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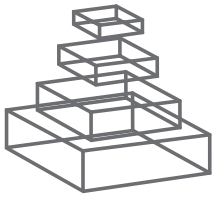

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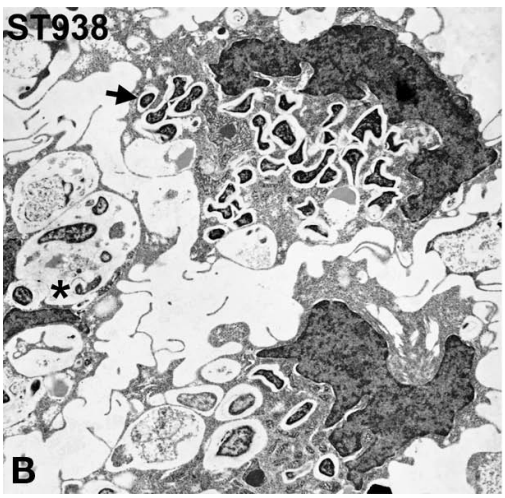
FRANCISELLA TULARENSIS AND TULAREMIA

Hosted By
Anders Sjöstedt, Department of Clinical Microbiology, Umeå University, Umeå, Sweden
anders.sjostedt@climi.umu.se



The bacterium today known as *Francisella tularensis* was first identified 99 years ago and, since then, much research has been devoted to study it and the resulting disease, tularemia. *F. tularensis* became the focus of an intense research effort during the first half of the 20th century, in particularly in the United States and Soviet Union, since the disease was fairly common. Due to its high infectivity, ease of spread, and severity of the resulting disease, it was one of the agents given the highest priority in the biological weapon programs of the United States and Soviet Union. After termination of these programs in the 1960s, the interest in *F. tularensis* diminished significantly, but after several decades of little attention, the last decade has led to resurgence in the research on *F. tularensis*. In 2003, the Science magazine phrased it as follows: "an obscure weapon of the cold war edges into the limelight". There were multiple reasons for this resurgence, one of which was an increase in the number of tularemia cases in several European countries and, moreover, the intense research effort on potential bioterrorist agents in the US post 9/11. Thereby, the number of annual publications on *F. tularensis* has tripled in 10 years, and many new research groups, in particular American, have entered the field. This has led to very rapid development of state-of-the-art research tools, and fast progress in the understanding of *F. tularensis*. A proof of the rapid progress was the publication of a comprehensive volume on *Francisella* and tularemia in 2007. Although only four years ago, the rapid pace of the research has led to many new discoveries since then and, to this end, we now present a collection of articles from leading scientists on the up-to-date knowledge regarding *F. tularensis*. The articles cover important areas such as molecular research tools, experimental models, genomics, virulence mechanisms, manipulation and subversion of host responses, host immune responses, and the ecology of *F. tularensis*.

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EDITORIAL
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Special topic on *Francisella tularensis* and tularemia

Anders Sjöstedt*

Department of Clinical Microbiology, Umeå University, Umeå, Sweden
**Correspondence: anders.sjostedt@climi.umu.se*

Francisella tularensis was first identified exactly a century ago in California as part of a plague surveillance program and the resulting disease, tularemia, is still referred to as "rabbit plague" in many languages. In nature, rodents and rabbits appear to be the species most frequently affected by tularemia but *F. tularensis* is a highly versatile pathogen and the disease has been identified in some 190 mammalian species. Moreover, the disease is commonly vector-borne and the bacterium has the capability to survive within arthropods and, in fact, one of its natural reservoirs appears to be protozoa. Thus, it is an extremely adaptive pathogen with an astounding wide range of potential vectors and target species. There has been a resurgent research interest in the pathogen during the last decade. This has resulted in numerous intriguing discoveries and this special topic summarizes our current knowledge about the bacterium and tularemia. Reviews written by experts in various aspects of tularemia research have provided comprehensive up-to-date summaries of the rapid progress recently achieved as well as providing perspectives on important conceptual questions that need to be addressed in the next years.

One timely review by Lederballe-Meibom and Charbit (2011) summarizes the relationship between *F. tularensis* virulence and metabolic and nutritional genes. They emphasize that there is close association between nutritional starvation and virulence gene expression via the stringent response and therefore conclude that expression of its virulence is intimately dependent on its ability to capture nutrients from the host and to regulate its metabolism in response to the nutrients available.

Chong and Celli (2011) recapitulate our present understanding of the complex intracellular lifecycle of *F. tularensis* in a variety of mammalian cell types. The authors discuss the data regarding the intracellular survival strategy of the pathogen and outline the consensus view of the intracellular lifecycle. Moreover, they also attempt to settle discrepancies that exist in our views of the *Francisella*–phagocyte interaction. Intimately related to many or most stages of the bacterium's intracellular life cycle is the *Francisella* pathogenicity island (FPI), which contains almost 20 genes. Many of these appear be essential for the intricate intracellular lifecycle and although our current understanding of the genes' functions are far from complete, this is one of the most active areas of *Francisella* research and the basis for a review by Bröms et al. (2010). The review emphasizes the fact that the suggested role of the FPI as a type VI secretion system (T6SS) is still far from clear since it is evolutionary distinct and shares few if any functions compared to all other described secretion systems. It is concluded that the FPI constitutes a system essential for the intracellular lifecycle and the virulence of the bacterium and that it shares some similarities to components of T6SS and T4SS.

The review by Dai et al. (2011) that summarizes the current knowledge of environmental adaptation by the *F. tularensis*, its transcriptional regulators and their relationship to animal virulence. On one hand, the authors point out that there are surprisingly few sensory and regulatory factors described for the pathogen, particularly in view of its proven ability to survive in a multitude of environmental niches. However, it is also noted that there has been rapid progress in elucidating the gene regulation and identifying novel regulatory factors. One reason for recent, rapid progress in understanding the genetics of *F. tularensis* has been the development of suitable tools for genetic manipulation of the organism. Recently developed genetic techniques, such as transposon mutagenesis and targeted gene disruption, described in the review by Zogaj and Klose (2011) have significantly improved our understanding of *F. tularensis* virulence.

Host responses during the intracellular lifecycle of *F. tularensis* are the focus of three reviews by Jones et al., Cremer et al., and Asare and Kwaik, *F. tularensis*, in particular *F. novicida*, has become one of the most studied model organisms for elucidating the function of the inflammasome and the review by Jones et al. (2011) describes mechanisms of its activation by the bacterium and the consequences *in vitro* and *in vivo*. Moreover, it also discusses the coordination between the inflammasome and other cytosolic host responses and how these are affected by *Francisella* virulence factors. The review by Cremer et al. (2011) addresses how signaling pathways of phagocytic cells are targeted by *Francisella*, with a focus on the phosphatidylinositol 3-kinase/Akt pathway but also the role of microRNAs, specifically miR-155, as a key regulator of host signaling and defense. The third review by Asare and Kwaik (2011) describes the mechanisms used for uptake and internalization of the pathogen and the various host signaling pathways that are engaged during infection. A review by Bosio (2011) describes the subversion of specific immune mechanisms, both innate and adaptive ones, by *F. tularensis*. It describes recent advances based on findings in the mouse model and *in vitro* studies with human cells that have identified specific bacterial and host compounds that mediate generalized suppression of the host immune response.

One of the most studied *F. tularensis* virulence factors has been the type IV pilus system (Tfp) described in the review by Salomonsson et al. (2011). The Tfp and assembly of PilA have been shown to be required for full virulence of strains, however, there is much genetic variation in the encoding genes and therefore the authors hypothesize that the variations reflect adaptation to different environmental niches of the subspecies and play a role in transmission of tularemia. Another virulence system, the *cap* system, is described in an original article by Su et al. (2011). They demonstrate that a *capBCA* mutant of Schu S4 demonstrated significant attenuation *in vivo* and even more so the corresponding mutant of *F. tularensis* LVS.

An original article by Soni et al. (2010) is based on a comprehensive analysis of the enigmatic, so called gray variant of *F. tularensis* LVS. It showed defective replication in human, rat, but not mouse macrophages and conferred no protection to a virulent strain in the mouse model. It displayed structural differences in both the

core and lipid A regions. The latter were identified as decreased galactosamine modifications and correlated to reduced transcription of the *flmF2* and *flmK* genes.

The review by Cowley and Elkins (2011) describes immune mechanisms operative to control tularemia, mainly in the widely used mouse model. Many controlling mechanisms are analogous to those operational against other intracellular pathogens, but there are also recently identified mediators such as IL-17A, Toll-like receptor 2, and the inflammasome. In addition, the authors elaborate on the important roles of CD4 and CD8 T cells for control of tularemia but also emphasize that the roles of new T cell subpopulations are beginning to be unraveled. They conclude that also B cells contribute in important ways to protection but this varies depending on the virulence of the *F. tularensis* strain. A number of studies of the targets of anti-*Francisella* antibodies have been published and the results are summarized in the review by Kilmury and Twine (2011). The authors state that the detailed information generated from the immunoproteomics studies have the potential to be exploited in the identification of new diagnostic or protective antigens and in the development of vaccines.

There has been much interest to develop alternative models for *F. tularensis* and one organism that has attracted special attention is *Drosophila melanogaster* as a model for arthropod vectors. The review by Akimana and Kwaik (2011) concludes that it is a useful vector amenable for the identification of *F. tularensis* virulence mechanisms, also for those operative in evolutionarily distant mammalian hosts. Another model for tularemia is described in the original article by Santic et al. (2011). They demonstrate that *F. novicida* survives and replicates within the ameba *Hartmannella vermiformis* but with a lifestyle that is distinct to the one within mammalian cells (Santic et al., 2011).

The review by Telford and Goethert (2011) summarizes the rather extensive but also to some extent conflicting knowledge regarding the ecology of *F. tularensis*. The authors argue that the epidemiological information regarding tularemia to some extent has been used incorrectly to postulate ecological models for the bacterium. They identify the need to develop quantitative models for the reproduction of *F. tularensis* and suggest that the models may be distinct for subspecies *holarctica* and *tularensis* since they exhibit fundamental differences in their ecology.

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Francisella tularensis metabolism and its relation to virulence

Karin Lederballe Meibom[1,2] and Alain Charbit[1,2]*

[1] *Faculté de Médecine Necker-Enfants Malades, Université Paris Descartes, Paris, France*
[2] *INSERM, Unit of Pathogenesis of Systemic Infections, Paris, France*

Edited by:
Anders Sjostedt, Umeå University, Sweden

Reviewed by:
Antje Flieger, Robert Koch Institute, Germany
Jürgen Heesemann, Max von Pettenkofer-Institute, Germany

***Correspondence:**
Alain Charbit, Faculté de Médecine Necker, 156, rue de Vaugirard, 75730 Paris Cedex 15, France.
e-mail: alain.charbit@inserm.fr

Francisella tularensis is a Gram-negative bacterium capable of causing the zoonotic disease tularaemia in a large number of mammalian species and in arthropods. *F. tularensis* is a facultative intracellular bacterium that infects and replicates *in vivo* mainly inside macrophages. During its systemic dissemination, *F. tularensis* must cope with very different life conditions (such as survival in different target organs or tissues and/or survival in the blood stream…) and may thus encounter a broad variety of carbon substrates, nitrogen, phosphor, and sulfur sources, as well as very low concentrations of essential ions. The development of recent genome-wide genetic screens have led to the identification of hundreds of genes participating to variable extents to *Francisella* virulence. Remarkably, an important proportion of the genes identified are related to metabolic and nutritional functions. However, the relationship between nutrition and the *in vivo* life cycle of *F. tularensis* is yet poorly understood. In this review, we will address the importance of metabolism and nutrition for *F. tularensis* pathogenesis, focusing specifically on amino acid and carbohydrate requirements.

Keywords: *Francisella tularensis*, pathogenesis, metabolism

INTRACELLULAR PATHOGENS AND METABOLIC REQUIREMENTS

The *in vivo* metabolism of pathogenic bacteria constitutes an important, and yet insufficiently studied, aspect of host–pathogen interactions. Metabolic pathways comprise: (i) degradative pathways (catabolism) of organic molecules, processes generally accompanied by the production of energy; and (ii) biosynthetic pathways (anabolism) that uses energy to build-up molecules. Both metabolic pathways require the sequential action of dedicated enzymes whose expression and activity may be tightly regulated in response to environmental changes. In order to survive and efficiently replicate in host cells, intracellular pathogens must adapt their metabolism to the available nutrients and physical conditions (including pH, oxygen availability, osmotic pressure, etc.). Metabolism is tightly associated with nutritional capacities, which involve nutrient availability and dedicated nutrient uptake systems. Indeed, pathogenic bacteria use the host organism as a macronutrient system that comprises many different specialized microenvironments. In particular, bacteria capable of systemic dissemination like *Francisella tularensis* have to cope with very different life conditions (such as survival in different target organs or tissues and/or survival in the blood stream, etc.) and may thus encounter a broad variety of carbon substrates, nitrogen, phosphor, and sulfur sources, as well as very low concentrations of essential ions such as magnesium, manganese, and iron (Eisenreich et al., 2010).

Many intracellular bacteria reside in a vesicular compartment (e.g., *Salmonella, Legionella, Brucella, Mycobacteria,* etc.). These bacteria encounter stressful conditions in these membrane-bound vacuoles (low pH, free-radicals, nutrient deprivation, antimicrobial compounds, etc.) and have therefore developed efficient defense mechanisms. For example, *S. enterica* is able to survive and replicate for extended periods in *Salmonella*-containing vacuoles (SVCs) of infected cells (preferentially macrophages) and thus can cause chronic infections. Notably, *S. enterica*, seems to undergo only limited replication cycles in SVCs. Although the nutritional content of SVCs is still poorly defined, transcriptomic and proteomic analyses have suggested that ions such as magnesium, manganese, and iron, could be limited in the SCV. Furthermore, *in vivo* studies have indicated that sugars, fatty acids, and acetate, could be used as carbon sources (García-del Portillo et al., 2008). Other intracellular bacteria, like *Francisella, Listeria, Shigella*, and *Rickettsia*, have chosen the cytosol as a replication niche (Casadevall, 2008). The host cell cytosol is generally viewed as a more permissive milieu than the phagosomal compartment.

In this review, we shall try to understand the importance of metabolism and nutrition for *F. tularensis* pathogenesis, focusing specifically on amino acid and carbohydrate requirements.

F. TULARENSIS NUTRITIONAL REQUIREMENTS

Francisella tularensis is a Gram-negative bacterium capable of causing the zoonotic disease tularaemia in a large number of mammalian species and in arthropods such as ticks, flies, and mosquitoes (Keim et al., 2007). It is a highly infectious bacterium that can be transmitted to humans in numerous ways, including contact with infected animals, inhalation, ingestion of contaminated water or food, or insect bites (Sjostedt, 2007). Four different subspecies (subsp.) of *F. tularensis* are generally recognized that differ in virulence and geographic distribution. These are four designated subsps. *tularensis* (type A), *holarctica* (type B), *novicida*, and *mediasiatica*, respectively. However, the classification of *novicida* as a subspecies is still a matter of debate (Huber et al., 2010; Johansson et al., 2010). *F. tularensis* subsp. *tularensis* is the most virulent subspecies causing a severe disease in humans, whereas *F. tularensis* subsp. *holarctica* causes a similar disease but of less severity (McLendon et al., 2006). Because of its high infectivity and lethality, *F. tularensis* is considered a potential bioterrorism agent (Oyston and Griffiths, 2009).

Francisella tularensis is a facultative intracellular bacterium that infects and replicates *in vivo* mainly inside macrophages, but which can also infect and survive in a variety of non-phagocytic mammalian cells such as hepatocytes, endothelial cells, epithelial cells, and fibroblasts (Santic et al., 2010). Remarkably, *F. tularensis* is also one of the rare bacteria that can survive within neutrophils (McCaffrey and Allen, 2006). *F. tularensis* subsp. *holarctica* live vaccine strain (LVS) has been shown to inhibit the respiratory burst by preventing NADPH oxidase assembly at the phagosomal membrane (McCaffrey and Allen, 2006). Attempts to identify LVS genes that affect neutrophil function have only led to the selection of uracil auxotrophs (*carA*, *carB*, and *pyrB*) whose intracellular growth defect are most likely pleiotropic.

Francisella tularensis strains, including highly virulent species, have also been reported to survive and multiply in amebae in the environment (Abd et al., 2003; El-Etr et al., 2009), suggesting a potential link between ameba–*Francisella* interactions and environmental persistence. The specific nutritional requirements of *F. tularensis sensu lato* in the ameba have not yet been studied.

The recent availability of complete genome sequences and the development of numerous genome-scale genetic methods have led to the identification of hundreds of genes participating to variable extents to *Francisella* virulence (Ahlund et al., 2010; Akimana et al., 2010; Asare and Abu Kwaik, 2010; Asare et al., 2010; Lai et al., 2010; Meibom and Charbit, 2010, and references therein; Moule et al., 2010; Peng and Monack, 2010). However, the specific contribution of only a limited number of these genes is currently understood at the molecular level. Although an important proportion of the genes identified are related to metabolic and nutritional functions, the relationship between nutrition and the *in vivo* life cycle of *F. tularensis* is yet poorly understood.

F. TULARENSIS BIOSYNTHETIC PATHWAYS AND VIRULENCE

Let us first consider the general relationship between the presence or absence of a biosynthetic pathway and its associated nutritional requirement. By definition, in a synthetic (or minimal) medium, a prototrophic (heterotrophic) facultative intracellular bacterium is able to synthesize all its components from the carbohydrate source provided. When one or several genes of a biosynthetic pathway are missing, or have been inactivated, supplementation of the medium by the substrate is required for growth (the bacterium is auxotroph for this substrate). In infected cells, two outcomes exist when a biosynthetic pathway is impaired in a mutant strain: (i) if the mutant bacteria require supplementation of the medium by the cognate substrate for intracellular growth, it is deduced that the intracellular milieu is depleted (or limited) for that substrate; (ii) alternatively, if the mutant bacteria grow like the wild-type strain, this is deduced the intracellular milieu is replete in that substrate (and implies that the bacterium is able to take-up enough substrate for growth).

A chemically defined growth medium was developed to support the growth of *F. tularensis,* which includes 13 amino acids (Traub et al., 1955; Nagle et al., 1960). Later, Chamberlain optimized the concentrations of the different components (amino acids, vitamins, ions) of this medium that is still widely used (Chamberlain, 1965). *F. tularensis* strains Schu S4 (subsp. *tularensis*) and LVS (subsp. *holarctica*) both require cysteine for growth, most likely due to a non-functional pathway for sulfate assimilation (Larsson et al., 2005). The absolute requirement for growth of the other 12 amino acids contained in the medium has not been experimentally confirmed. Functional biosynthetic pathways have been identified in the Schu S4 genome for the seven non-essential amino acids (alanine, asparagine, glutamate, glycine, glutamine, phenylalanine, and tryptophan). Evidence was also found in the Schu S4 genome for biosynthetic pathways for 8 of the 13 amino acids that are supplied in the synthetic medium (see **Table 1**; Larsson et al., 2005). However, of these eight, the biosynthetic pathways for isoleucine, valine, and threonine are predicted to have missing steps and therefore to be non-functional (due to presence of pseudogenes encoding enzymes catalyzing the missing steps in the pathways). Altogether, the pathways for arginine, histidine, lysine, tyrosine, methionine, cysteine, threonine, valine, and isoleucine biosynthesis seem to be incomplete or absent. *F. tularensis* subsp. *tularensis* strain Schu S4 is hence auxotroph for these amino acids. It remains to be determined whether one or more of the other four amino acids for which pathways were predicted to be present, but supplied by the synthetic medium (i.e., serine, aspartate, leucine, and proline), are absolutely required for growth.

Aromatic amino acid biosynthetic pathways

The shikimate pathway (**Figure 1**) is the common pathway for the biosynthesis of chorismate, which is the precursor for the generation of aromatic amino acids, *para*-aminobenzoic acid (*p*ABA,

Table 1 | Nutritional requirements of *F. tularensis*.

Amino acids	Biosynthetic pathway predicted	Pathway score*
REQUIRED FOR GROWTH IN SYNTHETIC BROTH		
Arginine	No	–
Histidine	No	–
Lysine	No	–
Methionine	No	–
Tyrosine	No	–
Cysteine (incomplete/required)	Yes	5/3/3
Isoleucine (incomplete/required)	Yes	5/4/1
Threonine (incomplete/required)	Yes	2/1/0
Valine (incomplete/required)	Yes	4/3/2
Serine (required ?)	Yes	3/2/2
Aspartic acid (required ?)	Yes	1/1/1
Leucine (required ?)	Yes	4/2/0
Proline (required ?)	Yes	4/2/1
NOT REQUIRED FOR GROWTH IN SYNTHETIC BROTH		
Alanine	Yes	3/2/2
Asparagine	Yes	2/1/0
Glutamate	Yes	1/1/1, 1/1/1, 1/1/1
Glutamine	Yes	2/2/1
Glycine	Yes	2/1/1, 1/1/1
Phenylalanine	Yes	3/2/2, 4/2/2, 3/2/2
Tryptophan	Yes	5/5/0

In the F. tularensis subsp. tularensis Schu S4 genome. Each predicted pathway, P, was assigned a score X/Y/Z, according to Larsson et al. (2005). P consists of X reactions; enzymes for Y reactions were identified in the genome; and Z of the Y reactions are used in other predicted pathways.

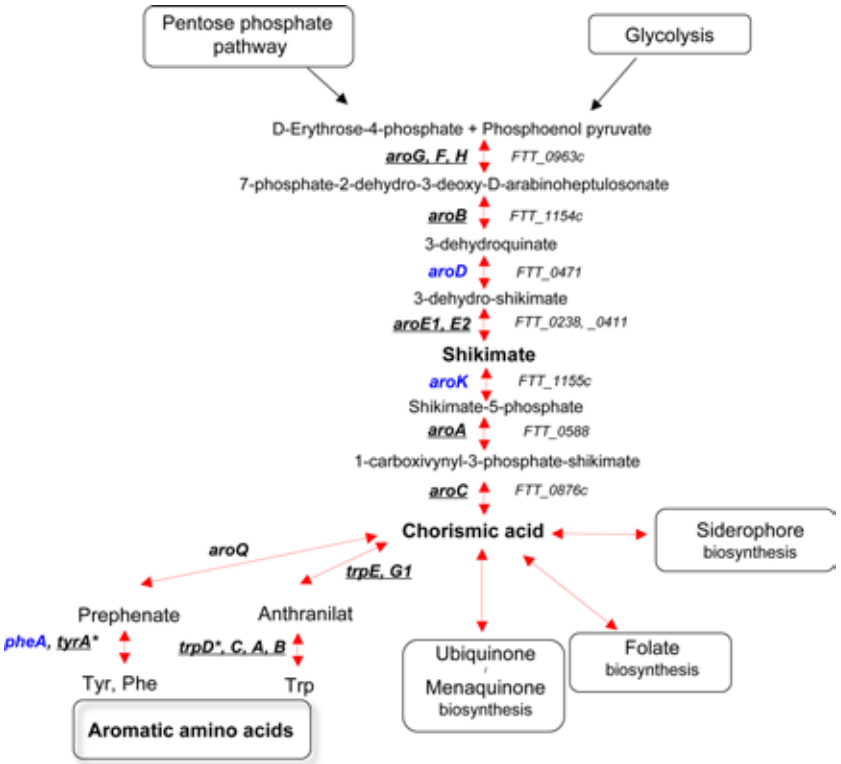

FIGURE 1 | The shikimate pathway. Genes that have been identified in genetic screens (*in vivo* or *in vitro*) are underlined. Genes that have not been hit in any screen are in blue. TrpD*, the gene is absent in the Schu S4 and LVS strains but present in the subsp. *novicida* (FTA_2078). TyrA*, the gene is absent in the Schu S4 strain but present in both LVS (FTL_048) and subsp. *novicida* (FTN_0055).

folate biosynthesis), 2,3-dihydroxybenzoic acid (DHB, biosynthesis of siderophores), ubiquinone, and menaquinone (Bentley, 1990; **Figure 1**). Notably, *F. tularensis sensu lato* has been shown to express a siderophore under iron limiting conditions. This siderophore, structurally similar to rhizoferrin, promotes the growth of both LVS and Schu S4 strains under iron limitation. The siderophore locus, designated *fsl* in *F. tularensis* subsp. *tularensis* (Schu S4) and *F. tularensis* subsp. *holarctica* (LVS), or *fig* in *F. tularensis* subsp. *novicida*, is involved in both synthesis and uptake of the siderophore (Sullivan et al., 2006; Kiss et al., 2008; Ramakrishnan et al., 2008; Crosa et al., 2009).

Bruce Stocker's pioneer work on the genetics of *Salmonella enterica* serovar Typhimurium (*S. typhimurium*) has demonstrated the crucial importance of the aromatic biosynthetic pathway for bacterial virulence. Mutations in different genes coding for the biosynthesis of aromatic amino acids have been used to reduce the virulence of *Salmonella* strains and for immunization of various animal species (Chatfield et al., 1994, 1995). The attenuation of *aro* mutants of *S. typhimurium* is thought to be due to the inability of the bacterium to generate *p*ABA and DHB from chorismate (Hoiseth and Stocker, 1981). Remarkably, *aroA* and *aroD* mutants of *S. enterica* serovar Typhi were also successfully tested as a vaccine against human typhoid (Tacket and Levine, 2007).

All of the necessary genes encoding the chorismate pathway enzymes are present in the genome of *F. tularensis* Schu S4 (i.e., *aroG, aroB, aroD, aroE, aroK, aroA*, and *aroC*). The genes (*pabA* and *pabB*) encoding the two components of *para*-aminobenzoate synthase have also been identified in the *F. tularensis* Schu S4 genome as well as the gene encoding FolP, FolC, and FolA, involved in folate biosynthesis. In recent screens, we and others (Su et al., 2007; Alkhuder et al., 2009) have selected mutants in gene *FTL_1240* (*aroG*), encoding DAHP synthase. This is the first enzyme of the aromatic amino acid biosynthetic pathway, which converts erythro-4-phosphate and phosphoenolpyruvate (PEP) to 3-deoxy-D-arabino-heptuosonate-7-phosphate. We have shown that an *aroG* mutant of LVS had only a slightly reduced intracellular growth capacity, in both J774 cells and in bone marrow-derived macrophages (BMM). Still, in the mouse model, the mutant strain was very severely attenuated. Of interest, wild-type *Escherichia coli* has been shown to produce three feedback inhibitor-sensitive DAHP synthase isoenzymes: a tyrosine-sensitive, a phenylalanine-sensitive, and a tryptophan-sensitive enzyme (encoded by genes genes *aroF, aroG*, and *aroH*, respectively). Hence, the functionality of a biosynthetic pathway does not only depend on the presence of intact genes but may also rely on the amount and activity of the enzymes.

This interplay between the availability of an amino acid and the activity of its cognate biosynthetic pathway might exist for other types of nutrients that the bacterium can either biosynthesize or acquire from its growth medium.

Additional genes of the *F. tularensis* aromatic amino acid biosynthetic pathway have been identified in genome-wide screens: *aroA, aroB, aroC, aroE1, tyrA, trpA, trpB, trpC*, and *trpE*. The gene

aroA is responsible for the sixth step of the biosynthetic pathway (converting shikimate-3-phosphate and PEP to 5-enolpyruvyl-shikimate-3-phosphate). An *aroA* mutant of *F. tularensis* Schu S4 strain was selected in a screen of transposon insertion mutants performed in the human hepatic carcinoma cell line HepG2 (Qin and Mann, 2006). The same screen also led to the identification of purine and pyrimidine auxotrophs (see below).

An *aroB* mutant of *F. tularensis* subsp. *novicida* (*FTN_1135*), encoding a putative 3-dehydroquinate synthetase, has been very recently identified in a screen in human macrophages (Asare and Abu Kwaik, 2010). The mutant strain, which was also deficient for growth in *Drosophila melanogaster*-derived S2 cells, localized to the cytosol in macrophages, indicating a cytosolic growth defect.

The gene *aroC* encodes chorismate synthase, the seventh step of the aromatic biosynthetic pathway. It was identified in a screen of transposon insertion mutants performed in *F. tularensis* subsp. *novicida*, searching for genes required for pulmonary and systemic infection in mice (Kraemer et al., 2009). The gene *tyrA* (*FTN_0055*), which encodes the enzyme prephenate dehydrogenase converting chorismate to tyrosine, was also hit in this *in vivo* screen, as was *aroC*. Notably, the *tyrA* gene is absent in the Schu S4 strain but present in LVS (*FTL_0048*). *aroC* encodes the enzyme performing the last step of chorismic acid synthesis. The gene *aroE1*, which encodes shikimate-5-dehydrogenase, the fourth step of the biosynthetic pathway, was identified in two screens of transposon insertion mutants. One screen was performed in *F. tularensis* subsp. *holarctica* LVS searching for auxotrophic mutants unable to grow on chemically defined medium (Maier et al., 2006) and the other was a screen of *F. tularensis* subsp. *novicida* for mutants unable to replicate intracellularly in macrophages (Asare and Abu Kwaik, 2010). Finally, five genes (*trpA, trpB, trpC, trpE,* and *trpG1*) encoding enzymes involved in the conversion of chorismic acid to tryptophan were identified in three *in vivo* selections using *F. tularensis* subsp. *novicida* (Weiss et al., 2007; Kraemer et al., 2009; Peng and Monack, 2010).

Altogether, almost every gene in the aromatic amino acids biosynthetic pathway has been identified in mutant screens, highlighting the importance this pathway for *F. tularensis* virulence. Interestingly, several of the genes have also been found to be important for growth/virulence in a non-mammalian model, *in vivo* in *D. melanogaster* or *in vitro* in *D. melanogaster*-derived cells (Asare et al., 2010; Moule et al., 2010).

Purine and pyrimidine biosynthetic pathways

In silico analysis reveals that the *F. tularensis* genomes encode all the enzymes necessary for the *de novo* synthesis of purines and pyrimidines. The two pathways are virtually identical to those in *E. coli*.

Pur pathway. Several pur auxotrophic mutants of *F. tularensis* (**Figure 2**), selected from banks of mutants or genetically engineered, have already been obtained and tested for virulence (Gray et al., 2002; Qin and Mann, 2006; Tempel et al., 2006; Quarry et al., 2007; Titball et al., 2007; Weiss et al., 2007; Kadzhaev et al., 2009; Asare and Abu Kwaik, 2010; Asare et al., 2010; Peng and Monack, 2010). The pur mutants with single gene mutations showed variable degrees of attenuation *in vivo* and growth defects *in vitro*, suggesting that the step at which the pathway is inactivated may have a distinct impact

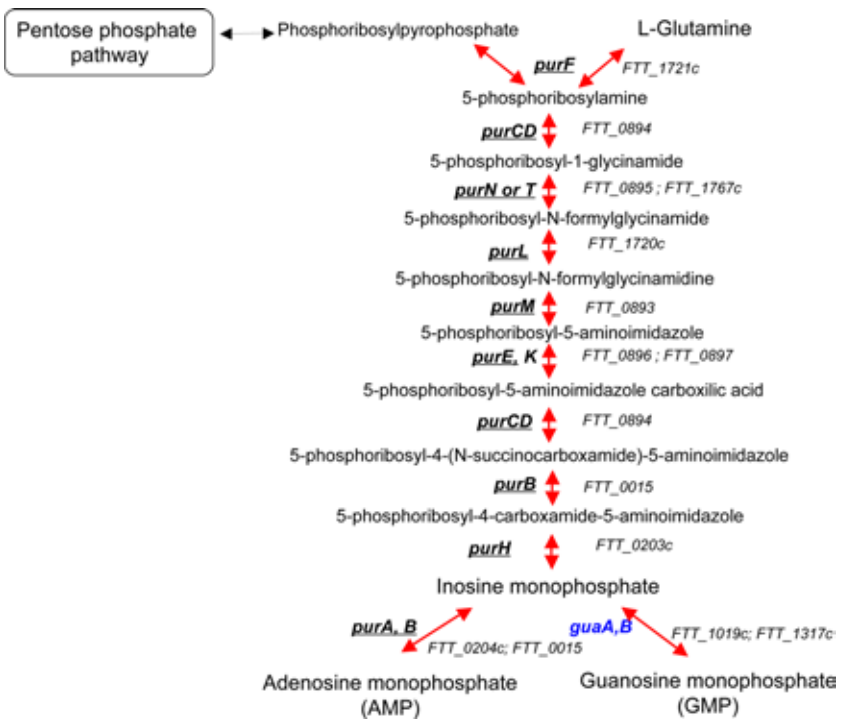

FIGURE 2 | The purine biosynthetic pathway. Genes that have been identified in genetic screens (*in vivo* or *in vitro*) are underlined. Genes that have not been hit in any screen are in blue.

on virulence. Remarkably, Pechous et al. showed that a triple mutant *ΔpurMCD* in both LVS (Pechous et al., 2006) and Schu S4 (Pechous et al., 2008) strains led to severe intracellular growth defects and strong attenuation in the mouse model. The fact that inability of these strains to synthesize purines *de novo* leads to a severe intracellular growth defect supports the notion that macrophages contain limiting concentrations of purines (Appelberg, 2006).

Pyr pathway. The pathway converting L-glutamine to uridine monophosphate (UMP) comprises six steps (**Figure 3**). Several mutants in this pathway have been identified in genetic screens (Qin and Mann, 2006; Weiss et al., 2007; Schulert et al., 2009; Asare and Abu Kwaik, 2010; Peng and Monack, 2010). Mutants unable to perform the first step, i.e., bacteria with mutations in genes *carA* or *carB* (encoding the two subunits of carbamoyl phosphate synthetase, converting L-glutamine to carbamoyl-P) were severely impaired in intramacrophage growth. However, conflicting results were reported regarding the phenotype of *pyrB* mutants. On one hand, a pyrB transposon insertion mutant of Schu S4 (Qin and Mann, 2006) showed reduced growth in HepG2 hepatocytes but normal growth in J774 cells, and was attenuated in mice and a similar mutant in subsp. *novicida* was shown to have a growth defect in human macrophages (Asare and Abu Kwaik, 2010). In contrast, another recent study (Kadzhaev et al., 2009) reports that a *pyrB* deletion mutant of Schu S4 is barely attenuated. In addition, mutants with transposon insertions in the *carA, carB*, and *pyrB* genes were selected upon screening of a bank of transposon mutants in LVS for mutants that failed to prevent the oxidative burst (Schulert et al., 2009). The three mutants appeared to grow normally in HepG2 and J774 cells but were killed by human monocytes and monocyte-derived macrophages. Transposon mutants of subsp. *novicida* with insertions in *pyrF* (encoding orotidine-5P decarboxylase, converting orotidine-5P to UMP) were identified in two *in vivo* screens (Weiss et al., 2007; Peng and Monack, 2010) and recently, a *pyrF* deletion mutant was generated in both *F. tularensis* subsp. *holarctica* (LVS) and *tularensis* (Schu S4). These *F. tularensis ΔpyrF* mutants were unable to replicate in primary human macrophages but retained full virulence in the mouse model (Horzempa et al., 2010).

These data clearly indicate that defects in the pyrimidine biosynthetic pathway affect *Francisella* virulence in a cell type- and strain-specific manner and suggest that *in vivo* the *pyr* pathway is not as important as the *pur* pathway for *F. tularensis* virulence.

