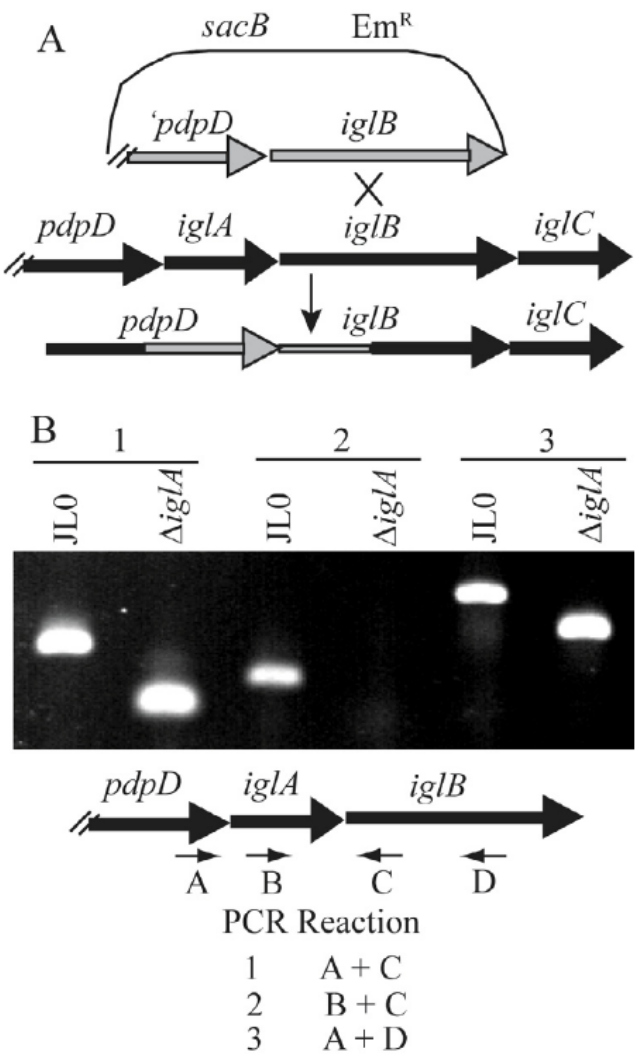


Figure 9. Deletion mutagenesis of *iglA*. Panel A. Diagram of steps used to construct an *iglA* deletion mutant. A fragment of *pdpD* was joined to *iglB* and these two fragments were ligated to an Em^R-*sacB* cassette. After transformation the recombinant construct integrated into the *F. novicida* chromosome. Plating the strain with the integrated fragment on sucrose selected for strains that had undergone an excision of the *sacB* and neighboring regions. Panel B. PCR confirmation of the deletion of *iglA*. The small arrows indicate the location of the primers used in the reactions.

We hypothesize that IglA and IglB are cytoplasmic, chaperone-like proteins that are involved in secretion of virulence factors. Therefore, the biological significance of IglAB interaction may be to secrete *Francisella* effector molecules. In other pathogens, secretion of virulence proteins often requires interaction between two cytoplasmic proteins. For example, in *Yersinia pestis*, a complex composed of SycN and YscB function as chaperones for YopN (Day and Plano 1998), which is secreted to the cell surface (Forsberg, Viitanen et al. 1991). Also, interaction of IcmS and IcmW is required for translocation of effector proteins via the Dot/Icm complex during *Legionella pneumophila* intracellular growth (Coers, Kagan et al. 2000; Ninio, Zuckman-Cholon et al. 2005). Hager *et al.* recently demonstrated protein secretion by *F. novicida* (Hager, Bolton et al. 2006). We did not observe any difference in secreted peptides between broth-grown wild type *F. novicida* and the Δ*iglA* strain by SDS-PAGE electrophoresis (data not shown). This observation is not surprising given the fact it has been demonstrated that secretion involving IAHPs is a highly regulated or an *in vivo*-induced process (Mougous, Cuff et al. 2006).

In summary, our results suggest that IglA and IglB are interacting cytoplasmic proteins that are required for intramacrophage growth. The significance of the interaction may be to secrete effector molecules that affect host cell processes.

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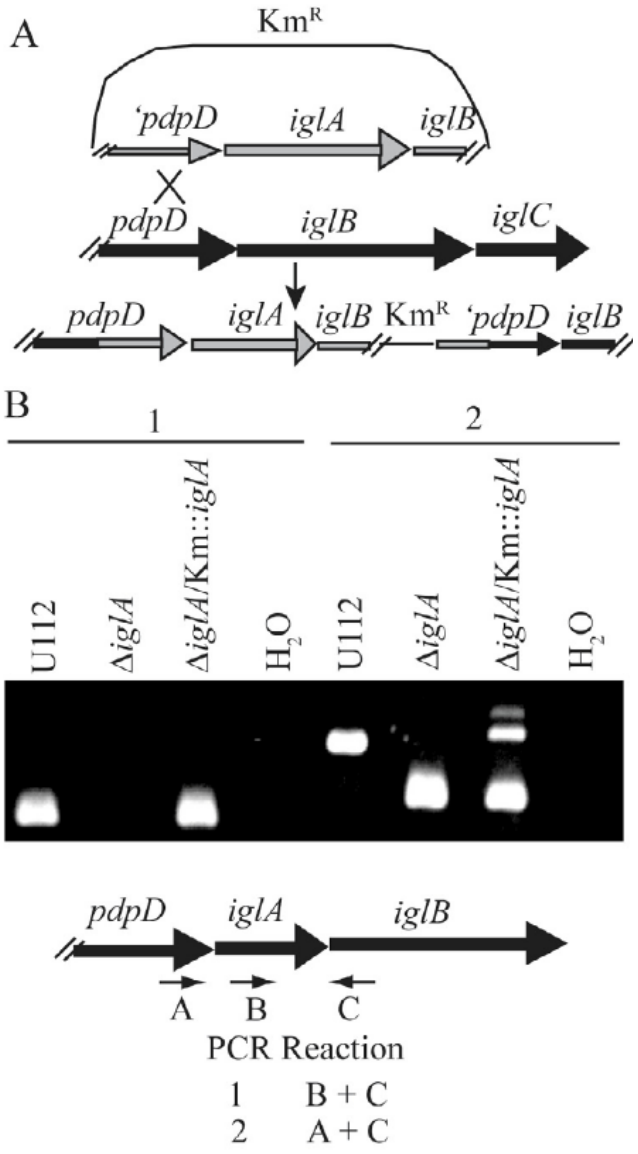

Figure 10. ***In cis*** **complementation of *iglA*.** Panel A. Diagram of complementation scheme. A PCR amplicon containing the *iglA* and neighboring regions was ligated to a Km^R cassette and used to transform a Δ*iglA* strain. Integration of the recombinant construct resulted in a strain with a chromosomally-integrated *iglA*. Panel B. PCR reactions demonstrating the presence of *iglA* in the complemented strain. Arrows in lower part of diagram indicate the location of the PCR primers used in the reactions.

Table 1. Strains used in the study of the role of *iglA* in *Francisella* intracellular growth

Name	Phenotype/Relevant Characteristics	Reference or Source
U112	*Francisella novicida* prototype strain.	ATCC
JL0	U112, Δsucrose hydrolase strain used to make deletion mutants.	Laboratory strain
ODB2	JL0, Δ*iglA*	This study
ODB7	U112, *iglA*::Em^R	This study
ODB1	U112, *iglB*::Em^R	This study
ODB5	Δ*iglA*/*iglA*:Km^R, *in cis* complementation of *iglA* in strain ODB2	This study
CG62	U112, *iglC*::Tn*Max*2	Gray *et al.* (2002)
GB2	U112, *mglA*	Baron *et al.* (1998)
GB6	U112, *mglB*::mTn*10*Km	Baron *et al.* (1998)

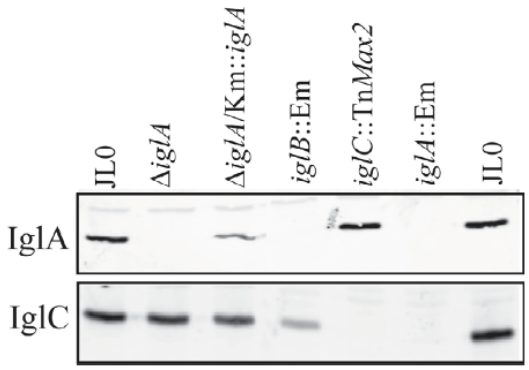

Figure 11. An *iglA* mutant lacks the expression of a 21 kDa protein. Western blot showing the lack of an anti-IglA serum reactive 21 kDa protein in the Δ*iglA* strain (top panel). Wild type levels of IglC are retained in the Δ*iglA* strain (bottom panel). In contrast, the *iglA*::Em mutant lacks expression of IglC. The expression of IglC is threefold lower in an *iglB*::Em strain than in JLO and Δ*iglA*. Fluorescence intensity was used to quantify relative amounts of protein.

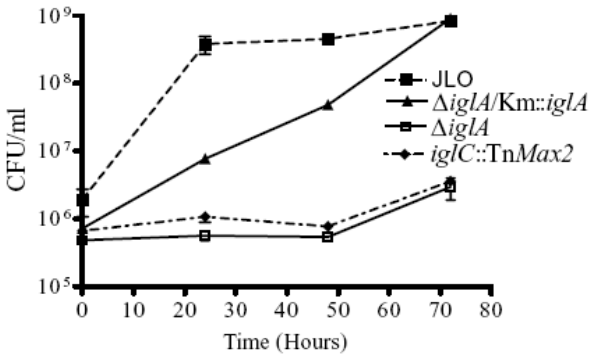

Figure 12. IglA is required for intracellular growth. Growth of Δ*iglA* strain in J774 mouse macrophage-like cells. Filled squares, parental strain JLO; open squares, Δ*iglA*; triangles, *in cis* complementation strain; diamonds, *iglC* transposon insertion mutant CG62. The experiments were done in triplicate and standard errors are shown by bars. This graph shows data from one of three independent experiments.

Chapter 3. The *Francisella* Pathogenicity Island Protein PdpD is required for full virulence and associates with homologues of the type VI secretion system.

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3.1 Introduction

Francisella tularensis, the causative agent of the zoonotic disease tularemia, is a gram negative, facultative intracellular bacterial pathogen (Ellis, Oyston et al. 2002). *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*") 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 (Bell, Owen et al. 1955; Olsufjev and Emelyanova 1963). 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 JE 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, Farlow 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 (Larsson, Oyston et al. 2005; Beckstrom-Sternberg, Auerbach et al. 2007; Guina, Radulovic 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) (Lauriano, Barker et al. 2004; Nano, Zhang et al. 2004; de Bruin, Ludu et al. 2007).

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, Zhang et al. 2004) (Fig. 2). 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 (Gray, Cowley et al. 2002; Abd, Johansson et al. 2003; Tempel,

Lai et al. 2006; de Bruin, Ludu et al. 2007; Santic, Molmeret et al. 2007; Weiss, Brotcke et al. 2007). 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 (Bladergroen, Badelt et al. 2003), including *Vibrio cholerae* (Pukatzki, Ma et al. 2006), and *Pseudomonas aeruginosa* (Mougous, Gifford et al. 2007). There is substantial evidence that T6SSs are tightly controlled and up-regulated during an infection (Mougous, Cuff et al. 2006; de Bruin, Ludu et al. 2007; Schell, Ulrich et al. 2007), and these properties may explain why proteins in *Francisella* that are secreted by its T6SS have not been identified in the past. Recent evidence suggest that the *V. cholerae* T6SS produces a secreted structure that is predicted to have cell-puncturing properties (Pukatzki, Ma et al. 2007), but the full picture of secretion by T6SS is yet to be elucidated.

As a gram negative bacterial pathogen *Francisella* is expected to have mechanisms to secrete proteinaous virulence factors to the surface of the bacterium or into the extracellular milieu. Gill and co-workers (Gil, Platz et al. 2006) found a *tolC* and a *tolC*-like homologue in *Francisella*, and showed that these genes contribute to resistance to bactericidal small molecules. 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 (Bina, Lavine et al. 2008) showed that an AcrB RND efflux pump contributes to drug resistance and virulence in the *F. tularensis*

LVS strain. Importantly, Hager *et al* (Hager, Bolton et al. 2006) showed true secretion of seven proteins in *F. novicida* that is dependent on genes that are homologous to type IV pili genes. 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.

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.

3.2 Materials and Methods.

The majority of the work presented in this chapter was performed by Jag Ludu in the laboratory of Francis Nano. Olle de Bruin contributed to this study by performing experiments and creating reagents relating to the T6SS of *Francisella*.

3.2.1 Strains and Growth Conditions.

The bacterial strains and plasmids used in this study are listed in Table 2. 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.

3.2.2 Transformation of *Francisella*.

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

3.2.3 SDS-PAGE and immunoblotting

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, 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 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.

3.2.4 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 h 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.

3.2.5 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.

3.2.6 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 (de Bruin, 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) (Guina, Radulovic 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 (LoVullo, Sherrill et al. 2006), and introduced this recombinant into *F. tularensis* LVS. The sequence of the primers used for this amplification and all others used in this work will be made available upon request.

3.2.7 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, 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 (Baron and Nano 1998), which does not grow in macrophages, was incorporated into all macrophage growth experiments.

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.

3.2.8 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, Cheung 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.

3.3 Results and Discussion

3.3.1 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 (Lauriano, Barker et al. 2004; Nano, Zhang 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.

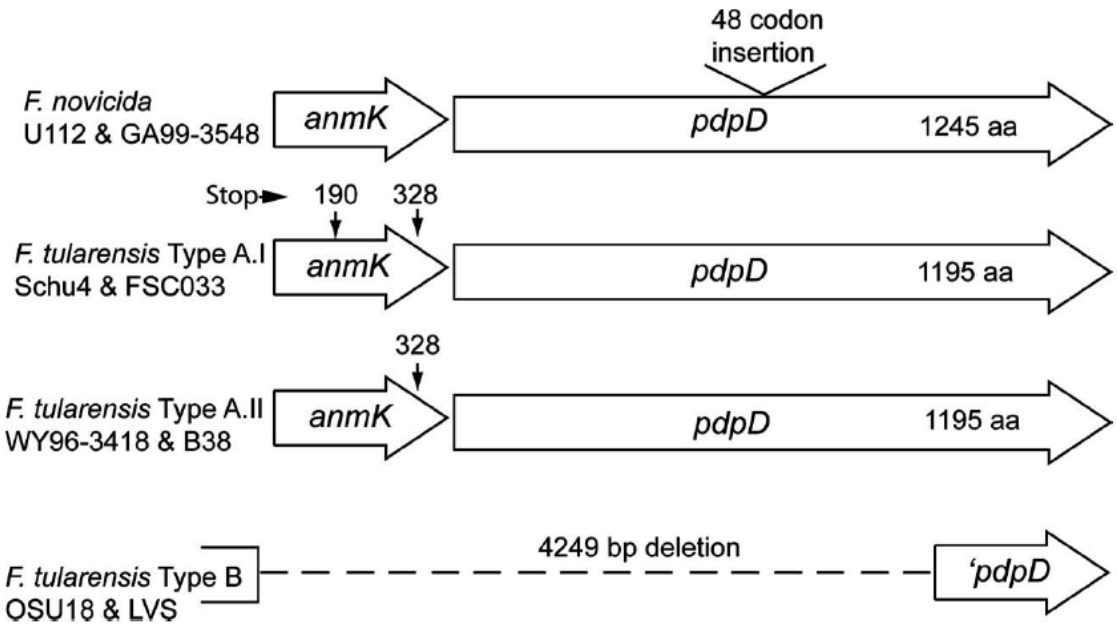

Figure 14. **The *anmK* and *pdpD* loci vary in *F. tularensis* subspecies.** The *F. novicida* form of the FPI differs from the type B biotype forms in having *anmK* and *pdpD*, while the type A biotypes have *pdpD* but two distinct, truncated forms of *anmK*. The *F. novicida* strains U112 and GA99-3548 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 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 (Fig. 14). 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*.

