Discovery and Analysis of Novel *Francisella* Virulence Factors

by

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CERTIFICATE OF APPROVAL

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List of Abbreviations

CD	circular dichroism
CDC	Centers for Disease Control and Prevention
CFU	colony forming unit
FCP	*Francisella*-containing phagosome
FCV	*Francisella*-containing vacuole
FPI	*Francisella* pathogenicity island
IAHP	IcmF associated homologous proteins
IF	intrinsic fluorescence
IMP	inner membrane protein
IS	insertion sequence
LD_{50}	lethal dose, 50%
LDH	lactate dehydrogenase
LPS	lipopolysaccharaide
LVS	live vaccine strain
MLVA	multiple-locus variable-number tandem repeat analysis
MS	mass spectrometry
OMP	outer membrane protein
PCR	polymerase chain reaction
SOE-PCR	splicing by overlapping extension PCR
TLR	toll-like receptor
T(n)SS	type (number) secretion system, example: T3SS
T4P	type four pili

Acknowledgements

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Most importantly, I would like to dedicate this work to my wonderful husband, Erik. He shared all of my frustrations and triumphs along the way, and his unconditional love and patience sustained me through this challenging journey.

Abstract

Francisella tularensis is a highly infectious bacterium whose mechanisms of pathogenesis are just beginning to be defined. Until recently, there were a very limited number of procedures available for studying *F. tularensis*; hence, only a handful of virulence factors and targets for vaccine development had been identified. Additionally, the process of *Francisella* entry is poorly understood.

The work presented in this thesis addresses these issues with the development of a transposon mutagenesis technique and its application for the discovery of several novel virulence factors in *F. tularensis* ssp. *novicida*. Subsequent studies demonstrated that immunization with mutant derivatives of four of these virulence factors provided protection against challenge with high doses of wild-type bacteria in a mouse model, thus showing promise as suitable candidates for vaccine development.

One of the transposon insertions disrupted an uncharacterized hypothetical gene. Sequence analysis revealed the presence of seven tandem repeat motifs in this gene in subspecies *novicida* that are present in one or two copies in other *F. tularensis* homologs. Expression and characterization of one of the repeat units showed that it is a stably folded domain capable of forming oligomers. Further studies suggest that this motif may have a specific role in *F. tularensis* entry, as it binds host surface factors associated with the E-cadherin complex and shares sequence and structural homology with repeat domains of a *Staphylococcus aureus* protein that is important for adherence and entry into host cells.

Together, the experiments described in this thesis provide information about the virulence strategies of *Francisella* and will facilitate future studies regarding vaccine development and elucidating the molecular mechanisms of *Francisella* pathogenesis.

Chapter 1

Introduction: An overview of *Francisella tularensis* history, infection, vaccine development, and research strategies

I. *Francisella tularensis*

Francisella tularensis is the Gram-negative, facultative, intracellular bacterial pathogen that causes tularemia, a debilitating and potentially fatal disease that affects humans and a wide range of animals. Infection can occur through many routes, and as few as ten bacteria are sufficient to cause a severe illness. Because of its high infectivity and ease of dissemination, *F. tularensis* is considered a prime candidate for use as a biological weapon. Efforts to generate an approved tularemia vaccine have been hindered by the relative paucity in knowledge about the molecular mechanisms of *F. tularensis* virulence. Several recent studies, including work presented in this thesis, have begun to unravel the processes that make *F. tularensis* such a successful pathogen.

A. Discovery and classification

F. tularensis was first isolated from ground squirrels suffering from "plague" in Tulare County, California in 1911 and was termed *Bacterium tularense* (McCoy 1912; Francis 1919). Upon serological analysis, the bacterium was designated as *Pasteurella tularensis,* but it was later discovered that the genus was not closely related to *Pasteurella* (Ritter and Gerloff 1966). In recognition of the work of Edward Francis, who revealed that seemingly disparate diseases in humans were all manifestations of tularemia, the bacterium was renamed *Francisella tularensis.* Subsequent studies of 16S rDNA sequences showed that *Francisella* taxonomically belongs to the γ-subclass of *Proteobacteria,* despite having no close relationships to other characterized genera in the same grouping (Forsman, Sandstrom et al. 1994).

The various reports on tularemia led to multiple classifications of the organism based upon the infectivity and epidemiology of the strains. It was particularly noted that different isolates exhibited distinct levels of virulence in several hosts (Olsufiev, Emelyanova et al. 1959). For example, a subset of *Francisella* isolated from infections in North America was highly infectious for the majority of species evaluated and caused the most severe form of disease in humans while a less virulent organism was identified in Europe, Asia, and North America. Based on the difference in virulence, the designation *F. tularensis* biovar tularensis was proposed for the most virulent organisms found only in North America, and the less virulent organisms were classified as *F. tularensis* biovar palaearctica.

A decade later, the same group recommended that the biovars be given the status of subspecies and suggested *F. tularensis* ssp. *tularensis* as the designation for the most virulent form of *Francisella* and *F. tularensis* ssp. *holarctica* for the less virulent form (Olsufjev 1970). These subspecies are also commonly known as type A and type B strains, respectively, and are considered to be the etiologic agents of human tularemia. Additionally, a third subspecies, *F. tularensis* ssp. *mediasiatica,* was proposed for strains originating from the Central Asia republics of the Soviet Union. In 1959, a *Francisella*-like bacterium originally labelled as a *Pasteurella* strain was designated *F. novicida* after being shifted to the *Francisella* genus (Olsufiev, Emelyanova et al. 1959). Subsequent analysis of biochemical properties and DNA relatedness resulted in the establishment of a fourth subspecies, *F. tularensis* ssp. *novicida* (Hollis, Weaver et al. 1989). To date, there have been no changes from these classifications; hence, there are currently four recognized subspecies of *Francisella*: *tularensis, holarctica, novicida,* and *mediasiatica*.

B. Genetics and Evolution

As mentioned, *Francisella* belongs to the γ-subclass of *Proteobacteria*. Studies of 16S rDNA and sequence homology indicate that members of this genus have no close pathogenic relatives and may be more related to arthropod endosymbionts (Forsman, Sandstrom et al. 1994). Indeed, subsequent phylogenomic analysis of *F. tularensis* and 15 other γ-proteobacterial genomes suggest that *F. tularensis* is the most divergent of the γ-proteobacteria (Figure 1-1) (Larsson, Oyston et al. 2005). Although *F. tularensis* shares lifestyle similarities with other γ-proteobacterial pathogens, its position in the phylogenetic tree suggests that it underwent independent evolution.

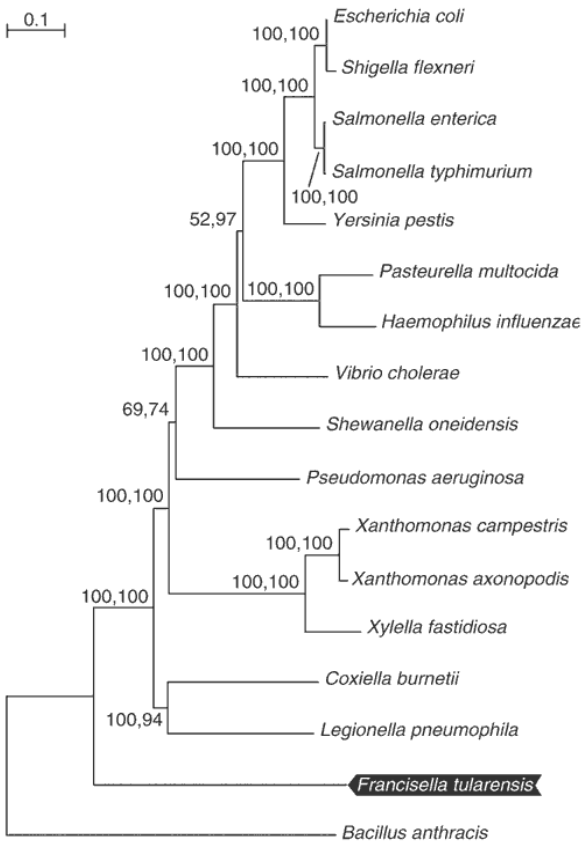

Figure 1-1. Phylogenetic relationship of 16 γ-proteobacterial species. The tree was created using a concatenated alignment of proteins encoded by *dnaA*, *ftsA*, *mfd*, *mraY*, *murB*, *murC*, *parC*, *recA*, *recG*, and *rpoC*. *B. anthracis* was used as the outgroup. The topology, branch lengths, and bootstrap support values are according to the reconstruction with the neighbor-joining method. The values indicated at the nodes are bootstrap support values for the neighbor-joining and maximum parsimony methods. [Reproduced with permission from (Larsson, Oyston et al. 2005)]

The genome of *F. tularensis* is contained on a single, circular chromosome and is comprised of approximately 1.9 megabases. The chromosome map from the first sequenced strain, *F. tularensis* ssp. *tularensis* Schu S4, is provided as an example in Figure 1-2 (Larsson, Oyston et al. 2005). Currently, data from six genome sequencing projects have been deposited into the

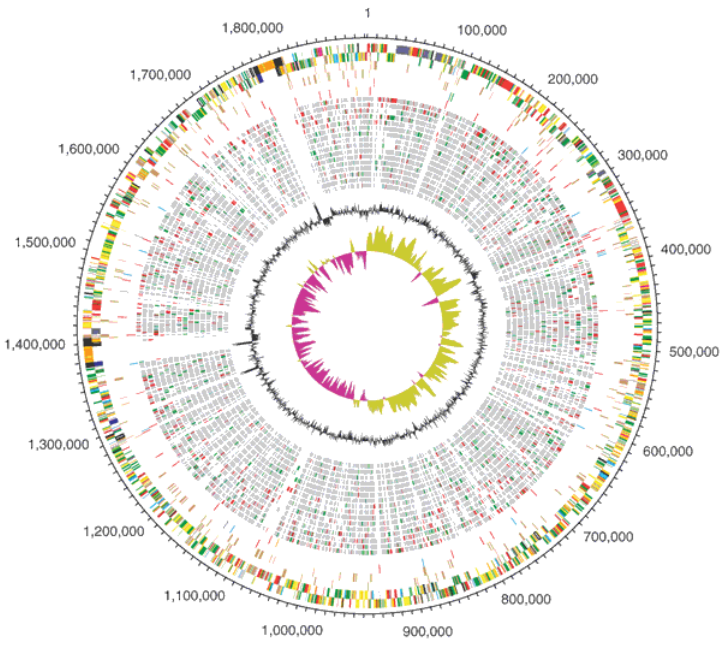

Figure 1-2. Circular map of the genome of *F. tularensis* ssp. *tularensis* Schu S4. The outer scale indicates base pairs. Numbering from the outside, circles 1 and 2 represent genes, circles 3 and 4 represent pseudogenes, and circles 5 and 6 show insertion sequence (IS) elements. The next 16 circles indicate the location of genes with matches to *L. pneumophila*, *P. aeruginosa*, *V. cholerae*, *C. burnetii* RSA 493, *B. anthracis* Ames, *S. oneidensis*, *E. coli* K12, *H. influenzae*, *P. mulocida*, *S. enterica* serovar Typhi, *S. enterica* serovar Typhimurium LT2, *X. aconopodis*, *X. capestris*, *Y. pestis*, *S. flexneri* 2a, and *X. fastidiosa*, respectively. Red is used to show the hits with highest homology, green shows the next best, and gray shows genes with sequence similarity less than 10^{-10}. The two innermost circles indicate G+C content (% in black) and GC deviation (G–C)/(G+C). [Reproduced with permission from (Larsson, Oyston et al. 2005)]

National Center for Biotechnology Information (NCBI) database: *F. tularensis* ssp. *tularensis* strains Schu S4 and FSC198; *F. tularensis* ssp. *holarctica* strains LVS, OSU18, and FSC200; and *F. tularensis* ssp. *novicida* strain U112. A summary of the genome characteristics of these strains can be found in Table 1-1. The number of genes ranges from 1781 to 2029, depending on the strain or subspecies. Differences in the number of coding regions may also be attributed to variations in annotation strategies from the research groups involved in the whole genome sequencing projects. Genome-wide microarray analysis of different strains demonstrated considerable DNA sequence identity (>95%) among the subspecies, with regions unique to the more virulent *tularensis* subspecies (Broekhuijsen, Larsson et al. 2003). Also consistent among the *Francisella* subspecies is the notably low G+C content of about 32%.

Table 1-1. *Francisella* genome characteristics.

Genome/strain	Length (bp)	G+C (%)	Coding (%)	Genes	Pseudogenes
FTT Schu S4	1,892,819	32	78	1804	201
FTH OSU18	1,895,727	32	74	1934	325
FTH FSC200	1,895,994[a]	NA	NA	2029[a]	NA
FTH LVS	1,895,994	32	82	2019	213
FTT FSC198	1,892,616	32	79	1852	199
FTN U112	1,910,031	32	89	1781	14

[a]Sequence not completed. Table adapted from Titball & Petrosino 2007
FTT: subspecies *tularensis*; FTH: subspecies *holarctica*; FTN: subspecies *novicida*

Interestingly, only a single plasmid has ever been recovered from an *F. tularensis* strain. pFNL10 was discovered in the subspecies *novicida*-like strain F6168 isolated from a 1984 human case of tularemia in California (Pavlov, Mokrievich et al. 1996). Analysis of pFNL10 revealed that it is 3990 bp in size and contains six open reading frames, two terminators, and an origin of replication (Pomerantsev, Golovliov et al. 2001). To date, all plasmids capable of replicating in *Francisella* are derivatives of pFNL10, including pKK202 that is commonly used in complementation studies (Norqvist, Kuoppa et al. 1996; Frank and Zahrt 2007).

Despite the high degree of sequence identity among the different strains of *F. tularensis*, comparative genomics and molecular typing methods suggest that the four subspecies are genetically distinct groups. Sequence analyses have revealed the presence of numerous DNA rearrangements, likely due to recombination between insertion sequence (IS) elements. For example, 51 syntenic blocks were rearranged between subspecies *tularensis* strain Schu S4 and subspecies *holarctica* strain OSU18, but no rearrangements were seen between two *F. tularensis* ssp. *holarctica* strains OSU18 and

LVS (Petrosino, Xiang et al. 2006). Each of the rearranged sequences was flanked by repeated DNA sequences corresponding to the IS elements found in high number in the *tularensis*, *holarctica*, and *novicida* subspecies genomes (Larsson, Oyston et al. 2005; Petrosino, Xiang et al. 2006). In all, there have been six different IS elements identified in *F. tularensis* subspecies (IS*Ftu1*-IS*FTu6*), and the IS genes account for as much as 5.8% of the coding sequence in the OSU18 strain (Larsson, Oyston et al. 2005; Petrosino, Xiang et al. 2006). Therefore, the high number of rearrangements observed among *F. tularensis* subspecies are likely a result of homologous recombination at these IS sites, as well as recombination at rRNA sites.

The genome sequence projects have allowed for the analysis of features such as unidirectional genomic deletion events, repeat structure, and single nucleotide polymorphisms (SNPs) to determine the evolutionary hierarchy of *F. tularensis*. Results from these studies indicate that the subspecies of *F. tularensis* evolved by vertical descent from a common ancestor (Titball and Petrosino 2007). Additionally, multiple-locus variable-number tandem repeat analysis (MLVA), examining the number of tandem repeats at 25 loci across the *F. tularensis* genomes, resolved the subspecies *tularensis* into two different clades and showed that subspecies *holarctica* strains have less heterogeneity in samples collected worldwide. This finding indicates that *holarctica* strains emerged more recently than the *tularensis* subspecies; a subsequent study using unidirectional deletion events and SNP analysis of seven *F. tularensis* loci supported the MLVA findings (Farlow, Smith et al. 2001; Farlow, Wagner et al. 2005; Svensson, Larsson et al. 2005). These data also suggested that subspecies *novicida* strains are evolutionarily the oldest and possibly the common ancestor of all *F. tularensis* strains.

Hence, it would appear that both a loss and gain of genetic information occurred during the evolution of *F. tularensis*, beginning with the relatively avirulent subspecies *novicida* (which also has the fewest pseudogenes) that evolved to the highly virulent *tularensis*, which then developed further into the lesser virulent *holarctica* subspecies.

Genetic comparisons among the different subspecies were also made possible by the availability of the *F. tularensis* whole genome sequences. In addition to determining evolutionary hierarchy and unique genetic motifs, this information is useful for establishing differences between the avirulent and virulent strains, such as the duplication of the *Francisella* pathogenicity island in *tularensis* and *holarctica* subspecies (discussed in a separate section). Such findings can be used to identify possible modes of attenuation in the less virulent strains or targets for drug and vaccine development in the highly virulent *tularensis* subspecies.

II. History and Epidemiology of Tularemia

Francisella infections in humans have been documented throughout the Northern Hemisphere for nearly a century (Figure 1-3). While the first description of tularemia was reported as disease in animals in 1911, the first documented human case occurred in Cincinnati, Ohio in 1914 (Wherry and Lamb 2004). Over the next decade, tularemia-like diseases were reported under various names from several regions of the United States. After the pioneering research of Edward Francis determined that these cases were all attributable to a single disease, reported tularemia cases increased to more than 800 incidents in the United States by 1929. By 1945, over 14,000 cases of tularemia had been reported in the U.S., with a peak incidence in 1939 of 2,291 cases (Sjostedt 2007). Many

of the reported tularemia cases occurred in Illinois and neighboring states, and a connection was soon discovered between the disease and the hunting and sale of imported cottontail rabbits. The association of tularemia with rabbits eventually led to the colloquial designation of tularemia as “rabbit fever”.

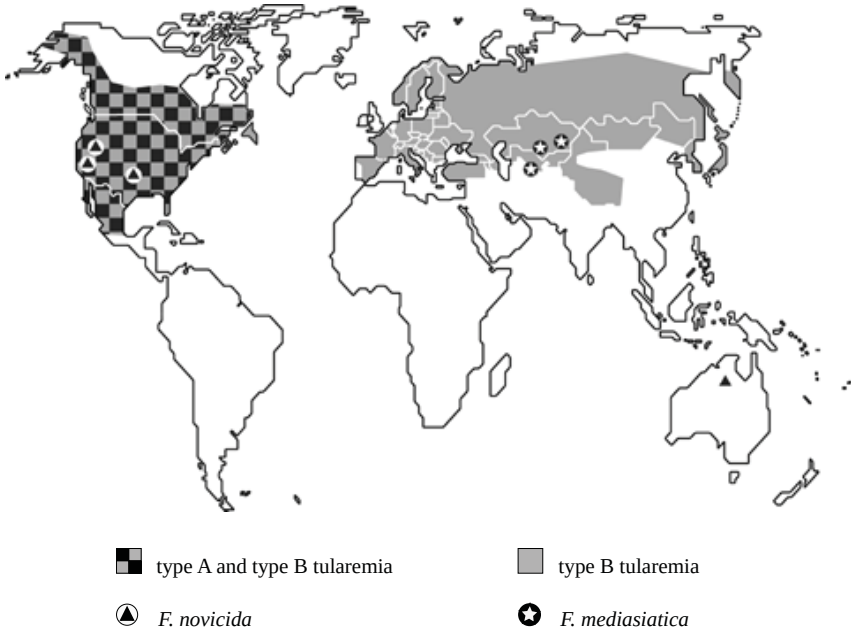

Figure 1-3. Worldwide incidence of tularemia in humans. The range of *F. tularensis* ssp. *tularensis* and *holarctica* (types A and B) in North America is indicated by the checkered pattern. The gray shading in Europe and Asia shows the distribution of *F. tularensis* ssp. *holarctica* (type B). The triangles in North America and Australia signify the rare occurrence of *F. tularensis* ssp. *novicida* and *novicida*-like infections, while the stars in Asia similarly mark isolations of *F. tularensis* ssp. *mediasiatica.* [Reproduced with permission from (Keim, Johansson et al. 2007)]

Another significant source of human tularemia infection is a bite from an arthropod vector, such as a tick or deer fly. It is suspected that these organisms acquire the infection from rabbits or rodents and then transfer the disease to humans. In fact, 56% of the 700 tularemia cases reported in Arkansas between 1938 and 1948 were identified as the result of tick bites, while only 31% of the reports cited contact with rabbits (Washburn and Tuohy 1949). This trend has held true over the years; in a summary of cases reported in the 1980s, 63% of more than 1000 cases in several Midwestern and Southern states featured an attached tick (Taylor, Istre et al. 1991). Besides the tularemia cases reported in Arkansas, Kansas, Louisiana, Missouri, Oklahoma, and Texas, the disease was also

notably present in the Western region of the U.S. In these states, particularly Montana and Nevada, human tularemia cases were shown to be associated with the deer fly (Keim, Johansson et al. 2007). As with ticks, deer flies are known to feed on rodents and rabbits, and both vectors appear to be common infectious cycles in their respective geographical regions.

Human tularemia was first reported in Europe in the Scandinavian countries of Norway (1929) and Sweden (1931) (Francis 1937), and the disease has remained endemic to that region. In Scandinavia and many areas of Europe, tularemia is predominantly associated with bites from arthropod vectors; as early as 1942, naturally infected mosquitoes were reported in Sweden (Sjostedt 2007). Other regularly reported transmission routes of tularemia in these countries are inhalation of aerosolized bacteria during farming activities and exposure to contaminated waterways. In Sweden, the majority of infections occur in areas that are in close proximity to rivers or lakes, but the transmission route has not been clarified. It has been suggested that *Francisella* may infect mosquitoes in the larval stage while they are feeding on infected animals and that lemmings and beavers may play a role in sustaining the aquatic niche of *F. tularensis* (Morner 1992; Sjostedt 2007). Another intriguing possibility is that the bacteria persist in waterways in association with protozoa (Berdal, Mehl et al. 1996). In Central Europe, tularemia was first reported in 1936 and, in contrast to disease in Scandinavia, is most often associated with hares and ticks (Sjostedt 2007). Figure 1-4 contrasts North American and European *Francisella* reservoirs and vectors that likely arose as a result of different niches.

QuickTime™ and a
TIFF (Uncompressed) decompressor
are needed to see this picture.

Figure 1-4. Host-vector associations in type A and type B tularemia. Type A (ssp. *tularensis*) tularemia is found only in North America and is associated with animals of a terrestrial origin. Sheep and rabbits are thought to harbor the bacteria, whereas ticks and flies are considered vectors of the disease. In contrast, type B tularemia, arising from infections with ssp. *holarctica*, is seen in both Eurasia and North America. This form of the disease is associated with animals having an aquatic niche, such as voles and beavers, and is thought to be transferred by ticks and mosquitoes. [Reproduced with permission from (Keim, Johansson et al. 2007)]

As with Scandinavian countries, aquatic origins of infection with *F. tularensis* ssp. *holarctica* are common in the former Soviet Union and Russia. In the former Soviet Union, tularemia was endemic in several regions, and extensive investigations of the occurrence were undertaken from the 1930s through the 1970s to discover the natural foci of the disease and implement preventive measures. From these studies, it was determined that the most prevalent sources of tularemia were voles; agricultural outbreaks were associated with common voles in the grasslands-meadowlands areas and vector-borne disease was attributed to water voles in the swamp-floodlands areas (Sjostedt 2007). Prophylactic measures in these regions included vaccination of at-risk humans, such as hunters and herdsmen, with a primitive live vaccine strain of *F. tularensis*, as well as poisoning of water rats and common voles. Organized hunting was implemented to limit the populations of potential animal sources of tularemia, and sanitary rules and regulations of water sources were enforced. As a result of these efforts, human tularemia cases in the former Soviet Union and Russia have decreased considerably over the last half-century.

It is difficult to determine whether tularemia is an emerging or disappearing disease. Indeed, the number of human cases has dramatically decreased in countries such as Russia and the United States, where only a few hundred cases are reported each year. Changes in societal activities, such as less hunting and farming and therefore less opportunity for exposure, as well as the successful implementation of preventative measures, may account for the decline of tularemia in these areas. Conversely, it is likely the action of humans that resulted in the handful of recent foci in the U.S. For example, tularemia was introduced to New England in the 1970s with the import of infected hares from the Midwest (Teutsch, Martone et al. 1979). The outbreaks on Martha's Vineyard in 1978 and 2000 may have been a consequence of this action. In 2002, prairie dogs infected with *F. tularensis* ssp. *holarctica* were imported to an exotic pet facility in Texas, and 14 of the 20 exposed personnel exhibited symptoms consistent with tularemia (Avashia, Petersen et al. 2004; Petersen, Schriefer et al. 2004).

Similarly, it is suspected that the source of the 1997 emergence of tularemia in Spain, a country with no previously reported cases of human disease, was the import of hares from Central Europe (Perez-Castrillon, Bachiller-Luque et al. 2001). The actions of humans may also account for the presence of tularemia in Kosovo in the late 1990s in that the emergence of tularemia corresponded to environmental repercussions of post-war living conditions (Reintjes, Dedushaj et al. 2002).

The most recent human cases of tularemia arose from the contamination or mislabeling of laboratory strains containing pathogenic *F. tularensis* ssp. *tularensis* that were transferred from Nebraska to Boston University (Barry 2005). Although it is not completely clear how the incident transpired, the researchers at BU failed to follow

guidelines for working with the bacteria in a BSL-2+ biosafety containment hood, presumably resulting in inhalation of the virulent strain and subsequent illness. Taken together, it appears that human behavior is responsible for the recent upsurges in tularemia and that future prevention of the disease lies largely in the hands of man.

III. *Francisella tularensis* Infection

A. Manifestations of disease

While tularemia usually presents within three to five days of infection, the diagnosis of the disease is often compounded by several factors. The onset of tularemia is rather nonspecific and includes fever, chills, malaise, and headache (Evans, Gregory et al. 1985). Because tularemia exhibits these flu-like symptoms, it is often misdiagnosed and thought to be underreported by patients and physicians who take the illness to be a more common ailment. Depending on the route of infection and the subspecies of *F. tularensis*, other specific symptoms may also be present.

1. Ulceroglandular tularemia

When infection is acquired through the skin or mucous membranes, ulceroglandular tularemia usually results. This is often the consequence of a bite from an arthropod vector or direct dermal contact with contaminated animals or aerosolized material. A primary ulcer is commonly present at the site of infection. Without an ulcer, this form of the disease is simply called glandular tularemia. In many cases, ulceroglandular tularemia is misrepresented when the initial ulcer is not recognized and heals undetected. After establishment of infection, the lymph nodes draining the ulcer

become enlarged and tender. In the absence of antibiotic therapy, the draining lymph nodes continue to expand, sometimes resulting in suppuration. This discharge of pus is one of the most common complications of tularemia caused by *F. tularensis* ssp. *holarctica* and can occur in 30 to 40% of all cases (Tarnvik and Chu 2007). While ulceroglandular tularemia is the predominant form of tularemia in subspecies *holarctica* (type B) cases, infection with all *F. tularensis* subspecies can manifest in this manner.

2. Respiratory tularemia

Another common form of tularemia is respiratory (or pneumonic) tularemia, which is contracted by inhalation of *F. tularensis*. It is thought that individuals become infected by contact with contaminated aerosols from animal carcasses or feces. This type of tularemia tends to be associated with farming and gardening activities, such as lawn mowing or brush cutting, but may form as a complication of any type of tularemia (Feldman, Enscore et al. 2001; Tarnvik and Chu 2007). While this form of the disease is often systemic and characteristically presents with fever, the lack of respiratory symptoms and diagnostic X-ray findings is not uncommon. It should also be noted that the infectious agent determines the outcome of the infection. Inhalation of *F. tularensis* ssp. *holarctica* usually causes a nonfatal respiratory infection, but infection with subspecies *tularensis* can result in a serious, acute form of tularemia that is characterized by high fevers, malaise, chills, and cough. Respiratory tularemia arising from *F. tularensis* ssp. *tularensis* is the most serious of all forms of tularemia; an infectious dose of as few as 10 bacteria is sufficient to cause a fatal disease in more than 50% of untreated cases (Dennis, Inglesby et al. 2001).

3. Other forms of tularemia

Besides ulceroglandular and respiratory tularemia, it is possible to contract oculoglandular, oropharyngeal, and typhoidal tularemia. Oculoglandular tularemia results from introduction of *F. tularensis* particles to the eye and is characterized by unilateral conjunctivitis, prominent swelling of tissues surrounding the eye, photosensitivity, and purulent secretion (Tarnvik and Chu 2007). Ingestion of contaminated food or water can cause oropharyngeal tularemia. This form of tularemia mainly affects the digestive tract and often presents with tonsillitis, diarrhea, vomiting, and fever. Tularemia that manifests in a severe systemic disease without a recognized port of entry is known as typhoidal tularemia.

4. Complications

In addition to the primary tularemia disease, complications such as meningitis, pericarditis, osteomyelitis, or pneumonia may arise. Bacterial meningitis is the infection of the fluid and membranes surrounding the spinal cord. Signs of this severe and sometimes deadly disease include fever, headache, stiff neck, and sensitivity to light. Without prompt treatment, brain damage or death may result. Similarly, pericarditis is the swelling and irritation of the membrane surrounding the heart. Often, mild conditions of pericarditis resolve without treatment; antibiotics may be required to clear more serious cases. Osteomyelitis, or bone infection, occurs when bacteria spread to the bones. Pain, decreased range of motion in adjacent joints, and sometime reddening or tenderness of the skin can accompany osteomyelitis. In many tularemia cases, the infection can spread to the lungs and cause pneumonia. Again, antibiotic therapy is usually sufficient to clear the infection.

5. Treatment

While the symptoms and seriousness of the multiple types of tularemia may vary from one another, all forms of the disease are effectively treated with common antibiotics. Due to its natural resistance to sulfa drugs and betalactams such as penicillin, infection with *F. tularensis* is treated with aminoglycoside and tetracycline antibiotics, with gentamicin and doxycycline being the first choices (Tarnvik and Chu 2007). The quinolone ciprofloxacin has also emerged as a new option for oral therapy of tularemia.

B. Immune Response to *Francisella*

1. Innate immune response

Since the symptoms of tularemia are so similar to those of other common illnesses, very little is known about the innate immune response to *F. tularensis* in humans – infected individuals seek medical attention after they have developed overt signs of disease. As a result, much of what is known about the innate immune response to *F. tularensis* has been learned from studies with animal models.

Experiments with mice sublethally infected with an attenuated *F. tularensis* ssp. *holarctica* strain showed that leukocytes accumulated at the site of infection, followed by chemokine and cytokine production (Conlan and Oyston 2007). In particular, tumor necrosis factor alpha (TNF-α) and gamma interferon (IFN-γ) were found to play important roles in the innate immune response to *F. tularensis* infection when depletion of these cytokines by neutralizing antibodies significantly reduced the LD_{50} (Leiby, Fortier et al. 1992). A subsequent study using IFN-γ knockout mice confirmed these results (Elkins, Rhinehart-Jones et al. 1996).

It is well-established that Gram-negative bacteria elicit an immune response through recognition of their lipopolysaccharide (LPS) by host cell toll-like receptor 4 (TLR4) (Miyake 2004; Miller, Ernst et al. 2005). However, *F. tularensis* LPS is not recognized by TLR4 and displays little or no endotoxic properties (Hajjar, Harvey et al. 2006). This is presumably due to unique properties of *F. tularensis* lipid A structure, which are discussed in a subsequent section. Although *F. tularensis* isn't recognized by TLR4, studies indicate that the innate immune response is mediated by TLR2, which recognizes peptidoglycans and lipoproteins, and that *F. tularensis* lipoproteins stimulate the TLR2/TLR6 heterodimer (Thakran, Li et al. 2008).

One aspect of the innate immune response to *F. tularensis* infection that is currently a subject of great interest is the activation of the inflammasome. The inflammasome is a multiprotein, cytosolic complex that leads to caspase-1 activation and release of the proinflammatory cytokine IL-1β (Weiss, Henry et al. 2007). In general, activation of the inflammasome is initiated when a bacterial pathogen is detected by a host receptor molecule, such as Nod-like receptor (NLR) or a pattern recognition receptor (PRR). These molecules oligomerize, and other proteins, including the ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain) adaptor protein and pro-caspase-1, are recruited to form the inflammasome. Pro-caspase-1 molecules oligomerize in the complex and undergo autocleavage to release p10 and p20 subunits, which form the heterotetrameric active complexes of caspase-1. The proinflammatory cytokines pro-IL-1β and pro-IL-18 are then cleaved into their active forms by caspase-1 and released from the cell (Weiss, Henry et al. 2007). Although it remains unknown how the host cell detects the presence of *F. tularensis*, it has been demonstrated that different

strains induce caspase-1 activation in a variety of host cell types (Mariathasan, Weiss et al. 2005; Gavrilin, Bouakl et al. 2006; Li, Nookala et al. 2006). Studies also indicate that *F. tularensis* must be in the cytoplasm for inflammasome activation, as use of inhibitors of bacterial internalization or mutants that don't escape the vacuole fail to induce IL-1β release (Mariathasan, Weiss et al. 2005; Gavrilin, Bouakl et al. 2006). Furthermore, experiments using macrophages from ASC knockout mice were resistant to *Francisella*-induced cell death and released significantly less IL-1β and IL-18, suggesting that ASC is required for inflammasome activation in response to *F. tularensis* infection (Mariathasan, Weiss et al. 2005). While these studies have provided insights into the host inflammatory cascades that are triggered in response to infection with *F. tularensis*, continued research in this area will further elucidate how this pathogen modulates the host defense pathway.

2. Adaptive immune response

In contrast to innate immune responses, cell-mediated immunity to *F. tularensis* has been studied in humans infected with subspecies *holarctica*. Individuals infected with subspecies *holarctica* developed long-lasting antibodies to a number of antigens, such as LPS, heat shock proteins, membrane proteins, and enzymes (Bevanger, Maeland et al. 1994; Porsch-Ozcurumez, Kischel et al. 2004). Subsequent testing of an infected population showed that a majority (>60%) still had *F. tularensis*-specific antibodies 8 years after exposure (Bevanger, Maeland et al. 1994). Further analyses showed that both $CD4^+$ and $CD8^+$ T cells were generated in response to infection with *F. tularensis*, that IL-12 and IFN-γ were produced upon stimulation with heat shock proteins or membrane proteins, and that these responses persisted for up to 25 years (Sjostedt, Eriksson et al. 1992; Ericsson, Kroca et al. 2001). In addition to these responses, *F. tularensis* also

induces expansion of γδT cells, which are usually associated with bacterial phosphoantigens (Poquet, Kroca et al. 1998).

Unfortunately, very little is known about antibody production or T cell responses in humans sublethally infected with *F. tularensis* ssp. *tularensis*. Furthermore, mice used in a research model of tularemia infection usually succumb to disease before an adaptive immune response can be mounted. However, it is known that mice that recover from a sublethal infection of subspecies *holarctica* generate specific antibodies, as well as $CD4^+$ and $CD8^+$ T cells (Elkins, Cowley et al. 2003; Havlasova, Hernychova et al. 2005). Additionally, experiments have shown that specific protective immunity can be adoptively transferred from immunized mice to naïve mice and that both $CD4^+$ and $CD8^+$ T cells are important for protection against subsequent infection with wild-type *F. tularensis* ssp. *novicida* (Heffron and Barry labs, unpublished data).

C. Infectious cycle

As a facultative intracellular pathogen, *F. tularensis* invades and replicates within host cells. Several cell types are permissive for *F. tularensis* infection, including type II alveolar epithelial cells, neutrophils, and mononuclear macrophages (McCaffrey and Allen 2006; Hall, Craven et al. 2007). Of these cells types, it has been long-established that mononuclear phagocytes are important host cells during *F. tularensis* infection (Nutter and Myrvik 1966; Anthony, Burke et al. 1991; Fortier, Green et al. 1994). Accordingly, *Francisella* research studies utilize mononuclear macrophages and macrophage-like cell lines. Figure 1-5 diagrams the intracellular life cycle of *Francisella*, including electron microscopy images from the various stages discussed below.

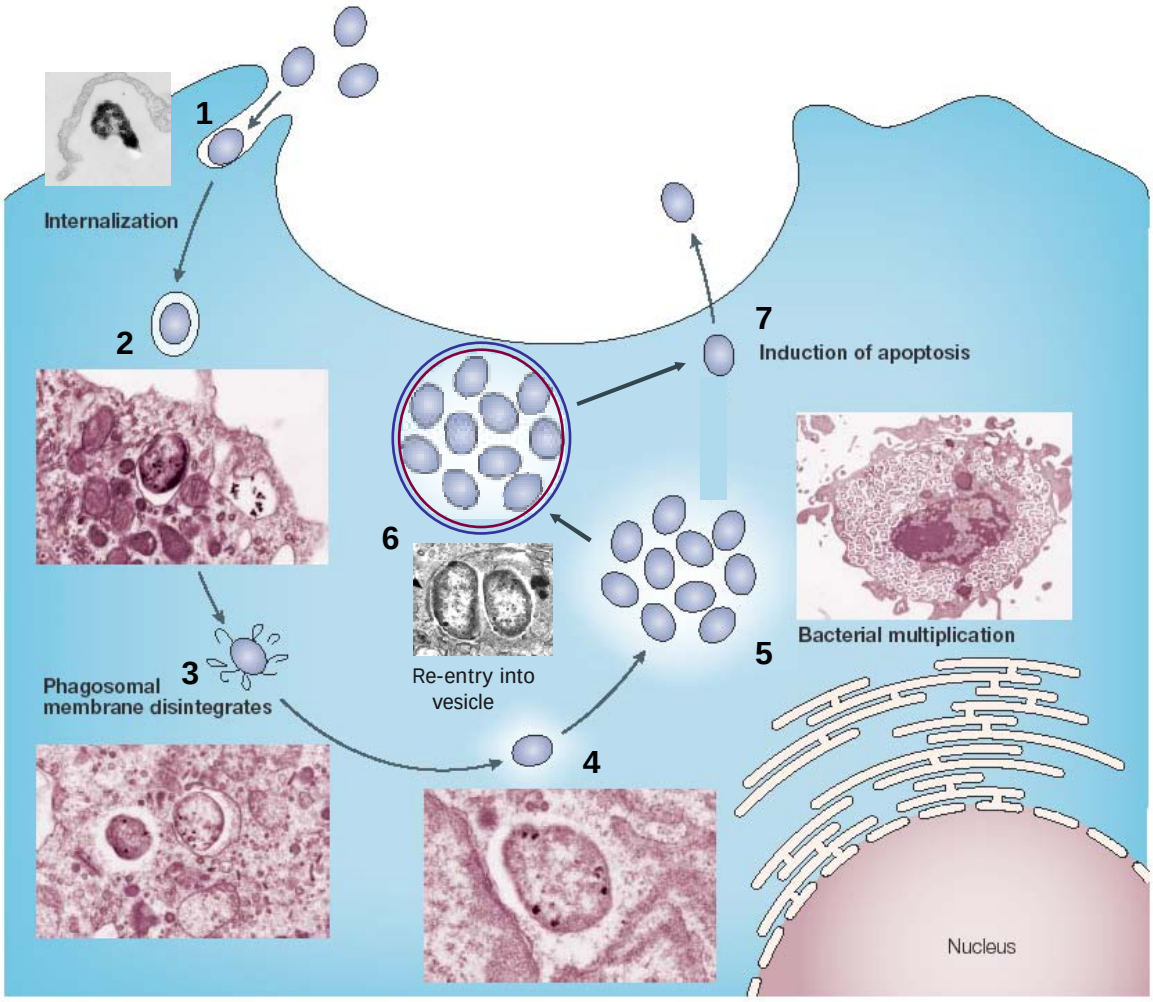

Figure 1-5. Intracellular lifecycle of *Francisella* in macrophages. *Francisella* is internalized by looping phagocytosis (1) and enters the macrophage in a phagosomal vesical (2). One to four hours after uptake, the phagosomal membrane is disrupted (3), and *Francisella* escapes into the cytoplasm (4). After replicating in the cytoplasm (5), the bacteria become re-enclosed in large, autophagosomally-derived vacuoles (6). Proliferation of *Francisella* inside the cell triggers the release of cytochrome c and activates caspases 3 and 9, thereby inducing apoptosis of the host cell (7). [Adapted from (Oyston, Sjostedt et al. 2004) with additions from (Clemens, Lee et al. 2005) (1) and (Checroun, Wehrly et al. 2006) (6)]

1. Uptake

While it is clear that mononuclear phagocytes are a principal host cell type, the particular mechanisms of the *F. tularensis* infectious cycle are still being elucidated. A recent study of the ultrastructural morphology of host cells during uptake of *F. tularensis* revealed that the bacteria enter host cells via a novel pathway termed "looping phagocytosis" (Clemens, Lee et al. 2005). Using monocyte-derived macrophages and the human macrophage-like cell line THP-1, the authors observed the majority of *F. tularensis* bacteria being engulfed by the macrophages in asymmetric extensions of the plasma membranes called pseudopod loops.