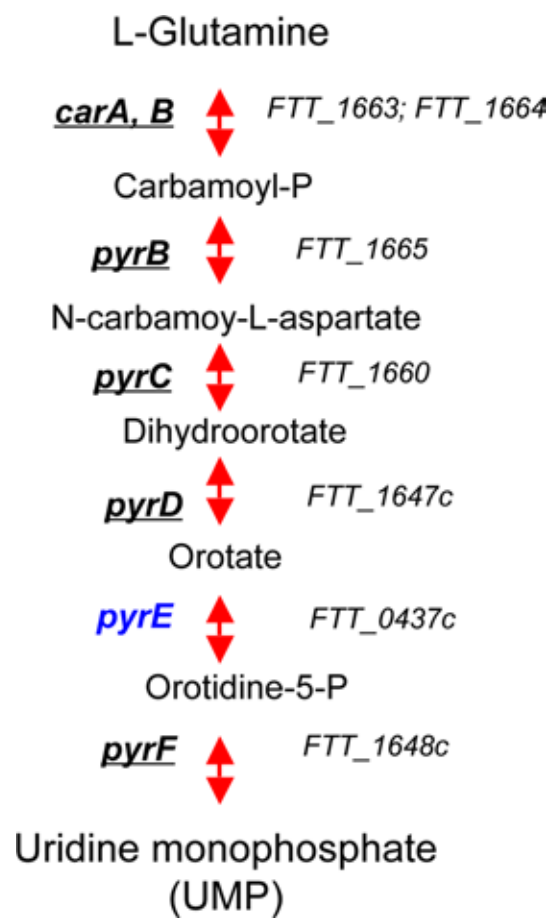

FIGURE 3 | The pyrimydine biosynthetic pathway. Genes that have been identified in genetic screens (*in vivo* or *in vitro*) are underlined. Genes that have not been hit in any screen are in blue.

F. TULARENSIS CARBON METABOLISM AND VIRULENCE

Each intracellular pathogen has adapted its intracellular metabolism to the nutrient supply of the host cell. Nevertheless, two bacterial species using the same host cell compartment (facing thus the same nutritional environment) may have quite different preferred carbon sources (and hence carbon metabolisms). Carbon catabolism provides the bacterial cell with energy and essential biosynthetic precursors such as glucose-6-phosphate (G6-P), fructose-6-phosphate (F6-P), 3-phosphoglycerate, PEP, and acetyl-CoA.

Glycolysis and gluconeogenesis pathways

Hexoses such as glucose are the preferred carbon and energy sources for many bacteria. The three best-characterized pathways of sugar catabolism in bacteria are glycolysis (**Figure 4**), the pentose phosphate pathway and the Entner–Doudoroff pathway. Each of these different pathways can be the preferred, or exclusive, carbon utilization pathway in a given pathogenic bacterial species. In the following we will discuss only genes encoding enzymes in glycolysis (and gluconeogenesis) and shown in **Figure 4** and not other carbohydrate metabolism pathways.

pckA. The enzyme PEP carboxykinase, encoded by the gene *pckA*, catalyzes the conversion of oxaloacetate to PEP. In *Mycobacterium bovis* BCG, a *pckA* mutant is attenuated both *in vitro* and *in vivo* (Liu et al., 2003), while this gene is dispensable in *Salmonella* (Tchawa Yimga et al., 2006). In *F. tularensis* subsp. *tularensis* Schu S4, Kadzhaev et al. (2009) observed no attenuation of a Δ*pckA* mutant in mice. The biological activity of this enzyme has not been experimentally established.

glpX. The glycolytic and gluconeogenic pathways comprise essentially the same set of enzymes that catalyze reversible reactions, except between F6-P and fructose-1,6-bisphosphate (F1,6-P2). The glycolytic reaction leading to the production of F1,6-P2 is catalyzed by phosphofructokinase, while the gluconeogenic reaction

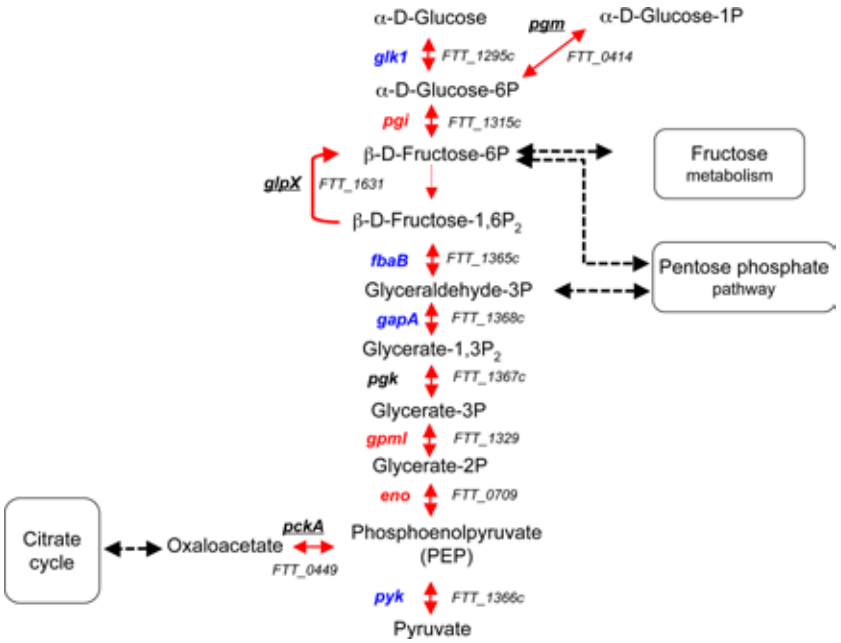

FIGURE 4 | The glycolytic and gluconeogenic pathways. GlpX is the only enzyme not promoting a reversible reaction. Genes that have been identified in genetic screens (*in vivo* or *in vitro*) are underlined. Genes that have not been hit in any screen are in blue. Genes predicted to be essential (according to Gallagher et al., 2007) are in red. The black dotted arrows indicate the connection between the glycolytic/gluconeogenic pathways and the pentose phosphate and fructose pathways. In each pathway, the gene name is indicated to the left of each reaction and the corresponding FTT number, to the right.

yielding F6P is catalyzed by fructose-1,6-bisphosphatase (FBP). The *F. tularensis* subsp. tularensis Schu S4 genome encodes apparently only a FBP (encoded by *glpX*) and lacks a *pfkA* gene encoding phosphofructokinase (Raghunathan et al., 2010). This suggests that *F. tularensis* uses the Embden–Meyerhof–Parnas pathway for gluconeogenesis rather than for glycolysis. However, the exact biological activity of the FBP enzyme remains to be experimentally established in *F. tularensis*. Mutants in the *glpX* gene have been repeatedly obtained in genetic screens, *in vivo* as well as *in vitro* (Maier et al., 2007; Su et al., 2007; Weiss et al., 2007; Kraemer et al., 2009; Peng and Monack, 2010). Moreover, a *glpX* deletion mutant of *F. tularensis* subsp. *tularensis* Schu S4 has been shown recently to be almost avirulent in the mouse model (Kadzhaev et al., 2009). Altogether, these observations strongly suggest that gluconeogenesis is critical for the full virulence of *F. tularensis*. At this stage, it cannot be excluded that the virulence defect of the *glpX* mutant could be due to another function of this enzyme, possibly regulatory. Supporting this hypothesis, none of the other genes involved in the conversion of glucose to PEP has been identified in previous genetic screens, but it is possible that some of these (*pgi, gpmI, eno*) are essential genes and therefore will not be found in screens (Gallagher et al., 2007). It remains to be determined by a systematic mutagenesis approach whether other enzymes in this pathway may also be involved in virulence.

pgm. The *pgm* gene encodes a predicted phosphoglucomutase, a glyconeogenic enzyme involved in the reversible conversion of glucose-1-phosphate (G1-P) to G6-P. A mutant in the *pgm* gene has been identified after an *in vivo* genetic screen in the *F. tularensis* subsp. *novicida* (Weiss et al., 2007).

The intracellular transcriptome of *F. tularensis* reveals that a number of genes involved in carbohydrate metabolism were up-regulated in BMM (Wehrly et al., 2009). In particular, genes of the glycolytic/gluconeogenic pathway have been found (*pgm, glk, fbaB,* and *pgk*; **Figure 4**), suggesting that this pathway is used during intracellular growth.

Very recently, a systems biology approach was applied to identify *F. tularensis* metabolic networks (Raghunathan et al., 2010). Integration of *in silico* metabolic reconstitutions and experimental data (including metabolic profiling and transcriptomic analyses) suggested that significant changes in carbohydrate metabolism occur during the intracellular growth phase. Gene expression profiling further supported the prediction that *F. tularensis* preferentially utilizes specific amino acids for energy and fatty acids as gluconeogenic substrates rather than relying on carbohydrate sources like glucose and fructose during infection.

NUTRIENT UPTAKE SYSTEMS OF *F. TULARENSIS*

Intracellular bacteria must possess dedicated nutrient uptake systems to capture their necessary host-derived nutrients. These systems must be particularly efficient for substrates available in limiting concentrations. The majority of the predicted transport systems present in *F. tularensis* are secondary carriers (**Table 2**). Secondary transporters encompass several major families, including: (i) the major facilitator superfamily (MFS, 31 proteins), predicted to participate in various functions including drug efflux, amino acids and sugar uptake; (ii) the amino acid-polyamine-organocation transporters (APC, 11 proteins); (iii) the hydroxy/aromatic amino acid permeases (HAAAP, 7 proteins); and (iv) the proton-dependent oligopeptide transporters (POT, 8 proteins).

Table 2 | The transport systems of *F. tularensis*.

ORF	Family ID[a]	Substrate	Experimental data[b]
ION CHANNELS			
FTT0685c	VIC	Potassium ion channel	Identified screen (Weiss et al., 2007)
FTT1775c	ClC	Chloride ion channel	–
FTT0475	MscS	Mechanosensitive channel	Identified screen (Asare et al., 2010)
FTT0992	MscS	Mechanosensitive channel	Identified screen (Asare et al., 2010)
FTT0133	MIP	Glycerol uptake	–
FTT1342	LIC	Glutamate-gated chloride channel	Identified screen (Asare and Abu Kwaik, 2010)
			Down-regulated in cells (Wehrly et al., 2009)
SECONDARY TRANSPORTERS			
FTT0006	MFS	Proline/betaine	–
FTT0026c	MFS	Drug efflux, Iron transport	Identified screen (Asare et al., 2010)
			Up-regulated in cells (Wehrly et al., 2009)
FTT0028c	MFS	Multidrug efflux, Iron transport	Identified screen (Weiss et al., 2007; Asare et al., 2010; Moule et al., 2010)
			Up-regulated in cells (Wehrly et al., 2009)
FTT0053	MFS	?	Identified screen (Maier et al., 2007; Weiss et al., 2007; Moule et al., 2010)
FTT0056c	MFS	?	Identified screen (Qin and Mann, 2006; Weiss et al., 2007; Moule et al., 2010; Peng and Monack, 2010)
FTT0070c	MFS	AmpG homolog	MglA regulated (Brotcke et al., 2006)
FTT0104c	MFS	?	Identified screen (Kraemer et al., 2009; Asare and Abu Kwaik, 2010)
FTT0127c	MFS	Multidrug efflux?	Identified screen (Kraemer et al., 2009)
FTT0129	MFS	?	Identified screen (Qin and Mann, 2006; Asare et al., 2010)
FTT0164c	MFS	Bicyclomycin efflux	Identified screen (Asare et al., 2010)
FTT0280c	MFS	Multidrug efflux?	–
FTT0442c	MFS	Bicyclomycin efflux	Down-regulated in cells (Wehrly et al., 2009)
FTT0444	MFS	Tetracycline efflux	Identified screen (Su et al., 2007)
FTT0488c	MFS	?	Identified screen (Asare et al., 2010)
			Down-regulated in cells (Wehrly et al., 2009)
FTT0671	MFS	?	–
FTT0708	MFS	?	Identified screen (Su et al., 2007; Asare et al., 2010)
			Up-regulated in cells (Wehrly et al., 2009)
FTT0719	MFS	Proline/betaine	Identified screen (Asare et al., 2010)
FTT0725c	MFS	Glycerol-3-phosphate	Identified screen (Asare and Abu Kwaik, 2010)
FTT0804	MFS	Proline/betaine	Down-regulated in cells (Wehrly et al., 2009)
FTT0931	MFS	Glucose/galactose	–
FTT0995	MFS	Proline/betaine	Identified screen (Asare et al., 2010; Moule et al., 2010)
FTT1148c	MFS	?	–
FTT1196c	MFS	Proline/betaine	Identified screen (Peng and Monack, 2010)
FTT1256	MFS	Multidrug efflux	Identified screen (Asare et al., 2010)
			Down-regulated in cells (Wehrly et al., 2009)
FTT1291	MFS	D-Galactonate	–
FTT1311	MFS	Multidrug efflux	Identified screen (Moule et al., 2010)
FTT1473c	MFS	Galactose/proton symport	Identified screen (Asare et al., 2010)
FTT1474c	MFS	Galactose/proton symport	Identified screen (Asare and Abu Kwaik, 2010)
FTT1683c	MFS	Multidrug efflux	–
FTT1727c	MFS	Chloramphenicol efflux	Down-regulated in cells (Wehrly et al., 2009)
FTT1783	MFS	Proline/betaine	Identified screen (Moule et al., 2010)
FTT0446	POT	Dipeptide/tripeptide/oligopeptide	Identified screen (Tempel et al., 2006)
			Down-regulated in cells (Wehrly et al., 2009)
FTT0572	POT	Dipeptide/tripeptide/oligopeptide	Up-regulated in cells (Wehrly et al., 2009)
FTT0651	POT	Dipeptide/tripeptide/oligopeptide	MglA-regulated (Brotcke et al., 2006)

(Continued)

Table 2 | Continued

ORF	Family ID[a]	Substrate	Experimental data[b]
FTT0686c	POT	Dipeptide/tripeptide/oligopeptide	Identified screen (Asare and Abu Kwaik, 2010) Up-regulated in cells (Wehrly et al., 2009)
FTT0953c	POT	Dipeptide/tripeptide/oligopeptide	Up-regulated cells (Wehrly et al., 2009)
FTT1005c	POT	Dipeptide/tripeptide/oligopeptide	Identified screen (Asare and Abu Kwaik, 2010; Moule et al., 2010)
FTT1233c	POT	Dipeptide/tripeptide/oligopeptide	–
FTT1253	POT	Dipeptide/tripeptide/oligopeptide	Identified screen (Kraemer et al., 2009) Up-regulated in cells (Wehrly et al., 2009)
FTT0219c	PiT	Phosphate	–
FTT1630c	SSS	Sodium ion/proline	Identified screen (Kraemer et al., 2009; Asare and Abu Kwaik, 2010)
FTT0598c	DAACS	Sodium ion/dicarboxylate	Up-regulated in cells (Wehrly et al., 2009)
FTT1337c	DAACS	C4-dicarboxylate	Identified screen (Asare et al., 2010)
FTT0849	BASS	Sodium ion/bile acid	Identified screen (Asare et al., 2010)
FTT0310	APC	Amino acid	MglA-regulated (Brotcke et al., 2006)
FTT0361c	APC	Glutamate/γ-aminobutyrate	Identified screen (Kraemer et al., 2009; Asare et al., 2010)
FTT0480c	APC	Glutamate/γ-aminobutyrate	Identified screen (Maier et al., 2007; Weiss et al., 2007; Kraemer et al., 2009; Peng and Monack, 2010)
FTT0881c	APC	Amino acid	Identified screen (Su et al., 2007)
FTT0968c	APC	Amino acid	Identified screen (Weiss et al., 2007; Su et al., 2007; Ahlund et al., 2010; Moule et al., 2010; Peng and Monack, 2010)
FTT0979c	APC	Amino acid	–
FTT1020c	APC	Amino acid	Identified screen (Moule et al., 2010)
FTT1149c	APC	Amino acid	Identified screen (Asare et al., 2010) Mutant constructed (Kadzhaev et al., 2009)
FTT1520c	APC	Glutamate/γ-aminobutyrate	–
FTT1633c	APC	Lysine	Identified screen (Weiss et al., 2007; Peng and Monack, 2010)
FTT1730c	APC	Amino acid	–
FTT1760	NhaA	Sodium ion/proton	Identified screen (Asare et al., 2010)
FTT0268	CPA1	Sodium ion/proton	Identified screen (Alkhuder et al., 2009; Kraemer et al., 2009)
FTT0604	CPA1	Sodium ion/proton	–
FTT1490	CPA1	Sodium ion/proton	Identified screen (Qin and Mann, 2006; Maier et al., 2007; Weiss et al., 2007; Moule et al., 2010)
FTT0669	CPA2	Potassium ion efflux	–
FTT1638	Trk	Potassium ion uptake	Identified screen (Alkhuder et al., 2010)
FTT0756	CDF	Cation efflux	Identified screen (Weiss et al., 2007; Asare and Abu Kwaik, 2010)
FTT0885	CDF	Cation efflux	–
FTT0827c	NCS2	Xanthine/uracil	–
FTT0115	CNT	Nucleosides	Identified screen (Moule et al., 2010)
FTT0116	CNT	Nucleosides	Identified screen (Moule et al., 2010)
FTT0712c	HAAAP	Serine	Identified screen (Weiss et al., 2007; Asare and Abu Kwaik, 2010)
FTT1126	HAAAP	Aromatic amino acid	–
FTT1502	HAAAP	Tyrosine	–
FTT1510c	HAAAP	Tyrosine	–
FTT1668	HAAAP	Serine	Identified screen (Asare and Abu Kwaik, 2010)
FTT1688	HAAAP	Tyrosine	Identified screen (Maier et al., 2007; Weiss et al., 2007; Asare et al., 2010)
FTT1732c	HAAAP	Tyrosine	Identified screen (Asare and Abu Kwaik, 2010)
FTT0853	ArsB	Arsenite (ArsB)	–
FTT1339c	SulP	Sulfate	–
FTT0105c	RND	Multidrug efflux	Identified screen (Su et al., 2007) Mutant constructed (Bina et al., 2008)
FTT1115c	RND	Protein-export (SecDF)	–

(Continued)

Table 2 | Continued

ORF	Family ID[a]	Substrate	Experimental data[b]
FTT1114c	RND	Protein-export (SecDF)	–
FTT1728	NhaD	Sodium ion/proton	Identified screen (Asare et al., 2010)
FTT0368c	MOP	Virulence factor MviN	Identified screen (Asare et al., 2010)
FTT1332	MOP	Polysaccharide	–
FTT1453c	MOP	O-antigen	–
FTT0157c	DMT	Choline uptake (LicB)	–
FTT0759	DMT	Drug/metabolite?	Identified screen (Su et al., 2007)
FTT1004c	DMT	Drug/metabolite?	Identified screen (Asare et al., 2010)
FTT1118c	DMT	Drug/metabolite?	Identified screen (Kraemer et al., 2009; Asare and Abu Kwaik, 2010)
FTT1399	DMT	Drug/metabolite?	Down-regulated in cells (Wehrly et al., 2009)
FTT1511	DMT	Drug/metabolite?	–
FTT1787c	LysE	Lysine efflux	–
FTT1431	RhtB	Threonine efflux	–
FTT0829c	AAE	Aspartate/alanine	Identified screen (Kraemer et al., 2009; Moule et al., 2010)
FTT0233c	Oxa1	OxaA homolog	Down-regulated in cells (Wehrly et al., 2009)
ATP-DEPENDENT			
FTT0109	ABC	Lipid A export	–
FTT0126	ABC	Oligopeptide	–
FTT0125	ABC	Oligopeptide	Mutant constructed, not attenuated (Kadzhaev et al., 2009) MglA-regulated (Brotcke et al., 2006)
FTT0175c	ABC	Nitrate/sulfonate/taurine	Identified screen (Asare et al., 2010) Up-regulated in cells (Wehrly et al., 2009)
FTT0265	ABC	Nitrate/sulfonate/taurine	–
FTT0266	ABC	Nitrate/sulfonate/taurine	Identified screen (Asare and Abu Kwaik, 2010)
FTT0209c	ABC	Manganese/zinc ion	Identified screen (Su et al., 2007; Asare et al., 2010) Up-regulated in cells (Wehrly et al., 2009)
FTT0208c	ABC	Manganese/zinc ion	Identified screen (Asare and Abu Kwaik, 2010)
FTT0207c	ABC	Manganese/zinc ion	–
FTT0405	ABC	Lipoprotein releasing	Identified screen (Weiss et al., 2007)
FTT0404	ABC	Lipoprotein releasing	Down-regulated in cells (Wehrly et al., 2009)
FTT0481	ABC	Putrescine	–
FTT0564	ABC	Putrescine	Identified screen (Kraemer et al., 2009)
FTT0563	ABC	Putrescine	–
FTT0562	ABC	Putrescine	Identified screen (Asare and Abu Kwaik, 2010)
FTT0728	ABC	Multidrug	–
FTT0729	ABC	Multidrug	–
FTT0793	ABC	Multidrug	Identified screen (Weiss et al., 2007; Asare and Abu Kwaik, 2010; Peng and Monack, 2010)
FTT0972	ABC	? (Fe–S assembly/SufBCD system)	–
FTT0971	ABC	? (Fe–S assembly/SufBCD system)	–
FTT0973	ABC	? (Fe–S assembly/SufBCD system)	Identified screen (Kraemer et al., 2009)
FTT1024c	ABC	? (YhbG)	–
FTT1125	ABC	D-Methionine	Identified screen (Maier et al., 2007; Su et al., 2007; Kraemer et al., 2009; Asare et al., 2010) Up-regulated in cells (Wehrly et al., 2009)
FTT1124	ABC	D-Methionine	Identified screen (Maier et al., 2007) Up-regulated in cells (Wehrly et al., 2009)

(Continued)

Table 2 | Continued

ORF	Family ID[a]	Substrate	Experimental data[b]
FTT1248	ABC	?	Identified screen (Asare et al., 2010)
FTT1247	ABC	?	–
FTT1608	ABC	Toluene tolerance	–
FTT1249	ABC	Toluene tolerance	Identified screen (Moule et al., 2010)
FTT1611	ABC	Toluene tolerance	Identified screen (Su et al., 2007; Asare et al., 2010)
FTT1609	ABC	Toluene tolerance	–
FTT1335	ABC	CydC/CydD homolog	–
FTT1336	ABC	CydC/CydD homolog	–
FTT1435c	ABC	Multidrug?	–
FTT1434c	ABC	Multidrug?	Identified screen
FTT1782c	ABC	?	Identified screen (Su et al., 2007; Weiss et al., 2007; Moule et al., 2010)
FTT0060	F-ATPase	Protons	–
FTT0064	F-ATPase	Protons	–
FTT0059	F-ATPase	Protons	–
FTT0063	F-ATPase	Protons	–
FTT0058	F-ATPase	Protons	–
FTT0062	F-ATPase	Protons	–
FTT0061	F-ATPase	Protons	Identified screen (Peng and Monack, 2010)
FTT0065	F-ATPase	Protons	Identified screen (Kraemer et al., 2009)
FTT1737c	P-ATPase	Potassium ion	Identified screen (Asare and Abu Kwaik, 2010; Moule et al., 2010)
FTT1738c	P-ATPase	Potassium ion	Identified screen (Asare et al., 2010)
FTT0120	IISP	SRP receptor FtsY	Identified screen (Moule et al., 2010)
FTT0964c	IISP	SRP protein Ffh	–
PTS			
FTT1280c	SSPTS	Nitrogen regulatory	–
UNCLASSIFIED			
FTT0668	PnuC	Nicotinamide mononucleotide	Identified screen (Asare et al., 2010; Moule et al., 2010)
			Up-regulated in cells (Wehrly et al., 2009)
FTT0707	PnuC	Nicotinamide mononucleotide	–
FTT1090	PnuC	Nicotinamide mononucleotide	MlgA-regulated (Brotcke et al., 2006)
FTT0249	FeoB	Ferrous ion	Identified screen (Su et al., 2007; Ahlund et al., 2010)

[a]*Transport family names: MFS, major facilitator superfamily; VIC, voltage-gated ion channel superfamily; CIC, chloride channel family; MscS, small conductance mechanosensitive ion channel; MIP, major intrinsic protein family; LIC, ligand-gated ion channel; POT, proton-dependent oligopeptide transporter family; PiT, inorganic phosphate transporter family; SSS, solute:sodium symporter family; DAACS, dicarboxylate/amino acid:cation (Na+ or H+) symporter family; BASS, bile acid:Na+ symporter family; APC, amino acid-polyamine-organocation family; NhaA, NhaA Na+:H+ antiporter family; CPA1, monovalent cation:proton antiporter-1 family; Trk, K+ transporter family; CDF, cation diffusion facilitator family; NCS2, nucleobase:cation symporter-2 family; CNT, concentrative nucleoside transporter family; HAAAP, hydroxy/aromatic amino acid permease family; ArsB, arsenite–antimonite efflux family; SulP, sulfate permease family; RND, resistance-nodulation-cell division superfamily; NhaD, NhaD Na+:H+ antiporter family; MOP, multidrug/oligosaccharidyl-lipid/polysaccharide flippase superfamily; DMT, drug/metabolite transporter superfamily; LysE, L-lysine exporter family; RhtB, resistance to homoserine/threonine family; AAE, aspartate:alanine exchanger family; oxa1, cytochrome oxidase biogenesis family; ABC, ATP-binding cassette superfamily; F-ATPase, H+- or Na+-translocating F-type, V-type and A-type ATPase superfamily; P-ATPase, P-type ATPase superfamily; IISP, type II (General) secretory pathway family; SSPTS, sugar specific PTS; PnuC, nicotinamide mononucleotide (NMN) uptake permease family; FeoB, ferrous iron uptake family.*

[b]*For each putative transporter it is indicated if the encoding gene has been identified in a cell-based or in vivo screen, is regulated by the major virulence regulator MglA, or has a changed expression after entry into macrophages.*

Several mutants in secondary transporters were identified in various genetic screens. For example, the gene *xasA*, encoding a predicted glutamate/γ-aminobutyrate transporter of the APC family (FTT_0480c, **Table 2**), has been identified in four different screens (Maier et al., 2007; Weiss et al., 2007; Kraemer et al., 2009; Peng and Monack, 2010), supporting a functional role in *F. tularensis* virulence. The gene *FTT_0708*, encoding a transporter of the MFS family has also been identified in an *in vivo* screen (Su et al., 2007), as were other transporters of this family.

The *F. tularensis* subsp. *tularensis* Schu S4 genome also encodes 15 complete transport ABC-type carriers, consisting of a membrane-spanning permease and an ATP-binding subunit (Atkins et al., 2006). These ABC transporters are predicted to participate in diverse functions, ranging from amino acid/peptide and ion uptake to multidrug efflux. Remarkably, mutants in one ABC transporter (FTT_1125) have been identified in several *in vitro* (Maier et al., 2007) and *in vivo* (Su et al., 2007; Kraemer et al., 2009) screens for attenuated mutants, indicating a direct contribution to *F. tularensis* virulence.

Supporting a role of transport systems in intracellular survival, transcriptional profiling of the *F. tularensis* subsp. *tularensis* Schu S4 strain in BMMs (Wehrly et al., 2009) revealed that genes encoding various transporters showed significantly altered expression (either up- or down-regulated) after host cell entry. Notably, five of the eight POT family members were up-regulated intracellularly. The amino acid identity between the POT family members does not exceed 47% (ranging from 22 to 31%, in most cases), suggesting that they might have distinct transport properties. Also, four of the POT transporters have been found in mutant screens (Tempel et al., 2006; Kraemer et al., 2009; Asare and Abu Kwaik, 2010), further indicating that these oligopeptide transporters and therefore amino acid metabolism is important during infection.

In addition, the *F. tularensis* subsp. *tularensis* Schu S4 genome encodes six putative ion channels but is devoid of any PEP-dependent phosphotransferase (PTS) system. At present, no biochemical data are available on any of the transporters present in *F. tularensis*.

One example of amino acid supply provided by the host cytosol

The cytosol of eukaryotic cells contains a high concentration (10 mM) of the tripeptide γ-glu-gly-cys named glutathione (in its reduced form, GSH; Alkhuder et al., 2009). GSH plays a pleiotropic and major role in mammalian cell homeostasis and GSH-deficiency has been associated with various severe diseases (Griffith, 1999; Wu et al., 2004; Franco et al., 2007). Biosynthesis of GSH is dependent on the availability of the amino acid precursors glutamate, glycine, and cysteine. The intracellular pool of cysteine is relatively small (0.10–0.25 mM) and cysteine is generally the limiting amino acid for GSH synthesis. The other two precursors, glycine and glutamate, are found in considerable higher intracellular concentration. As mentioned earlier, *F. tularensis* subspecies requires cysteine for growth. We have recently demonstrated that gene *FTL_0766* encodes a genuine γ-glutamyl transpeptidase (GGT) involved in the metabolism of γ-glutamyl-containing peptides. GGT allows the utilization of γ-glutamyl peptides as a source of cysteine during intracellular multiplication of the *F. tularensis* subsp. *holarctica* strain LVS, and is thus critical for its virulence (Alkhuder et al., 2009). This work represents the only direct experimental evidence of nutrient utilization by intracellular *F. tularensis*. The transporter of GSH and the molecular mechanism of crossing the bacterial envelope remain to be discovered.

STARVING THE INVADING BACTERIA AS A HOST CELL DEFENSE MECHANISM

The capacity of a macrophage to deprive intracellular pathogens of required nutrients (Appelberg, 2006) can be viewed as an intrinsic antimicrobial innate immune defense mechanism. Thus, microbial killing may not rely only on a toxic environment (such as low pH and oxidative stress in the phagosomal compartment) but also may result from the scarcity of nutrients in the cellular compartment it occupies (transiently or permanently). At any rate, one must keep in mind that the notion of a limiting concentration of nutrient may vary considerable from one intracellular pathogen to another.

Two distinct mechanisms of nutrient deprivation exist: (i) constitutive mechanisms, such as that mediated by the divalent cation transporter Nramp1, present in the membrane of endosomal compartment and participating to iron depletion (Cellier et al., 2007); and (ii) induced mechanisms, such as those triggered by the cytokine IFN-γ in activated macrophages, which may also affect iron availability (Mulero and Brock, 1999).

Notably, another pathway triggered by IFN-γ has been shown to play a role in the nutritional control of several intracellular pathogens (Taylor and Feng, 1991). Activation of the enzyme indoleamine 2,3-dioxygenase (ID) which degrades L-tryptophan by IFN-γ thus leads to tryptophan deprivation. Monack and co-workers very recently showed that tryptophan auxotrophs of *F. tularensis* subsp. *novicida* were severely affected in intracellular survival and multiplication and were attenuated in the mouse model (Peng and Monack, 2010). Interestingly, tryptophan metabolism appeared to be important only for bacterial colonization of the lungs, suggesting an organ specificity of this metabolic need. The authors found that this antimicrobial starvation mechanism mediated by the enzyme ID was effective against both auxotrophic and prototrophic microbes. These observations support the notion that, for bacteria, amino acid biosynthesis is more energetically costly than their capture from the environment. The necessity to biosynthesize amino acid in a depleted environment may thus reduce the capacity of the bacterium to replicate.

CONCLUDING REMARKS

Genetic screens have clearly established the critical importance of the aromatic amino acids biosynthetic pathway for *F. tularensis* virulence. The fact that inactivation of almost every gene in this pathway lead to reduced virulence suggests that the available pool of aromatic amino acids is limiting in the infected host. Similarly, the severe intracellular growth defect of mutants unable to synthesize purines *de novo*, also suggest that macrophages contain limiting concentrations of purines.

Altogether, experimental data and predictive models favor the notion that *F. tularensis* preferentially utilizes specific amino acids for energy and fatty acids as gluconeogenic substrates rather than carbohydrate sources during cytosolic multiplication. Intracellular transcriptomic studies revealed that significant changes occurred in the expression of genes encoding enzymes involved in carbohydrate metabolism as well as in genes encoding putative amino acid and carbohydrate transporters. Hence, the importance of nutrition in *F. tularensis* virulence can be seen as a fine balance between its ability to capture nutrients from the host (transport) and the regulation of its metabolism in response to the amounts of nutrients available (metabolism). Mutations affecting either one or the other (or both) of these two functions lead to an impaired fitness and are likely to cause a reduced virulence.

Intracellular pathogens often co-regulate their metabolic needs and the production of dedicated virulence factors by using pleiotropic (mainly transcriptional) regulators. The stringent response is a stress response that occurs in bacteria in reaction to amino acid or carbon starvation. The stringent response is signaled by the alarmone ppGpp. Notably, the expression of many virulence regulators is mediated by ppGpp, thereby coupling pathogenesis to metabolism (Dalebroux et al., 2010). *F. tularensis* also uses ppGpp to control the activity of its major regulator of virulence, MglA (Charity et al., 2009). In particular, ppGpp

was shown recently to promote physical interactions between the MglA–SspA complex and the putative DNA binding factor PigR (also designated FevR) to control the PigR-dependent activation of the *Francisella* pathogenicity island (Charity et al., 2009). These data suggest a link between nutrient availability and virulence. They are in agreement with the transciptomic analysis of a *mglA* knock-out mutant of *F. tularensis* subsp. *novicida* (Brotcke et al., 2006). Indeed, among the 102 MglA-regulated genes identified, 20 were predicted to play a role in metabolism, particularly in amino acid metabolism.

A systematic mutational analysis of *F. tularensis* metabolic pathways, coupled to thorough biochemical and biophysical characterization of its metabolic capacities will be required to fully understand the complex interplays between metabolism and virulence.

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The *Francisella* intracellular life cycle: toward molecular mechanisms of intracellular survival and proliferation

Audrey Chong and Jean Celli*

Tularemia Pathogenesis Section, Laboratory of Intracellular Parasites, Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Hamilton, MT, USA

Edited by:
Anders Sjostedt, Umeå University, Sweden

Reviewed by:
Lee-Ann H. Allen, University of Iowa, USA
Luiz Bermudez, Oregon State University, USA

****Correspondence:***
Jean Celli, Laboratory of Intracellular Parasites, Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, National Institutes of Health, 903 South 4th Street, Hamilton, MT 59840, USA.
e-mail: jcelli@niaid.nih.gov

The tularemia-causing bacterium *Francisella tularensis* is a facultative intracellular organism with a complex intracellular lifecycle that ensures its survival and proliferation in a variety of mammalian cell types, including professional phagocytes. Because this cycle is essential to *Francisella* pathogenesis and virulence, much research has focused on deciphering the mechanisms of its intracellular survival and replication and characterizing both bacterial and host determinants of the bacterium's intracellular cycle. Studies of various strains and host cell models have led to the consensual paradigm of *Francisella* as a cytosolic pathogen, but also to some controversy about its intracellular cycle. In this review, we will detail major findings that have advanced our knowledge of *Francisella* intracellular survival strategies and also attempt to reconcile discrepancies that exist in our molecular understanding of the *Francisella*–phagocyte interactions.

Keywords: *Francisella*, macrophage, phagosome, pathogenesis

INTRODUCTION

Many pathogenic microorganisms are characterized by an intracellular lifestyle and their ability to enter, survive, proliferate, and/or persist within cells of the infected host, therefore avoiding recognition and elimination by extracellular immune surveillance mechanisms. To achieve this, these pathogens have evolved sophisticated strategies to circumvent intracellular recognition and microbicidal processes, ultimately ensuring their survival and proliferation within the host. The Gram-negative bacterium *Francisella tularensis* is the causative agent of tularemia, a vector-borne zoonosis of the Northern Hemisphere that can affect humans and cause severe morbidity and mortality if untreated or misdiagnosed. Human tularemia is a fulminant disease that can be contracted by exposure to as few as 10 bacteria, the pneumonic form of which is the most severe (Oyston et al., 2004). Three subspecies of *F. tularensis*, *F. tularensis* subsp. *tularensis* (Type A), *F. tularensis* subsp. *holarctica* (Type B), and *F. tularensis* subsp. *mediasiatica* are recognized, among which strains from subspecies *tularensis* and *holarctica* can cause tularemia in humans (Oyston et al., 2004). Additionally, *F. novicida*, an avirulent *Francisella* species in humans that retains high virulence in rodents, has been used extensively as a surrogate model for *F. tularensis* due to lower biocontainment requirements (Baron and Nano, 1998; Lauriano et al., 2004; Santic et al., 2005a,b, 2007, 2008; Brotcke et al., 2006; de Bruin et al., 2007; Mohapatra et al., 2007a,b, 2008, 2010; Weiss et al., 2007; Brotcke and Monack, 2008; Barker et al., 2009a,b; Schmerk et al., 2009a,b; Al-Khodor and Abu Kwaik, 2010; Asare and Abu Kwaik, 2010; Asare et al., 2010). Essential to the development of tularemia is the bacterium's ability to infect and proliferate within mononuclear phagocytes, such as macrophages and dendritic cells, although this bacterium can also infect polymorphonuclear neutrophils, hepatocytes, epithelial, and endothelial cells (Oyston et al., 2004).