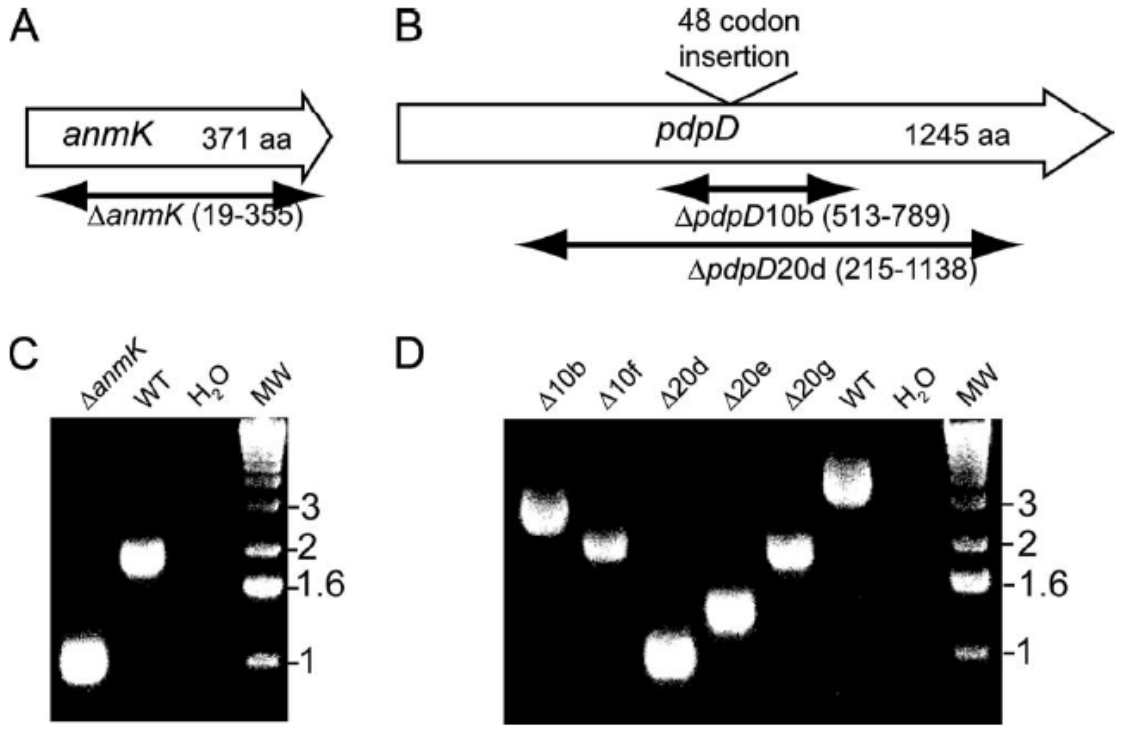

Figure 15. Mutagenesis of *pdpD*. Panels A and B. The extent of the deletions in *anmK* (A) and *pdpD* (B) are diagrammed. Unless otherwise stated, all reference to a Δ*pdpD* mutant in this work refers to mutant Δ*pdpD20d*. Panels C and D. The *anmK* and five in-frame deletion mutants of *pdpD* were 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.

The *F. novicida pdpD* 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.

3.3.2 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, Reichelova et al. 2006; Twine, Mykytczuk 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 (Fig. 15). 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 (Fig. 15). 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.

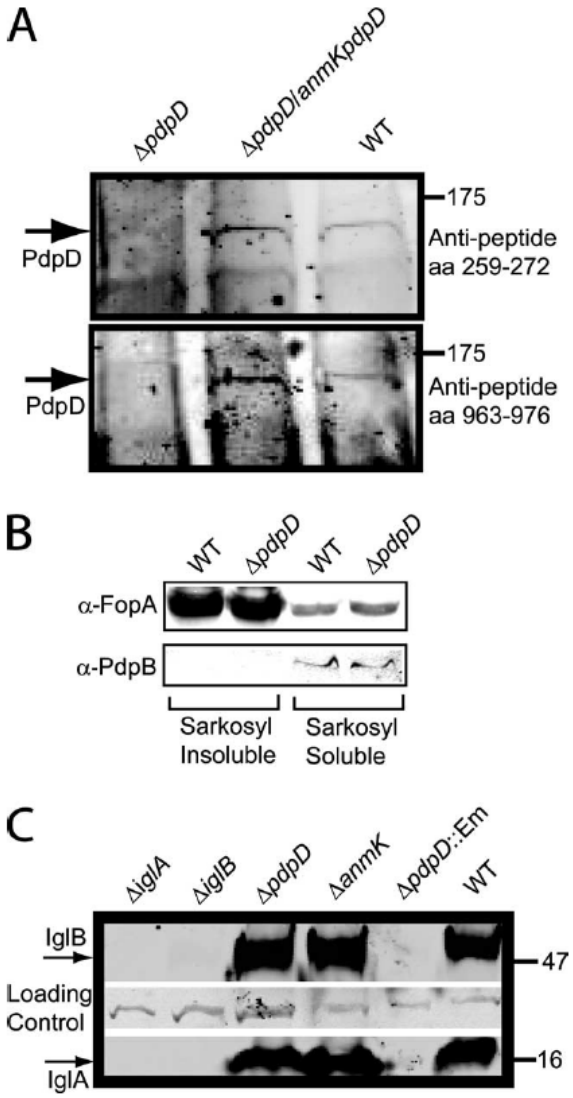

Figure 16. Immunoblot analysis of *pdpD* mutants. Panel A. Immunoblots developed with anti-PdpD peptide antibodies. Samples were normalized to 15 µg protein per lane prior to separation on a 4-12% gradient gel. Panel B. Sarkosyl solubilization separates markers for the inner and outer membrane. Membrane fractions were separated by sarkosyl solubilization and subjected to Western blotting. All lanes were loaded with 5µg of protein. Panel C. IglA and IglB expression profiles in *F. novicida* mutants.

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 bioinformatics 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 (Fig. 16A). As well, over-expression of PdpD occurred in strains carrying the complementation construct, pJL-SKX::*anmKpdpD*, and this complementation allowed enhanced detection of PdpD (Fig. 16A). 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 (Fig. 16B). 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* (Fig. 16C). 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* (Fig. 16C). The lack of both IglA and IglB in both the Δ*iglA* and Δ*iglB* mutants (first two lanes of Fig. 16C) highlights the previous finding (de Bruin, Ludu et al. 2007) that the lack of expression of one protein leads to the apparent degradation of the other.

3.3.3 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 (Melillo, Sledjeski et al. 2006) 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. 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.

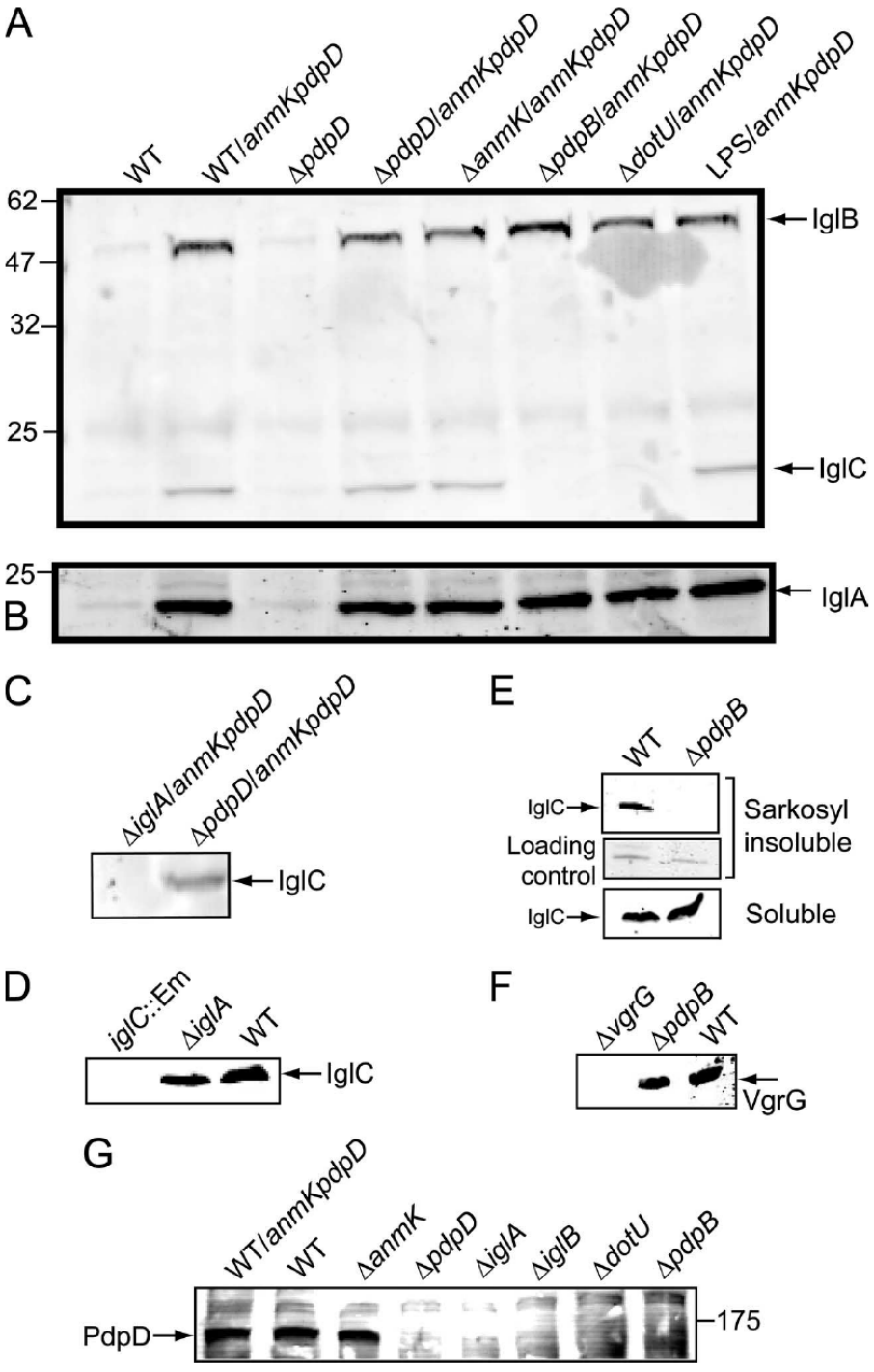

Figure 17. Effect of PdpD over-expression on surface biotinylation of IglA, IglB, and IglC. Panel A. Reactivity of surface-exposed biotinylated proteins with anti-IglB and anti-IglC monoclonal antibodies. Panel B. Reactivity with anti-IglA polyclonal serum. Panel C. Surface biotinylation of IglC in an Δ*iglA* strain. Panel D. Control for Panel C. IglC is found in the cytoplasm in the Δ*iglA* strain. Panel E. IglC fails to localize to the outer membrane in a Δ*pdpB* background. Panel F. The PdpB deletion does not affect expression of the downstream gene *vgrG*. Panel 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.

IglA and IglB are homologues of proteins that are part of type VI secretion systems. 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 (Fig. 17). 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 (Fig. 5A-B). However, the amount of IglC exposed to biotinylation was dramatically affected by the absence of *pdpB* and *dotU* (Fig. 17A). 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 (Fig. 17A). Similarly, in an Δ*iglA* or Δ*iglB* background IglC failed to be biotinylated, even when pJL-SKX::*anmKpdpD* was present (Fig. 17C and data not shown) even though IglC was shown to be in the soluble fraction of the wild type and Δ*iglA* strains (Fig. 5D). Biotinylation of IglA, IglB or IglC was identical to the wild type strain in an O-antigen mutant strain of *F. novicida* (Fig. 17A, far right). Previous work had shown that IglC was primarily localized to the cytoplasm (Golovliov, Ericsson et al. 1997), with a small amount being found in the outer membrane. 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 (Fig. 17E). 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 (Fig. 17E). 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, Ericsson 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 (Fig. 17F) 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 (Fig. 17G). 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 bioinformatics 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, Ludu 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.

3.3.4 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 (Lauriano, Barker et al. 2004). We noted that the insertion depressed the expression of the downstream-encoded IglA protein, which was later shown to be needed for intracellular growth and virulence (de Bruin, Ludu 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 (data not shown). 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 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 (data not shown).

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 suggests 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.

3.3.5 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 (data not shown). Complementation of the genetic defect using the integrating recombinant pJL-SKX::*anmKpdpD* completely restored virulence. Similarly, genetic complementation using a plasmid vector restored virulence, but a mock complementation with the plasmid without the *anmKpdpD* insert did not. Complementation of Δ*pdpD* strain with SKX::*anmK* alone did not restore virulence. 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*. A strain that we use for a universal negative control, GB2 (*mglA*) showed its usual low virulence. 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 (Fig. 18). 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, Ericsson et al. 1997). Subsequent work has shown that mutants with a deleted or disrupted *iglC* gene fail to grow in macrophages (Abd, Johansson et al. 2003), are deficient for escape from phagosomes (Lindgren, Golovliov et al. 2004), and fail to down-regulate pro-inflammatory response in macrophages (Telepnev, Golovliov et al. 2005). 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 (Golovliov, Ericsson et al. 1997), and this is consistent with the data presented in this work. 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, Austin 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.

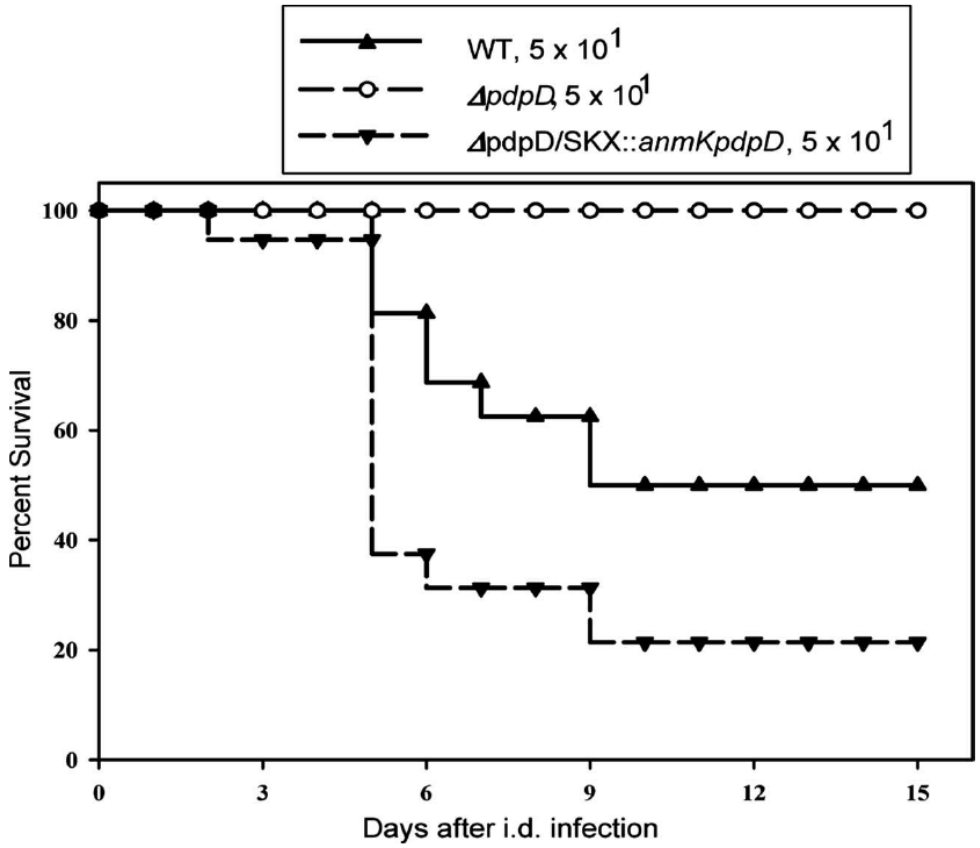

Figure 18. Survival patterns of mice infected intradermally with a low dose of *F. novicida* or *pdpD* mutants of *F. novicida*. BALB/cByJ male mice were infected with 5 x 10^1 of the indicated bacterial strain intradermally, and monitored for survival. Results are expressed as percentage of surviving 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.