This process of bacterial uptake differs from conventional phagocytosis and coiling phagocytosis in that there is no close juxtaposition of the bacterium with the host cell membrane, indicating a lack of interaction between bacterial surface ligands and host cell receptors (Griffin, Griffin et al. 1975; Griffin, Griffin et al. 1976; Horwitz 1982; Horwitz 1984). However, it should be noted that the data regarding pseudopod loops rely mainly upon static electron microscopy images and that transient bacterium-host cell interactions may not have been observed. Looping phagocytosis is also dissimilar to membrane ruffling and triggered macropinocytosis seen in *Salmonella*; the pseudopod loop structures are highly asymmetric, and uptake does not appear to be mediated by the bacteria since heat-killed *F. tularensis* are still engulfed in pseudopod loops (Francis, Ryan et al. 1993; Clemens and Horwitz 2007). Additionally, membrane ruffling is associated with a Type III secretion system (T3SS), which is not found in the *F. tularensis* genome (Larsson, Oyston et al. 2005). Thus, the host actin remodeling that occurs during *Francisella* entry is a unique mechanism for bacterial uptake.

2. Receptors

Although it has not been reported to be involved in asymmetric pseudopod loop formation, it has been demonstrated that the uptake of *F. tularensis* into the macrophage may be mediated by the macrophage complement receptor 3 and Fcγ receptors (Balagopal, MacFarlane et al. 2006). A number of intracellular pathogens exploit this pathway to enter macrophages, thereby blocking the oxidative burst. Other components shown to be involved in the uptake of *F. tularensis* are the macrophage mannose receptor (Balagopal, MacFarlane et al. 2006; Schulert and Allen 2006) and class A scavenger receptors (Pierini 2006).

The role of specific membrane domains in *F. tularensis* infection was recently addressed in a study that found cholersterol-rich host cell membrane domains, or lipid rafts, to be targeted by *F. tularensis* LVS for entry into macrophages (Tamilselvam and Daefler 2008). As with *Francisella*, cholesterol-rich lipid rafts are critical for uptake and intracellular survival of the bacterial pathogen *Listeria* (Seveau, Bierne et al. 2004). E-cadherin is known to be the host cell receptor for *Listeria* (Mengaud, Ohayon et al. 1996); in Chapter 3, we provide data that suggest *Francisella* may also interact with the E-cadherin complex.

3. Entry

Once the bacteria are enveloped by the pseudopod projections, the loops fuse with the plasma membrane to create *Francisella*-containing phagosomes (FCP) at the cell surface. While the initial phagosomes are spacious, they are remodeled into tight phagosomes within seconds to minutes of uptake into the macrophage (Clemens, Lee et al. 2005). At about five minutes after entry, the FCPs mature to early endosomes, as evidenced by their co-localization with the marker early endosome antigen-1 (EEA-1)

(Clemens, Lee et al. 2004; Checroun, Wehrly et al. 2006). This stage is transient and is followed by acquisition of the late endosomal/lysosomal markers lysosomal-associated membrane protein 1 and 2 (LAMP-1 and LAMP-2), which indicates a progression of the FCPs along the endocytic pathway (Clemens, Lee et al. 2004; Santic, Molmeret et al. 2005; Checroun, Wehrly et al. 2006).

4. Phagosomal escape

Whether *F. tularensis* remains in the phagosome or escapes from the FCP to replicate in the cytoplasm has been an area of much discussion in the field; only recently has strong evidence emerged to support the latter argument. Electron microscopy images suggested the dissolution of the phagocytic membrane, and subsequent studies directly measuring the pH of the FCP 3 to 4 hours after infection show that the acidity of the FCP is the same as the host cell cytosol (Golovliov, Baranov et al. 2003; Clemens, Lee et al. 2004; Clemens and Horwitz 2007). Taken together, these data indicated disruption of the FCP. Recently, a dual fluorescence microscopy assay, with sequential use of digitonin and saponin to differentially label cytoplasmic or phagosomal bacteria, showed that the majority of *F. tularensis* escaped from the phagosome by 1 to 4 hours after uptake (Checroun, Wehrly et al. 2006). As with *Listeria*, *F. tularensis* escape into the cytoplasm requires acidification of the phagosome. A current report showed that this was achieved through a proton vacuolar ATPase pump (Santic, Asare et al. 2008). While the mechanism of FCP membrane degradation remains to be determined, it is possible that hemolysins or phospholipases are involved, as this is the case with other cytoplasmically replicating pathogens.

5. Re-entry into autophagosomally-derived vacuoles

After replicating in the cytoplasm, the bacteria appear to become re-enclosed in large, endocytic, double-membraned vacuoles. Immunostaining revealed that these *Francisella*-containing vacuoles (FCV) were surrounded by a LAMP-1 positive membrane that also co-localized with the autophagic probe monodansylcadaverine (MDC) and the autophagosomal membrane protein LC3, indicating an autophagic origin for the FCVs (Checroun, Wehrly et al. 2006). This phenomenon of autophagy-mediated re-entry into a vacuole is unique to *Francisella*, and it appears to be a bacterial-driven process.

6. Induction of apoptosis

After proliferating inside the host, *F. tularensis* causes high levels of cytopathology and induces apoptosis (Lai, Golovliov et al. 2001). The cytopathogenic effect is dependent on the number of bacteria, as the percentage of macrophages exhibiting decreased membrane integrity or detachment (both signs of cell death), increased with the multiplicity of infection of *F. tularensis*. Apoptosis was shown to be the cause of cell death by the presence of surface-exposed phosphatidlyserine, the appearance of terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL)-positive infected cells, DNA fragmentation, and the release of nucleosomes, all of which are characteristic of apoptosis. In a subsequent study, the authors demonstrated that the mechanism of *Francisella*-induced apoptosis involves host cell mitochondria by triggering the release of cytochrome c and activated caspases 3 and 9, indicating of activation of the intrinsic apoptotic pathway (Lai and Sjostedt 2003). Additionally, it was shown that the entry and intracellular replication of viable *F. tularensis* was required to trigger apoptosis and that IglC expression is necessary for

intracellular growth and induction of apoptosis (Lai, Golovliov et al. 2001; Lai, Golovliov et al. 2004). Although the function of *Francisella*-mediated apoptosis remains unknown, a possible explanation is that the bacteria may induce apoptosis to kill the host cell to escape when nutrients become scarce.

D. Molecular basis of pathogenesis

While *F. tularensis* is a highly infectious pathogen capable of causing diverse manifestations of disease in a broad range of mammalian hosts, the mechanisms that account for its virulence remain largely unknown. *F. tularensis* is known to survive and replicate inside the highly bactericidal environment of macrophages, so much of the research pertaining to its virulence has focused on identifying factors used to gain entry into host cells and avoid macrophage killing. Although no toxin has been associated with *F. tularensis*, a global regulator of virulence (MglA) and a cluster of virulence-associated genes known as the *Francisella* pathogenicity island (FPI) have been identified. Also, *F. tularensis* has several surface features that may contribute to its virulence, including a distinct lipopolysaccharide (LPS) structure and capsule, type IV pili, and unique outer membrane proteins. Further identification and characterization of *Francisella* virulence genes will continue to shed light upon the pathogenesis of the organism.

1. MglA – the global regulator of *Francisella* virulence

Currently, the MglA protein is considered the primary regulator of virulence gene expression in *F. tularensis*. It was originally identified as a spontaneous mutant that lacked acid phosphatase activity and decreased the expression of several other genes (Baron and Nano 1998). Its attenuation for intracellular replication and requirement for

growth in host cells led to the gene designation *mglA* for macrophage growth locus A. Further studies with an allelic exchange *ermC* mutant of *mglA* showed that it was attenuated for growth in amoebae, as well as virulence in mice, suggesting that MglA is a regulator of factors necessary for both virulence and environmental persistence (Lauriano, Barker et al. 2004). The same group also demonstrated that MglA, which is encoded outside of the FPI, regulates intracellular growth locus genes *iglA, iglC,* and *iglD,* and pathogenicity determinant protein genes *pdpA* and *pdpD,* all of which are found on the pathogenicity island (Lauriano, Barker et al. 2004). A subsequent microarray study examining global transcriptional responses in *Francisella* found 102 MglA-regulated genes, including the entire FPI (Brotcke, Weiss et al. 2006). Most recently, MglA, along with IglC and IglD, was shown to be critical for phagosomal escape and intracellular replication in *F. tularensis* ssp. *holarctica* LVS (Bonquist, Lindgren et al. 2008).

Interestingly, MglA is an ortholog of the *E. coli* stringent starvation protein A (SspA), sharing 21% identity at the amino acid level (Barker and Klose 2007; Charity, Costante-Hamm et al. 2007). In *E. coli*, SspA is thought to interact with RNA polymerase to regulate gene expression under conditions of stress (Williams, Ouyang et al. 1994). Sequencing of a *Francisella* genome revealed the presence of a second *mglA*-like gene that was annotated as *sspA* (Larsson, Oyston et al. 2005). Expression analyses showed that SspA also regulates virulence genes in *Francisella*, many of which overlap with MglA-regulated genes (Charity, Costante-Hamm et al. 2007). The molecular mechanism of MglA regulation was recently elucidated with a series of experiments demonstrating that MglA and SspA cooperate with one another to associate with RNA polymerase and positively control virulence gene expression in *F. tularensis* (Charity, Costante-Hamm et

al. 2007). The recent identification of a third protein, FevR (for *Francisella* effector of virulence Regulation), that acts in parallel to MglA and is required for the expression of the FPI genes and other genes in the MglA/SspA regulon demonstrates that there is yet more to be uncovered regarding virulence gene regulation in *Francisella* (Brotcke and Monack 2008).

2. The *Francisella* pathogenicity island

The genomes of many intracellular pathogens contain one or more clusters of genes required for entry and/or survival within the host cell. These so-called pathogenicity islands are often characterized by deviant G+C content and are commonly flanked by tRNA or direct repeat sequences. The *Francisella* pathogenicity island (FPI) comprises about 30 kpb, has a G+C content of less than 27% (about 6% below the average content of the genome), and encodes genes necessary for intramacrophage growth (Figure 1-6) (Nano, Zhang et al. 2004). Intriguingly, two virtually identical copies of the FPI are found in separate locations of the human-virulent *tularensis* and *holarctcia* subspecies genomes, while subspecies *novicida* has only a single copy of the FPI (Larsson, Oyston et al. 2005; Nano and Schmerk 2007). With the exception of a truncated *pdpD* gene in the *holarctica* FPI, the regions are highly conserved among the different subspecies (Nano and Schmerk 2007).

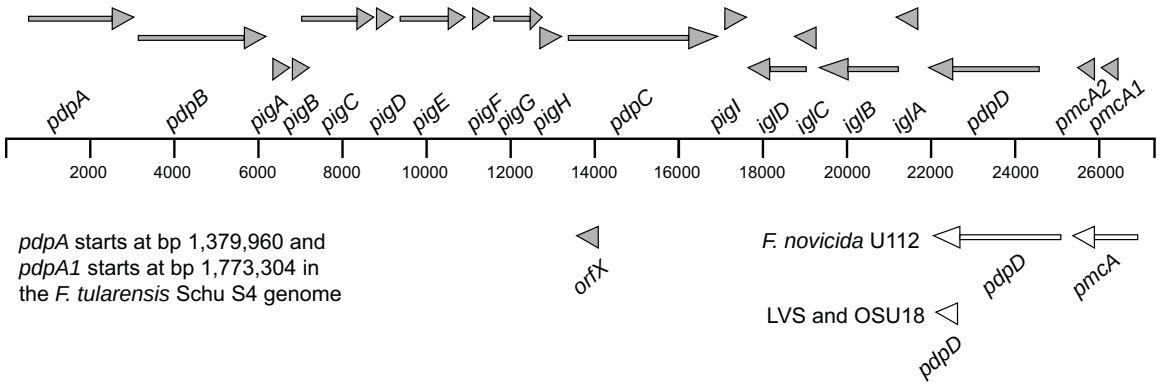

Figure 1-6. Organization of the *Francisella* Pathogenicity Island. The gene names from the annotated *F. tularensis* ssp. *tularensis* Schu S4 genome are shown below the arrows. The variation in the *pmcA-pdpD* region in *F. novicida* and *F. holarctica* strains LVS and OSU18 is shown below the size scale. The directions of the arrows indicate orientations of the open reading frames, which are drawn approximately to size relative to the other ORFs. [Adapted from (Nano and Schmerk 2007)]

The main components of the FPI are two large convergently transcribed operons: the *pdpDiglABCD* operon and the *pdpA* operon, which consists of an additional 11 genes, including *pdpBC* and the newly designated *pig* genes (for pathogenicity island gene) (Nano and Schmerk 2007). Each of these genes has been inactivated in *F. tularensis* ssp. *novicida*, and every gene has been shown to be necessary for growth inside macrophages and virulence in mice except for *pdpD* (Gray, Cowley et al. 2002; Lauriano, Barker et al. 2004; Nano, Zhang et al. 2004; Mariathasan, Weiss et al. 2005; Tempel, Lai et al. 2006). While the function of many of the FPI genes remains unknown, bioinformatics studies have revealed homology between several FPI genes and genes that have been identified as being involved in the recently described type IV secretion systems of *Pseudomonas aeruginosa* and *Vibrio cholera* (Mougous, Cuff et al. 2006; Pukatzki, Ma et al. 2006). Additionally, four related FPI genes, the *pdp* genes, exhibit some homology to the rhoptry proteins in protists, which are involved in secretion and invasion of host cells (Kats, Black et al. 2006).

The single most researched FPI gene is *iglC*. The gene product was first identified as one of the most upregulated *Francisella* proteins during macrophage infection and was subsequently shown to be required for growth in macrophages and virulence in mice in the *tularensis*, *holarctica*, and *novicida* subspecies (Golovliov, Ericsson et al. 1997; Gray, Cowley et al. 2002; Golovliov, Sjostedt et al. 2003; Lauriano, Barker et al. 2003; Santic, Molmeret et al. 2005). Furthermore, IglC plays a role in *Francisella* escape from the phagosome and avoiding phagosome-lysosome fusion in macrophages (Lindgren, Golovliov et al. 2004; Santic, Molmeret et al. 2005). The induction of apoptosis in macrophages after *Francisella* infection has also been shown to be dependent on IglC (Lai, Golovliov et al. 2004). Finally, IglC has been implicated in modulating the immune response by downregulating Toll-like receptor signaling (Telepnev, Golovliov et al. 2003). Although IglC clearly has a significant impact on the survival and virulence of *Francisella*, it remains unknown how many of these roles are direct and how many are consequences of downstream effects due to a lack of IglC function.

3. Surface components

a. Lipopolysaccharide

Lipopolysaccharide, or LPS, is the main component of the outer membrane of Gram-negative bacteria. It is comprised of a lipid portion (lipid A or endotoxin) that anchors it to the membrane, a polysaccharide core, and an oligo- or polysaccharide chain (O-antigen) that extends from the core. In addition to its role in bacterial membrane integrity, LPS is commonly a significant factor in host immune response stimulation because it is detected by pattern recognition receptors.

Although LPS structure and stimulatory potential varies among bacterial species, *Francisella* LPS is particularly unique from that of other bacteria in both structural properties and in its inability to stimulate a strong host immune response. Lipid A is the component of LPS that is recognized by the host innate immune system, and this occurs primarily through a Toll-like receptor 4 (TLR4) complex and requires an accessory LPS binding protein (LBP). Neither *Francisella* lipid A nor LPS act as agonists for human or mouse TLR4-mediated immune responses, and *F. holarctica* live vaccine strain (LVS) LPS does not bind LBP (Hajjar, Harvey et al. 2006). The inability of *Francisella* LPS to elicit an immune response by signaling through the typical TLR4 pathway may be due to key structural differences of *Francisella* lipid A. Where the highly inflammatory lipid A from enteric bacteria is commonly phosphorylated at the 4' site, is hexa-acylated, and has acyl side chains of 12-14 carbons in length, *Francisella* lipid A lacks a 4' phosphate on the glucosamine backbone dimer, is tetra-acylated, and has acyl side chains of 16-18 carbons (Vinogradov, Perry et al. 2002; Phillips, Schilling et al. 2004; Wang, Ribeiro et al. 2006).

In addition to its distinct lipid A structures, *Francisella* also exhibits blue/gray LPS phase variation. This phenotypic diversity was first reported in 1951 and has been associated with changes in virulence and immunogenicity, with rough and/or gray colonies being less virulent and immunogenic than blue and/or smooth variants (Eigelsbach, Braun et al. 1951). The occurrence of the variants appears to involve the age of the culture, pH, inoculum size, and other yet unestablished factors. Recognition of the LPS of an *F. holarctica* LVS gray mutant with monoclonal antibodies against *F. novicida* LPS O-antigen indicates that the O-antigen plays a role in phase variation, as the O-

antigens from these two subspecies are distinct from one another (Cowley, Myltseva et al. 1996). In the case of a different gray variant of *F. holarctica* LVS, the O-antigen was lacking completely, and the strain was attenuated in macrophages and non-protective in mice (Hartley, Taylor et al. 2006). Taken together, these findings show that while *Francisella* LPS is unable to elicit a robust immune response, it is required for virulence of the organism.

b. capsule

The presence of an electron dense layer surrounding *Francisella*, and the lack of this layer after treatment with acridine orange, suggests that the pathogen possesses a capsule, but this structure is an especially poorly understood aspect of the organism. The rough colonies that resulted from *F. holarctica* LVS treatment with acridine orange were attenuated in mice and were more sensitive to serum killing but survived inside polymorphonuclear leukocytes better than wild-type *Francisella* (Sandstrom, Lofgren et al. 1988). Capsule mutants arising from treatment with Tween-80 were also reported to have increased susceptibility to serum killing and greater levels of phagocytosis by macrophages (Barker and Klose 2007). Because the capsule mutants studied to date arose from spontaneous mutations, it is not possible to assign specific functions to the capsule. Additional research using defined capsule mutants will allow for a better understanding of the role the capsule plays in *Francisella* virulence.

c. type IV pili

Type IV pili (T4P) are filamentous surface organelles expressed by several Gram-negative bacteria, including *Neissera* species, *P. aeruginosa*, and *V. cholerae* (Tweedy, Park et al. 1968; Weiss 1971; Wistreich and Baker 1971; Punsalang and Sawyer 1973).

The genes that encode T4P are generally conserved among bacteria expressing T4P, and the nomenclature proposed for *Francisella* components corresponds to that used for *Neisseria pil* genes, with a few exceptions (Gil, Benach et al. 2004). The pilus structure consists of repeating pilin subunits arranged in a left-handed helix. It is extruded through and anchored by a secretin pore situated in the outer membrane. T4P also contain at least one minor protein of unknown function. Over twenty proteins play a role in T4P biogenesis. Of note are PilF, which polymerizes pilin subunits into the fiber, and PilT, which causes the fiber to retract. While the biochemistry of T4P biogenesis has not been defined, TFP retraction is known to exert tremendous force on the substrate to which the T4P is attached (Merz, So et al. 2000). This force triggers many host cell responses. T4P retraction is important for many biological processes of bacteria, including twitching motility, DNA uptake, and interactions with the host cell (Burrows 2005).

The first evidence that *Francisella* expresses T4P arose from an analysis of *F. holarctica* LVS ultrastructure by electron microscope that revealed long, thin fibers on the surface of the bacteria (Gil, Benach et al. 2004). Sequence analysis confirmed the presence of *pil* genes in both LVS and *F. tularensis* strain Schu S4, and further studies using RT-PCR demonstrated that several *pil* genes were expressed in LVS (Gil, Benach et al. 2004; Larsson, Oyston et al. 2005). In *F. novicida*, a subset of *pil* genes was found to be involved in the secretion of proteins into culture medium, indicating that the *Francisella* has a T4P-mediated secretion system (Hager, Bolton et al. 2006). Several proteins involved in T4P biogenesis and regulation are homologous to proteins required for type II secretion (T2S) in Gram-negative bacteria; the potential for such a secretion system in *Francisella* is discussed in a subsequent section.

A deletion derivative of one T4P gene, *pilE1* (*pilA* in *Neisseria*), was shown to be attenuated for infection in mice using a subcutaneous route of infection, indicating that T4P are involved in virulence in *Francisella* (Forslund, Kuoppa et al. 2006). Most recently, the roles of *pilF* and *pilT* in *F. holarctica* LVS were examined using deletion mutants of the genes (Chakraborty, Monfett et al. 2008). *pilF* (*pilB* in *Neisseria*) encodes a nucleotide-binding protein that is required for pilus assembly and secretion, and the *pilT* gene product is an ATPase motor protein necessary for pilus retraction and twitching motility (Wolfgang, Lauer et al. 1998). Using transmission electron microscopy, the authors demonstrated that expression of T4P in LVS requires *pilF* and *pilT* genes. Further, it was shown the *Francisella* T4P is important in host cell adherence (or uptake), but not for intracellular replication. These mutants were also highly attenuated in a mouse model of infection, corroborating previous findings that T4P are important virulence factors of *Francisella* (Forslund, Kuoppa et al. 2006; Chakraborty, Monfett et al. 2008). Research on *Francisella* T4P is still in its early stages; further studies to identify and characterize the pilus assembly components and pathways will contribute to the delineation of the molecular mechanisms of *Francisella* pathogenesis.

d. outer membrane proteins

As the first point of contact with the host, outer membrane proteins (OMPs) are often critical for promoting bacterial invasion and can also have roles in immune evasion, virulence, and intracellular survival. Bacterial OMPs have been identified as antigens and used successfully in vaccine studies in a number of diseases (McSorley and Jenkins 2000; Cassataro, Estein et al. 2005; Pal, Peterson et al. 2005; Philipovskiy, Cowan et al. 2005). The first *Francisella* OMP was identified by probing extracts of bacteria with antisera

collected from infected humans after a tularemia outbreak in Norway (Bevanger, Maeland et al. 1988). The protein was designated FopA (*Francisella* outer membrane protein) based on its colocalization with *E. coli* OMPs when expressed in *E. coli* (Nano 1988). Further studies showed that FopA did not provide protection against *F. tularensis* or *F. holarctica* LVS challenge (Fulop, Manchee et al. 1995; Fulop, Manchee et al. 1996). A second *Francisella* OMP, TUL4, was identified in a study examining T lymphocyte stimulation by *Francisella* membrane proteins (Sandstrom, Tarnvik et al. 1987; Sjostedt, Sandstrom et al. 1990). TUL4 was subsequently shown to stimulate the proliferation of T cells and increase host cell production of interleukin-2 (IL-2) and gamma interferon (IFN-γ) (Surcel, Sarvas et al. 1989; Sjostedt, Sandstrom et al. 1990; Sjostedt, Sandstrom et al. 1990). In these studies, several other *Francisella* membrane proteins were identified, yet none were further characterized.

In a recent investigation, multiple bioinformatic analyses were used to identify membrane proteins of *F. tularensis* Schu S4 and *F. holarctica* LVS (Huntley, Conley et al. 2007). Sixteen putative OMPs, including FopA and TUL4, and two inner membrane (IM) regions, SecY and LolC, were identified and cloned into *E. coli* for expression and generation of polyclonal antisera. *Francisella* membranes were isolated and enriched from other cellular components by spheroplasting and osmotic lysis, followed by sucrose density gradient ultracentrifugation. Probing with the polyclonal antibodies revealed fifteen of the putative OMPs localized to the OM fractions and confirmed that both SecY and LolC fractionated with IMs. When OMPs were incubated with sera from mice infected with *Francisella*, several proteins exhibited immunoreactivity. A subsequent investigation by the same authors demonstrated that immunization with *Francisella*

OMPs induced high levels of TNF-α, IL-2, and IL-10, and that OMPs act as protective antigens against pulmonary *Francisella* infection in mice (Huntley, Conley et al. 2008). These studies mark the first time that *Francisella* OMPs have been enriched and physically separated into OM and IM fractions, as well as the first report characterizing OMPs for use as a subunit vaccine.

4. Secretion system and secreted effectors

Several Gram-negative bacteria employ secretion systems to control their environment. Secreted effector proteins can serve to increase nutrient uptake and control the population of other bacteria in the niche. They may also influence several phases of the bacterial infectious cycle, such as subversion of host cell processes, entry into host cells, immune evasion, intracellular survival and replication, phagosomal escape, and resistance to host cell killing. There are currently eight described secretion pathways in Gram-negative bacteria (Forsberg and Guina 2007). However, bioinformatic analysis of the *Francisella* genome showed no evidence of the type III and type IV secretion systems (T3SS and T4SS) commonly found in pathogenic bacteria (Larsson, Oyston et al. 2005).

A recent study identified seven *F. novicida* proteins that were secreted into the culture supernatant. The secreted proteins were identified as a protease (PepO), two chitinases (ChiA and ChiB), a chitin binding protein (CbpA), a predicted beta-glucosidase (BglX), and two proteins that do not share homology to any other known proteins (designated Fsp58 and Fsp53 for *Francisella* secreted protein with molecular weights of 58 kDa and 53 kDa) (Hager, Bolton et al. 2006). The open reading frames encoding these proteins have predicted signal sequence domains in their N-terminal

regions, suggesting that they are translocated to the periplasm and secreted via the Sec system, possibly in conjunction with a type II secretion system (T2SS).

As previously mentioned, *Francisella* has been shown to encode and express genes for T4P formation, and several of the genes required for secretion and assembly of the T4P share homology with T2S genes (Gil, Benach et al. 2004; Burrows 2005). When specific components of the T4P system (the ATPase PilB, the IMP PilC, and the OM pore-forming PilQ) were mutated, a lack of secretion resulted (Hager, Bolton et al. 2006). These pilus system proteins share significant homology with T2SS components; the failure to secrete proteins in their absence suggests that *Francisella* may employ a type II-like secretion system. Furthermore, T2S and T4P appear to have evolved from a common system, as they are structurally homologous, and T2SS are known to secrete bacterial chitinases (Cianciotto 2005; Hansen and Forest 2006). However, the *Francisella* genome does not contain the genes, or homologs thereof, for the inner membrane and periplasmic T2SS components that distinguish this secretion system from T4P: *gspC*, *gspL*, and *gspM* (Larsson, Oyston et al. 2005; Hager, Bolton et al. 2006). Taken together with the loss of secretion in T4P mutants, the lack of genes required for T2SS-mediated secretion indicate that protein secretion in *Francisella* is mediated, at least in part, by homologs from the T4P system.

Another suggestion is that *Francisella* utilizes the newly described T6SS, the essential components of which are a protein with an IcmF-motif and two linked genes that correspond to *iglA* and *iglB* (Pukatzki, Ma et al. 2006). IglA and IglB are found on the FPI. A recent study showed that IglAB homologs in other bacteria are found in a cluster of genes encoding proteins known as IcmF-associated homologous proteins

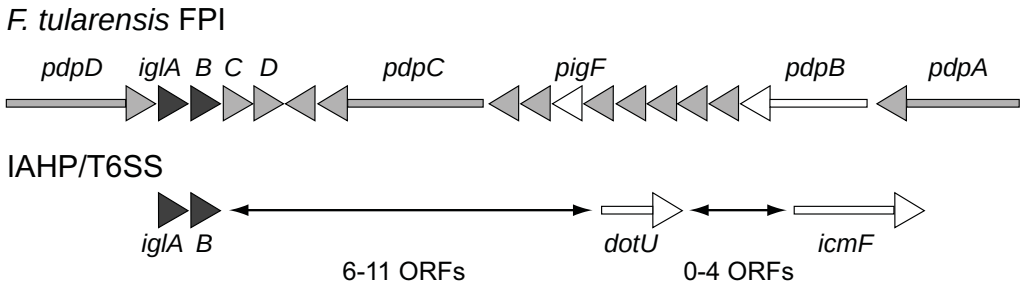

Figure 1-7. Similarity of FPI to IAHP or T6SS gene clusters. The amino acid sequences of IglAB have high identity to orthologs in other species, and the C-terminal third of PdpB contains a IcmF-like region. PigF shows weak similarity to the conserved orthologous group (COG) that contains DotU, a protein encoded on IAHP regions. [Adapted from (Nano and Schmerk 2007)]

(IAHPs) (Das and Chaudhuri 2003; de Bruin, Ludu et al. 2007). BLASTP analysis showed that the C-terminal third of another FPI protein, PdpB, contains an IcmF region. While the arrangement of IAHP components on the FPI is strikingly similar to other IAHP virulence gene clusters (Figure 1-7) and indicates that *Francisella* may carry a T6SS, further research is needed to elucidate the secretion mechanisms of *Francisella* (Nano and Schmerk 2007).

Of the seven identified *Francisella* secreted proteins, only PepO has been characterized in terms of a role in virulence. Examination of culture supernatants from an *mglA* mutant strain indicated that *pepO* is positively regulated by MglA (Hager, Bolton et al. 2006). Interestingly, infection of mice with a *pepO* transposon mutant resulted in an increased splenic bacterial burden and higher mortality compared to mice infected with wild-type *F. novicida* (Hager, Bolton et al. 2006). A possible explanation for this result is that PepO secretion limits *Francisella* infection by increasing vasoconstriction around the infection site or by cleaving a *Francisella* factor that contributes to spread to systemic sites. PepO exhibits similarity to the M13 family of zinc metalloproteases that cleave vasoactive peptides, such as the endothelin-converting enzyme ECE-1; the carboxy-terminal end of PepO has all of the conserved active site residues in the same alignment as that of ECE-1 and bacterial PepO (Hager, Bolton et al. 2006). The human-infectious *Francisella* ssp. *tularensis* and *holarctica* exhibit a rearrangement affecting the *pepO*

codons that encode the N-terminal secretion signals, resulting in a loss of PepO secretion. Furthermore, the *F. holarctica* genomes contain a point mutation that introduces a premature stop codon, resulting in inactivation of the protease domain of PepO (Forsberg and Guina 2007). It has been suggested that these mutations played a role in the evolution of the more pathogenic, human-virulent *Francisella* subspecies (Hager, Bolton et al. 2006; Forsberg and Guina 2007).

IV. Vaccine Strategies

A. The need for an approved tularemia vaccine

F. tularensis is one of the most infectious bacteria known and is currently classified as a category A select agent by the Centers for Disease Control and Prevention (CDC). *Francisella* has received attention for its potential as a bioweapon and has in fact been tested and developed for use as such by several countries. Naturally acquired infections occur throughout the world as a result of contact with arthropod vectors, infected animals, ingestion of contaminated water or food, or inhalation of aerosolized infectious particles. Despite these factors, no approved vaccine against tularemia exists, and the development of one remains an international research focus.

1. *Francisella* as a biological weapon

Inhalation of as few as 10 *F. tularensis* ssp. *tularensis* bacteria is sufficient to cause disease with a mortality rate of 30-60% if left untreated (Dennis, Inglesby et al. 2001). Besides infection through the aerosol route, *F. tularensis* can also be easily mixed into food and water supplies for ingestion. The high infectivity, coupled with the ease of dispersal, led to an interest in *F. tularensis* for development as a biological weapon. In fact, the pathogen was studied at Japanese germ warfare research units in Manchuria that were in operation between 1932 and 1945 (Harris 1992). According to a former Soviet Union weapons biologist, *F. tularensis* was one of the organisms studied and used by the former Soviet Union during World War II (Alibek 1999). Not to be left out, the United States developed weapons for aerosol dissemination of *F. tularensis* during the 1950s and 1960s (Christopher, Cieslak et al. 1997).

The potential of *F. tularensis* to be exploited as a biological weapon led to studies by the World Health Organization: in 1969, a WHO committee estimated that an aerial dispersal of 50 kg of virulent *F. tularensis* over a city of 5 million people would result in 250,000 incapacitating illness, including 19,000 deaths (Dennis, Inglesby et al. 2001). That same year, President Nixon announced the termination of the US biological weapons program, and it is presumed that stockpiles of biological weapons, including *F. tularensis*, were subsequently destroyed. It is unclear if the same occurred in other countries; the former Soviet Union reportedly continued to work with *F. tularensis* in the capacity of a bioweapon until the 1990s and are thought to have created strains that are resistant to antibiotics or even strains that can cause disease in vaccinated individuals (Oyston, Sjostedt et al. 2004). Recent events such as the terrorist attacks of 9/11 and the subsequent use of anthrax as a biological weapon have renewed concerns about the

deliberate use of *F. tularensis* to cause disease. In addition to the concerns about health, a bioterrorist attack would have a dramatic impact on federal finances: a 1997 analysis placed economic health damages from a tularemia attack in excess of $5 billion per 100,000 people affected (potential military response cost not included) (Kaufmann, Meltzer et al. 1997).

2. Naturally occurring tularemia

Tularemia affects individuals throughout the Northern Hemisphere, especially those residing in North America and Eurasia. In the United States, tularemia has been seen in every state except Hawaii, with the majority of cases occurring in the South-Central and Western states of Missouri, Arkansas, Oklahoma, South Dakota, and Montana (Dennis, Inglesby et al. 2001; Sjostedt 2007). Tularemia is also endemic on the island of Martha's Vineyard, which is the only place in the United States to have two recognized outbreaks (1978 and 2000) (Matyas, Nieder et al. 2007). Both times, 15 people were infected with *F. tularensis*, with the majority presenting with primary pulmonary tularemia. However, the 2000 outbreak saw one fatality and subsequent outbreaks totaling 59 reported cases continued through 2006. In contrast, only two or fewer cases were reported each year after the 1978 outbreak (Matyas, Nieder et al. 2007). In Eurasia, the disease is widely endemic, with the greatest number of cases reported from Scandinavian countries and countries of the former Soviet Union.

In these instances, tularemia is a predominantly rural disease associated with outdoor activities and occurring mostly in males during the summer months. In the 2000 Martha's Vineyard outbreak, the majority of infected individuals were landscapers; subsequent studies showed that almost 10% of professional landscapers on the island

were seropositive for *F. tularensis*, compared to 0-1% seropositivity in the control groups (Feldman, Stiles-Enos et al. 2003). The conclusion of an investigation by the Massachusetts Department of Public Health was that lawn mowing and brush cutting activities were the most likely source of exposure for those with pneumonic tularemia, as these activities can result in aerosolized particles (Matyas, Nieder et al. 2007). In Eurasia, as well as in the United States, hunters and farmers have a higher chance of being exposed to *F. tularensis* infection by virtue of their occupation or recreational activities. Several routes of infection – bites from flies, ticks, or mosquitoes, contact with infected animals, and ingestion of contaminated food or water – favor the outdoor lifestyles of landscapers, hunters, and farmers.

3. Tularemia among persons at risk

In addition to the deliberate use of *F. tularensis* as a biological weapon and to the increased chance of infection for those with outdoor occupations and recreational activities, veterinary, laboratory, and clinical workers would also benefit from an approved tularemia vaccine. A powerful example of when such a vaccine would have been useful is exemplified by the 2004 incident when three Boston University researchers became ill with pneumonic tularemia and were hospitalized (Barry 2005; Lawler 2005). They were unknowingly using a strain of *F. tularensis* that was apparently contaminated with virulent subspecies *tularensis* bacteria.

Although the current live vaccine remains unlicensed, several scientists with access to the vaccine have been immunized and continue to administer the vaccine to personnel in their lab because it at least offers some level of protection against tularemia.

The fact that researchers and military personnel worldwide are using an unlicensed live vaccine from the 1960s serves to highlight the importance of developing an FDA-approved vaccine against tularemia.

B. Rationally attenuated live vaccines

1. LVS - The type B live vaccine strain

The current live vaccine strain, LVS, was generated from an *F. tularensis* ssp. *holarctica*-derived Russian vaccine strain received by the United States from the former Soviet Union in the 1960s as part of a formal scientific exchange program. Subsequent passage of the strain on peptone cysteine agar gave rise to blue and gray colony variants (Eigelsbach and Downs 1961). Mice infected intraperitoneally with gray colony variants exhibited an LD_{50} of 10^9 colony forming units (CFU), compared to 10^6 CFU with the blue variant. However, when the mice were subcutaneously vaccinated with 10^2-10^6 CFU of the blue variant or 10^8 CFU of the gray variant, almost all vaccinated with the blue variant were completely protected against subcutaneous challenge of 10^3 CFU wild-type *F. tularensis* ssp. *tularensis* Schu S4 while none of the mice vaccinated with the gray variant survived the challenge (Eigelsbach and Downs 1961).