Because of the importance of its intracellular lifestyle, the interactions of *F. tularensis* with host cells have been studied thoroughly, with a particular emphasis on those with macrophages. Using a variety of *Francisella* strains, such as *F. novicida*, the attenuated *holarctica* vaccine strain LVS, or virulent strains such as SchuS4 or clinical *tularensis* isolates, and host cell models, including murine or human primary macrophages and macrophage-like cell lines, several laboratories have defined the intracellular cycle of *Francisella* (**Figures 1 and 2**). Following phagocytic uptake, *Francisella* initially resides within a phagosome called the *Francisella*-containing phagosome (FCP) that interacts with early and late compartments of the endocytic pathway (Clemens et al., 2004; Santic et al., 2005a; Checroun et al., 2006; Chong et al., 2008), prior to phagosomal membrane disruption that allows bacterial release in the macrophage cytosol (Golovliov et al., 2003; Clemens et al., 2004; Santic et al., 2005a; Checroun et al., 2006; Chong et al., 2008; Wehrly et al., 2009). Once cytosolic, bacteria undergo extensive replication that culminates in the apoptotic and pyroptotic deaths of the infected cells (Lai et al., 2001; Lai and Sjostedt, 2003; Mariathasan et al., 2005; Santic et al., 2010) and/or reentry into the endocytic compartments in *Francisella*-containing vacuoles (FCV) in murine primary macrophages via an autophagy-mediated process (Checroun et al., 2006; Wehrly et al., 2009). Our current knowledge of the *Francisella* intracellular cycle indicates that this bacterium can be considered a cytosolic pathogen, yet cycles through a variety of intracellular compartments with distinct environments to ensure survival, proliferation, and eventual release. For this purpose, it has likely evolved a battery of mechanisms to subvert various host cell processes. In this review, we will focus on *Francisella*–macrophage interactions and discuss recent findings about both bacterial and host factors that contribute to the intracellular pathogenesis of this bacterium.

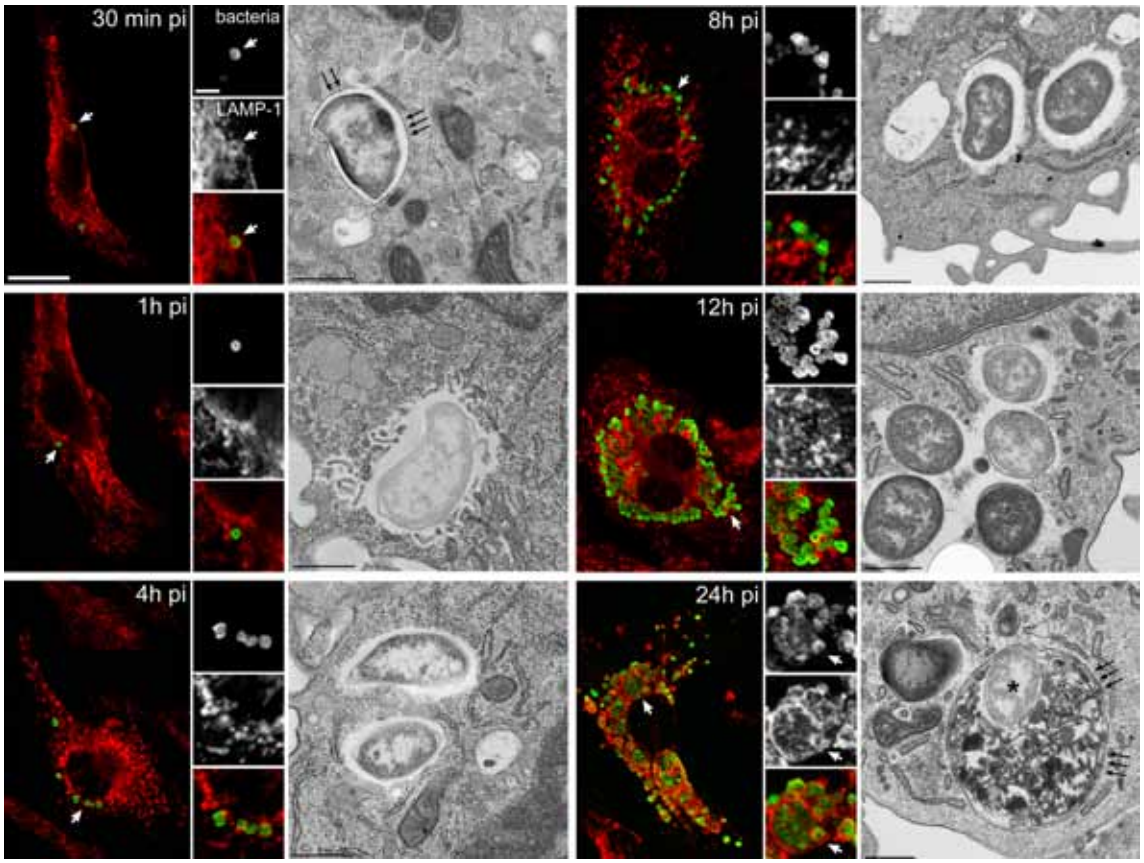

FIGURE 1 | Infection cycle of *F. tularensis* Schu S4 within C57BL/6J murine bone marrow-derived macrophages (BMMs). BMMs were infected with Schu S4 and processed at various times post infection (pi) for immunofluorescence or transmission electron microscopy. Representative confocal and electron (TEM) micrographs of intracellular SchuS4 at 30 min, 1, 4, 8, 12, and 24 h pi, showing the early phagosomal stage, phagosomal disruption, bacterial release in the cytosol, cytosolic replication, and FCV formation. Bacteria appear in green and endosomal, LAMP-1-positive membranes appear in red. White arrowheads indicate either regions of interest in whole images or bacteria enclosed within a LAMP-1-positive compartment in insets. Black arrows in TEM micrographs indicate single or double membranes surrounding intracellular bacteria. Scale bars, 10 and 2 μm (confocal images) or 0.5 μm (TEM images). Reproduced with permission from John Wiley and Sons Publishing.

ENTRY INTO MAMMALIAN CELLS

An essential step in the lifestyle of intracellular pathogens is binding and entry into host cells where they can undergo survival and proliferation. Such interactions require engagement of receptors that triggers specific signaling cascades, phagosome maturation events, and host microbicidal responses devoted to the destruction of the microorganism (Underhill and Ozinsky, 2002). Thus, the mode of uptake likely impacts the intracellular fate of a given pathogen.

The underlying mechanisms that are important for the phagocytosis of *Francisella* by macrophages are beginning to be defined. Uptake of *Francisella* into macrophages is markedly enhanced by serum opsonization (Clemens et al., 2004, 2005; Balagopal et al., 2006; Schulert and Allen, 2006). In an ultrastructural analysis of the entry process of a clinical *F. tularensis* subspecies *tularensis* isolate and the attenuated *Francisella* live vaccine strain (LVS) into human monocyte derived macrophages (MDMs) or THP-1 cells, Clemens et al. (2005) observed bacteria engulfed within asymmetric pseudopod loops, a process that was dependent on filamentous actin, serum with intact complement factor C3, and complement receptors. Several studies have also described the contribution of complement and the complement receptor CR3 in the phagocytosis of serum-opsonized *F. novicida* and LVS by human and murine macrophages, as well as human neutrophils and dendritic cells (Balagopal et al., 2006; Ben Nasr et al., 2006; Pierini, 2006; Schulert and Allen, 2006; Barker et al., 2009a). Fcγ receptors, scavenger receptor class A (SR-A), nucleolin, and the lung surfactant protein A (SP-A) are also involved in the uptake of serum-opsonized *F. novicida* and LVS into human and murine macrophages (Balagopal et al., 2006; Pierini, 2006; Barel et al., 2008), yet the predominant entry pathway appears to be mediated by complement receptor CR3 (**Table 2**). Although it remains to be determined whether serum opsonization of *Francisella* is important during pathogenesis *in vivo*, CR3-mediated phagocytosis may contribute to the bacterium's intracellular survival, since this route of uptake does not induce the macrophage oxidative burst (Underhill and Ozinsky, 2002) and could therefore limit antimicrobial activities by the infected cells. Compared to opsonized bacteria, phagocytosis of non-opsonized *F. novicida* and LVS by human MDMs is mediated, in part, by the mannose receptor (MR; Balagopal et al., 2006; Schulert and Allen, 2006). The contribution of this receptor

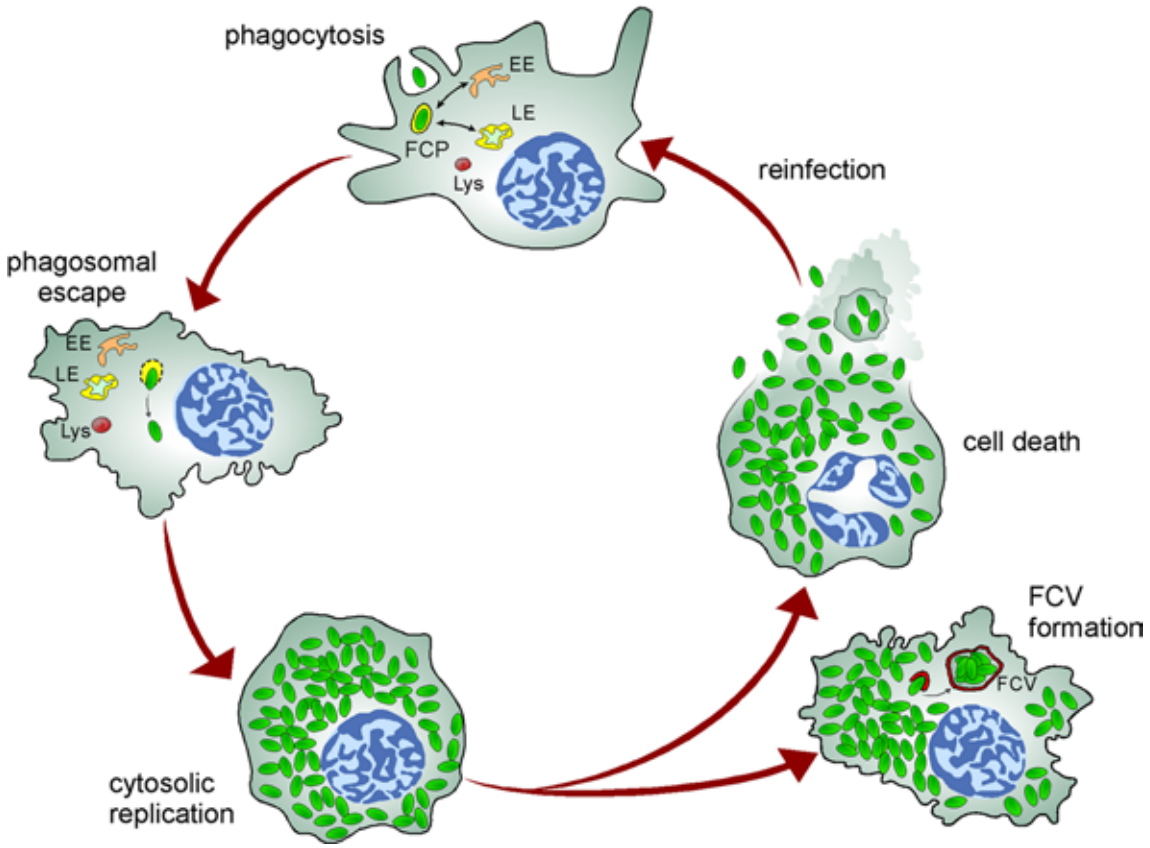

FIGURE 2 | Model of the *Francisella* intracellular cycle in macrophages. Upon phagocytosis, bacteria reside in an early phagosome (FCP) that interacts with early (EE) and late (LE) endocytic compartments but not lysosomes (Lys). Bacteria rapidly disrupt the FCP membrane and reach the cytosol where they undergo extensive replication, a process followed by cell death and bacterial release or reentry of cytosolic bacteria within *Francisella*-containing vacuoles (FCV) via autophagy in murine macrophages.

was further confirmed by the enhanced entry of LVS into J774A.1 macrophage-like cells expressing the MR and human MDMs with upregulated expression of the MR (Schulert and Allen, 2006).

A variety of pathogens target lipid rafts for internalization into host cells to avoid intracellular degradation (Zaas et al., 2005). *Francisella* appears to exploit this portal of entry, since cholesterol and caveolin-1, two components of lipid rafts, are incorporated into the newly formed FCP and cholesterol-rich lipid domains are required for uptake of LVS into the murine macrophage-like cell lines J774A.1 and RAW264.7 (Tamilselvam and Daefler, 2008). *Francisella* may target lipid rafts via the engagement of receptors that preferentially reside within these membrane signaling platforms, such as CR3 and Fcγ receptors (Peyron et al., 2000; Beekman et al., 2008). Consistently, depletion of lipid raft-associated GPI-anchored proteins abolished LVS entry and intracellular survival, suggesting a requirement for signaling events mediated through these proteins. Intracellular signals that accompany phagocytosis of *Francisella* involve the tyrosine kinase Syk and the MAP kinase ERK pathway (Parsa et al., 2008). Syk was activated upon infection of RAW264.7 cells with non-opsonized *F. novicida* (Parsa et al., 2008). Inhibition of Syk blocked uptake of *F. novicida* whereas overexpression enhanced uptake, demonstrating the involvement of this kinase in promoting bacterial entry (Parsa et al., 2008). Further analysis revealed that Syk regulates the activation of ERK and PI3K/Akt pathways. Inhibition of ERK, but not the PI3K/Akt pathway, decreased phagocytosis of *F. novicida* by macrophages (Parsa et al., 2008). Thus, the ERK pathway appears to be important for the phagocytosis of *Francisella*, but neither the precise mechanism of ERK activation nor the phagocytic pathways initiating this signaling have been determined.

FRANCISELLA INTRACELLULAR SURVIVAL STRATEGIES

Regardless of their replication niche, most intracellular pathogens initiate their intracellular cycle within the membrane-enclosed environment of the nascent phagosome. Newly formed phagosomes typically undergo a maturation process involving sequential interactions with the endosomal–lysosomal network that progressively modify their composition into a degradative compartment (Haas, 2007). To survive the destructive nature of a mature phagolysosome, intracellular pathogens must either evade or withstand this hostile environment (Haas, 2007). Although *Francisella* disrupts its phagosome to proliferate in the macrophage cytosol, the bacterium is likely outfitted to combat the microbicidal arsenal of this compartment during its interim phagosomal stage.

INTRACELLULAR SURVIVAL: INHIBITION OF NADPH OXIDASE ACTIVITY

Among the microbicidal agents encountered by newly ingested pathogens are the reactive oxygen species (ROS) generated by NADPH oxidase complexes assembled at the nascent phagosomal

membrane. NADPH oxidase assembly involves phosphorylation of cytosolic p47^{phox}, which is complexed with p67^{phox} and p40^{phox}, and the entire heterotrimer translocates to cell membrane sites where the gp91^{phox} and p22^{phox} subunits accumulate. Participation of a small G-protein, either Rac1 or Rac2, is also required for NADPH oxidase function (Babior, 2004). *Francisella* avoids this early killing mechanism by blocking NADPH oxidase activation on the newly formed FCP in human neutrophils, as well as human and murine macrophages (McCaffrey and Allen, 2006; Buchan et al., 2009; Schulert et al., 2009; McCaffrey et al., 2010; Mohapatra et al., 2010). Whereas virulent strains of the *tularensis* (strain Schu S4) and *holarctica* (strain 1547-57) subspecies and the attenuated *holarctica* LVS strain exclude gp91^{phox}/gp22^{phox} heterodimers from FCPs and limit p47^{phox} phosphorylation (McCaffrey and Allen, 2006; McCaffrey et al., 2010), *F. novicida* appears to mainly depend upon dephosphorylation of Phox components to disrupt NADPH oxidase function (Mohapatra et al., 2010). Furthermore, SchuS4 and the virulent *holarctica* strain FSC200 are more resistant to killing by ROS *in vitro* than the avirulent LVS, via mechanisms independent of the catalase KatG (Lindgren et al., 2007). These data highlight the subtle differences in intracellular survival mechanisms used by different *Francisella* species. Additionally, *Francisella* impairs ROS production by subsequent heterologous stimulation (McCaffrey and Allen, 2006; Edwards et al., 2010; McCaffrey et al., 2010; Mohapatra et al., 2010). McCaffrey et al. (2010) determined that LVS does so by retaining dysfunctional NADPH oxidase complexes on the phagosomal membranes. Altogether, these studies clearly document that *Francisella* affects pre- and post-NADPH oxidase assembly mechanisms to inhibit the phagocyte respiratory burst (McCaffrey et al., 2010), a process that likely contributes to its survival within the FCP.

More controversial, species-specific findings have been made on the role of other *Francisella* proteins in inhibiting antimicrobial processes. An early study showed that the acid phosphatase AcpA, purified from an attenuated *F. tularensis* type A strain (ATCC6223), inhibits the oxidative burst of activated porcine neutrophils (Reilly et al., 1996). Consistent with this report, Mohapatra et al. (2010) found that loss of AcpA function in *F. novicida* resulted in ROS production and impaired intracellular survival in human neutrophils and MDMs. Moreover, infection with a quadruple acid phosphatase deletion mutant (Δ*acpA*ΔacpBΔacpCΔhap) triggered even higher levels of oxidant generation and increased bacterial killing than infection with a Δ*acpA* mutant, illustrating the cumulative contribution of acid phosphatases to evasion of oxidative killing (Mohapatra et al., 2010). Contrary to the observations regarding AcpA in *F. novicida*, studies in SchuS4 revealed that AcpA activity is dispensable for inhibition of the phagocyte oxidative defense, indicating that observations made using *F. novicida* do not always apply to *F. tularensis* subspecies strains (McCaffrey et al., 2010). Other bacterial factors have also been implicated in impairing oxidase activation. Pyrimidine biosynthesis genes (*carA, carB*, and *pyrB*) were identified in LVS by a random transposon mutagenesis screen for factors affecting neutrophil NADPH oxidase activation (Schulert et al., 2009). These uracil auxotroph mutants triggered an oxidative burst in neutrophils and were killed in phagosomes containing superoxide (Schulert et al., 2009). Additionally, these mutant strains were unable to block subsequent neutrophil activation by heterologous stimuli; however, the effects on neutrophil activation may be due indirectly to the diminished fitness of these mutants (Schulert et al., 2009). Interestingly, these auxotrophic mutants did not elicit a respiratory burst in human monocytes or MDMs but were, nonetheless, unable to survive within these phagocytes (Schulert et al., 2009). Together, these data suggest that *Francisella* evades oxidative killing by different mechanisms in monocytes and macrophages versus neutrophils: in the former, by utilizing uptake receptors (e.g., CR3) that do not trigger an oxidative burst, and in the latter, by actively inhibiting NADPH oxidase assembly and activation (Schulert et al., 2009). Two *Francisella* virulence gene regulators, *fevR* and *migR*, were also shown to be required by LVS for regulating NADPH oxidase activity in neutrophils (Buchan et al., 2009; McCaffrey et al., 2010), suggesting they regulate expression of specific effector proteins. Studies in SchuS4 confirmed that *fevR* is required to block NADPH oxidase activity, but two proteins encoded by the *Francisella* pathogenicity island (FPI) affecting phagosomal escape, *iglI* and *iglJ* (see below), are not. These observations demonstrate that distinct genes within the *fevR* regulon are required for inhibiting oxidative burst and disrupting the FCP (McCaffrey et al., 2010). Altogether, these data indicate that *Francisella* applies a multi-factorial, multi-pronged strategy to circumvent phagocyte oxidative defenses.

INTRACELLULAR SURVIVAL: BACTERIAL ESCAPE FROM THE *FRANCISELLA*-CONTAINING PHAGOSOME

Although an early study suggested that *Francisella* inhabit atypical phagosomes within rodent macrophages (Anthony et al., 1991), recent findings by several laboratories have established that various *Francisella* strains escape from their initial phagosome into the cytosol of human and murine primary macrophages (Golovliov et al., 2003; Clemens et al., 2004; Santic et al., 2005a; Checroun et al., 2006), yet with conflicting kinetics since phagosomal escape has been shown to occur within one to several hours post infection (pi). In several ultrastructural studies, bacteria were observed in intact phagosomes until 2–4 h pi, from which point the phagosome membrane was gradually degraded and the bacteria eventually became cytosolic (Golovliov et al., 2003; Clemens et al., 2004; Lindgren et al., 2004; Santic et al., 2005a; McCaffrey and Allen, 2006; Schulert et al., 2009). Other studies, using ultrastructural analysis and phagosome integrity assays based upon the delivery of anti-*Francisella* antibodies to the cytosol of infected cells, have reported more rapid escape, where the majority of bacteria are cytosolic by 1 h pi (Checroun et al., 2006; Santic et al., 2007, 2008; Chong et al., 2008; Wehrly et al., 2009). While such variations in phagosomal escape kinetics may be a reflection of strain differences, as illustrated by side-by-side comparison of phagosomal escape kinetics of various *Francisella* strains (Chong et al., 2008), technical differences and differential sensitivity in assays and criteria used to assess phagosomal disruption may have also led to these different conclusions (Clemens et al., 2004, 2009; Santic et al., 2005a; Checroun et al., 2006; Chong et al., 2008). In particular, different opsonic and non-opsonic infection models used by various laboratories may have contributed to these discrepancies in the kinetics of *Francisella* phagosomal escape. A systematic analysis of how the mode of uptake affects phagosomal escape should address this question.

Prior to phagosomal escape, *Francisella* initially resides within a phagosome that interacts with early and late endocytic compartments (Clemens et al., 2004; Checroun et al., 2006; Chong et al., 2008; Santic et al., 2008; Wehrly et al., 2009). While the kinetics of acquisition of endosomal markers differ depending on the infection models used, confocal and electron microscopy studies have shown that nascent phagosomes containing *F. tularensis*, LVS, or *F. novicida* transiently acquire the early endosomal antigen 1 (EEA1) followed by acquisition of late endosomal markers CD63, LAMP1, LAMP2, and the Rab7 GTPase, the eventual loss of which correlates with phagosomal disruption (Golovliov et al., 2003; Clemens et al., 2004; Santic et al., 2005a, 2008; Checroun et al., 2006; Bonquist et al., 2008; Chong et al., 2008). These early trafficking events resemble those of a normal maturation process, although the FCP does not mature into a phagolysosome as it fails to acquire the acid hydrolase cathepsin D or lysosomal tracers (Anthony et al., 1991; Clemens et al., 2004; Santic et al., 2005a; Bonquist et al., 2008). Whether this results from *Francisella* actively inhibiting fusion with terminal lysosomes or from the loss of phagosomal integrity during bacterial escape still remains debatable. Santic et al. (2005b) have proposed that the process of phagosomal escape prevents fusion with lysosomes, since a phagosomal escape-deficient, *F. novicida* mutant in the FPI gene *iglC* (see below) is delivered to a Cathepsin D-positive, lysosomal compartment. However, Bonquist et al. (2008) have observed limited fusion of vacuoles containing various phagosomal escape-deficient mutants of LVS, including mutants in *iglC* and *iglD*, arguing that some inhibition of fusion with terminal lysosomes may still occur despite a lack of phagosomal escape. It is possible that phagosomes containing mutants deficient in phagosomal escape still undergo some residual phagosomal membrane alteration that, although not disruptive enough to ensure bacterial release, affects FCP maturation and lysosomal fusion. While these discrepancies could be due to differential phagosomal escape defects between the mutant strains examined, mutants in the same genes (*iglC*, *iglD*) were used in these studies, making these findings difficult to reconcile at this stage.

Phagosomal acidification, a process dependent upon the activity of the vacuolar ATPase (vATPase) pump, is critical for the fission and fusion of membranes that promote maturation and for the optimal activity of a number of microbicidal enzymes; thus, acidification is both a requirement and a consequence of phagosomal maturation (Huynh and Grinstein, 2007). The extent of FCP acidification and whether acidification influences *Francisella* phagosomal escape are contentious topics. In some studies FCPs harboring LVS, SchuS4, or a clinical isolate of *F. tularensis* subspecies *tularensis* in human macrophages and J774A.1 cells resisted acidification and acquired only limited amounts of the vATPase pump, suggesting that *Francisella* impairs phagosome maturation (Clemens et al., 2004, 2009; Bonquist et al., 2008). Moreover, the efficiency of *Francisella* phagosomal escape was unaffected in the presence of a vATPase pump inhibitor (Clemens et al., 2009). Other studies in human and murine macrophages reported that FCPs harboring *F. novicida* or SchuS4 became acidified and acquired the vATPase pump (Chong et al., 2008; Santic et al., 2008) prior to phagosomal disruption. Inhibition of phagosome acidification significantly delayed, but did not block, phagosomal disruption, indicating that FCP maturation is important for optimal bacterial escape to the cytosol (Chong et al., 2008; Santic et al., 2008). These studies suggest that the FCP acidic pH may provide conditions that induce and/or activate factors that promote the phagosomal disruption process, some of which may be related to pH-dependent, phagosomal iron availability (Fortier et al., 1995), although this needs to be formally demonstrated. Interestingly, studies reporting a lack of FCP acidification were performed using serum-opsonized bacteria, while those reporting transient FCP acidification were performed using non-opsonic conditions, regardless of the bacterial strains used, suggesting that the mode of uptake may affect FCP maturation.

DETERMINANTS OF INTRACELLULAR SURVIVAL – THE *FRANCISELLA* PATHOGENICITY ISLAND

Phagosomal escape is an essential aspect of the intracellular survival strategy of *Francisella*, since it is a process conserved among all *Francisella* strains studied to date and is a pre-requisite for cytosolic replication. Mutants impaired in phagosomal escape are defective for intracellular growth (Lindgren et al., 2004; Santic et al., 2005b; Bonquist et al., 2008; Schmerk et al., 2009a), and delaying translocation to the cytosol impedes the onset of replication (Chong et al., 2008). Thus, considerable effort has focused on determining the factors that contribute to phagosomal disruption. Several genes encoded within the FPI, an approximately 30 kb locus of 17 genes with similarity to components of the recently described type VI secretion system of *Vibrio cholera* and *Pseudomonas aeruginosa* (Mougous et al., 2006; Pukatzki et al., 2006), are involved in the phagosomal escape and intracellular survival of *Francisella* (Nano et al., 2004) (**Table 1**). Two identical copies of the FPI are present in the genomes of *F. tularensis* subsp. *tularensis* and subsp. *holarctica* and a single copy is present in *F. novicida* (Nano et al., 2004), although whether FPI copy number is a factor in the virulence differences between *Francisella* species remains unclear. The FPI-encoded gene *iglC* encodes a 23-kDa hypothetical protein that is prominently induced during the intracellular growth of LVS in J774A.1 cells (Golovliov et al., 1997) and SchuS4 in primary bone marrow-derived macrophages (BMMs; Wehrly et al., 2009). Disruption of *iglC* in LVS and *F. novicida* impairs phagosomal escape and intracellular growth (Lindgren et al., 2004; Santic et al., 2005b; Bonquist et al., 2008; Chong et al., 2008). In LVS, this defect was partially reversed by *in trans* complementation. In *F. novicida*, phagosomal escape was restored only with the expression of both IglC and IglD in the *iglC* mutant due to the polar effect of the mutation on IglD expression (Chong et al., 2008). Therefore, observations drawn from the use of this *F. novicida* mutant regarding the role of IglC (Santic et al., 2005b) need to be re-evaluated in the absence of genetic complementation. In addition, disruption of IglCD functions significantly reduced, but did not completely block, FCP disruption, indicating the contribution of additional factors in the phagosomal escape of *Francisella* (Chong et al., 2008). In J774A.1 cells, the *iglD* mutant of LVS demonstrated an increased and persistent propensity for colocalization with the late endosomal marker LAMP-1 which was reversed by complementation with *iglD*, indicating that IglD function is required for escape from the FCP (Bonquist et al., 2008). Contradictory to this observation, Santic et al. (2007) reported that the *iglD* mutant of *F. novicida* rapidly escaped into the cytosol but was unable to replicate, suggesting

Table 1 | Bacterial factors that contribute to the *Francisella* intracellular cycle.

Intracellular stage	Bacterial factors	Proposed function	References
Phagosomal escape	*iglC*	Unknown	Lindgren et al. (2004), Santic et al. (2005b), Bonquist et al. (2008), Chong et al. (2008)
	iglD[a]	Unknown	Bonquist et al. (2008)
	pdpA	Unknown	Schmerk et al. (2009a)
	iglI	Unknown	Barker et al. (2009b)
	vgrG	Unknown	Barker et al. (2009b)
	mglA	Transcriptional regulator	Baron and Nano (1998), Santic et al. (2005b), Bonquist et al. (2008)
	fevR	Transcriptional regulator	Brotcke and Monack (2008), Buchan et al. (2009), Wehrly et al. (2009)
	migR	Transcriptional regulator	Buchan et al. (2009)
	FTT1103	Unknown	Qin and Mann (2006), Qin et al. (2009)
	FTT1676	Unknown	Wehrly et al. (2009)
	carA, carB, pyrB	Uracil biosynthesis	Schulert et al. (2009)
	acpA[a], *acpB*[a], *acpC*[a], *Hap*[a]	Acid phosphatases	Baron et al. (1999), Mohapatra et al. (2007a, 2008), Child et al. (2010)
Cytosolic replication	*iglD*[a]	Unknown	Santic et al. (2007)
	FTT0369c	Unknown	Wehrly et al. (2009)
	purMCD	Purine biosynthesis	Pechous et al. (2006, 2008)
	ggt	γ-Glutamyl transpeptidase	Alkhuder et al. (2009)
	FTT0989[a]	Unknown	Brotcke et al. (2006), Wehrly et al. (2009)
	ripA	Unknown	Fuller et al. (2008)
	htpG[b]	Chaperone	Tempel et al. (2006), Weiss et al. (2007)
	dsbB[b]	Disulfide bond formation	Maier et al. (2006), Tempel et al. (2006), Weiss et al. (2007), Qin et al. (2008)
	iglA[b]	Unknown	de Bruin et al. (2007)
	iglB[b]	Unknown	Gray et al. (2002)
	pdpB[b]	Unknown	Brotcke et al. (2006), Tempel et al. (2006)
	cds2[b]	Unknown	Brotcke et al. (2006)
	mglB[b]	Transcriptional regulator	Baron and Nano (1998)
	pmrA[b]	Orphaned two-component response regulator	Mohapatra et al. (2007b), Bell et al. (2010)

[a]*Contradictory findings obtained.*
[b]*Role in phagosomal escape not examined.*

that IglD does not play a role in phagosomal disruption but is required for cytosolic proliferation. The distinct *iglD* mutant trafficking phenotypes reported may be a result of methodological and strain differences. It is however difficult to envision how highly conserved genes within a major virulence locus such as the FPI can have different functions between *Francisella* species. Additional mutagenesis studies in *F. novicida* have identified other FPI proteins required for intracellular survival: PdpA (Schmerk et al., 2009a), VgrG (Barker et al., 2009b), and IglI (Barker et al., 2009b) are required for phagosomal escape and intramacrophage growth; *iglA* (de Bruin et al., 2007), *iglB* (Gray et al., 2002), *pdpB* (Brotcke et al., 2006; Tempel et al., 2006), and *cds2* (Brotcke et al., 2006) are also required for intramacrophage growth, although their contributions to intracellular trafficking have not been examined. Despite the lack of information regarding the precise functions of the FPI genes, these data highlight the importance of the FPI-encoded proteins in the intracellular pathogenesis of *Francisella*.

OTHER BACTERIAL DETERMINANTS OF INTRACELLULAR SURVIVAL

While the FPI is to date the most prominent virulence locus required for phagosomal escape, intracellular survival and growth of *Francisella*, other proteins encoded outside the FPI have also been implicated in the proper translocation of *Francisella* into the cytosol and subsequent replication. Intracellular trafficking and survival defects of mutants in the transcriptional regulators *mglA* (Baron and Nano, 1998; Santic et al., 2005b; Bonquist et al., 2008), *fevR* (Brotcke and Monack, 2008; Buchan et al., 2009; Wehrly et al., 2009), and *migR* (Buchan et al., 2009) have been documented. These are likely a result of the dysregulation of effector proteins that contribute to phagosomal escape and cytosolic proliferation, rather than a direct participation, since these transcription factors regulate virulence genes both within and outside of the FPI (Brotcke et al., 2006; Brotcke and Monack, 2008). Other loci, such as FTT1103 (Qin et al., 2009) and FTT1676 (Wehrly et al., 2009),

both encoding hypothetical lipoproteins, are required for the phagosomal escape of SchuS4 in J774A.1 cells and murine BMMs, respectively. Three genes involved in pyrimidine biosynthesis, *carA, carB,* and *pyrB*, were identified in LVS to also play a role in phagosome disruption in human MDMs (Schulert et al., 2009), although their mechanistic contribution to phagosomal escape is elusive and may be indirect.

The contribution of acid phosphatases to the intracellular pathogenesis of *Francisella* has been a subject of much investigation and remains controversial. *Francisella* spp. contain at least six acid phosphatase-encoding genes, the conservation of which varies depending on the *Francisella* species (Child et al., 2010). While the most conserved genes are *acpA*, *acpB*, and *acpC*, others have been disrupted through genome rearrangements in virulent Type A and Type B strains, raising the question of their importance for *Francisella* virulence (Child et al., 2010). An *acpA* truncation mutant in *F. novicida,* lacking phosphatase activity but retaining phospholipase activity, was found to exhibit wild-type level replication in J774A.1 cells (Baron et al., 1999), indicating it is not essential to intracellular growth. Citing differences in mutant constructs, Mohapatra et al. (2007a) found that deletion of the entire *acpA* gene in *F. novicida* resulted in bacteria that were impaired for phagosomal escape and intracellular growth in human MDM and THP-1 macrophages. These defects were more pronounced with the accumulation of *acp* deletions (*acpA, acpB, acpC*, and *hapA*) indicating a cumulative role for these proteins (Mohapatra et al., 2008). In *F. tularensis* subspecies *tularensis*, however, neither the deletion of *acpA* nor the combined deletion of *acpA, acpB,* and *acpC* had an effect on the phagosomal escape or cytosolic proliferation in murine BMMs or human MDMs (Child et al., 2010). These contradictory findings between *F. novicida* and *F. tularensis* may illustrate species-specific differences. Yet, one would expect that proteins involved in an essential virulence process such as phagosomal escape to be highly conserved and not subjected to evolutionary genome reduction. While these functional differences require future clarification, they emphasize again that observations made from less virulent strains do not necessarily reflect those from virulent *F. tularensis* subspecies.

Our current knowledge of bacterial factors required for phagosomal escape indicates that it is a multi-factorial process. Consistently, a recent genetic screen performed in human macrophages using a *F. novicida* transposon mutant library identified 91 genes, encoding a variety of functions, as required for phagosomal escape (Asare and Abu Kwaik, 2010). Despite progress in identifying the factors involved in this crucial stage of *Francisella* trafficking, the actual mechanism of phagosome membrane disruption remains elusive and effector proteins of phagosomal disruption have yet to be characterized at the functional level.