Table 2. Strains and plasmids used in the study of the contribution of PdpD to virulence and T6S-like secretion of *Francisella*

Bacterial Strains	Relevant Characteristics	Source and/or Reference
U112	Wild type *Francisella novicida*	(Larson, Wicht 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.	(de Bruin, Ludu et al. 2007)
GB2	U112 with point mutation in the global virulence regulator, *mglA*	(Baron and Nano 1998)
SC92	O-antigen mutant of U112	(Cowley, Gray et al. 2000)
JL12	An *ermC* allelic exchange mutant of *pdpD*	(Lauriano, Barker et al. 2004)
ODB2	JL0 with deletion of *iglA*	(de Bruin, Ludu 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 integrating pJL-SKX::*pdpD* construct	This work
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	This work

	with the integrating pJL-SKX::*anmKpdpD* construct	
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.	American Type Culture Collection (Eigelsbach and Downs 1961)
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	Invitrogen (Hanahan 1983)
Plasmids		
pWSK29	Low copy cloning vector, Amp^R.	(Wang and Kushner 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	(de Bruin, Ludu et al. 2007)
pMP633	*Francisella* shuttle plasmid, Hyg^R.	(LoVullo, Sherrill et al. 2006)
pJL-ES-X	An *ermCsacB* cassette with flanking Xho I restriction sites	(de Bruin, Ludu et al. 2007)
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

Chapter 4. The *Francisella* pathogenicity island encodes a unique secretion system

(Submitted for publication)

4.1 Introduction

The Gram negative bacterium *Francisella tularensis* is the etiologic agent of tularemia, a zoonotic and severe febrile disease. Some researchers divide *F. tularensis* into four subspecies namely *tularensis, holarctica, novicida* and *mediasiatic*. Of these, *tularensis* and *holarctica* are clinically relevant to humans, whereas *novicida* causes tularemia-like disease in mice (Larsson, Oyston et al. 2005). A major virulence strategy of *F. tularensis* is its capacity to survive and grow in host immune cells (Anthony, Burke et al. 1991). *F. tularensis* subspp. *novicida* (hereafter referred to as *F. novicida*) is indistinguishable from *tularensis* and *holarctica* in this regard, and *F. novicida* is therefore considered a valid model for studying the biology of intracellular growth of *Francisella* (Santic, Molmeret et al. 2005).

The intracellular life style of *F. tularensis* differs from any other pathogen studied to date. After phagocytosis, *F. tularensis* is able to delay maturation of the phagosome, specifically by inhibiting the acquisition of lysosomal hydrolases (Clemens, Lee et al. 2004; Santic, Molmeret et al. 2005). However, soon after uptake by a macrophage the phagosomal membrane enclosing *F. tularensis* undergoes degradation allowing the bacterium to gain access to the cytosol, which is followed by robust replication leading to eventual host cell lysis (Abd, Johansson et al. 2003; Clemens, Lee et al. 2004; Checroun, Wehrly et al. 2006). Late during infection, *Francisella* has been observed to enter a

double membranous compartment with the characteristics of an auto-phagosome (Checroun, Wehrly et al. 2006). The significance of the delay in phagosomal maturation and the role of autophagy are not clear, however, phagosomal escape is critical to the virulence of this pathogen (Lindgren, Golovliov et al. 2004; Mariathasan, Weiss et al. 2005).

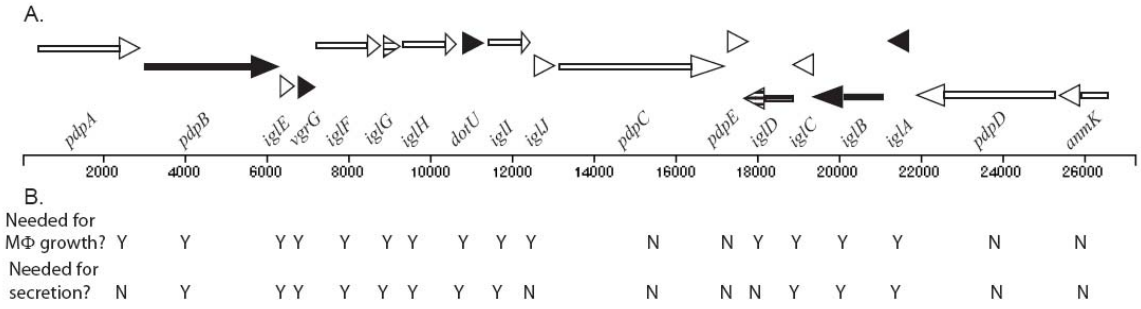

Figure 19. Organization of FPI genes and summary of their roles in secretion of IglC and in intracellular growth. Black arrows show genes with clear orthologues, and striped arrows indicate genes with distantly related orthologues in other T6SSs. In legend below, arrows indicate if genes are needed for intramacrophage growth or needed for secretion of IglC, Y indicating a requirement, whereas N means dispensability.

The molecular mechanisms contributing to the intracellular survival strategies of *Francisella* are poorly understood and sequencing of its genome suggests an absence of many classical virulence factors such as toxins and type III and type IV secretion systems (Larsson, Oyston et al. 2005). Mutagenesis approaches have been useful in identifying a number of genes that when inactivated renders *F. tularensis* unable to replicate intracellularly (Gray, Cowley et al. 2002; Tempel, Lai et al. 2006; Maier, Casey et al. 2007). Several of these genes are located in a genomic region named the *Francisella*

pathogenicity island (FPI) (Lauriano, Barker et al. 2004; Nano, Zhang et al. 2004). The FPI is found in all *Francisella* species and strains, and is exactly duplicated in all of the human-virulent biovars of *F. tularensis*. In contrast, *F. novicida* only harbors one copy of the FPI, which makes this species attractive for creation of isogenic FPI deletion mutants.

The FPI gene *iglC* is one of the most highly induced genes during intracellular growth (Golovliov, Ericsson et al. 1997). Deletion of *iglC* causes a defect in the ability of *Francisella* to escape from the phagosome during infection of monocytes (Lindgren, Golovliov et al. 2004), as well as failure to induce apoptosis and to down-regulate host cell signaling (Lai, Golovliov et al. 2004; Telepnev, Golovliov et al. 2005). The current understanding is that the latter two are consequences of failure to escape into the macrophage cytosol. Exactly how IglC contributes to phagosomal-escape is not known and the solved crystal structure of IglC offered limited clues regarding its function (Sun, Austin et al. 2007). Indeed, most FPI proteins do not show similarity to any other protein by BLAST analysis making predictions regarding their function difficult. Overall, it is clear that the FPI encodes an important virulence mechanism, but the function of FPI-encoded proteins has remained elusive.

Protein secretion is a fundamental process of bacterial life. Gram negative bacteria use specialized secretion systems for diverse tasks such as nutrient uptake, motility, conjugation and virulence. In these bacteria, membrane-associated machineries involved in protein transport are categorized into six different systems according to evolutionary origin and structure. Recent studies suggest type six secretion systems constitute important bacterial virulence or survival factors, however, only basic aspects of

this system have been characterized (Bingle, Bailey et al. 2008; Cascales 2008; Pukatzki, McAuley et al. 2009). Semi-conserved gene clusters, which include homologues of IcmF, DotU and IglA and IglB (DUF770/877), are signatures bacteria have potential to encode a type six secretion system (Bingle, Bailey et al. 2008). In some species, these systems have been shown to be responsible for secretion of novel proteins, including Hcp and VgrG, which lack an N-terminal signal peptide (Mougous, Cuff et al. 2006; Pukatzki, Ma et al. 2006; Schell, Ulrich et al. 2007; Zheng and Leung 2007).

In this work, we systematically deleted the genes of the *F. novicida* FPI and determined the contribution of individual genes to intracellular growth. Under the course of this study we identified at least 7 of the deduced gene products of the FPI as similar to those of T6SSs; hence, we tested the hypothesis that the FPI encodes a secretion system. We found that the FPI-encoded protein IglC is secreted into the growth supernatant, and its secretion is dependent on several of the FPI genes including, but not limited to, T6SS homologues. Furthermore, during infection of macrophages IglC is secreted in an FPI-dependent manner. Because the FPI harbors several novel genes required for secretion, and the secreted protein, IglC, are unique among bacteria, our data demonstrate this pathogen has evolved a specialized secretion system related to T6SSs essential to its ability to cause disease.

4.2 Materials and Methods.

The data presented in this chapter is the result of a collaboration between the Nano lab of the University of Victoria, Canada, and the Hueffer lab of the Univeristy of Fairbanks-Alaska, USA. All biochemical experiments and data presented in this and

subsequent chapters were performed by Olle de Bruin. FPI mutants listed in table 4 were constructed by Jag Ludu and Olle de Bruin. Complementation of these mutants involves the work of Eli Nix, Rebbeckah Hare-Sanford and Olle de Bruin. Microscopy was performed by Rebbeca Hare-Sanford and Olle de Bruin. Although Olle de Bruin created all strains used in macrophage infection studies, Crystal Schmerk contributed substantially to this study by conducting intramacrophage growth experiments of mutant and complementation strains. Rebbeckah Hare-Sanford is a graduate student at the Univeristy of Fairbanks-Alaska, USA, whereas Eli Nix, Jag Ludu and Crystal Schmerk are members of the Nano lab of the University of Victoria, Canada.

4.2.1 Strains and growth conditions.

All strains used in this study are listed in table 4. *Francisella* was routinely cultured in trypicase soy broth supplemented with 0.1% cysteine (TSBC). Erythromycin (15 μg/ml), kanamycin (15 μg/ml) or 10% sucrose was added as needed. *E. coli* was grown in Luria-Bertani broth and kanamycin (30 μg/ml) or ampicillin (200 μg/ml).

4.2.2 Subcellular localization of *F. novicida* proteins.

F. novicida was grown to stationary phase in 20 ml TSB-C harvested by centrifugation, resuspended in 1.0 ml phosphate buffered saline (PBS) containing protease inhibitor (EMB bioscience) and lysed by intermitten sonication (Sonic Dismembrator Model 100, Fisher brand) and cooling on ice ten times for a total of 300 seconds. Unbroken cells were removed by centrifugation 8,000 rpm, 4^{o}C, 20 min (Thermo IEC Micromax). Soluble and insoluble fractions were separated by

ultracentrifugation as described (de Bruin, Ludu et al. 2007). The soluble fraction was ultracentrifuged again to remove residual membrane proteins and the insoluble pellet was washed once in ice cold PBS. For preparation of outer membrane fractions, Sarkosyl (Sigma) was added to cleared lysates to a final concentration of 1% followed by ultracentrifugation (Beckman optima TLX ultracentrifuge, TLA-100.3 rotor) for 1 h at 100,000 x *g* at 4^{o}C to pellet the outer membrane. Outer membranes were washed once in PBS to remove excess Sarkosyl. To normalize loading during SDS-PAGE and Western immunoblotting, protein from equal amount of bacteria was analysed for each sample. Purity of fractions was determined with anti-PdpB (inner membrane) and anti-FopA (outer membrane) antibody.

4.2.3 Co-immunoprecipitation of *Francisella* proteins.

12 ml of overnight cultures of *F. novicida* were sonicated and cleared as described above. Soluble protein was used in immunoprecipiatation reactions using FLAG Tagged Protein Immunoprecipitation kit (Sigma, FLAGIPT-1) according to manufacturer's instructions.

4.2.4 Secretion assays.

20 ml of overnight *F. novicida* culture was harvested and resuspended in 1.2 ml of ice cold 20mM Tris, pH 8.0 containing protease inhibitors. Bacterial cells were lysed by sonication and unbroken cells carefully removed by centrifugation 8,000 rpm, 20 min, 4^{o}C. Sarkosyl (Sigma) was added to lysates to a final concentration of 1% and outer membranes pelleted by ultracentrifugation 80 min, 100,000 x *g* (Beckman L8-M ultracentrifuge, Type 70 Ti rotor). To analyse secretion of IglC to the outer membrane of

strains, outer membranes were resuspended in 500 µl of ice-cold 20 mM Tris, pH 8.0, and subjected to western immunoblotting.

To detect proteins in the culture growth supernatant of strains, 15 ml of overnight cultures were centrifuged at 8,000 rpm, 20 min, 4^{o}C and supernatants passed through a 0.45 µM filter. Supernatant proteins were precipitated by addition of ice cold TCA (10% final concentration) in Pyrex Sorvall centrifuge tubes (Du Pont Instruments) for 90 min on ice, pelleted by centrifugation 11,000 rpm, 45 min, 4^{o}C, and washed twice in ice cold acetone. The final pellet was resuspended in 30 µl of 67 mM Tris, pH 6.8.

4.2.5 Macrophage infection assays.

Macrophage infection assays were performed as previously described (de Bruin, Ludu et al. 2007).

4.2.6 Immunoprecipitation of *Francisella* protein from infected macrophages.

75 cm^2 flasks of J774.1 mouse macrophage-like cells were infected with *F. novicida* strains, washed, harvested and pelleted in a Thermo IEC Centra CL2 centrifuge. Cells were resuspended in 1 ml of ice cold homogenization buffer containing 250 mM sucrose, 3 mM imidazole (pH 7.4), 0.5 mM EDTA and protease inhibitor, and broken by repeat passage through a 27 ^{½} gauge needle on ice. The sample was centrifuged 8500 rpm, 20 min, 4^{o}C to remove bacteria, unbroken macrophages and debris in a Thermo IEC Micromax centrifuge. The soluble fraction containing macrophage proteins and secreted *F. novicida* proteins were subjected to FLAG-immunoprecipitation as outlined above. Since Δ*pdpB* fails to replicate intracellularly, infections were stopped 3 h post infection, a

time point before wild type *F. novicida* starts to replicate. Bacterial pellets from the infection (also containing macrophage debris) were analysed by Western immunoblotting using anti-FLAG antibody to ensure similar amount of bacteria were present in each sample.

4.2.7 Western immunoblot analysis.

SDS-PAGE and Western blotting was performed according to standard techniques as previously described (de Bruin, Ludu et al. 2007). Membranes used for secretion blots were stained with Ponceau S (Sigma) to ensure equal loading of samples. Anti-IglA, anti-IglB and anti-IglC antibodies were used at dilutions of 1:5,000, anti-PdpB and anti-FopA at 1:1,000, and anti-VgrG, anti-FLAG (Sigma) at 1:10,000. The hybridomas producing anti-IglB, anti-IglC and anti-PdpB have been deposited with the American Type Culture Collection's Biodefence and Emerging Infections Resources program.

4.2.8 Construction of deletion strains.

Primers used to construct mutant strains are listed in table 5.

Unmarked deletion mutants of *iglB, vgrG, pdpB* and *dotU* were constructed using the *sacB* counter-selection method as previously described (de Bruin, Ludu et al. 2007). Briefly, flanking regions were PCR amplified, joined by ligation, cloned into pWSK29 or pCR2.1, excised and ligated to a *sacB*-erythromycin resistance cassette. The construct was transformed into *F. novicida* essentially as previously described (Mdluli, Anthony et al. 1994) with the minor modification that cells were grown in presence of 2% glucose

overnight before plating on tryptic soy agar plates supplemented with 0.1% cysteine (TSAC) containing erythromycin. Merodiploid colonies were identified by PCR and tested for sensitivity to sucrose. Sensitive colonies were grown in TSBC containing 10% sucrose to resolve the *sacB*-erythromycin resistance cassette, and plated on TSAC-sucrose plates. Resistant colonies were screened by PCR to identify bacteria harboring desired gene deletions.

To construct deletions of *iglC, iglD* and *pdpE,* amplicons encompassing all three genes were generated using primers x-clone3-L and x-clone3-R, and cloned into the *Xho*I site of pWSK29 as one insert (clone 3) (Wang and Kushner 1991). Clones of *iglE, iglF* and *iglG* (clone 1)*,* and *iglH, iglI* and *iglJ* (clone 2) were constructed using primers x-clone1-L and x-clone1-R, and x-clone2-L and x-clone2-R, respectively, and cloned into pWSK29. Flanking regions of each gene were amplified by PCR from the respective clone using primers with incorporated *BamH*I sites. Each amplicon was then digested with *BamH*I and joined by ligation to produce deletion constructs. Constructs were excised by digestion with *Xho*I and ligated to a *sacB*-erythromycin resistance cassette and used to create deletions as described above.

4.2.9 Construction of FLAG-epitope tagged FPI proteins

Primers used for construction of FLAG-epitope tagged genes are listed in table 5. To construct plasmids carrying FLAG-epitope tagged FPI genes, FPI genes were PCR amplified and ligated into pKH3, a pFNTLP derivative with a 3X FLAG insert. Constructs were used to transform *F. novicida* U112R$^-$ to kanamycin resistance. Strains

were analysed by Western immunoblotting for production of FLAG-tagged proteins of the expected size.