Because *F. tularensis* ssp. *tularensis* is not found in Eurasia, LVS had only been tested as a vaccine against subspecies *holarctica* strains when it was presented to the U.S. Therefore, further testing to assess the efficacy of LVS protection against subspecies *tularensis* infection was conducted with dermal and respiratory routes using mice, guinea pigs, rabbits, monkeys, and humans (Conlan and Oyston 2007). In human volunteers, individuals immunized with LVS via scarification were shown to have greater protection

against challenge with subspecies *tularensis* Schu S4 than those immunized with killed bacteria or unimmunized: only 17% of LVS-vaccinated volunteers developed overt disease upon inhalation of 10-52 particles of Schu S4, compared to 60% immunized with killed bacteria and 75% who were not immunized (Saslaw, Eigelsbach et al. 1961; Conlan and Oyston 2007). Subsequent studies with human volunteers demonstrated that aerosol immunization with LVS provided better protection against aerosol challenge with Schu S4 than immunization via scarification, but that LVS was more virulent in humans when administered as an aerosol (Hornick and Eigelsbach 1966; Conlan and Oyston 2007). Because it retains virulence as an aerosol, LVS has only ever been approved for administration by scarification. The use of LVS at the United States Army Medical Research Institute for Infectious Disease highlights the value of the vaccine strain: compared to the killed bacteria vaccine, immunization by scarification with LVS reduced the incidence of laboratory-acquired tularemia from 5.7 to 0.27 cases per 1000 at-risk employee-years (Burke 1977).

Despite the obvious protective benefits of vaccination with LVS, the strain will likely remain unlicensed as a tularemia vaccine. Genetic analysis of the strain exposed multiple defects, including point mutations and large deletions, compared to clinical isolates of subspecies *holarctica* (Rohmer, Brittnacher et al. 2006). It is unknown how the individual defects contribute to the overall attenuation of the strain, or what the potential for reversion may be. Also, the mechanism of protection is not well-defined, and the exact parent strain is unknown. Furthermore, the safety of administering LVS to immunocompromised individuals has yet to be assessed. However, until a suitable tularemia vaccine is available, LVS will continue to be used by those at risk.

2. Other attenuated and protective live strains

In the past few years, live attenuated and protective *F. tularensis* strains in addition to LVS have begun to be generated and studied as the basis of an approved tularemia vaccine. In one report, a spontaneous mutant of *F. tularensis* ssp. *tularensis* Schu S4 was found to have two proteins of unknown function that were defective. The engineered deletion of one of these genes, ΔFTT0918, resulted in an attenuated strain with an intradermal (i.d.) LD_{50} of $>10^5$ CFU in mice, whereas the wild-type LD_{50} is <10 CFU (Twine, Bystrom et al. 2005). Mice immunized i.d. with 10^5 CFU of ΔFTT0918 showed no outward signs of illness. When challenged i.d. with 500 CFU of the virulent *F. tularensis* ssp. *tularensis* strain FSC033, mice were protected for >35 days. However, aerosol challenge with ~10 CFU of the same strains yielded only 35% protection (Twine, Bystrom et al. 2005). In the same investigation, i.d. vaccination with 10^6 CFU of a Δ*iglC* mutant in an *F. tularensis* ssp. *tularensis* background failed to provide protection against either i.d. or aerosol challenge, with the Δ*iglC*-vaccinated mice surviving only 1-3 days longer than unvaccinated controls (Twine, Bystrom et al. 2005). Additional studies identifying potential vaccine candidates are described in Chapter 2 of this thesis. Briefly, we created and tested an *F. tularensis* ssp. *novicida* transposon mutant library, which led to the discovery of four highly attenuated transposon mutant strains that protected mice against intraperitoneal challenges of up to one million times the wild-type LD_{50}.

Historically, mutating the aromatic amino acid or purine biosynthetic pathways are common ways to generate good live vaccine candidates in pathogenic bacteria, as these generate the building blocks for proteins and nucleic acids. However, this approach has met with mixed results in *F. tualrensis*. A subspecies *novicida* strain carrying a

deletion in *purA* was found to be over-attenuated and not able to protect against challenge with wild-type *F. tularensis* ssp. *novicida*, while in the same study, a *purF* mutant was well-attenuated and provided protection against subsequent challenge with the parental strain in mice but was unable to protect against challenge with *F. tularensis* ssp. *tularensis* Schu S4 (Quarry, Isherwood et al. 2007). In another study using *F. tularensis* ssp. *holarctica* LVS, a *purMCD* mutant was attenuated and protective against the parental strain. In our work with *F. tularensis* ssp. *novicida*, mutants with transposon insertions in *purCD* and *purM* were attenuated but failed to protect against wild-type parental challenge in mice (Tempel 2003; Pechous, Celli et al. 2006). These apparent conflicts could be attributed to the significantly higher challenge dosage used in our study. Further studies using an in-frame deletion mutant of *purMCD* constructed in *F. tularensis* ssp. *tularensis* Schu S4 showed that this mutant was highly attenuated in mice vaccinated via intranasal or intradermal routes and offered good protection against subspecies *tularensis* or *holarctica* intradermal challenge but not intranasal challenge (Pechous, McCarthy et al. 2008). Regardless of the differences, rational mutations of the purine biosynthetic pathway appear to have an overall attenuating effect on the virulence of *F. tularensis* and may yet yield an acceptable vaccine if the proper combination of mutations is achieved.

C. Subunit vaccines

Numerous *F. tularensis* antigens have been tested for the capacity to act as a tularemia vaccine, yet this approach has met with very limited success. The O-antigen component of LPS was found to provide protection against systemic challenge with attenuated (LVS) and virulent subspecies *holarctica* strains but not against subspecies *tularensis* challenge or against aerosol delivery of either type (Fulop, Mastroeni et al.

2001; Conlan, Shen et al. 2002; Thomas, Titball et al. 2007). Mice immunized with LPS purified from *F. tularensis* ssp. *novicida* were protected against challenge with subspecies *novicida* organisms but succumbed to disease when challenged with subspecies *holarctica*, demonstrating a lack of cross-protection by LPS vaccination (Thomas, Titball et al. 2007). It should be noted that the O-antigen is identical between subspecies *tularensis* and *holarctica* but altered in subspecies *novicida*.

Previous studies using highly immunogenic proteins, such as the outer membrane proteins FopA and Tul4, indicated that these antigens were unable to induce protective immunity (Fulop, Manchee et al. 1995; Golovliov, Ericsson et al. 1995). However, recent experiments using native outer membrane proteins (OMPs) purified from *F. tularensis* ssp. *tularensis* Schu S4 showed that such antigens may be able to elicit a protective immune response. Mice immunized with OMPs exhibited a 50% survival rate 20 days post-challenge with Schu S4 compared to naïve mice (Huntley, Conley et al. 2008).

The lack of success with finding an acceptable subunit vaccine against tularemia has many feasible explanations. It is possible that there are only a limited number of proteins sufficient to elicit a protective immune response, and these may not have been discovered yet. Sublethal infection with *F. tularensis* ssp. *novicida* does not protect mice against subsequent challenge with subspecies *tularensis* or *holarctica* strains, so the protective antigens may be specific to these more virulent strains (Shen, Chen et al. 2004). Perhaps a combination of antigens is needed to provide protection, yet the proper cocktail hasn't been concocted yet. Finally, antigens need to be delivered in the context of an adjuvant or carrier – if this aspect of the experiment is not formulated correctly, it could interfere with the immune response. Despite these obstacles, precise identification

of protective antigens and careful consideration of experimental parameters may still yield a suitable subunit vaccine against tularemia.

D. Killed vaccines

One of the first methods to combat human tularemia was using killed bacteria to vaccinate individuals. These vaccines, developed in the 1940s by Dr. Lee Foshay, were comprised of whole phenol-killed bacteria or acetone extracts thereof. They were used as protection for U.S. Army personnel involved in tularemia research, a population that was considered high-risk and had a high incidence of laboratory-acquired infections (Conlan and Oyston 2007). Although the vaccine was able to protect against both ulceroglandular and typhoidal tularemia, many recipients developed infections severe enough to require therapeutic intervention. These killed-bacteria vaccines also performed poorly in mice, guinea pigs, and monkeys; it is presumed that they were unable to elicit the robust immune response that is necessary for protection against *F. tularensis.*

V. Tools for *Francisella* Research

A. *in vitro* manipulation of *Francisella* genetics

1. DNA delivery into *Francisella*

While the initial studies of *Francisella* mutants concentrated on natural variants and chemically induced mutations, later endeavors turned to achieving targeted genetic manipulations. Introducing DNA into bacteria serves to create defined genetic mutations

or to complement previously existing mutations. Several approaches were developed or modified for use in *Francisella*. The methods described below outline the general techniques of chemical transformation, cryotransformation, electroporation, and conjugation; often the parameters are adjusted by individual researchers in an attempt to improve DNA uptake efficiency.

a. Chemical transformation

The chemically induced increase in cell membrane permeability to promote uptake of naked DNA by bacteria is called chemical transformation. In general, chromosomal or plasmid DNA is purified from the donor bacteria and mixed with chemically competent recipient bacteria. This mixture is then incubated on agar or in liquid medium. *Francisella* cells are commonly made competent by growth on glucose cysteine blood agar, cysteine heart agar, or Chamberlain's defined medium (Chamberlain 1965; Tyeryar and Lawton 1969). The bacteria are then concentrated in a buffered saline solution, incubated with the DNA to be introduced in a calcium chloride transformation buffer with slow shaking, and then incubated in Chamberlain's medium to allow for gene expression prior to being plated on selective agar medium. Although no special reagents or equipment are required for chemical transformation, the only reports of successful use with this method of DNA uptake have been using the *F. novicida* subspecies (Frank and Zahrt 2007).

b. Cryotransformation

Another method of chemical transformation that involves rapid freezing of the bacteria to induce DNA uptake is cryotransformation. With this technique, *Francisella* are grown on agar or in liquid medium and concentrated in a potassium chloride solution.

The cells are then incubated with the DNA at room temperature in a magnesium sulfate buffer before being rapidly frozen in liquid nitrogen. The cells are gradually thawed to 37ºC and incubated on agar medium to allow phenotypic expression before being transferred to selective agar medium to isolate recombinants. Although cryotransformation has been used successfully in *F. novicida* and *F. holarctica* subspecies, the use of liquid nitrogen in this procedure may be a constraint for those strains restricted to biosafety level 3 (BSL-3) facilities (Frank and Zahrt 2007).

c. Electroporation

Applying a brief, controlled pulse of electricity to bacteria temporarily destabilizes the cell membrane, allowing for the entry of purified DNA through transient pores; this is called electroporation. *Francisella* can be made electrocompetent by growing to mid-log phase in liquid medium or to confluency on solid agar plates, followed by several washes with 0.5M sucrose +/- 10% glycerol. Purified DNA is incubated with the electrocompetent *Francisella* cells on ice, and the mix is electroporated in a cuvette. The typical range of settings is 12.5-15 kV/cm, resistances of 200 to 400 Ω, and a capacitance of 25μF (Baron, Myltseva et al. 1995; Kawula, Hall et al. 2004; Maier, Havig et al. 2004). After electroporation, the cells are incubated in liquid medium in the absence of a selective agent to allow for phenotypic expression and then plated onto selective agar for selection of recombinants. There are several advantages of this method over others for use in *Francisella*: the efficiency of DNA uptake is higher; a large quantity of electrocompetent cells can be prepared at once; aliquots can be stored at -80ºC for extended periods of time; the technique has been applied successfully in *F.*

tularensis, *F. holarctica*, and *F. novicida* subspecies; and it can be adapted for use in BSL-3 laboratories.

d. Conjugation

Another common method for introducing DNA into bacteria involves the direct transfer of DNA from a donor organism to a recipient; this technique is known as conjugation. The donor bacteria possesses conjugative genes on a plasmid, which code for the pili to contact the recipient bacterium, as well as for proteins that open a channel between the bacteria for DNA exchange. For experiments with *Francisella*, the *E. coli* strain S-17 is commonly used as the donor. The DNA to be transferred is marked with a mobilization sequence (*ori*T or *mob*) and introduced into the *E. coli* strain. The mating is conducted by incubating the *E. coli* and *Francisella* cells together at room temperature on nonselective agar medium for 18 hours before transferring the conjugation mixture to selective medium to isolate recombinants. The selective medium must also carry a counter-selection against the donor bacteria, as both the donor and desired recombinants will carry the DNA of interest. Polymixin B (50-100 µg/ml) can be used to select against *E. coli* without affecting the growth of recipient *Francisella*. Like electroporation, this method has been successfully used in a variety of *Francisella* subspecies, can produce high transfer frequencies under optimal conditions, and is safe for use under BSL-3 conditions (Golovliov, Sjostedt et al. 2003; Twine, Bystrom et al. 2005; Frank and Zahrt 2007; Thomas, Titball et al. 2007).

2. Transposon mutagenesis

When little is known about the roles or identities of genes in a particular organism, genetic studies are hindered. Introducing random mutations into the genome of

an organism is a way to assign functions to previously uncharacterized genes. Among the first insertional mutagenesis methods used for *F. tularensis* research was cassette mutagenesis. In this technique, *F. tularensis* DNA is digested to completion, self-ligated, partially re-digested with a separate restriction enzyme to allow for random insertion of a kanamycin resistance gene, and then transformed into *F. tularensis* bacteria. Homologous recombination with the DNA flanking the kanamycin gene inserts the cassette into the chromosome, resulting in kanamycin-resistant colonies that can be screened for any number of phenotypes. The identification of *minD*, which is required for intracellular replication, was achieved using this strategy (Anthony, Cowley et al. 1994). However, instability of the kanamycin marker, a low number of random mutations, and duplication of the transforming DNA limits the success of this technique.

Another type of insertional mutagenesis that has seen more success in *F. tularensis* is shuttle mutagenesis. In this technique, *F. tularensis* DNA is cloned into standard *E. coli* vectors and mutagenized in *E. coli* (Anthony, Gu et al. 1991). Transformants exhibiting expression of the desired marker are then pooled for plasmid extraction, and the plasmids are transformed into *F. tularensis*. Integration of the mutant DNA results in gene inactivation, and the resulting mutant library can be screened for various phenotypes. This method of mutagenesis led to the identification of three LPS biosynthetic genes, the *clpB* heat-shock protease gene, and the intracellular growth locus operon, *iglABCD* (Cowley, Gray et al. 2000; Gray, Cowley et al. 2002). Recently, driven by the lack of an available *F. tularensis* shuttle plasmid, a hybrid plasmid (pFNLTP1) that can be used for genetic procedures in both *F. tularensis* and *E. coli* was constructed (Maier, Havig et al. 2004).

The technique of using transposon-transposase, or transposome, complexes for mutagenesis was concurrently adapted for use in *F. tularensis* ssp. *holarctica* by Kawula *et al.* and for use in subspecies *novicida* by our research group, based on our success using the approach in *Salmonella* (Tempel 2003; Kawula, Hall et al. 2004; Geddes, Worley et al. 2005; Tempel, Lai et al. 2006). This technique is independent of recombination and is achieved by electroporating transposomes directly into competent bacteria. Transposon integration is driven by activation of transposase activity by intracellular magnesium ions, creating stable, random insertions, as shown in Figure 1-8. Colonies expressing the appropriate marker are selected and screened for the desired phenotype. The transposome mutagenesis method was subsequently used to generate a library of subspecies *tularensis* Schu S4 mutants, in which the authors modified the

technique to include a rifampicin resistance marker and R6K origin of replication (Qin and Mann 2006). This technique also generated one of the most significant tools to date for the study of *F. tularensis* genetics: the construction of a comprehensive *F. tularensis* ssp. *novicida* transposon mutant library, with an average of >9 insertions in each non-essential gene (Gallagher, Ramage et al. 2007).

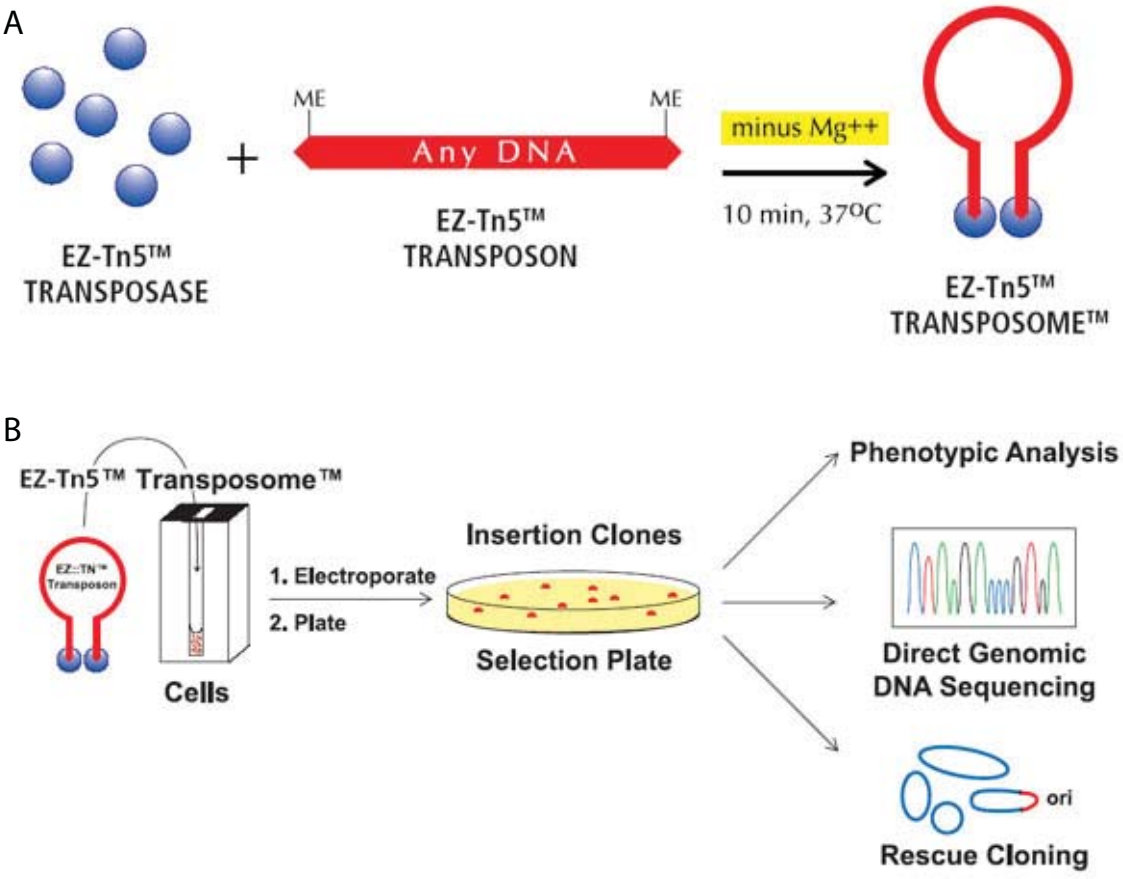

Figure 1-8. Transposome mutagenesis technique. Example of a commercially available method for transposome mutagenesis. Transposase is mixed with a transposon in the absence of Mg^{++} to form a transposome (A). The transposome complex is then introduced into the cells by electroporation (B). In the presence of Mg^{++} in the cytoplasm, the transposase becomes activated. Insertion mutants can be identified by plating on selective medium. Further analyses may include phenotypic analysis, DNA sequencing to identify the disrupted genes, and rescue cloning. Method adapted from EpiCentre Biotechnologies, Madison, WI.

3. Allelic exchange

Targeted insertion or deletion of specific genes can be accomplished by allelic replacement. This technique has been performed successfully in several *F. tularensis* strains, using both one-step and two-step allelic exchange protocols. The one-step method is generally used to create antibiotic-marked gene deletions where the gene of interest is replaced with an antibiotic resistance cassette. This is accomplished by generating either a PCR fragment or suicide vector carrying a DNA sequence in which an antibiotic resistance cassette is flanked by regions of homology upstream and downstream to the gene to be deleted. The recombinant DNA is transformed into the recipient *F. tularensis*, and transformants are isolated by growth on selective medium. PCR-based mutants in *mglA, iglC, bla,* and *tul4* have been made using this method in *F. tularensis* ssp. *novicida* (Lauriano, Barker et al. 2003).

The two-step method of allelic exchange is mainly used to create unmarked deletions in *F. tularensis*, although a marked gene deletion is an option (Golovliov, Baranov et al. 2003). The technique involves the transient integration of plasmid vector DNA into the *F. tularensis* chromosome. As with one-step allelic exchange, regions of homology flanking the gene of interest are amplified and cloned into the backbone of a nonreplicating vector, which also contains a selectable marker as well as a counterselectable marker. In contrast to the one-step method, the flanking regions may be cloned adjacent to one another, omitting the antibiotic resistance cassette that would otherwise replace the gene of interest. When the plasmid vector is transformed into the recipient *F. tularensis*, a single crossover event integrates the plasmid DNA into the chromosome. Merodiploids (cells carrying two copies of the target gene as a result of the

integration event) are isolated based on expression of the selectable marker. The second crossover event will resolve the plasmid, looping out either the wild-type or mutant copy of the gene. Recombinants that have completed the second crossover event can be selected with the counterselectable marker, and subsequent PCR analysis can be used to identify if the resulting strain contains the wild-type or mutant copy of the gene. This method has been used with success in *F. tularensis* ssp. *tularensis*, *holarctica*, and *novicida* and is particularly valuable, as it can also be used to complement mutant strains *in cis*. Appendix 1 of this thesis describes in detail the generation of plasmids for creating unmarked deletions in *F. tularensis* ssp. *tularensis* Schu S4 by two-step allelic exchange.

B. Cell culture models

As discussed in a previous section, macrophages are the preferred host cell type for *F. tularensis* (Fortier, Green et al. 1994). Recent experimentation has shown that *F. tularensis* has the ability to infect other cell types such as alveolar type II (AT2) epithelial lung cells, neutrophils, dendritic cells, and hepatocytes (Bosio and Dow 2005; McCaffrey and Allen 2006; Hall, Craven et al. 2007; Chakraborty, Monfett et al. 2008). However, because it is well-established that mononuclear phagocytes are the host cells infected during natural infections, these are the cell types most often used for *ex vivo* and *in vitro* studies of *F. tularensis* infection. Common cells and cell lines used include: human peripheral blood monocytes, monocyte-derived macrophages (MDM), the THP-1 human monocyte-like cell line, mouse bone marrow-derived macrophages (BMDM), and the J774 and RAW 264.7 mouse macrophage-like cell lines.

C. Animal models

F. tularensis is known to infect and replicate in over 200 species of mammals, as well as within insects, birds, fish, and reptiles (Farlow, Wagner et al. 2005). In a laboratory setting, *F. tularensis* has been studied using human, primate, rabbit, guinea pig, rat, and mouse models of infection. Human models of tularemia were used as late as the 1950s and 1960s to study the efficacy of various vaccines, as well as to test different delivery methods for better protection (Lyons and Wu 2007). As with many human diseases, primate models were developed in an attempt to define the pathology associated with human tularemia infection. Due to regulations with human testing today, primates are the animal model of choice for advanced vaccine trials.

Because of its association as a vector of the disease, the rabbit was once studied as a model of tularemia. Studies describing the effect of *F. tularensis* ssp. *tularensis* Schu S4 on rabbits were conducted in the 1970s and showed that inhalation of the bacteria caused a systemic illness, with death by five days post-infection (Baskerville and Hambleton 1976). Similar experiments using the subspecies *holarctica*-derived LVS in rabbits indicated that there was an increased LD_{50} with this strain, but no pathological results were described (Hambleton, Harris-Smith et al. 1977). Studies using a guinea pig model of infection were mainly performed in the 1960s for the purpose of evaluating the efficacy of LVS; the same is true of the rat model of tularemia. Neither of these animal models has been used to study *F. tularensis* for over 25 years.

Initial studies with mice as a model for tularemia infection began in the late 1940s to analyze the effectiveness of the killed *F. tularensis* vaccine (Ruchman and Foshay 1949). In the following decade, the mouse model was used to isolate what has become

the only tularemia vaccine used in the United States to date – the subspecies *holarctica*-derived LVS strain (Eigelsbach and Downs 1961). Many studies have since been conducted with several strains of *F. tularensis* in various lines of mice to test the efficacy of tularemia vaccines, as well as to study the pathology of the disease and gain insight into the mammalian immune response to *F. tularensis* infection. Because of the multiple molecular tools that exist for working with mice, the number of inbred and transgenic lines available, and the low cost per animal, the mouse model of tularemia remains the most attractive whole animal research tool for studying *F. tularensis.* However, it must be noted that while *F. tularensis* ssp. *holarctica* LVS causes a tularemia-like disease in mice, there are differences between mouse infection and human infection; care must be taken when extrapolating information gleaned from mouse experimentation to disease in humans.

Recent research efforts have attempted to identify other potential animal models for tularemia infection in addition to the mouse. Low doses of wild-type and mutant derivatives of subspecies *holarctica* and *novicida* strains were used to assess the capacity of chick embryos as a *F. tularenis* model of virulence (Nix, Cheung et al. 2006). The first invertebrate model for tularemia was recently proposed when it was shown that *F. tularensis* is capable of replicating inside *Drosphila melanogaster* (Vonkavaara, Telepnev et al. 2008). A *Drosophila* model has many advantages in that flies are easy to cultivate in large numbers, the intracellular signaling pathways are well-described, and their relative transparency would facilitate *in vivo* studies using GFP-labeled bacteria; it is a particularly intriguing model for tularemia since *F. tularensis* is thought to be transferred via an insect vector.

VI. Thesis Overview

Francisella tularensis is the etiologic agent of tularemia, a debilitating and potentially fatal disease that affects humans and a wide range of animals. Infections can be contracted through a number of routes, the most dangerous of which is inhalation of as few as 10 bacteria. Because of its high infectivity and ease of aerosol transmission, *F. tularensis* has been modified for use as a biological weapon by several countries and is classified as a Category A select agent by the CDC. Despite these developments, there is no approved vaccine available in the United States or Europe. Additionally, the molecular mechanisms of *F. tularensis* pathogenesis are relatively obscure, and few strategies have been devised for researching this fastidious pathogen. This dissertation describes the development and application of a transposon mutagenesis technique to identify virulence factors that may be used in the development of a rationally attenuated live vaccine strain against tularemia, as well as the biochemical and biophysical characterization of a novel virulence factor identified using this method.

Although the pathogenesis of *Francisella* is not fully understood, it has been established that replication in human and animal macrophages is central to the organism's ability to cause tularemia. Manuscript 1 describes the application of a technique I designed to identify genes in *F. novicida* that are required for invasion and growth in macrophages. Using a transposon-transposase complex, random mutations were introduced into the *Francisella* genome by electroporation to generate a library of nearly 800 mutants. These strains were screened for attenuation in macrophages and then for ability to provide protection against wild-type infection in a mouse model, which resulted

in the identification of five attenuated and protective transposon mutants. These mutants were evaluated for *in vivo* attenuation, dissemination from the site of the infection, and lack of persistence, as well as specific levels of entry and attenuation in different cell lines. In addition, the conservation of the mutated genes and their functions between *F. tularensis* subspecies was confirmed by complementing *F. novicida* mutant strains with wild-type *F. tularensis* genes. Overall, my research resulted in the identification of four candidates for designing a rationally attenuated live vaccine against tularemia.

The FDA requirements for constructing a live vaccine are that the strain is genetically defined, the mutation(s) must not be able to revert at any frequency, and the final vaccine strain cannot express antibiotic resistance. Because the *F. tularensis* ssp. *tularensis* is the most virulent in humans, the most effective tularemia vaccine would likely be derived from a subspecies *tularensis* parent strain. Appendix 1 serves to summarize our work toward making such live vaccine strains in a type A *F. tularensis* background and is a continuation of the work described in Manuscript 1. I constructed in-frame deletions of each of the four genes identified in Manuscript 1 in a background suitable for allelic exchange in *F. tularensis.* At the time of this writing, we are working in collaboration with Thomas Zahrt's group at the Medical College of Wisconsin to introduce these deletions into the genome of *F. tularensis* strain Schu S4. Once the deletion strains are confirmed, the mutant strains will be tested for attenuation and protection in murine and non-human primate models. These experiments are a major step in the development of an approved tularemia vaccine.

One of the protective and attenuating mutations identified in the vaccine screen is in a putative gene (FTN0715) that has not been characterized. The work presented in

Manuscript 2 provides information pertaining to the structure and function of the gene product. BLAST analysis of FTN0715 showed that it is unique to *Francisella* and that homologs are present in all other sequenced *F. tularensis* strains, thus resulting in the discovery of a novel *Francisella* virulence factor family (designated Pat for pathogenicity factor). Interestingly, further sequence analysis revealed the presence of seven adjacent 102 amino acid repeat domains (designated TAARP for tandem amino acid repeat) in two subspecies *novicida* homologs that are present in only one or two copies in other *Fracisella* homologs. To further elucidate the structure and function of these repeat motifs, I expressed and purified a recombinant version of the *F. tularensis* Schu S4 TAARP domain and subjected it to numerous biochemical analyses. These studies indicated that this repeat motif is a stably folded domain capable of homo-oligomerization. Additionally, the TAARP domain may play a role in *Francisella* entry, as it is structurally related to a bacterial adhesion factor and appears to bind host cell factors associated with the E-cadherin complex.

Overall, the goal of this thesis was to contribute to the body of knowledge concerning *F. tularensis* virulence and to shed some light on what makes this bacterium such a successful pathogen. With respect to the continued efforts of other scientists in the field and new scientists yet to be realized, I hope that the work represented here will prove to be beneficial for unraveling the mechanisms of *Francisella* pathogenicity.

Chapter 2

Manuscript 1: Attenuated *Francisella novicida* transposon mutants protect mice against wild-type challenge

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Preface

This work was published in the September 2006 issue of Infection and Immunity by Rebecca Tempel, Xin-He Lai, Lidia Crosa, Briana Kozlowicz, and Fred Heffron.

My contributions to the manuscript include conception of the project, experimental design, developing a transposon mutagenesis technique for *F. novicida*, generating the mutant library, assessing the mutants for attenuation and survival in cell lines and primary cells, fluorescence microscopy of infected cells, Southern blot analysis of transposon insertions, assisting with mouse infections and harvesting animal tissues, primer design and cloning to create *F. novicida* complementation strains, data analysis, and preparation of all figures, tables, and the manuscript.

Xin-He Lai contributed to this work by conducting mouse infections and harvesting tissues for vaccination, challenge, and dissemination studies, calculating the LD_{50} values for the protective *F. novicida* mutants, performing the lactate dehydrogenase release assay, and as an experienced consultant for working with *Francisella*.

Lidia Crosa contributed to this study by helping manage the *F. novicida* transposon mutant library strain collection, assisting in the elecroporation experiments, and harvesting bone marrow-derived macrophages.

Briana Kozlowicz contributed to this work by electroporating the FTT0742 complementation vector into the corresponding transposon mutant for complementation studies.

Fred Heffron contributed to this study by providing advice and suggestions on experimental design and data analysis, as well as the space, equipment, and supplies with which to perform the experiments.

Summary

Francisella tularensis is the bacterial pathogen that causes tularemia in humans and a number of animals. To date, there is no approved vaccine for this widespread and life-threatening disease. The goal of this study was to identify *F. tularensis* mutants that can be used in the development of a live attenuated vaccine. We screened *F. novicida* transposon mutants to identify mutants that exhibited reduced growth in mouse macrophages, as these cells are the preferred host cells of *Francisella* and an essential component of the innate immune system. This approach yielded 16 *F. novicida* mutants that were 100-fold attenuated for virulence in a mouse model than the wild-type parental strain. These mutants were then tested to determine their abilities to protect mice against challenge with high doses of wild-type bacteria. Five of the 16 attenuated mutants (with mutations corresponding to *dsbB*, FTT0742, *pdpB*, *fumA*, and *carB* in the *F. tularensis* SCHU S4 strain) provided mice with protection against challenge with high doses (>8 × 10^5 CFU) of wild-type *F. novicida*. We believe that these findings will be of use in the design of a vaccine against tularemia.

Introduction

Francisella tularensis is a Gram-negative, facultative intracellular pathogen that causes tularemia, a debilitating and potentially fatal disease that affects humans and a wide range of animals. Infections can be acquired through bites from an arthropod vector, skin lesions, ingestion of contaminated food or water, and, most dangerously, inhalation of as few as 10 bacteria (Dennis, Inglesby et al. 2001). The low dose required to cause tularemia by aerosol route resulted in the development of *F. tularensis* for use as a biological weapon by several national weapons programs. This has led the U.S. Centers for Disease Control and Prevention (CDC) to classify *F. tularensis* as a Category A bioterrorism agent; members of this category are considered the organisms that pose the most serious risk to national security (http://www.bt.cdc.gov/Agent/Agentlist.asp). There is currently no approved vaccine available in the United States or Europe. Thus, the development of a vaccine against *F. tularensis* has become an international research priority.

Although the molecular mechanisms of *F. tularensis* pathogenesis remain relatively obscure, it has been established that replication in human and animal macrophages is central to this organism's ability to cause tularemia (Fortier, Green et al. 1994). Several *F. tularensis* genes associated with intracellular growth have been identified, including *iglB, iglC, mglA, pdpD,* and a *clpB* homolog (Baron and Nano 1998; Gray, Cowley et al. 2002; Golovliov, Sjostedt et al. 2003; Lai, Golovliov et al. 2004; Lauriano, Barker et al. 2004). Additionally, it is thought that many of the genes in the recently described *F. tularensis* pathogenicity island (FPI) contribute to the survival and growth of this organism in macrophages (Nano, Zhang et al. 2004; Larsson, Oyston et al.

2005). Of these, only *iglC* has been studied as the basis for a potential vaccine strain. Pammit, *et al.* recently reported that intranasal vaccination with a *F. novicida* carrying an *iglC* deletion resulted in greater than 50% protection against challenges with the wild-type organism (Pammit, Raulie et al. 2006). However, the capacity of mutant derivative strains with mutations in other FPI genes to confer protection against challenge with wild-type bacteria has not been studied.

Four main subspecies of *F. tularensis* are commonly recognized: *F. tularensis* ssp. *tularensis* (type A), *F. tularensis* ssp. *holarctica* (type B), *F. tularensis* ssp. *novicida* and *F. tularensis* ssp. *mediasiatica*. All of these biotypes exhibit more than 95% DNA sequence identity (Broekhuijsen, Larsson et al. 2003). Although type A and type B strains are highly infectious, only type A strains cause significant mortality in humans. The current live vaccine strain (LVS) is an attenuated type B strain that provides varying levels of protection against challenge with type A *F. tularensis* strains depending on route of immunization, the route of challenge, and the genetic background of the host (Chen, Shen et al. 2003; Chen, KuoLee et al. 2004; Shen, Chen et al. 2004; Conlan, Shen et al. 2005; Green, Choules et al. 2005; Wu, Hutt et al. 2005). Because the molecular basis for LVS attenuation is not known, this strain is not licensed as a tularemia vaccine.

F. novicida U112 provides an ideal model for studying *Francisella* pathogenesis for several reasons. While *F. novicida* is not considered a human pathogen, it exhibits a similar degree of virulence in mice to that of *F. tularensis* subspecies (Kieffer, Cowley et al. 2003; Shen, Chen et al. 2004). Moreover, *F. novicida* is easier and less dangerous to manipulate genetically than *F. tularensis*. In addition to the considerable genomic similarity (>95%), the close relationship between *F. novicida* and *F. tularensis* is further

highlighted by their nearly identical 16S rDNA sequences (Forsman, Sandstrom et al. 1994). The degree of genetic identity suggests that the two organisms utilize similar virulence genes and that *F. novicida* is thus an apt platform for the development of a tularemia vaccine.

In this study, we used transposon mutagenesis to identify *F. novicida* genes required for intracellular growth. The resulting mutant strains were screened for attenuation in macrophages and mice and tested for the ability to provide protection against a wild-type challenge in mice. Five *F. novicida* mutant strains were found to protect mice against challenge with $>8 \times 10^5$ CFU of wild-type *F. novicida*. These results will be used in the future construction of a *Francisella* vaccine.

Results

Transposon mutagenesis and identification of disrupted loci

An ongoing challenge to the establishment of a suitable set of genetic tools for *F. tularensis* is the difficulty of creating stable mutations in the genome. To address this issue, we developed a transposon mutagenesis technique independent of phages and shuttle vectors that yielded as many as 150 transposon mutants from a single electroporation procedure (see materials and methods). Our method, although independently developed, is quite similar to the technique used by the Kawula laboratory in LVS (Tempel 2003; Kawula, Hall et al. 2004). The bacteria were electroporated with a transposase/transposon complex that completes the transposition event once inside the bacteria. This approach yielded the library of 779 *Francisella* transposon mutants used in this study.

Macrophages are the primary host cell type for *Francisella* in both humans and animals (Fortier, Green et al. 1994). It follows that one approach to developing a tularemia vaccine would be to discern which *Francisella* genes are necessary for growth in macrophages. Thus, we screened our *F. novicida* transposon mutant library for those that exhibited a reduced ability to grow in macrophages, as described in the materials and methods section. Of more than 700 *F. novicida* transposon mutants screened, 34 exhibited reduced growth in RAW macrophages. We obtained sequences for 28 of these mutant strains and identified the disrupted ORFs by comparison to the SCHU S4 sequence (Table 2-1).

Table 2-1. *F. novicida* transposon mutant strains generated in this study

Mutant	Corresponding SCHU S4 FTT	Gene disrupted	% identity to SCHU S4[a]	Nucleotide location of Tn insert[b]	% survial rate[c]
1	FTT0107c	*dsbB*	100	114151	100
2	FTT0145	*rpoC*	99	163108	0
3	FTT0203c	*purH*	99	222340	100
4	FTT0334	*rpsQ*	100	342324	0
5	FTT0356	*htpG*	95	356504	100
6	FTT0504c	*sucC*	97	524250	0
7	FTT0583	*fopA*	98	599781	0
8	FTT0742	hypothetical[d]	89	765155	100
9	FTT0893	*purM*	98	901556	100
10	FTT0893	*purM*	97	901647	100
11	FTT0893	*purM*	98	901848	100
12	FTT0894	*purCD*	99	904045	100
13	FTT0894	*purCD*	99	904160	100
14	FTT0917	*maeA*	98	926193	33
15	FTT1165c	*aspC2*	99	1179264	0
16	FTT1222	*dedA2*	98	1240288	33
17	FTT1241	*glyA*	97	1261475	67
18	FTT1269c	*dnaK*	100	1291446	100
19	FTT1345/1700	*pdpB*[e]	98	1384141/1777485	100
20	FTT1369c	*tktA*	97	1416905	100
21	FTT1535c	*ocd*	99	1597434	0
22	FTT1535c	*ocd*	97	1597841	0
23	FTT1600c	*fumA*	98	1667516	100
24	FTT1629c	hypothetical[f]	99	1692570	0
25	FTT1664	*carB*	99	1730805	100
26	FTT1720c	*purL*	98	1804171	100
27	FTT1720c	*purL*	97	1805882	100
28	FTT1769c	*clpB*	96	1858564	0

[a] using our fragment sequence [b] corresponding to SCHU S4
[c] at 7 days after infection with 6 X 10^3 CFU, groups of 3 mice [d] lipoprotein
[e] SCHU S4 contains two copies of *pdp* [f] membrane protein

Sixteen *F. novicida* mutants exhibit attenuation in mice

To narrow our study to those mutant strains that retained an attenuated phenotype in an animal model, we infected wild-type BALB/c mice with the 28 *F. novicida* mutants that were attenuated for growth in macrophages. Mice were injected intraperitoneally with 6×10^3 bacteria in 150 µl of PBS, which is about 100 times greater than the wild-type *F. novicida* LD_{50} in mice. At 28 days post-infection, 16 of the 28 groups of mice had a 100% survival rate, indicating that 16 of the insertion mutants were highly attenuated in this animal infection model (Table 2-1).