INTRACELLULAR REPLICATION OF *FRANCISELLA*

Besides the importance of phagosomal escape, another critical aspect of *Francisella* intracellular pathogenesis is its ability to proliferate extensively within the cytosol of host cells (Oyston et al., 2004). Because phagosomal escape is pre-requisite to cytosolic proliferation, the identification of factors specifically required for cytosolic proliferation using mutagenesis approaches has been challenging. Since phagosomal escape-deficient mutants are also affected in intracellular growth, assigning a specific role in cytosolic replication to a gene requires characterization of the intracellular trafficking of the corresponding mutant. A vast number of genes affecting the intracellular survival and proliferation of *Francisella* has indeed been identified through mutagenesis studies (Brotcke et al., 2006; Qin and Mann, 2006; Tempel et al., 2006; Su et al., 2007; Weiss et al., 2007; Alkhuder et al., 2009; Schulert et al., 2009; Wehrly et al., 2009; Asare and Abu Kwaik, 2010). Yet, few have been confirmed by targeted mutagenesis, and even fewer have been characterized and specifically implicated in cytosolic replication. Alternative approaches, such as transcriptional profiling of intracellular *Francisella*, have also revealed genetic determinants of intracellular survival based on their upregulation during the intracellular cycle (Wehrly et al., 2009). What has emerged from this wealth of data suggests that *Francisella* uses specific mechanisms to successfully adapt to its intracellular lifestyle, because many candidate virulence determinants encode hypothetical proteins of unknown function. Highlighted here are the determinants for which a contribution to intracellular replication has been confirmed via targeted mutagenesis, or specifically demonstrated.

Bacterial factors implicated in the intramacrophage growth of *Francisella* that have been confirmed by targeted mutagenesis studies include *pmrA* (Mohapatra et al., 2007b; Sammons-Jackson et al., 2008), FTT0989 (Brotcke et al., 2006), *htpG* (Tempel et al., 2006; Weiss et al., 2007), *dsbB* (Maier et al., 2006; Tempel et al., 2006; Weiss et al., 2007; Qin et al., 2008), *iglD* (Santic et al., 2007), *iglA* (de Bruin et al., 2007), *iglB* (Gray et al., 2002), *pdpB* (Brotcke et al., 2006; Tempel et al., 2006), *cds2* (Brotcke et al., 2006), and *mglB* (Baron and Nano, 1998) (**Table 1**). While the role of FPI genes, such as *iglD*, *iglA*, *iglB*, *pdpB,* and *cds2*, has been discussed above with regard to phagosomal escape, the specific roles in cytosolic replication of the transcriptional regulators PmrA (Mohapatra et al., 2007b; Sammons-Jackson et al., 2008) and MglB (Baron and Nano, 1998), the stress response protein HtpG (Tempel et al., 2006; Weiss et al., 2007), the inner membrane disulfide bond forming protein DsbB (Maier et al., 2006; Tempel et al., 2006; Weiss et al., 2007; Qin et al., 2008) and the putative transglutaminase FTT0989 (Brotcke et al., 2006) remain speculative until intracellular trafficking studies of the mutants are performed. The roles of these genes in intracellular growth may indeed be an indirect or non-specific consequence of their contribution to other aspects of the bacterium's intracellular adaptation.

More extensive studies have nonetheless identified genes required for cytosolic proliferation of *Francisella*. Characterization of purine auxotroph mutants (Δ*purMCD*) in LVS and SchuS4 revealed no defects in phagosomal escape, but bacteria failed to replicate in the cytosol of J774A.1 or primary murine macrophages (Pechous et al., 2006, 2008), indicating that *Francisella* cannot scavenge nucleotides from the macrophage and must synthesize them. Another metabolism-related gene, *ggt*, encoding a γ-glutamyl transpeptidase, was identified using an *in vitro* negative screen of a library of LVS transposon mutants (Alkhuder et al., 2009). Disruption of *ggt* in LVS impaired cytosolic replication in murine J774A.1, RAW264.7, BMMs, and human THP-1 macrophages. Although the efficiency of escape was not thoroughly investigated, *ggt* mutant bacteria were observed in the cytoplasm of infected RAW264.7 cells at 24 h pi by ultrastructural analysis, suggesting that mutant bacteria were

able to breach the FCP. Further characterization showed that *ggt* is required for bacterial acquisition of cysteine from glutathione (the most abundant source of this amino acid in the cytosol) and γ-glutamyl peptides to promote intracellular growth in murine macrophages (Alkhuder et al., 2009). Consistently, genes involved in the catabolism of amino acids and encoding small peptide transporters are upregulated intracellularly in SchuS4, further indicating nutrient requirements and source for cytosolic *Francisella* (Wehrly et al., 2009). The contribution of *ripA*, which encodes an inner membrane protein, to the intracellular growth of LVS was identified through random mutagenesis and confirmed by characterization of a targeted deletion mutant (Fuller et al., 2008). Δ*ripA* mutants escaped from their phagosomes with similar efficiency to wild-type LVS, but were unable to replicate in murine lung epithelial cells, TC-1, or J774A.1 cells (Fuller et al., 2008). The expression of *ripA* was upregulated during the early cytosolic replication stage during infection of J774A.1 macrophages, corroborating its role at this stage of the *Francisella* intracellular cycle (Fuller et al., 2009). Lacking homology to any previously characterized genes, the function of this *Francisella* specific factor remains to be uncovered. The FTT0369c locus, encoding a hypothetical protein with Sel1-family tetratricopeptide (TPR) repeat domains, was identified as transcriptionally upregulated during the cytosolic stage of SchuS4 infection of murine BMMs (Wehrly et al., 2009). Upon deletion of FTT0369c, SchuS4 bacteria were not able to replicate in the cytosol of murine BMMs, but retained phagosomal escape kinetics similar to wild-type SchuS4 (Wehrly et al., 2009), indicating a specific role of this locus in cytosolic proliferation.

As accentuated by the short list of determinants discussed above, characterization of the exact functions of bacterial factors specifically required for cytosolic proliferation has only just begun.

HOST FACTORS AFFECTING *FRANCISELLA* INTRACELLULAR SURVIVAL

Understanding *Francisella*–macrophage interactions not only requires the characterization of the bacterial determinants but also that of host factors that either contribute to, or interfere with, the bacterium's intracellular cycle (**Table 2**). Despite its ability to survive and proficiently replicate within quiescent macrophages, *Francisella* is unable to do so within interferon (IFN)-γ-activated macrophages (Anthony et al., 1992; Fortier et al., 1992; Polsinelli et al., 1994), indicating that host innate immune responses are

Table 2 | Host factors that contribute to the *Francisella* intracellular cycle.

Intracellular stage	Host factors	Function	References
Entry	Complement component C3	Activation of the complement system	Clemens et al. (2005), Ben Nasr et al. (2006), Barker et al. (2009a)
	Surfactant protein A (SR-A)	Immunomodulatory pulmonary surfactant	Balagopal et al. (2006)
	Complement receptor 3 (CR3)	Receptor for complement component C3, facilitate phagocytosis	Clemens et al. (2005), Balagopal et al. (2006), Ben Nasr et al. (2006), Pierini (2006), Clemens and Horwitz (2007), Barker et al. (2009a)
	FcγR	Receptor for antibody Fc region, facilitates phagocytosis	Balagopal et al. (2006)
	Scavenger receptor A (SR-A)	Pattern recognition receptor, facilitates phagocytosis	Pierini (2006)
	Mannose receptor (MR)	Pattern recognition receptor, facilitates phagocytosis	Balagopal et al. (2006), Schulert and Allen (2006)
	Nucleolin	Ribosome biogenesis	Barel et al. (2008)
	Syk	Signaling tyrosine kinase	Parsa et al. (2008)
	ERK	Extracellular signal-regulated kinase	Parsa et al. (2008)
Phagosomal escape	CDC27	Ubiquitin ligase	Akimana et al. (2010)
	Akt	Serine/threonine protein kinase	Rajaram et al. (2009)
	SHIP	Inositol 5′ phosphatase	Rajaram et al. (2009)
Cytosolic replication	IFNγ	Immunomodulatory and immunostimulatory cytokine	Anthony et al. (1992), Fortier et al. (1992), Polsinelli et al. (1994), Lindgren et al. (2004, 2005, 2007), Santic et al. (2005a), Bonquist et al. (2008), Edwards et al. (2010)
	PI4KCA	Phosphatidylinositol 4 kinase	Akimana et al. (2010)
	USP22	Ubiquitin hydrolase	Akimana et al. (2010)
	Ras	Small GTPase activating protein, activates several intracellular signaling pathways	Al-Khodor and Abu Kwaik (2010)
	SOS2	Ras guanine nucleotide exchange factor	Al-Khodor and Abu Kwaik (2010)
	GrB2	Adaptor protein that regulates receptor tyrosine kinase signal transduction	Al-Khodor and Abu Kwaik (2010)
	PKCα	Serine/threonine protein kinase	Al-Khodor and Abu Kwaik (2010)
	PKCβ1	Serine/threonine protein kinase	Al-Khodor and Abu Kwaik (2010)

capable of antagonizing *Francisella* intracellular proliferation. However, the mechanisms induced by this cytokine to control infection by *Francisella* are unclear. Several studies have ascribed the killing of LVS in IFN-γ-activated murine peritoneal exudates cells (PECs) to the actions of the inducible nitric oxide (NO) synthase (iNOS), which generates reactive nitrogen species (RNS; Anthony et al., 1992; Fortier et al., 1992; Lindgren et al., 2005, 2007). In IFN-γ-activated murine alveolar macrophages, NO was determined to play a minimal role in inhibiting the intracellular growth of LVS (Polsinelli et al., 1994). Addition of superoxide dismutase, catalase, excess iron or L-tryptophan did not reverse the growth inhibition of LVS observed in IFN-γ-activated alveolar macrophages, indicating that the antibacterial activity was not mediated by ROS or limiting concentrations of L-tryptophan or iron (Polsinelli et al., 1994). Similar to LVS, control of SchuS4 replication in IFN-γ-activated human and murine macrophages did not involve ROS, L-tryptophan sequestration or iron limitation, nor did it seem to involve autophagy (Edwards et al., 2010). In studies using SchuS4, the restriction on bacterial growth was reported to be iNOS-dependent in murine PECs (Lindgren et al., 2007), but was iNOS-independent in primary human and murine macrophages (Edwards et al., 2010).

In examining the interactions of LVS with IFN-γ-activated PECs, Lindgren et al. (2004) found that IFN-γ treatment reduced, but did not completely block, LVS escape into the macrophage cytosol. Phagosomal escape of LVS in IFN-γ-activated J774A.1 cells was however not affected, but its intracellular growth remained restricted (Bonquist et al., 2008). Consistent with these findings, IFN-γ treatment did not hinder phagosomal escape of SchuS4 in murine BMMs and human MDMs (Edwards et al., 2010). In contrast, Santic et al. (2005a) found that *F. novicida* is unable to escape from the FCP and is killed within phagolysosomes in IFN-γ-activated human MDMs. Altogether, these results indicate that inhibition of intracellular growth by IFN-γ is exerted at the cytosolic stage of the *F. tularensis* intracellular cycle, whereas growth restriction is determined during the phagosomal stage for *F. novicida*. Thus, the IFN-γ-induced mechanisms inhibiting *Francisella* intracellular replication seem to be dependent on macrophage type, as well as the infecting strain, although one cannot exclude that variations in the experimental designs of these studies have yielded contrasting conclusions. Regardless, the actual effector mechanisms induced by IFN-γ remain elusive.

Alternative activation of macrophages by mast cells has been shown to effectively control the intramacrophage growth of LVS (Ketavarapu et al., 2008). Both contact-dependent and secreted factors from mast cells contribute to this inhibitory effect since entry and replication of LVS within murine BMMs were diminished when co-cultured with infected murine bone marrow-derived mast cells or in the presence of infected mast cell-spent media. Among the factors secreted by mast cells, IL-4 was determined to be an important component mediating bacterial growth inhibition in BMMs. An earlier report demonstrated that IL-4 mediated upregulation of MR enhanced *Francisella* uptake by macrophages (Schulert and Allen, 2006); this mechanism of entry may ultimately be detrimental to the intracellular survival of *Francisella* as Rodriguez et al. (2010) found that restriction of intramacrophage replication by IL-4 was associated with increased ATP production, upregulated MR expression and prolonged FCP acidification, but how IL-4 affects *Francisella* intracellular fate remains to be directly demonstrated. Taken together, however, these data reflect the idea that mode of entry affects the intracellular fate of the invading pathogen.

INTERACTIONS OF *FRANCISELLA* WITH THE AUTOPHAGIC PATHWAY

The cytosolic location of *Francisella* during its intracellular proliferation makes this pathogen a target for innate immune recognition and delivery to lysosomes via the autophagic pathway, a process known to recover and eliminate various cytosolic pathogens (Deretic, 2006). Yet, surprisingly, this bacterium can proliferate within the cytosol without any evidence for an autophagic response, suggesting it is either capable of preventing its recognition by the autophagic machinery or of inhibiting the autophagic process. Nonetheless, it was shown that a subset of cytosolic bacteria reentered the endocytic compartment in an autophagy-dependent process following replication in murine BMMs (Checroun et al., 2006), arguing for a belated autophagic response from the macrophage. While it remains unclear whether FCVs play a role in the intracellular cycle of *Francisella* or if they are a macrophage response to contain bacterial proliferation, their discovery indicated an involvement of autophagy in *Francisella*–macrophage interactions. Interestingly, FCV formation does not occur in human MDMs (Akimana et al., 2010; Edwards et al., 2010). While this suggests that their formation may require murine-specific signaling, one cannot exclude that higher and earlier *Francisella*-induced cytotoxicity observed in human MDMs may precede, hence prevent, FCV formation (Edwards et al., 2010). Additional evidence that autophagy may be manipulated by *Francisella* is that several autophagy-related genes, including *beclin1*, *ATG5*, *ATG12*, *ATG16L*, *ATG7*, and *ATG4a*, were down regulated during infection of human monocytes with SchuS4 or *F. novicida*, suggesting that *Francisella* suppresses the host autophagy response at the gene expression level (Butchar et al., 2008; Cremer et al., 2009). It was therefore proposed that by delaying autophagy, *Francisella* gains time in the macrophage cytosol to allow for replication and/or development of resistance to the acidic environment of the FCV (Cremer et al., 2009). However, whether autophagy is still functional in infected macrophages was not tested in these studies, so it remains to be demonstrated whether *Francisella* can actually inhibit the autophagic pathway during its intracellular cycle.

SIGNALING PATHWAYS IN *FRANCISELLA* INTRACELLULAR PATHOGENESIS

While innate immune processes that control *Francisella* intracellular growth have been extensively studied, additional host factors that influence the intracellular fate of *Francisella* have recently come to light from studies using *F. novicida* as a model system (**Table 2**). The PI3-Kinase/Akt signaling pathway is activated early during *F. novicida* infection (Parsa et al., 2006; Rajaram et al., 2009). Although this pathway was irrelevant for the phagocytosis of *F. novicida* (Parsa et al., 2008), continuous activation of Akt or deletion of the inositol phosphatase SHIP, which downregulates the PI3K/Akt pathway, inhibited phagosomal disruption,

and increased FCP fusion with lysosomes in murine BMMs and RAW264.7 cells (Rajaram et al., 2009), suggesting that activation of this pathway is restrictive to *Francisella*. Indeed, numerous gene members of the PI3K/Akt pathway were substantially down-regulated in SchuS4 and *F. novicida*-infected human monocytes (Butchar et al., 2008), suggesting that *Francisella* may down modulate the PI3-K/Akt pathway.

Another signaling pathway initiated by *F. novicida* involves the small G-protein Ras. Intracellular *F. novicida* transiently activated Ras within 5 min of infection of human embryonic kidney cell line HEK293T through a signaling cascade involving SOS2, Grb2, PKCα, and PKCβ1 (Al-Khodor and Abu Kwaik, 2010). Knockdown of these components eliminated Ras activation, and impaired *F. novicida* intracellular replication without affecting the efficiency of phagosomal escape (Al-Khodor and Abu Kwaik, 2010). Furthermore, silencing of these signaling molecules had no impact on the phosphorylation of Akt or ERK, indicating that Ras activation does not trigger signaling through these pathways (Al-Khodor and Abu Kwaik, 2010). A recent genome-wide RNAi screen was used to identify 186 host factors that influence the intracellular replication of *F. novicida* in *Drosophila melanogaster*-derived S2R+ cells (Akimana et al., 2010). Among these, three were confirmed by knockdown of their corresponding mammalian homologues to impact the intracellular survival of *F. novicida*. Knockdown of the ubiquitin ligase CDC27 diminished the capacity of *F. novicida* to disrupt its phagosome and to replicate in the host cytosol, while silencing of the type III PI4-kinase α subunit PI4KC and the ubiquitin specific peptidase USP22 resulted in a cytosolic replication specific defect in HEK293T cells (Akimana et al., 2010). These data indicate that *F. novicida* exploits multiple host signaling pathways, as well as the host ubiquitin system, to ensure its intracellular survival. Future work should examine whether these signaling pathways and host machineries are also relevant to the intracellular pathogenesis of virulent *Francisella* species.

CONCLUDING REMARKS

Because the ability of *Francisella* spp. to survive and proliferate within mammalian cells is essential to their virulence, much research has focused over the last few years on understanding the intracellular cycle of these pathogens. While there is a consensus on the overall survival strategy of this pathogen, much controversy remains on more specific aspects of its intracellular cycle, such as the maturation of the FCP and the bacterial factors of phagosomal escape. Additionally, recent genomic and functional evidence indicates that caution should be exerted when assuming that conclusions drawn from studies using species of low virulence apply to virulent species of *Francisella*. A plethora of genetic screens for bacterial and host factors required for intracellular pathogenesis of various *Francisella* species has generated a wealth of information and identified key determinants of the *Francisella*–host cell interaction. Despite all these efforts, the molecular mechanisms governing the various stages of the *Francisella* intracellular cycle remain elusive. Future research on *Francisella* intracellular pathogenesis must now focus on characterizing the molecular functions of the bacterial and host factors identified, in order to gain a much-needed understanding of this bacterium's pathogenesis at the molecular level.

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The role of the *Francisella tularensis* pathogenicity island in type VI secretion, intracellular survival, and modulation of host cell signaling

Jeanette E. Bröms†, Anders Sjöstedt* and Moa Lavander†

Department of Clinical Microbiology, Clinical Bacteriology, and Laboratory for Molecular Infection Medicine Sweden, Umeå University, Umeå, Sweden

Edited by:
John S. Gunn, The Ohio State University, USA

Reviewed by:
Thomas C. Zahrt, Medical College of Wisconsin, USA
Tonyia Eaves-Pyles, University of Texas Medical Branch, USA

****Correspondence:***
Anders Sjöstedt, Department of Clinical Microbiology, Clinical Bacteriology, Umeå University, SE-901 85 Umeå, Sweden.
e-mail: anders.sjostedt@climi.umu.se

†Jeanette E. Bröms and Moa Lavander contributed equally to this article.

Francisella tularensis is a highly virulent gram-negative intracellular bacterium that causes the zoonotic disease tularemia. Essential for its virulence is the ability to multiply within host cells, in particular monocytic cells. The bacterium has developed intricate means to subvert host immune mechanisms and thereby facilitate its intracellular survival by preventing phagolysosomal fusion followed by escape into the cytosol, where it multiplies. Moreover, it targets and manipulates numerous host cell signaling pathways, thereby ameliorating the otherwise bactericidal capacity. Many of the underlying molecular mechanisms still remain unknown but key elements, directly or indirectly responsible for many of the aforementioned mechanisms, rely on the expression of proteins encoded by the *Francisella* pathogenicity island (FPI), suggested to constitute a type VI secretion system. We here describe the current knowledge regarding the components of the FPI and the roles that have been ascribed to them.

Keywords: *Francisella tularensis*, phagosomal escape, intracellular growth, FPI, T6SS

INTRODUCTION

Francisella tularensis is a highly infectious gram-negative intracellular bacterium that causes the disease tularemia (Oyston et al., 2004). Two main subspecies, *F. tularensis* subsp. *tularensis* and *F. tularensis* subsp. *holarctica* (also known as type A and type B, respectively), are responsible for the majority of infections and disease, which are particularly severe if caused by type A strains. Importantly, *F. tularensis* has the ability to infect a multitude of hosts, including mammals, fish, amphibians, protozoa and arthropods (reviewed in Mörner and Addison, 2001; Keim et al., 2007). Although primarily a vector-transmitted disease, *F. tularensis* infections can also occur through inhalation of contaminated materials, ingestion and skin abrasions (Dennis et al., 2001). From the site of infection, the bacteria will spread to regional lymph nodes, liver and spleen, where they rapidly grow and overwhelm the host's immune defenses (Fortier et al., 1991).

The pathogenicity of *F. tularensis* is believed to depend on its ability to replicate within host cells, particularly macrophages (Tärnvik, 1989). *F. tularensis* enters host macrophages by triggering the formation of asymmetric, spacious pseudopod loops (Clemens et al., 2005). It then modifies the endocytic pathway of the macrophage, preventing phagolysosomal fusion, upon which it escapes into the cytosol and multiplies (Golovliov et al., 2003a; Clemens et al., 2004; Santic et al., 2005a). Several factors that regulate or facilitate phagosome escape and intracellular survival of *F. tularensis* have been identified (Dean et al., 2009; Meibom and Charbit, 2009; Qin et al., 2009; Mohapatra et al., 2010), however, the underlying molecular mechanisms still remain largely unknown. A gene cluster frequently encountered in screens aimed at identifying elements important for intracellular growth and/or virulence, is the *Francisella* pathogenicity island (FPI; reviewed in Nano et al., 2004; Nano and Schmerk, 2007; Meibom and Charbit, 2009), which has been suggested to encode a type VI secretion system (de Bruin et al., 2007; Nano and Schmerk, 2007; Bingle et al., 2008; Ludu et al., 2008; Barker et al., 2009; Bröms et al., 2009).

Note: F. tularensis subsp. *novicida* will be referred to as *F. novicida* and the FPI nomenclature proposed by Ludu et al. (2008) will be used throughout (**Table 1**; **Figure 1**).

THE *FRANCISELLA* PATHOGENICITY ISLAND

DUPLICATION OF THE FPI IN STRAINS OF *F. TULARENSIS*

The 33-kb pathogenicity island, designated FPI (**Figure 1**), consists of 16–19 ORFs and its crucial role in the virulence of *F. tularensis* is well established (Golovliov et al., 2003b; Nano et al., 2004; Twine et al., 2005; Tempel et al., 2006; Santic et al., 2007, 2009; Weiss et al., 2007; Bönquist et al., 2008; Ludu et al., 2008; Vonkavaara et al., 2008; Bröms et al., 2009; Kraemer et al., 2009; Schmerk et al., 2009b; Åhlund et al., 2010; Bröms et al., unpublished). It was first identified in 2004 (Nano et al., 2004) by bioinformatic analysis of the partly sequenced genomes of *F. tularensis* subsp. *tularensis* strain Schu S4 (Karlsson et al., 2000; Prior et al., 2001) and *F. novicida* strain U112 (Nano et al., 2004). All subsequently sequenced *Francisella* genomes have confirmed the presence of the FPI, suggesting that it is part of the core genome (Larsson et al., 2009). However, in contrast to the rest of the genome, the FPI has lower G + C content, indicating horizontal gene transfer (Nano et al., 2004).

Interestingly, the FPI is duplicated in all the subsp. of *F. tularensis* (*F. tularensis* subsp. *holarctica*, *mediasiatica*, and *tularensis*), but is present in a single copy in *F. novicida* and *F. philomiragia* (Nano et al., 2004; Larsson et al., 2009).

Larsson et al. (2009) recently suggested that this duplication event was facilitated by the insertion of ISFtu1 insertion sequence element adjacent to the FPI and an rRNA operon of an ancestral genome,

Table 1 | FPI gene nomenclature.

Strains and FPI gene loci[1]					Gene names and references[2]			
Schu S4		LVS		U112	Nano et al. (2004)	Nano and Schmerk (2007)	Ludu et al. (2008)	Barker et al. (2009)
Locus 1	Locus 2	Locus 1	Locus 2	Locus 1				
FTT1699	FTT1344	FTL_0126	FTL_1172	FTN_1309	*pdpA*	*pdpA*	*pdpA*	*pdpA*
FTT1700	FTT1345	FTL_0125	FTL_1171	FTN_1310	*pdpB*	*pdpB*	*pdpB/icmF*	*pdpB/icmF*
FTT1701	FTT1346	FTL_0124	FTL_1170	FTN_1311	–	*pigA*	*iglE*	*iglE*
FTT1702	FTT1347	FTL_0123	FTL_1169	FTN_1312	–	*pigB*	*vgrG*	*vgrG*
FTT1703	FTT1348	FTL_0122	FTL_1168	FTN_1313	–	*pigC*	*iglF*	*iglF/clpV*
FTT1704	FTT1349	FTL_0121	FTL_1167	FTN_1314	–	*pigD*	*iglG*	*iglG*
FTT1705	FTT1350	FTL_0120	FTL_1166	FTN_1315	–	*pigE*	*iglH*	*iglH*
FTT1706	FTT1351	FTL_0119	FTL_1165	FTN_1316	–	*pigF*	*dotU*	*dotU*
FTT1707	FTT1352	FTL_0118	FTL_1164	FTN_1317	–	*pigG*	*iglI*	*iglI*
FTT1708	FTT1353	FTL_0117	FTL_1163	FTN_1318	–	*pigH*	*iglJ*	*iglJ*
FTT1709	FTT1354	FTL_0116	FTL_1162	FTN_1319	*pdpC*	*pdpC*	*pdpC*	*pdpC*
FTT1710	FTT1355	FTL_0115	FTL_1161	FTN_1320	–	*pigI*	*pdpE*	*pdpE/hcp*
FTT1711	FTT1356	FTL_0114	FTL_1160	FTN_1321	*iglD*	*iglD*	*iglD*	*iglD*
FTT1712	FTT1357	FTL_0113	FTL_1159	FTN_1322	*iglC*	*iglC*	*iglC*	*iglC*
FTT1713	FTT1358	FTL_0112	FTL_1158	FTN_1323	*iglB*	*iglB*	*iglB*	*iglB*
FTT1714	FTT1359	FTL_0111	FTL_1157	FTN_1324	*iglA*	*iglA*	*iglA*	*iglA*
FTT1715	FTT1360	FTL_0110, FTL_0109	FTL_1156, FTL_1155	FTN_1325	*pdpD*	*pdpD*	*pdpD*	*pdpD*
FTT1716	FTT1361	–	–	FTN_1326	*pmcA*	*pmcA* (*pmcA1*, *pmcA2* in Schu S4)	*anmK*	*anmK*

[1]*Adapted from http://www.francisella.org/*

[2]*anmK (anhydro-N-acetylmuramic acid kinase); clpV (virulent strain); dot (defect in organelle trafficking); hcp (hemolysin co-regulated protein); icm (intracellular multiplication); igl (intracellular growth locus); pdp (pathogenicity determinant protein); pig (pathogenicity island gene); pmcA (plasma membrane Ca^{2+} ATPase); vgr (valine–glycine repeat).*

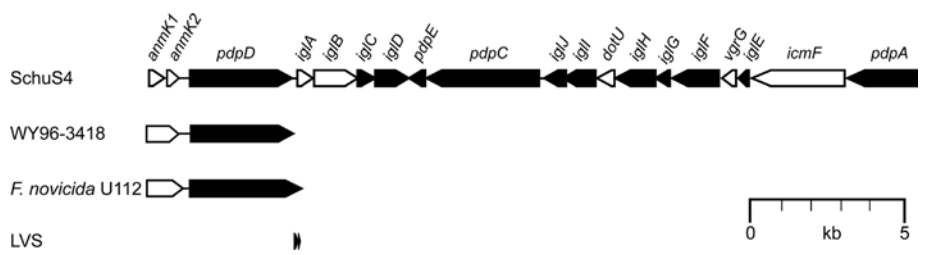

FIGURE 1 | The *Francisella* pathogenicity island (FPI). A schematic representation of the *Francisella* tularensis Schu S4 variant of the FPI with a consensus nomenclature adapted from Ludu et al. (2008). The ORFs of the FPI are essentially the same in all subspecies, with the exception of the *anmK–pdpD* region. In type A strains like Schu S4 (clade A.I) or WY96-3418 (clade A.II), *pdpD* encodes a 1,195 aa protein, which is somewhat larger in *F. novicida* (1,245 aa). The anmK gene product (371 aa in *F. novicida*) is truncated in WY96-3418, and may be expressed as two separate ORFs in Schu S4 (*anmK1* and *anmK2*). *F. tularensis* type B strains, e.g., LVS, lack anmK and most of the pdpD gene due to a large deletion within this region. The ORFs are drawn to scale, and their relative sizes indicated. Black arrows indicate gene products with no significant homology to other proteins; while white arrows represent products with homologs in other bacterial systems (see text for details).

which enabled duplication of the FPI by non-reciprocal recombination. Interestingly, as deduced from the genomic locations of the units, one of them was identified as ancestral whereas the second, more recently acquired, has integrated in various locations in the different genomes. As a consequence of the duplication, FPI null mutants are easier to generate in *F. novicida*, which is also the species most extensively used to analyze the role of individual FPI proteins.

Noteworthy, there is a gene cluster in *F. novicida*, encompassing ORFs FTN_0040–FTN_0052, which show homology to the FPI, albeit rudimentary, and with a different organization of the "true" FPI genes. The similarities are particularly pronounced for the core components IglA, IglB, PdpB/IcmF, and DotU where FTN_0042, FTN_0043, FTN_0040, and FTN_0051, respectively, show high (IglA and IglB) or intermediate (PdpB and DotU) protein sequence

homology (Larsson et al., 2009). Further, protein-BLAST (http://blast.ncbi.nlm.nih.gov/) reveals that there are genes encoding products with weak homology to PdpA, PdpC, PdpD, IglD, IglH, and IglI, interspersed with ORFs that reveal no FPI similarity. This gene cluster has not been widely discussed, nor its functionality investigated and its role in *F. novicida* virulence remains unknown.

Interestingly, combined work by several groups suggests that the second FPI locus may be functionally redundant in strains of *F. tularensis*, as transposon or deletion mutants for which only one of the two gene copies have been targeted are unaffected for virulence (Golovliov et al., 2003b; Kawula et al., 2004; Qin and Mann, 2006; Nano and Schmerk, 2007), in contrast to mutants for which both gene copies have been removed by targeted mutagenesis (Golovliov et al., 2003b; Bönquist et al., 2008; Bröms et al., 2009). In fact, Schu S4 can tolerate the loss of one of its two FPI loci (targeted deletions of regions encompassing FTT_1699–FTT_1715 or FTT_1344–FTT_1360), without substantial loss of virulence toward mice infected by the intradermal route (A. Sjöstedt and W. Conlan, unpublished). Contradicting these results is a study by Su et al. (2007), which suggests that both gene copies of *iglA, iglB,* or *iglC* are required for full virulence of LVS in a mouse model of respiratory tularemia and a study by Maier et al., demonstrating that *HimarFT* insertion mutants in one of the *iglB*, *iglC*, or *iglD* loci result in only partial loss of J774 monolayer integrity upon infection, in contrast to when parental LVS was used (Maier et al., 2007). Thus, it cannot be ruled out that the duplication event, and hence doubling of gene dosage, may provide some benefit to the pathogenic *F. tularensis* type A and B strains, and that this may have contributed to their differentiation from the human non-pathogenic, or rarely pathogenic, *F. novicida* and *F. philomiragia* strains, although there is little data to support this. It should be noted that FPI mutants even in highly virulent subspecies of *F. tularensis*, e.g., in the Schu S4 strain, are avirulent. Thus, despite that one CFU of the wild-type strain kills mice irrespective of route of inoculation, the lethal dose of the *iglB*, *iglC*, and *iglD* Schu S4 mutants are $\gg 10^7$ CFU. Presumably, the lack of phagosomal escape renders them avirulent (Twine et al., 2005; Kadzhaev et al., 2009). Moreover, they are non-immunogenic since mice infected with the *iglC* mutant of Schu S4 are not protected against a subsequent infection with a subspecies *tularensis* strain (Twine et al., 2005).

GENETIC DIFFERENCES WITHIN THE FPI CLUSTER

The ORFs of the FPI are essentially conserved in all subspecies, with the exception of the *anmK–pdpD* region (**Figure 1**). In *F. novicida*, *anmK* (originally denoted *pmcA*) is suggested to encode a 371 aa gene product, AnmK, which shows high identity to members of COG2377 (predicted molecular chaperone distantly related to HSP70-fold metalloproteases) (Nano and Schmerk, 2007). However, in type A strains like Schu S4 and FSC033 (clade A.I), *anmK* may be expressed as two separate ORFs due to the presence of premature stop codons at positions 190 and 328, followed by an ATG start codon at position 194. In type A strains like WY96-3418 and B38 (clade A.II), AnmK has a premature stop codon at position 328, suggesting that this group encodes a truncated form of AnmK (Nano and Schmerk, 2007). How these genetic differences affect AnmK function has not been addressed so far, but AnmK of *F. novicida* has been shown to contribute to virulence, albeit to a small degree (Nano and Schmerk, 2007; Ludu et al., 2008). Supporting the notion that AnmK is not critical for *Francisella* pathogenicity is the fact that *F. tularensis* type B strains, virulent strains as well as LVS, lack the *anmK* gene due to a deletion that extends from 107 bp upstream of *anmK* and encompasses most of the adjacent *pdpD* gene (Nano et al., 2004; Champion et al., 2009). In *F. novicida*, *pdpD* encodes a 1,245 aa (1,195 aa in type A strains) outer membrane protein that has been shown to be important for virulence in mice (Ludu et al., 2008), while the deletion within type B strains is presumed to result in two minor ORFs (corresponding to residues 992–1124 and 1129–1195 of PdpD from type A strains) that are likely to be non-functional (Ludu et al., 2008; Champion et al., 2009). It has been speculated that the presence or absence of *pdpD* may account, in part, for the strain-to-strain specific difference in virulence between type A and type B strains (Nano et al., 2004).

FPI NOMENCLATURE – NAMING OF COMPONENTS

Over the years, the nomenclature of the FPI genes has been altered in attempts to adapt to the increasing knowledge of the function of individual proteins (**Table 2**). Already in 1997, the first FPI gene (later named *iglC*) was identified, and its expression shown to be prominently induced during intracellular growth (Golovliov et al., 1997). The gene was later recognized as a member of a four gene operon, which, due to its requirement for intramacrophage growth, was denoted *iglABCD* (*intracellular growth locus*) (Gray et al., 2002; Golovliov et al., 2003b, **Figure 1**; **Tables 1 and 2**). In 2004, at the time of the discovery of the FPI, a handful of the predicted ORFs were named, including *pmcA* (today *anmK*), which share homology to molecular chaperones, and the large ORFs *pdpA*, *pdpB*, *pdpC,* and *pdpD* (*pathogenicity determinant protein*) (Nano et al., 2004; **Table 1**). Later, *pdpB* was named IcmF, based on its homology to this crucial T6SS core component (Ludu et al., 2008). In 2007, the remaining ORFs of the island were recognized as most likely functional genes and thus denoted *pigA* to *I* (*pathogenicity island gene*) (Nano and Schmerk, 2007; **Table 1**). Subsequently, limited homologies have been identified between *pigB*, *pigC*, *pigF*, *pigI,* and the T6SS core components *vgrG*, *clpV*, *dotU,* and *hcp*, respectively, so these *Francisella* genes have been renamed accordingly (Ludu et al., 2008; Barker et al., 2009; **Table 1**). Also the remaining *pig* genes, i.e., *pigA*, *pigD*, *pigE*, *pigG*, and *pigH* have been renamed *iglE*, *iglG*, *iglH*, *iglI,* and *iglJ* respectively (Ludu et al., 2008; Barker et al., 2009; **Table 1**), suggesting that these genes, similar to *iglABCD*, are required for intracellular growth. However, with the exception of *iglI* of *F. novicida* (Barker et al., 2009), there is no published data to support that this is the case (**Table 3**). In fact, our data strongly suggest that *iglG* is dispensable for intracellular growth of *F. tularensis* strain LVS in both J774 macrophages and mouse peritoneal exudate (PEC) cells (Bröms et al., unpublished) (**Table 3**). Moreover, an *iglI* null mutant of LVS is able to efficiently grow within J774 cells, which is in contrast to the results observed by Barker et al. (2009) for *F. novicida* (above) (Bröms et al., unpublished; **Table 3**). This is an exciting finding, since it suggests that the phenotypes of FPI mutants may be species-specific, but it clearly complicates the use of a common FPI nomenclature; thus, the name IglG (Intracellular growth locus G) and IglI (Intracellular growth locus I) may perhaps be appropriate for *F. novicida*, but evidently not for LVS. Also, we do not see any obvious reason to distinguish *pdpA* from the *iglABCD*

Table 2 | Effects of FPI gene mutations on *Francisella* pathogenicity.