4.2.10 Complementation of FPI gene deletions

Strains and plasmids used to complement deletion mutants are listed in table 4. Plasmids carrying FLAG-epitope tagged FPI constructs were transformed into mutant strains for *in trans* complementation of mutants. To identify complementing clones, kanamycin resistant colonies were tested for production of FLAG protein of the expected size by Western immunoblotting and for growth in macrophage infection assays.
In cis complementation was performed using the integration vector pJL-SKX (Ludu, Nix et al. 2008). The *pdpA* promoter was cloned into pJL-SKX to drive expression of genes. *In cis* complementation of *ΔdotU* was performed using a *sac*B erythromycin cassette leading to re-constitution of the wild type allele after counter selection on sucrose. Briefly, *dotU* including its promoter region was amplified by PCR, digested and ligated to *sac*B-Em^R cassette and bacteria transformed to erythromycin resistance. Erythromycin resistant colonies were grown in sucrose to resolve the cassette leading to either the wild type or the deletion. Erythromycin-sensitive, sucrose-resistant colonies were screened by PCR to identify wild type reconstituted clones and screened for growth in macrophages.

4..3 Results

4.3.1 Similarity of seven FPI proteins to other T6SS-associated proteins.

We previously identified the deduced amino acid sequence of FPI-encoded proteins IglAB, VgrG, PdpB and DotU as similar to those of T6SS components found in other bacteria (chapter 2). Recently, two analyses of sequenced bacterial genomes revealed more than 100 T6SS-like loci, and both of these studies concluded the FPI-encoded secretion systems was either a distant outlier of the T6SS group or perhaps an entirely different secretion system (Bingle, Bailey et al. 2008; Boyer, Fichant et al. 2009). The identification of many new T6SSs gene clusters allowed us to carry out further bioinformatics analysis to reveal that IglD and IglG also are similar to proteins found in T6SSs (Fig 19). BLASTP analysis (http://blast.ncbi.nlm.nih.gov) showed IglG has similarity (e-value, 8 e^{-09}) to proteins containing the COG4104/pfam05488 (PAAR) motif, which is found in proteins in a subset of T6SS. The motif is often in predicted membrane proteins. The BLASTP results were supported by analysis of IglG by the HHpred server (http://toolkit.tuebingen.mpg.de/hhpred), which also showed a Pfam PAAR motif (90.4 probability) and similarity to COG4104 (96.2 probability). In addition, the HHpred predicts IglD to be similar to VCA0114 of *Vibrio cholerae* (with 99.9 probability). Homologues of VCA0114 are found in the majority of identified T6SS gene clusters. Our previous annotation of the fourth ORF of the *pdpA* operon of the FPI as encoding a VgrG homologue is also supported by analysis using HHpred. The VgrG proteins in well-characterized T6SS are predicted to form a structure similar to the hole-puncturing devices of bacteriophages (Pukatzki, Ma et al. 2007). Similarly, HHpred

analysis of the *Francisella* VgrG protein shows it is related to bacteriophage base plate gp5. The *Francisella* VgG protein is predicted to form a barrel-like protrusion, but lacks the gp27/Mu44 domain, and an effector domain, which are predicted for some VgrG proteins (Pukatzki, Ma et al. 2007; Pukatzki, McAuley et al. 2009). Bioinformatics analysis therefore suggests at least seven of the FPI-encoded proteins are orthologues of protein commonly associated with T6SSs.

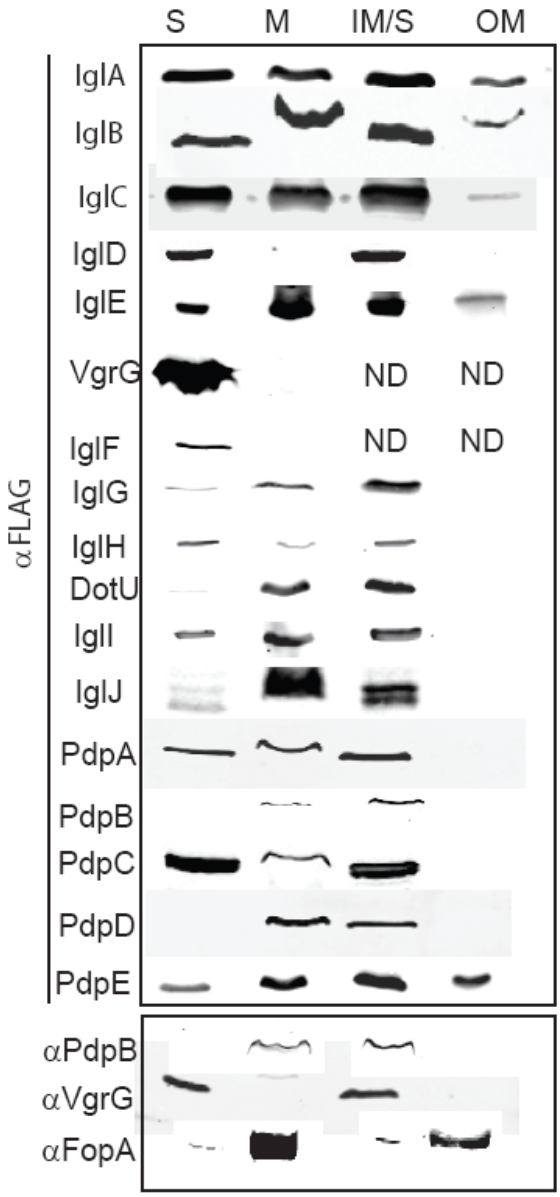

Figure 20. Membrane-association of FPI-encoded components. Strains carrying products of 3X FLAG-tagged FPI genes were separated into soluble and membrane associated fractions, or inner membrane (sarkosyl soluble membrane) plus soluble and outer membrane (sarkosyl insoluble) fractions and immunoblotted. Upper panel shows collection of blots reacted with anti-FLAG antibody and lower panel show blots reacted with antibodies reactive with each specified protein. ND, not determined.

4.3.2 Solubility of FPI-encoded proteins.

The supposition many of the FPI-encoded proteins are part of a secretion apparatus suggest many of these will be membrane associated. To help us discern the function of FPI-encoded proteins we determined their solubility properties. In order to facilitate protein localization by antibody-detection, we tagged all of the FPI-encoded proteins with the 3X FLAG epitope (Fig. 21). For most strains, two Western blotting experiments were performed on bacterial cell fractions. First, cells were separated between soluble (cytoplasm and periplasm) and insoluble (whole membrane) fractions (Fig 20, columns 1 & 2). If α-FLAG reactive material was found in the membrane fraction then a second experiment was performed, where cells were separated into two fractions, one consisting of soluble plus sarkosyl soluble proteins and another fraction, containing sarkosyl insoluble proteins, which are generally outer membrane proteins (Fig. 20, columns 3 & 4).

Our results present evidence IglD, IglF, and VgrG are soluble proteins, whereas IglH and PdpC are mostly soluble with some protein found in the insoluble fraction (Fig. 20). Homologues of VgrG are known to form homo-multimetric SDS-resistant high molecular mass complexes (Kanamaru, Leiman et al. 2002). In *F. novicida,* a soluble, high molecular mass form of circa 200 kDa is found when immunoblots are reacted with anti-VgrG or anti-FLAG antibody for the unmodified and FLAG-tagged forms of VgrG respectively (Fig. 21). In contrast, PdpB (IcmF-containing protein) and DotU associate with the inner membrane, as does IglJ, and to a lesser extent IglG and IglI (Fig. 20). We have previously shown IglA, IglB and IglC are mostly soluble, but that they have a minor portion localized to the outer membrane, and our results show the same localization

pattern. In addition IglE and, to a lesser extent, PdpE showed substantial localization to the outer membrane (Fig. 20). HHPred analysis and the deduced amino-terminal amino acid sequence of IglE has the characteristics of a lipoprotein (table 3, and not shown), which is consistent with IglE localization to the outer membrane.

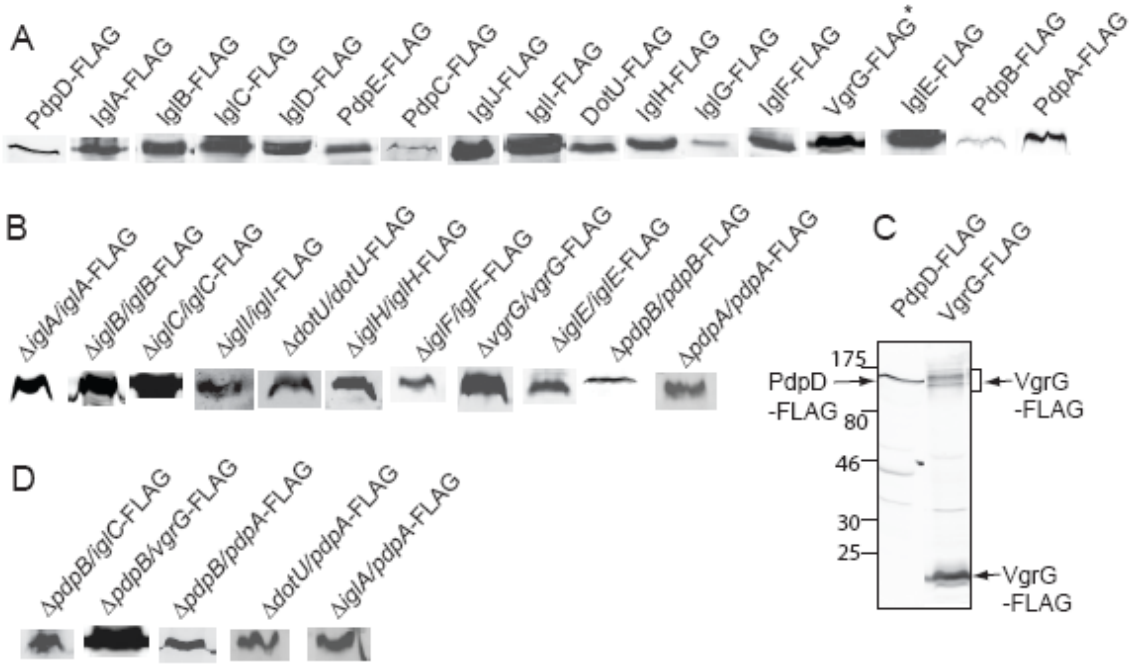

Figure 21. Expression of 3 X FLAG epitope-tagged FPI proteins in *F. novicida.*

Panel A. Expression of indicated FLAG-tagged proteins in WT bacteria. Panel B. Expression of FLAG-tagged proteins used for complementation of deletion strains. Panel C. VgrG proteins form a SDS-stable high molecular mass complex similar to its homologue, the hole-poking device of bacteriophages. Panel D. Expression of indicated proteins in strains carrying deletions in T6SS homologues.

Although the vast majority of FPI-encoded proteins are predicted to be soluble our results indicate several are membrane-associated, some even capable of spanning the cell envelope. These results are consistent with the hypothesis FPI-encoded proteins constitute a secretion machinery.

4.3.3 DotU stabilizes PdpB.

IcmF and DotU proteins appear to be integral proteins of T6SSs. To test whether the IcmF and DotU proteins encoded by the FPI interact, we examined their properties. In extracts of a Δ*dotU* mutant, anti-PdpB antibody detected two reactive bands suggesting PdpB is proteolytically cleaved in the absence of DotU (Fig. 22A). This phenotype could be reversed when the expression of DotU was restored from a plasmid or from a chromosomal integrating vector (Fig. 22A). To determine if this degradation of PdpB is dependent on other FPI gene products, we analyzed whole cell lysates of all FPI deletion mutants and found that only deletion of *dotU* affected PdpB stability (data not shown). We reasoned this specific phenotype may be due to an interaction between PdpB and DotU. Therefore, we performed anti-FLAG immunoprecipitation experiments using FPI proteins carrying a FLAG-tag. Western blot analysis revealed PdpB could be co-precipitated from a strain expressing DotU-FLAG, but not from wild type (Fig. 22B). Further, immunoprecipitation of VgrG-FLAG or IglC-FLAG did not co-precipitate PdpB suggesting these proteins do not interact strongly with PdpB, and furthermore, indicates the co-precipitation of PdpB with DotU-FLAG is not due to non-specific binding of PdpB to the FLAG-tag. Cumulatively, our data indicates PdpB and DotU of *F. novicida* are interacting inner membrane proteins.

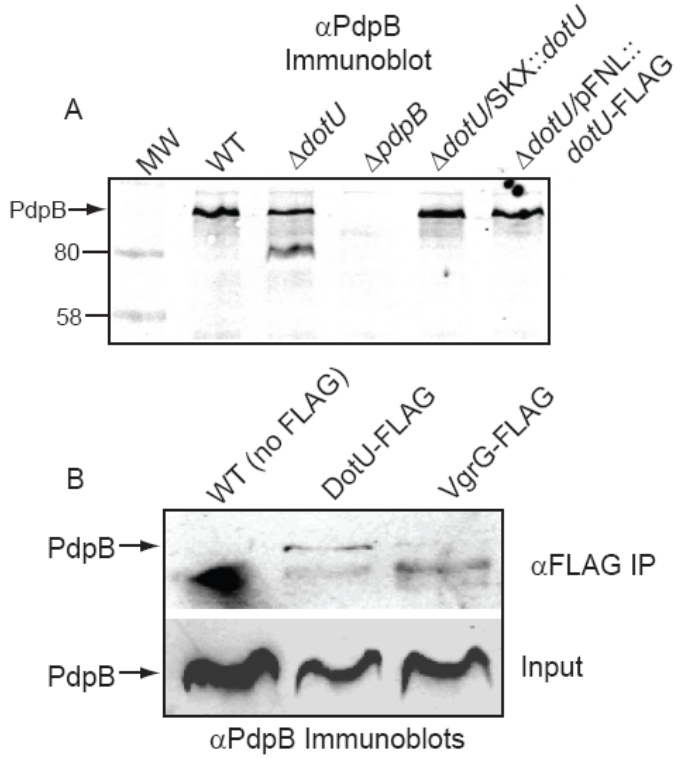

Figure 22. PdpB and DotU physically interact. Panel A. Immunoblot showing full-length form of PdpB in a *wild type* background and the appearance of a lower relative molecular mass form in a *ΔdotU* genetic background. Control lanes show a reactive band absent in Δ*pdpB* strain and that only full-length forms present in Δ*dotU* strain complements *in cis* (SKX::*dotU*) or *in trans* (pFNL::*dotU*-FLAG). Panel B. Co-immunoprecipitation of PdpB with DotU. Upper portion shows anti-PdpB immunoblot of samples .immunoprecipitated with anti-FLAG antibody. Lower portion shows anti-PdpB immunoblot of bacterial extracts used in the immunoprecipitations.

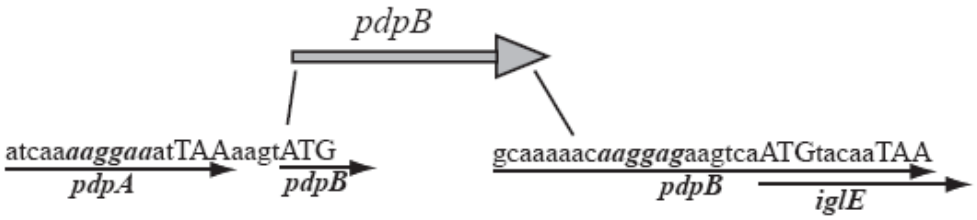

Figure 23. Representative diagram of the gene deletion strategy. For *pdpB* the structural gene was removed except for the 3'-end except for the last 22 nucleaotides which were retained in order to have the ribosome binding and start region of the downstream gene (*iglE*).

4.3.4 Deletion mutagenesis and genetic complementation reveals requirement of individual FPI genes for intramacrophage growth.