Five *F. novicida* mutants protect mice against challenge

The ideal living vaccine strain produces an asymptomatic infection that provides complete protection against subsequent exposures to the wild-type organism. To determine if any of our 16 attenuated *F. novicida* transposon mutants could confer protection against wild-type infection, we next challenged the surviving vaccinated mice with the wild-type parental strain. Four weeks after infection with mutant *F. novicida* strains, surviving mice were intraperitoneally challenged with 8×10^5 CFU *F. novicida* U112. We considered this to be a very stringent challenge, as the dose was greater than 10,000 times the LD_{50} observed for wild-type infection. At 28 days post-challenge, 5 of the 16 mutants demonstrated 100% protection after a single vaccination: *dsbB*, the ORF corresponding to FTT0742 (henceforth referred to as FTT0742), *pdpB*, *fumA*, and *carB* (Table 2-2). In these same experiments, all wild-type control infections led to 0% survival.

Table 2-2. *F. novicida* transposon mutants attenuated in mice

***F. novicida* mutants**	**% Survival[a, b]**
dsbB	100
purH	0
htpG	0
FTT0742	100
purM	0
purM	0
purM	33
purCD	0
purCD	0
dnaK	0
pdpB	100
tktA	66
fumA	100
carB	100
purL	0
purL	33

[a] After challenge with 8 X 10^5 CFU wild-type U112
[b] Groups of three mice were inoculated intraperitoneally

LD_{50} values for *F.novicida* mutants

Further infections in mice with the *F. novicida* transposon mutants were conducted to determine the 50% lethal dose (LD_{50}) of the 5 protective strains. In our studies, the *F.novicida* U112 parental strain was observed to have an LD_{50} of 66.25 CFU (Table 2-3). Our *carB* mutant exhibited the least attenuation with an LD_{50} of 6.75×10^3 CFU. The LD_{50} values for our *dsbB* and *fumA* mutants were 6.625×10^5 CFU and 6.17×10^5 CFU, respectively. The mutant strains with the highest level of attenuation in an animal infection model were FTT0742 and *pdpB*, both of which were observed to have LD_{50} values of $>6 \text{ X } 10^7$ CFU. Taken together, these results show that our 5 *F. novicida* transposon mutants are significantly attenuated in a mouse infection model in comparison to the wild-type parental strain.

Table 2-3. LD_{50} values for protective *F. novicida* mutants

Strain	LD_{50} (CFU)[a]
wild-type U112	66.25
dsbB	6.625×10^5
FTT0742	$>6 \times 10^7$
pdpB	$>6 \times 10^7$
fumA	6.17×10^5
carB	6.75×10^3

[a] LD_{50} when mice were inoculated intraperitoneally

***F. novicida* mutants are highly attenuated for growth in mouse macrophage cell lines**

To calculate the levels of entry and attenuation in macrophages, wild-type *F. novicida* U112 and the five mutants that conferred protection were quantified for growth

in macrophage cell lines and primary mouse macrophages (BMDM). RAW and J774 macrophage-like cells and BMDM were infected in triplicate wells in duplicate plates with an input MOI of 100, as described in the materials and methods section. One plate of each cell type was lysed at 2 hours after infection to determine the ability of the transposon mutants to enter host cells, and the lysing of the second plates at 24 hours allowed us to quantify the intracellular growth of the mutant strains.

In J774 mouse macrophage-like cells, the level of entry was quite similar between the mutant and wild-type strains, with the exception of the *pdpB* mutant (Figure 2-1a). The *dsbB* mutant exhibited the highest level of attenuation in J774 cells, and the FTT0742 mutant derivative also showed a decrease in CFUs after 24 hours. Although fewer *pdpB* mutants entered J774 cells in comparison with the other strains, this mutant still displayed an attenuation phenotype. The *fumA* mutant showed very little change between the level of entry and the level of replication at 24 hours, indicating no death, no replication, or a balance between the two.

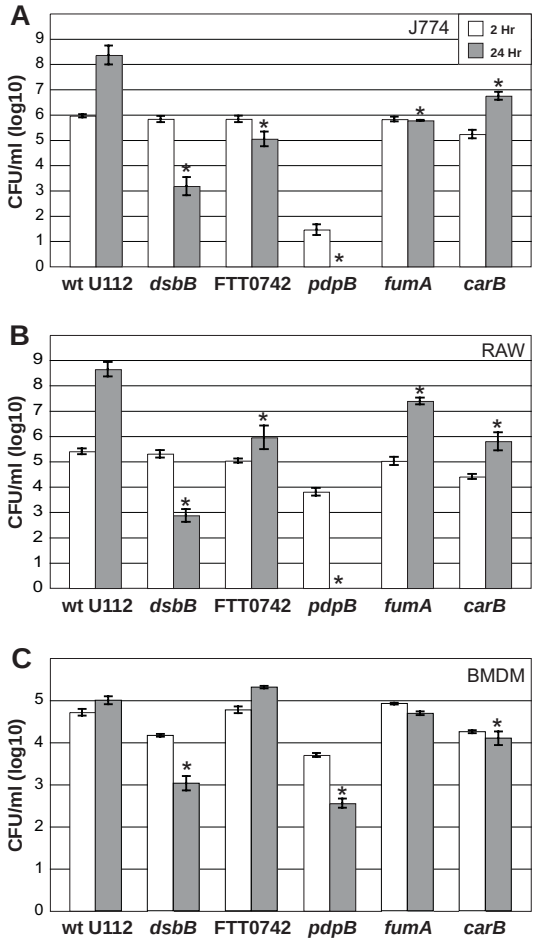

Figure 2-1. Five *F. novicida* mutants are attenuated for growth in macrophages. J774 (A) and RAW (B) macrophage cell lines, and mouse BMDM (C) were infected with the five *F. novicida* mutants and wild-type strain U112 at an MOI of 100 for 2 h and 24 h. Cells were lysed and serial dilutions of the lysates were plates onto CHA/Kan20 (mutants) or CHA (U112 and mock infection controls). Colonies were counted and CFU/ml were calculated and converted to log scale. Each column shows the average for three individual infections. No bacteria were detected in J774 or RAW cells infected with the *pdpB* mutant after 24 h. Asterisks indicate the statistically significant results for 24 h (J774 cells, $P < 0.005$; RAW cells $P < 0.05$; BMDM, $P < 0.01$).

Interestingly, the *carB* mutant displayed an ability to replicate within J774 cells, albeit at a slower rate than the wild-type control. Each of the mutants exhibited statistically significant attenuation in J774 cells at 24 hours p.i. ($p < 0.005$).

The level of entry between the transposon mutants and the wild-type bacteria was similar in RAW mouse macrophage-like cells (Figure 2-1b). Again, the *dsbB* and *pdpB* mutant derivatives exhibited the strongest attenuation phenotype. Intriguingly, the FTT0742, *fumA*, and *carB* mutant strains all demonstrated the ability to replicate in RAW cells, whereas only wild-type and the *carB* mutant replicated in the J774 cell line. Compared to wild-type, each of the mutants strains was significantly impaired for replication inside RAW cells at 24 hours p.i. ($p < 0.05$).

As in the J774 and RAW cells, the *dsbB* and *pdpB* mutants were the most attenuated strains in primary murine BMDM (Figure 2-1c). Despite their high LD_{50} values in mice, the FTT0742 mutant derivative was not impaired in the ability to enter or replicate within BMDM cells, and both the *fumA* and *carB* strains displayed only slight attenuation. This apparent disparity underlies the differences in infection among various cell populations. It should also be noted that the BMDM were not stimulated prior to infection and were likely less microbicidal than tissue-resident macrophages in the mouse model. In BMDM, only the *dsbB*, *pdpB*, and *carB* strains were significantly attenuated for growth at 24 hours p.i. ($p < 0.01$).

To visually assess the replication of *F. novicida* transposon mutants in macrophages, fluorescence microscopy was performed. J774 macrophages were infected and prepared for microscopy as indicated in the materials and methods section. As expected, macrophages infected with *F. novicida* U112 contained a greater number of

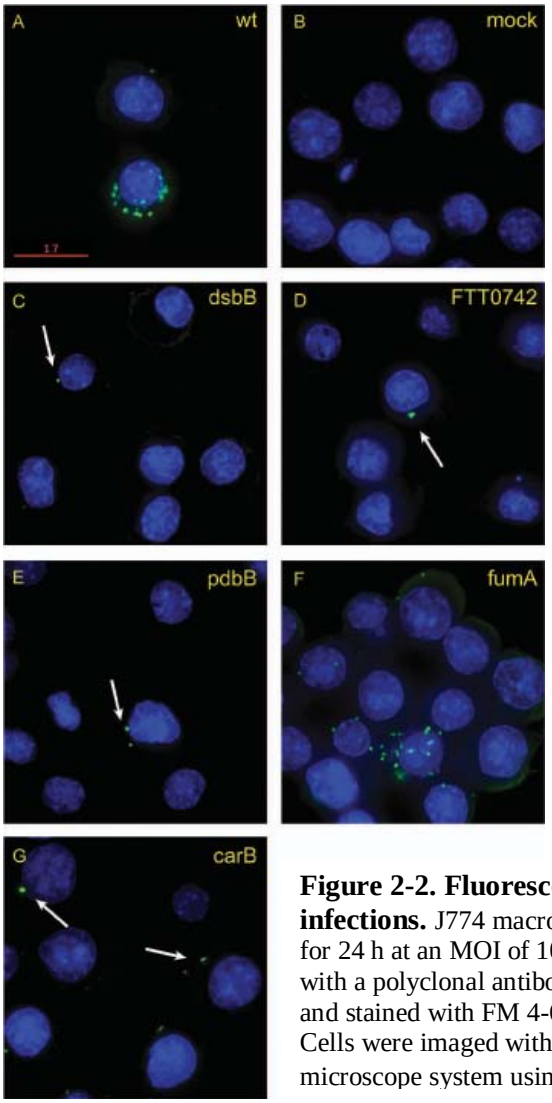

Figure 2-2. Fluorescence microscopy of macrophage cell line infections. J774 macrophages were infected in four-chamber microscopy plates for 24 h at an MOI of 100. The cells were fixed in 4% paraformaldehyde, probed with a polyclonal antibody against *Francisella* (conjugated to Alexa 488, green) and stained with FM 4-64 (membrane, not shown), and Draq 5 (DNA, blue). Cells were imaged with an Applied Precision DeltaVision deconvolution microscope system using a ×60 objective. Arrows indicate individual bacteria.

bacteria compared to those cells infected with our mutants (Figure 2-2). Although several bacteria were observed inside host cells infected with the *fumA* mutant (2-2f), infections with the *dsbB*, FTT0742, *pdpB*, and *carB* mutant strains resulted in only one or two intracellular bacteria at 24 h p.i. (white arrows). These findings corroborated our data that the *F. novicida* transposon mutants were defective for replication or survival inside macrophages. Furthermore, although the macrophages were initially seeded at the same concentration, fewer cells remained in the wells after infection with wild-type U112 when compared to the mutants and uninfected controls. This observation indicated that host cell death occurred during the course of the wild-type infection but not during infection with attenuated mutant strains.

To address the possibility that the attenuation phenotypes of our *F. novicida* mutant strains could be due to overall defects in replication, a simple growth curve was determined for each mutant. While the *dsbB*, FTT0742, and *pdpB* mutants replicated at levels similar to that of wild-type U112, both *fumA* and *carB* mutants exhibited defects in

replication after 4 hours of growth (data not shown). At 24-hours post-inoculation, these two strains grew to approximately OD_{600} 1.5, and the others grew in the range of OD_{600} 2.5 to 3.3. Interestingly, this phenotype was not rescued by supplementing the media with malate (*fumA*) or arginine (*carB*) (data not shown). It is possible that the addition of exogenous substrates cannot complement the defects because these enzymes are part of multienzyme complexes in which additional intermediates are excluded. Nonetheless, the *fumA* and *carB* mutants remained potential vaccine candidates on the basis that they protect mice against wild-type challenge (Table 2-2).

Infection with *F. novicida* mutants does not reduce host cell integrity

The observed attenuation phenotypes could be a result of increased host cell killing, which would yield less live infected macrophages and thus fewer bacteria, as they would be killed by the gentamicin in the extracellular media (Kudelina 1978). One method for determining the degree of cytotoxicity that results from bacterial infection is to measure cell lysis by quantifying the release of the stable cytosolic enzyme lactate dehydrogenase (LDH). J774 macrophages were infected with either wild-type U112 bacteria or one of the five mutant strains for 48 h at an input MOI of 100. The levels of LDH in the supernatant were then recorded. As shown in Figure 2-3, the five mutant strains are significantly impaired in their abilities

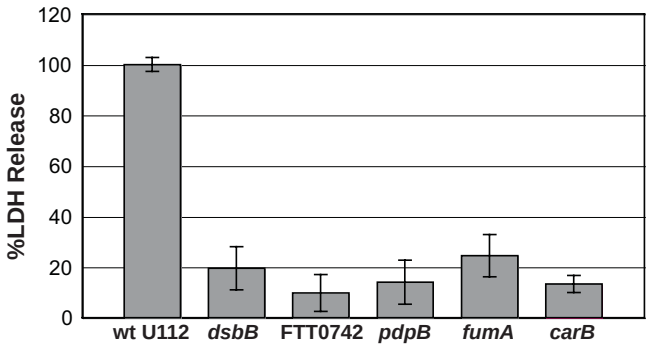

Figure 2-3. Infection with *F. novicida* mutants does not reduce host cell integrity. J774 macrophages were infected with the five *F. novicida* mutants and wild-type strain U112 (wt U112) at an MOI of 100 for 48 h. The levels of LDH in the extracellular medium were determined. The level of LDH release for the wild-type infection was defined as 100%, and the levels of LDH release for the five mutant strains were normalized to this level. Each column shows the average for three individual infections.

to cause cell lysis in comparison with wild-type *F. novicida*. With the levels of LDH released during wild-type infection normalized to 100%, the LDH released from infection with the five attenuated mutants ranged from 9.75% (FTT0742) to 24.52% (*fumA*). These results indicate that the attenuation phenotypes are not due to increased killing of host cells by the transposon mutants and that these strains are indeed compromised for intracellular replication.

Each *F. novicida* mutant harbors a single transposon insertion

Transposon mutagenesis has the potential to produce strains with more than one transposon insertion and thus multiple causes for an observed phenotype. Each of our 5 protective *F. novicida* transposon mutants were subjected to Southern blot analysis to ensure that the attenuation phenotypes of the mutant strains were the result of a single transposition event. To quantify the number of inserts, a DNA probe that spans a unique *Hin*dIII site in the transposon was designed such that digestion of chromosomal DNA harboring a single transposon insert would yield two targets for this probe. Chromosomal DNA from each of the five protective mutant strains, as well as wild-type U112 and a *Salmonella* strain known to contain a single copy of the transposon, was prepared and probed as indicated in the materials and methods section. The presence of two bands demonstrated that the *F. novicida* mutant strains each harbored a single copy of the transposon insert, as seen in Figure 2-4.

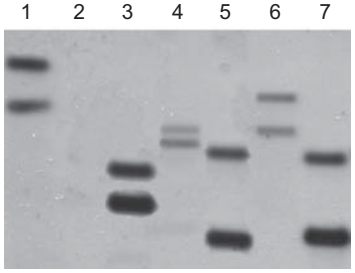

Figure 2-4. Each *F. novicida* transposon mutant harbors a single transposon. Chromosomal DNA preparations from a *Salmonella* strain known to carry the mini-Tn*5* cycler transposon (lane 1), wild-type U112 (lane 2), and the *dsbB* (lane 3), FTT0742 (lane 4), *pdpB* (lane 5), *fumA* (lane 6), and *carB* (lane 7) mutant strains were digested with HindIII. The DNA was transferred to a membrane and probed with a digoxigenin-labeled DNA probed that spanned a HindIII site in the transposon. The membrane was exposed to film for 2 min (lanes 1, 2, 3, 5, and 7) or 8 min (lanes 4 and 6). The presence of two bands indicates a singe transposon insertion event.

F. novicida mutants disseminate to the liver, spleen, and lungs and are subsequently cleared

Acceptable vaccine candidates ideally infect mice transiently and are cleared before challenge with the parent strain. We injected groups of 15 BALB/c mice (i.p.) with each mutant at 0.1 × LD_{50}. Thus, for these infections, the vaccination dose varied from one strain to another. Three mice from each group were sacrificed at 1, 3, 5, 7, and 28 days after vaccination, and their spleen, liver, and lungs were harvested. As can be seen in Figure 2-5, each mutant, with the possible exception of *carB*, disseminates to all three organs (spleen, liver, and lungs) from the original site of inoculation. Two of the five strains, *dsbB* and *fumA*, were completely cleared by day 28 following infection. Although a relatively low number of bacteria remained in the spleen at day 28 after infection with FTT0742 and *pdpB*, it is possible that these organisms would have cleared in the vaccination experiments because a lower dose (10 to 1000 fewer bacteria) was used for that procedure (Tables 2-2 and 2-4).

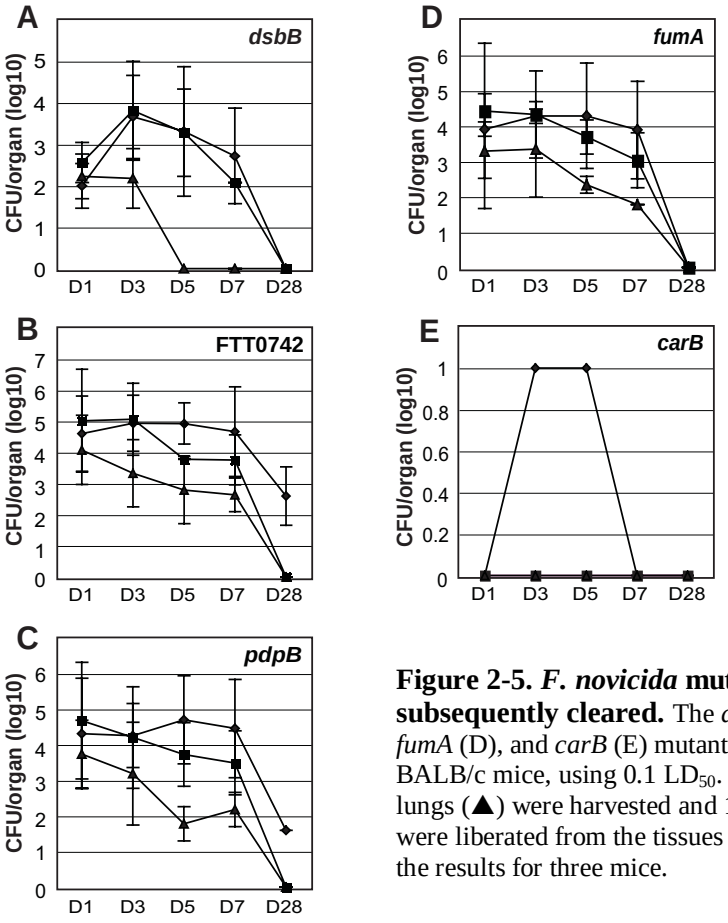

Figure 2-5. *F. novicida* mutants disseminate and are subsequently cleared. The *dsbB* (A), FTT0742 (B), *pdpB* (C), *fumA* (D), and *carB* (E) mutant derivatives were infected i.p. into BALB/c mice, using 0.1 LD_{50}. The spleen (◆), liver (■), and lungs (▲) were harvested and 1, 3, 5, 7, and 28 days p.i. Bacteria were liberated from the tissues and enumerated. Each point shows the results for three mice.

Expressing full-length genes *in trans* complements the attenuation phenotype

While the Southern hybridization experiments strongly indicated that each mutant derivative contained only a single transposon insertion, we wished to determine if cloned copies of the genes could complement the observed virulence defects. This would serve as additional evidence that the attenuation phenotype of each strain is a result of a single mutation, marked by the transposon insertion.

The *dsbB* gene was amplified from SCHU S4 DNA by PCR and cloned into the plasmid pKK202 (Kuoppa, Forsberg et al. 2001). Following transformation into the *dsbB* mutant, the abilities to replicate within macrophages and cause disease in mice were determined. As can be seen in Figure 2-6, *in trans* expression of the cloned *dsbB* gene provided nearly complete complementation of the virulence defect in three different cell types. Further analysis showed the LD_{50} value to be 60.25 CFU, which is comparable to the wild-type LD_{50} value of 66.25 CFU.

In a parallel experiment, *in trans* expression of the full-length FTT0742 gene in the respective mutant derivative resulted in incomplete complementation. In RAW cells, the complemented FTT0742 strain exhibited a 10-fold increase in intracellular replication over the mutant but was still nearly two orders of magnitude shy of the wild-type enumeration (Figure 2-6d).

Like *dsbB*, complementation of the *fumA* mutation with the full-length gene restored the level of intracellular growth to that of wild-type *F. novicida* (figure 2-6e). Taken together, these findings show that the observed attenuation phenotypes are due to mutations in *dsbB*, FTT0742, and *fumA*.

Complementation of *pdpB* will be attempted in the future, as the transposon insertion is located in the second gene of a 12-gene operon and is undoubtedly polar on expression of downstream genes. Based on its *in vitro* growth defect, comparatively low LD_{50}, relative lack of intracellular attenuation, and questionable dissemination patterns, we felt that the *carB* mutant was not a strong enough candidate to include in further development of a vaccine against tularemia and therefore did not attempt to complement the *carB* gene.

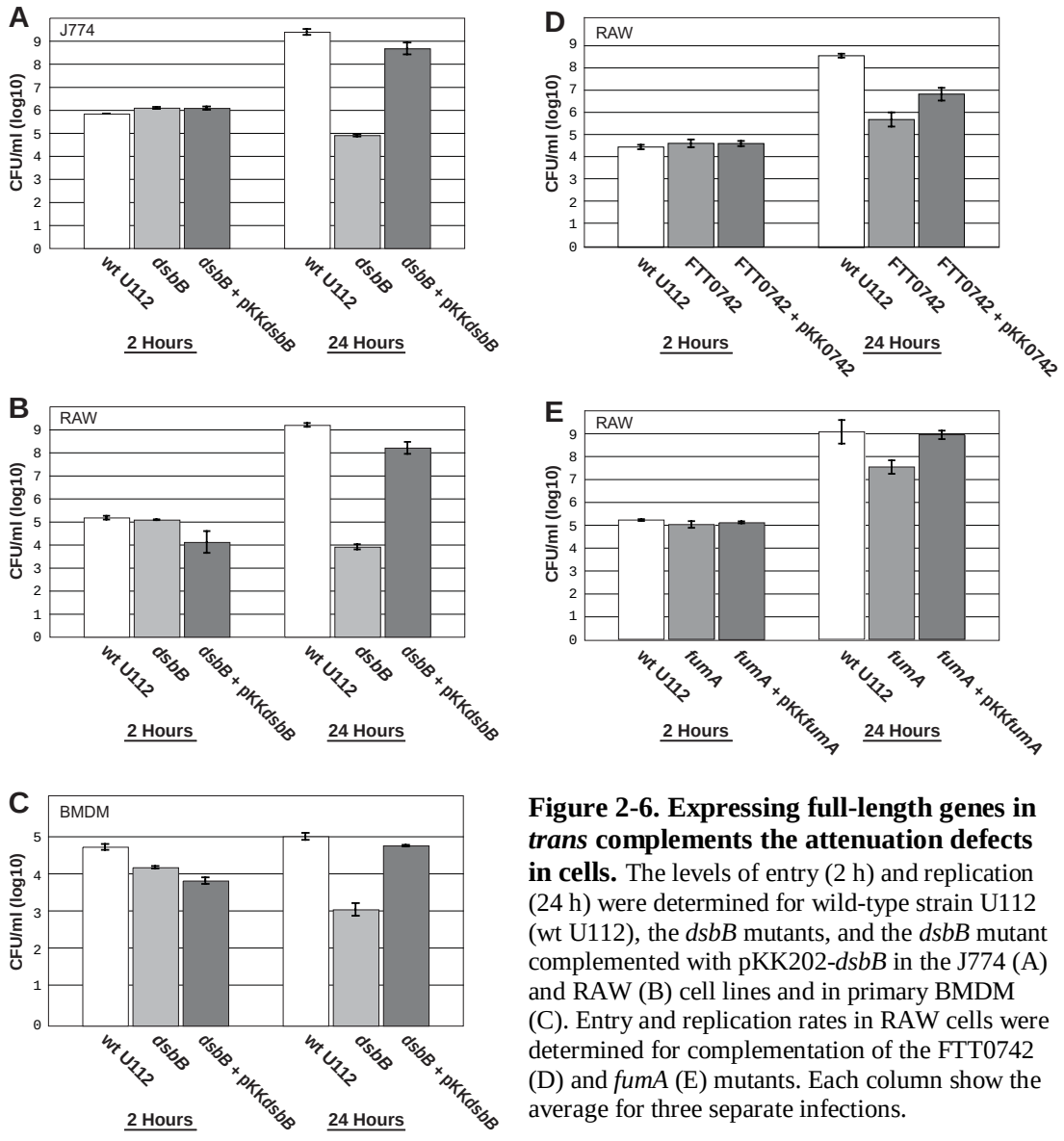

Figure 2-6. Expressing full-length genes in *trans* complements the attenuation defects in cells. The levels of entry (2 h) and replication (24 h) were determined for wild-type strain U112 (wt U112), the *dsbB* mutants, and the *dsbB* mutant complemented with pKK202-*dsbB* in the J774 (A) and RAW (B) cell lines and in primary BMDM (C). Entry and replication rates in RAW cells were determined for complementation of the FTT0742 (D) and *fumA* (E) mutants. Each column show the average for three separate infections.

Mutant strains protect mice against very high doses of wild-type bacteria

To further assess the level of protection afforded by the *F. novicida* transposon insertion mutants, we decided to challenge vaccinated mice with higher doses of the wild-type U112 parental strain. The *dsbB*, FTT0742, *pdpB*, and *fumA* mutant strains were used to infect groups of 5 mice with doses of 6×10^5, 6×10^6, and 6×10^7 CFU (see Table 2-4). Mice infected with each of the three doses of our FTT0742 and *pdpB* mutants had a 100% survival rate, as did the animals infected with the lowest doses of the *dsbB* and *fumA* mutants. Four weeks after vaccination, surviving animals were challenged with 6×10^7 CFU of wild-type *F. novicida* U112, which is approximately 1 million-fold the observed LD_{50} for wild-type infection. All of the challenged mice survived without any symptoms of tularemia. These results indicated that four of our *F. novicida* transposon mutants are capable of protecting mice against infection with very high levels of the wild-type organism. Overall, our findings indicate that *F. tularensis* strains carrying mutations in these genes are candidates for a vaccine against tularemia.

Table 2-4. Results of challenge studies after vaccination with *F. novicida* transposon mutants

Mutant strain	Vaccine dose (CFU)	% Survival (5 mice)	Challenge dose (CFU)	% Survival
dsbB	6×10^5	100	6×10^7	100
	6×10^6	20	6×10^7	100
	6×10^7	0	ND	ND
FTT0742	6×10^5	100	6×10^7	100
	6×10^6	100	6×10^7	100
	6×10^7	100	6×10^7	100
pdpB	6×10^5	100	6×10^7	100
	6×10^6	100	6×10^7	100
	6×10^7	100	6×10^7	100
fumA	6×10^5	100	6×10^7	100
	6×10^6	0	ND	ND
	6×10^7	0	ND	ND

[a] ND, not determined

Discussion

The categorization of *F. tularensis* as a Class A bioterrorism agent by the CDC demonstrates that this organism is widely acknowledged as a potential threat to national security. Thus, an immediate need exists for an approved tularemia vaccine. The lack of genetic tools with which to manipulate *F. tularensis* remains a great barrier to developing such a vaccine. Hence, we developed a transposon mutagenesis technique to create random insertions in the *F. novicida* genome and analyzed the resulting mutant strains for intracellular growth defects in macrophages, attenuation in mice, and the ability to confer protection against wild-type infection. We identified 28 *F. novicida* transposon mutants that have a defect in intracellular growth in macrophage cell lines, sixteen of which exhibited 100% attenuation in mice at greater than 100-fold the wild-type LD_{50}. Upon challenge with the wild-type organism, 5 transposon mutant strains were found to protect mice against infection with high doses of parental *F. novicida* U112. The disrupted genes in our 5 protective *F. novicida* mutants correspond to *dsbB*, FTT0742, *pdpB*, *fumA*, and *carB* in the *F. tularensis* strain SCHU S4.

Disulfide bond formation protein B is encoded by *dsbB*. This integral membrane protein is part of a pathway that leads to disulfide bond formation between cysteines in periplasmic proteins in *E. coli* and other bacteria (Kadokura, Katzen et al. 2003). The functional folded conformation of a protein often relies upon correct disulfide pairing of the cysteine residues. Thus, one explanation for why our *dsbB* mutant strain is attenuated is that a protein(s) required for replication inside host cells is not achieving its active conformation. It is also exciting to speculate that, with its potential influence on periplasmic proteins, the *dsbB* gene product may be involved in the secretion of virulence

factors, possibly to ensure correct folding of components of a secretion apparatus.

The ORF FTT0742 codes for a hypothetical lipoprotein that is predicted to have transmembrane regions. Therefore, it is possible that the gene product is a component of the *F. novicida* cell wall and/or may be involved in molecule transport. Because *in vitro* growth and entry into the host cell were not compromised, we can speculate that FTT0742 affects a function necessary for virulence and growth inside macrophages. Characterization of the FTT0742 protein will further clarify its role in virulence.

The gene product of *pdpB* is an uncharacterized protein encoded on the FPI that exhibits some similarity to the conserved bacterial protein IcmF (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). It has been shown that *icmF* is required for *Legionella pneumophila* intracellular growth, so we may hypothesize that *pdpB* plays a similar role in *F. novicida* intracellular growth (Zusman, Feldman et al. 2004). Also of note, this gene displays some similarity to the rhoptry proteins of the parasite *Plasmodium*, which mediate attachment to host red blood cells (Sam-Yellowe 1992). Although *pdpB* mutant did not display defects during *in vitro* growth, it was significantly compromised in the ability to enter host cells and displayed prominent intracellular attenuation in that no colony forming units were detected in the lysates of RAW and J774 cells at 24 hours p.i. Because *pdpB* is the second ORF in a 12-gene operon, it is likely that the tranposon insert in this gene has polar effects on downstream genes. These findings indicate that *pdpB* or other genes in the *pdp* operon are needed for both entry into and replication within host cells. As yet, the function of each gene in the *pdp* operon remains to be elucidated.

Fumarate hydratase A, the component of the Kreb's cycle (citric acid cycle/CAC)

that converts fumarate to malate, is encoded by *fumA* (Tseng, Yu et al. 2001). The CAC is one of the three metabolic pathways of cellular respiration and is necessary for fuel catabolism and ATP production. Precursory molecule for compounds such as amino acids are also generated by the CAC. Thus, the observation that our *fumA* mutant expressed lower levels of *in vitro* replication, in comparison to wild-type U112, may be a result of energy deficiency or a lack of molecules needed for replication.

The *carB* gene encodes the large subunit of heterodimeric enzyme carbamoyl phosphate synthase, which is required for pyrimidine biosynthesis (Koonin and Galperin 2003). As pyrimidines are absolutely required for replication, it is clear that a mutation in this biosynthesis pathway would lead to both *in vitro* and intracellular growth defects. Indeed, we did observe an *in vitro* growth defect with this mutant strain. Nevertheless, our *carB* mutant was able to infect macrophages and protect mice against challenges with the wild-type organism, which suggests that mutations in the pyrimidine pathways of *F. novicida* have some merit as potential vaccine strains. However, in comparison to the other *F. novicida* mutants in this study, the *carB* mutant derivative had a lower LD_{50}, a lesser degree of intracellular attenuation, and did not appear to disseminate from the initial site of infection. For these reasons, we have decided not to pursue this mutant as a possible vaccine candidate.

Although we saw only partial complementation of the FTT0742 mutant phenotype, we still consider this strain to be a vaccine candidate. Similar complementation experiments to those described in this work, carried out in *Salmonella*, have also met with mixed success because the copy number of the plasmid, as well as the regulation of the gene itself, can influence complementation. In fact, the Forsberg group

recently demonstrated the importance of correct gene regulation during complementation in *Francisella* with their studies on *pilA*: functional complementation was achieved *in cis* but expression of PilA was barely detectable in the strain complemented *in trans* (Forslund, Kuoppa et al. 2006). The situation is further compounded in our studies because the *F. novicida* genome is not yet published; therefore, the genes used for complementation were amplified from SCHU S4 DNA and may be incompatible with *F. novicida* due to variations between the subspecies.

Intriguingly, another finding of this study was the lack of protection conferred by *pur* mutants in a murine model. It has been previously postulated that mutations affecting the *F. tularensis* purine systhesis pathway could be used to generate a live attenuated tularemia vaccine (Karlsson, Prior et al. 2000). In fact, defined allelic replacement mutants disrupting this pathway have been used to produce vaccine strains attenuated for replication in host cells in a variety of other bacterial species (Oyston and Quarry 2005). Our *F. novicida* transposon library contained 8 unique *pur* mutants: *purA*, a *purCD* fusion (2 strains), *purL* (2 strains), and *purM* (3 strains). Each of these strains exhibited 100% attenuation in mice with 6×10^3 CFU, yet all of them failed to protect against a wild-type parental challenge with 8×10^5 CFU. Although these results do not necessarily preclude purine biosynthesis mutants as potential live vaccines, we found it noteworthy that these mutants did not protect against challenge with wild-type *F. novicida* U112.

As an alternative to live vaccines, the viability of developing a sub-unit vaccine must also be examined. Studies evaluating the efficacy of whole killed cells as a crude tularemia vaccination demonstrated the feasibility of a sub-unit vaccine and prompted research in the area of identifying antigens that induce protective immunity (Coriell 1948;

Kadull 1950). Although several polysaccharide and protein components of *F. tularensis* have been shown to react with convalescent sera or T cells (Isherwood, Titball et al. 2005), the only antigen that has demonstrated the ability to induce a protective immune response against tularemia is lipopolysaccharide (LPS) (Oyston and Quarry 2005). However, this protection was only effective against *holarctica* strains and was incomplete (Fulop, Manchee et al. 1995; Fulop, Mastroeni et al. 2001; Conlan, Shen et al. 2002). Protection against a highly virulent *F. tularensis* type A strain likely depends on a Th1-mediated cellular immune response (Tarnvik 1989). As yet, there is no means of administering antigens or killed bacteria that is as effective as a living attenuated vaccine — a point that has been highlighted by work from the Pamer laboratory showing that the immune system distinguishes between living and dead bacteria (Pamer 2004).

In contrast, a live attenuated vaccine would be effective in inducing the appropriate cellular responses (Oyston and Quarry 2005). In fact, the type B LVS strain provides the only current means of tularemia vaccination. However, several limitations prevent the licensing of this vaccine. Foremost of these, the genetic basis of LVS attenuation and protection remains unknown. Secondly, culturing LVS under certain conditions can lead to poorly immunogenic colony variants, which demonstrates this organism's inherent genetic instability (Eigelsbach and Downs 1961; Cowley, Myltseva et al. 1996). Also, this vaccine does not confer protection to every vaccinated individual (McCrumb 1961; Saslaw, Eigelsbach et al. 1961). Finally, LVS protection against aerosol challenge is variable and depends on the route of immunization as well as the host (Chen, Shen et al. 2003; Chen, KuoLee et al. 2004; Shen, Chen et al. 2004; Conlan, Shen et al. 2005). This last point is especially critical when considering *F. tularensis* as a biological

weapon, as aerosol dispersal is the most likely route of delivery. Taken together, these limitations clearly show that the development of an approved tularemia vaccine requires the development of a rationally attenuated, non-reverting live vaccine strain.

While this manuscript was in preparation, the Sjöstedt group published the first report of a defined gene deletion mutant of a type A strain that protected mice against challenge with the wild-type organism (Twine, Bystrom et al. 2005). Indeed, the ideal live vaccine strain would be derived from a virulent *F. tularensis* strain; however, we used *F. novicida* as a model for preliminary analysis of potential tularemia vaccines because this subspecies is more amenable to genetic manipulation without the danger of infection. Furthermore, because all of the *Francisella* subspecies are closely related (Broekhuijsen, Larsson et al. 2003), genes necessary for intracellular growth in *F. novicida* are likely to have the same function in *F. tularensis*. Consequently, *F. novicida* strains afford researchers a relatively safe, genetically significant organism with which to conduct exploratory investigations prior to studies with the more virulent *F. tularensis* subspecies.

Here, we report the discovery of five *F. novicida* transposon mutants that exhibit attenuation in macrophages and are capable of protecting mice against infection with the wild-type parental strain at doses of up to one million times the observed wild-type LD_{50}. An approved tularemia vaccine will need to be a highly attenuated non-reverting derivative of a type A strain, as these are most likely to be used in a bioterrorism attack and there is no certainty that one subspecies will protect against another. Accordingly, we will now focus on extending this work into *Francisella* type A strains by creating deletions of each of the genes identified and assaying for virulence in a mouse model.

Materials and Methods

Bacterial strains and culture

F. novicida strain U112 was a kind gift from Dr. Fran Nano (University of Victoria). All *Francisella* strains were cultured at 37ºC in tryptic soy broth supplemented with 0.1% cysteine (TSBC; Becton, Dickinson and Company [BD], Sparks, MD) or on cysteine heart agar (CHA; Difco/BD) plates. Kanamycin was added to a final concentration of 20 µg/ml to these media for selection of U112 strains carrying the transposon (TSBC/Kan20 or CHA/Kan20). *E. coli* Genehogs (Invitrogen, Carlsbad, CA) used in subcloning for the purpose of sequencing were transformed, according to manufacturer's directions. Colonies containing the transposon were selected by growth at 37ºC on LB plates containing 60 µg/ml kanamycin (LB/Kan60). The *Salmonella* strain used in the Southern blot was grown in Luria-Bertani (LB) broth and on LB plates at 37ºC.