Requirement for:	Intracellular growth	Phagosome escape	Cytopathogenicity	Virulence		
				Chick embryos	Mice	Drosophila
pdpA	Y[1,3,20]	Y[1]	Y[1]	Y[1]	Y[1,18]	Y[3]
pdpB/icmF	Y[3,8,9,20]	NT	Y[8]	NT	Y[8,9,18]	Y[3]
iglE	Y[3,6]	NT	NT	NT	Y[18]	Y[3]
vgrG	Y[3,5]	Y[5]	NT	NT	Y[5,18]	Y[3]
iglF	Y[3,7]	NT	NT	NT	Y[18]	Y[3]
iglG	N[17], Y[3,7]	N[17]	Y[17]	NT	Y[17,18]	Y[3]
iglH	Y[3,7]	NT	NT	NT	Y[18]	Y[3]
dotU	Y[3]	NT	NT	NT	Y[18]	Y[3]
iglI	N[17], Y[3,5]	Y[5]	Y[17]	NT	Y[5,17,18]	Y[3]
iglJ	Y[3,8]	NT	Y[8]	NT	Y[8,18]	Y[3]
pdpC	N[3,20]	NT	NT	NT	Y[18]	N[3]
pdpE	N[3,17]	N[17]	N[17]	NT	N[17,18]	N[3]
iglD	Y[3,15,20]	Y[15]	Y[15]	NT	Y[18]	Y[3,21]
iglC	Y[3,10,11, 14–16,19,20,22]	Y[14–16]	Y[10,15,22]	NT	Y[10,11,18]	Y[3,21]
iglB	Y[3,12,20]	Y[12]	NT	NT	Y[18]	Y[3,21]
iglA	Y[3,12,13,20]	Y[12]	N[17]	Y[13]	Y[12,18]	Y[3]
pdpD	N[2,3,20]	NT	NT	Y[2]	Y[2,18]	N[3]
anmK	N[3]	NT	NT	Y[2]	Y[18]	N[3]

"Y" indicates that the genes is required for the phenotype tested (gray boxes), "N" indicates that it is not (black boxes). NT, not tested (white boxes). For mutants, such as iglG and iglI, contradicting results exist (dark gray boxes). For strains which carry duplications of the FPI genes, the stated phenotypes and references refer to mutants that contain deletions in both copies of the FPI gene.

Supporting data from: [1]Schmerk et al. (2009a), [2]Ludu et al. (2008), [3]Åhlund et al. (2010), [4]Kraemer et al. (2009), [5]Barker et al., (2009), [6]Rodriguez (2010), [7]Asare and Abu Kwaik (2010), [8]Brotcke et al. (2006), [9]Tempel et al. (2006), [10]Golovliov et al., (2003a), [11]Lauriano et al., (2004), [12]Bröms et al. (2009), [13]de Bruin et al. (2007), [14]Santic et al. (2005b), [15]Bönquist et al. (2008), [16]Lindgren et al. (2004), [17]Bröms et al. (unpublished), [18]Weiss et al. (2007), [19]Gray et al. (2002), [20]Read et al. (2008), [21]Vonkavaara et al. (2008), [22]Lai et al. (2004).

genes required for intracellular growth, since a *pdpA* mutant of *F. novicida* also clearly exhibits very limited replication in host cells (Schmerk et al., 2009a). Moreover, the homologies reported between some of the *Francisella* proteins and T6SS core components, e.g., PigC and ClpV (Barker et al., 2009), are weak and do not provide any convincing evidence for their relatedness, nor do they provide support when it comes to assigning a function to the specific FPI protein (Bröms et al., unpublished).

For these reasons, we believe that the existing FPI nomenclature should be modified to better reflect the current knowledge of FPI protein function, but also to take into account that for many of these proteins we still have no assigned functions. In fact, since the *Francisella* T6SS is so different from all other T6SS described so far (Bingle et al., 2008) it is not unlikely that some of the FPI proteins will exhibit functions distinct from their homologous T6SS core components. For simplicity, we therefore propose that all FPI genes should be named *igl*, regardless of function, but this will be discussed more thoroughly elsewhere.

THE FPI – ITS ROLE IN PHAGOSOME BIOGENESIS, INTRACELLULAR GROWTH AND VIRULENCE

Similar to other intracellular pathogens like *Listeria monocytogenes*, *Shigella flexneri*, and *Rickettsia* spp. (Ray et al., 2009), *Francisella* rapidly escapes from the phagosome into the host cell cytosol where it replicates (Clemens et al., 2004; Santic et al., 2005a,b; Bönquist et al., 2008). The *Francisella* containing phagosome (FCP) transiently acquires early (EEA-1 and Rab5) and late (Rab7, CD63, LAMP-1, and LAMP-2) endosomal markers, but no/little cathepsin D, suggesting limited fusion to lysosomes (Clemens et al., 2004; Santic et al., 2005a, 2008; Bönquist et al., 2008). Inhibition of the proton ATPase pump within 15–60 min post infection has been shown to result in a significant delay in phagosomal escape (Chong et al., 2008; Santic et al., 2008), indicating the importance of acidification for FCP disruption and bacterial escape. Importantly, these results have recently been challenged by Clemens et al. (2009).

A vast number of studies have identified the FPI as a key player in the ability of *F. tularensis* to modulate phagosome maturation and escape into the host cell cytosol. This is likely to be the consequence of T6SS-mediated translocation of bacterial effector protein(s) directly involved in modulation of phagosome biogenesis and lysis of the phagosomal membrane. However, none of the FPI-encoded proteins required for escape so far hold any properties of cytolysins, pore-forming toxins, or hydrolytic enzymes commonly used by intracellular pathogens to disrupt the phagosomal membrane (Ray et al., 2009). This suggests that *Francisella* may employ novel mechanisms to promote bacterial escape into the cytosol, or merely that the effector proteins are encoded outside of the FPI cluster.

Table 3 | *In silico* analysis of FPI proteins, demonstrated protein localization and protein–protein interactions.

	Bioinformatic predictions based on protein sequence						Experimental data	
Prediction	**Amino acids[1] Schu S4 (LVS/U112)**	**Size (kDa)[2]**	**Secretion signal[3]**	**TM domains[4]**	**Bacterial localization[5]**	**COGs[7]**	**Bacterial localization**	**Interaction partner**
pdpA	820	95.4	N	0	OM		Soluble[8]	
pdpB/*icmF*	1093	127.6	N	2	OM	COG3523	IM[9,16]	
iglE	125	14.5	Y	1	Unknown (lipid attachment site[6])		IM, OM (latter requires a lipid attachment site)[10]	
vgrG	164	17.5	N	0	Unknown		surface[11], secreted[12]	
iglF	574, (554/576)	67.6	N	0	Unknown			
iglG	173	18.4	N	0	Unknown		OM[16]	
iglH	476	55.3	N	0	Unknown			
dotU	207	24.6	N	1	Unknown	COG3455		
iglI	383	44.6	N	0	Unknown		Secreted[12], Soluble, IM, OM[16]	
iglJ	257	30.9	N	0	Unknown			
pdpC	1328, (1328/1325)	156.2	N	0	Unknown			
pdpE	191, (191/188)	22.1	Y	0	Unknown		OM[16]	
iglD	401, (398/398)	46.5	N	0	Unknown			
iglC	211, (209/209)	22.4	N	0	Unknown		CP[13], surface[9]	
iglB	514, (506/506)	58.9	N	0	Unknown	COG3517	CP[14], surface[9]	IglA[14,15]
iglA	196, (184/184)	21.0	N	0	CP	COG3516	CP[14], surface[9,11]	IglB[14,15]
pdpD	1195, (67; 133/1245)	135.4	N	0	Unknown		OM[9]	
anmK	189, 134 (NA/381)	20.4 + 15.2	N	0	OM	COG2377		

[1]Number of amino acids predicted for FPI proteins from F. tularensis. subsp. tularensis Schu S4. Sizes for homologous proteins from F. tularensis subsp. holarctica LVS and F. novicida U112 are only indicated when they deviate from the Schu S4 sequence in length.
[2]Predicted size in kDa of proteins from Schu S4 according to SAPS.
[3]SignalP 3.0 prediction of the presence (Y) or absence (N) of a secretion signal.
[4]Number of transmembrane (TM) domains predicted with TMHMM 2.0.
[5]Protein localization predicted by PSortb.
[6]A lipid attachment site predicted (Rodriguez, 2010).
[7]Relatedness to COGs (cluster of orthologous groups; Tatusov et al., 2000).
[8]Schmerk et al. (2009a).
[9]Surface localization of IglA, IglB, and IglC occurs upon overexpression of PdpD (Ludu et al., 2008).
[10]Mutation of the putative site of lipidation (C22G) prevents OM localization of IglE–FLAG (Rodriguez, 2010).
[11]Melillo et al. (2006).
[12]Barker et al. (2009).
[13]Golovliov et al. (1997).
[14]de Bruin et al. (2007).
[15]Bröms et al. (2009).
[16]Bröms et al. (unpublished).
NA, not applicable.
OM, outer membrane; IM, inner membrane; CP, cytoplasmic.

Since FPI mutants that fail to escape also fail to multiply, it has been difficult to determine whether the encoded gene products also contribute to multiplication, or to later stages of infection. Interestingly, FPI genes reach their maximal expression at the end of cytosolic replication, which may suggest a role for the FPI during late stages of infection, or perhaps, may provide an advantage ("ready and armed") to bacteria that are about to infect new cells upon lysis of their current host cell (Wehrly et al., 2009).

IglC is the best characterized FPI protein so far, together with MglA, a positive regulator encoded outside of the FPI. Both have been shown to be essential for the ability of *F. tularensis* to modulate the biogenesis of the phagosome to avoid lysosomal fusion (Santic et al., 2005b; Bönquist et al., 2008), to escape into the host cytosol (Lindgren et al., 2004; Santic et al., 2005b), and to induce apoptosis of J774 cells (Lai et al., 2004; **Table 3**). Consequently, *mglA* and *iglC* mutations diminish intracellular survival and growth in many different macrophage-like cell lines (Gray et al., 2002; Golovliov et al., 2003a; Lauriano et al., 2003; Lindgren et al., 2004; Santic et al., 2005b), as well as ameba (Lauriano et al., 2004) and insect cells (Read et al., 2008; Vonkavaara et al., 2008; Santic et al., 2009; **Table 3**). To date, most of the genes located in the FPI, such as *iglA*, *iglB*, *iglC*, *iglD*, *iglI*, *iglG*, *pdpA*, *pdpB*, and *pdpD*, have been demonstrated to be required either for intramacrophage growth and/or virulence of at least one subspecies (Golovliov et al., 2003b; Lai et al., 2004; Nano et al., 2004; Tempel et al., 2006; de Bruin et al., 2007; Santic et al., 2007; Weiss et al., 2007; Bönquist et al., 2008; Chong et al., 2008; Ludu et al., 2008; Bröms et al., 2009; Schmerk et al., 2009b; Bröms et al., unpublished; **Table 3**). Interestingly,

some gene products, such as IglG, have been shown to be required for virulence but dispensable for intracellular growth, while PdpE appears to be dispensable for both processes (Bröms et al., unpublished; **Table 3**). Noteworthy, many of the earlier FPI studies lacked the tools for precise genetic analysis and proper complementation controls, and this should be taken into account when discussing the phenotypes of *Francisella* strains with lesions in FPI genes.

FPI-MEDIATED MANIPULATION OF THE HOST INFLAMMATORY RESPONSE

The pathogenesis of tularemia appears to be critically dependent on the ability of *F. tularensis* to modulate the host immune response by a number of mechanisms, including degradation of serum antibodies (Crane et al., 2009), induction of immunosuppressive cytokines and prostaglandin E2 in the lungs of infected mice (Bosio et al., 2007; Woolard et al., 2008), suppression of responses to secondary stimuli *in vivo* (Bosio and Dow, 2005), inhibition of the respiratory burst in human neutrophils (McCaffrey and Allen, 2006), impairment of phosphatidylinositol 3-kinase-mediated (Parsa et al., 2006) and IFN-γ-mediated signaling (Parsa et al., 2008) and downregulation of several TLRs in monocytes (Telepnev et al., 2003), rapid induction of apoptosis in J774 cells and mouse tissue macrophages (Lai et al., 2004; Wickstrum et al., 2009), activation of the ASC inflammasome (Mariathasan et al., 2004; Fernandes-Alnemri et al., 2010; Jones et al., 2010; Rathinam et al., 2010), as well as rapid activation of Ras (Al-Khodor and Abu Kwaik, 2010). Several of these mechanisms are directly or indirectly linked to FPI proteins since specific FPI mutants or mutants within *mglA* show phenotypes deviant from the parent strain.

Already within 15 min after monocytic ingestion of wild-type *F. novicida*, recruitment of PKCα and PKCβI to the SOS2/GrB2 complex occurs, leading to activation of Ras (**Figure 2**). None of these events take place after infection with an *iglC* mutant of *F. novicida*, instead rapid activation of caspase-3 and cell death follows (Al-Khodor and Abu Kwaik, 2010; **Figure 2**). Thus, Ras activation is advantageous to the bacterium since it allows a prolonged replication in the cytosol. As it occurs so rapidly, the phagosomal localization of the *iglC* mutant may not necessarily be the reason for the aberrant Ras activation, instead, the effect may directly or indirectly relate to the function of IglC.

The proinflammatory response to *F. tularensis* in monocytic cells is TLR2-dependent (Cole et al., 2006, 2007; Katz et al., 2006; Li et al., 2006; Thakran et al., 2008; Abplanalp et al., 2009). Moreover, it has been demonstrated that *Francisella* colocalizes with TLR2 and MyD88 inside macrophages (Cole et al., 2007; **Figure 3**), signals through TLR2 from within the phagosome (Cole et al., 2008;

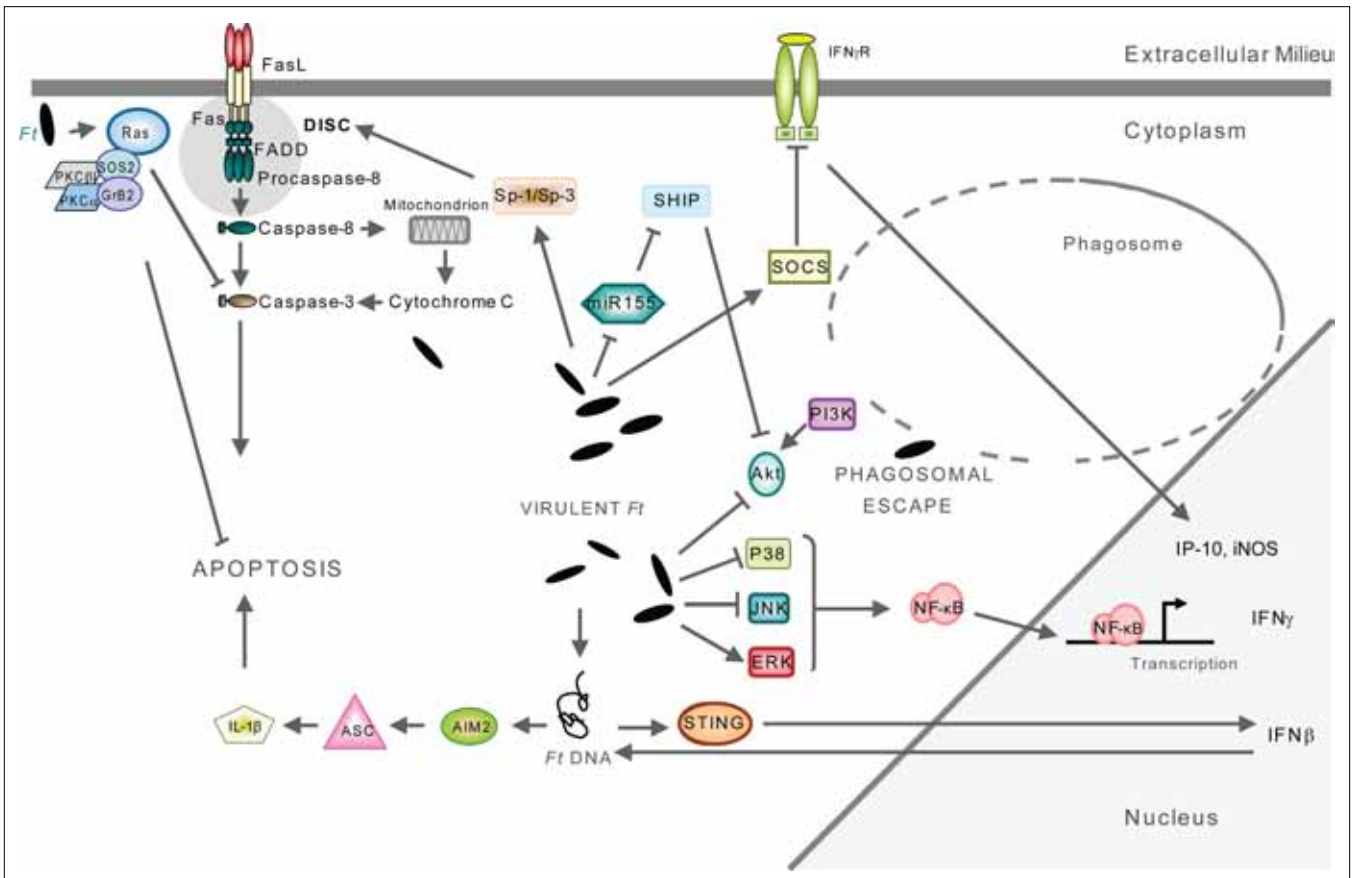

FIGURE 2 | Signaling triggered by *F. tularensis* located in the cytosol. Host cell signaling occurs after *F. tularensis* escapes from the phagosome to reside and multiply within the cytoplasm of the monocytic cells. The infection eventually leads to apoptosis of the host cell. The engagement of a multitude of signaling pathways have been described, for details see references in the text. Only pathways that have been experimentally verified are included in the figure.

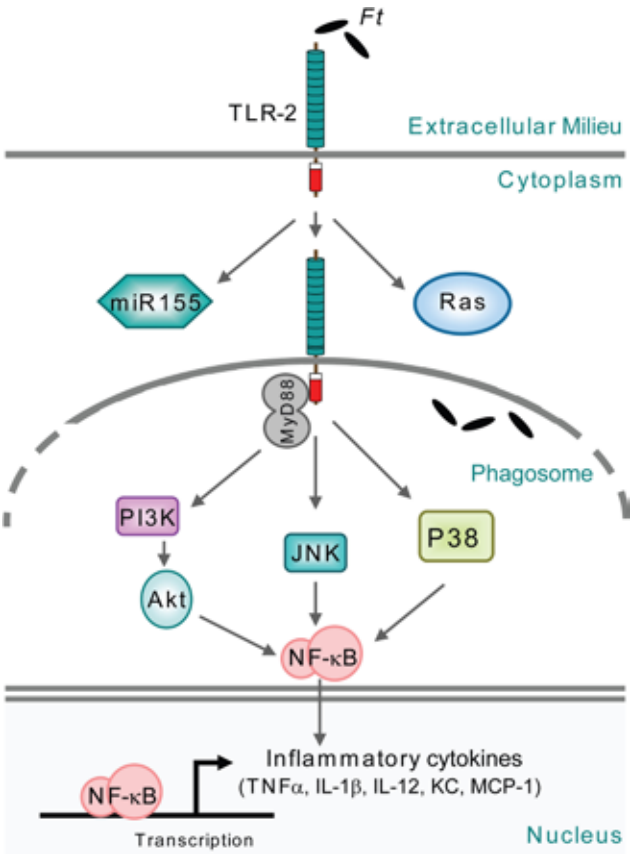

FIGURE 3 | Signaling triggered by *F. tularensis* located in the phagosome. The signaling occurs during the brief period after uptake of the bacteria by monocytic cells and before they escape into the cytoplasm. Moreover, the signaling also occurs during infection with FPI mutants, e.g., within *iglC*, which are unable to escape from the phagosome.

Figure 3), and that phagosome-contained *F. tularensis* demonstrate a distinct signaling pattern (Cole et al., 2008). In the latter case, macrophages infected with an *iglC* mutant showed increased expression of a subset of TLR2-dependent, proinflammatory genes, but decreased expression of other genes encoding, e.g., IFN-γ, iNOS, and IP-10 (Cole et al., 2008). The latter subset was dependent on IFN-β, indicating that this cytokine is required for full induction of the macrophage proinflammatory response (**Figure 2**). While the *iglC* mutant greatly increased IL-1β mRNA expression in wild-type macrophages, IL-1β secretion was not observed (Cole et al., 2008). Thus, TLR2-dependent expression of IFN-β, IFN-β-stimulated genes, and IL-1β secretion requires bacterial escape from the phagosome. Noteworthy, in the absence of IFN-β, intracellular bacterial numbers were increased showing the central role of the cytokine in modulating the host response and controlling *F. tularensis* infection (Cole et al., 2008).

A detailed characterization of the *F. tularensis*-induced IFN-β signaling has been revealed in a series of recent publications, resulting in the identification of *F. novicida* as a prototypic agent for AIM2-mediated inflammasome activation (Fernandes-Alnemri et al., 2010; Jones et al., 2010; Rathinam et al., 2010; Ulland et al., 2010). AIM-2 activation involves recognition of cytosolic DNA, which triggers formation of the AIM2 inflammasome by inducing AIM2 oligomerization (Fernandes-Alnemri et al., 2009; **Figure 2**). The AIM2-DNA complex then recruits and subsequently oligomerizes the caspase-1-activating adaptor protein ASC into the large ASC pyroptosome, which in turn activates caspase-1 leading to cleavage of IL-1β and subsequent apoptosis (Fernandes-Alnemri et al., 2007; **Figure 2**). By infecting AIM2-deficient mice with *F. novicida*, it was concluded that cytosolic invasion by *F. tularensis* leads to STING-mediated production of type I interferons, such as IFN-β, which is a prerequisite for the subsequent AIM2 activation, leading to caspase-1 activation, IL-1β and IL-18 secretion and inflammasome-mediated cell death (Gavrilin et al., 2006; Mariathasan et al., 2006; Henry et al., 2007; Cole et al., 2008; **Figure 2**). This chain of events critically depends on a cytosolic localization of bacteria as shown by infections with FPI mutants unable to escape the phagosome, such as *iglC*, or mutants within *mglA*, the positive regulator encoded outside of the FPI (Gavrilin et al., 2006; Cole et al., 2008). Similarly, *F. novicida vgrG* and *iglI* mutants, both unable to escape from the phagosome, were recently shown to be defective for IL-1β release (Barker et al., 2009). Thus, for *F. novicida*, there appears to be a strong correlation between the degree of phagosomal escape and inflammasome activation. In view of this, it was somewhat surprisingly that one of the studies found that the endosomal-acidification inhibitor bafilomycin completely prevented the production of type I interferons, implying the phagosome as the source of DNA for inflammasome activation (Fernandes-Alnemri et al., 2010). However, the divergent findings may be reconciled if it is assumed that the amounts of *F. novicida* DNA initially released into the cytosol upon escape *per se* are not sufficient to activate AIM2 but still can trigger production of type I interferons such as IFN-β. This will lead to monocyte activation and enhanced bacterial degradation in the phagosome, resulting in increased DNA release into the cytosol. Thereby, the accumulation of cytosolic DNA may reach a threshold required for AIM2 inflammasome activation. The role of bafilomycin in this context may be to prevent the type I interferon-enhanced killing of *F. tularensis* in the phagosome.

Francisella tularensis infection leads to interference of several signaling pathways crucial for the control of intracellular infection. One such example is the IFN-γ signaling pathway, which is essential although alone not sufficient to control *F. tularensis* infection of monocytic cells (Anthony et al., 1989; Lindgren et al., 2004; Edwards et al., 2009). The role of this pathway has been analyzed by studies of the IFN-γ-induced STAT1 activation in human and murine monocytic cells (Parsa et al., 2008; Roth et al., 2009). The interference with IFN-γ *per se* was dependent on upregulation of SOCS3, a well-known inhibitor of IFN-γ-mediated signaling, and accompanied by the suppression of IP-10 and iNOS production, resulting in increased intracellular bacterial survival (**Figure 2**). This signaling interference is independent of phagosomal escape and replication since mutants in *mglA* or *iglD* were still able to efficiently suppress IFNγ-induced STAT1 (Parsa et al., 2008). The important role of IFN-γ has been corroborated by determining its role when added to *F. tularensis*-infected cell cultures. Then, it was observed that treatment of mouse PEC cells conferred a static effect on wild-type LVS bacteria, whereas the treatment had a bactericidal effect on the *iglC* mutant demonstrating that the

effect of IFN-γ is dependent on the subcellular localization of *Francisella* (Lindgren et al., 2004). There are conflicting data on the mechanisms effectuated by IFN-γ since one study reported that IFN-γ treatment did not affect the phagosomal escape and subcellular localization of Schu S4 in murine bone marrow-derived macrophages or human monocyte-derived macrophages (Edwards et al., 2009); two others that the subcellular localization of LVS was marginally affected in murine PEC (Lindgren et al., 2004), but not in murine J774A.1 cells (Bönquist et al., 2008); and a fourth that *F. novicida* was completely confined to the phagosome of human monocyte-derived macrophages (Santic et al., 2005a). Some of the discrepancies may relate to the use of different infection models and strains of *F. tularensis*, but additional studies are required to clarify this.

Mitogen-activated protein kinase (MAPK) pathways, such as p42/p44 (Erk1/2), c-Jun terminal kinase (JNK) and p38, of monocytic cells are also modulated by *F. tularensis* during infection. Hrstka et al. (2005) observed that apoptosis induced by LVS was associated with reduced p38 MAPK activity and activation of p42/p44 kinases (**Figure 2**). Moreover, Telepnev et al. (2005) showed that LVS is able to rapidly downregulate the initial activation of p38 and c-Jun, and hence cytokine secretion and that IglC is critical for this process. Thus, unlike the *iglC* mutant, LVS eventually downregulates the initial proinflammatory response in monocytic cells.

Moreover, phagosomal escape has been shown to be accompanied by marked downregulation of the phosphoinositol 3 kinase PI3K/Akt pathway and subsequent suppression of NF-kB-mediated production of inflammatory cytokines (Parsa et al., 2006; Rajaram et al., 2006; **Figure 2**). Suppression does not occur when an *mglA* mutant is used; instead it leads to increased proinflammatory production and lower IL-10 release (Rajaram et al., 2006; **Figure 3**). The pathway is under negative control of the inositol phosphatase SHIP, which becomes phosphorylated upon infection by *F. tularensis* (Parsa et al., 2006; **Figure 2**). Interestingly, the microRNA-155 (MiR-155) that targets and negatively regulates SHIP is induced by *F. novicida* but not Schu S4 (**Figure 2**), thereby leading to an enhanced proinflammatory response in the former case (Cremer et al., 2009). Furthermore, the expression of the PI3K/Akt pathway members is preferentially downregulated in macrophages infected with *F. tularensis* Schu S4 compared to *F. novicida* (Butchar et al., 2008).

The PI3K/Akt pathway is also related to host-cell death. Phagosomal escape of *F. novicida* leads to activation of Sp-1/Sp-3, upregulation of Fas and recruitment of the DISC complex (Rajaram et al., 2009), which in turn activates caspases-3 and -8 leading to apoptosis (Lai and Sjöstedt, 2003; Santic et al., 2009; **Figure 2**). Since activation of PI3K/Akt promotes phagosome–lysosome fusion (Rajaram et al., 2009), this should limit intracellular bacterial growth and host-cell death. In corroboration of this scenario, it was found that constitutive activation of Akt, or deletion of SHIP, promotes phagolysosomal fusion and limits bacterial replication in the host cytosol, as well as preventing subsequent induction of Fas expression and cell death (Rajaram et al., 2009).

Akt is also involved in phagosome biogenesis through activation of Rab5 (Barbieri et al., 1998) and the assembly of NADPH oxidase leading to triggering of the oxidative burst (Chen et al., 2003; Hoyal et al., 2003). Thereby, modulation of the PI3K/Akt pathway by *F. tularensis* may serve multiple purposes; downmodulation of the proinflammatory response and the oxidative burst, induction of host-cell death and limiting the biogenesis of the FCP.

The exact roles of the FPI proteins in modulating essentially all of the aforementioned pathways described is enigmatic since it is possible that the bacterium, once in the cytosol, mediates the effects via non-FPI-dependent mechanisms, e.g., via signaling through cytosolic receptors. Thus, a possible scenario is that the essential role of the FPI proteins simply is to enable the phagosomal escape.

Regulation of the FPI – a complex story

Studies in pathogens such as *Burkholderia pseudomallei*, *Salmonella enterica*, *Vibrio cholerae*, *Pseudomonas aeruginosa,* and enteroaggregative *Escherichia coli* have led to the identification of regulatory determinants and mechanisms underlying expression of T6SS gene clusters. These complex transcriptional regulatory networks involve two-component systems (Ishikawa et al., 2009; Syed et al., 2009), quorum sensing systems (Deziel et al., 2005; Ishikawa et al., 2009; Lesic et al., 2009), alternative sigma factor σ^{54} (Pukatzki et al., 2006; Ishikawa et al., 2009; Syed et al., 2009), transcriptional activators (Deziel et al., 2005; Dudley et al., 2006; Lesic et al., 2009; Sun et al., 2010), and histone-like proteins (Lucchini et al., 2006; Castang et al., 2008). In addition, upregulation of most T6S clusters has been shown to depend on contact with, or intracellular growth within, the host cell during infection (Das et al., 2000; Parsons and Heffron, 2005; Chugani and Greenberg, 2007; Shalom et al., 2007). In *F. tularensis*, this is consistent with the demonstration that FPI genes are induced during growth within macrophages (Golovliov et al., 1997; Baron and Nano, 1998; Twine et al., 2006; de Bruin et al., 2007; Chong et al., 2008; Wehrly et al., 2009). To date, the environmental stimuli and mechanisms that govern regulation of the FPI genes and thus virulence of *Francisella* spp. are as yet unknown, although iron depletion (Deng et al., 2006; Lenco et al., 2007), and oxidative stress (Lenco et al., 2005) have been shown to induce expression of FPI genes.

Combined data from several research groups suggest the involvement of at least six regulatory proteins in controlling FPI gene expression; MglA, SspA, FevR (PigR), MigR, Hfq, and PmrA (**Figure 4**; Baron and Nano, 1998; Lauriano et al., 2004; Brotcke et al., 2006; Charity et al., 2007; Mohapatra et al., 2007; Buchan et al., 2009; Meibom et al., 2009). Some of these also regulate expression of genes outside the FPI, however, whereas MglA, SspA, and FevR appear to regulate the same set of genes, PmrA and Hfq control separate groups of genes (Brotcke et al., 2006; Charity et al., 2007; Guina et al., 2007; Mohapatra et al., 2007; Brotcke and Monack, 2008; Meibom et al., 2009).

Macrophage growth locus (MglA) and SspA are members of the stringent starvation protein A (SspA) family (Baron and Nano, 1998) and interact to form heterodimers that bind to the RNA polymerase, inducing the transcription of numerous genes, including *fevR* (Brotcke et al., 2006; Charity et al., 2007). FevR (*Francisella* effector of virulence regulation) has been shown to physically interact with MglA/SspA in a manner facilitated by the alarmone ppGpp, although there are conflicting data concerning this interaction (Brotcke and Monack, 2008; Charity et al., 2009). FevR positively autoregulates its own expression (Charity et al., 2009) and exhibits some homology to the helix-turn-helix region

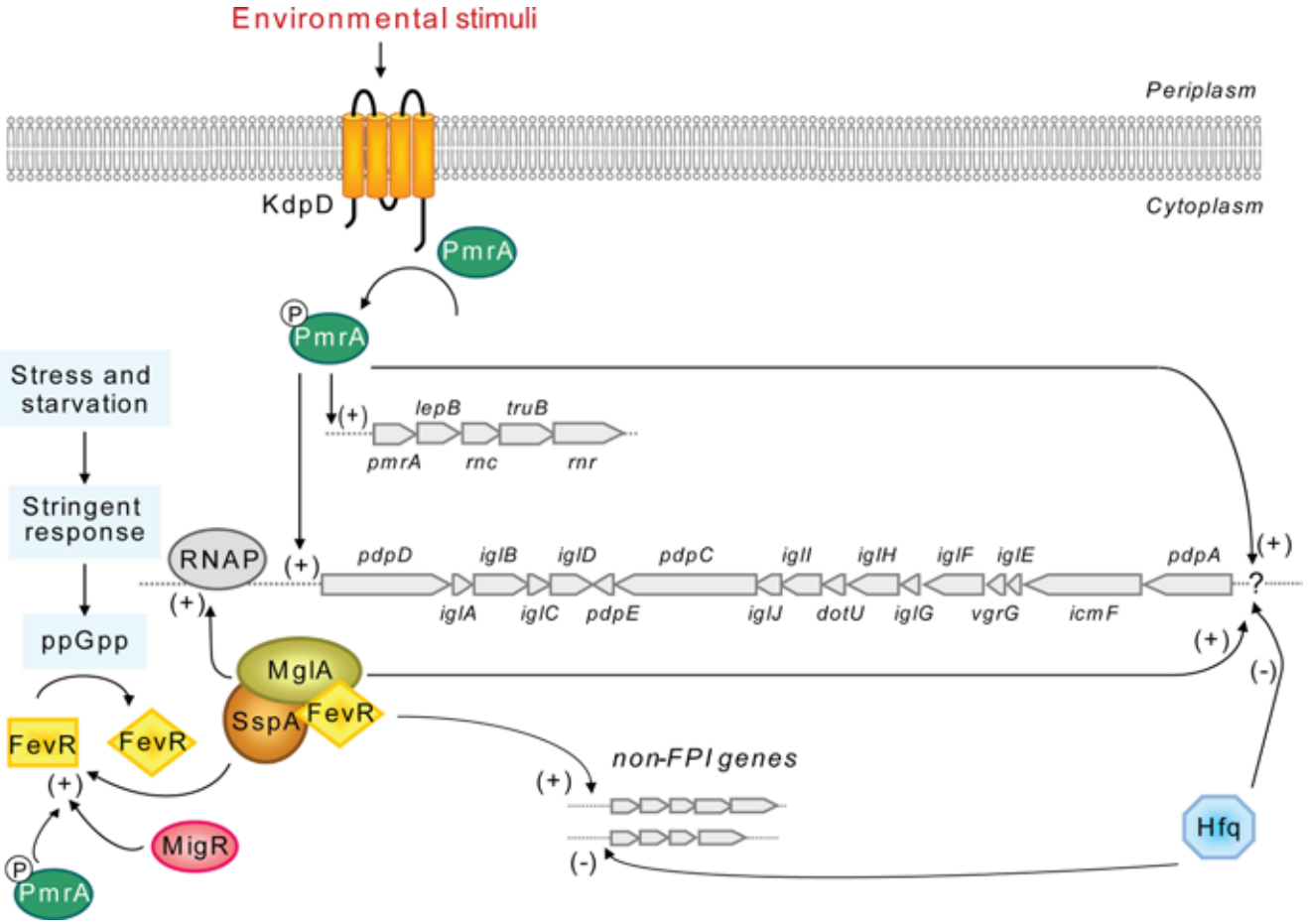

FIGURE 4 | A model for the regulation of the FPI locus. Preceding expression of the FPI locus is an unknown environmental stimulus that leads to KdpD-mediated phosphorylation of PmrA, allowing it to bind to promoter regions. The promoter upstream of *pdpD* has been experimentally confirmed, while the promoter upstream of *pdpA* (?) is hypothetical. PmrA binding may recruit the MglA/SspA/FevR complex, which directly interacts with the RNA polymerase (RNAP) to initiate transcription. For FevR to efficiently bind to MglA/SspA, it first needs to be activated by the alarmone ppGpp of the stringent response, while *fevR* expression requires PmrA, MglA, SspA as well as MigR. PmrA and MglA/SspA/FevR also regulate many additional genes outside of the FPI cluster, mainly in a positive manner, however their target genes appear to be different. At an unknown signal, the Hfq repressor specifically inhibits the expression of the putative *pdpA* to *pdpE* operon, presumably by binding to the predicted promoter upstream of *pdpA*. Hfq also regulates expression of genes outside of the FPI, mainly by acting as a repressor.

of the MerR family of transcriptional activators, suggesting that it may bind to target DNA (Brotcke and Monack, 2008). MigR (Macrophage intracellular growth regulator) may exert its positive effect on FPI gene expression indirectly through FevR, since it has been shown to be required for *fevR* expression, in contrast to that of *mglA*, *sspA*, or *pmrA* (Buchan et al., 2009). Moreover, *fevR* expression also requires the presence of MglA, SspA as well as PmrA (Brotcke et al., 2006; Charity et al., 2007; Mohapatra et al., 2007; **Figure 4**).