Several studies have examined the phenotypes of insertion mutants in FPI genes, and these mutants were found to be defective for intracellular growth (Gray, Cowley et al. 2002; Lauriano, Barker et al. 2004; Tempel, Lai et al. 2006; Maier, Casey et al. 2007). Since we now know many FPI insertion mutations affected the expression of downstream genes a thorough re-analysis of the role of individual FPI genes in intracellular growth was needed. To help determine the role of each FPI gene in *Francisella* intracellular growth, we constructed a marker-less deletion mutant strain for each FPI gene. In most cases the majority of the gene was deleted, and only the 3'-end that contained the predicted ribosome binding site of the downstream gene was retained (Fig. 23). In some cases, the extent of the deletion was determined by the requirement of primers used to make the deletions. Each deletion mutation was also analyzed by creating strains

containing genetic complements in the form of plasmid-borne copies of the cognate wild type gene containing a region encoding the FLAG-tag or from a chromosomal integrating vector. As shown in figure 24 (and data not shown), deletion mutants of *iglBCD, pdpB, vgrG, iglEFGHIJ* were defective for intramacrophage growth. We have previously analyzed marker-less deletion mutants and their complements for *iglA*, *pdpA* and *pdpD,* and we found that *iglA* and *pdpA* are required for intracellular growth, whereas *pdpD* is not required for intracellular growth (de Bruin, Ludu et al. 2007; Ludu, de Bruin et al. 2008; Schmerk, Duplantis et al. 2009). Additionally, deletion of *pdpC* or *pdpE* does not affect intracellular growth (Nix et al, unpublished).

Genetic complementation of each mutation restored intracellular growth, except in the case of the Δ*iglE* mutant. The restoration of the wild type intracellular growth phenotype was not always complete but in every case, except Δ*iglE*, the growth of the complemented mutant was statistically different from the mutant (Fig. 24). Despite that lack of complementation, we are confident the phenotype of the Δ*iglE* mutant reflects the loss of IglE expression alone as the gene products of genes downstream of *iglE* appeared to be at wild type levels in the Δ*iglE* mutant (data not shown). From our analysis, we conclude *pdpA*, *pdpB, dotU, vgrG,* and *iglABCDFGHIJ* are required for intracellular growth, whereas *pdpCDE* are not.

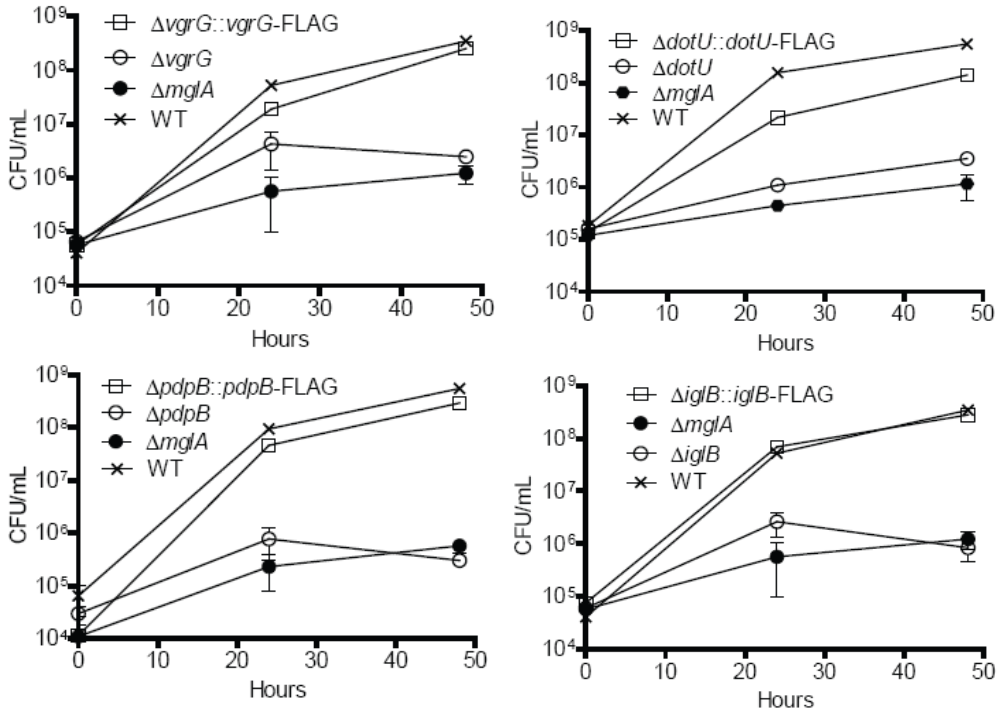

Figure 24. *PdpB, vgrG, iglB* and *dotU* are needed for growth in macrophages. J774 mouse macrophage-like cells were infected with indicated strains and harvested at 0, 24 and 48 h post infection, and the number of intracellular bacteria determined as described in materials and methods. Δ*mglA* has a mutation in a global regulator required for FPI gene expression and fails to grow in macrophages. Representative results from three replicates are shown. Similar results were obtained for a number of FPI deletion strains not shown. See text for details.

4.3.5 Outer membrane localization of IglC is dependent on a subset of the FPI genes.

We and others previously showed that the bulk of IglC is localized to the bacterial cytoplasm, but a small proportion of IglC localizes to the outer membrane. We also demonstrated IglC localization to the outer membrane is dependent upon FPI-encoded proteins IglB, PdpB and DotU (Ludu, de Bruin et al. 2008), which are predicted to have

similarity to canonical T6SS components (see Table 3) . To identify other FPI proteins involved in T6SS-like translocation of IglC, we analyzed our FPI deletion mutant strains for IglC localization to the outer membrane. In deletion mutants of *iglA, iglB, iglF, iglG, iglI, pdpB, dotU* and *vgrG*, IglC failed to localize to the outer membrane (Fig. 25A & B and Fig. 19). Deletion of *iglE* and *iglH* resulted in reduced amount of IglC in the outer membrane, whereas deletion of *iglD, iglJ, pdpA, pdpC, pdpD* and *pdpE* did not affect IglC localization at detectable levels (Fig. 25A & B). The observed defects in localization were not due to lack of expression of IglC since soluble IglC was detected in cell lysates of all strains (Fig 25A and data not shown) except in the Δ*iglG* mutant. No IglC was found in the growth-supernatant of the deletion mutant strains ruling out the possibility absence of IglC in the outer membrane in these strains is due to hyper-secretion of IglC or dissociation of IglC from the outer membrane. Genetic complementation of the Δ*pdpB* and the Δ*iglA* strains restored the localization of IglC to the outer membrane (Fig. 25). These results suggest IglABEFGHI, DotU, VgrG and PdpB participate in the localization of IglC to the outer membrane, whereas IglDJ and PdpACDE are not involved in IglC outer membrane localization.

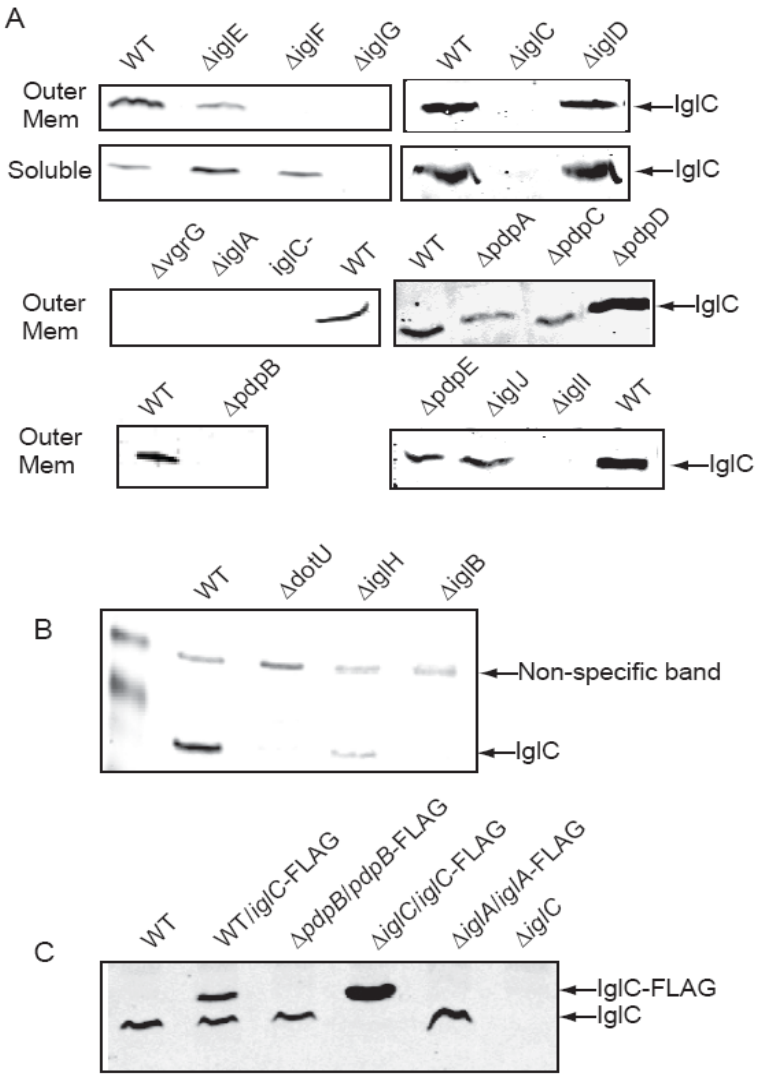

Figure 25. IglC secretion depends on FPI-encoded genes. Panels A and B. Analysis of IglC localization to outer membrane of FPI deletion strains. Panel C. Complementation restores wild type IglC localization. Outer membranes were isolated as described in materials and methods. Protein from equal amount of bacteria in each experiment were separated on a SDS-PAGE gel, transferred to a nitrocellulose membrane and probed with anti-IglC antibody. To ensure even loading between strains, membranes were stained with ponceau S. Outer membranes were free of the inner membrane protein PdpB (not shown but see figure 20).

4.3.6 IglC is secreted into the growth supernatant in a FPI-dependent manner.

As mentioned above, we did not find IglC in the growth supernatants of any of the FPI mutant strains. We reasoned this may be due to the low amounts of IglC secreted, and thus tested whether IglC could be detected in *F. novicida* strains, which have more abundant IglC associated with the outer membrane. We previously demonstrated a *F. novicida* strain over-expressing recombinant PdpD has increased amounts of IglC on its surface (Ludu, de Bruin et al. 2008). Therefore, we introduced the recombinant plasmid expressing PdpD into *F. novicida* strains with wild type, Δ*iglA*, Δ*iglC*, Δ*dotU,* Δ*iglE,,* Δ*vgrG* and Δ*iglF* genetic backgrounds and attempted to detect IglC in concentrated growth supernantants. As shown in Figure 26, IglC could be detected in the supernatant of wild type *F. novicida* over-expressing PdpD, but not in any of the FPI mutant strains over-expressing PdpD. No IglB was found to be secreted into the growth supernatant even though hyper-expression of PdpD increases the surface localization of IglB (Fig. 26B). These results suggest the presence of IglC in the supernatant is not due to leakage of soluble proteins from the bacteria, nor sloughing off of surface-exposed proteins, and demonstrate the secretion of IglC is dependent on several of the FPI-encoded proteins

4.3.7 IglC is secreted into the macrophage cytosol during *F. novicida* infection.

We hypothesized IglC may also be secreted into macrophages during infection by *F. novicida*. Testing this hypothesis we infected J774 macrophage-like cells with IglC-FLAG could, lyzed macrophages at 3 hours post infection, removed bacteria from macrophage extracts by centrifugation, and used anti-FLAG-coated agarose beads to precipitate reactive proteins, and immunoblotted the resulting precipitate. Our results

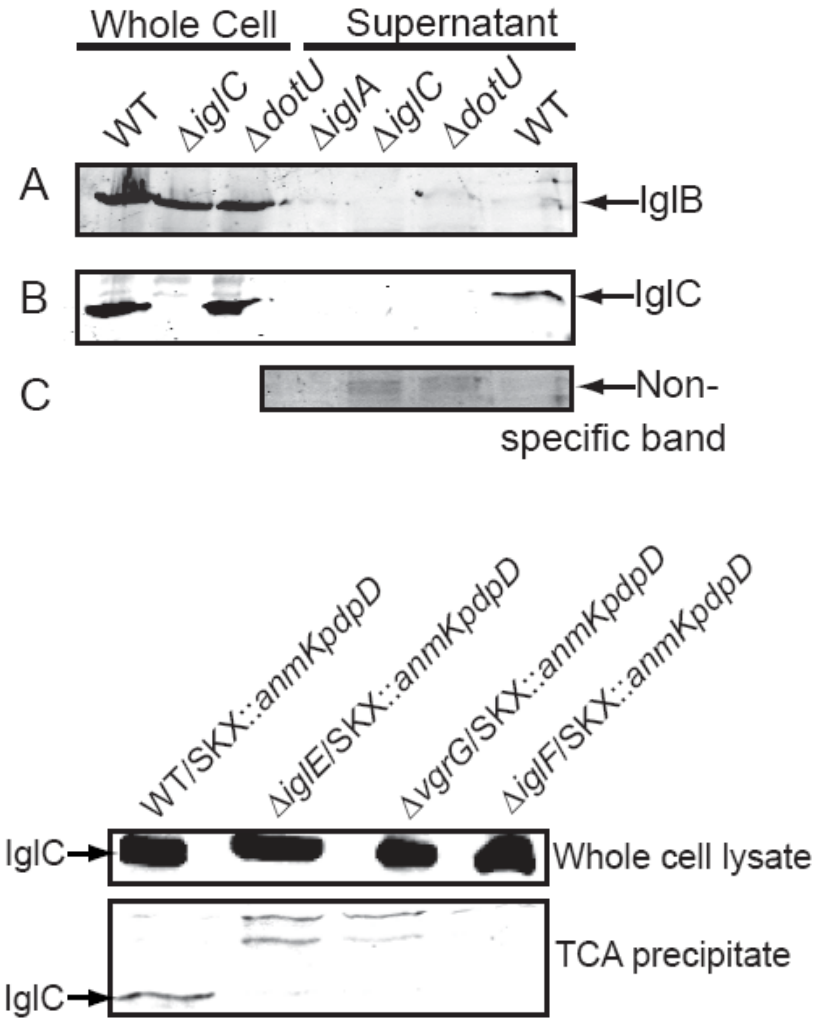

Figure 26. FPI-dependent secretion of IglC into the growth supernatant in strains over-expressing *pdpD*. Growth-supernatant proteins of *anmKpdpD* over-expressing strains were precipitated with TCA and analysed by Western immunoblotting. IglC is secreted into growth supernatant in this strain, whereas no IglB was found to be secreted into the growth supernatant (panels A and B). Although IglC could still be found inside the bacteria of deletion strains of *dotU, iglE, vgrG, iglA* and *iglF* over-expressing *anmKpdpD*, no IglC was detected in their growth supernatant.

show an anti-FLAG reactive band of the appropriate relative molecular mass for IglC-FLAG was precipitated from the macrophage lysate (Fig. 27). During co-infection of macrophages with *iglB*-FLAG and *iglC*-FLAG strains of *F. novicida*, IglC-FLAG was immunoprecipitated from the macrophage lysate, whereas negligible, if any, IglB-FLAG was precipitated (Fig. 27).

These results suggest IglC, but not IglB, is secreted into the macrophage. The residual amounts of IglB precipitated are not surprising given IglB is secreted to the *F. novicida* surface. To determine whether the presence of IglC in the macrophage lysate is dependent on components of the putative FPI-encoded T6SS we introduced the plasmid encoding IglC-FLAG into a Δ*pdpB F. novicida* mutant strain, and used this strain to infect macrophages. Whereas IglC was precipitated from macrophages infected with wild type *F. novicida* expressing IglC-FLAG protein, no IglC could be precipitated from the lysate of macrophages infected with IglC-FLAG/Δ*pdpB* suggesting PdpB is required for IglC secretion into macrophages (Fig. 27). Importantly, similar amounts of IglC-FLAG protein were found in bacteria recovered from macrophages infected with wild type and Δ*pdpB* strains expressing IglC-FLAG.

As a separate approach to detect IglC-FLAG in the host cell, we performed immunofluorescence microscopy of macrophages infected with *F. novicida iglC*-FLAG and *iglA*-FLAG. To distinguish between intra-bacterial proteins and those found outside of the bacterial cell, macrophages were treated with 0.1% saponin, which does not permeabilized the *Francisella* outer membrane (Checroun, Wehrly et al. 2006).