Generation of bacterial transposon mutant strains

A library of *F. novicida* transposon insertion mutants was created by electroporating mini-Tn5 transposon/transposase complexes into appropriately prepared *F. novicida.* Although independently developed, our technique was similar to that of Kawula, *et al.* (Tempel 2003; Kawula, Hall et al. 2004, Tempel, 2003 #54). The mini-Tn5-cycler transposon was constructed as previously described (Geddes, Worley et al. 2005). The transposon/transposase complex was prepared as described by Goryshin *et al.* (Goryshin, Jendrisak et al. 2000). *F. novicida* U112 were grown to confluency on CHA plates at 37ºC and resuspended with 5 ml of ice-cold 10% glycerol/500mM sucrose

buffer. Aliquots of 1 ml were transferred to 1.5 ml microcentrifuge tubes, pelleted at 12K × g for 5 min at 4ºC, and resuspended in 1 ml of buffer. This wash step was repeated for a total of 4 washes. After the final wash, each aliquot was resuspended in 100µl buffer. One microliter of transposon/transposase complex was added to each tube, and the samples were electroporated in 1 mm gap cuvettes at 1.5 to 1.7 kV, 200 Ω, 25 µF. The bacteria were recovered in 1 ml TSBC in glass tubes for 4 hours in a 37ºC rotator and plated on CHA/Kan20 plates. The frequency of isolating transposon insertion mutants was relatively low (about 10-100 insertions per 10^9 cells following electroporation).

Culture and infection of cell lines and primary macrophages

The J774A.1 and RAW264.7 murine macrophage cell lines (American Type Culture Collection [ATCC], Manassas, VA) were cultured in Dulbecco's modification of Eagle's medium (DMEM; Gibco-BRL, Rockville, MD) supplemented with 10% fetal bovine serum (FBS; Gibco-BRL), 1mM non-essential amino acids (Gibco-BRL), and 0.2mM sodium pyruvate (Gibco-BRL) at 37ºC with 5% CO_2. Bone marrow-derived macrophages (BMDM) were collected by flushing the femurs of BALB/c mice with serum-free DMEM and cultured in DMEM supplemented with 20% L929 and pen-strep. For infections, bacteria were added to 50% confluent cells in 24- or 96-well culture dishes (Corning, Corning, NY) or 4-chamber microscope plates (Nalge Nunc, Naperville, IL) at the indicated multiplicity of infection (MOI), and the cells were centrifuged at 1K × g for 5 min at room temperature and incubated at 37ºC with 5% CO_2. One hour post-infection, the cells were washed twice with phosphate-buffered saline (PBS), and DMEM containing 100 µg/ml of gentimicin was added to prevent the growth of any extracellular

bacteria (Kudelina 1978). Two hours post-infection, cells were washed twice with PBS and either lysed or incubated in the presence of 10 µg/ml gentimicin for an additional 22 hours. Cells were lysed with TSBC containing 0.5% saponin (Sigma) for 30 min at 37ºC with 5% CO_2.

Screening for reduced growth in macrophages

RAW macrophages were seeded to 50% confluency in 96-well tissue culture plates and infected (MOI ≈ 1000) with overnight cultures of *F. novicida* mutant strains that were grown in stationary 96-well tissue culture plates, as described above. At 24 h post-infection, the macrophages were washed and lysed as described. Three percent of each lysate was plated onto CHA/Kan20 plates and incubated overnight at 37ºC. Mutants that exhibited growth defects were identified visually. To eliminate false positives, the potentially attenuated mutants were subjected to another round of selection by infecting RAW macrophages in 24-well plates, as above, with an input MOI of 100, which corresponds to about 1 bacterium per macrophage. After lysing, 50 µl of each lysate was plated onto CHA plates and incubated overnight at 37ºC. *F. novicida* mutants compromised for growth in macrophages were identified visually by comparison to wild-type U112 infection lysates, with the attenuated mutants yielding individual colonies while the wild-type bacteria grew to confluency.

Sequencing of mini-Tn-5 insertion sites and sequence analysis

The method described by Geddes, *et al*. was followed (Geddes, Worley et al. 2005). Briefly, chromosomal DNA from *F. novicida* mutants exhibiting reduced growth

in macrophages was prepared (Ausubel 2002), digested with *Eco*RI, and subcloned into pACYC184. Ligation reactions were electroporated into GeneHogs *E. coli* cells (Invitrogen) and selected for growth on LB/Kan60. Plasmids from kanamycin-resistant colonies were purified according to the manufacturer's instructions using the QIAprep Spin miniprep kit (Qiagen, Valencia, CA). The DNA sequence of the fusion junction was obtained using a primer complementary to base pairs 166-190 of the 5' end of mini-Tn5-cycler (5' GTTGACCAGGCGGAACATCAATGTG 3'). Sequence analysis was performed using MacVector^{TM} 7.2.3 software and the NCBI Blast server at http://www.ncbi.nlm.nih.gov/BLAST/.

Mouse studies

Six- to 8-week old female BALB/c mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Animals were fed autoclaved food and water *ad libitum*. All experiments were performed in accordance with Animal Care and Use Committee guidelines. For vaccination and challenge studies, mice were injected intraperitoneally (i.p.) with bacteria in a total volume of 150 µl PBS. Mice were vaccinated with the indicated colony forming units (CFU). Surviving mice were challenged 28 days later with the indicated doses. Dissemination and clearance of the bacteria were determined by harvesting the lung, liver, and speen at the indicated days post-infection, homogenizing the organs with a stomacher, and plating serial dilutions. The 50% lethal dose (LD_{50}) values were calculated according to the method of Reed and Muench (Reed 1935). Mice were checked for signs of illness or death twice each day following infection.

Bacterial growth curves in liquid media

Overnight cultures of *F. novicida* were diluted into 10 ml of TSBC to an optical density at 600 nm (OD_{600}) of 0.1. Optical density readings were then recorded at the specified timepoints. It should be noted that the cultures were diluted 1:10 for OD_{600} >1 for accuracy. We previously determined by plating that OD_{600} $1 \approx 4 \times 10^9$ bacteria/ml.

Quantification of bacterial entry and growth in macrophages

J774 and RAW cell lines, and BMDM were seeded in triplicate to 50% confluency in 24-well tissue culture plates and infected as described with *F. novicida* mutant strains at an input MOI of 100. Cells were lysed at 2 or 24 hours p.i. Serial dilutions of the lysates were plated onto CHA/Kan20 or CHA (wild-type and mock infection controls) plates. After overnight incubation at 37ºC, the colonies on each plate were counted. Means and standard deviations were calculated using Microsoft® Excel X for Mac®. The 24 h data were statistically analyzed by paired, two-tailed t-tests using Microsoft® Excel X for Mac®.

Southern blot analysis

F. novicida chromosomal DNA was prepared using the CTAB (cetyltrimethylammonium bromide) method (Ausubel 2002), and 250 ng of each preparation were digested to completion with *Hin*dIII. Digested DNA was run on a 0.8% agarose gel for 2 h at 90 kV and then transferred to a positively charged nylon membrane (Roche) using a standard capillary transfer method (Ausubel 2002). DNA was crosslinked to the membrane at 120,000 µjoules/cm^2 using the Stratalinker 1800 UV

Crosslinker (Stratagene, La Jolla, CA). The digested bacterial DNA was probed with a digoxigenin (DIG) labeled probe using the DIG High Prime DNA Labeling and Detection Starter Kit II (Roche, Indianapolis, IN), and the membrane was exposed to film (Kodak, Rochester, NY) for 2 or 8 minutes as indicated. By using a DNA probe that spans a *Hin*dIII site in the transposon and hybridizes to two separate locations of the *Hin*dIII-digested chromosomal DNA, we were able to determine the number of transposon inserts in each strain.

Cytotoxicity assay

A cytotoxicity assay was conducted as in van der Velden, *et al.* (van der Velden, Lindgren et al. 2000). Briefly, J774 cells seeded in 96-well culture plates were infected in triplicate with either the transposon mutants or wild-type *F. novicida* U112 at an input MOI of 100. After 48 h, the supernatants were removed and assayed for released lactate dehydrogenase (LDH) using the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI). Cytotoxicity was determined for each mutant strain by calculating the LDH released as a percentage of the maximal release from macrophages infected with wild-type U112.

Microscopy

J774 cells were infected at an input MOI of 100, as previously described, in 4-well chamber plates (Nalge Nunc). After 24h, the cells were washed twice with PBS, fixed for 1 hr with 4% paraformaldehyde, and stored in PBS at 4ºC. After washing 3 × 10 min in PBS, the cells were permeabilized with 0.5% Triton X-100 (Sigma Chemical) in

PBS for 20 min at room temperature, blocked with 5% FBS in PBS for 30 minutes, and incubated for one hour at 4ºC with a polyclonal antibody against *F. tularensis* (BD). After washing 3 × 10 minutes in PBS, the cells were again blocked with 5% FBS. A goat-anti-rabbit antibody conjugated to Alexa 488 (Molecular Probes, Eugene, OR) was applied to the cells overnight at 4ºC. The cells were once again washed 3 × 10 min in PBS and incubated with a 1:1000 dilution of FM 4-64 membrane stain (Molecular Probes) and 1:000 Draq5 DNA stain in PBS (Alexis Biochemicals, San Diego, CA) for 10 min at room temperature. The cells were washed twice with PBS, mounted in Fluormount-G® antifade solution (Southern Biotechnology, Birmingham, AL), and imaged with an Applied Precision DeltaVision Deconvolution microscope system (Advanced Precision Instruments, Issaquah, Wa.). All images were taken using a 60× objective. Stacks of 10 Z plane images spaced 1 micron apart were captured at 1024 × 1024 pixels and deconvolved for 7 iterations. Selected images were saved in TIFF format and imported into Adobe Photoshop to be formatted for publication.

Complementation of disrupted genes

The plasmid pKK202 (Kuoppa, Forsberg et al. 2001) was modified to include unique *Not*I, *Sfi*I, and *Xho*I restriction sites by digestion with *Cla*I and *Xba*I, followed by ligation with a DNA fragment. Oligos CGGCGGCCGCTTGGCCTCGAGGGCC and CTAGGGCCCTCGAGGCCAAGCGGCCGC were annealed to yield a double-stranded product encoding the new restriction sites. Using SCHU S4 DNA as a template, full-length genes were amplified via PCR. The *dsbB* gene was cloned using the primers GCGGCCGCCTTCTTAACGTCCACAGTTTTGTCC and

GGCCCTCGAGGCCCTTTCTGATGGTTTGTCATTTCTCC, FTT0742 was cloned using primers GCGGCCGCGCAGCATTACCTGGAATTACAAG and GGCCCTCGAGGCCCAAACAGCAAATAAATATACAACACC, and *fumA* was cloned using primers GCGGCCGCTAGTGATAAAATTAGCGAGG and GGCCCTCGAGGCCATTAACTATAATGCCGAG. The modified pKK202 vector and the PCR products were digested with *Not*I and *Xho*I and ligated. pKK202-*dsbB* was electroporated into the *dsbB* mutant *F. novicida* strain and used to infect J774 and RAW cell lines, primary macrophages, and mice as indicated. pKK202-FTT0742 and pKK202-*fumA* were similarly tested in RAW cells.

Acknowledgements

We thank the OHSU Core Facility and Aurelie Snyder for sequencing and microscopy assistance. We would also like to acknowledge Jeff Vandehey and Chris Langford for technical assistance. We are grateful to Dr. Fran Nano for the *Francisella novicida* U112 strain. The mini-Tn5-cycler transposon was a kind gift from Kaoru Geddes. We appreciate the helpful comments of Dr. Jean Gustin for revision of the manuscript. This work was supported by NIH R21 grant number EB000985 to FH, as well as the National Science Foundation Graduate Research Fellowship and the OHSU Tartar Trust Fellowship, both to RT.

Chapter 3

Manuscript 2: Characterization of a novel *Francisella* outer membrane protein family that contains a highly stable repeat domain with implications for pathogen-host cell interaction

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Manuscript in preparation

Preface

This work is currently in preparation by Rebecca Tempel, Jason Burkhead, Fred Heffron, and Ujwal Shinde.

My contributions to the manuscript include experimental design, performing sucrose density gradient ultracentrifugation and Western analysis of *F. novicida* membrane proteins, cloning to create *F. novicida patB* complementation strain and infections to show complementation in cell culture, expression and purification of the TAARP domain, circular dichroism spectroscopy, intrinsic fluorescence spectroscopy, denaturation of the TAARP domain with urea, acrylamide quenching, gel filtration analysis, performing the co-purification assay, analyzing data, and preparing several figures and the manuscript.

Jason Burkhead contributed to this work by suggesting experiments, participating in experimental design, providing advice and instruction for the circular dichroism spectrometer, performing gel filtration analysis and intrinsic fluorescence spectroscopy, and analyzing data.

Fred Heffron contributed to this study by providing advice and suggestions on experimental design and results, as well as the space, equipment, and supplies with which to perform the experiments.

Ujwal Shinde contributed to this manuscript by providing expert advice on experimental design, performing molecular modeling and clustal alignments of the TAARP and EAP domains, fitting the CD and IF data for the melting curve of TAARP, preparing figures 3-5, 3-6, 3-7, 3-10, and 3-11, analyzing results, and providing equipment and supplies with which to perform the experiments.

Summary

In a previous study, we identified an uncharacterized ORF (FTT0742/FTN0715) whose mutant derivative was highly attenuated and protective in a mouse model of tularemia. Genes with high similarity to FTN0715 are present in all sequenced strains of *Francisella* currently available. Based on the attenuated phenotype of the FTN0715 mutant in previous studies, we designated these genes "*pat*" for pathogenicity factor. Proteins in the Pat family are predicted to be lipoproteins, and two *F. novicida* homologs purified with the outer membrane fraction during sucrose density gradient ultracentrifugation. Intriguingly, Pat homologs all contain one, two, or seven copies of an internal 102 aa domain. In this study, we investigate the structure and function of this 102 amino acid domain, designated TAARP for Tandem Amino Acid RePeat, for its potential role in pathogenicity. We provide evidence that the repeat motif is highly stable, independently folded, and forms a homo-oligomer. Furthermore, we show that the TAARP domain has sequence and structural homology to the EAP repeat domain of the extracellular adhesion protein (Eap) of *Staphylococcus aureus*. Lastly, TAARP directly binds host factors in the E-cadherin complex *in vitro*, suggesting a role in *Francisella* entry and intracellular survival.

Introduction

Francisella tularensis is a highly virulent gram-negative bacterium that causes tularemia, a serious and sometimes deadly disease. Tularemia is contracted through numerous routes, the most dangerous of which is inhalation of as few as 10 aerosolized bacteria (Dennis, Inglesby et al. 2001; Oyston, Sjostedt et al. 2004). Due to its high infectivity and ease of aerosol transmission, *F. tularensis* has been modified as a biological weapon and is classified by the CDC as a Category A select agent, which can pose a serious threat to national security (Oyston, Sjostedt et al. 2004). In addition to its potential use in bioterrorism, *F. tularensis* is found throughout the Northern Hemisphere, and the incidence of tularemia is especially high in farmers and hunters in Scandinavian countries (Sjostedt 2007). Despite these factors, there is currently no approved tularemia vaccine available. Hence, the identification of potential virulence factors and protective antigens remains a primary focus of *Francisella* research.

Four main subspecies of *F. tularensis* are commonly recognized: *tularensis* (type A), *holarctica* (type B), *novicida*, and *mediasiatica*. All of these biotypes share greater than 95% DNA sequence identity (Broekhuijsen, Larsson et al. 2003). While both type A and type B strains are considered infectious for humans, only type A strains cause significant mortality (Gill and Cunha 1997). The only current means of protection against infection with *Francisella* is vaccination with the attenuated type B live vaccine strain (LVS). However, because the molecular basis for LVS attenuation remains unknown, this strain is not licensed as a tularemia vaccine. While *F. novicida* is not considered a human pathogen, it causes a tularemia-like disease in mice and hence provides an excellent

alternative to highly virulent type A and B strains for research using a mouse model system (Kieffer, Cowley et al. 2003).

Virulence is often mediated through bacterial outer membrane proteins (OMPs), which act at the host-pathogen interface and are significant contributors towards host cell invasion, immune evasion, and intracellular survival. Many OMPs have antigenic properties and are potentially useful as protective vaccines for several bacterial diseases (Udhayakumar and Muthukkaruppan 1987; Murphy, Kirkham et al. 1999; Pal, Peterson et al. 2005; Pillai, Howell et al. 2005; Huntley, Conley et al. 2008). Despite the high degree of virulence of *Francisella*, little is known about the OMPs and the role these proteins play in the pathogenesis of tularemia. The further identification and characterization of OMPs can provide clues about the mechanisms of pathogenesis, as well as information to improve medical diagnostic tools and environmental detection of *Francisella*.

Recent strategies for the characterization of *Francisella* OMPs have combined bioinformatics techniques to predict OMP-targeting motifs and proteomics techniques to directly identify *Francisella* OMPs (Huntley, Conley et al. 2007). Our previous efforts to discover *F. novicida* transposon mutants that were attenuated and protective against subsequent challenge with the wild-type strain in a mouse model of tularemia revealed an uncharacterized ORF (FTN0715, published as the ORF corresponding to FTT0742), that contained a putative lipobox domain in the N-terminal region (Tempel, Lai et al. 2006). The presence of the lipobox domain is highly suggestive of a membrane location (Madan Babu and Sankaran 2002). This gene has homologs within all *Francisella* biotypes and is

unique to *Francisella* species. Because of its previously defined role in virulence, we designated the protein Pat for "Pathogenicity factor".

This work demonstrates that the full-length FTN0715 (PatB) and FTN0714 (PatC) proteins are localized to the outer membrane of *F. novicida.* An informatics based analysis indicates that both PatB and PatC sequences in the *F. novicida* homolog contain seven Tandem 102 Amino Acid RePeat domains (TAARP domains), while Pat family members from the human pathogenic *Francisella* strains contain either one or two copies of the TAARP domain. The TAARP domain is a novel sequence with little sequence identity with all other proteins from the SwissProt Database. Our detailed biophysical and biochemical studies on the recombinant TAARP domain from the type A *F. tularensis* Schu S4 strain demonstrate that the TAARP domain adopts a stable independent fold. The TAARP domain contains significant β-sheets with evidence for stability through an aromatic cluster, which also plays a role in protein oligomerization. *Ab initio* based modeling using Rosetta provides a structure that is consistent with the solution structure of the TAARP domain. Structural comparison of the TAARP domain with structures from the PDB database suggest that the TAARP domain adopts a fold similar to the EAP domains found in Eap (Extracellular adherence protein), a virulence factor from *Staphylococcus aureus* that also displays a modular architecture. A preliminary screen for TAARP-interacting mammalian proteins suggests that the TAARP domain may interact with components of the E-cadherin pathway, providing a possible mechanism through which *F. tularensis* may infect host cells.

Results

Identification of a novel virulence factor family in *Francisella*

In a previous study, we identified four *F. novicida* genes whose mutant derivatives were attenuated and protective against wild-type challenge with the parental strain in a mouse model (Tempel, Lai et al. 2006). One of the insertions occurred in an uncharacterized open reading frame; however, because only the *F. tularensis* ssp. *tularensis* Schu S4 sequence was available at the time of the study, the corresponding *F. novicida* transposon mutant was designated as the homolog of FTT0742 (Larsson, Oyston et al. 2005). Subsequent availability of the *F. novicida* U112 sequence showed that the transposon insertion occurred in FTN0715 (Rohmer, Fong et al. 2007). Comparison of FTT0742 to FTN0715 revealed that the two ORFs are closely related, with the exception of seven tandem repeat domains of 102 amino acids in the central region of FTN0715 that is present as a single domain in FTT0742 (Figure 3-1). Further analysis resulted in the discovery of an upstream adjacent homolog in *F. novicida*, FTN0714, as well as homologs in other *Francisella* subspecies. Interestingly, homologs in the type B *holarctica* subspecies (LVS, OSU18, and FTNF002-00) were

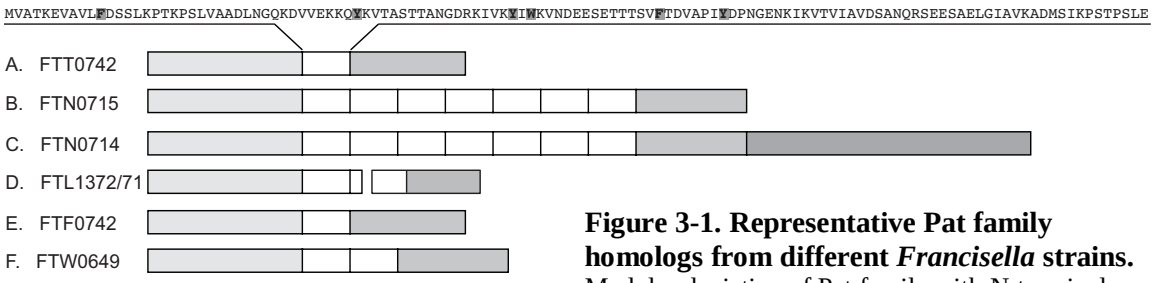


Figure 3-1. Representative Pat family homologs from different *Francisella* strains. Modular depiction of Pat family with N-terminal regions in light gray, central repeat domains in white, C-terminal domains in darker gray. A. FTT0742 from *F. tularenis* ssp. *tularensis* strain Schu S4, 662 a.a.; B. FTN0715 from *F. tularensis* ssp. *novicida* strain U112, 1256 a.a.; C. FTN0714 from *F. tularensis* ssp. *novicida* strain U112, 1852 a.a.; D. FTL1372/1371 from *F. tularensis* ssp. *holarctica* strain LVS, 449/287 a.a. in complement orientation; E. FTF0742 from *F. tularenis* ssp. *tularensis* strain FSC198, 662 a.a.; F. FTW0649 from *F. tularenis* ssp. *tularensis* strain WY96-3418, 756 a.a. The sequence for the Schu S4 repeat domain is shown.

annotated as comprising two open reading frames, as represented by the depiction of LVS in Figure 3-1. Because of the high attenuation and protection seen in the *F. novicida* mutant derivative, we designated this protein family "Pat" for Pathogencity factor. In strains having only one homolog, the protein is PatA; in *F. novicida*, FTN0715 is PatB and FTN0714 is PatC. The repeat motifs were termed TAARP domains for Tandem Amino Acid RePeat.

Based on the multiple TAARP motifs present in Pat homologs in *F. novicida* strain U112 and *F. tularensis* strain WY98-3416, proteins in the Pat family can be divided into three domains: an N-terminal region of 311 to 344 amino acids, the central region with one or more repeat motifs, and a C-terminal domain that varies in length. With the exception of a consensus lipobox sequence, the N-terminal domains did not exhibit homology to any previously described proteins or motifs by BLAST analysis. Among the *Francisella* subspecies, residues in these regions displayed >95% sequence identity. As with the N-terminal regions, BLAST analysis of the TAARP domains indicated that these regions are also unique to *Francisella*, as there were no similarities to other annotated proteins. In the *F. novicida* Pat homologs with seven TAARP domains, these regions are >95% homologous to one another. However, when comparing the TAARP domain among all Pat family members, the level of homology ranges from 65% to 95% at the amino acid level. The C-terminal regions of the *F. novicida* Pat homologs share 39% homology with the *F. tularensis* and *F. holarctica* C-terminal regions, which appear unrelated to any known proteins. The C-terminal region of FTN0714, or PatC, contains an additional ~600 amino acids of unknown function that are not found in any of the other Pat homologs.

F. novicida PatB and PatC localize to the outer membrane fraction

The Pat family proteins are annotated as hypothetical lipoproteins and are shown to contain a consensus LAGC lipobox domain in the N-terminal region by analysis with the Database of Bacterial Lipoproteins (DOLOP). Additionally, *F. novicida* PatB and PatC are predicted to localize to the outer membrane (OM) by the computational program PSORTb with scores of 9.52 and 9.83 on a 10.00 scale, respectively. For these reasons, it was hypothesized that Pat family proteins would localize to the OM of *Francisella.* To investigate the location of PatB and PatC in *F. novicida*, sucrose density gradient ultracentrifugation was performed using the modifications described for *Francisella* by the Norgard group (Huntley, Conley et al. 2007). Cells were grown to early log phase to avoid membrane blebbing, and membrane preparations were prepared by spheroplasting followed by osmotic lysis. The membranes were separated by ultracentrifugation in a discontinuous sucrose gradient ranging from 55% to 25% in steps of 5%. Western blot analysis with a polyclonal antibody against Pat revealed that both PatB and PatC localize to the outer membrane of *Francisella* (Figure 3-2). Antibodies against known *Francisella* outer (FopA) and inner (SecY) membrane proteins were used as controls (Huntley, Conley et al. 2007).

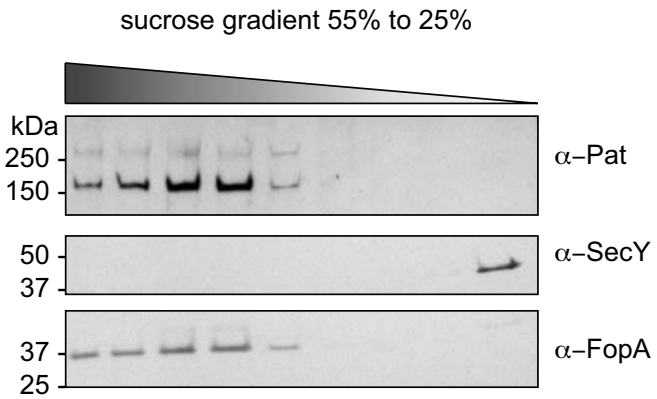


Figure 3-2. Outer membrane localization of *F. novicida* Pat homologs. Western blot analysis of sucrose density gradient fractions. Membrane fractions of *F. novicida* were separated and collected as explained in Materials and Methods. Proteins were separated by SDS-PAGE on a pre-cast gradient gel (4%-20%), transferred to PVDF membrane, and probed with antibodies against Pat, SecY (inner membrane control), or FopA (outer membrane control). PatB (~175 kDa) and PatC (~275 kDa) were observed in the same fractions as FopA, indicating that they localize to the outer membrane of *Francisella.*

Expression of full-length *F. novicida* PatA *in trans* complements an attenuating mutation in the corresponding gene

In a previous study, a *F. novicida* PatB transposon mutant was shown to be attenuated and protective against wild-type challenge in mice. Expression of *F. tularensis* PatA *in trans* in the *patB*::Tn background partially restored intracellular survival to that of wild-type levels in cell culture (Tempel, Lai et al. 2006). To investigate if the differences between Pat homologs were responsible for the incomplete complementation, we conducted the experiment with *F. tularensis* PatA and *F. novicida* PatB in parallel. As in the previous study, *F. novicida patB* was amplified from genomic DNA by PCR and cloned into plasmid pKK202 for expression in the *F. novicida patB* mutant. RAW macrophage-like cells were infected with wild-type *F. novicida* U112, *patB*::Tn, *patB*::Tn + p*FTTpatA*, or *patB*::Tn + p*FTNpatB*, and entry and intracellular replication levels were determined by CFU counts (Figure 3-3). Comparison of the ratio of entry to survival at 24 hours post-infection shows that expression of the strain-appropriate Pat homolog restores intracellular replication to that of wild-type levels, indicating that differences among the Pat family members may affect their roles in virulence.

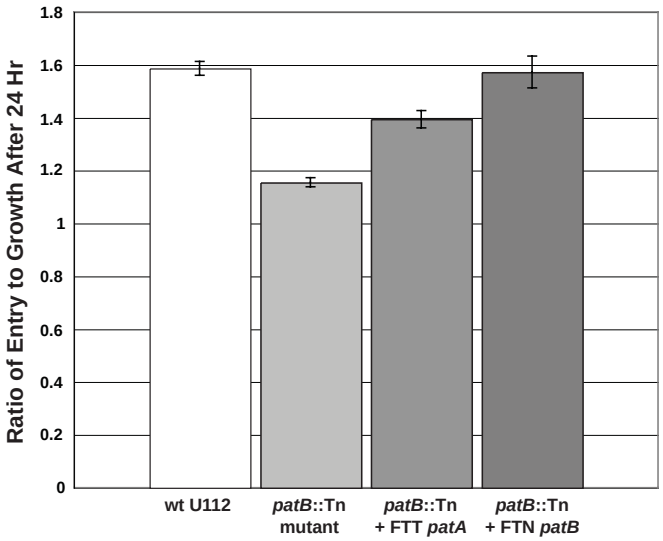


Figure 3-3. Complementation of *F. novicida* U112 *patB* transposon mutant with *F. tularensis* Schu S4 *patA* or *F. novicida* U112 *patB*. The ratio of entry (2 h p.i.) to survival after 24 h in J774 macrophage-like cells is shown for wild-type *F. novicida* U112 (white), the *patB*::Tn mutant (light gray), *patB*::Tn complemented with full-length *F. tularensis* Schu S4 *patA* (medium gray), and *patB*::Tn complemented with full-length *F. novicida* U112 *patB* (dark gray). Compared to wild-type, *patB*::Tn is attenuated for growth and is partially complemented by expression of *F. tularensis patA*, while expression of *F. novicida patB* fully restores virulence. Each column represents three independent infections.

Expression and purification of the TAARP domain

One of the prominent differences between the Pat family homologs is the occurrence of seven unique tandem repeats in the *F. novicida* homologs, which are present in duplicate in the *F. tularensis* WY98-3416 strain and in single copy in other *Francisella* subspecies. While it is not unusual for proteins to contain tandem repeats of a particular motif, the 102 amino acid size of this repeat was intriguing, as was the variation in number of TAARP domains among the different Pat homologs. To further investigate the structure and function of this domain, the sequence encoding the TAARP domain in *F. tularensis* Schu S4 (Figure 3-1) was cloned into an IPTG-inducible *E. coli* expression vector to generate a C-terminal 6His-tagged recombinant version of the TAARP domain. Subsequent analysis with SDS-PAGE and Coomassie staining revealed that the domain was present in the soluble fraction and was visible at the predicted 15 kDa size (Figure 3-4). Purification using the HisPur cobalt resin routinely yielded purified samples of greater than 0.5 mg/ml concentration.

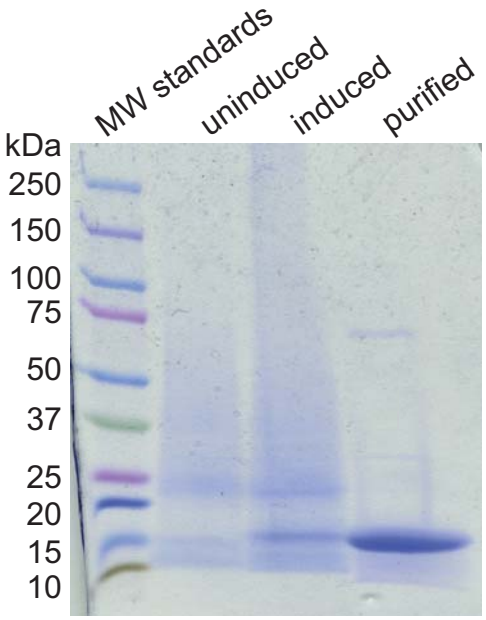


Figure 3-4. Expression and purification of the TAARP domain. The sequence of the *F. tularensis* Schu S4 TAARP domain was cloned into the IPTG-inducible *E. coli* expression vector pET-28a and transformed into BL21 cells. Cells were left untreated (uninduced) or expression of TAARP was induced with 500 mM IPTG (induced). The TAARP domain was purified on HisPur cobalt resin as described in Materials and Methods (purified). Samples from each preparation were separated by SDS-PAGE and stained with Coomassie blue. The TAARP domain is apparent at the expected size of approximately 15 kDa.

CD and intrinsic fluorescence indicate that the TAARP domain is stably folded

To assess the secondary structure of the TAARP motif, a circular dichroism spectrum was acquired in the far-UV range at pH 7.0 (Figure 3-5a). The resulting spectrum is typical of a folded protein with β-sheets as indicated by the broad negative between 200 and 220 nm. The peak at 227 nm is suggestive of aromatic stacking, as the aromatic composition of the domain includes three tyrosines, two phenylalanines, and one tryptophan. Preliminary analysis by nuclear magnetic resonance (NMR) spectroscopy corroborates these structural findings (data not shown). When the far-UV CD spectrum was acquired in the presence of 4 M urea, the structure was lost (Figure 3-5a). This unfolding indicates that the domain is independently folded and is capable of achieving structure in the absence of the full-length Pat protein.

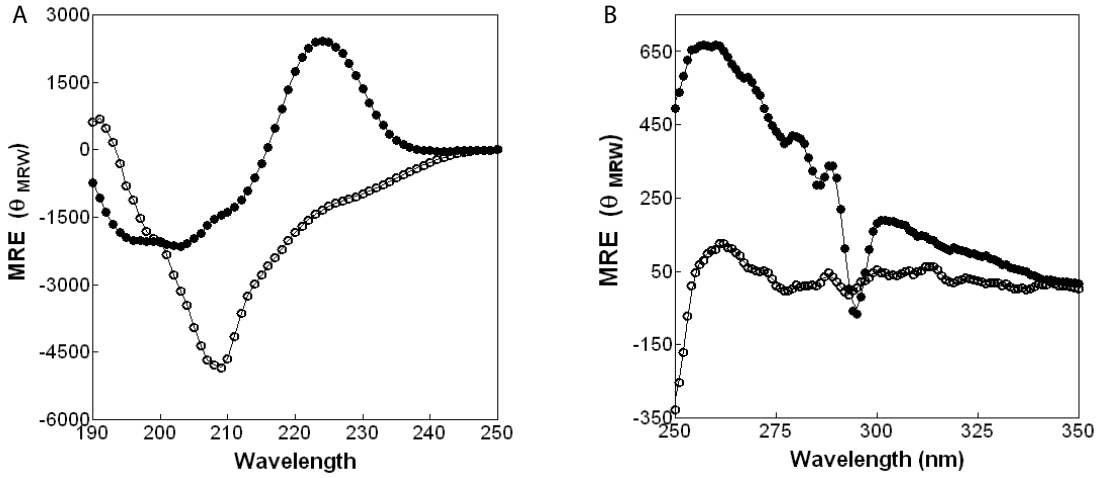


Figure 3-5. Circular dichroism spectroscopy of the TAARP domain. CD spectra were acquired for native (●) and denatured (○) TAARP in far-UV (A) and near-UV (B) ranges. The far-UV spectrum suggests a secondary structure with β-sheets and aromatic stacking. The significant signal observed in the near-UV range indicates that TAARP retains a tertiary structure. Denaturation with 4 M urea resulted in a loss of CD signal in both far- and near-UV ranges. Native TAARP was measured at pH 7.0. Data for each point was collected three times and averaged, and the signal contribution from the buffer was subtracted.

Intrinsic fluorescence and near-UV CD spectra acquired at pH 7.0 reveal that the TAARP domain retains tertiary structure (Figures 3-5b and 3-6). Addition of urea to a final concentration of 4 M caused a loss of intensity and a red shift in the intrinsic fluorescence emission spectra acquired with excitation at both 280 and 295 nm (Figure 3-6). While excitation at 280 nm targets all aromatic residues, the signal from phenylalanine is often obscured in the presence of tyrosine and tryptophan; therefore, the signal observed from excitation with 280 nm is likely due to contributions from only the tyrosine and tryptophan residues. By contrast, excitation with 295 nm targets only tryptophan residues. In both spectra, the maximum peaks shifted from 320 nm to the 345-350 nm range, which is typical of the fluorescence maximum of tryptophan in unfolded proteins. Both the red shift and the decrease in fluorescence signal indicate a loss of tertiary structure as the aromatic residues become more exposed to the buffer solution.

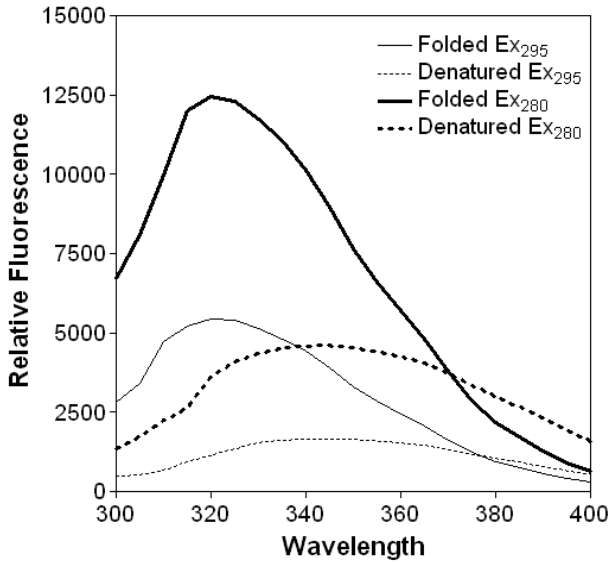


Figure 3-6. Intrinsic fluorescence of the TAARP domain. Fluorescence emission of TAARP was recorded in native (pH 7.0) and denatured (4 M urea) conditions with excitation at either 280 nm (targeting Y and W) or 295 nm (targeting W). The decrease in fluorescence signal in the presence of 4 M urea indicates a loss of tertiary structure. Each point represents the average of three measurements.

The native TAARP domain exhibits a significant CD signal in the near-UV range; changes in this signal with the addition of urea can provide information about changes in structure with respect to aromatic packing. As previously mentioned, the domain includes

a single tryptophan, two phenylalanines, and three tyrosines. In the near-UV CD spectrum acquired at pH 7.0, the peak at 290 nm can be assigned to the tryptophan, the peaks in the 275-280 nm range are characteristic of tyrosine residues, and the phenylalanines likely contribute to the peaks observed in the 250-270 nm range. When the TAARP domain is denatured with urea to a final concentration of 4 M, these peaks are greatly diminished, indicating a loss of native protein folding and aromatic packing (Figure 3-5b).

Urea denaturation shows TARRP to be very stable and suggests a two-step melting process

To examine the unfolding mechanism of the domain, intrinsic fluorescence and CD spectra were acquired at 21 (IF) or 35 (CD) different urea concentrations ranging from 0 M to 4.6 M (Figure 3-7). CD data were collected at the 227 nm wavelength, as this was the maximum peak observed in the native far-UV spectrum and is associated with aromatic stacking. Intrinsic fluorescence emission signals were collected over the spectrum of 300-400 nm, following excitation at either 280 nm or 295 nm. The maximum peak of 320 nm was used to construct the melting curve. Comparison of the

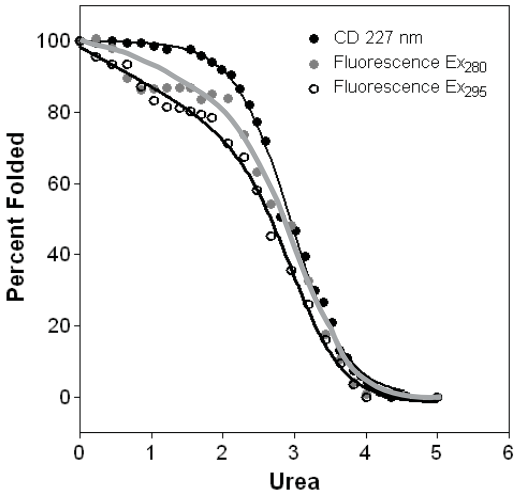


Figure 3-7. Melting curve of the TAARP domain during denaturation with urea. CD and fluorescence spectra were acquired for the TAARP domain as urea was titrated to a final concentration of 5 M. CD measurements were recorded at 227 nm to observe the changes in aromatic stacking. Intrinsic fluorescence was recorded with excitation at 280 nm or 295 nm; the curve shown is the maximum emission peak of 320 nm. The presence of a shoulder in the intrinsic fluorescence curves indicates a two-step melting process. Each point represents the average of three to six separate measurements.