Importantly, current data suggest a connection between MglA/SspA/FevR and a two-component regulatory system consisting of PmrA/KdpD (Bell et al., 2010). PmrA, which positively regulates its own transcription and that of the FPI, was originally assumed to be an orphan response regulator (Mohapatra et al., 2007; Sammons-Jackson et al., 2008). However, Bell et al. (2010) recently demonstrated that PmrA, upon phosphorylation by the histidine kinase KdpD, efficiently binds to regulated promoters of the *pmrA* operon and FPI-encoded *pdpD*. Moreover, using coimmunoprecipitation experiments it was revealed that PmrA, MglA, and SspA are part of the same protein complex (Bell et al., 2010). Thus, although the sequence of protein binding to the regulatory complex is not clear, phosphorylated PmrA may bind to FPI gene promoters recruiting free or RNA polymerase-bound MglA, SspA and FevR, to initiate FPI gene transcription (**Figure 4**).

Finally, Hfq differs from all the other FPI regulators identified so far in that it seems to act primarily as a repressor of gene expression, both for genes located within the FPI as well as genes located outside (Meibom et al., 2009). Moreover, Meibom et al. (2009) were able to show that only a subset of FPI genes (*pdpA* to *iglJ*) are negatively regulated by Hfq (**Figure 4**), leading the authors to speculate that there is differential regulation of the two putative divergent operons in the FPI (*pdpD* to *iglD* and *pdpA* to *pdpE*). The G + C content of these two regions are significantly different from one another: 30.6% (*pdpD* to *iglD*) vs 26.6% (*pdpA* to *pdpE*),

compared to an average of 33% for the *Francisella* chromosome (Nano and Schmerk, 2007), which supports the notion of two different origins for these regions. Nevertheless, this operon organization must still be shown experimentally and putative promoter(s), in addition to that located within the intergenic region of *pdpD* and *anmK* (Bell et al., 2010), identified. Importantly, the intergenic regions between *pdpD* and *iglA* (80 nt) and *iglJ* and *pdpC* (39 nt) are also, in theory, large enough to harbor promoters, suggesting the possibility of multiple overlapping promoters within the FPI (Nano and Schmerk, 2007).

Clearly, much more work is needed in order to understand the intricate regulatory network behind *Francisella* FPI gene expression, especially how the many regulators are able to coordinate this process. The fact that these also regulate genes outside of the FPI suggests there is cross-talk between T6S and other virulence determinants, which adds further complexity to the situation. Defining how these networks are coordinated and identifying the environmental signals that induce FPI expression and T6S may also help to identify the elusive effector proteins secreted by this system. All together, this will shed light on the critical role of the FPI in *Francisella* pathogenesis.

T6S – WHAT CAN WE LEARN ABOUT THE *F. TULARENSIS* SYSTEM FROM OTHER PATHOGENS?

To date, there is limited information about the functions of the putative T6SS encoded by the *Francisella* FPI gene cluster. However, as a phenomenon highly related to pathogenesis, bacterial secretion has received great interest from the scientific society. Today several comprehensive studies on T6S in various pathogens exist, which have contributed to the understanding of these complex secretion systems. An overview is presented below.

TYPE VI SECRETION SYSTEM GENE CLUSTERS ARE UBIQUITOUS AMONG GRAM-NEGATIVE BACTERIA

When first discovered in *V. cholerae*, the T6SS gene clusters were denoted IAHP (IcmF associated homologous protein), since they include a gene with homology to *icmF* of the *Legionella pneumophila* Dot/Icm type IV secretion system (T4SS) (Das and Chaudhuri, 2003). Since then, similar clusters encompassing 15–25 ORFs have been identified by *in silico* methods in approximately 100 bacterial species (~ ¼ of all bacterial genomes sequenced to date), some encoding more than one system (Bingle et al., 2008; Shrivastava and Mande, 2008; Boyer et al., 2009). Subsequently, the importance of T6SSs for virulence has been established for pathogens like *V. cholerae*, *B. mallei*, *S. typhimurium*, *Edwardsiella tarda*, *Aeromonas hydrophila*, enteroaggregative *E. coli*, and *P. aeruginosa* (Folkesson et al., 2002; Dudley et al., 2006; Mougous et al., 2006; Pukatzki et al., 2006; Schell et al., 2007; Zheng and Leung, 2007; Suarez et al., 2008). Importantly, T6SSs are not restricted to pathogens, since they are also found in symbiotic bacteria as well as bacteria with no known association with eukaryotes, the latter suggesting that they could be used by bacteria to adapt to environmental conditions and/or acquire food nutrients (Bingle et al., 2008; Shrivastava and Mande, 2008; Boyer et al., 2009).

There is large heterogeneity among T6SS gene clusters, which are suggested to form four to five major phylogenetic groups (Bingle et al., 2008; Boyer et al., 2009). However, the phylogenetic analysis by Bingle et al. (2008), based exclusively on IglA (DUF770) and IglB (DUF877) sequences, does not include the FPI in any of these groups, instead it is suggested to form an outgroup, indicating that it is unique from all other T6SSs. This is further accentuated by the fact that this is the only bioinformatic approach that actually identifies the FPI as a putative T6SS cluster. The current understanding of T6SSs reveals that they are related to both bacterial T4SSs and the cell-puncturing devices utilized by bacteriophages for DNA delivery (below).

CORE COMPONENTS AND SUBSTRATES OF T6SSs

T6SSs are, like T4SSs, complex multi-subunit machineries that can deliver macromolecules from the bacterial cytoplasm and into a eukaryotic host cell, traversing both bacterial and eukaryotic membranes. Such machineries include structural components of the secreton, secreted effector proteins, energizers of the secretion process as well as regulators that control the expression of these components. Most gene products of T6SS gene clusters are likely to be structural components, since when mutated, T6S is abolished. For example, in *E. tarda* 13 out of 16 genes are required for functional secretion, however their specific roles are largely unknown (Dudley et al., 2006; Schell et al., 2007; Zheng and Leung, 2007; Suarez et al., 2008; Wu et al., 2008; Pukatzki et al., 2009).

Due to the heterogeneity between the T6SS gene clusters, and the fact that empiric evidence exist from only a limited number of bacteria, it is not a trivial task to define the core T6SS components. However, most systems encode homologs to *V. cholerae* IcmF, DotU, ClpV, VipA, VipB, VgrG, and Hcp proteins (Boyer et al., 2009).

While contributing to stabilization of the *L. pneumophila* T4SS, but not being essential for its function (Sexton et al., 2004), IcmF–DotU homologs are absolutely required for functional T6S. These membrane proteins have been shown to interact and are found in all phylogenetic groups of T6SSs, suggesting that they are essential components of the secretion apparatus (Zheng and Leung, 2007; Boyer et al., 2009; Ma et al., 2009). In *Francisella*, the suggested IcmF homolog PdpB has been shown to be essential for functional T6S of IglI (Barker et al., 2009).

Another defining trait is a bicistronic locus encoding homologs of VipA and VipB (denoted IglA and IglB in *Francisella*). These are only found in T6SS clusters and are suggested to form a functional pair, since they have been shown to interact in a number of bacterial species including *F. tularensis* and to depend on each other for stability (de Bruin et al., 2007; Bröms et al., 2009; Aubert et al., 2010). Interestingly, VipA–VipB of *V. cholerae* and the corresponding proteins $BcsL_B$–$BcsK_C$ from *B. cenocepacia* assemble into tubular structures suggested to span the bacterial membranes (Bönemann et al., 2009, 2010; Aubert et al., 2010). Whether this also occurs for *Francisella* IglA and IglB is not known, especially since it lacks a ClpV homolog, with which the *V. cholerae* and *B. cenocepacia* homologs have been shown to interact. ClpV is a ClpB-like AAA + family adenosine triphosphatase (ATPase) which is required for secretion of T6SS substrates. It forms a hexameric complex at the inner membrane, and requires an intact ATPase domain to support secretion suggesting that it is the main energizer of T6S (Schlieker et al., 2005; Mougous et al., 2006; Zheng and Leung, 2007; Bönemann et al., 2009). Intriguingly, ClpV is required for assembly of $BcsL_B$–$BcsK_C$ tubules and for disassembly of VipA–VipB tubules. The latter is hypothesized to enable transport of VipA–VipB across

the inner membrane before reassembling into the tubular structure. Further, the VipA–VipB tubule structurally resembles the T4 bacteriophage tail sheath. This sheath forms an outer layer to the rigid internal tail tube that, when the sheath is contracted, penetrates the outer bacterial membrane and intermembrane peptidoglycan (Kostyuchenko et al., 2005; Leiman et al., 2009). It is conceivable that the mechanism for T6S occurs in a similar manner, since also other T6SS components bear resemblance to phage proteins (below).

The two most well studied T6SS components are VgrG (valine–glycine repeat protein G) and Hcp (hemolysin co-regulated protein). Principally, a functional T6SS is defined by its ability to export these proteins into the extracellular milieu and they represent a class of secreted substrates that also are structural components of the secretion machinery. Hcp, which is the major secreted protein of T6SSs, forms hexameric donut-shaped rings that stack on top of each other to form tubules. These have large inner diameters, forming a conduit for delivery of macromolecules, and show great resemblance to phage tail tube structures (Mougous et al., 2006; Ballister et al., 2008; Leiman et al., 2009; Pell et al., 2009). VgrG contains two domains that structurally resemble the bacteriophage T4 gp27 and gp5 proteins (**Figure 5**). These assemble into $(gp27)_3$–$(gp5)_3$ needle complexes where the gp5 trimer forms the needle that pierces the bacterial membrane. Presumably, a VgrG trimer forms a similar structure with the ability to puncture both bacterial and eukaryotic membranes (Dudley et al., 2006; Mougous et al., 2006; Pukatzki et al., 2006, 2007, 2009; Schell et al., 2007; Zheng and Leung, 2007; Suarez et al., 2008; Wu et al., 2008; Chow and Mazmanian, 2010).

Apart from the needle complex domains, some evolved VgrGs have C-terminal functional extensions that upon delivery into the host cell can interfere with cellular functions, e.g., the actin cross-linking domain of *V. cholerae* VgrG1 (Pukatzki et al., 2007). Besides Hcp and VgrG, there is a small number of T6SS exported substrates that are not themselves required for functional secretion and hence are more similar to the "classical" effectors of T3SSs and T4SSs, e.g., EvpP of *E. tarda* (Zheng and Leung, 2007; Pukatzki et al., 2009).

A HYPOTHETICAL MODEL FOR TYPE VI SECRETION

Based on the current knowledge of T6S, and guided by the structural resemblance to phage tail-spike organelles, models of how the system functions have been proposed (Filloux, 2009; Pukatzki et al., 2009; Bönemann et al., 2010). A set of core components are suggested to form a pore complex in the bacterial plasma membrane, through which Hcp and VgrG are delivered to the periplasm. Here, Hcp assembles into a tubular structure, capped by the VgrG trimer and sheathed by the VipA–VipB tubule. Upon elongation of the Hcp tubule and contraction of the VipA–VipB sheath, VgrG pushes through the bacterial membrane. Depending on the localization of the bacterium, the VgrG tip may also puncture either the outer or the vesicular membrane of the host cell, allowing the delivery of the VgrG effector domain in the target cell cytosol. It is not known whether the active domain remains attached to VgrG or if it is cleaved off before it reaches its final destination. Further, the VgrG tip is too narrow to allow secretion and hence VgrG detaches from the Hcp tubule to allow Hcp and other components with effector functions to be delivered into the host cell cytosol. The hexameric ATPase ClpV has been suggested to localize to the membrane pore complex where it energizes the T6S process, controlling disassembly/assembly and export of T6S components.

EVIDENCE OF A *FRANCISELLA* PATHOGENICITY ISLAND ENCODED SECRETION SYSTEM

As discussed above, many of the components of the FPI are required for pathogenicity-related phenotypes such as phagosome escape, intracellular replication, cytopathogenicity and virulence in animal models (summarized in **Table 2**). Although information concerning the specific roles these proteins play in *Francisella* pathogenicity is scarce, they are likely to form part of a T6SS or a T6SS-like system (de Bruin et al., 2007; Bingle et al., 2008; Ludu et al., 2008; Barker et al., 2009).

IN SILICO ANALYSIS OF THE PATHOGENICITY ISLAND GENE PRODUCTS

A bioinformatic analysis of the FPI gene products was employed to predict the presence of secretion signals and transmembrane regions, protein localization, homology to known proteins and functional domains. However, this analysis (summarized in **Table 3**) only gives so much away. Most FPI components reveal no homology to any known proteins, display no functional domains and the predicted localization is primarily "unknown." Nevertheless, five of them belong to different COGs (clusters of orthologous groups) (Tatusov et al., 2000; **Table 3**). COG2377, represented by AnmK, includes members of the anhydro-*N*-acetylmuramic acid

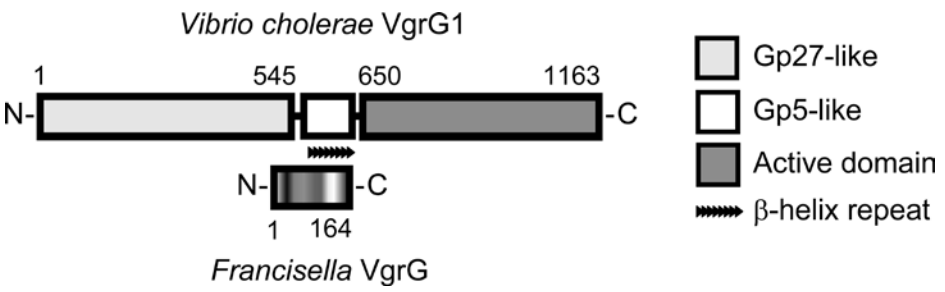

FIGURE 5 | A comparison of *V. cholerae* VgrG1 and *Francisella* VgrG. VgrG1 schematics are as presented by Pukatzki et al. (2007). The gp27- and gp5-like domains are homologous to bacteriophage T4 tail-spike complexes utilized to pierce bacterial membranes. VgrG1 is an evolved VgrG, with a C-terminal extension encoding an actin cross-linking domain. The significantly smaller VgrG of *Francisella* lacks a C-terminal active domain and aligns to the central parts of VgrG1, showing homology to the predicted cell-puncturing parts of this protein.

kinase family, which contribute to the fitness of pathogenic and non-pathogenic bacteria alike but is unrelated to T6S. However, the other four COGs do indeed define proteins found in T6SS clusters, i.e., the canonical functional pairs IglA–IglB (COG3516, COG3517) and PdpB/IcmF–DotU (COG3523, COG3455) (**Figure 1**; **Table 3**). Moreover, both PdpB/IcmF and DotU display predicted transmembrane domains, which is logical if they, like the homologous protein pair of the *Legionella* T4SS, localize to the inner membrane. While the predicted localization for DotU is "unknown," PdpB is predicted to localize to the outer membrane. However, fractionation experiments have revealed that PdpB is found exclusively in the bacterial inner membrane, a localization that better agrees with its homology to IcmF (Bröms et al., unpublished; Ludu et al., 2008). Together with the requirement of PdpB/IcmF and DotU for *Francisella* virulence (**Table 2**), this suggests that the functions of these core components, whatever they may be, are likely to be conserved in *Francisella*.

Two FPI members, PdpE and IglE, are predicted to possess Sec secretion signals (**Table 3**). Both localize to the bacterial outer membrane (Bröms et al., unpublished; Rodriguez, 2010) and in the case of IglE, this localization depends on the presence of a lipid attachment site suggesting that IglE is a lipoprotein (Rodriguez, 2010).

Protein alignment studies suggest that the FPI encodes a VgrG homolog, which is significantly smaller than any known VgrG. A comparison to VgrG1 from *V. cholerae* reveals that *Francisella* VgrG aligns to the central part of this protein, corresponding to the most C-terminal part of protein gp27 and to the greater part of the membrane puncturing gp5 protein of bacteriophage T4 (**Figure 5**). Needless to say, the diminutive FPI VgrG has no C-terminal active domain. However, it cannot be ruled out that it still possesses an effector function, since VgrG export into the host cell cytosol was recently demonstrated (Pukatzki et al., 2007; Ludu et al., 2008; Barker et al., 2009).

It has also been suggested that PdpE encodes an Hcp-like protein (Barker et al., 2009), but this homology is faint and there has been no mechanistic evidence of PdpE executing similar functions as Hcp. In addition, Hcp-like proteins are often encoded by a monocistronic operon separated from the T6SS gene cluster. In the same study, IglF was proposed to be equivalent to ClpV, but it lacks the key ATPase domain of ClpV proteins. Overall, there is some homology between FPI members and T6SS gene clusters, but mechanistic data supporting the notion that the FPI truly encodes a T6SS is scarce.

IglA–IglB – FORMATION OF A TRANSMEMBRANE TUBULAR STRUCTURE?

The FPI components with the greatest homology to T6SS proteins are IglA and IglB, which have been shown to interact and also depend on each other for protein stability (de Bruin et al., 2007; Bröms et al., 2009). This interaction is vital for virulence and relies on a conserved α-helix within IglA (Bröms et al., 2009). Furthermore, interactions between homologous components of pathogens like *V. cholerae, P. aeruginosa*, *Y. pseudotuberculosis*, and uropathogenic *E. coli*, also depend on the same α-helical domain suggesting that the mechanism of interaction is conserved (Bröms et al., 2009). Hence, it is conceivable that IglA–IglB, similar to *V. cholerae* VipA–VipB and *B. cenocepacia* $BcsL_B$–$BcsK_C$, form a tubular transmembrane structure that encases the internal components of a secretion conduit (Bönemann et al., 2009; Bröms et al., 2009; Aubert et al., 2010). There is, however, no existing evidence of IglA–IglB multimerization beyond dimers, and they primarily localize to the bacterial cytosol (**Table 3**), where they have been suggested to function as a chaperone complex (de Bruin et al., 2007). Nevertheless, in support of the notion that IglA and IglB may form a membrane spanning structure are reports of surface location of these components (Melillo et al., 2006; Ludu et al., 2008).

IglC – A UNIQUE EFFECTOR PROTEIN?

A comparative study of protein expression in *Francisella* grown in liquid culture or in J774 cells revealed significant induction of a 23-kDa protein, later named IglC, during intracellular growth (Golovliov et al., 1997; Gray et al., 2002). IglC has no homology to any characterized bacterial proteins (**Table 3**), suggesting that it may be a novel virulence factor. Furthermore, it possesses no recognizable functional motifs but adopts a unique beta-sandwich structure, with weak structural similarity to gp27, a cell-puncturing device component of bacteriophage T4 (Sun et al., 2007).

Although predominantly cytoplasmic (Golovliov et al., 1997), a small fraction of IglC localizes to the bacterial surface as determined by a biotinylation assay (Ludu et al., 2008). This led the authors to speculate that IglC could play a role in a secretion channel in the outer membrane of *Francisella*, or alternatively, in forming a pore in the host cell membrane. The biological relevance of this observation is, however, not easily assessed since IglC surface localization was demonstrated upon overexpression of PdpD (Ludu et al., 2008; **Table 3**). Nonetheless, IglC-export did not occur in *F. novicida* strains mutated for the T6SS core components *pdpB*, *dotU*, *iglA,* or *iglB*, suggesting that it was T6SS dependent. This was in contrast to PdpD-mediated export of IglA and IglB, which occurred also in the absence of *pdpB* or *dotU* (Ludu et al., 2008; **Table 3**). Still, if IglC depends on the outer membrane protein PdpD for export, and this export is an important feature of T6S, it is difficult to comprehend that a *F. novicida pdpD* mutant is able to grow as efficiently as the wild-type strain in all of 11 eukaryotic cell lines tested (Ludu et al., 2008).

SECRETION OF VgrG AND IglI IN MACROPHAGES

The first evidence of translocation of FPI proteins was provided in a recent study by Barker et al. (2009) who demonstrated delivery of *F. novicida* VgrG and IglI, the latter lacking homology to known bacterial proteins, into the cytosol of infected J774 macrophages. VgrG and IglI export was demonstrated by several means: translocation of C-terminal CyaA fusions was observed based on the accumulation of cAMP in the host cell cytosol, and N-terminally FLAG-tagged proteins were visualized using immunofluorescence microscopy. Moreover, the latter proteins could also be detected in the culture supernatants of bacteria grown in broth (Barker et al., 2009).

Intriguingly, in all assays, VgrG secretion occurs in an FPI-independent manner, as seen when expressed in a ΔFPI *F. novicida* strain. In contrast, export of IglI within macrophages depends on the FPI and does not occur in Δ*vgrG*, Δ*icmF,* or ΔFPI backgrounds. However, IglI–FLAG is efficiently secreted to the extracellular medium of broth-grown Δ*vgrG* and ΔFPI mutants. Since neither VgrG nor IglI is secreted when expressed *in trans* in *E. coli* DH5α, this

suggests that they can exit via an FPI-independent but *Francisella*-specific export mechanism during growth in liquid culture (Barker et al., 2009). It would be interesting to investigate if the rudimentary gene cluster (FTN_0040–FTN_0052), which share homology with core components of the FPI (as described above), contributes to this export. Immunofluorescence staining of VgrG and IglI during infection shows that VgrG, when expressed in a Δ*vgrG* background, is released into the host cell cytosol forming a plume extending from the bacterium (Barker et al., 2009). In contrast, when secreted from an ΔFPI mutant, VgrG is localized more closely to the bacterium. Similarly, staining for IglI–FLAG in Δ*iglI*, Δ*vgrG*, or ΔFPI backgrounds also reveals a close association between the protein and the bacteria. These data could be interpreted to suggest that VgrG is truly secreted while IglI is primarily exported to the bacterial surface. Combined with the data from Ludu et al. (2008) and Melillo et al. (2006) this may imply that the FPI-encoded secretion system exports proteins to form a surface located complex including IglA, IglB, IglC, IglI, and VgrG (Barker et al., 2009).

DOES THE FPI ENCODE A TRUE T6SS?

As discussed above, there is increasing evidence that *Francisella* does encode a T6SS-like system that may export FPI proteins such as IglI but this system truly appears to be unique from all other systems described so far. First of all, it is evolutionary distinct (Bingle et al., 2008), and only 3 out of the 17 FPI proteins share significant homology to T6SS core components, i.e., IglA, IglB, and DotU (Nano and Schmerk, 2007; Boyer et al., 2009). So far, no convincing homolog of ClpV, the AAA + ATPase and putative energizer of T6SSs (Mougous et al., 2006), has been identified in the genome, although Barker et al suggested that FTT1348 (IglF) may be a candidate (Barker et al., 2009). Moreover, while a protein with a short IcmF-related portion (PdpB) and a potential VgrG homolog have been recognized in the FPI (Nano and Schmerk, 2007; Barker et al., 2009; Boyer et al., 2009), the Walker A box commonly present in IcmF homologs is missing from *F. tularensis* PdpB (Zheng and Leung, 2007; Barker et al., 2009) and the *F. tularensis* VgrG homolog is much smaller than VgrG homologs from other species, and it also lacks the C-terminal extensions found in, e.g., the *V. cholerae* VgrG-1 and VgrG-3 proteins (Pukatzki et al., 2007; Barker et al., 2009). Altogether, these differences would suggest that the *Francisella* FPI system is clearly different compared to other T6SS. Further supporting this notion is the observation that *F. tularensis* VgrG, in contrast to all VgrG proteins reported so far, is exported into the extracellular milieu in an FPI-independent fashion (Barker et al., 2009), suggesting a novel mechanism of export. While IglI-translocation into host cells is dependent on FPI core components, it can also be secreted via an FPI-independent mechanism during *in vitro* growth.

Together, these observations raise the question whether the FPI actually encodes a true T6SS or just a system implicated in virulence that shares some components with T6SSs and T4SSs. Much more work is needed to distinguish between these possibilities.

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Regulation of *Francisella tularensis* virulence

Shipan Dai[1†], Nrusingh P. Mohapatra[1†], Larry S. Schlesinger[1,2,3] and John S. Gunn[1,2,3]*

[1] Center for Microbial Interface Biology, The Ohio State University, Columbus, OH, USA
[2] Department of Molecular Virology, Immunology and Medical Genetics, The Ohio State University, Columbus, OH, USA
[3] Division of Infectious Diseases, Department of Internal Medicine, The Ohio State University, Columbus, OH, USA

Edited by:
Anders Sjostedt, Umeå University, Sweden

Reviewed by:
Simon Daefler, Mount Sinai School of Medicine, USA
Jörgen Johansson, Umeå University, Sweden

***Correspondence:**
John S. Gunn, The Ohio State University, Biomedical Research Tower, Room 1006, 460 West 12th Avenue, Columbus, OH 43210, USA.
e-mail: gunn.43@osu.edu

†Shipan Dai and Nrusingh P. Mohapatra have contributed equally to this work.

Francisella tularensis is one of the most virulent bacteria known and a Centers for Disease Control and Prevention Category A select agent. It is able to infect a variety of animals and insects and can persist in the environment, thus *Francisella* spp. must be able to survive in diverse environmental niches. However, *F. tularensis* has a surprising dearth of sensory and regulatory factors. Recent advancements in the field have identified new functions of encoded transcription factors and greatly expanded our understanding of virulence gene regulation. Here we review the current knowledge of environmental adaptation by *F. tularensis*, its transcriptional regulators and their relationship to animal virulence.

Keywords: *Francisella*, transcriptional regulators, *Francisella* pathogenicity island, two-component regulatory systems

INTRODUCTION

Francisella tularensis is a Gram-negative non-motile intracellular pathogen and the causative agent of the zoonotic human disease tularemia. Tularemia, also known as rabbit fever, is usually transmitted to humans by arthropod bites, oral consumption of contaminated food or water, or handling of infected animal carcasses (Evans et al., 1985; Thomas and Schaffner, 2010).

Francisella tularensis is divided into several subspecies (Staples et al., 2006; Kugeler et al., 2009). These subspecies are associated with important differences in geographic distribution. *F. tularensis* subsp. *tularensis* (Type A) is found exclusively in North America. It is highly virulent, with less than 10 bacteria causing disease in humans via the respiratory route, and if untreated, demonstrates a high mortality rate of 30–60% (Dienst, 1963). *F. tularensis* subsp. *holarctica* (Type B) is less virulent in humans and is predominant in Europe and Japan, but also found in North America. *F. tularensis* subsp. *mediasiatica* is found primarily in Asia and Russia, and is rarely associated with disease in humans. *F. novicida* is normally considered as the fourth subspecies of *F. tularensis*; however, recent genome-wide polymorphism analysis suggests that it is an independent species highly virulent in mice but not in humans (Johansson et al., 2010). An attenuated strain of *F. tularensis* subsp. *holarctica*, the live vaccine strain (LVS), was developed and is used as a live-attenuated vaccine in the former Soviet Union and in some US laboratory workers (Tigertt, 1962; Burke, 1977). Because *F. tularensis* is able to infect through the respiratory route and cause disease with a very small dose, can be easily disseminated, results in a high mortality rate, and has the potential to cause panic among the public, *F. tularensis* is given the highest priority classification by the Centers for Disease Control and Prevention as a Category A select agent and is a potential bioweapon.

Completed genome sequencing for different subspecies has revealed that despite high nucleotide identity, there are many DNA rearrangements, gene acquisitions, and gene losses between and even within different subspecies (Titball and Petrosino, 2007; Champion et al., 2009; Nalbantoglu et al., 2010; Sjodin et al., 2010). These differences are thought to reflect the environmental adaptation of these subspecies and are associated with their differences in virulence. Most of our current understanding about the genetics of *F. tularensis* pathogenesis have been acquired with *F. novicida*, as this subspecies can be genetically manipulated with relative ease and causes a disease in mice similar to that caused by the human virulent *F. tularensis* subsp. *tularensis*. However, due to the genetic, phenotypic, and host range variations between the species, care should be taken in extrapolating data from one subspecies to another. With the recently developed genetic tools for the human virulent subspecies *tularensis* (LoVullo et al., 2006, 2009), it is now possible and necessary to carry out studies in these strains, which is required gain a full understanding the molecular basis of *Francisella* pathogenesis. Nevertheless, the studies with *F. novicida* and LVS have provided and will continue to provide valuable information on the molecular pathogenesis of tularemia, and the results have been generally applied to *F. tularensis*.

Francisella tularensis exists naturally in a variety of environments. It can survive in water, wet soil, and animal carcasses for several weeks. *F. tularensis* has also been found in a broad range of hosts, including mammals, insects, arthropods, and fresh water protozoans (Ellis et al., 2002). Not only does *F. tularensis* infect different hosts, as an intracellular pathogen, *F. tularensis* also goes through various microenvironments within host cells, including the macrophage phagosome and the host cell cytosol after phagosomal escape (Santic et al., 2010). Essential to the success of *F. tularensis*

as an intracellular pathogen is its ability to adapt to a wide variety of environments and host cell types. This is achieved via timely activation/repression of dedicated patterns of gene expression. Most of the virulence genes of *F. tularensis* identified so far localize to the *Francisella* pathogenicity island (FPI). This is a cluster of 17 genes that is critical for the intracellular survival and virulence of *F. tularensis* (Nano and Schmerk, 2007). FPI genes were the first identified *Francisella* virulence factors and are affected by essentially all environmental cues and transcriptional virulence regulators identified thus far, demonstrating their importance in *F. tularensis* virulence.

Environmental changes are often sensed and relayed into bacteria through two-component regulatory systems (TCS). These systems are conserved and ubiquitous in bacteria, typically composed of a histidine sensor kinase, regulated by environmental stimuli, and a response regulator that activates downstream responses (Stock et al., 2000). In contrast to the variety of environments *F. tularensis* naturally encounters and opposite to most Gram-negative pathogens, which have numerous TCS, *F. novicida* has very few TCS encoded in the genome, and other subspecies of *F. tularensis*, such as subsp. *tularensis* lack any classically arranged (adjacent and co-transcribed) TCS. Orphaned TCS members KdpD, QseC, and PmrA are the only TCS factors discovered so far that affect virulence/virulence factor regulation (Mohapatra et al., 2007; Bell et al., 2010). In addition, *F. tularensis*, unlike many other bacteria, encodes only one alternative sigma factor (Fuller et al., 2009). This makes *F. tularensis* a unique pathogen with relatively few easily identifiable regulatory factors to survive in diverse environments. It is possible that some regulators exist that do not show homology to know regulators in the database or that alternative means of regulation (e.g., sRNA, post-transcriptional) may predominate in the *Francisella*.

ENVIRONMENTAL STIMULI

To survive in different environments, *F. tularensis* has to be able to sense and respond to signals from its surroundings to precisely regulate virulence gene expression. In fact, the protein profiles of *F. tularensis* (LVS) grown in broth versus inside macrophages revealed the altered expression of several proteins (Golovliov et al., 1997). In addition, global transcriptional profiling of *F. tularensis* Type A strain Schu S4 within infected macrophages confirmed that the expression of 658 genes was significantly changed, among which 298 were up-regulated and 360 were down-regulated, depending on the stage of the infection (Wehrly et al., 2009). These observations support the notion that *F. tularensis* is able to sense and respond to changing environments during the course of infection. Although the molecular mechanisms that underlie gene regulation are not fully understood, studies thus far have suggested that environmental stimuli including temperature, limited iron source, oxidative stress, and host intramacrophage components, lead to altered expression profiles.

TEMPERATURE

A number of pathogenic bacteria that encounter a temperature shift during their life cycle have the ability to respond to this environmental cue with enhanced virulence. *Shigella flexneri* increases *virB* expression in response to elevated temperature (Tobe et al., 1991, 1995). *Borrelia burgdorferi* and *Yersinia pestis* have also adapted ways to change the expression profile of host-specific virulence genes according to an alteration in temperature (Konkel and Tilly, 2000). As a pathogen with different hosts, *F. tularensis* will go through a temperature shift upon entering a warm-blooded animal. When growing at 25 versus 37°C, *F. novicida* changes its outer surface by modifying the lipid A of lipopolysaccharide (Shaffer et al., 2007). Also, a recent transcriptomic study compared the global expression profiles of *F. tularensis* LVS cultivated in defined media at 26°C (environmental temperature) versus 37°C (mammalian body temperature; Horzempa et al., 2008). Approximately 11% of the genes in the entire LVS genome had significant changes in expression. Notably, around 40% of the genes that were up-regulated were previously identified or predicted to be important for intracellular growth and/or virulence (Horzempa et al., 2008). Another temperature change proteomic study with LVS and the Type A strain Schu S4 confirmed a significant increase in the level of FPI-encoded IglC, IglD, and PdpC, when the temperature shifted from 25 to 37°C (Lenco et al., 2009).

The regulatory mechanisms that underlie these temperature-dependent changes are not yet clear, but a few studies have suggested the involvement of an alternative sigma factor and its induction of heat-shock proteins, which is a mechanism used by many other bacteria (Ericsson et al., 1994; Meibom et al., 2008; Grall et al., 2009). However, unlike most other bacteria that have multiple sigma factors, *F. tularensis* encodes only one alternative sigma factor, RpoH, which is a sigma factor σ^{32} (Grall et al., 2009). Transcription profiling under heat-stress conditions in *F. tularensis* LVS showed that a number of heat-shock proteins and proteins involved in virulence were up-regulated. However, among all the genes that were regulated upon heat-stress, only 13 of them, which did not include FPI genes, had a putative σ^{32} binding site in their promoter region (Grall et al., 2009). This suggested that σ^{32} might not be acting directly on FPI genes.

IRON

Iron acquisition is a critical requirement for the survival of bacteria as it is involved in many metabolic processes. As one of the most common elements in the Earth's crust, iron is easily accessible for bacteria in the environment. However, for intracellular pathogens, their ability to acquire iron is critical because of the limited nature of freely available iron in mammalian host cells. Iron availability has a great impact on both virulence of bacteria pathogens and the host immune response (Wang and Cherayil, 2009; Skaar, 2010).