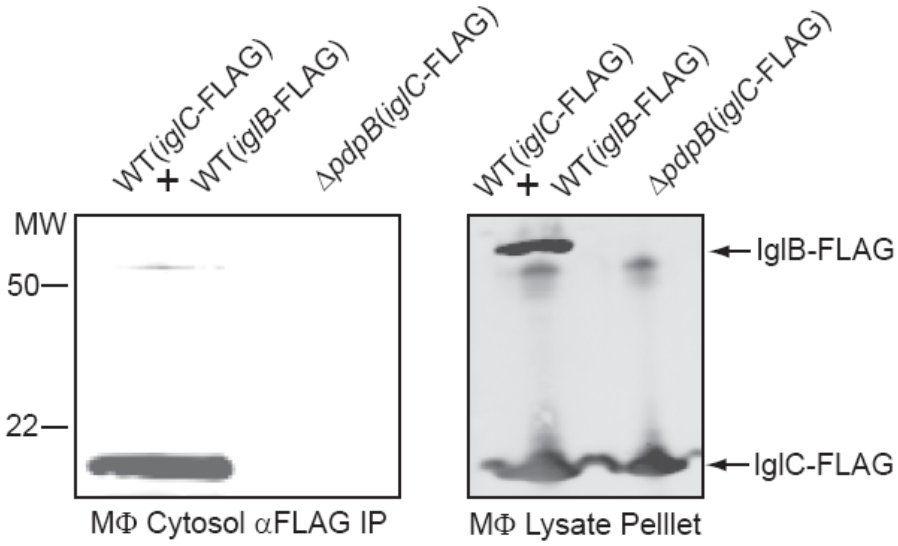

Figure 27. IglC is secreted into macrophages during *F. novicida* infection. IglC-FLAG is immunoprecipitation of from lysates of macrophages infected with WT *F. novicida* expressing IglC-FLAG, but not from cells infected with Δ*pdpB(iglC*-FLAG*)*.

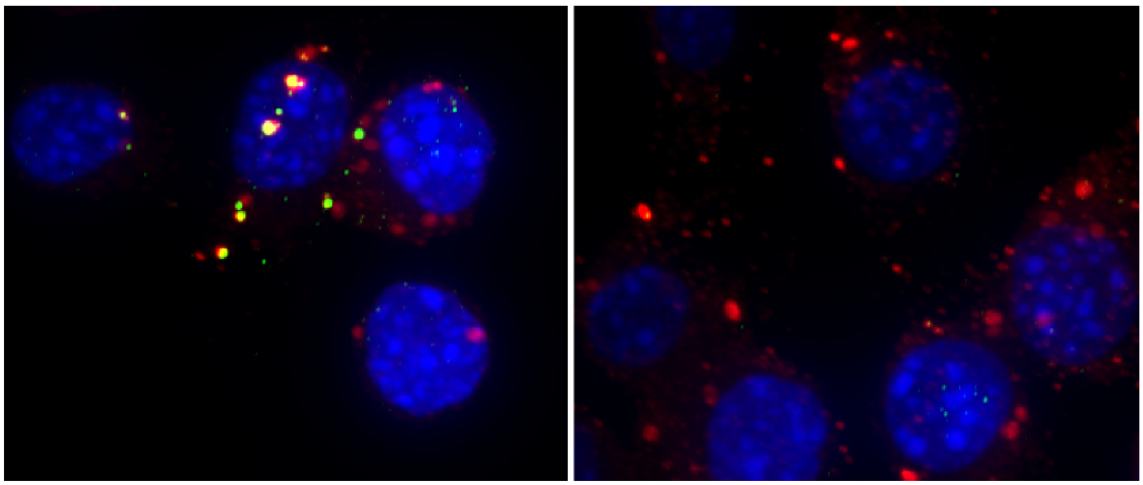

Figure 28. IglC-FLAG is secreted during *F. novicida* infection of macrophages. Fluorescence microscopy of FLAG-tagged material during infection of macrophages with either WT(*iglC*-FLAG), left, or WT(*iglA*-FLAG), right. Green represents anti-FLAG reactive material; red, bacteria; blue, host nuclei. The experiment was repeated at least three times with identical results.

Following saponin treatment, anti-FLAG reactive signal was detected in the IglC-FLAG infected macrophages, but not in macrophages infected with wild type *F. novicida* or an *iglA*-FLAG expressing strain (Fig. 28 and 29). These results support our biochemical detection of IglC-FLAG in the macrophage cytosol infected with the wild type strain expressing IglC-FLAG. Overall, these results demonstrate IglC is secreted into the host cell in a T6SS-dependent manner.

4.4 Discussion.

It is apparent that the biology of *Francisella* intracellular parasitism of macrophages is dependent on many of the gene products encoded by the FPI. However, currently no information is available on the molecular mechanism of any of the FPI-encoded proteins. A number of the FPI-encoded gene products show similarity to those found in gene clusters encoding T6SSs of other organisms. Of these, genes similar to *iglAB, pdpB* and *dotU* are found in every T6SS identified to date (Bingle, Bailey et al. 2008). However, several of the FPI genes do not show similarity at the deduced amino acid level to T6SS-encoded proteins; furthermore, the FPI apparently lacks genes encoding the T6SS components Hcp and ClpV. Given only limited similarity between T6SS and FPI genes, the assumption the FPI encodes a T6SS was recently challenged (Boyer, Fichant et al. 2009). Here we have used bioinformatic, genetic, biochemical and cell biology approaches providing evidence the FPI encodes a secretion system.

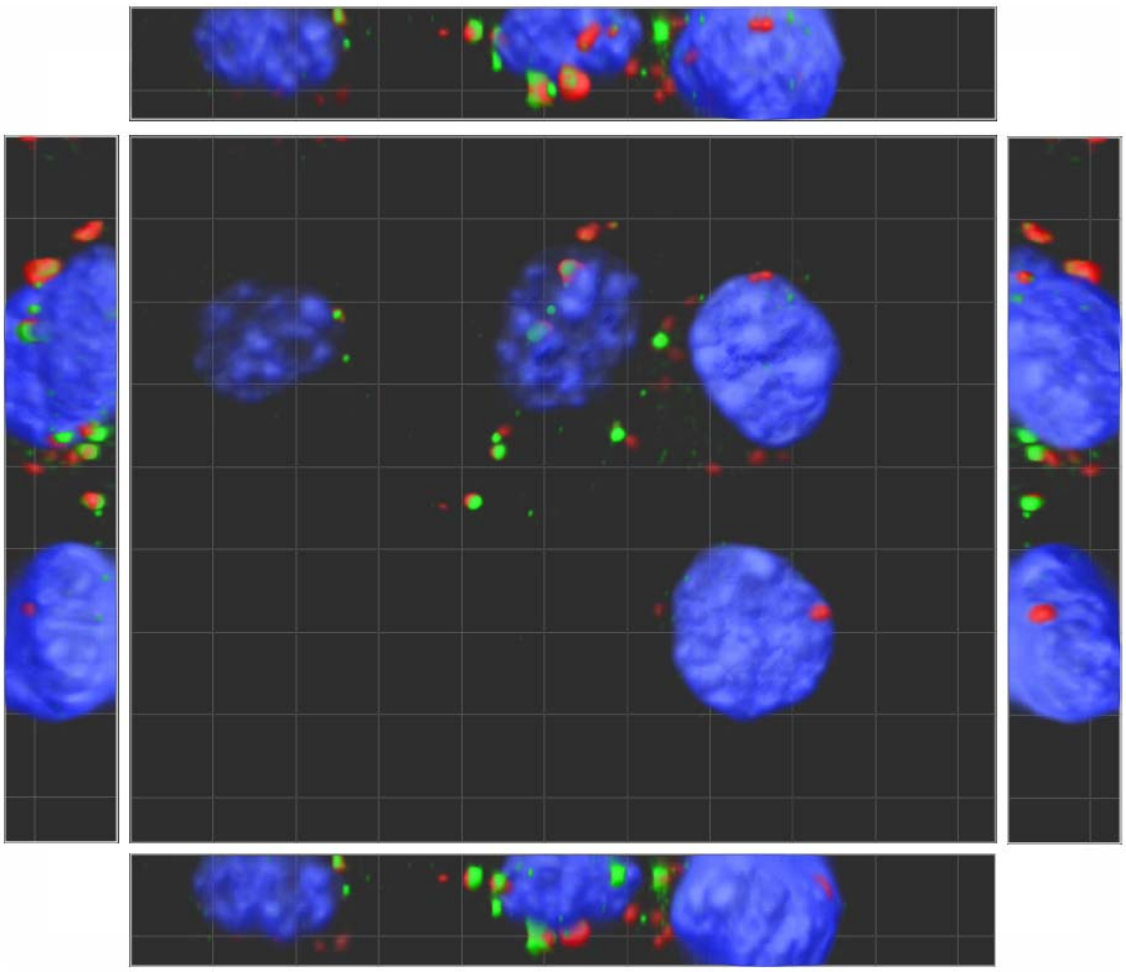

Figure 29. Flattened 3D representation of IglC-FLAG secretion during infection of macrophages. Panels flanking central micrograph are side-views of the micrograph from respective direction. Red, *F. novicida*; Green, anti-FLAG reactive material; Blue, indicates nuclei.

In our hands, the FPI is responsible for secretion of the FPI-encoded protein IglC, a novel protein not significantly similar to any other protein found in databases; therefore, the FPI-encoded secretion system appears unique among organisms. Analysis of deletion mutations in each of the FPI genes showed 14 genes of the FPI, *pdpAB,*

iglABCDEFGHIJ, vgrG and *dotU,* are required for *Francisella* intracellular growth. All of these genes, except pdpA and iglDEJ, are needed for secretion of IglC. It is possible one or more of the gene products of *pdpA* and *iglDEJ* play a minor role in secretion of IglC that we could not detect, or play a role in secretion of a yet to be identified secreted protein. Alternatively, it may be that one or more of the gene products of *pdpA* and*, iglDEJ* are secreted effector proteins. Overall, the observation 10 FPI-encoded products are required for secretion agrees well with what is found in T6SSs in other bacteria.

At present there is little information on the interaction of FPI-encoded proteins. We previously showed that IglAB could be co-immunoprecipitated and that the absence of one led to the presumed degradation of the other, which has later been observed in T6SSs. In a *Legionella pneumophila* strain lacking *icmF*, the DotU protein is missing presumably due to protease degradation (Sexton et al. 2004). Here, we provided genetic and biochemical evidence DotU and PdpB interact. We also found the deletion of *iglG* lead to the loss of detectable IglC, and this phenomenon suggest a physical interaction or an effect on transcription or translation of *iglC*; however, attempts to detect an IglC-IglG interaction by co-immunopreciptiating IglG-FLAG and IglC failed to provide supporting evidence of an interaction.

Unlike most other secretion systems, the majority of T6SS components are predicted to encode soluble proteins (Cascales 2008). Our analysis suggests that the majority of the FPI proteins do indeed associate with the bacterial membranes, which is expected of components of a secretion system (see Fig. 30 for a model of the FPI-encoded secretion system). Previous proteomics studies of *Francisella* have found IglE, DotU and PdpE in the *Francisella* membrane, which is in agreement with our results

(Twine, Bystrom et al. 2005; Pavkova, Reichelova et al. 2006). Notably, many of the FPI proteins also have a soluble portion, which may have significance related to the assembly of the system. For example, a pool of intracellular protein may be cytoplasmically-localized during broth growth becoming membrane-associated after contact with host cells. Alternatively, these soluble proteins may be localized to periplasmic space of the bacterium; however, given these proteins lack a signal peptide required for export this would be difficult to explain.

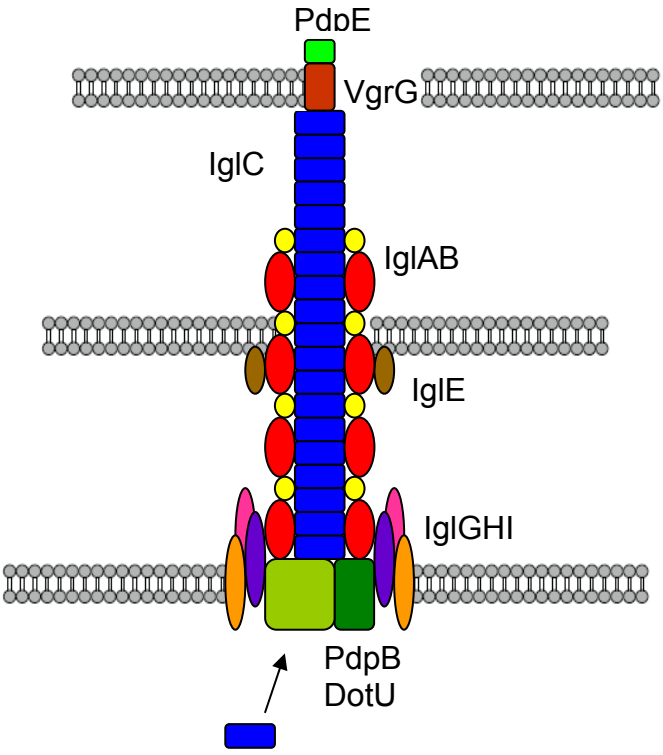

Figure 30. Model of an FPI-encoded secretion system. The model is based upon intracellular growth and secretion assays, subcellular localization experiments and homology analysis. IglAB form a cell-envelope spanning secretion channel, which IglC and possibly putative effector proteins are secreted through. IglC secretion may be accomplished according to the proposed model shown in fig. 4.

The FPI gene products which do not clearly contribute to IglC secretion or intracellular growth become candidates as substrates of the secretion system. Interestingly, we identified genes that were not required for IglC secretion, but were required for intramacrophage growth. One possibility is this set of gene products may be

needed for secretion of different secreted substrates required for proper T6SS assembly and/or intracellular growth. Such differential requirement has been shown in the secretion of *Mycobacterium* ESX-1 virulence factors (McLaughlin, Chon et al. 2007). Hidden Markov analysis of IglD protein showed it has similarity to a conserved orthologous group of proteins associated with a number of T6SSs, suggesting it may play a subtle role in secretion. Analysis of IglJ shows some similarity to bacteriophage tail proteins. Given the bacteriophage origin of a number of proteins in T6SSs it is conceivable IglJ is a secretion apparatus protein possibly sitting on top of IglC or having a role otherwise not affecting IglC secretion.

In this work we showed that the products of *pdpCDE* and *anmK* do not play a role in secretion, and we have shown previously that AnmK and PdpD are not needed for intracellular growth; further, PdpC and PdpE are also not needed for intracellular growth (Nix and Nano, unpublished). AnmK has high identity to members of COG2377, which are thought to be responsible for the utilization of exogenous or recycled 1,6-anhydro-*N*-acetylmuramic acid. Since AnmK is present in many non-pathogens and is absent from many strains of *Francisella* we have not afforded it much attention as a virulence factor. It is conceivable that it plays a minor role in restruction of the peptidoglycan to accommodate the FPI-encode secretion machinery. The case of PdpD is especially curious as deletion of *pdpD* does not affect secretion of IglC or affect intramacrophage growth, but over-expression of PdpD leads to increased amounts of IglABC on the surface of *F. novicida*. These data suggest PdpD affects the localization of IglABC, perhaps through a physical interaction. Experimental data suggests PdpE, PdpC and PdpD are candidate effectors of the FPI-encoded secretion system. Deletion of either of

these genes does not affect intracellular growth of *F. novicida;* however, PdpC and PdpD are required for virulence. Although this eludes to an important function of the proteins during infection, further studies are needed to elucidate the function of the FPI-secreted effector proteins. Furthermore, these proteins do not show similarity to any other known protein, which is a hallmark of most effector proteins (Galan, 2009). Interestingly, the observed phenotypes of *pdpCDE* mutants are similar to those described for effectors of other secretion systems such as the *Legionella pneumophila* type IV (Clemens, Lee et al. 2004; Bardill, Miller et al. 2005), the *Salmonella* SPI-2 type III (Knodler, Vallance et al. 2003), and the mycobacterium ESX-1 secretion system (MacGurn and Cox 2007). Our analysis shows PdpC, PdpD and PdpE localizing to the bacterial membranes during *F. novicida* broth growth. Effectors of type IV secretion systems in *Legionella pneumophila* (Cambronne and Roy 2007) and *Helicobacter pylori* (Couturier, Tasca et al. 2006) are known to associate with the bacterial membrane during *in vitro* growth conditions.