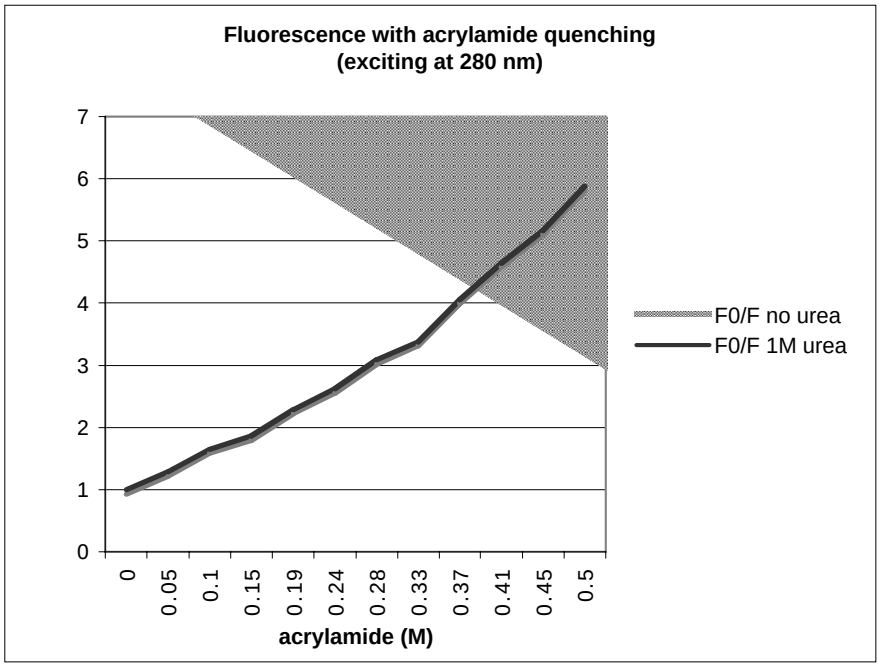
fitted melting curves from the CD and IF spectra suggests a two-step melting process, as indicated by the presence of shoulders in the intrinsic fluorescence spectra.

Native TAARP contains non-uniformly exposed residues

Acrylamide is a polar, uncharged molecule that is a dynamic quencher of intrinsic fluorescence. Having minimal penetration into the tightly packed interior of a folded protein, acrylamide can be used to assess the exposure of fluorescent residues to solvent. This readout can be used to gain insight into unfolding dynamics or interactions between subunits of an oligomer by observing the differences in fluorescence signal as a measure of conformational change. Figure 3-8 shows the Stern-Volmer plot for the TAARP domain. In the absence of urea, the plot is not linear, indicating non-uniform exposure of the residues to solvent and acrylamide quenching. By contrast, in the mildly denaturing conditions of 1 M urea, the Stern-Volmer plot is linear. In previous experiments, we demonstrated that the TAARP motif is not denatured at this concentration of urea (Figures 3-5 and 3-7). Instead, this result suggests disruption of a multimer to monomers or a shift in the conformation that resulted in equal exposure of fluorescence residues to the surrounding solution and a linear Stern-Volmer plot.

The TAARP domain is capable of oligomerization

The TAARP motif was subjected to gel filtration chromatography to investigate whether it is present as an oligomer in its native state. Under denaturing conditions, the domain was observed to migrate with a molecular mass of 15 kDa (Figure 3-4). Gel



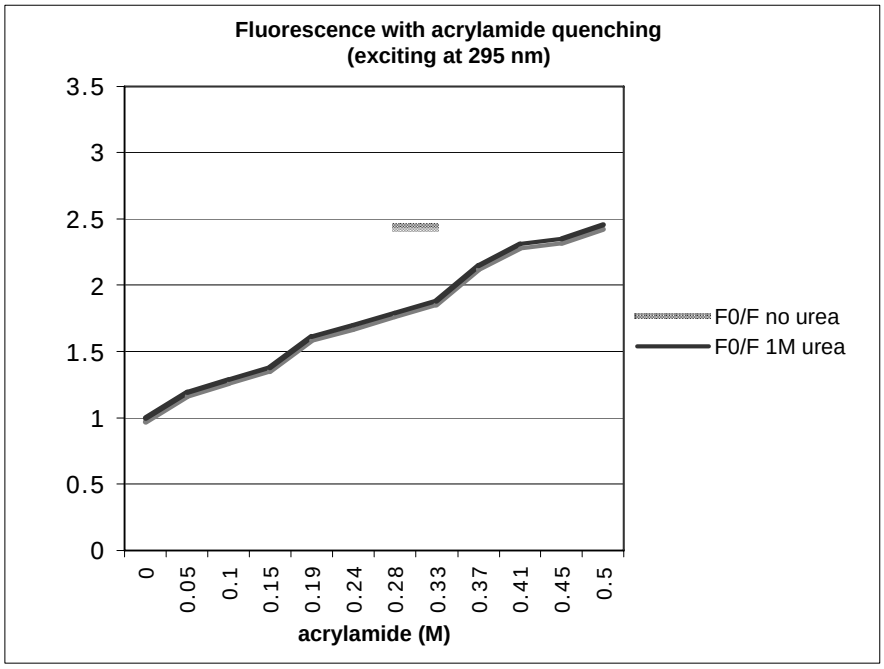


Figure 3-8. Stern-Volmer plots of TAARP domains in 0 versus 1 M urea. Intrinsic fluorescence spectra were acquired for the TAARP domain in the absence of urea or under mildly denaturing conditions with 1 M urea. Samples were excited at 280 nm or 295 nm as indicated and the emission spectra were collected from 300 nm to 400 nm. The peak emission of 320 nm was used to construct the plot. The Stern-Volmer plots were calculated as indicated in the materials and methods section. The nonlinear plot in the absence of urea indicates non-uniform exposure of residues to solution, while the linear plot in 1 M urea suggests uniform exposure of aromatic residues.

filtration analysis of the native sample resulted in a single peak eluting at 24.9 minutes, corresponding to a dimer with an approximate molecular mass of 30 kDa (Figure 3-9).

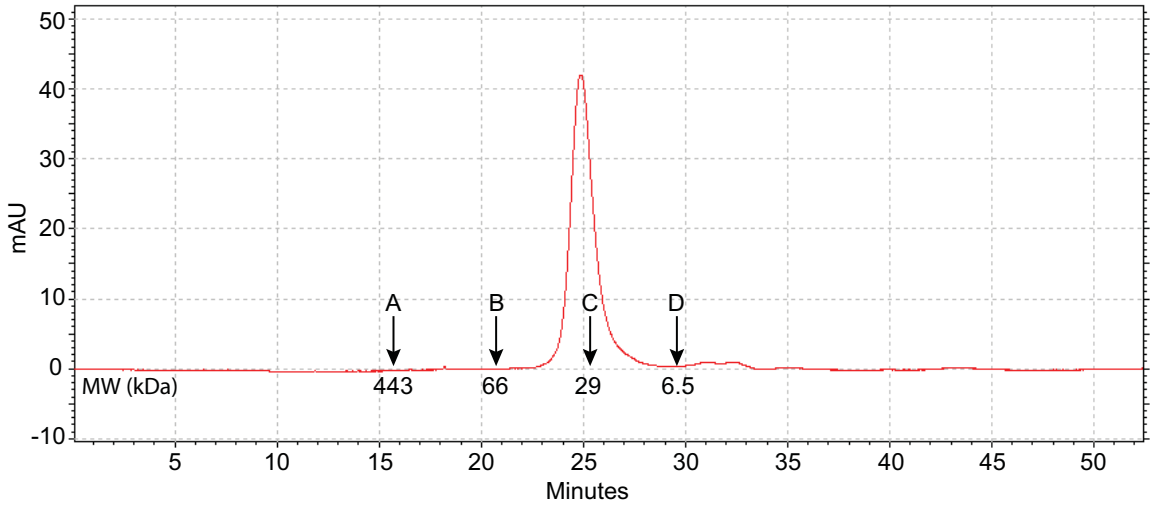


Figure 3-9. Gel filtration chromatography of TAARP indicates dimerization. Native TAARP domain was subjected to size exclusion chromatography as indicated in the materials and methods section. Apoferritin (A), albumin (B), carbonic anhydrase (C), and aprotinin (D) were used as standards. Their respective elution peaks are represented by arrows, and their molecular weights are provided for reference. The TAARP domain eluted from the column just prior to the 29 kDA carbonic anhydrase standard, indicating dimer formation, as the molecular weight of TAARP under denaturing conditions is ~15 kDa (Figure 3-4).

Homology to *S. aureus* EAP domain sequence and structure

The computer modeling program Rosetta was used to generate a structure consistent with the solution structure of the TAARP domain (Figure 3-10a). Comparison of the TAARP domain structure with structures from the PDB database indicated that the TAARP domain shares structural homology to the ~110 a.a. EAP domains found in Eap (Extracellular adherence protein) of the bacterial pathogen *Staphylococcus aureus* (Figure 3-10b) (Harraghy, Hussain et al. 2003). The Eap protein has been shown to bind a number of extracellular matrix proteins, including fibronectin, laminin, and the pro-inflammatory cell surface receptor intercellular adhesion molecule 1 (ICAM-1) (Palma, Haggar et al. 1999; Chavakis, Hussain et al. 2002). Although Eap lacks a transmembrane

region, ~30% of the protein was found to be associated with the bacterial cell surface due to protein-protein interactions (Flock and Flock 2001), uncharacterized membrane structures (Kreikemeyer, McDevitt et al. 2002), and Eap multimerization (Palma, Haggar et al. 1999). The EAP repeat domains were discovered upon analysis of the deduced primary structure sequence of Eap (Jonsson, McDevitt et al. 1995). Depending on the strain of *S. aureus*, Eap is comprised of either four or six repeats of the EAP domain. Sequence comparison of the TAARP domains from the Pat homologs (represented in Figure 3-1) with EAP domains showed that there are regions of shared sequence homology (Figure 3-11).

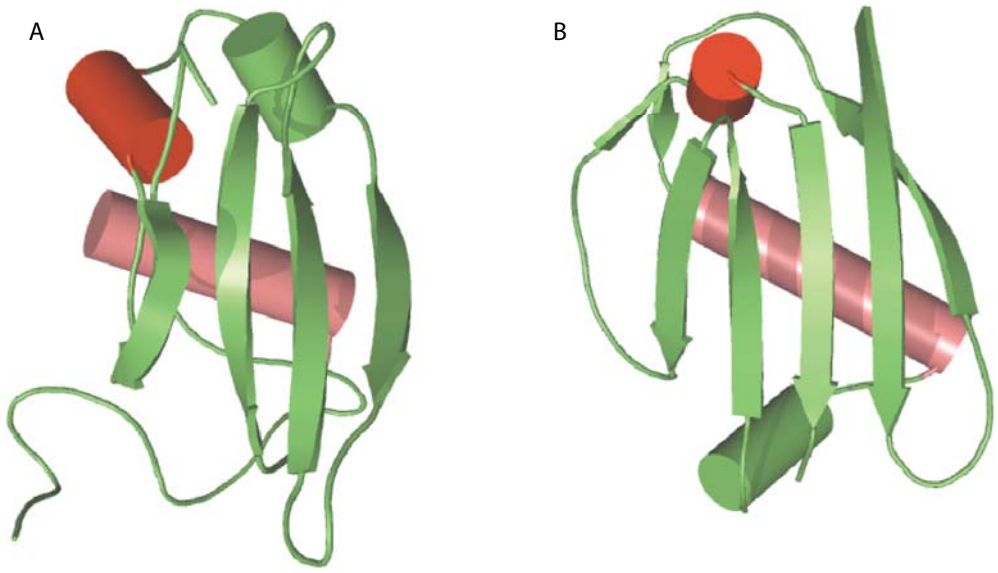


Figure 3-10. Structural comparison of the EAP domain with the TAARP domain. Rosetta was used to generate a structure for the TAARP domain from *F. tularensis* Schu S4 (A). Comparison with structures in the PDB database revealed similarity to the EAP domain from the *S. aureus* extracellular adhesion protein (B).

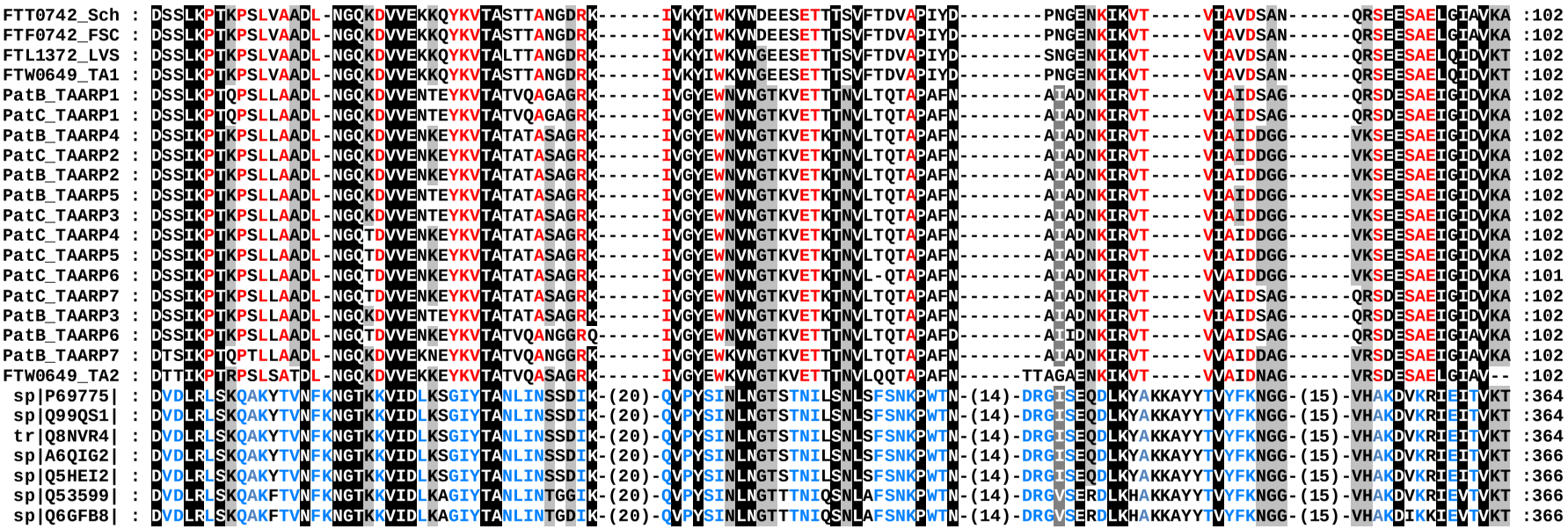


Figure 3-11. Sequence comparison of the TAARP domains with the EAP domains. Amino acid sequences of *Francisella* TAARP domains from the homologs represented in figure 1 were aligned with the amino acid sequences of *S. aureus* EAP domains obtained from SwissProt (sp). The dashes represent gaps in the sequences, with the number of residues present in the EAP domains shown in parentheses. The colored residues are conserved in their respective species, red for *Francisella* and blue for *Staphylococcus*. The residues shaded in black are conserved in >90% of the sequences, and those shaded in gray are similar. Although an exact analysis cannot be conducted due to the gaps from additional residues in the *S. aureus* domains and minor differences between individual repeat domains, the sequences can be considered highly conserved.

The TAARP domain binds host factors

F. novicida PatB was initially identified as a virulence factor when a transposon mutant derivative exhibited significant attenuation in macrophages and mice, as well as protection against wild-type challenge in a mouse model of infection. The transposon insert occurred in the sixth repeat motif of PatB, which could have disrupted oligomerization or yielded an incorrectly folded protein, thus negating its function. To investigate if the native TAARP domain directly interacts with host factors, a co-purification assay was performed. Extracts from J774 or THP-1 macrophage-like cells were incubated with the repeat domain immobilized on HisPur resin. The bound protein samples were purified using high salt washes (300mM) to select for strong protein-protein interactions. Subsequent analysis with SDS-PAGE and Coomassie staining revealed the presence of several protein bands not observed in the purified motif or J774 extract + resin alone lanes (Figure 3-12). Mass spectrometry analysis of the eluates from the co-purification assay was performed to identify host factors that bind the TAARP motif. Of particular interest was the presence of δ-catenin, a component of the E-cadherin complex. Interaction between the TAARP domain and the E-cadherin complex could be a mechanism for entry, as this is the pathway that the bacterial pathogen *Listeria* exploits for uptake into host cells (Mengaud, Ohayon et al. 1996). Further studies to explore this possibility are currently underway.

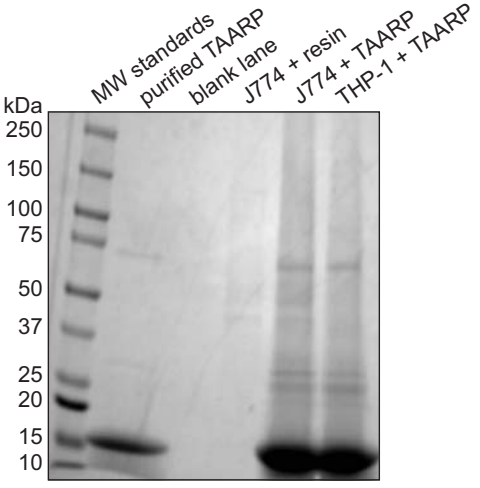


Figure 3-12. Co-purification of TAARP with host cell factors. TAARP was bound to HisPur resin and incubated with extracts from J774 or THP-1 macrophages. J774 extracts were incubated with HisPur resin in the absence of TAARP as a negative control. After elution, the samples were separated by SDS-PAGE and stained with Coomassie blue.

Discussion

It is widely accepted that outer membrane proteins of bacterial pathogens serve important functions for attachment and invasion of host cells, as well as immune evasion (Radolf 1994; Beuscher, Rodel et al. 1995; Boyle and Finlay 2003; Cullen, Haake et al. 2004). Indeed, these proteins are often the first site of contact with the host and may interact with host factors throughout the course of infection. As surface components, their accessibility also makes them attractive targets for drug therapy and vaccine development. Here, we report the discovery of a new family of *Francisella* outer membrane proteins, designated "Pat" for Pathogenitcity factor. PatB was initially identified as the *F. novicida* homolog of *F. tularensis* Schu S4 PatA whose transposon mutant derivative was attenuated and protective in mice against high doses of wild-type *F. novicida* challenge (Tempel, Lai et al. 2006). Further investigation led to the discovery of Pat homologs in other *Francisella* strain sequences, and subsequent comparison of the homologs revealed the presence of the unique TAARP domain, as well as the variation in the number of repeats. In this work, we examined the structure and stability of the Schu S4 TAARP domain to provide insights into the role of this novel *Francisella* virulence factor family.

Through far-UV circular dichroism (CD) spectra and intrinsic fluorescence (IF) studies, we demonstrated that the TAARP domain adopts a stably folded conformation in the absence of the rest of the Pat protein, while IF and near-UV CD spectra showed that the TAARP domain retained tertiary structure. Further preliminary analysis by nuclear magnetic resonance corroborated the presence of β-sheeted motifs and aromatic stacking.

IF and CD spectra collected after denaturation with urea revealed that the domain is very stable and suggested a two-step melting process.

That the TAARP domain is present in seven copies in *F. tularensis* ssp. *novicida* PatB and PatC while Pat homologs in other *Francisella* subspecies contain only one or two TAARP domains is intriguing. Moreover, *novicida* is the only subspecies that appears to possess two Pat homologs in its genome. *F. tularensis* ssp. *novicida* is evolutionarily the oldest of the *Francisella* subspecies (Svensson, Larsson et al. 2005). Perhaps additional copies of the TAARP domain were lost in the many rearrangement events that occurred among the *Francisella* subspecies during evolution. It could be speculated that the domain's ability to oligomerize, as shown by gel filtration chromatography in Figure 3-9, precluded the requirement for multiple copies of the domain. Following this line of thought, one explanation for the nonlinear Stern-Volmer plot in the absence of urea may be that homo-oligomerization of the TAARP domain results in non-uniform exposure of residues, and the addition of 1M urea caused dissociation of these multimers to monomers, resulting in uniform exposure of the aromatic residue and the observed linear Stern-Volmer plot seen in Figure 3-8.

Interestingly, Pat homologs in the *F. tualrensis* ssp. *holarctica* strains LVS, OSU18, and FTNF002-00 were found to span two open reading frames, with a frameshift mutation disrupting the region that codes for the second TAARP domain. The PatA amino acid sequences are identical for these three proteins; however, since they are the only *holarctica* PatA sequences currently available, it would be premature to assume that PatA is similarly affected in all *holarctica* strains. Despite the truncation, these genes may still yield functional proteins. In LVS, *pilT*, which plays a role in expression of type

IV pili, is divided into two ORFs, FTL1770 and FTL1771 (Chakraborty, Monfett et al. 2008). The first ORF is truncated by a premature stop codon followed by a stretch of 20 amino acids that are not translated before the second ORF begins and was therefore thought to be nonfunctional. Surprisingly, a transposon insertion in the first *pilT* ORF resulted in a loss of pili and increased survival in mice, which was rescued by complementation with the complete *pilT* locus (Chakraborty, Monfett et al. 2008). Although further research is required to determine if *patA* in LVS and other *F. tularensis* ssp. *holarctica* strains is functional, the finding that the truncated LVS *pilT* gene retains activity indicates that this is a possibility.

The Gram-positive bacterial pathogen *Staphylococcus aureus* secretes the virulence factor Eap (extracellular adherence protein) to mediate interactions between the bacterial cell surface and extracellular host proteins (Palma, Haggar et al. 1999; Harraghy, Hussain et al. 2003). Eap was initially identified as Map (major histocompatibility complex class II analog protein) for its homology with the peptide binding groove of the β chain of MHC class II proteins but was later designated Eap for its broad binding activity and role as an adherence enhancement protein (Jonsson, McDevitt et al. 1995; Palma, Haggar et al. 1999). Despite lacking a defined transmembrane domain, ~30% of Eap is found associated with the bacterial cell surface by protein-protein interactions, uncharacterized membrane structures, and Eap multimerization (Palma, Haggar et al. 1999; Flock and Flock 2001; Kreikemeyer, McDevitt et al. 2002). Eap has been shown to have roles as an anti-inflammatory molecule, in enhancing bacterial internalization into eukaryotic cells, and in activating expression of proinflammatory cytokines and is known to bind host cell factors such as

fibronectin and prothrombin (Palma, Haggar et al. 1999; Chavakis, Hussain et al. 2002; Haggar, Hussain et al. 2003; Scriba, Sierro et al. 2008). Structurally, Eap appears to be comprised of four to six repeat domains of approximately 110 amino acids, termed EAP domains (Jonsson, McDevitt et al. 1995; Harraghy, Hussain et al. 2003). Using predicted structure computations and subsequent sequence comparisons, we discovered several structural similarities, as well as a significant number of conserved residues between the EAP and TAARP domains. These findings are intriguing for several reasons, as the homology between EAP and TAARP domains not only defines a link to another protein of known function but perhaps provides insight into the specific role that Pat and the TAARP domains may play in *Francisella* pathogenicity.

Co-purification studies followed by mass spectrometry (MS) analysis indicated that TAARP binds host factor δ-catenin (p120). Interaction with this protein may be part of a host cell entry mechanism. Although found on the intracellular side of the host cell membrane, δ-catenin has been shown to form a complex with E-cadherin, which spans the eukaryotic membrane to interact with extracellular factors and is in fact the receptor by which *Listeria* gains entry into the host cell (Mengaud, Ohayon et al. 1996). It is possible that δ-catenin was identified by MS as part of the E-cadherin complex and that E-cadherin was present but not detected. Indeed, preliminary studies indicate that E-cadherin does interact with TAARP, and further experiments are currently underway to investigate this possibility. On the intracellular side, the E-cadherin/δ-catenin complex has been shown to bind to cortactin, which can trigger actin polymerization via the actin related protein Arp2/3 (Boguslavsky, Grosheva et al. 2007; Ireton 2007). If Pat proteins do indeed have a role in *Francisella* entry through binding E-cadherin, this interaction

could result in restructuring of the host actin cytoskeleton as seen in pseudopod loops (Clemens, Lee et al. 2005).

Furthermore, in a recent report, *Francisella* was shown to target cholesterol-rich host membrane domains, or lipid rafts, for entry into macrophages (Tamilselvam and Daefler 2008). Depletion of the cholesterol resulted in a significant reduction in *Francisella* entry and intracellular replication. A subpopulation of E-cadherin has been shown to associate with lipid raft domains of the plasma membrane (Golub, Wacha et al. 2004). When these rafts were disrupted by removing cholesterol, binding and entry of *Listeria* was inhibited. While the lack of cholesterol did not affect the surface levels of E-cadherin in the lipid rafts, it affected the clustering of E-cadherin around bacteria (Seveau, Bierne et al. 2004). These findings, along with the structural and sequence homology to the Eap adhesion factor, allow for speculation that *Francisella* may gain entry into host cells via the TAARP domain by binding E-cadherin in a manner similar to that of *Listeria*. Here, we provide the first description of a novel *Francisella* virulence factor family with evidence for an interaction between *Francisella* and members of the E-cadherin complex and implications for host cell entry.

Materials and Methods

Sucrose density gradient fractionation

Following a similar procedure to that developed by Huntley and co-workers, *Francisella novicida* strain U112 was inoculated from overnight culture at a dilution of 1:50 and grown to OD_{600} 0.3 in 1 L tryptic soy broth with 0.1% cysteine at 37ºC with shaking (Huntley, Conley et al. 2007). Cultures were centrifuged at 5520 × g (6000 rpm in a Beckman JA-14 rotor) for 30 min at 10ºC to pellet the cells, the supernatant was decanted, and the cells were suspended in 35 ml of 0.75 M sucrose in 5 mM Tris pH 7.5 and transferred to a sterile 250 ml flask with a stir bar. Over the course of 10 min, 70 ml of 10 mM EDTA in 5 mM Tris pH 7.8 was added to the cell suspension with gentle stirring. Following 30 min of incubation at room temperature, 11 ml of 2 mg/ml lyzozyme was slowly added to a final concentration of 200 µg/ml. Following 30 min of incubation at room temperature, the cells were osmotically lysed by slowly diluting them into 520 ml of Cellgro molecular grade distilled water in a 1 L flask over the course of 10 min with gentle stirring. Following 30 min of incubation at room temperature, the lysis solution was separated into 40 ml aliquots and centrifuges at 6000 × g (7500 rpm in a Beckman JA-20 rotor) for 30 min at 10ºC to remove intact cells and debris. Supernatants were then removed and centrifuged at 31,500 rpm in a Beckman SW32 rotor (Beckman-Coulter, Fullerton, CA) for 1 hour at 4ºC to collect total membranes. Supernatants were removed, and total membrane pellets were resuspended in 5-6 ml of resuspension buffer (25% sucrose, 5 mM Tris, 30 mM $MgCl_2$, complete EDTA-free protease inhibitor cocktail, DNase, RNase) and incubated with gentle inversion for 30 min at room temperature. Linear sucrose gradients were prepared by layering 1.67 ml each of sucrose

solutions (in 5 mM EDTA pH 7.5) into 14- by 89-mm ultracentrifuge tubes in this order: 55%, 50%, 45%, 40%, 35%, and 30%. Total membranes were layered on top of each gradient, with no more than 1.5 mg of total protein to avoid saturating the gradient. Sucrose gradients were centrifuged in a Beckman SW41 swinging bucket rotor (Beckman-Coulter, Fullerton, CA) at 38,500 rpm for 14 h at 4ºC. After centrifugation, 500 µl fractions were collected by gravity flow from the bottom of each gradient after puncturing the tubes with a 21-gauge needle.

Antibody production and purification

A polyclonal peptide-specific antibody against the peptide QTYVDQLVKKVMISEVRHDC (α-Pat) was raised in rabbits (Lampire Biological Laboratories, Pipersville, PA) and purified for total IgG. Serum was centrifuged for 10 min at 5000 × g and 291 mg/ml solid ammonium sulfate was slowly added to the serum supernatant with stirring. Following 30 min of incubation at 4ºC with stirring, the mixture was centrifuged at 5000 × g for 10 min at 4ºC. The supernatant was decanted and the pellet was resuspended in PBS pH 7.4 to the original volume. The purified IgG was separated into 50 µl aliquots and stored at -80ºC until used. Antibodies against SecY and FopA were kindly provided by the Norgard laboratory.

Western blot analysis

Fractions from the sucrose density gradient procedure were mixed with sample buffer, heated to 95ºC on a heat block for 20 min, separated by SDS-PAGE on a pre-cast 4%-20% gradient gel (Pierce Biotechnology, Rockford, IL), and transferred to PVDF

membrane. Blots were blocked either overnight at 4ºC or for 1 h at room temperature in blocking buffer (0.05% Tween and 5% milk in PBS). Blots were incubated with antiserum (1:1500 to 1:3000 for Pat, 1:500 for SecY, 1:50,000 to 1:100,00 for FopA) either overnight at 4ºC or for 1 h at room temperature in blocking buffer, followed by four washes of 10 min each with PBST (PBS with 0.05% Tween). Blots were incubated in secondary antibody (1:50,000 to 1:100,000 goat anti-rabbit IgG-HRP or 1:20,000 goat anti-rat IgG-HRP) for 1 h at room temperature, followed by three washes of 10 min with PBST. Blots were incubated with Western Lightning Chemiluminescence substrate (PerkinElmer, Waltham, MA) for 2-5 min and exposed to film.

Complementation of Tn-*patB* mutant strain

Construction of the pKK202-FTT0742 (Schu S4 *patA*) used to complement the *F. novicida patB* transposon mutant has already been described (Tempel, Lai et al. 2006). Primers GCGGCCGCTGAGATGCTGAAATAAATTGAGC (forward) and GGCCCTCGAGGCCGCAAACTAAAGTTAGAAGAACACCC (reverse) were used to amplify the full-length *patB* gene from the *F. novicida* genome by PCR. The pKK202 vector (previously modified to include *Not*I, *Sfi*I, and *Xho*I sites) and the PCR product were digested with *Not*I and *Xho*I and ligated. pKK202-*patB* was electroporated into *F. novicida* Tn-*patB* and used to infect RAW264.7 macrophage-like cells as previously described (Tempel, Lai et al. 2006).

Cloning, expression, and purification of recombinant *F. tularensis* Schu S4 (FTT0742) TAARP domain

The amino acid sequence VATKEVAVLFDSSLKPTKPSLVAADLNGQKDVV EKKQYKVTASTTANGDRKIVKYIWKVNDEESETTTSVFTDVAPIYDPNGENKIKV TVIAVDSANQRSEESAELGIAVKADMSIKPSTPS was codon optimized for expression in *E. coli* and inserted into pET-28a using the *Nco*I and *Xho*I sites to generate a C-terminal 6His-tagged IPTG-inducible construct (Celtek Genes, Nashville, TN). The expression vector was transformed into *E. coli* strain BL21(DE3) and grown in 2 L LB broth to OD_{600} 0.9, after which protein expression was induced overnight at 25ºC wtih the addition of 500 mM isopropyl 1-thio-β-D-galactopyranoside (IPTG.) Cells were pelleted and washed 3 times with wash buffer (150 mM NaCl and 20mM Tris pH 7). Cell pellets were aliquoted and frozen. To purify the TAARP domain, cells were resuspended (using 3 ml per 100 ml pellet) in wash buffer containing DNase I and protease inhibitors and sonicated. Samples were centrifuged briefly to remove unlysed cells and supernatants were centrifuged for 1 h at 4ºC to separate the pellet and soluble fractions. The protein of interest was determined to be in the soluble fraction by SDS-PAGE analysis followed by Coomassie staining. Sample supernatants were incubated with equilibrated HisPur cobalt resin (Pierce Biotechnology, Rockford, IL) at 4ºC in 15 ml tubes on an end-over-end rotator for 30 min, followed by 5 washes with wash buffer (50 mM $NaPO_4$, 300 mM NaCl, 10 mM imidazole), centrifuging at 700 × g for 2 min between each wash. Purified protein was eluted with elution buffer (50 mM $NaPO_4$, 300 mM NaCl, 150 mM imidazole) and dialyzed overnight against 50 mM $NaPO_4$ to remove excess imidazole. Following dialysis, the samples were subjected to ultracentrifugation in a Beckman TLA 45 rotor (Beckman-Coulter, Fullerton, CA) at 45,000 rpm for 20 min at 4ºC to remove any remaining precipitates.

Circular dichroism spectroscopy

Measurements were carried out on an AVIV-215 spectrophotometer (Lakewood, NJ). Spectra in the far UV range were recorded over the wavelength range from 190 to 260 nm while spectra in the near UV range were recorded over the wavelength range from 250 to 350 nm. All spectra were measured with a 1 nm wavelength step and 3.0 s averaging time. Samples were measured in a 1 mm (far UV) or 1 cm (near UV) path length quartz cuvette, with a sample volume of 300 µl (far UV) or 2-3 ml (near UV), at room temperature. Protein stability was measured by adding aliquots of 8 M urea to the sample. Three scans were averaged for each sample, and the appropriate background contribution from $NaPO_4$ buffer was subtracted from the spectra.

Intrinsic fluorescence spectroscopy

To measure intrinsic fluorescence, samples were excited at 280 or 295 (tryptophan only) nm and emission spectra were recorded between 300 and 400 nm using a SpectraMax M2^e fluorimeter (Molecular Devices, Sunnyvale, CA). Dissociation and unfolding was measured by adding aliquots of 8 M urea to the samples. Three to six scans were averaged for each reading, and the measurements were corrected for dilution.

Fitting of CD and IF melting curves

CD and IF spectra were acquired during the course of urea denaturation. As previously described, the transitions a protein undergoes during unfolding are represented by at least three states: the native (N), intermediate (I), and unfolded (U) states (Subbian, Yabuta et al. 2004). These are related by the equilibrium constants K_{NI} and K_{IU} during the

unfolding transitions as shown in equation 1.

$$\mathrm{N} \xleftrightarrow{K_{\mathrm{NI}}} \mathrm{I} \xleftrightarrow{K_{\mathrm{IU}}} \mathrm{U} \qquad (1)$$

The observed ellipticity (CD) or fluorescence emission (IF) [$A_{\mathrm{obs}}(c)$] at any concentration of urea is given by the sum of the contributions from the three states, as shown in equation 2.

$$A_{\mathrm{obs}}(c) = \frac{A_{\mathrm{N}} + A_{\mathrm{I}} \exp[-(\Delta G_{\mathrm{NI}}^{\mathrm{H_2O}} - m_{\mathrm{NI}}c)/RT] + A_{\mathrm{U}} \exp[-(\Delta G_{\mathrm{NU}}^{\mathrm{H_2}0} - m_{\mathrm{NU}}c)/RT]}{1 + \exp[-(\Delta G_{\mathrm{NI}}^{\mathrm{H_2O}} - m_{\mathrm{NI}}c)/RT] + \exp[-(\Delta G_{\mathrm{NU}}^{\mathrm{H_2}0} - m_{\mathrm{NU}}c)/RT]} \qquad (2)$$

Here, $f_{\mathrm{N}}(c)$, $f_{\mathrm{I}}(c)$, and $f_{\mathrm{U}}(c)$ are the fractions of the three states at a urea concentration of c ($f_{\mathrm{N}} + f_{\mathrm{I}} + f_{\mathrm{U}} = 1$) and A_{N}, A_{I}, and A_{U} are the values of the pure N, I, and U states, respectively. The f_{N}, f_{I},and f_{U} terms are related to the equilibrium constants of K_{NI} and K_{NU} of the unfolding transitions from N to I and N to U. $\Delta G_{\mathrm{NI}}^{\mathrm{H_2O}}$ and $\Delta G_{\mathrm{NU}}^{\mathrm{H_2O}}$ are ΔG_{NI} and ΔG_{NU} at 0 M urea, respectively. The terms $m_{\mathrm{NI}}c$ and $m_{\mathrm{NI}}c$ represent the dependence of the free energy changes on c. The data were fitted using Prism Graphpad.

Acrylamide quenching

Acrylamide quenching measurements were carried out by adding aliquots of a 5M solution of acrylamide to the sample and measured as above. The reciprocal of the fluorescence intensity as a function of quencher concentration [Q] was linear in the presence of 1M urea and was fitted to the Stern–Volmer equation

$$F_0/F = 1 + K_{\mathrm{SV}}[\mathrm{Q}] \qquad (3)$$

in which F_0 is the initial fluorescence intensity, F the fluorescence intensity in the presence of an added quencher concentration [Q], and K_{SV} is the Stern–Volmer constant.

Gel filtration chromatography

The SEC was performed on a Sephadex G-75 column (Sigma-Aldrich, St. Loius, MO) with 50 mM $NaPO_4$ at pH 7 as the mobile phase. Absorbance of the fractions was measured using a System Gold 168 Detector (Beckman-Coulter, Fullerton, CA). Apoferratin (8 mg/ml, MW 443 kDa), albumin (8 mg/ml, MW 66 kDa) carbonic anhydrase (2 mg/ml, MW 29 kDa), and aprotinin (4 mg/ml, MW 6.5 kDa) were passed through the column individually to define molecular weight elution standards. The sample was dialyzed against 50 mM $NaPO_4$ before analysis.

Molecular modeling and multiple sequence alignments of TAARP

Ab initio structure prediction was carried out on a locally installed Rosetta ab initio software version 2.3 licensed through the University of Washington (the web-based version of this program is known as "Robetta"); the fragment libraries were generated using the web version of the Rosetta fragment server. In the fragment selection, the "homs" option was included while all remaining parameters were set as defaults. Using "ab initio" mode after decoy population filtering 1,000 structures had been obtained, which were clustered using the Rosetta clustering program. The center of the most populated cluster was selected and minimized using CharmM and then validated as previously described (Subbian, Yabuta et al. 2004). The validated structure was then used to search structurally similar proteins from the PDB database using the least-squares fit superposition program (lsqfit.pl) from the Multiscale Modeling Tools for Structural Biology (MMTSB) package. The structures were displayed using open-source PYMOL version 0.99rc6. The amino acid sequence from the target hit from the PDB database

(1YN3) along with its homologues were compared with all known TAARP domains using a locally installed multiple sequence alignment program, CLUSTALW. The alignment was analyzed and annotated using the program GeneDoc.