Pathogens respond to iron-restriction conditions by up-regulation of not only iron acquisition machinery but also virulence determinants (Ratledge and Dover, 2000; Skaar, 2010). A general mechanism for iron sensing is controlled by the ferric uptake regulator (Fur). Fur forms a complex with ferrous iron and represses gene expression by binding to the Fur box upstream of iron-regulated genes, which is then derepressed under iron-limiting conditions. In *F. tularensis,* an iron acquisition operon (*fsl* for *F*t siderophore *l*ocus or *fig* for *F*t *i*ron *g*enes) involved in siderophore production is located downstream of *fur,* and is induced under iron-restriction conditions in a Fur-dependent

manner (Deng et al., 2006; Sullivan et al., 2006). A Fur box is also found upstream of *iglC* and *pdpB*, two FPI genes (Deng et al., 2006). Microarray analysis found that approximately 80 genes, including FPI genes, are differentially expressed in iron-starved *F. tularensis* LVS (Deng et al., 2006). This was confirmed by a proteomics analysis in *F. tularensis* LVS, which showed that the levels of a significant number of proteins change under iron-limiting conditions, which again included those in the FPI (Lenco et al., 2007). Although it did not appear in these two previously mentioned studies, the FPI-encoded PdpA is also up-regulated under iron-limiting conditions (Schmerk et al., 2009). Moreover, gallium, a transition metal that competes with iron for uptake and/or utilization, inhibits *F. tularensis* LVS and *F. novicida* growth in broth and in macrophages, and inhibits *F. novicida* virulence in a mouse model of pneumonic tularemia (Olakanmi et al., 2010), providing further evidence for an important role of iron in regulating *F. tularensis* virulence factors.

OXIDATIVE STRESS

One of the essential host cell innate immune defenses against invading pathogens is the generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS; Fang, 2004). Many bacterial pathogens are able to sense the oxidative stress inside host cells and have ways to combat it. *F. tularensis* is in this group of pathogens as it is able to inactivate the phagocyte NADPH oxidase through a mechanism likely involving dephosphorylation of host kinases and *phox* components by bacterial enzymes/bacterial-mediated host signaling cascades (McCaffrey and Allen, 2006; Schulert et al., 2009; McCaffrey et al., 2010; Mohapatra et al., 2010). A screen for *F. tularensis* LVS proteins responding to heat and hydrogen peroxide identified chaperones DnaK, GroEL, and GroES (Ericsson et al., 1994). In addition to these proteins, Lenco et al. (2005) detected approximately 20 proteins that were up-regulated upon exposure to oxidative stress in *F. tularensis* LVS. In searching for the molecular mechanisms that underlie the response to oxidative stress, a proteomics assay was carried out comparing the protein profiles of wild-type *F. novicida* and an *mglA* (macrophage growth locus) mutant. In addition to virulence factors, homologs of oxidative, and general stress response proteins were identified in this study, including the previously identified chaperons, suggesting a role for MglA, a regulator of *F. tularensis* virulence, in the oxidative response (Guina et al., 2007).

OTHER ENVIRONMENTAL CUES

In addition to reactive oxygen and nitrogen species, other components of the host environment may also be signals sensed by *F. tularensis*. A recent study identified spermine, a polyamine produced only in eukaryotic cells, as an intramacrophage component sensed by *F. tularensis* (Carlson et al., 2009). Infection with *F. tularensis* LVS or Schu S4 cultured in the presence or absence of spermine induced different patterns of cytokine production in macrophages. A genome-wide microarray assay indicated that the presence of spermine affected a significant portion of the *F. tularensis* transcriptome. This is in part mediated by the induction of the *Francisella* insertion sequence (IS) elements IS*Ftu1* and IS*Ftu2*, which is sufficient to induce the expression of downstream genes in response to spermine (Carlson et al., 2009). This observation suggested a model of gene regulation in which intramacrophage compounds elicit substantial changes in *Francisella* gene expression, and result in low cytokine production and immune evasion.

As *F. tularensis* resides temporally in phagosomal vacuoles after entering the host cell, the decrease in pH inside the phagosome may play a role in the induction of virulence factors. In support of this, the expression of RipA, a cytoplasmic membrane protein involves in *F. tularensis* intracellular replication and host immune evasion, is regulated by pH with increased expression at neutral rather than acidic environment (Fuller et al., 2008, 2009; Huang et al., 2010). However, the early acidification of the phagosome is not singularly involved in FPI gene induction as virulence protein expression is increased even upon inhibition of phagosome acidification (Chong et al., 2008).

Another environmental change that might play an important role in the induction of *F. tularensis* virulence is stress/starvation. The host cell phagosome/cytosol is known to be a nutrient poor and stress-inducing environment for bacteria. Several of the *F. tularensis* virulence regulators have been implicated in the stringent response, i.e., a microbial reaction to starvation. For example, SspA is a stringent starvation transcriptional regulator that is highly related to and physically interacts with MglA in *F. tularensis* LVS and *F. novicida* (Charity et al., 2007). In addition, guanosine pentaphosphate (ppGpp), an alarmone that is the global regulator of gene expression during the stringent response, is also involved in *F. tularensis* virulence gene expression by promoting the interaction of FevR/PigR with the MglA–SspA–RNA polymerase (RNAP) complex (Charity et al., 2009). Moreover, RelA, the enzyme involved in ppGpp production, is also involved in *F. tularensis* LVS virulence and intracellular survival (Dean et al., 2009). These findings suggest that starvation plays a critical role in the induction of *F. tularensis* LVS virulence.

TRANSCRIPTIONAL REGULATORS

Bacteria typically utilize specialized transcription factors that bind non-specifically or specifically to DNA recognition sequences. The location and nucleotide composition of transcription factor binding sites in part determines whether a transcription factor represses or activates the expression of a certain gene. An interesting feature of *Francisella* is that very few transcription factors have been identified by experimentation or homology searches (**Table 1**), but these few factors likely control the virulence cascades of this pathogen. While translational or post-translational regulation may play a role, the majority of evidence points to regulation at the level of transcription/mRNA transcripts. The FPI is the primary site of virulence-related genes; thus the remainder of this review will focus on transcription factors or regulators that have been identified to play a role in FPI gene regulation.

RNA POLYMERASE

Bacteria react to various environmental conditions by employing different modes of regulation. *F. tularensis* can replicate in a wide range of hosts and cell types, which suggests an extensive ability to adapt to different niches through variable gene

Table 1 | Transcription factors of *Francisella tularensis* identified by protein relatedness to known transcription factors/transcription factor motifs.

			Conservation in other *Francisella*					Experimentally studied in:		
FTT# (Type A; Schu S4)	**Gene**	**Description**	**W96-3418 (Type A)**	**FSC198 (Type A)**	**OSU18 (Type B)**	**LVS (Type B)**	**U112 (*F. novicida*)**	**Schu S4 (Type A)**	**LVS (Type B)**	**U112 (*F. novicida*)**
30	*fur*	Fe transcriptional regulator	+	+	+	+	+	Ramakrishnan et al. (2008)	Deng et al. (2006)	Kiss et al. (2008)
94	*qseC*	Sensor histidine kinase	+	+	+	+	+	Rasko et al. (2008)		Weiss et al. (2007), Durham-Colleran et al. (2010)
112		Homolog of Bvg acc. factor	+	+	+	+	+			
383	*fevR/ pigR*	Transcriptional regulator	+	+	+	+	+	Wehrly et al. (2009)	Charity et al. (2007, 2009)	Brotcke and Monack (2008)
458	*sspA*	Stringent starvation protein A regulator	+	+	+	+	+		Charity et al. (2007, 2009)	
492	*lysR*	Homolog of LysR	+	+	+	+	+			
556	*oxyR*	Oxidative stress regulator	+	+	+	+	+			Moule et al. (2010)
748		Homolog of TtgV	+	+	+	+	+	Mortensen et al. (2010)	Mortensen et al. (2010)	Weiss et al. (2007), Mortensen et al. (2010)
864		LysR family regulator	+	+	–	–	+			
932		ROK family regulator	–	+	–	+	+			
970		Transcriptional repressor	+	+	+	+	+			
977		MarR-like regulator	+	–	–	–	–			
981		Sensor kinase	+	+	+	+	+			
985		Transcriptional regulator	+	+	+	+	+			
1010		Cro family regulator	–	–	–	–	–			
1035	*rpoD*	Sigma factor	+	+	+	+	+		Grall et al. (2009)	
1076	*hipA*	Homolog of HipA	–	+	+	+	–			
1112	*rpoH*	Sigma factor	+	+	+	+	+		Grall et al. (2009)	
1119		LysR family regulator	+	+	+	+	+			
1172		Cold shock protein	+	+	–	+	–			
1202		LysR family regulator	+	+	–	+	+			

(Continued)

Table 1 | Continued

			Conservation in other *Francisella*					Experimentally studied in:		
FTT# (Type A; Schu S4)	**Gene**	**Description**	**W96-3418 (Type A)**	**FSC198 (Type A)**	**OSU18 (Type B)**	**LVS (Type B)**	**U112 (*F. novicida*)**	**Schu S4 (Type A)**	**LVS (Type B)**	**U112 (*F. novicida*)**
1255		AraC family regulator	+	+	+	+	+			
1267		LysR family regulator	+	+	+	+	+			
1275	*mglA*	Macrophage growth locus regulator	+	+	+	+	+		Bonquist et al. (2008), Charity et al. (2007, 2009)	Baron and Nano (1998), Brotcke et al. (2006), Guina et al. (2007)
1281	*rpoN*	Sigma factor	–	–	–	–	–			
1285		LysR family regulator	+	+	–	+	+			
1392		Homolog of Bvg acc. factor	+	+	+	+	+			
1401		LexA homolog	+	+	+	+	+			
1543		Response regulator	+	+	–	–	+			
1557c	*pmrA*	Response regulator	+	+	+	+	+		Sammons-Jackson et al. (2008)	Bell et al. (2010), Mohapatra et al. (2007)
1594		LysR family regulator	+	+	+	+	–			
1652		LysR family regulator	+	+	+	+	+			
1613		Homolog of BolA	–	–	–	–	–			
1684		Transcriptional regulator	+	+	–	+	+			
1736	*kdpD*	Sensor kinase KdpD	+	+	–	+	+			Bell et al. (2010), Weiss et al. (2007

expression. Transcription is the process of transcribing DNA into RNA and is performed primarily by a complex of proteins that form the RNAP. RNAP binding involves the alpha subunit recognizing the upstream sequences (40 to −70 base pairs) of the gene, as well as the σ factor recognizing the −10 to −35 regions of the promoter. *Francisella* RNAP is unique, as it contains two distinct forms of the alpha subunit. Two separate genes encode these proteins and they are both incorporated into the enzyme (Charity et al., 2007). Bacterial σ factors recognize different promoter elements upstream of genes allowing the cell to respond to various environmental conditions by directing the RNAP to unique sets of genes. However, in contrast to *Escherichia coli* and *Bacillus subtilis* which have six to eight alternative sigma factors that are used under various circumstances, as mentioned previously, *F. tularensis* has only two (the housekeeping σ^{70} and the stress-related σ^{32}).

MglA AND SspA

Baron et al. was the first to identify genes that are required for the replication of *F. novicida* within macrophages, where they described *mglA* and *mglB* (Baron and Nano, 1998). As discussed previously, MglA is highly up-regulated upon infection of macrophages. An *mglA* mutant of *F. novicida* is unable to escape from macrophage phagosomes and is highly attenuated in the mouse model but does not provide protection against homolgous or heterologous *Francisella* subsp. challenge (Lauriano et al., 2004; Santic et al., 2005; Brotcke et al., 2006). MglA has been characterized as a transcription factor and is responsible for regulating ~100 genes including those of the FPI (Lauriano et al., 2004; Brotcke et al., 2006; Guina et al., 2007), while proteomic analysis indicates that the abundance of ~350 proteins is altered in the absence of MglA. In addition to the FPI, other virulence factors such as the metalloprotease PepO are regulated by MglA (Guina

et al., 2007; West et al., 2008). MglA is similar to SspA (Baron and Nano, 1998; Brotcke et al., 2006), a transcription factor in *E. coli* that responds to nutrient limitation and is involved in regulation of genes contributing to the stringent starvation response. SspA of *Francisella* is important for the regulation of virulence genes, and the regulons of MglA and SspA are overlapping, including those genes within the FPI (Charity et al., 2007). MglA and SspA physically interact and both bind *Francisella* RNAP in a heterodimer, which is required for FPI gene activation (Charity et al., 2007).

FevR/PigR

An additional regulator of the FPI that is essential for intramacrophage replication and virulence in the mouse model has been named *Francisella* effector of virulence regulation or FevR (also called PigR; Buchan et al., 2008; Charity et al., 2009). Microarray analysis comparing *fevR*/*pigR* and *mglA* mutant strains of *F. novicida* to their parental strains revealed identical regulons for these two transcription factors (Brotcke and Monack, 2008), suggesting that FevR/PigR works in parallel with MglA and SspA to modulate gene expression. FevR/PigR has poor homology to DNA binding proteins, has not been demonstrated to bind to any specific DNA sequences, and appears to act upstream of MglA, SspA, and RNAP binding (Brotcke and Monack, 2008). Studies have varied on whether FevR/PigR, MglA, and SspA physically interact, but recently Charity et al. (2009) demonstrated that ectopic expression of *fevR*/*pigR* in LVS restored expression of the MglA/SspA-regulated genes to wild-type levels in a *fevR*/*pigR* mutant strain, failed to restore expression of the MglA/SspA-regulated genes in a *mglA* mutant strain, and partially restored expression of the MglA/SspA-regulated genes in a Δ*relA* Δ*spoT* mutant strain, which suggested that PigR/FevR functions together with the MglA–SspA complex downstream of ppGpp (Charity et al., 2009). Detailed studies of ppGpp and RelA/SpoT gene regulation under stress conditions are described in a later part of this review.

MigR/CaiC

Another regulator, macrophage intracellular growth regulator (MigR, also called CaiC), is a large protein with a putative AMP-binding domain and homology with acyl-transferase/ligase proteins. This regulator was identified from a random pool of LVS mutagenized with the Tn5 transposon as one that regulates the *iglABCD* and *fslABCD* operons (Buchan et al., 2009). In *F. tularensis* LVS, the mutation of *migR* demonstrated a defect of growth and intracellular trafficking in macrophages (Buchan et al., 2009). However, mutation of *migR* in the Schu S4 strain retained its regulatory effect but growth inside macrophages was unaffected. MigR positively regulates *fevR*/*pigR*, and controls the expression of the FPI genes, *iglABCD*. The lack of a DNA binding domain in MigR calls into question its role as a transcriptional regulator, instead it seems likely that the regulatory effect of MigR on *fevR*/*pigR* is indirect. MigR activity could affect RelA or SpoT (stringent response regulators) to induce the synthesis of (p)ppGpp, which has been demonstrated to contribute to the expression of *fevR*, and *iglABCD*, and thus is involved in the pathogenesis of *F. tularensis* (Charity et al., 2009).

(p)ppGpp

Under nutrient starvation conditions, certain bacterial species resist environmental stress by the production of hyperphosphorylated guanosine diphosphate and triphosphate analogs, collectively termed (p)ppGpp. During this process, uncharged tRNA molecules bind to the ribosome, which results in ribosome stalling, activation of ribosome-associated RelA, and production of (p)ppGpp, which is subsequently converted to ppGpp. These ppGpp small molecules bind to RNAP to affect expression of a wide range of physiological systems. In some bacteria, inactivation of both *relA* and *spoT* is required to completely abolish (p)ppGpp synthesis; SpoT is a bifunctional enzyme capable of both synthesis and degradation of the signal molecule, thereby preventing uncontrolled accumulation of (p)ppGpp (Jain et al., 2006). It has been proposed that the RelA-dependent response is linked to amino acid starvation and that the SpoT-dependent response is linked to fatty acid metabolism in bacteria (Battesti and Bouveret, 2009). In some pathogens, regulation of (p)ppGpp synthesis is dependent on SpoT, and virulence in *Legionella pneumophila* is critically dependent on the degradation of (p)ppGpp by SpoT (Dalebroux et al., 2009). Both *relA* and *spoT* genes have been annotated in the *F. novicida* U112 (Rohmer et al., 2006) and *F. tularensis* Schu S4 (Larsson et al., 2005; Dean et al., 2009) genome sequences. Inactivation of the *relA* gene in *F. novicida* rendered it defective for (p)ppGpp production, and showed increased biofilm formation and reduced resistance to *in vitro* stress (Dean et al., 2009). The mutant was attenuated in the J774.1 macrophage cell line and in the mouse model, and induced a protective immune response against homologous challenge. The double deletion of *relA* and *spoT* in *F. tularensis* LVS resulted in complete disappearance of (p)ppGpp production and increased the robustness of the *relA* mutant phenotypes. ppGpp promotes interaction of FevR/PigR with the MglA–SspA complex and RNAP by an unknown mechanism (Charity et al., 2009).

PmrA AND KdpD

Bacteria have evolved sensory systems including two-component systems to sense and respond to their immediate surroundings. TCS typically consist of a membrane-bound sensor kinase and a cytoplasmic response regulator whose genes are usually adjacent in a single transcription unit. The communication between the sensor kinase and response regulator involve phosphotransfer at conserved residues.

The *F. tularensis* genome encodes only two such systems, neither of which is chromosomally paired (Mohapatra et al., 2007). One of these unpaired response regulators is homologous to PmrA in *Salmonella enterica,* where it is primarily involved in the induction of genes that mediate LPS modification (Gunn, 2008). Inactivation of this gene in *F. novicida* led to increased bacterial susceptibility to killing by antimicrobial peptides, decreased intramacrophage growth, lack of phagosomal escape in macrophages and attenuation in the mouse model while providing protection against homologous wild-type challenge (Mohapatra et al., 2007). Microarray analysis of this mutant revealed a change in expression of 65 genes when compared to wild-type bacteria grown under the same conditions (Mohapatra et al., 2007). PmrA

is a DNA binding protein that is phosphorylated at Asp51 primarily by the unlinked putative histidine kinase KdpD, forming the first TCS described for *F. tularensis* (Bell et al., 2010). Phosphorylation of PmrA is complicated in *F. novicida* by the presence of a secondary KdpD target, KdpE, and an additional kinase, FTN1453, both of which negatively affect the phosphorylation state of PmrA. However, neither FTN1453 or KdpE is present/functional in the *F. tularensis* Schu4 strain, resulting in increased net phosphorylation of PmrA (Bell et al., 2010).

Coimmunoprecipitation assays suggested that PmrA, MglA, and SspA are a part of the same protein complex and likely physically interact (Bell et al., 2010). The *pdpD* gene is regulated by PmrA, MglA, and SspA; however, *pmrA* is positively autoregulated by PmrA but not affected by MglA or SspA (Bell et al., 2010). Phosphorylation aids PmrA binding to regulated promoters (Bell et al., 2010). A strain expressing PmrA D51A retains some DNA binding, but exhibits reduced expression of the PmrA-regulon, a deficiency in intramacrophage replication and attenuation in the mouse model. Thus, *Francisella* gene regulation includes a TCS consisting of KdpD and PmrA. Once phosphorylated, PmrA binds to regulated gene promoters recruiting free or RNAP-bound MglA and SspA to initiate FPI gene transcription. Interestingly, recent published and unpublished data suggest that the gene regulation by PmrA varies between *Francisella* subsp. and even within different isolates of the same subspecies (Sammons-Jackson et al., 2008). The external signals that are required for KdpD and PmrA two-component system activation remain to be identified but are a focus of current investigation.

hfq AND SMALL RNA

It is increasingly apparent that in most living organisms, small non-coding RNAs (sRNA) are responsible for wide range of changes in gene expression (Gottesman and Storz, 2010). The regulatory mechanism of these sRNAs is based on pairing with complementary sequences within mRNA messages (Papenfort and Vogel, 2010), though in some cases the regulatory RNA directly affects protein function (Yang et al., 1996). The sRNA–mRNA interaction impacts mRNA stability and translational efficiency. Sometimes sRNA activity is dependent upon interaction with a specific protein, which can aid in stabilization of the regulatory RNA or in targeting to a specific mRNA (Thomas and Schaffner, 2010). One such protein, host factor for bacteriophage Q β replication (Hfq), has been identified in many bacterial species, including *F. tularensis*, and often is involved in the regulation of a wide range of genes (Meibom et al., 2009). Not surprisingly, mutation of *hfq* in *F. tularensis* LVS resulted in pleiotropic effects including an increased sensitivity to elevated salt concentrations, detergents, and high temperatures (Meibom et al., 2009). In addition to these *in vitro* phenotypes, the *hfq* mutant was attenuated for growth in several types of macrophages and demonstrated reduced virulence in mice. Microarray and qRT-PCR analysis of the mutant revealed an effect on the expression of 104 genes, including 10 genes within the FPI. Strikingly, Hfq negatively regulates only one of two divergently expressed putative operons in the FPI, in contrast to the other known regulators that appear to affect all FPI-encoded genes. Because of the large size of the Hfq regulon, it is difficult to attribute specific phenotypes of the mutant to specific genes or to assess the impact of reduced FPI gene expression on virulence in this mutant.

A recent study identified several sRNAs in the *F. tularensis* (LVS) genome (Postic et al., 2010) by *in silico* predictions and experimental assays. These non-coding regulatory RNAs, include members of well conserved families of structural and housekeeping RNAs as well as less conserved RNAs that may have novel functions/regulate novel targets in *Francisella*. Thus, the combined work on Hfq and the discovery of these sRNAs suggest a non-protein based means of gene regulation in the *Francisella* that may somewhat explain the paucity of protein regulatory factors.

CONCLUSION

Transcription factors are important regulators of virulence in virtually all known bacterial pathogens. However, virulence gene regulation in *Francisella* spp. is unique with regard to the fact that it has only one alternative sigma factor, no classically arranged TCS, and the involvement of MglA, SspA, PmrA, MigR, Hfq, sRNA, and FevR/PigR in regulatory control of the FPI. The published literature suggests that MglA, SspA, PmrA, MigR, Hfq, and FevR/PigR co-operate with each other to regulate the *Francisella* virulence network. Only one identified two-component system exists in virulent Type A *F. tularensis*, where the sensor kinase KdpD becomes phosphorylated by an unidentified environmental signal and relays that signal to the response regulator PmrA through phosphorylation of D51. PmrA binds to regulated promoters (including those within the FPI) and interacts with the MglA–SspA–RNAP complex to regulate virulence gene transcription (Bell et al., 2010). Microarray data have demonstrated that *fevR/pigR* is transcriptionally regulated by *pmrA*, *mglA*, and *sspA* (**Figure 1**). Thus, several environmental cues are likely integrated to activate *fevR/pigR*, which then plays a key role in FPI-mediated transcription. Additionally, several nutrient starvation and stress response proteins, such as RelA and SpoT (ppGpp), and sRNA species/sRNA carrier proteins are involved in virulence

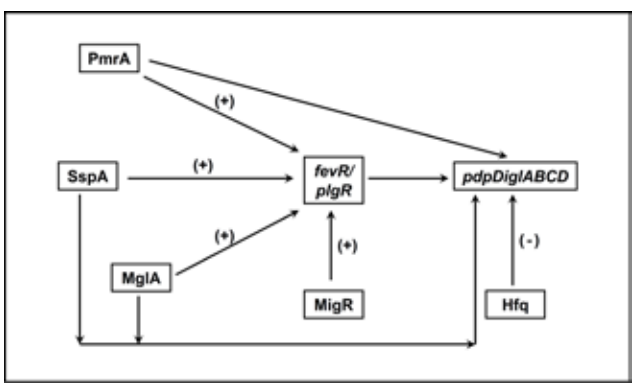

FIGURE 1 | Interactions between transcriptional regulators of *Francisella* that control FPI gene expression. PmrA, SspA, MglA, and MigR all positively regulate expression of *fevR/pigR*. The *fevR/pigR* product then plays a role, in conjunction with all of the other illustrated factors, in FPI gene transcription. PmrA, MglA, and SspA also directly regulate the genes of the FPI (see **Figure 2**). (+), Positive transcriptional regulation. (–), Negative transcriptional regulation.

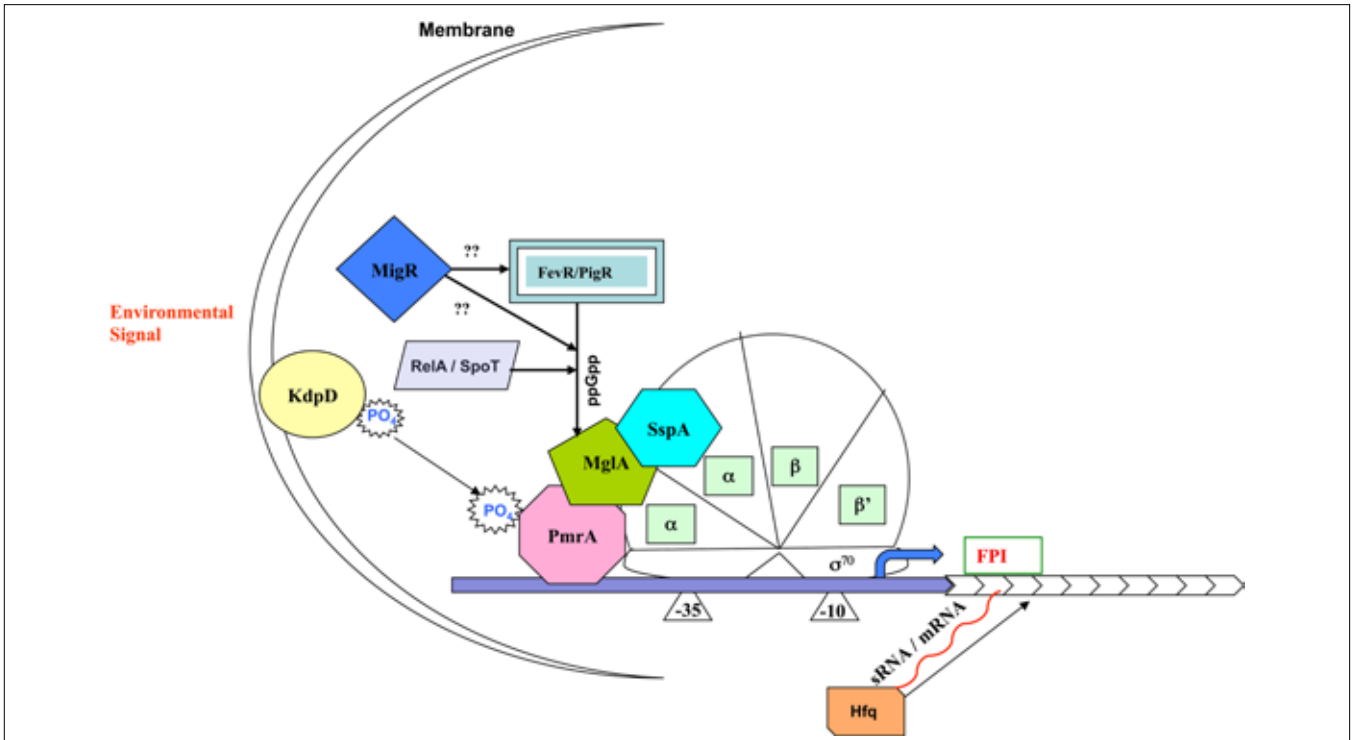

FIGURE 2 | Model for regulation of *Francisella* pathogenicity island gene expression. Unknown environmental signals are sensed by KdpD and relayed to the DNA binding protein PmrA. MglA and SspA heterodimerize and associate with RNA polymerase (RNAP). The regulatory activity of the MglA/SspA/RNAP complex is dependent on association of PmrA to the complex as well as the production of ppGpp, which promotes interaction of FevR/PigR with the MglA/SspA/RNAP complex.

gene regulation in *Francisella* (see **Figure 2** for model). Present and future studies will investigate the molecular interactions between the known FPI regulators and identify new regulators/DNA binding proteins that aid *Francisella* pathogenesis in various host and non-host niches.

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The subversion of the immune system by *Francisella tularensis*

Catharine M. Bosio*

Immunity to Pulmonary Pathogens Section, Laboratory of Intracellular Parasites, Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Hamilton, MT, USA

Edited by:
Anders Sjostedt, Umeå University, Sweden

Reviewed by:
Tom Kawula, University of North Carolina at Chapel Hill, USA
Stefanie Vogel, University of Maryland, USA

****Correspondence:***
Catharine M. Bosio, Immunity to Pulmonary Pathogens, Laboratory of Intracellular Parasites, Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Hamilton, MT 59840, USA.
e-mail: bosioc@niaid.nih.gov

Francisella tularensis is a highly virulent bacterial pathogen and the causative agent of tularemia. Perhaps the most impressive feature of this bacterium is its ability to cause lethal disease following inoculation of as few as 15 organisms. This remarkable virulence is, in part, attributed to the ability of this microorganism to evade, disrupt, and modulate host immune responses. The objective of this review is to discuss the mechanisms utilized by *F. tularensis* to evade and inhibit innate and adaptive immune responses. The capability of *F. tularensis* to interfere with developing immunity in the host was appreciated decades ago. Early studies in humans were the first to demonstrate the ability of *F. tularensis* to suppress innate immunity. This work noted that humans suffering from tularemia failed to respond to a secondary challenge of endotoxin isolated from unrelated bacteria. Further, anecdotal observations of individuals becoming repeatedly infected with virulent strains of *F. tularensis* suggests that this bacterium also interferes with the generation of adequate adaptive immunity. Recent advances utilizing the mouse model for *in vivo* studies and human cells for *in vitro* work have identified specific bacterial and host compounds that play a role in mediating ubiquitous suppression of the host immune response. Compilation of this work will undoubtedly aid in enhancing our understanding of the myriad of mechanisms utilized by virulent *F. tularensis* for successful infection, colonization, and pathogenesis in the mammalian host.

Keywords: *Francisella tularensis*, antibody, complement, oxidative burst, macrophage, dendritic cell, inflammation, suppression

INTRODUCTION

Francisella tularensis is a small, non-motile, Gram negative bacterium, and the causative agent of tularemia. It is also a facultative intracellular pathogen. There are four primary subspecies of *F. tularensis*. *F. tularensis* subsp. *mediasiatica* and *novicida* are attenuated in humans. *F. tularensis* subsp. *holarctica* causes a mild disease in people. *F. tularensis* subsp. *tularensis* causes severe disease in humans and other mammals following exposure to small numbers (<15 bacteria) of bacteria. *F. tularensis* can be transmitted following exposure to aerosols, contaminated biological products, e.g., animal carcasses, ingestion of contaminated water, or from the bite of infected arthropod vector (as reviewed, Nigrovic and Wingerter, 2008). Once inside the host *F. tularensis* can invade multiple cell types. However, antigen presenting cells (APC) such as macrophages and dendritic cells appear to be the primary cell types targeted by the bacterium at the outset of infection (Bosio and Dow, 2005; Bosio et al., 2007; Hall et al., 2007, 2008; Bar-Haim et al., 2008).

As an intracellular pathogen, *F tularensis* must confront antimicrobial defenses present in the host at multiple steps during infection. Subversion of host immune responses begins at the site of infection. Depending on the route of entry, and prior to meeting a desirable host target cell, the bacterium must first evade killing by serum components designed to eliminate pathogens in the extracellular space. These serum components can include complement present in both naïve and immune hosts. In the vaccinated host, or those previously exposed to *F. tularensis*, serum antibodies may also participate in extracellular detection of the invading organism. Once in contact with a suitable host cell, the bacterium faces additional hurdles in place to control bacterial replication. Two of the most formidable host defense systems faced by *F. tularensis* are the reactive oxygen and reactive nitrogen systems, ROS and RNS respectively. ROS and RNS can be triggered by multiple mechanisms. Thus, the bacterium is forced to possess an arsenal of evasion strategies to either prevent triggering and/or, in some circumstances, dismantling the machinery of ROS and RNS in the host. In this review we will discuss specific strategies utilized by *F. tularensis* to successfully evade detection by the host in the extracellular space as well as disruption of the ROS and RNS in the intracellular compartment that facilitates replication, dissemination, and virulence of this bacterium.

INTERFERENCE WITH HOST RESPONSE IN THE EXTRACELLULAR SPACE

SERUM MEDIATED KILLING

Depending on the immune status of the host, serum, and/or plasma can mediate killing of bacteria via two often intertwined pathways. First, both naïve and immune animals possess the complement system. The complement system, as originally described by Jules Bordet, is comprised of heat-labile components present in plasma that enhance phagocytosis and killing of microorganisms. Today, we understand that complement can act independently, or

in conjunction with, antibodies to control pathogens. The complement system itself is made up of three pathways: the classical pathway, the mannose-binding lectin (MBL) pathway, and the alternative pathway. Each of these pathways can interact directly with pathogens, although the initial proteins and complexes that bind bacterial surfaces vary. Regardless of the pathway or proteins that initially target the microorganism, the pathways converge with the generation of C3 convertase, an enzyme that cleaves C3 to C3b. C3b is the primary effector of the complement system. This protein can act in two ways. First, C3b may directly opsonize pathogens to facilitate their phagocytosis and clearance from the host. Second, C3b plays a role in the generation of C5b. C5b forms the base of the membrane attack complex (MAC) which, when assembled, can induce direct lysis of the bacterium. These pathways are extremely well studied and there are many excellent reviews and textbook chapters that discuss them in detail (Janeway et al., 2005). Thus, only aspects of these systems that have been shown to be directly involved in control of *F. tularensis* will be discussed here.

During the early years of research on immunity to *Francisella*, it was noted that these microorganisms were relatively resistant to killing following exposure to human serum (Lofgren et al., 1983). This suggested that one mechanism of immune evasion by virulent *F. tularensis* was resistance to the assembly of the MAC on their outer membrane. The specific mechanism by which *F. tularensis* is resistant to the assembly of MAC is not completely clear. In one study, the presence of capsule contributed to the evasion of killing by serum components (Sandstrom et al., 1988). However, it has also been shown that *F. tularensis* binds another serum component, Factor H (Ben Nasr and Klimpel, 2008). In that study, Factor H served to cleave C3b to its inactive form iC3b. Generation of the iC3b under these conditions led to inefficient assembly of the MAC. Importantly, generation of iC3b served a second function for the bacterium. Inactive C3b serves as an opsonin for pathogens present in the host vascular system. Pathogens coated in iC3b are targeted for phagocytosis by various cell types which encode the receptors that are capable of interacting with this protein, including macrophages, dendritic cells, and neutrophils (Plow and Zhang, 1997). The two primary receptors noted to interact with iC3b are the complement receptor complex 3 (CR3) which consists of CD11b and CD18 and complement receptor complex 4 (CR4) that consists of CD11c and CD18. Indeed, *F. tularensis* is more efficiently phagocytosed by macrophages and dendritic cells following opsonization with iC3b and it has been shown to utilize both CR3 and CR4 in this process (Ben Nasr et al., 2006). Thus, the ability of *F. tularensis* to participate in the generation of iC3b serves at least two roles. First, it interferes with the deposition of the MAC on the surface of the bacterium and second it facilitates uptake by cells which are favored by the bacterium for replication.

In addition to complement proteins, the immune host may also possess *Francisella* specific antibodies. These antibodies can interfere with the ability of bacteria to infect host cells. Although the role for antibody in *Francisella* immunity has been controversial, classical studies by Foshay demonstrated that passive transfer of hyperimmune serum into humans infected with virulent *F. tularensis* greatly enhanced their recovery (Foshay et al., 1947). Further, this therapy was at least as effective as administration of streptomycin. Importantly, as described in Foshay's manuscript, passive transfer of immune serum was an effective treatment for patients regardless of the form, e.g., pneumonic versus ulceroglandular, of tularemia they had. More recent studies using the mouse model of pneumonic tularemia have demonstrated that passive transfer of both immune serum and specific monoclonal antibodies can protect against a lethal challenge of the Live Vaccine Strain (Kirimanjeswara et al., 2007; Savitt et al., 2009). However, passive transfer of antibodies failed to increase survival of animals challenged with fully virulent, Type A strains of *F. tularensis* although extended mean time to death was noted (Kirimanjeswara et al., 2008).