At present it is not clear whether any of the FPI proteins are secreted effector proteins influencig host cell responses enabling infection. A candidate effector of many T6SSs is VgrG. Like other VgrG proteins, *F. novicida* VgrG is similar to the gp5 component of bacteriophage tail-spikes, but uncharacteristically lacks a gp27 domain. Similarly to some VgrG proteins, such as VgrG-2 of *V. cholerae,* VgrG of *F. novicida* does not appear to posses an evolved effector domain. Therefore, it appears *F. novicida* VgrG and VgrG-2 of *V. cholerae* are apparatus components. Consistent with this hypothesis, both these proteins are required for T6SS-related secretion; further, *vgrG* of *F. novicida* is needed for *Francisella* intracellular growth, and *vgrG-2* is needed for intra-amoebal growth of *V. cholerae.* In the live vaccine strain of *F. holarctica*, VgrG has

been found to be surface-exposed (Melillo, Sledjeski et al. 2006), and our analysis also indicates VgrG is surface-exposed in *F. novicida.* Fractionation experiments of the bacterial cell show VgrG is found in the soluble fraction, which suggests VgrG is surface-exposed but distant from the bacterial membrane. These observations are consistent with the hypothesis VgrG is a part of the hole-poking device of this secretion system and possibly attached at the top of a surface-exposed secretion apparatus.

The major secreted protein of most T6SSs investigated to date is Hcp or an Hcp-like protein. The FPI-encoded system is considerably different from other T6SSs; therefore, it is not surprising a different protein than Hcp, namely IglC, is secreted by a *Francisella.* However, there are several similarities between IglC and Hcp or their encoding gene, which suggest these proteins have similar functions. Gene synteny can be a clue to the function of the encoded proteins (Hain, Steinweg et al. 2006). It is therefore noteworthy, like *iglC*, many *hcp* genes are found immediately downstream of homologues of *iglB.* The positioning of *hcp* and *iglC* immediately downstream of *iglB* homologues on the chromosome, together with the observation both are secreted, may point to a conserved function of these proteins. An interesting observation is IglC affects IglA and IglB outer membrane localization suggesting a role of IglC as an apparatus protein. Therefore, another commonality between Hcp and IglC are they both are required for secretion of other proteins. One further similarity is several isoforms of both Hcp and IglC exist in the bacterial cell (Pukatzki, Ma et al. 2006; Lenco, Hubalek et al. 2007; Lenco, Link et al. 2009). Given these genetic and biochemical observations, one possibility is the function of IglC in a *Francisella*-encoded secretion system is analogous

to that of Hcp and Hcp-like proteins in T6S. Consistent with this hypothesis, like Hcp, biochemical data suggests IglC spans both bacterial membranes.

The vast majority of the knowledge on IglC has been deduced from the contrasting behavior of an isogenic *iglC* deletion mutant compared to wild type in a variety of cell biology assays. Indeed, such studies have contributed significantly to the understanding of the cell biology of wild type *Francisella* infection; however, it is difficult to assign a function or role of the IglC protein solely from these types of experiments. For example, unlike the wild type strain, an *iglC* deletion mutant fails to induce apoptosis and to inhibit activation of cell signaling pathways of J774 macrophage-like cells (Telepnev, Golovliov et al. 2005); however, it is not clear how IglC contributes to these phenotypes. Additionally, a deletion mutant of *iglC* is defective for intracellular growth and impaired in its ability to escape from the phagosome of macrophages (Lai, Golovliov et al. 2004). Overall, the pleiotropic intracellular defects associated with absence of *iglC* suggest its inactivation interferes with an important virulence mechanism of this bacterium. Our biochemical and microscopic analysis offers an explanation to these observations. In wild type *F. novicida*, IglC is secreted to the surface of the bacterium, whereas its secretion is abolished in strains carrying deletions of homologues of T6SS genes. These data indicate *Francisella* has a T6SS-like secretion system and that IglC is a secreted component required for intracellular growth and phagosomal escape. Our observation several isogenic FPI mutants have identical virulence-defects suggest deletion of these individual gene results in a failure of the secretion system to assemble or function properly. The pleitropic virulence defect of Δ*iglC* may be

explained by a failure of this strain to assemble a functional secretion system essential to the intracellular life style of *Francisella*.

In conclusion, we have shown the FPI encodes a secretion system; although there are similarities between the FPI-encoded secretion system and T6SSs, the secretion system identified in this study appears to be unique and different from those previously identified.

Table 3. Results of HHpred[1] Homology Detection Analysis of FPI encoded proteins

FPI Protein	Orthologue[2]	Probability	E-value	Comment
IglA	DUF770 (T6SS)	100	0	
	FlgG, flagellar basal body protein	88.5	2.1	Central region of IglA
IglB	DUF877 (T6SS)	100	0	
	Phage Sheath	84.96	28	C-terminal 2/3
IglC	2QE4 (IglC)	100	0	
	Phage Attachment head-tail	68	19	
IglD	DUF876/T6SS	97.6	0.024	
	Phage T7 endonuclease	78.56	1.2	
PdpA	PilB type IV ATPase	92.48	0.18	
	Fibronectin	91.27	5.2	N-terminal 100 aa
PdpB	COG3523 (IcmF)	100	3.9 e^{-38}	Over aa 240-1078
IglE	Lipoprotein	92.2	0.28	N-terminal 20%
VgrG	Bacteriophage gp5	99.0	9.4 e^{-14}	
IglF	Borrelia OspD	77.8	10	
IglG	COG4104/T6SS	95.4	0.036	C-terminal 50%
	Pfam 05488 PARR motif	77.6	3.6	C-terminal 22 aa
DotU	DUF2077 (DotU)	100	8 e^{-42}	
IglJ	gp14 bacteriophage tail	43.84	57	
	Capsid protein	72.24	9.4	
PdpC	Viral glycoprotein	65.54	14	
PdpE	Glycosyl hydrolase	94.71	0.22	Over aa 107-164

1. Available on-line, http://toolkit.tuebingen.mpg.de/hhpred
2. T6SS indicates DUF or COG is associated with T6SS clusters

Table 4. Strains and plasmids used in the study of a T6S-like system of *Francisella*

Name	**Genotype/Relevant characteristics**	**Source or Reference**
U112	*Francisella novicida* prototype strain	ATCC
U112R$^-$	U112, Δrestriction genes	(Gallagher, McKevitt et al. 2008)
Δ*iglA*	U112, Δ*iglA*	(de Bruin, Ludu et al. 2007)
Δ*iglB*	U112, Δ*iglB*	This study
Δ*iglC*	U112, Δ*iglC*	This study
Δ*iglD*	U112, Δ*iglD*	This study
Δ*iglE*	U112, Δ*iglE*	This study
Δ*iglF*	U112, Δ*iglF*	This study
Δ*iglG*	U112, Δ*iglG*	This study
Δ*iglH*	U112, Δ*iglH*	This study
Δ*iglI*	U112, Δ*iglI*	This study
Δ*iglJ*	U112, Δ*iglJ*	This study
Δ*pdpA*	U112, Δ*pdpA*	(Schmerk, Duplantis et al. 2009)
Δ*pdpB*	U112, Δ*pdpB*	This study
Δ*pdpC*	U112, Δ*pdpC*	Nix, unpublished
Δ*pdpD*	U112, Δ*pdpD*	(Ludu, de Bruin et al. 2008)
Δ*pdpE*	U112, Δ*pdpE*	This study
Δ*vgrG*	U112, Δ*vgrG*	This study
Δ*dotU*	U112, Δ*dotU*	This study
U112/pJL-SKX::*amnK-pdpD*	U112/pJL-SKX::*amnK-pdpD*	This study
Δ*iglA*/pJL-SKX::*amnK-pdpD*	Δ*iglA*/pJL-SKX::*amnK-pdpD*	This study
Δ*iglC*/pJL-SKX::*amnK-pdpD*	Δ*iglC*/pJL-SKX::*amnK-pdpD*	This study
Δ*iglE*/pJL-SKX::*amnK-pdpD*	Δ*iglE*/pJL-SKX::*amnK-pdpD*	This study
Δ*iglF*/pJL-SKX::*amnK-pdpD*	Δ*iglF*/pJL-SKX::*amnK-pdpD*	This study

Δ*vgrG*/pJL-SKX::*amnK-pdpD*	Δ*vgrG*/pJL-SKX::*amnK-pdpD*	This study
Δ*pdpB*/pJL-SKX::*amnK-pdpD*	Δ*pdpB*/pJL-SKX::*amnK-pdpD*	(Ludu, de Bruin et al. 2008)
Δ*dotU* /pJL-SKX::*amnK-pdpD*	Δ*dotU* /pJL-SKX::*amnK-pdpD*	(Ludu, de Bruin et al. 2008)
Δ*dotU* /*dotU*	*in cis* comp	This study
Δ*iglB*::pKH18	*in trans* comp	This study
Δ*dotU*::pKH13	*in trans* comp	This study
Δ*vgrG*::pKH10	*in trans* comp	This study
Δ*pdpB*::pKH24	*in trans* comp	This study
Δ*iglF*::pKH26	*in trans* comp	This study
Δ*iglH*::pKH12	*in trans* comp	This study
Δ*iglI*::pKH14	*in trans* comp	This study
Δ*iglC/*pJL-SKX::*iglC*	*in cis* comp	This study
Δ*iglD/*pJL-SKX::*iglD*	*in cis* comp	This study
Δ*iglG/*pJL-SKX::*iglG*	*in cis* comp	This study
Δ*iglJ/*pJL-SKX::*iglJ*	*in cis* comp	This study
U112R$^-$ ::pKH4	*F. novicida* expressing FLAG-tagged IglC protein	This study
U112R$^-$ ::pKH5	*F. novicida* expressing FLAG-tagged PdpC protein	This study
U112R$^-$ ::pKH6	*F. novicida* expressing FLAG-tagged IglD protein	This study
U112R$^-$ ::pKH7	*F. novicida* expressing FLAG-tagged PdpD protein	This study
U112R$^-$ ::pKH8	*F. novicida* expressing FLAG-tagged IglA protein	This study
U112R$^-$ ::pKH9	*F. novicida* expressing FLAG-tagged IglE protein	This study
U112R$^-$ ::pKH10	*F. novicida* expressing FLAG-tagged VgrG protein	This study
U112R$^-$ ::pKH11	*F. novicida* expressing FLAG-tagged IglG protein	This study
U112R$^-$ ::pKH12	*F. novicida* expressing FLAG-tagged IglH protein	This study
U112R$^-$ ::pKH13	*F. novicida* expressing FLAG-tagged DotU protein	This study

U112R$^-$ ::pKH14	*F. novicida* expressing FLAG-tagged IglI protein	This study
U112R$^-$ ::pKH15	*F. novicida* expressing FLAG-tagged IglJ protein	This study
U112R$^-$ ::pKH16	*F. novicida* expressing FLAG-tagged PdpE protein	This study
U112R$^-$ ::pKH18	*F. novicida* expressing FLAG-tagged IglB protein	This study
U112R$^-$ ::pKH22	*F. novicida* expressing FLAG-tagged PdpA protein	This study
U112R$^-$ ::pKH24	*F. novicida* expressing FLAG-tagged PdpB protein	This study
U112R$^-$ ::pKH26	*F. novicida* expressing FLAG-tagged IglF protein	This study

Table 5. Primers used to construct strains and plasmids.

Designation	Nucleotide sequence
iglB-F	cCTCGAGggggatctacagaagttga
iglB-R	ttaCTCGAGccgaataattctggttcaagc
iglB RR	ccgtcgactcaaaggcttttggaaatcaa
iglB LF	ccgtcgacgaagaagataattcttcttctgaaaccg
iglB LR	CCTCGAGattgtcataacaaaatcctctctactt
iglB RF	cctcgagtgactatagatactaggcttgaacca
iglB comp F	ccctcgagTCTTTTGATTTTGAGGCACCA
iglB comp R	ccctcgagACCCCATGCTTCATCAGTTTT
dotU comp F	ccctcgagGAATATGTATTCAAAAACAACATTCCT
dotU comp R	ccctcgagTTTTCCATTTCTCAGAGCCTTG
fvp comp F	ccctcgagTTAAATCTTAAAACAGGAAAGAATGAA
fvp comp R	ccctcgagGAATAATATCGCTAGCTAAAAGACGA
Fvp RL Eag	GGCGGCCGcaagccttgctcttctaagttga
Fvp RR Xho	CCCTCGAGtaactcggctgcaacaaatg
Fvp LL Xho	CCCTCGAGaactttgttaataagtttgctggaga
Fvp LR Eag	GGCGGCCGgcagcaacaatggttggata
Fvp His Xho R	ccCTCGAG ttaTTATCCAACCATTGTTGCTG
Fvp His Nde F	ccCATATG TCAAAAGCAGACCATATTTTCAA
6LF	CGCGTCGACTCTGCTTTTATACCCACGAACC
6RR	CGCGTCGACTGTATTTGGGAGCACATTGAA
6LR	ATCTCGAGTTCTAATATCCTTTATATAGAGTTATTT AAAACAAA
6RF	ATCTCGAGAGCTGGTAATAAGGGTAAGAGGAGA
pdpB RR	ATGTCGACATGTGAAAATATCTACGGCA
pdpB LF	GCGTCGACATATCTTGTAAAGGGAAATC
pdpB LR	CTCGAGAGAAGTCAATGTACAATAAATTATTGAA
pdpB RF	CTCGAGTTGATGATTTTTAATAAAATTCATACTT

Designation	Nucleotide sequence
pdpA promoter UP	aaggatccttagaacataactaaagtcattttacataagcac
pdpA promoter TT-16S	aaagatctagtttaattctcaaattctgactctaactactg
iglD-R	gggaattcttaagaaaaggctataaagaaatc
iglD-L	aaggatccggatatattgcagctgcatagt
IglE-R	gggaattcttaatctttttctatgctgctatc
iglE-L	aaggatccattcttacaagtattaggatggc
iglG-R	gggaattcctaagatgtttttacatttatttgtc
iglG-L	aaggatcccaaataatccgatcattgatagtat
iglJ-R	gggaattctcataaattaaaataacctagatatatc
iglJ-L	aaggatccaaaaccagaatgattcggtag
pdpD_C_termFLAG_F_EcoR1	ggcgaGAATTCgtaagagtagtaagtatggatcaag
pdpD_C_termFLAG_B_N	ggcgaCCATGGGaacccagatcattggtctatac

co1	
iglA_C_term_F_EcoR1	agagGAATTCgtaaaaaaaggacaataagatg
iglA_C_term_B_Nco1	ggaaCCATGGCcttatcatctacttg
iglB_C_term_F_Nde1	gagaCATATGgtagagaggattttgttatg
iglB_C_term_B_Nco1	agagCCATGGCgttattatttgtacc
iglC_C_term_F_EcoR1	gaGAATTCaaaggagaatgattatgagtgag
iglC_C_term_B_Nco1	gaCCATGGGtgcagctgcaatatatcc
iglD_C_term_F_EcoR1	ggcagGAATTCaagatcggagttgattctaatg
iglD_C_term_B_Nco1	ggcgaCCATGGGagaaaaggctataaagaaatc
pigI_C_term_F_EcoR1	gaagGAATTCcttaaggatgcaaaaatatg
pigI_C_term_B_Nco1	aggcCCATGGCtattatagtaattttcttttc
pdpC_C_term_F_EcoR1	ggcgaGAATTCgataaattaaggaagtacatatg
pdpC_C_term_B_Nco1	ggcagCCATGGGtgacgatatttttttaaaaaagtc
pigH_C_term_F_EcoR1	caagGAATTCcaaatgagatagatg
pigH_C_term_B_Nco1	agcgCCATGGCtaaattaaaataacc
pigG_C_term_F_EcoR1	cgagGAATTCgggtaagaggagatttatatg
pigG_C_term_B_Nco1	gaagCCATGGCtatgtcaaaaagatcttc
pigF_C_term_F_EcoR1	aggaGAATTCctatataaaggatattagaaatg
pigF_C_term_B_Nco1	aggaCCATGGCccagcttaataaaattag
pigE_C_term_F_EcoR1	agagGAATTCcttagaaggtcattatcatg
pigE_C_term_B_Nco1	agagCCATGGCtatagagttatttaaaacaatc
pigD_C_term_F_EcoR1	agagGAATTCgaagcttattggaaaatttaaatg
pigD_C_term_B_Nco1	agagCCATGGCagatgtttttacatttatttg
pigC_C_term_F_Nde1	gcagCATATGcaatggttggataataatatg
pigC_C_term_B_Nco1	agcaCCATGGCattttccaataagcttcttgcttgc
pigB_C_term_F_EcoR1	gagaGAATTCgattaaggggatattcttatg
pigB_C_term_B_Nco1	agagCCATGGCtccaaccattgttgctgcggaacc
pigA_C_term_F_EcoR1	aggaGAATCCggcaaaaacaaggagaagttaatg
pigA_C_term_B_Nco1	gacgCCATGGCatctttttctatgctgctatc
pdpB_C_termFLAG_F_KpnI1	agGGTACCcaaaaggaaattaaaagtatg
pdpB_C_termFLAG_B_Nco1	agCCATGGGttgtacattaacttctccttg
pdpA_C_termFLAG_F_Nde1	ggcagCATATGctaattaagtagacaatgatagc
pdpA_C_termFLAG_B_Nco1	ggcagCCATGGGatttccttttgatttatatc