Co-purification of TAARP with mammalian cell extracts

The human THP-1 monocyte cell line and J774 mouse macrophage-like cells were purchased from American type Culture Collection (ATCC) (Manassas, VA) and grown in RPMI 1640 and DMEM media, respectively. Both media were supplemented with 10% heat-inactivated fetal bovine serum (FBS). One T-75 flask (15 ml) of THP-1 cells was collected by centrifugation at 700 × g for 5 min at 20ºC, washed twice with PBS, and resuspended in 2 ml of lysis buffer (PBS containing 0.5% Triton X-100, 1mM PMSF, 1mM Na_3VO_4, and 1 tablet complete EDTA-free protease inhibitors). One T-75 flask of confluent J774 cells was washed twice with PBS and scraped into 2 ml of lysis buffer. Both samples were then incubated on ice for at least 30 min to allow lysis to proceed. Following incubation, unlysed cells and debris were removed by centrifugation for 20 min at 10,000 × g at 4ºC, and four volumes of PBS was added to reduce the Triton concentration to 0.1%. Lysates were added to columns containing TAARP bound to HisPur resin (washed with 50mM $NaPO_4$, 300mM NaCl, and 10mM imidazole and equilibrated with 0.1% Triton X-100 in PBS). Samples were incubated with rocking for 1 h and washed first with 0.1% Triton X-100 in PBS and then with 0.1% Triton X-100 and 200mM NaCl in PBS. Proteins were eluted from the resin with 50mM NaPO4, 300mM NaCl, 150mM imidazole, pH 7.4.

Protein identification with mass spectrometry

For dialysis, samples were transferred from the storage vial to a Pierce Slide-A-Lyzer® (Pierce is now part of Thermofisher Scientific, Rockford, IL) mini dialysis unit (Pierce part #69570), and were dialyzed against 1 L of diH_2O overnight at 4ºC. After dialysis the sample was transferred to a 1.5 ml eppendorf tube and dried in a Speed Vac. Dry samples were brought up in SDS-Page running buffer and transferred into an Invitrogen 10% Bis-Tris gel (Carlsbad, CA) by electrophoresis (200 V for 5 min) to further isolate the protein from potential contaminates. The proteins were excised from the gel and reduced, alkylated with iodoacetamide, and digested via an overnight incubation with trypsin in preparation for analysis on the mass spectrometer. LC-MS/MS Analysis was performed on a Thermofinnigan LTQ mass spectrometer using data dependant settings and an exclusion list to select MS peaks for fragmentation and analysis. The LC gradient was delivered using an Agilent 1100 series pump (Santa Clara, CA) to elute peptides from an Agilent Zorbax C18 column, and sample introduction was accomplished using an Agilent 1100 series autosampler. The LC system delivered a gradient from 5-30% Acetonitrile over the course of 100 min. All MS/MS spectra were analyzed using Sequest (ThermoFinnigan, San Jose, CA; version 27, rev. 12). Sequest was set up to search the Swiss-Prot database (version 51.6) with the TAARP amino acid sequence added in (Boeckmann, Bairoch et al. 2003; UniProt 2007). Sequest was searched with a fragment ion mass tolerance of 1.00 Da and a parent ion tolerance of 2.5 Da. Iodoacetamide derivative of cysteine was specified in Sequest as a fixed modification. Oxidation of methionine was specified in Sequest as a variable modification. Scaffold (version Scaffold_2_01_01, Proteome Software Inc., Portland,

OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 90.0% probability as specified by the Peptide Prophet algorithm (Keller, Nesvizhskii et al. 2002). Protein identifications were accepted if they could be established at greater than 95.0% probability and contained at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm (Nesvizhskii, Keller et al. 2003). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.

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Chapter 4

Discussion and Conclusions

I. Summary

The field of *Francisella* has seen much growth in the past five years, including the development of new molecular tools and techniques, identification of several virulence factors, characterization of host cell entry and intracellular events, advancements in vaccine development, availability of whole genome sequences, understanding gene regulation, and insights about host immune response. However, much remains unknown about *Francisella* pathogenesis, and we are likely just beginning to elucidate the mechanisms of this organism.

The work presented in this thesis contributes to many aspects of *Francisella* research. In Chapter 2, we describe the use of transposome mutagenesis to identify several new virulence factors whose mutant derivatives were attenuated in macrophages and mice. Further studies revealed that four of these strains protected mice against subsequent challenge with wild-type bacteria, thereby contributing to the development of a rationally attenuated vaccine. The continuation of this project to create in-frame, non-polar deletions of these genes in a human pathogen strain of *Francisella* is described in Appendix 1. One of the transposon insertions from Chapter 2 occurred in an uncharacterized ORF that was annotated as a hypothetical lipoprotein. Chapter 3 chronicles the analyses we performed to characterize that ORF, which led to the discovery of a novel protein family, Pat, members of which contain a motif with homology to a repeat domain in a known *Staphylococcus aureus* adherence factor, Eap. In this chapter, I discuss our results in the context of those of other research groups, as well as the implications and future directions of the studies presented in this thesis.

II. Transposome mutagenesis

The first attempts to create transposon mutants in *Francisella* used shuttle mutagenesis or cassette mutagenesis (Anthony, Cowley et al. 1994; Cowley, Gray et al. 2000; Gray, Cowley et al. 2002). Although these methods led to the identification of such *F. tularensis* virulence genes as *minD, clpB,* and the intracellular growth locus *iglABCD,* the techniques were time-consuming and involved assembling libraries, sub-cloning, and recombination. A low number of random insertions and instability of the kanamycin marker were additional drawbacks of the cassette mutagenesis method.

After seeing the relative simplicity and success of using a transposon-transposase (transposome) complex for genome mutagenesis and identification of secreted proteins in *Salmonella* (Geddes, Worley et al. 2005), I decided to apply the technique to *F. tularensis* ssp. *novicida*. Although we did not publish the technique of transposome mutagenesis in *F. tularensis,* we were the first to introduce the idea as a successful means to creating a library of random, stable transposon mutants. This was presented as a poster at the 4th International Meeting on Tularemia in 2003 (Tempel 2003; Kawula, Hall et al. 2004).

The technique of transposome mutagenesis has many benefits over other transposon mutagenesis methods. Creating the transposon-transposase complexes is straight-forward and provides a purified transposase enzyme, forgoing the need for transposase expression by *F. tularensis.* The transposome can be directly electroporated into bacterial cells, upon which the transposase becomes activated by contact with intracellular magnesium ions. The resulting insertions are random and stable, and the transposon can be modified with markers or promoter sequences to suit the researcher's aims.

The *F. tularensis* ssp. *novicida* mutant library described in Chapter 2 was a modest collection of less than 800 mutants, yet we identified over 30 isolates that were attenuated for growth in macrophages. Sixteen of these strains resulted in 100% survival rates in a mouse model of infection with doses 100 times greater than the LD_{50} (6×10^3 CFU versus 66 CFU). When challenged with 8×10^5 CFU of wild-type *F. tularensis* ssp. *novicida*, five mutant strains provided complete protection in mice. With results such as these from a small library, it is probable that there are several other such virulence factors yet to be discovered – it is hardly likely that our findings are simply attributable to luck.

The genes that were disrupted in our protective transposon mutants were *carB*, *dsbB*, *fumA*, the ORF corresponding to FTT0742, and *pdpB*. Although the *carB* mutant was excluded from further vaccine development efforts based on subsequent findings, its mode of attenuation is apparent in that it is required for pyrimidine biosynthesis (Nyunoya and Lusty 1983). Similarly, that the *dsbB* and *fumA* mutants are attenuated is no surprise, as they encode proteins that are required for disulfide bond formation and therefore periplasmic protein folding, and fumarase A, a component of the citric acid cycle, respectively (Ueda, Yumoto et al. 1991; Kadokura, Katzen et al. 2003). Interestingly, while our *F. novicida dsbB* transposon mutant was attenuated and protective in mice, a *dsbB* mutant in the *F. tularensis* ssp. *tularensis* Schu S4 background was also attenuated in mice but failed to protect against wild-type intranasal (i.n.) challenge (Qin, Scott et al. 2008). These results are further discussed in the next section.

Because only the *F. tularensis* ssp. *tularensis* Schu S4 sequence was available at the time of our study, we were unable to assign a potential function or mode of attenuation to the mutant that corresponded to the uncharacterized FTT0742. The ORF

was annotated as a hypothetical lipoprotein, so we speculated that it might have a role in the cell wall or molecule transport. When the *F. tularensis* ssp. *novicida* sequence became available, we found that the insertion occurred in the ORF FTN0715. Characterization of this gene and its product are presented in Chapter 3, and its potential role in *Francisella* pathogenesis is discussed later in this section.

The mechanism of *pdpB* attenuation was not determined, nor was complementation of this mutant attempted due to its location as the second gene in a 12-gene operon on the *Francisella* pathogenicity island (FPI). While it is likely that the insertion in *pdpB* exerted polar effects on downstream genes, it is interesting to note that the C-terminal region of PdpB shares homology with IcmF, a protein that is important for intracellular growth and trafficking in *Legionella* (VanRheenen, Dumenil et al. 2004; Zusman, Feldman et al. 2004). As mentioned in Chapter 1 and shown in Figure 1-6, the IglAB proteins at the opposite end of the FPI are homologs of IcmF-associated homologous proteins (IAHPs); together with the IcmF motif in PdpB, these form the essential components of a type VI secretion system (T6SS) (Das and Chaudhuri 2003; Pukatzki, Ma et al. 2006; de Bruin, Ludu et al. 2007). Thus, it is possible that the insertion in *pdpB* interfered with a yet-undefined secretion apparatus. Further experiments to test if a clean deletion of *pdpB* abrogates protein secretion would answer this question and perhaps reveal whether *Francisella* carries a T6SS.

In addition to our work, the transposome mutagenesis technique has been utilized by other research groups to identify genes required for *Francisella* virulence. Weiss *et al.* used a microarray-based negative selection method that led to the discovery of 44 previously unidentified genes that were required for *Francisella* virulence *in vivo* in an *F.*

tularensis ssp. *novicida* background (Weiss, Brotcke et al. 2007). Concurrent identification of many known virulence factors served to validate this approach. Importantly, the study revealed a class of *Francisella* genes that modulate the host ASC/caspase 1 pathway, providing clues about immune evasion and ways by which bacteria subvert the host immune response (Weiss, Henry et al. 2007). Another group screened a library of nearly 4000 *F. tularensis* ssp. *holarctica* LVS transposome mutants for attenuation in a mouse model of respiratory tularemia by signature-tagged mutagenesis (Su, Yang et al. 2007). This work resulted in the discovery of 341 individual mutants, mapping to 95 genes, that were attenuated for lung infection. Although a subset of the mutations occurred in genes known to be associated with *Francisella* virulence, the majority of insertions were in novel virulence factors. These studies illustrate the usefulness of transposome mutagenesis as an approach to identify new bacterial virulence factors.

Perhaps one of the most important *Francisella* research tools to emerge in recent years is a result of the transposome mutagenesis technique. Gallagher *et al*. exploited the technology in combination with a Genetix Qpix2 robot to build a library of 16,508 unique insertions in an *F. tularenis* ssp. *novicida* background (Gallagher, Ramage et al. 2007). The mutational coverage is nearly twice that of the greatest previously achieved for any bacterial species, with an average of >9 insertions in each nonessential gene. Mutant derivatives were recovered for 1,490, or 84%, of the predicted genes. In addition to the large original library, a second, smaller library consisting of two mutants per allele (the "two-allele set") was constructed to facilitate genome-scale mutant screens in *F. tularensis* ssp. *novicda*. This library is available by request from the University of

Washington and has been distributed to at least five research groups at the time of this writing, including OHSU.

In addition to screening mutant libraries to identify new virulence factors and determine variations among different infection conditions, transposons can be modified to allow for identification of specific types of proteins. For example, my original goal was to identify secreted *Francisella* effectors using transposome mutagenesis in the same way that a fellow graduate student applied the technique in *Salmonella* (Geddes, Worley et al. 2005). In this method, first used in *Yersinia*, the active portion of the *Bordatella pertussis* adenylate cyclase gene (*cyaA'*) is used as a marker to indicate secretion of a bacterial protein (Sory and Cornelis 1994). *cyaA'* catalyzes the formation of cyclic AMP in the presence of calmodulin, which is not found in bacteria. If a transposon carrying *cyaA'* made a translational fusion with a gene encoding a secreted protein, there will be a significant increase in levels of cyclic AMP in the host cell (Figure 4-1). This event can easily be detected using an enzyme-linked immunosorbent assay (ELISA).

In fact, data have been presented in which the *cyaA'* marker was used to indicate that IglC was secreted in *Francisella* (Barker 2006). However, although the ELISA assay was positive for increased cAMP levels, this result was not convincing for IglC secretion. Fluorescence images of *Francisella*-infected macrophages showed IglC to localize around the perimeter of the bacterium rather than throughout the macrophage, raising the question of whether IglC is secreted or surface-exposed. Indeed, this is something to be considered when using *cyaA'* as a marker for secreted proteins. Nevertheless, the matter can be clarified with fractionation experiments to see if the protein remains associated with bacteria or if it is found in the host cell cytoplasm.

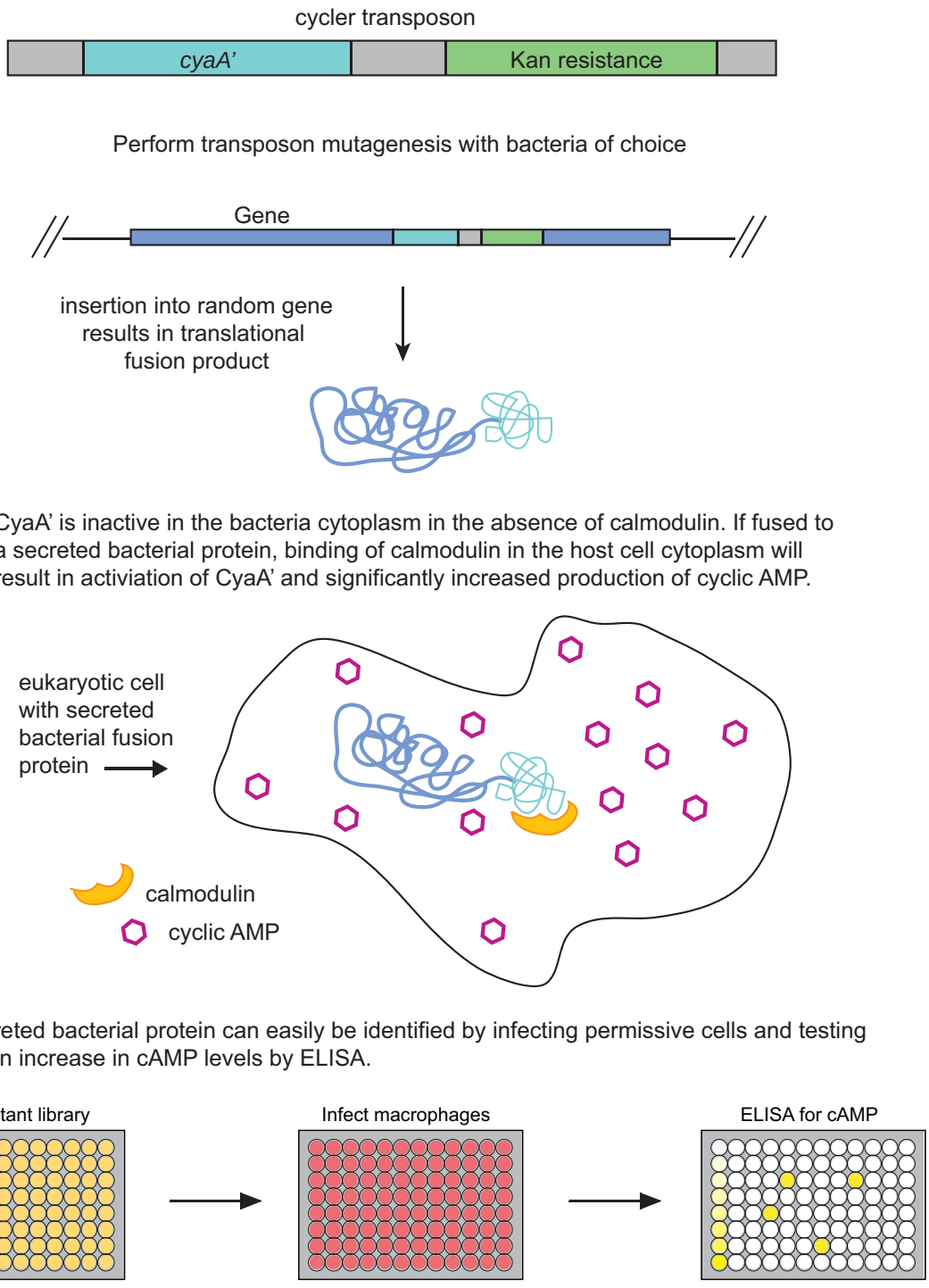

Figure 4-1. Method for identifying novel secreted effectors by fusion to CyaA'. A transposon carrying the active region of the adenylate cyclase gene (*cyaA'*) from *B. pertussis* may be used to identify unknown secreted bacterial proteins. CyaA' is only active in the presence of calmodulin, which is not found in bacteria. Insertion of a *cyaA'* transposon, such as cycler, to form a translational fusion with a secreted bacterial protein would result in high levels of cyclic AMP in the presence of host cell calmodulin. Production of cAMP can be detected by an enzyme-linked immunosorbent assay.

In conclusion, our work in Chapter 2 demonstrates the value and ease of using transposome mutagenesis to identify virulence genes and potential vaccine strains of *Francisella.* Other research groups have since applied this methodology to discover additional virulence factors and gain insight into *Francisella* pathogenicity. While the development of a comprehensive *Francisella* transoposon mutant library is a very valuable research tool, the use of modified transposomes to identify secreted or surfaced-expressed *Francisella* proteins remains virtually untapped and would likely be a fruitful research endeavor.

III. Development of a live tularemia vaccine

As discussed in the introduction, approaches to a tularemia vaccine have included killed vaccines, subunit vaccines, and live attenuated *Francisella* strains that provide protection against subsequent exposure. Killed vaccines, although able to protect against ulceroglandular and typhoidal tularemia, were dangerous to the recipients and sometimes caused tularemia (Conlan and Oyston 2007). Subunit vaccines have seen limited success and rely upon a consistent adjuvant for proper delivery (Thomas, Titball et al. 2007; Huntley, Conley et al. 2008). In contrast, live vaccine strains have been moderately successful, with some causing no outward signs of illness in a mouse and providing full protection against subsequent wild-type challenge (Twine, Bystrom et al. 2005; Pechous, Celli et al. 2006; Tempel, Lai et al. 2006). For these reasons, I am focusing my discussion of the development of a tularemia vaccine on live vaccines strains.

While interesting to ponder, it is difficult to accurately predict which genes or pathways should be mutated to make a live vaccine. In Chapter 2, we identified 16 *F.*

tualrensis ssp. *novicida* mutants that were attenuated in mice. Half of these carried unique insertions in genes in the purine synthesis pathway, yet none of the *pur* mutants fully protected against wild-type challenge. We found this result particularly notable because it had been widely speculated that mutations in this pathway would provide the basis for a live *Francisella* vaccine and because defined allelic mutations disrupting purine synthesis had already been developed for use as vaccine strains in other bacterial species (Karlsson, Prior et al. 2000; Oyston and Quarry 2005). Indeed, while our manuscript was in press, another report was published describing a *purMCD* mutant that was attenuated and protective in a mouse model of tularemia infection (Pechous, Celli et al. 2006). Although this mutant was in the *F. tularensis* ssp. *holarctica* LVS strain background and mice in the experiments were challenged with significantly fewer bacteria (5×10^4 vs. our 6×10^7), it still raised the question of whether any of our *pur* mutants would have been protective if challenged at a lesser dose and if this would be sufficient for the basis of a live attenuated tularemia vaccine.

In addition to our five mutant strains discussed in Chapter 2 and the *purMCD* mutant mentioned above, few *Francisella* strains have been tested for efficacy as a live vaccine against tularemia. Among these are two other *pur* mutants, *purA* and *purF*, as well as the deletion derivatives of the uncharacterized ORF FTT0918, *iglC*, and *dsbB* in the *F. tularensis* ssp. *tularensis* background. Table 4-1 summarizes the immunization and challenge routes and doses and the percent of surviving mice for these strains. For brevity, only the largest challenge dose with 100% survival is shown if serial doses were used. It is interesting to note that the threshold challenge dose for other groups was often only 100 times the LD_{50}, while we saw full protection using challenge doses of 10^6 times

Table 4-1. Efficacy of *Francisella* mutant strains as potential live vaccines in mice.

Vaccine used	Strain background	Vaccine dose (CFU) / route	% Survival	Challenge dose (CFU) / route	% Survival
dsbB	*F. novicida* U112	6×10^5 i.p.	100	6×10^7 i.p.	100
		6×10^6 i.p.	20	6×10^7 i.p.	100
FTN0715 (*patB*)	*F. novicida* U112	6×10^7 i.p.	100	6×10^7 i.p.	100
pdpB	*F. novicida* U112	6×10^7 i.p.	100	6×10^7 i.p.	100
fumA	*F. novicida* U112	6×10^5 i.p.	100	6×10^7 i.p.	100
carB	*F. novicida* U112	6×10^5 i.p.	0	ND	ND
ΔFTT0918[a]	*F. tularensis* Schu S4	10^5 i.d.	100	500 i.d.	100
		10^5 i.d.	100	~10 aerosol	33
iglC[a]	*F. tularensis* Schu S4	10^6 i.d.	100	500 i.d.	0
		10^6 i.d.	100	~10 aerosol	0
purMCD[b]	*F. holarctica* LVS	5×10^1 i.p.	100	5×10^1 i.p.	100
		5×10^1 i.p.	100	5×10^2 i.p	66
		5×10^1 i.p.	100	5×10^3 i.p	0
		5×10^2 i.p.	100	5×10^2 i.p	100
		5×10^2 i.p.	100	5×10^3 i.p	33
		$5 \times 10^{3,4,5,\text{or } 6}$ i.p.	100	$5 \times 10^{1,2,\text{ or } 3}$ i.p	100
purMCD[c]	*F. holarctica* LVS	10^6 i.n. no boost	100	Mice were challenged with Schu S4: a. 100 i.d. b. 2000 i.d. c. 100 i.n. d. 2000 i.n.	0
		10^6 i.n. w/ boost	100		100, 100, 100, 33
	F. tularensis Schu S4	10^4 i.n. no boost	100		66, 100,14, 0
		10^4 i.n. w/ boost	100		100, 100, 70,0
purA[d]	*F. novicida* U112	3.3×10^2 i.p.	NI	170 i.p.	40
		$3.3 \times 10^{3,4,5,\text{or } 6}$ i.p.	NI	170 i.p.	20
purF[d]	*F. novicida* U112	7×10^2 i.p.	NI	170 i.p.	40
		7×10^5 i.p.	NI	170 i.p.	100
		? s.c.	NI	170 i.p.	0
		? i.p or s.c.	NI	$100 \times LD_{50}$ Schu S4	0
dsbB[e]	*F. tularensis* Schu S4	6.8×10^3 i.n.	100	13, 1300, or 13000 i.n.	0
		6.8×10^3 i.n.	100	130	25

[a](Twine, Bystrom et al. 2005) [b](Pechous, Celli et al. 2006) [c](Pechous, McCarthy et al. 2008) [d](Quarry, Isherwood et al. 2007) [e](Qin, Scott et al. 2008) i.p. – intraperitoneal; i.d. – intradermal; i.n. – intranasal; NI – not indicated

the LD_{50}. In our experiments, all of the mice that survived the vaccination survived the challenge, regardless of the dose. Even in the studies using minimal challenge doses, 100% survival after challenge was not a common result, with the exception of the *purMCD* studies that incorporated a boost dose prior to challenge (Pechous, McCarthy et al. 2008). Together, these results indicate that our approach of testing attenuation with an initially high vaccination allowed for the discovery of more robust strains that can induce stronger immune responses and protect against larger challenge doses.

There are several parameters that must be considered in creating a rationally attenuated live vaccine strain. As the mutations must be non-reverting and non-polar on downstream genes, they would best be in-frame deletion mutants achieved by allelic exchange. The engineered strains must also not include any introduced antibiotic resistance, so care must be taken designing the molecular techniques to generate the mutations. The vaccine with the highest chance of licensure would comprise multiple attenuating mutations but not be over-attenuated. Because there appears to be limited cross-protection among the different subspecies and because *F. tularensis* ssp. *tularensis* is the most virulent, the parental strain for the basis of the vaccine would likely be a subspecies *tularensis* strain such as Schu S4. However, the ideal live vaccine would afford cross-protection between different subspecies of *Francisella*, as well as by various routes of inoculation and challenge, so challenge with non-parental subspecies and by multiple routes would be necessary to test. For example, intranasal vaccination with a subspecies *tularensis*-based vaccine should be tested by i.p. challenge with *F. tularensis* ssp. *holarctica*. Another consideration is that of safety: research with *F. tularensis* ssp. *tularensis* requires working under BSL-3 conditions with highly infectious bacteria.

Because of the similarity of the *Francisella* genomes, preliminary vaccine strains can be identified in lesser virulent strains such as *F. novicida* or the current live vaccine strain LVS. As described in Chapter 2 and Appendix 1, we used *F. novicida* to identify protective mutants and are making antibiotic-sensitive in-frame deletion derivatives in *F. tularensis* ssp. *tularensis* Schu S4 to pursue the development of a rationally attenuated live vaccine strain.

Although it is safer for researchers to initially identify potential vaccine candidates by screening in a lesser virulent strain, it must be remembered that what is protective in *F. novicida* may not necessarily have the same phenotype in a more virulent strain, as seen with *dsbB* mutant derivatives (Table 4-1) (Tempel, Lai et al. 2006; Qin, Scott et al. 2008). On the other hand, the *F. tularensis* ssp. *tularensis* Schu S4 *dsbB* mutant tested for protection was not a clean deletion but a transposon mutant that did not appear to disseminate to the spleen, liver, or lungs (Qin, Scott et al. 2008). Dissemination from the point of inoculation and clearance by the time of challenge is necessary for a good vaccine candidate because it indicates that the vaccine dose causes an immune response in the host. Also, while *in trans* expression of the wild-type gene mostly complemented the growth defect in cells, there could still be polar effects on downstream genes. Therefore, it may be worthwhile to continue creating an in-frame deletion of *dsbB* for testing as a potential vaccine candidate.

As discussed, few other *Francisella* strains have been tested for use as a live vaccine. Interestingly, some mutant strains that are attenuated in mice were not tested for protection against wild-type challenge. Most recently, a study found Δ*pilF* and Tn-*pilT* mutants in an LVS background to be highly attenuated in mice when infected i.d. with

10^6 or 10^7 CFU, yet there was no mention of challenging the surviving mice with wild-type LVS (Chakraborty, Monfett et al. 2008). This is disappointing, as the experiment to test these mutants for efficacy as potential vaccines would not have involved mice other than those already infected, would have taken minimal time and effort, and would have provided information about the protective capacity of *pil* mutants.

There are doubtless many yet unidentified targets for generating an attenuated and protective strain of *F. tularensis*. The lack of complete protection with the *pur* mutants exemplifies the difficulties with predicting which mutations may make good vaccine candidates, as this pathway was suspected to be a prime target for vaccine development. Therefore, the approach we took of creating random mutations in the genome and testing those strains that were attenuated in cells and mice for protection against wild-type challenge is the method most likely to yield the best vaccine candidates because it relies on phenotypic results instead of educated guesses and extrapolations from other pathogens. With continued identification of *Francisella* virulence factors and complete testing of attenuated mutants for protection against subsequent wild-type challenge, it is quite plausible that a licensable live tularemia vaccine will be engineered.

IV. Discovery of the TAARP domain and implications for host cell entry

At the time of our transposon mutagenesis study, only the *F. tularensis* ssp. *tularensis* Schu S4 genome sequence was available. As a result, the protective *F. novicida* strains we discovered were identified as having insertions in genes corresponding to *dsbB*, *pdpB*, *fumA*, *carB*, and FTT0742. When the *F. novicida* U112

genome sequence became available, we confirmed that the transposon insertions occurred in the homologous genes, and that FTN0715 was the correlating ORF for FTT0742.

Further comparison of FTT0742 with the *F. novicida* genome sequence revealed the presence of seven tandem repeat domains comprised of 102 amino acids in the FTN0715 translated ORF that occurred in single copy in the FTT0742 ORF. Additionally, a second homolog in *F. novicida*, FTN0714, was located immediately adjacent to and upstream of FTN0715. FTN0714 also contained seven tandem repeats, as well as a longer C-terminal domain with no homology to other proteins of known function. Investigation of other *Francisella* sequences resulted in the discovery of highly similar FTT0742 homologs in every sequence available, thus constituting a protein family, designated Pat for "Pathogenicity factor". The repeat domain was found in one or two copies in subspecies *tularensis* or *holarctica* strains and was named the TAARP domain for "Tandem Amino Acid RePeat". Neither the translated sequence of the full-length homologs, nor the TAARP domain was found to have homology to any proteins of known function by BLAST analysis.

Intrigued by the novelty of this repeat motif, we decided to express a recombinant His-tagged version of the *F. tularensis* Schu S4 TAARP domain in an IPTG-inducible *E. coli* vector for further analysis. As discussed in Chapter 3, we used circular dichroism spectroscopy, intrinsic fluorescence, and gel filtration analysis to glean information about the structure and stability of the TAARP domain. Results from these studies showed that the TAARP motif is a stable, independently folded domain with β-sheets and aromatic stacking that is capable of homo-oligomerization.

In addition to the structural data, potential roles for the TAARP domain during *Francisella* infection were also discovered. Using the Rosetta computer modeling program, we generated a structure that is consistent with the solution structure of the TAARP domain. Comparison of the TAARP domain with structures from the PDB database suggested that the TAARP domain was similar to the EAP domains found in Eap (Extracellular adherence protein), a virulence factor secreted by *Staphylococcus aureus* that also displays a modular architecture (Figure 3-11). Although it lacks a defined transmembrane domain, ~30% of Eap is found in association with the bacterial cell surface by protein-protein interactions, uncharacterized membrane structures, and Eap oligomerization (Palma, Haggar et al. 1999; Flock and Flock 2001; Kreikemeyer, McDevitt et al. 2002). While relatively little is known about its role in *S. aureus* virulence, Eap has been shown to inhibit neutrophil recruitment and appears to be involved in activating expression of the proinflammatory cytokines IL-6 and TNF-α, which may contribute to septic shock and fever (Chavakis, Hussain et al. 2002; Scriba, Sierro et al. 2008). Additionally, Eap has been shown to enhance bacterial internalization into eukaryotic cells and is known to bind host cell factors such as fibronectin and prothrombin, indicating a role in host cell entry (Palma, Haggar et al. 1999; Haggar, Hussain et al. 2003). Given the sequence and structural similarities between the EAP and TAARP domains, potential follow-up experiments to examine the role of the TAARP domain might include investigating if it induces cytokine production, binds specific host factors such as fibronectin and prothrombin, or acts as an anti-inflammatory molecule by inhibiting recruitment of neutrophils.

In our co-purification assay and and subsequent mass spectrometry analysis, we identified δ-catenin (p120) as a host protein that binds the TAARP domain. As discussed in Chapter 3, δ-catenin interacts with the intracellular regions of E-cadherin and is thought to play a role in regulating the adhesive properties of this receptor (Gumbiner 2005). E-cadherin is a membrane-spanning adhesion molecule that is important for the stability of adherens junctions (AJ) in epithelial cells. Although *Fracisella* infection is predominantly associated with macrophages, it has been shown that these bacteria infect alveolar type II epithelial cells in the lungs (Hall, Craven et al. 2007). Thus, a possible result of the interaction between δ-catenin and the TAARP domain in the host cell cytoplasm could be disruption of junctions between adjacent epithelial cells. This mechanism would allow *Francisella* access to underlying tissues, as well as potentially inhibiting co-infection with other pathogens, such as *Listeria*, that use E-cadherin as a receptor for host cell entry. In support of this hypothesis, a recent report indicated that infection with the arthropod-borne intracellular bacterial pathogen *Rickettsia rickettsii* increased microvascular permeability, presumably by interfering with AJs (Woods and Olano 2008). If *Francisella* TAARP binds the δ-catenin/E-cadherin complex to disrupt epithelial junctions during the course of infection, this could be measured by infecting a monolayer of cells and testing for changes in conductivity.

Although δ-catenin is a cytoplasmic protein, its interaction with TAARP may have implications for a *Francisella* entry mechanism through the E-cadherin pathway. While it wasn't detected by mass spectrometry analysis, it is possible that E-cadherin interacts with the TAARP domain and was not recognized because of the overabundance of the TAARP domain in the samples or due to a lack of cleavage by trypsin.

Nevertheless, re-probing of the co-purification samples with an E-cadherin antibody indicates that E-cadherin is present and does therefore bind the TAARP domain. Further studies are currently underway to explore this possible interaction.

The hypothesis that *Francisella* can enter host cells via an E-cadherin pathway is further substantiated by the bacterial pathogen *Listeria* using this mechanism to cause infection (Mengaud, Ohayon et al. 1996). As shown in Figure 4-2, E-cadherin is the host cell receptor for the *Listeria* surface protein Internalin A (InlA) (Ireton and Cossart 1997). Although InlA binding to E-cadherin results in the engulfment of *Listeria*, and E-cadherin interacts with several components involved in actin polymerization and remodeling, it is unknown if the actin polymerization that drives *Listeria* uptake has the same mechanisms as the cytoskeletal remodeling for forming cell-cell junctions. If E-cadherin were found to be an entry receptor for *Francisella*, it would be interesting to investigate the roles of the different components of actin polymerization during bacterial uptake to see if they are involved in forming pseudopod loops.

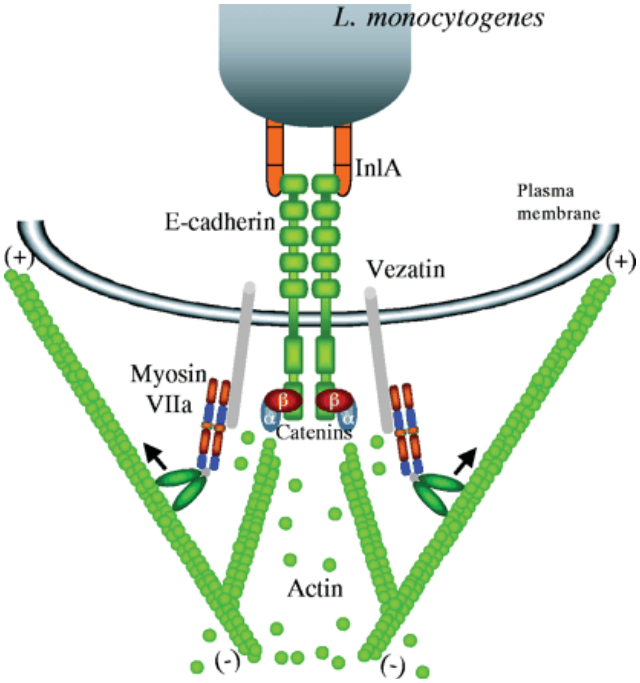

Figure 4-2. Model for InlA-dependent entry of *Listeria monocytogenes*. *Listeria* InlA binds the extracellular domain of host cell E-cadherin. Proteins known to play a role in entry are indicated and include E-cadherin, α- and β-catenins, vezatin, myosin VIIa, and actin. This model highlights new findings that indicate how myosin VIIa could influence actin polymerization leading to host cell membrane rearrangements during *Listeria* entry. [Reproduced with permission from (Sousa, Cabanes et al. 2004)]

A recent report showed that *Francisella* interacts with cholesterol-rich domains, or lipid rafts, on the host cell membrane (Tamilselvam and Daefler 2008). When cholesterol was depleted from these regions, entry and intracellular replication of *Francisella* was significantly reduced. Likewise, the disruption of lipid rafts by depletion of cholesterol inhibited binding and entry of *Listeria* (Seveau, Bierne et al. 2004). A subpopulation of E-cadherin was found to be associated with cholesterol-rich domains of the host membrane (Golub, Wacha et al. 2004). When cholesterol was depleted, the surface levels of E-cadherin were not diminished, but clustering of E-cadherin around *Listeria* was affected, suggesting that lipid raft composition has an effect on the binding properties of E-cadherin (Seveau, Bierne et al. 2004). With the recent data regarding *Francisella*'s interaction with cholesterol-rich lipid rafts, it is exciting to speculate that *Francisella* may employ an entry mechanism similar to that of *Listeria*. The notion raises several points, such as the presence and localization of E-cadherin in lipid rafts during *Francisella* infection and how or if that is affected by depletion of cholesterol.

Although our *F. novicida patB* transposon mutant was not defective for entry in macrophages (Figure 2-1), this result could be explained by the transposon inserting in the sixth TAARP domain or by the expression of *patC*. *F. novicida* is the only *Francisella* subspecies so far that appears to have two homologs of *pat.* An interesting experiment would be to make a clean deletion of both ORFs or a clean deletion of *patA* in another subspecies and test if entry is affected. However, it should be kept in mind that *Francisella* may have multiple factors for binding host cell receptors and that Pat and the TAARP domain may not be the sole mechanism for *Francisella* entry. Another worthwhile experiment would be to delete only the TAARP domain(s) to assess its

specific role in host cell entry. If TAARP were shown to have a role in host cell entry or intracellular survival, additional studies could include generating point mutations of the TAARP domain to determine which residues are required for acheiving the correct structure and stability for the domain to be functional.

If Pat and the TAARP domain were indeed found to be a mechanism by which *Francisella* enters host cells, that would be a very significant finding. With the sequence and structural homology of the TAARP and EAP domains, the MS identification of δ-catenin as a binding partner of TAARP, the preliminary detection of E-cadherin in the co-purification assay, and the similarities between *Francisella* and *Listeria* uptake with regard to cholesterol-rich lipid raft domains in host cells, a model for TAARP-mediated *Francisella* entry via the E-cadherin pathway can be constructed (Figure 4-3).

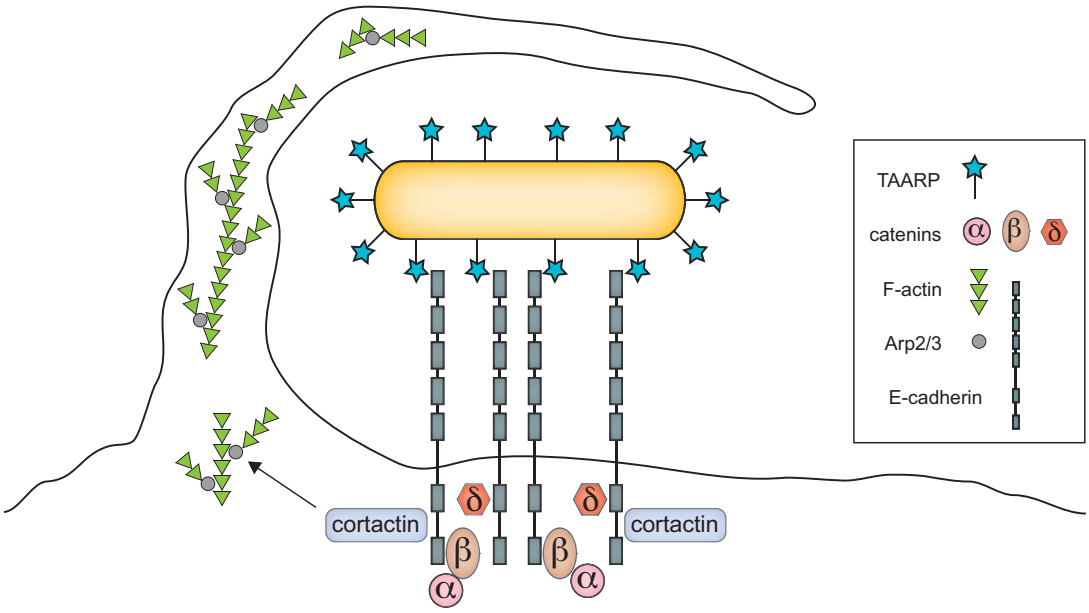

Figure 4-3. Model for TAARP-mediated *Francisella* entry via E-cadherin. The *Francisella* TAARP domain of *Pat* has been shown to interact with δ-catenin, and preliminary data indicate that TAARP binds E-cadherin. The cytoplasmic domains of E-cadherin are known to interact directly with δ-catenin and β-catenin, as well as cortactin. Activated cortactin recruits Arp2/3 complex proteins, which provide nucleation sites for the formation of filamentous actin (F-actin). The polymerization of actin results in cytoskeletal rearrangements, as seen in pseudopodia. *Francisella* is thought to be engulfed by a mechanism involving pseudopod loops.

V. Conclusions

The past five years have been an exciting time for *Francisella* researchers. We have seen tremendous growth in all aspects of the field and are continuing to unravel the processes of *Francisella* pathogenesis. The research presented in this dissertation contributes to the areas of genetic manipulation of *Francisella*, identification of unique attenuated and protective virulence factors, design of an approved tularemia vaccine, and suggests a potential entry mechanism similar to that of *Listeria*. Together, these data have shed some light on the mechanisms behind *Francisella* pathogenicity. More importantly, they open the door to more research questions and are the beginning point for several future projects, thus perpetuating the chain of discovery.

Appendix 1

Generation of in-frame deletions in virulence factor genes in the type A *F. tularensis* strain Schu S4 via allelic replacement: steps toward a rationally attenuated live tularemia vaccine

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Preface

This work is currently in preparation by Rebecca Tempel, Roger Pechous, Travis McCarthy, Thomas Zahrt, and Fred Heffron.

My contributions to the study include conception of the project, modification of the suicide vector plasmid for use with *Francisella*, primer design for the SOE-PCR, generating the in-frame deletion mutant constructs in *E. coli*, analyzing data, and preparing the figures and the manuscript to date.

Roger Pechous and Travis McCarthy contributed to this work by performing conjugation between *E. coli* and *F. tularensis* Schu S4 to achieve the merodiploid strains.

Thomas Zahrt contributed to this study by providing the alternate *sacB* cassette under control of a native *Francisella* promoter, preparing the photographs in figure A1-5, advising on experimental design, and providing space, equipment, and supplies with which to perform the conjugations.

Fred Heffron contributed to this work by providing advice and suggestions on experimental design and results, as well as the space, equipment, and supplies with which to perform the experiments.

Summary

Francisella tularensis is the causative agent of tularemia, a debilitating and potentially fatal disease that affects humans and a broad range of animals. Despite its high infectivity and classification as a category A select agent, there is no approved vaccine available. In a recent study, we devised a transposon mutagenesis technique to aid in the discovery of novel *Francisella* virulence factors, resulting in the identification of several attenuated strains of *F. novicida.* Four of these strains were highly attenuated and protective against wild-type infection in a mouse model of tularemia and are thus a good basis for the development of a rationally attenuated live tularemia vaccine. Here, we summarize our efforts to introduce in-frame deletions of these genes into the chromosome of the type A *F. tularensis* strain Schu S4 to create non-polar, non-reverting, antibiotic resistant potential live vaccine strains.

Introduction

Tularemia is the incapacitating and potentially lethal disease caused by the bacterial pathogen *Francisella tularensis*. Infections can be acquired through multiple routes, which include bites from an arthropod vector such as a tick or deerfly, skin lesions, ingestion of contaminated food or water, and, most dangerously, by inhalation of as few as 10 bacteria (Dennis, Inglesby et al. 2001). The low dose required to cause tularemia by aerosol route resulted in the development of *F. tularensis* for use as a biological weapon by several national weapons programs (Alibek 1999; Dennis, Inglesby et al. 2001). In 1969, the World Health Organization estimated that an aerosol dispersal of 50 kg of virulent *F. tularensis* over a metropolis of 5 million people would result in 19,000 deaths and 250,000 cases of serious illness, as well as enormous economic damage (Kaufmann, Meltzer et al. 1997; Dennis, Inglesby et al. 2001). Currently, the U.S. Centers for Disease Control and Prevention (CDC) classifies *F. tularensis* as a Category A select agent, members of which are considered most serious in posing a risk to national security. In addition to its potential role in a bioterror attack, *F. tularensis* is found throughout the Northern Hemisphere, and the incidence of tularemia is especially high in farmers and hunters in Scandinavian countries (Keim, Johansson et al. 2007; Sjostedt 2007). Despite these factors, there is no approved tularemia vaccine available in the U.S. or Europe, making the development of a vaccine against *F. tularensis* an international research priority.

Four main subspecies of *F. tularensis* are commonly recognized: *tularensis* (type A), *holarctica* (type B), *novicida*, and *mediasiatica*. All of these biotypes share greater than 95% DNA sequence identity (Broekhuijsen, Larsson et al. 2003). Although type A

and type B strains are highly infectious, only type A strains cause significant mortality in humans. The current live vaccine strain (LVS) is an attenuated type B strain that provides varying levels of protection against challenge with type A *F. tularensis* strains depending on route of immunization, route of challenge, and genetic background of the host (Chen, Shen et al. 2003; Chen, KuoLee et al. 2004; Shen, Chen et al. 2004; Shen, Chen et al. 2004; Conlan, Shen et al. 2005; Green, Choules et al. 2005; Wu, Hutt et al. 2005). Because the molecular basis for LVS attenuation remains unknown, this strain is not licensed as a tularemia vaccine. Although *F. novicida* is not considered a human pathogen, it displays a similar degree of virulence in mice as other *F. tularensis* subspecies (Kieffer, Cowley et al. 2003; Shen, Chen et al. 2004). With its genetic similarity to other *Francisella* subspecies and avirulence in humans, *F. novicida* provides an excellent alternative to virulent type A and B strains for preliminary vaccine studies in a mouse model system. The fourth subspecies, *F. mediasiatica*, is considered infectious for humans but is not well studied (Keim, Johansson et al. 2007).

While the molecular mechanisms of *F. tularensis* pathogenesis are yet to be fully elucidated, it is understood that replication in macrophages is central to the organism's ability to cause disease (Fortier, Green et al. 1994). Accordingly, we designed a technique to identify genes in *F. novicida* that are required for invasion and growth in macrophages. We generated a library of over 700 *F. novicida* transposon mutants and screened them for attenuation in macrophages and avirulence in mice. The mutant strains that exhibited attenuation in mice were further tested to determine if they could provide protection against subsequent infection with wild-type bacteria. These studies led to the identification of five mutant *F. novicida* strains that are attenuated and protective in mice.

Sequence analysis showed that the disrupted genes in these five strains corresponded to four known genes (*dsbB*, *fumA*, *pdpB*, and *carB*) and one hypothetical gene (designated FTT0742, since renamed *patA*) in the *F. tularensis* type A Schu S4 strain. The mutant derivatives were further assayed for growth defects in enriched medium, number of transposon insertions, and specific levels of entry and attenuation in different macrophage cell lines. In addition, we examined the conservation of the mutated genes and their functions between *F. tularensis* subspecies by complementing the attenuation defects with wild-type Schu S4 genes. Using a mouse model system, we also tested the mutant strains for *in vivo* attenuation levels (LD_{50} values), dissemination from the site of the infection, and lack of persistence. Due to lower levels of attenuation in cells and mice and its inability to disseminate to the liver and lungs, the *carB* mutant was dismissed as a potential vaccine candidate. However, the remaining four mutant strains show great promise as the basis for a rationally attenuated live vaccine against tularemia.

Because type A strains are the most virulent for humans and thus the likely basis for a weaponized *Francisella* strain, the most effective tularemia vaccine should also be derived from a type A strain. Further parameters in constructing an approved vaccine are that 1) any mutations in the identified genes cannot be polar on downstream genes, 2) the mutations must not be able to revert at any frequency, and 3) the final vaccine strain cannot express antibiotic resistance. To satisfy each of these requirements, we used an allelic exchange method that was optimized for *Francisella* (Golovliov, Sjostedt et al. 2003). Here, the work conducted towards the generation of these potential tularemia vaccine strains is summarized.

Results

Allelic exchange technique

To generate non-polar, non-reverting, antibiotic sensitive mutant strains of the virulence genes we identified in a previous study, we aimed to replace the wild-type genes with in-frame deletion derivatives via allelic exchange. In this approach, a suicide vector carrying a mutant copy of the target gene is introduced into the recipient strain. The mutated gene, a deletion derivative in our case, becomes inserted into the chromosome via homologous recombination. Merodiploids can be selected for by growth on media containing an antibiotic (or other selective marker), as the integration of the vector DNA may provide transient antibiotic resistance. Subsequent growth under counter-selective conditions will select those colonies that have resolved the plasmid DNA, resulting in a loss antibiotic resistance and one copy of the gene by a second recombination event. Figure A1-1 provides a schematic representation of this technique.

For our purposes, the plasmid pDM4 was chosen, as it can replicate in *E. coli* but not in *F. tularensis*, carries the *sacB* counter-selective marker, and has the mobilization element mobRP4 to allow transfer by conjugation (Figure A1-2). Levansucrase is encoded by *sacB*, making growth in sucrose toxic for bacteria carrying the gene because they accumulate polymers of fructose (levans) in the periplasm during sucrose hydrolysis (Pelicic, Reyrat et al. 1996). Therefore, after the first recombination event has occurred to insert the plasmid DNA into the *F. tularensis* chromosome, growth on media containing sucrose will ensure that the second recombination event has taken place to resolve the vector DNA. PCR analysis can then be performed to assess if the full-length or deleted copy of the target gene remains on the chromosome (as diagrammed in Figure A1-1).

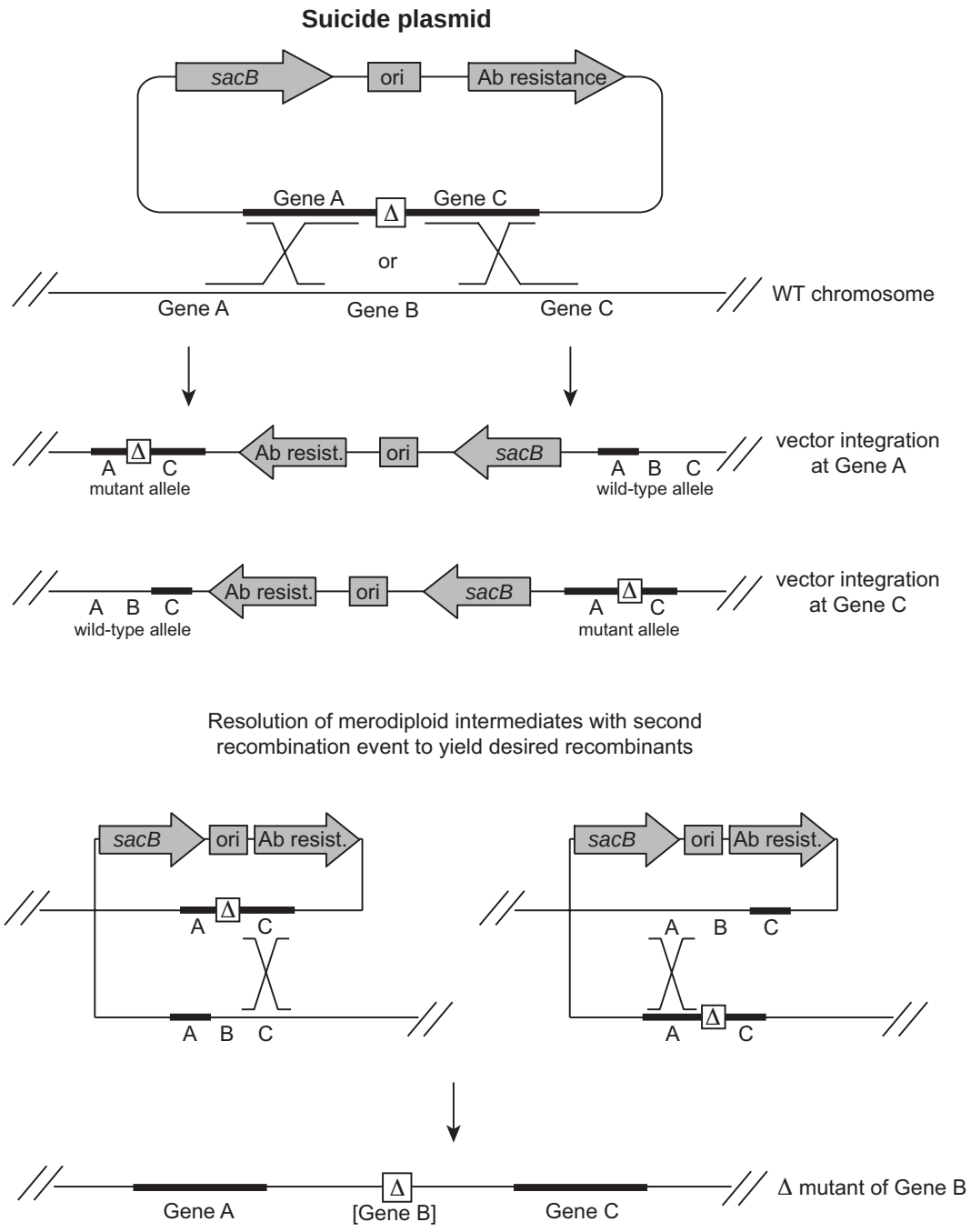
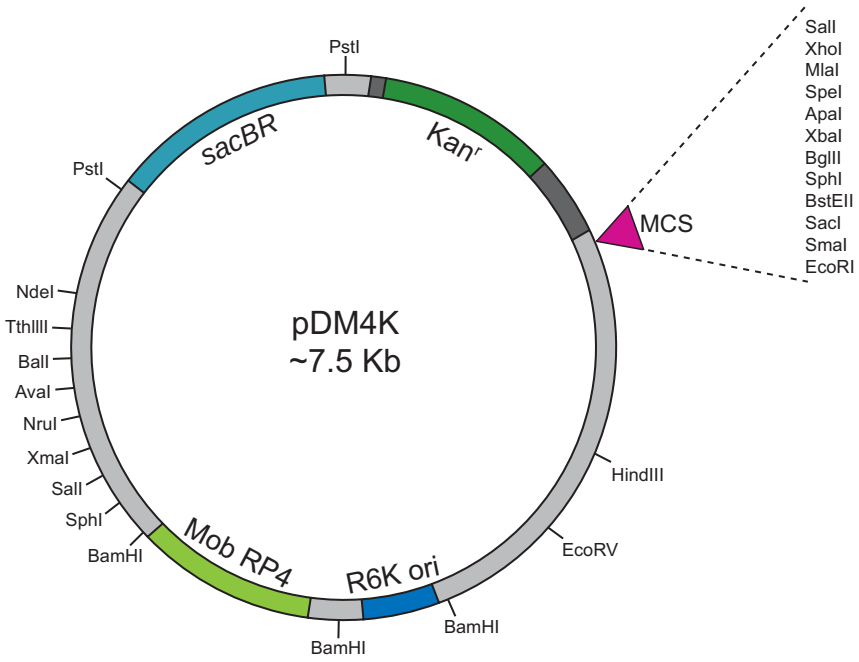


Figure A1-1. Allelic exchange to generate unmarked deletions. A suicide plasmid carrying an in-frame deletion of the gene of interest (Gene B in this figure) with large upstream flanking regions is introduced into bacteria. The vector is integrated into the chromosome via homologous recombination between the flanking regions (Gene A or C). Merodiploids can be identified by growth on selective medium. A second cross-over event resolves the vector sequence, including the antibiotic resistance marker. Recombinants are selected for by growth in sucrose, which is toxic to bacteria expressing *sacB*. The result is an unmarked deletion in place of the wild-type gene.



Lineage of pDM4K

pBR322 (Amp^r) + R6K ori
- pRK703 (both R6K ori and ori E1)

remove ori E1
- pJM703

add MobRP4
- pJM703.1

add MCS of M13tg131
- pGP704

replace Amp^r with Cm^r (CAT gene)
- pNQ705

add SacBR genes
- pDM1

repair MCS
- pDM4

insert Kan^r into CAT, remove part of CAT
- pDM4K (Kan^r, Cm^s)

Figure A1-2. Map and lineage of pDM4K. A schematic of pDM4K, including the multiple cloning site (MCS), is shown. Significant features of this suicide vector, such as the R6K origin of replication, a mobilizable element (Mob RP4), the levansucrase cassette (*sacBR*), and kanamycin resistance cassette (Kan^r) are labeled. The modifications and resulting plasmid names leading to pDM4K are shown.

Modification of pDM4

The original antibiotic marker of pDM4 was a chloramphenicol (Cm) resistance cassette. Because this drug is used to treat tularemia, creating type A *F. tularensis* strains with chloramphenicol resistance, even transiently, is not permissible. Therefore, pDM4 was modified to be sensitive to chloramphenicol and harbor kanamycin (Kan) resistance. The Cm cassette was disrupted by inserting a Kan cassette via blunt-end cloning with *Pvu*II sites (Kan) and *Sca*I sites (Cm). Subseqent digestion with *Bsm*I, followed by end repair and re-ligation, deleted a significant region of the Cm cassette. Resulting colonies exhibited Cm sensitivity and Kan resistance, and the plasmid was designated pDM4K (Figure A1-2).

Further modification of pDM4K proved to be necessary when post-conjugative merodiploids failed to resolve after growth on sucrose. It was suspected that the promoter from original *B. subtilis sacB* cassette was not sufficient in *F. tularensis*, so it was replaced with a *sacB* cassette containing the *sacB* gene under control of the groE promoter from *Francisella*.

In-frame deletions of *dsbB, pdpB, fumA*, and FTT072

The in-frame deletions were constructed by splicing by overlapping extension PCR (SOE-PCR). In this technique, 1 to 1.5 kb regions up- and downstream of the target gene, including part of the target gene, are amplified by PCR. The primers sets for the two regions were designated “outer forward and inner reverse” (OF/IR) and “inner forward and outer reverse” (IF/OR) and are listed in Table A1-1. The IR and IF primers were designed to share a significant number of overlapping bases that allowed the PCR

Table A1-1. List of primers for generating in-frame deletions in Schu S4.

Primer name	Sequence (5' to 3')
pdpB	
OF	CTCGAGAGCACTTTGGACTAAGCACAAACC
IR	TGCTTGATTACCCAGGTACATGCATTTTTTATGGTCTTTGAGGCAG
IF	CAAAGACCATAAAAAATGCATGTACCTGGGTAATCAAGCACAAAG
OR	AGATCTCCAACCATTGTTGCTGTAGAACC
Up1	AGAACAACCCGTTTTATGGC
Down1	ACTAATACAACTGCTAAGCCCTATG
Up2	AATCAAAAGGAAATTAAAAGTATG
Down2	TTGCTAAATAAATTCTCAAACC
FTT0742	
OF	CTCGAGATGGTATAGATACACCCCAGCCAG
IR	GTTCGCCGTGTGTGGAATATAACCGCAGCCAACTAAAGCAATAGGTAG
IF	ATTGCTTTAGTTGGCTGCGGTTATATTCCACACACGGCGAACG
OR	AGATCTACCCTGATCTATCCAACGTGATGG
Up1	CCATTATTCCTCTCTCAGACC
Down1	GCTTGCAGATATTTGGTGAC
Up2	AAAATCGGCGATGAGTTC
Down2	AAATTGGAATAGCCTGGC
fumA[a]	
OF	CTCGAGGCTCACCAATTAGTGACCATCCTC
IR	TATGAAGCATATCAGCGTGATCAGCAAGGTCCTAAACTTTGGAAAC
IF	AAAGTTTAGGACCTTGCTGATCACGCTGATATGCTTCATACATTG
OR	AGATCTAATTAGCGAGGTTGGCAAGAGGA
Up1	GGCTAAAGAAAGCTATTGTATTC
Down1	ACTAACTCTTGCATAGATTAGACC
Up2	ATTAAATAAATGGCTGTTATC
Down2	TTGATATGCTAAAACCTCAC
dsbB	
OF	GGGCCCTGGCGCCGTTAGAGATATGTT
IR	GGTGATTGATTTACACATGGGACATGGTTTCCAA
IF	CCCATGTGTAAATCAATCACCGGAACAATC
OR	GTCGACATTTGCATATGTTGCTTGAACA
Up1	GCAGAAAATGCCATAAATGCT
Down1	TCCACAGTTTTGTCCCACTATT
Up2	AAACACGCTAAAGCAGCTAAA
Down2	TCTTTCATTACCGGTTCTAGA

[a]*fumaA* is in complement, so Up primers are in reverse and Down primers are in forward orientation

products to be joined in a subsequent PCR reaction, as diagrammed in Figure A1-3. The resulting final PCR product yielded a deletion of the target gene, which was engineered to be in-frame and thus non-polar on downstream genes. The *dsbB* deletion construct was generated in Umeå, Sweden where the SOE-PCR and allelic replacement technique were learned in *Francisella*, but the PCR products containing in-frame deletions of *pdpB*, *fumA*, and FTT0742/*patA* are shown in Figure A1-4. The deletion constructs were subcloned into common, commercially available cloning vectors and then transferred into pDM4K via the multiple cloning site (MCS).

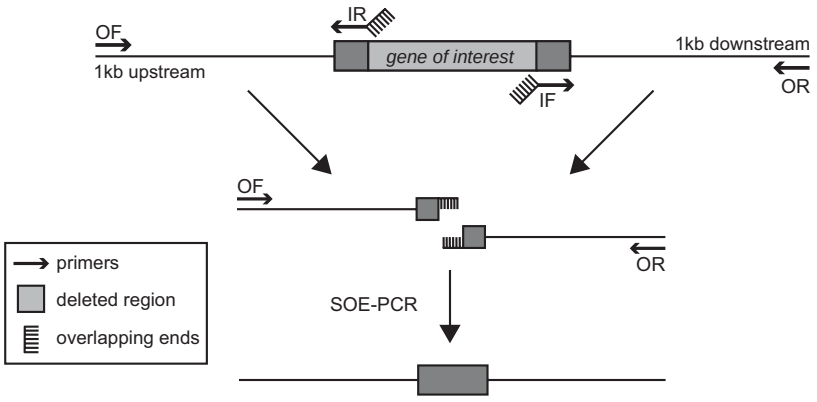


Figure A1-3. SOE-PCR technique to generate in-frame deletions of target genes. Outer forward (OF) and inner reverse (IR) and inner forward (IF) and outer reverse (OR) primer sets are used to amplify up- and downstream regions of the gene to be deleted. The IR and IF primers share a significant number of overlapping base pairs, usually at least 20. A second PCR reaction using the products generated in the first reaction as templates and OF and OR primers is performed to yield the final deletion product.

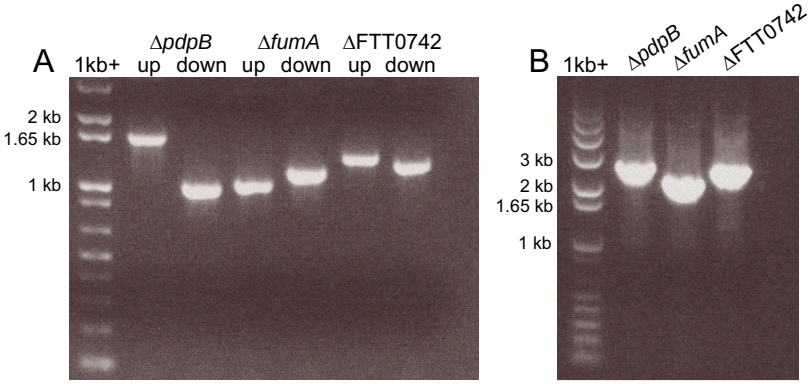


Figure A1-4. Generation of in-frame deletion constructs by SOE-PCR. PCR with OF/IR and IF/OR primer sets were used to amplify regions up- and downstream of the gene to be deleted (A). As shown in Figure A1-3, the IF and IR primers were designed to contain regions of overlapping sequence. Using the up- and downstream arm from the first PCR as templates, a second PCR was performed with OF/OR primers to create the in-frame deletion constructs (B).

Conjugation with Schu S4 resulted in integration of plasmid DNA

E. coli S17 strains carrying pDM4KΔ*pdpB*, pDM4KΔ*fumA*, or pDM4KΔFTT0742/*patA* were mated with *F. tularensis* Schu S4 under BSL-3 conditions as described in the Materials and Methods section. Colonies that exhibited kanamycin resistance were subjected to PCR analysis to determine if integration of the plasmid DNA had occurred. Because the recombination event can result in insertion of the vector DNA either up- or downstream of the native copy of the target gene, additional "Up/Down" primer sets were designed to determine the orientation of insertion (Table A1-1). Figure A1-5 shows that recombination occurred after conjugation between *E. coli* and Schu S4, and the resulting merodiploids integrated a deletion copy of the target gene.

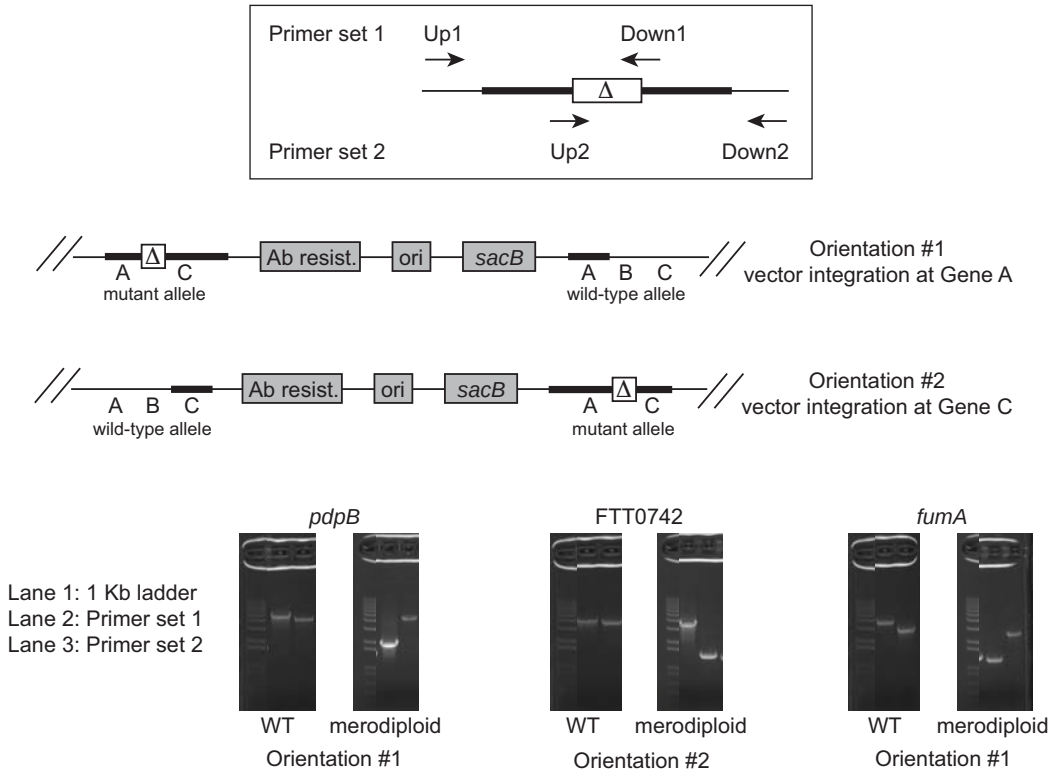


Figure A1-5. Confirmation of deletion vector insertion in Schu S4. The suicide vector can integrate into the genome in two ways. Primer sets 1 and 2, shown in the box above, amplify the deleted region and either the upstream (1) or downstream gene (2) to indicate the orientation of integration. Comparison of the PCR products from the merodiploids to the wild-type genome reveal the occurrence and orientation of integration. Results from Δ*pdpB*, ΔFTT0742 (*patA*), and Δ*fumA* are shown.

Discussion

The work shown here lays the foundation for the development of deletion mutants of *F. tularensis* Schu S4 in genes that were previously identified as necessary for full virulence in a mouse model in the closely related subspecies *novicida* U112 strain. We generated in-frame deletions of the *dsbB*, *fumA*, *pdpB*, and FTT0742/*patA* genes in the pDM4K suicide vector and introduced the constructs into the *F. tularensis* type A Schu S4 strain via conjugation with *E. coli*. Recombination occurred between the up- and downstream regions flanking the genes of interest and the homologous regions on the chromosome. The first crossover event resulted in integration of the vector DNA, and both wild-type and deleted copies of the target gene were present on the chromosome.

Although we were not able to resolve the first round of merodiploids by growth on sucrose-containing media, we believe that this was due to an incompatible promoter and insufficient *sacB* expression. The issue was addressed by cloning the *Francisella* groE promoter-*sacB* cassette into pDM4K in place of the original *B. subtilis sacB* cassette. After conjugation and recombination with these new pDM4K deletion constructs, subsequent growth on sucrose-containing media should cause the vector DNA to resolve. This will remove the kanamycin resistance gene, as well as either the wild-type or deleted copy of the target gene. Positive strains that retain the deletion and loop out the full-length gene can be identified by PCR. The final result will be an in-frame deletion in the chromosome of the type A Schu S4 strain that is unable to revert to wild-type and is sensitive to antibiotics. Because *pdpB* is on the *Francisella* pathogenicity island (FPI), which is present in two copies in type A strains, two rounds of allelic exchange will be necessary to delete both copies of the gene. The deletion of *iglC*, which

is also on the FPI, was achieved in such a manner (Golovliov, Baranov et al. 2003). In fact, additional rounds of conjugation and selection can be conducted to produce multiple different deletions in a single bacterial strain and may be preferable over a singly deleted mutant.

After the Schu S4 deletion mutants are created, they will need to be tested for attenuation in cell culture and protection in a mouse model of tularemia using a variety of vaccination and challenge routes before being considered for further vaccine studies. Even though a strain confers protective immunity in some studies, it must be thoroughly examined to determine if it is suitable for testing in a primate vaccine model. For example, a subspecies *holarctica* LVS *purMCD* mutant was shown to be attenuated and protective in mice with challenge doses of 5×10^3 CFU wild-type bacteria (Pechous, Celli et al. 2006). When the same *purMCD* gene locus was deleted in the type A Schu S4 strain, it remained highly attenuated in mice when delivered intranasally or intradermally, but intranasal delivery appeared to damage the lungs and provided protection only against intradermal challenge with type A and type B strains (Pechous, McCarthy et al. 2008). Therefore, it is important to investigate the cross-protection of the deletion mutants with various combinations of infection and challenge routes and with challenge with other subspecies of *Francisella*. It should be noted that the initial challenge dose of the LVS *purMCD* mutant was 5×10^3 CFU while we saw full protection with challenge doses of up to 6×10^7 CFU of wild-type subspecies *novicida* in our studies (Tempel, Lai et al. 2006). Furthermore, intradermal delivery of the *purMCD* Schu S4 mutant resulted in a limited dissemination to the lung, perhaps accounting for the lack of protection against intranasal challenge; all of our subspecies *novicida* mutants displayed the capacity to

disseminate to the lung following intraperitoneal delivery. We also used genes derived from Schu S4 for complementation studies with our transposon mutant strains, further indicating a conservation of gene product and function between the two subspecies.

Failure to provide protection as a single mutant would not necessarily exclude a gene from further vaccine studies, as it may be useful in the context of a multiply deleted strain. In fact, the ideal vaccine strain would carry multiple gene deletions to further reduce the unlikely possibility of reversion to wild-type; however, it remains to be seen if multiple mutations would yield an over-attenuated strain that is incapable of eliciting an immune response. On the other hand, it is possible that a second mutation in a strain such as the *purMCD* mutant may have resulted in a vaccine strain capable of protecting against intranasal challenge. Speculation aside, the four deletion mutants constructs described in this work were generated as a result of promising mouse infection studies using parallel mutants in subspecies *novicida* and may form the basis of a rationally attenuated live tularemia vaccine.

Materials and Methods

Bacterial strains and culture

F. tularensis strain Schu S4 was obtained from the Centers for Disease Control and Prevention. All work with Schu S4 and its derivatives was conducted under BSL3 conditions and was approved by the Institutional Biosafety Committees at the Oregon Health and Science University (OHSU) and the Medical College of Wisconsin (MCW). OHSU and MCW are registered with the Centers for Disease Control and Prevention to work with virulent strains of *F. tularensis*. Schu S4 was grown aerobically at 37°C in Chamberlain's medium or modified Mueller-Hinton (MH) broth, or on agar (Difco) supplemented with 1% proteose peptone, 2.5% fetal bovine serum (Invitrogen), 0.25% ferric pyrophosphate, 1% dextrose, and 2% IsoVitalex. When required, kanamycin (10 µg/ml), polymixin (50 µg/ml), or sucrose (10%) was added to media. *Escherichia coli* strain DH5α was used for basic cloning procedures, TransforMax pir-116 electrocompetent *E. coli* (Epicentre, Madison, WI) were used for manipulations of pDM4 and production of pDM4K-derived deletion constructs, and *E. coli* strain S17 was used for conjugation with Schu S4. *E. coli* strains were grown at 37°C in Luria-Bertani (LB) broth or agar, supplemented with 50 µg/ml kanamycin when required.

Splicing by overlapping extension PCR (SOE-PCR) to make in-frame deletions

Primers were designed using MacVector 7.2.3 software. Approximately 1 to 1.5 kb regions up- and downstream of each gene of interest was amplified using Phusion DNA polymerase (Finnzymes, Woburn, MA). The tail of reverse primer from the upstream amplification contained 40 complementary bases to the tail of forward primer from the

downstream amplification such that the two PCR products could be joined in a second PCR reaction using Roche Expand Long Template DNA polymerase (Indianapolis, IN). The hybrid PCR product was cloned into pCR4Blunt (Invitrogen, Carlsbad, CA) for restriction analysis and sequencing. In-frame deletions were transferred into pDM4K via restriction sites. Standard miniprep, PCR clean-up, and gel purification kits were used (Qiagen, Valencia, CA).

Construction of pDM4K

pDM4 was a kind gift from Debbie Milton. The chloramphenicol resistance cassette was disrupted by insertion of a kanamycin resistance cassette from pMODEZTn (Tom Kawula, UNC Chapel Hill), using *Pvu*II sites in pMODEZTn and *Sca*I sites in pDM4. The chloramphenicol resistance marker was further inactivated by deletion with *Bsm*I, followed by end repair and re-ligation. The resulting plasmid exhibited Kan^r/Cm^s and was named pDM4K. The original *sacB* gene from *B. subtilis* was replaced with a *sacB* cassette (from pTZ535) containing the *sacB* gene under control of the groE promoter from *Francisella*. *Pst*I was used to remove *sacB* from pDM4K, *Bgl*II was used to isolate the groE-*sacB* cassette from pTZ535, and *Pst*I- *Bgl*II linkers were used to insert the cassette into pDM4K. All restriction enzyme reactions were conducted per manufacturer's instructions, and all ligation reactions took place at 16ºC overnight.

Conjugation and selection of merodiploids

Liquid cultures of *E. coli* S17 and *F. tularensis* Schu S4 were inoculated from overnight liquid cultures and grown to mid-log phase. 500 µl of Schu S4 and 10 µl of S17

were centrifuged for 2 min at ~16,000 × g and resuspended in 25 µl phosphate buffered saline (PBS) solution + 10 mM $MgSO_4$. The samples were mixed, spotted onto LB plates, and incubated overnight at 25ºC in the dark. The following day, conjugation mixtures were resuspended in 300 µl PBS + 10 mM $MgSO_4$ and 100 µl was spread onto 3 plates containing kanamycin and polymixin to counterselect S17. After incubation at 37ºC for three days, the colonies were checked for pDM4K integration by PCR.

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Appendix 2

Outline of potential future experiments

I. Transposome mutatgenesis applications

A. Test for secreted or surface exposed *Francisella* proteins

1. Modify cycler (*cyaA'*) transposon to have kanamycin marker under control of a *Francisella* promoter such as GroE
2. Generate transposon library
3. Infect macrophages and assay for cAMP with ELISA

B. Differentiate between secreted and surface exposed proteins

1. Infect macrophages, lyse cells, collect soluble and pellet fractions
2. Probe for protein (antibody to CyaA' or ELISA)

II. Allelic replacement in Schu S4 and vaccine testing

A. Complete deletion derivatives in Schu S4 as discussed in Appendix 1

1. Must do two rounds of allelic exchange for Δ*pdpB* mutant

B. Test for entry and attenuation in macrophages as in Chapter 2

C. Test for attenuation and protection in mice

1. Use different combinations of vaccination and challenge routes
2. Determine LD_{50} values
3. Test for dissemination and clearance from inoculation site
4. Test for protection against other subspecies
5. May test with other pathogens to see if protection is specific

D. Make strains with multiple deletions and test as above

E. Begin vaccine trials in non-human primate rhesus macaque model

III. Role of Pat family proteins and the TAARP domain

A. Test for interaction of TAARP with E-cadherin

1. Repeat TAARP and macrophage extract co-purification experiment resin as in Chapter 3 and assay for presence of E-cadherin
2. Conduct direct binding studies (Western co-IP) with cell line transfected with construct expressing His-tagged TAARP

B. Determine role of Pat/TAARP with deletion derivatives

*using Δ*patA* or Δ*patBC* (in *F. novicida*) or ΔTAARP

1. Look at role in entry vs. wild-type with macrophage infections
    a. Can make stepwise deletions to determine necessary region(s)
    b. Investigate what host cell components are required for entry by use of siRNA in cells or KO mice (cortactin, formin, cadherins)
2. Test for role as an anti-inflammatory molecule (as with EAP domains)
    a. Assay for induction of cytokine production in host cells with deletion vs. wild-type (activate T cells and/or B cells?)
    b. Test for inhibition of neutrophil recruitment
3. Assess ability to disrupt epithelial junctions (as with *Rickettsia*)

a. Infect monolayer and test for changes in conductivity

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