iglE-F X-clone1-L	AAACTCGAGATCCGAAACCAATTCATCTAAAAA
iglG-R X-clone1-R	AAACTCGAGCTTTTTAATAAAGCTAAAGGTGTTTTG
iglE-L deletion Bam-pigA-L end	AAGGATCCAAAAATGATAGCAGCATAGAAAAAGA
iglE-R deletion Bam-pigA-R start	AAGGATCCTACAACTGCTAAGCCCTATGATTG

iglF-L deletion Bam-pigC-L end	AAGGATCCCCGATCATTGATAGTATAAAAAGCAA
iglF-R deletion Bam-pigC-R start	AAGGATCCTTCGTTGAGTATCTTACCTCTTCAA
iglG-L deletion Bam-pigD-L end	AAGGATCCTCCGGGTGGTATAGTTGATTTTAT
iglG-R deletion Bam-pigD-R start	AAGGATCCTTATTTCTTCCAAGCGTTTTAAGG
iglH-F X-clone2-L	AAACTCGAGCCAATTATTAAAGGATATGGAACAAAA
iglJ-R X-clone2-R	AAACTCGAGAAATGTTTCACCAAGCCAAATAAT
iglH-L deletion Bam-pigE-L end	AAGGATCCCACCTTTAGCTTTATTAAAAAGATTGT
iglH-R deletion Bam-pigE-R start	AAGGATCCTGCTAGCATCATTCGTACTTAAATC
iglI-L deletion Bam-pigG-L end	AAGGATCCCCAGAATGATTCGGTAGAAAAA
iglI-R deletion Bam-pigG-R start	AAGGATCCCGTTTTCATATTTTGCTTGTATTTG
iglJ-L deletion Bam-pigH-L end	AAGGATCCAAATCTCCCATTTAAGATAGAACTTG
iglJ-R deletion Bam-pigH-R start	AAGGATCCCAAAAAGATCTTCAAAATAGTCTTCA
pdpE/iglC-F X-clone3-L	AAACTCGAGTAGAGTTTCACCAGAATTACTTAACAA
iglC/pdpE -R X-clone3-R	AAACTCGAGAAAAGGACAATAAGATGGCAAAAA
iglC-L deletion Bam-iglC-L start	AAGGATCCACATGCAGTAGGATCAGTTCTCAC
iglC-R deletion Bam-iglC-R end	AAGGATCCTGGTCTTACAACATCTCAAGGAAG
iglD-L deletion Bam-iglD-L start	AAGGATCCAACCATCTTCCCAATAAATCCTTT
iglD-R deletion Bam-iglD-R end	AAGGATCCTATTAGCGGATAGTAGTGCGGTTT
pdpE-L deletion Bam-pdpE-L end	AAGGATCCGGCTATCCCTCATTATGAAAAGAA
pdpE-R deletion Bam-pdpE-R start	AAGGATCCTTGTATTACTAAATGTTTGTTGGAACG

Chapter 5. Conclusions and future studies

Of the six kingdoms of life it can easily be argued that the kingdom of Bacteria is the most dominating of all; these organisms are ever present and affect all parts of our daily lives. We often equate bacteria with disease, and for a good reason: despite increase in research activity bacterial infections remain a leading cause of death worldwide. While the advent of bacterial antibiotic resistance can only compound this problem, continued research casting light upon basic aspects of bacterial disease mechanisms provides an untapped resource for exploring new areas of disease treatment.

Horizontally-acquired fitness islands confer antibiotic resistance and novel virulence factors to bacteria. T6SSs are poorly characterized, but appear to play important functions to over 90 bacterial species, many which are pathogens. We have identified a pathogenicity island encoding a unique secretion system related to T6SSs as a major virulence factor of *Francisella tularensis*. In *Francisella,* this secretion system secretes the FPI-encoded protein IglC and is required for intracellular growth and phagosomal escape. Systematic deletion mutagenesis determined the contribution of individual FPI-encoded proteins to intramacrophage growth and IglC secretion. Intracellular growth was dependent on 14 of the 17 genes in the FPI, and secretion of IglC was absolutely dependent on 10 FPI genes. It appears two other FPI gene products contributed partially to secretion of IglC. Hence, the FPI-encoded T6SS appears to have component numbers similar to those found in T6SSs.

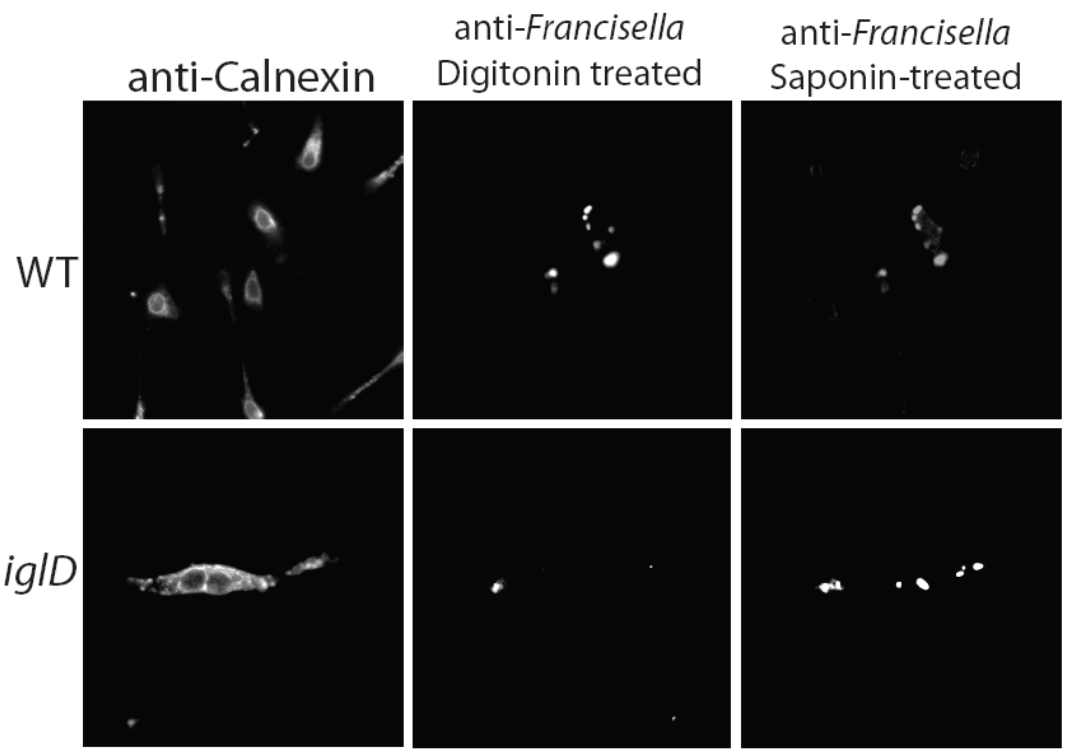

Figure 31. A Δ*iglD* mutant is defective in phagosomal escape. Mouse macrophages were infected for 4 h with indicated strains and processed for fluorescence microscopy after sequential treatment with digitonin and saponin. Digitonin permeabilization allows antibody to reach the macrophage cytosol, and saponin treatment allows antibody access to the inside of phagosomes. After digitonin treatment all WT bacteria are labeled indicating cytosolic localization, whereas only a small fraction of Δ*iglD* mutant bacteria are labeled suggesting localization in the phagosome of most Δ*iglD* bacteria. Anti-calnexin antibody was used to identify infected macrophage cells.

Evidence gathered from multiple avenues supports the hypothesis the FPI encodes a specialized T6SS. In addition to secretion of IglC into the growth supernatant of

Francisella, IglC was detected in the cytosol of infected macrophages from secretion-competent *F. tularensis,* but not from secretion-negative *F. tularensi*s. Fluorescence microscopy showed FLAG-tagged IglC, but not FLAG-tagged IglA, nor IglB, is secreted into macrophages during *Francisella* infection of immune cells. Cumulatively, it appears IglC is an important apparatus component of the FPI-encoded secretion system. *iglC* deletion mutants of *F. novicida* and *F. holarctica* are defective for intramacrophage growth in the host cytosol; additionally, Δ*iglC* mutants of *F. holarctica* LVS are defective for phagosomal escape (Lindgren, Golovliov et al. 2004). Our preliminary analysis indicate other FPI deletion strains needed for intracellular growth, including Δ*iglD* and Δv*grG,* are also defective for phagsosomal escape (Fig. 31). Therefore, a main function of the FPI-encoded secretion system is to overcome host cell defenses, such as phagosomal maturation, enabling the bacteria to gain access to the host cytosol.

It is not known what intracellular stimuli expression of the FPI responds to, but intracellular transcriptional profiling analysis suggests the FPI is up-regulated early during infection before phagosomal escape, as well as at a late stage associated with re-entry of *Francisella* into an autophagosome. Our analysis of protein levels of IglA and IglB support up-regulation of FPI-encoded products at an early stage of infection. Perhaps surprisingly, acidification of the *Francisella*-laden phagosome is not required for FPI expression (Chong, Wehrly et al. 2008). As inhibition of acidification delays bacterial escape, FPI-independent escape mechanisms could therefore exist. In view of current data, one possibility is the FPI encodes a secretion system required for multiple virulence-associated phenotypes; however, inactivation of FPI genes encoding proposed secretion apparatus components renders the bacterium defective in phagosomal escape

precluding genetic studies of other virulence-associate FPI functions using secretion-deficient mutants in a macrophage-infection model.

In a number of organisms, T6SSs are tightly regulated, and this appears to be the case for *Francisella* as well (Pukatzki, Ma et al. 2006). Genetic manipulation was necessary to identify T6S in *Pseudomonas aeruginosa* and *Burkholderia mallei* (Mougous, Cuff et al. 2006; Schell, Ulrich et al. 2007), and here a similar approach identified secretion of a T6S-substrate into the bacterial growth-supernatant. Over-expression of PdpD causes increased amounts of IglC on the surface and IglC secretion into the supernatant. The mechanism explaining this phenomenon is not presently known. Future studies could ascertain whether PdpD interacts with IglC, possibly as an effector molecule, or whether the function of PdpD is regulatory.

Investigation of individual secreted effectors of the FPI-encoded secretion system promises to reveal how *Francisella* is able to be such a successful intracellular pathogen. Our preliminary data show PdpE is secreted during macrophage infection (Fig. 32). Given the outer membrane localization of PdpE and the observation this protein is not required for IglC secretion, it should be considered a possibility PdpE is attached at the tip of an FPI-encoded secretion apparatus conferring an effector function. The mode of effector delivery is not clear at present; however, our results for PdpE are consistent with a recently proposed mechanism for T6SS effector-delivery (Leiman, Basler et al. 2009; Pukatzki, McAuley et al. 2009). According to this model, effectors are attached at the tip of the secretion machinery and are then displaced away from the apparatus. It should also be considered a strong possibility genes encoding effectors of this secretion system are localized elsewhere on the chromosome outside of the FPI. Identification of novel

genes co-regulated with the FPI should facilitate identification of such effectors. Apart from an involvement in phagsosomal degradation, effector functions of the FPI may include interference with host cell signaling pathways, delay of phagosomal maturation, modulation of an autophagic response, and possibly inhibition of inflammasome activation and other cytosolic killing mechanisms of the host.

Proteins found in the supernatant during broth growth of bacteria are often deemed as secreted; however, the abundance of outer membrane proteins largely contributes to the so-called secretome of a bacterium. In truth, most proteins found in the growth supernatant of a given bacterium have fallen off the outer membrane of the bacterium. Thus, *bone fide* bacterial secreted proteins are surface-exposed proteins. We found IglC to be surface-exposed and exposed on the outer membrane of *F. novicida.* We took advantage of this observation to assay our FPI deletion mutant strains for their contribution to IglC secretion. Supporting the validity of this approach to measure secretion, many proteins found in the growth supernatant are indeed surface-localized secretion apparatus components, which are shed into the supernatant during broth-growth (Kimbrough and Miller 2000; Freeman, Rappl et al. 2002). *In vitro* secretion of Hcp proteins by T6SSs has been proposed to occur by a similar mechanism, where Hcp proteins form a membrane-spanning channel ultimately exposed on the bacterial surface resulting in shedding of Hcp into the extracellular milieu (Mougous, Gifford et al. 2007; Filloux, Hachani et al. 2008).

The structure of Hcp is remarkably similar to the tail tube of bacteriophages (Kostyuchenko, Chipman et al. 2005). It is therefore possible that Hcp forms a structure related to bacteriophage tail tubes, which would form a membrane-spanning tunnel

(Leiman, Basler et al. 2009). Indeed, biochemical and cell biology evidence have shown Hcp may form a membrane-spanning tube (Ballister, Lai et al. 2008; Wu, Chung et al. 2008). The localization pattern of IglC to all compartments of the bacterial cell, as well as its surface-exposure, suggest IglC has the capacity to form a similar structure.

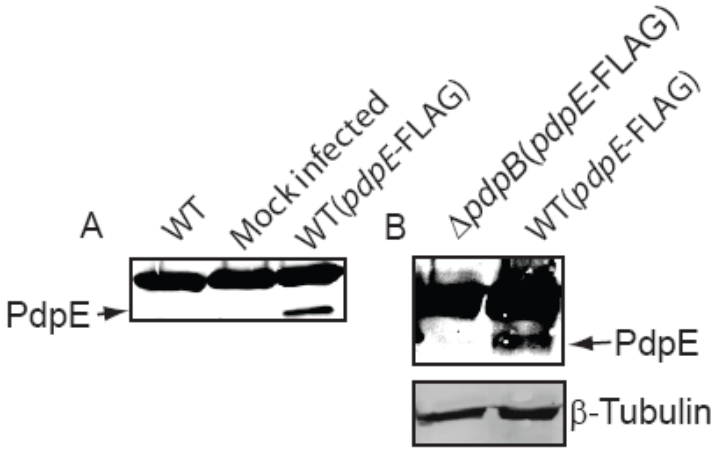

Figure 32. Western blot analysis of FLAG-precipitated proteins from macrophage lysates infected with indicated *F. novicida* strains. Panels A and B. PdpE was precipitated from lysates of macrophages infected with wild type *pdpE*-FLAG bacteria; no detectable amounts of PdpE were precipitated from macrophage cells infected with Δ*pdpB*(*pdpE*-FLAG) or wild type bacteria not carrying a FLAG-tag. Anti-tubulin indicates comparable amount of macrophage lysate was loaded in both lanes. Comparable amounts of PdpE in the bacterial pellet of both strains indicate the amount of bacteria in each infection were similar (not shown).

In this work several experiments demonstrate FPI-dependent IglC secretion. The amino acid sequence of IglC offers few clues to its function; however, it is noteworthy that IglC previously has been found to be one of the most prominently up-regulated proteins during infection of mice (Twine, Mykytczuk et al. 2006). Our data supports IglC together with IgA and IglB form a secretion tube. Consistent with this hypothesis, it is noteworthy the crystal structure of IglC does show some distant similarity to gp27 of bacteriophages suggesting a structural role. Finally, VgrG and PdpE are located on top of the secretion channel-forming tube. According to our model, we speculate contraction of IglA and IglB polymers, as observed in *V. cholerae,* is responsible for secretion of IglC. In addition to identifying and characterizing effectors of the FPI-encoded secretion system, future studies could be aimed at characterizing the structure and interaction of FPI secretion system apparatus components. Overall, the results of this study could inspire research projects focusing on the FPI and its encoded proteins in *Francisella* intracellular growth and virulence for years to come.

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