The failure of antibodies to contribute to survival of Type A infections in mice is not understood. One explanation may lie with the bacterium itself. Recently, our laboratory has demonstrated that antibody mediated opsonization of LVS and the Type A strain *F. tularensis* subsp. *tularensis* strain SchuS4 resulted in similar phagocytosis of each strain of bacterium by mouse macrophages. Importantly, we also demonstrated that phagocytosis of antibody opsonized bacteria provoked a pro-inflammatory response from infected macrophages (Crane et al., 2009). This was notable since it is widely accepted that one of the primary virulence mechanisms possessed by *F. tularensis* is its ability to infect host cells without eliciting pro-inflammatory cytokines such as TNF-α and IL-6 (Telepnev et al., 2003; Bosio and Dow, 2005; Chase et al., 2009). Production of these cytokines during infection is important because they could contribute to the activation of the ROS and RNS pathways that participate in control of *F. tularensis* infection (Fortier et al., 1992). Thus, the ability of both antibody opsonized LVS and SchuS4 to be phagocytosed by host cells and induce cytokine production from infected macrophages did not explain the failure of passively transferred antibody to protect against SchuS4 infections in mice.

To address this issue we turned to factors available in the host, but not routinely present among *in vitro* culture systems, which may influence the interaction of *F. tularensis* with antibody. One such factor is the host serine protease plasmin. Plasmin is generated following interaction of components of the host plasminogen system. The host plasminogen system is a key proteolytic system for dissolution of fibrin clots, migration of host cells through tissues, and the penetration of those cells through protein barriers (as reviewed, Plow et al., 1995). Many bacteria have been noted to bind plasminogen and plasmin and utilize these host proteins to enhance virulence (Lahteenmaki et al., 2001). Indeed, virulent *F. tularensis*, but not attenuated LVS, bound the active protease plasmin. Further, plasmin coated *F. tularensis* inhibited opsonization by *Francisella* specific antibody. The reduced ability of antibody to opsonize plasmin coated, virulent *F. tularensis* resulted in production of significantly less cytokines compared to opsonized, non-plasmin coated controls (Crane et al., 2009). These data suggested that an additional mechanism by which virulent *F. tularensis* subverts host immune responses is by utilizing host proteolytic machinery to degrade antibodies that may participate in protective responses.

INTERFERENCE WITH HOST RESPONSE AT THE CELLULAR LEVEL

INTERACTION WITH CELL SURFACE RECEPTORS

Once the bacterium has traversed the serum and contacted a target host cell, a series of new hurdles that may interfere with successful colonization of the host confront *F. tularensis*. Cellular defense often

begins with the interaction of the pathogen with receptors present on the host cell surface. The vast majority of receptors that interact with invading pathogens are termed pattern recognition receptors (PRR) due to their ability to recognize and bind conserved motifs present on multiple microorganisms. These PRR include, but are not limited to, scavenger receptors (SR), mannose receptors (MR), C-type lectins, and toll-like receptors (TLR). In some circumstances co-receptors and/or binding partners are required to optimally induce signaling though these receptors. For example, CD14 is a promiscuous co-receptor that acts to enhance signaling through TLR4 and TLR2 (Ulmer et al., 1999; Jiang et al., 2000).

Typically, engagement of many PRR results in the secretion of multiple cytokines and chemokines by the host cell. These soluble mediators then activate anti-microbial pathways and facilitate migration of effector cells to the site of infection. Thus, there are two strategies a pathogen may adopt to avoid engagement of these receptors. One is to possess ligands that fail to interact with the receptor or do so inefficiently. Another strategy is to engage receptors that fail to promote strong inflammatory responses. Both of these strategies are utilized by *F. tularensis*.

For example, as found in other Gram negative bacteria, *F. tularensis* possesses lipopolysaccharide (LPS) as part of its outer membrane. However, unlike LPS associated with *E. coli* or *Salmonella* species, the LPS associated with Type A subspecies of *Francisella* is an extremely weak TLR4 agonist (Phillips et al., 2004; Duenas et al., 2006) The poor stimulatory activity of LPS associated with this subspecies is attributed to the presence of only four acyl groups on their LPS (Phillips et al., 2004). Optimal signaling of LPS through TLR4 requires at least six acyl groups (Park et al., 2009). Thus, another mechanism by which *F. tularensis* evades detection by the host is modulation of ligands present on its surface to poorly interact with PRR that aid in altering the host cell to invading pathogens.

In addition to LPS, *Francisella* possess other TLR agonists. For example, Tul4 is lipoprotein that induces signaling responses via TLR2 (Thakran et al., 2008). Tul4 is present on the surface of the bacterium and thus represents a ligand that could alert the host cell to the presence of *F. tularensis* prior to phagocytosis of the bacterium. Yet, despite the presence of Tul4 as an available TLR2 agonist, strong inflammatory responses are not observed in host cells infected with virulent *F. tularensis*. One explanation for lack of detection of Tul4 on the surface of *F. tularensis* by specific host cells may lie in the absence of co-receptors present on select target cells. As stated above CD14 acts as a co-receptor to enhance interaction of microbial ligands for several PRRs including TLR2. At least two primary target cells of *F. tularensis*, alveolar macrophages and dendritic cells fail to express or, only express minimal concentrations of CD14 on their surface. Indeed, when dendritic cells were supplemented with soluble CD14 they secreted several pro-inflammatory cytokines following infection with *F. tularensis* (Chase and Bosio, 2010). *In vivo*, addition of CD14 at the time of intranasal infection induced production of TNF-α and IL-6 and was correlated with control of bacterial replication and dissemination (Chase and Bosio, 2010). However, it should be noted that despite early control of *F. tularensis* infection in mice receiving CD14, supplementation of this receptor *in vivo* did not result in increased survival (Chase and Bosio, 2010). Thus, while early detection of *F. tularensis* and induction of modest inflammatory response can impact replication the bacterium can ultimately overcome this response. The potential mechanisms by which *F. tularensis* overcomes the inflammatory response is discussed below.

A second strategy used by *F. tularensis* to evade detection at the level of the host cell surface is to engage receptors that fail to induce and/or suppress inflammatory responses. Utilization of the MR and CR3 are considered fairly innocuous routes of entry for intracellular pathogens since neither are associated with induction of signaling cascades that result in production of pro-inflammatory cytokines (Aderem and Underhill, 1999; Zhang et al., 2005). It has been demonstrated that, depending on the environment or conditions of infection, *F. tularensis* can utilize both MR and CR3 for entry into host cells. As discussed above, when opsonized with serum *F. tularensis* binds iC3b and gains entry into host cells via the CR3 receptor. Under non-opsonizing conditions, similar to that found in airways, *F. tularensis* utilizes the MR for entry in macrophages (Schulert and Allen, 2006). Therefore, *F. tularensis* evades detection at the point of entry in the host in three ways: (i) the bacterium has modified cell surface structures that enable it to avoid interaction with host receptors that are associated with induction of inflammation, e.g., TLR4; (ii) it targets cells that lack co-receptors which facilitate binding to receptors that could alert the host cell to invasion; and (iii) it utilizes receptors that fail to initiate production of pro-inflammatory cytokines.

THE INTRACELLULAR COMPARTMENT

Host defense by invading pathogens is not limited to detection of the microorganism at the surface of the cell. Mammals also possess an array of defense complexes, intracellular receptors, and signaling pathways that enable the host to control and eliminate the unprepared pathogen. Thus, as an intracellular pathogen, survival and replication of *F. tularensis* relies on its ability to interfere or modulate these intracellular defense mechanisms.

As observed in other bacterial infections, *F. tularensis* is susceptible to killing by reactive oxygen and reactive nitrogen species generated by the host (Fortier et al., 1992; Bosio and Elkins, 2001; Ireland et al., 2010). Thus, it is unsurprising that *F. tularensis* is capable of evading destruction by these toxic molecules. The evasion of products associated with an oxidative burst can be partially attributed to the neutralization of oxidative species by enzymes encoded by *F. tularensis*, e.g., catalase and superoxide dismutase (Lindgren et al., 2007). However, there is also evidence that *F. tularensis* is capable of directly interfering with the assembly of complexes in the host cell responsible for generating oxidative species. Allen and colleagues demonstrated that, following phagocytosis of opsonized *F. tularensis* by polymorphonuclear cells (PMN), the generation of superoxide anions via the NAPDH oxidase was actively inhibited. They then demonstrated that *F. tularensis* directly interfered with the phosphorylation of the p47 subunit of the NADPH oxidase. More recently, it was shown that interference with NADPH oxidase assembly occurred at two separate points. In addition to inhibiting activation of the p47 subunit, virulent *F. tularensis* also inhibited accumulation of the $gp91^{phox}$/$gp22^{phox}$ heterodimer (also known as flavocytochrome b_{558}) in neutrophils. Further, virulent *F. tularensis* also suppressed the ability of human neutrophils to assemble the NADPH oxidase following exposure to unrelated stimuli (McCaffrey et al., 2010).

In addition to directly interfering with assembly of the machinery responsible for focusing degradative enzymes at invading pathogens, *F. tularensis* has the capability of modulating this response in an indirect fashion. It has been suggested that attenuated strains of *F. tularensis* induce alternative activation of macrophages (Shirey et al., 2008). One property of alternatively activated macrophages is a dampened ability to activate reactive nitrogen species (Gordon, 2003). As described above RNS can contribute to the control of *F. tularensis* infections. Thus, provoking a state of alternative activation in host cells could give the invading bacterium an advantage for unrestricted replication in the host cell. However, it is not known if virulent strains of *F. tularensis* can provoke a similar response in resting macrophages. A more recent report provided evidence that *F. tularensis* inhibited oxidative burst via antioxidant scavenging systems associated with the bacterium (Melillo et al., 2010).

Pro-inflammatory cytokines such as TNF-α and IL-12 have been shown to contribute to the control of *F. tularensis* infections (Elkins et al., 1996, 2002). Some of these cytokines, e.g., TNF-α, may act directly on the APC to induce oxidative burst. Alternatively, other pro-inflammatory cytokines, e.g., IL-12, may act indirectly via the activation of effectors cells such T cells or NK cells to produce IFN-γ which then activates the APC. Thus, inhibition of the production and secretion of these pro-inflammatory cytokines by infected cells could also limit anti-microbial oxidative burst. The first evidence that *F. tularensis* was capable of modulating host cell production of cytokines was observed in humans infected with *F. tularensis* followed by exposure to endotoxin. Unlike uninfected controls, humans suffering from pneumonic tularemia failed to mount an inflammatory response following administration of endotoxin isolated from unrelated bacteria (Greisman et al., 1963). Later, *in vitro* studies using the murine macrophage cell line J774 demonstrated that *F. tularensis* actively suppressed the ability of host cells to produce TNF-α in response to *E. coli* LPS (Telepnev et al., 2003). In this report, a 23-kDa protein was found to be essential for suppression of TNF-α and IL-1β production in J774 cells, although the specific mechanism by which this protein interfered with host cell function was not described. Later studies conducted by our laboratory demonstrated that the *F. tularensis* mediated suppression of cytokine production also occurred following *in vivo* pulmonary infection (mirroring the original observations in humans) and, importantly, was extended to human dendritic cells (Bosio et al., 2007; Chase et al., 2009). The specific mechanism(s) by which virulent *F. tularensis* interferes with the ability of host cells to mount inflammatory responses has not been fully elucidated, although it is a subject of intense study by our lab and others. Recent work by Huang and colleagues demonstrated that LVS lacking a gene previously identified to be required for intracellular proliferation (RipA) provoked secretion of greater quantities of IL-1β and TNF-α in mouse macrophages and the human monocytic cell line, THP-1 cells (Fuller et al., 2008; Huang et al., 2010). It was not determined if RipA encoded a protein that directly interfered with induction of inflammatory responses in these cells or if the heightened inflammatory response observed in LVSΔripA mutants was a result of poorly replicating bacteria. Similarly, LVSΔmviN bacteria also elicited a stronger inflammatory response in mouse macrophages compared to wild type LVS (Ulland et al., 2010). However, unlike RipA, mviN was not required for intracellular replication of LVS. Thus, the effect mviN had on elicitation of inflammation was presumably not due to bacteria that were compromised for growth in the intracellular compartment. The role of RipA and mviN in infections mediated by virulent *F. tularensis* has not been explored. However, these data generated with LVS provided promising evidence that novel genes encoded by *F. tularensis* can contribute to the immunosuppression host cells.

CONCLUDING REMARKS

Francisella tularensis is a remarkable bacterial pathogen. In the early days of *F. tularensis* research, when scientists were first characterizing the bacterium, it was apparent that the "success" of this pathogen was tied to its ability to modulate and evade the immune system. This modulation was evident in two central observations. First, the ability of this microorganism to cause acute, lethal, disease in the mammalian host following exposure to relatively miniscule numbers of bacteria suggested it was capable of readily evading innate host defense mechanisms present at the outset and throughout the infection. Second, the lack of development of long lived immunity in laboratory workers who had survived a primary infection pointed to manipulation of adaptive immunity (Jellison, 1974). As discussed herein, it is clear that *F. tularensis* possesses a myriad of mechanisms by which to manipulate immunity. Further, current data suggests that this subversion begins in the extracellular compartment and continues throughout the intracellular life cycle of the bacterium. We have just begun to uncover the collection of immune evasion strategies embodied by this organism. Due to the multiple pathways *F. tularensis* influences as it traverses host environment, continued research into the specific mechanisms by which *F. tularensis* evades, modulates, and suppresses the host immune response will undoubtedly enhance our understanding of tularemia, infectious disease, and regulation of host immunity as a whole.

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Type IV pili in *Francisella* – a virulence trait in an intracellular pathogen

Emelie Näslund Salomonsson[1], Anna-Lena Forslund[1] and Åke Forsberg[2]*

[1] *CBRN Defence and Security, FOI Swedish Defence Research Agency, Umeå, Sweden*
[2] *Umeå Centre for Microbial Research (UCMR), Laboratory for Molecular Infection Medicine Sweden (MIMS), Department of Molecular Biology, Umeå University, Umeå, Sweden*

Edited by:
Anders Sjostedt, Umeå University, Sweden

Reviewed by:
David Thanassi, Stony Brook University, USA
Lisa Craig, Simon Fraser University, Canada

***Correspondence:**
Åke Forsberg, Department of Molecular Biology, Umeå University, 901 87 Umeå, Sweden.
e-mail: ake.forsberg@molbiol.umu.se

Francisella tularensis is a highly virulent intracellular human pathogen that is capable of rapid proliferation in the infected host. Mutants affected in intracellular survival and growth are highly attenuated which highlights the importance of the intracellular phase of the infection. Genomic analysis has revealed that *Francisella* encodes all genes required for expression of functional type IV pili (Tfp), and in this focused review we summarize recent findings regarding this system in the pathogenesis of tularemia. Tfp are dynamic adhesive structures that have been identified as major virulence determinants in several human pathogens, but it is not obvious what role these structures could have in an intracellular pathogen like *Francisella*. In the human pathogenic strains, genes required for secretion and assembly of Tfp and one pilin, PilA, have shown to be required for full virulence. Importantly, specific genetic differences have been identified between the different *Francisella* subspecies where in the most pathogenic type A variants all genes are intact while several Tfp genes are pseudogenes in the less pathogenic type B strains. This suggests that there has been a selection for expression of Tfp with different properties in the different subspecies. There is also a possibility that the genetic differences reflect adaptation to different environmental niches of the subspecies and plays a role in transmission of tularemia. This is also in line with recent findings where Tfp pilins are found to be glycosylated which could reflect a role for Tfp in the environment to promote survival and transmission. We are still far from understanding the role of Tfp in virulence and transmission of tularemia, but with the genomic information and genetic tools available we are in a good position to address these issues in the future.

Keywords: *Francisella tularensis*, type IV pili, virulence, type II secretion

INTRODUCTION

Francisella tularensis, the causative agent of tularemia, has attracted significant attention over the years. A major reason is that the most pathogenic variant, subspecies *tularensis* also known as type A, causes severe infections that without rapid therapeutic intervention shows high mortality rates. These strains have also been recognized to have potential for development of biological weapons. Type A strains are found exclusively in North America while the less pathogenic subspecies *holarctica*, also known as type B strains, is more broadly distributed in the Northern hemisphere (Petersen and Schriefer, 2005). Still, *Francisella* remained an understudied pathogen and this did not really change until the first genome sequence became available and genetic systems were developed (Golovliov et al., 2003; Larsson et al., 2005). When the first genome sequence became accessible it was somewhat of a disappointment to note that the number of genes with homology to known virulence determinants in other pathogens were relatively few (Larsson et al., 2005). One of the exceptions was the gene clusters predicted to encode a type IV pili (Tfp) system. Tfp have been identified as a major virulence determinant in many different pathogens even if it was not obvious what role a pilus adhesin could have for an intracellular pathogen like *Francisella*. In this focused review we summarize and discuss the main findings regarding the biological role of genes encoding the Tfp system in *F. tularensis* and the significance of the distinct differences seen for specific Tfp genes between different subspecies.

TYPE IV PILI – DYNAMIC ADHESIVE SURFACE STRUCTURES

Type IV pili are multifunctional, flexible filamentous appendages that have been assigned specific virulence traits in several important pathogens. These properties include adhesion, twitching motility, biofilm formation, and competence for DNA transformation and are important for host colonization and virulence in pathogens like *Pseudomonas aeruginosa*, *Neisseria* spp, *Vibrio cholerae*, and *Moraxella catarrhalis* (Mathis and Scocca, 1984; Bergström et al., 1986; Taylor et al., 1987; Sato et al., 1988; Catlin, 1990; Marrs and Weir, 1990; O'Toole and Kolter, 1998). The nomenclature of the Tfp gene clusters have not been harmonized between systems and pathogens (Craig and Li, 2008), and also for *F. tularensis* different research groups use different nomenclature. Here we have chosen to mainly use the nomenclature adapted for *P. aeruginosa*. In **Table 1** the nomenclature used for the Tfp genes discussed in this review are listed.

Type IV pili biogenesis is a process whereby a single protein subunit, the so called major pilin, is processed and translocated across the inner membrane where it forms a dynamic multimeric filament. Multiple proteins sharing structural

Table 1 | Nomenclature and presence of functional Tfp genes in different strains.

Gene name	Alternative gene name	Putative function	Type A/*F. novicida* strains	Type B strains	Type B LVS
pilA, FTT0890	*pilE*, *pilE1*	Type IVa pilus subunit	+[1]	+	–
pilE, FTT0889	*pilE2*	Type IVa pilus subunit	+	–	–
pilV, FTT0888	*pilE3*	Type IVa pilus subunit	+	–	–
FTT0861	*pilE4*	Type IVb pilus subunit	+[2]	+[2]	+[2]
FTT0230	*pilE5*	Type IVa pilus subunit	+	+	+
FTT1314		Type IVa pilus subunit	+	+	+
pilD		Peptidase	+	+	+
pilQ		Secretin	+	+	+
pilB	*pilF*	ATPase, pilus extension	+	+	+
pilT		ATPase, pilus retraction	+	–[3]	–[3]
pilC	*pilG*	Transmembrane protein	+	+	+

[1]*pilA differs in the 3′-end in F. novicida.*
[2]*FTT0861 encodes a mutation in the stop codon resulting in a longer gene in F. novicida and type B strains.*
[3]*pilT is truncated due to a non-sense mutation.*

similarities with this major pilin subunit, known as minor pilins, are also required for proper Tfp function and/or assembly, but their exact role is not completely understood (Alm and Mattick, 1996; Winther-Larsen et al., 2005; Helaine et al., 2007). Tfp are further divided into two subclasses, type IVa and type IVb pilins, based on the presence of specific conserved motifs (Strom and Lory, 1993; Kachlany et al., 2001; Craig et al., 2004). Type IVb pili are commonly found in pathogens colonizing the human intestine like *V. cholerae*, *Salmonella typhi*, and enteropathogenic *Escherichia coli* (EPEC) (Faast et al., 1989; Girón et al., 1991; Zhang et al., 2000).

The major pilin, PilA, is processed by a specific peptidase, PilD, and thereafter translocated across the inner membrane, followed by assembly into a multimeric pilus fiber on the periplasmic side of the inner membrane. The pilus fiber is then secreted across the outer membrane via the secretin pore PilQ (**Figure 1**; Strom and Lory, 1993; Drake and Koomey, 1995). The assembly and extension of Tfp is facilitated by the PilB ATPase and *pilB* mutants are negative for Tfp (Turner et al., 1993). In several of the bacteria expressing Tfp a second ATPase PilT promotes disassembly and retraction of Tfp and in this case *pilT* mutants are hyperpiliated (Wolfgang et al., 2000). PilT is also required for motility on solid surfaces – a phenomenon denoted twitching motility seen in several bacteria expressing PilT (Whitchurch et al., 1991; Maier et al., 2004). Another key component of the Tfp biogenesis is PilC, an inner membrane protein of unknown function (Nunn et al., 1990). Several *F. tularensis* Tfp related genes also show homology to genes involved in type II secretion system (*T2SS*) (Peabody et al., 2003). These include the inner membrane associated proteins PilB and PilC, the secretin PilQ, and the pilin peptidase PilD (Nunn and Lory, 1991). In addition, Tfp pilins show homology to T2SS pseudopilins (Peabody et al., 2003). It has been shown that the Tfp subunit PilA of *P. aeruginosa* is also required for efficient secretion of T2S substrates (Lu et al., 1997). In addition, there is evidence that Tfp in some cases can promote protein secretion by a mechanism similar to T2SS (Kennan et al., 2001; Kirn et al., 2003; Han et al., 2007).

F. TULARENSIS SUBSPECIES SHOW DISTINCT GENETIC DIFFERENCES IN Tfp GENES

The genomes of the different *F. tularensis* subspecies; *tularensis* (type A), *holarctica* (type B), and *novicida*, all encode Tfp clusters including six putative pilin genes; *pilA*, *pilE*, *pilV*, FTT0861, FTT0230, and FTT1314 (Gil et al., 2004; Larsson et al., 2005; Forslund et al., 2006). Interestingly, there are some distinct differences in these pilin genes between the subspecies (Larsson et al., 2005). In virulent type B strains, *pilE* and *pilV* are non-functional due to non-sense mutations, and the type IVb pilin gene, FTT0861, harbors a mutation in the stop codon resulting in a longer gene. Furthermore, some type B strains harbor an additional frame-shift mutation in the FTT0861 gene resulting in an even longer open reading frame. The three remaining pilin genes; *pilA*, FTT0230, and FTT1314, are essentially identical between type A and type B strains. In contrast, all six pilin genes are intact and functional in type A strains and *F. novicida*. Interestingly, the *pilA* gene in *F. novicida* differs in the 3′-end compared to *pilA* in type A and type B strains (Zogaj et al., 2008). The Tfp genes encoded by the different subspecies are listed in **Table 1**.

Genomic analysis early revealed the presence of several regions of difference (RDs), flanked with direct repeat sequences that could mediate deletions of certain genes or regions (Broekhuijsen et al., 2003; Svensson et al., 2005). One of these regions, RD19, encodes the pilin gene *pilA*. Interestingly, several attenuated type B strains, like the live vaccine strain (LVS) and an isolate from a hare, have lost the *pilA* gene due to homologous recombination involving the direct repeats (Svensson et al., 2005; Forslund et al., 2006; Salomonsson et al., 2009b). Another distinct difference between the subspecies is that there is a unique, single non-sense mutation that truncates the *pilT* gene in type B strains (Gil et al., 2004). Taken together, all these distinct genetic differences indicate that the biological properties of Tfp expressed by the different subspecies could be significantly different.

Tfp GENES ARE REQUIRED FOR VIRULENCE OF *F. TULARENSIS*

Among the different pilin genes only *pilA* has been found to be required for virulence in both type A and type B strains. The above-mentioned hare type B isolate possessing the spontaneous *pilA*

deletion showed reduced virulence at levels comparable to that of the LVS strain, which also lacks *pilA* and several pilus assembly genes (**Table 1**; Forslund et al., 2006; Salomonsson et al., 2009b). Furthermore, *pilA* has been demonstrated to be important for full virulence in the pathogenic type B strain FSC200 and the highly virulent type A strain SCHU S4 (Forslund et al., 2010; Näslund Salomonsson et al., unpublished results). When we compared the importance of *pilA* for virulence in different strains and subspecies, we found that *pilA* had less impact on virulence in the more virulent strains (**Table 2**). As seen in column 3 of **Table 2**, the infection dose is very low in virulent type A and type B strains, making it problematic to measure small differences in attenuation of strains. Hence, the infection dose experiments are not sensitive enough for the highly virulent strains. Therefore, when using strains that are highly virulent to mice we performed competitive infection experiments where mice were simultaneously infected with the *pilA* mutant and the isogenic wildtype in order to be able to verify small differences in virulence. The competitive index (CI) was calculated by dividing the ratio of mutant/wt after infection with the mutant/wt ratio before infection. The low CI value, 0.004, for the infection with the type B strain FSC200, is consistent with the result from the single infection experiment where the infection dose was 40-fold higher for mice infected with the *pilA* negative strain. When studying the highly virulent type A strain and the influence of *pilA* in virulence, we were unable to measure any difference regarding the infection dose in the single infection study, but the relatively low CI value verifies that *pilA* is also needed for full virulence in the most pathogenic subspecies (**Table 2**).

Regarding *F. novicida*, the Tfp encoding genes are, overall very similar to type A strains and therefore it is interesting to note that virulence data between these subspecies are so diverse. There are also conflicting results between different *F. novicida* studies. In one study a *F. novicida pilA* mutant was found to be even more virulent than the wildtype strain (Hager et al., 2006), while Zogaj et al. (2008) found that *pilA* mutants were attenuated compared to the wildtype. Hager and colleagues suggested the enhanced virulence to be a result of abolished secretion of the protein PepO, a protease involved in vasoconstriction which limits the spread of *F. novicida*. Since *pepO* is non-functional or lacking in type A and type B strains, it is not possible to directly compare the different subspecies regarding these defects. In addition, there are genetic findings indicating that *F. novicida* displays Tfp-mediated secretion, similar to the type IV toxin coregulated pilus-dependent secretion of TcpF in *V. cholerae*, rather than a T2SS mediated secretion (Kirn et al., 2003; Hager et al., 2006). In conclusion, there is convincing evidence for a role of a functional Tfp in virulence where most data so far support a role for PilA as well as some of the assembly/secretion factors. The evidence for Tfp-mediated secretion in *F. novicida* is convincing, while it is somewhat less clear if secretion is common to all subspecies or what the role of functional Tfp-mediated secretion could play in virulence.

So far, FTT0861 is the only pilin besides PilA that has been reported to be virulence associated. Zogaj et al. (2008) showed that FTT0861 was required for full virulence in mice infected with *F. novicida* via the intradermal route, while the pilin had no influence on intracellular survival and growth in macrophages. Of particular interest is that this pilin belongs to the type IVb pilin family found in enteropathogens like EPEC and *V. cholerae*. Our lab has not found a link to virulence for any of the other pilin genes in type B strains but we have data suggesting that the pseudopilins/minor pilins encoded by FTT1621 and/or FTT1622 are required for full virulence of type B strains (Näslund Salomonsson et al., unpublished).

Several genes are involved in secretion and assembly of Tfp and there are evidence supporting that some of these genes are also associated with virulence of *F. tularensis*. The Tfp biogenesis genes *pilC* and *pilQ* both contribute to virulence in the highly virulent type A strain SCHU S4 (Forslund et al., 2010). These proteins are of fundamental importance for biogenesis and function of Tfp in other pathogens

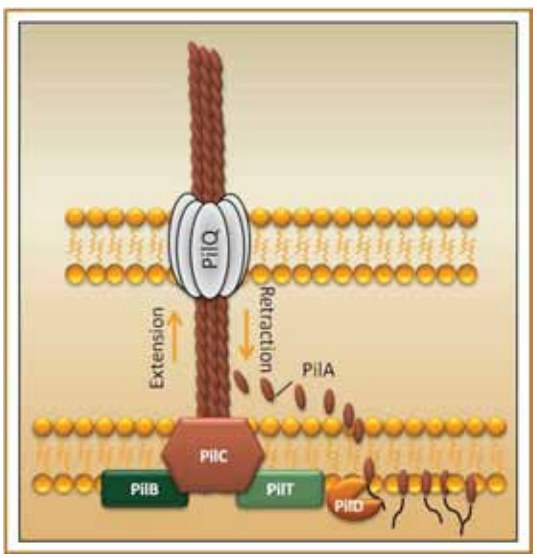

FIGURE 1 | Schematic overview of the type IV pili system. The pilus fiber is mainly composed of the major pilin subunit (denoted PilA in *P. aeruginosa*), which is expressed as a prepilin that upon cleavage by the prepilin peptidase PilD allows for proper pilus assembly and function (Strom et al., 1993). The major pilin is translocated across the inner membrane where it forms a dynamic multimeric filament that is secreted via the pore-forming secretin PilQ to the bacterial surface (Marceau et al., 1998). PilC is a transmembrane protein found in the inner membrane (Nunn et al., 1990). The two ATPases, PilB and PilT, mediates extension and retraction respectively of the pilus (Whitchurch et al., 1991; Maier et al., 2004).

Table 2 | Comparison in infection doses and CI values for *pilA* positive and *pilA* negative strains.

Strain	*pilA*	CFU lethal doses – single infection	CI
Type B LVS *cis*-complemented	*pilA*$^+$	5×10^3	
Type B LVS (wt)	*pilA*$^-$	1×10^6	
Type B FSC200 (wt)	*pilA*$^+$	<5	
Type B FSC200 Δ*pilA*	*pilA*$^-$	1.8×10^2	0.004*
Type A SCHU S4 (wt)	*pilA*$^+$	<10	
Type A SCHU S4 Δ*pilA*	*pilA*$^-$	<10	0.14*

**Competitive index (CI) is the ratio between the pilA mutant and the isogenic wildtype strain for bacteria isolated from spleens of mice simultaneously infected with the two strains.*

and provide indirect evidence suggesting that *Francisella* expresses functional Tfp. Furthermore, PilT has previously been shown to be of importance of virulence in other pathogens, e.g., *P. aeruginosa* and *Dichelobacter nodosus* (Comolli et al., 1999; Han et al., 2008). Chakraborty et al. (2008) demonstrated that mutations in either *pilB* or *pilT* in LVS resulted in attenuation in a mouse infection model. These results are somewhat puzzling as *pilT* is a pseudogene in type B strains where there is a missense mutation that would be expected to result in a truncated protein of only one-third in size compared to the full length protein. In an attempt to verify this hypothesis we introduced a FLAG-tag at the 3′-end of both an intact *pilT* and *pilT* that harbors the stop codon, and looked for a read-through. The FLAG-tag was expressed from the intact *pilT* gene but not from the gene carrying the stop codon, suggesting that suppression of this nonsense mutation does not occur and *pilT* is therefore non-functional in type B strains (Näslund Salomonsson et al., unpublished). The influence of *pilT* in virulence has also been studied in the type A strain SCHU S4 where *pilT* is intact, but in this case *pilT* was found not to be involved in mouse virulence (Forslund et al., 2010).

Even if it has been established that *pilA* contributes to virulence in *F. tularensis*, the overall picture is that the pilin is not required for intracellular survival or replication in type A or type B strains (Forslund et al., 2006), with the exception of *F. novicida* where it was found that *pilA* mutants were impaired for intracellular growth (Zogaj et al., 2008). The mechanism whereby PilA promotes infection has not been elucidated, but there is evidence suggesting that PilA is required for optimal spread of the bacterium from the initial peripheral site of infection to cause a systemic infection (Forslund et al., 2006). This suggests that PilA mediates specific interactions with host cells/tissue that facilitates spread of the infection.

PHYSICAL EVIDENCE FOR Tfp

In a study published by Gil et al. (2004), LVS was demonstrated to express Tfp-like structures on the surface (**Figure 2**). In a following study, mutations in either the *pilB* gene, involved in pilus extension, or the *pilT* gene, involved in pilus retraction, resulted in loss of surface fibers as well as a defect in adherence to host epithelial cells, arguing that both proteins are involved in pilus assembly (Chakraborty et al., 2008). The *pilB* results are in agreement with other Tfp expressing pathogens while the *pilT* results were somewhat unexpected as in other pathogens, like *Neisseria* spp, deletion of *pilT* results in a hyperpiliated phenotype (Wolfgang et al., 2000). Interestingly, in *Clostridium* spp., the only known Gram-positive that harbors Tfp genes, it has been shown that a *pilT* mutant can still produce pilins, but are unable to assemble the pili on the surface of the cell (Varga et al., 2006). The reason for this unexpected phenotype of the *pilT* mutant suggests that PilT may have a different role than retraction for Tfp in *Francisella*. However, the *F. tularensis* PilT results were even more surprising due to the fact that the *pilT* gene is only intact in type A strains while it is non-functional in all type B strains (Gil et al., 2004; Näslund Salomonsson et al., unpublished). Overall, the role of PilT in Tfp biogenesis and virulence in different subspecies is still unclear.

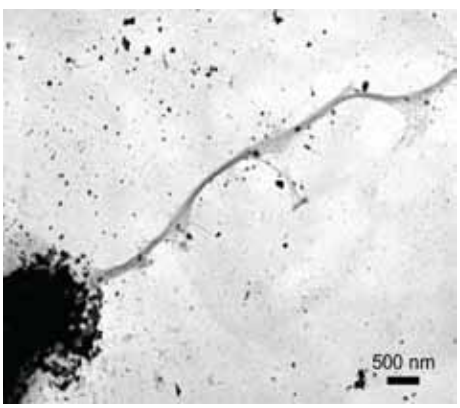

FIGURE 2 | The live vaccine strain was demonstrated to express Tfp-like structures on the bacterial surface with negative stained electron microscopy (Gil et al., 2004). Reprinted with permission. Copyright 2004, ASM press.

In a different study, *F. novicida* was also confirmed to assemble filamentous structures on the bacterial surface (Zogaj et al., 2008). In this case, a mutation in the secretin-encoding gene *pilQ* resulted in decreased but not complete lack of pili. Furthermore, a *pilC* mutant had no impact on piliation. This is again surprising, since both PilQ and PilC are expected to be essential for secretion and assembly of Tfp (Tønjum et al., 1995; Helm et al., 2007). Similar to what was found in LVS, *pilB* and *pilT* mutants were totally deficient for Tfp-like structures in *F. novicida*, indicating that PilB and PilT are required for Tfp assembly. Interestingly, Zogaj et al. (2008) suggested the type IVb pilin FTT0861 to be the major Tfp subunit in *F. novicida*, as they showed that a FTT0861 mutant lack surface fibers, which was not seen for any of the other pilin mutants. Still, some of the other pilin mutants expressed significantly fewer surface fibers indicating that the mutated genes could encode minor pilins that somehow influence the assembly and expression of the pilus. PilA, on the other hand, was shown to be required for secretion in *F. novicida* but had no impact on pili expression.

Indeed, even if Tfp-like structures have been identified on the surface of LVS and *F. novicida*, these structures have still not been verified to be composed of one of the Tfp pilins. In one of our studies the PilA protein was FLAG-tagged in the C-terminus and analyzed with electron microscopy in order to facilitate detection on the surface of type B strains (Forslund et al., 2006). However, in that study no filamentous structures were detected, but the finding that PilA is exported to the bacterial surface was confirmed (**Figure 3**), though we could not correlate *pilA* to adhesion to epithelial cells (Forslund et al., 2006). In a further study we used a different strategy to verify if any of the *F. tularensis* pilin genes could form pili structures. Here a trans-species complementation assay was used where *F. tularensis* pilins were expressed in *N. gonorrhoeae* (Salomonsson et al., 2009a). Interestingly, out of all pilins tested only PilA was able to complement Tfp related functions in the heterologous system. Both PilA derived from the type A strain SCHU S4 and from *F. novicida* promoted assembly of Tfp-like structures in the *N. gonorrhoeae* background (**Figure 4**; Salomonsson et al., 2009a). Further support for the ability of these two PilA proteins to form pilus-like multimers was their capacity to restore competence for DNA uptake in a *N. gonorrhoeae* pilin mutant strain. According to these findings, PilA seems to be the only *F. tularensis* pilin able to form functional filaments. The type II secretion pseudopilins

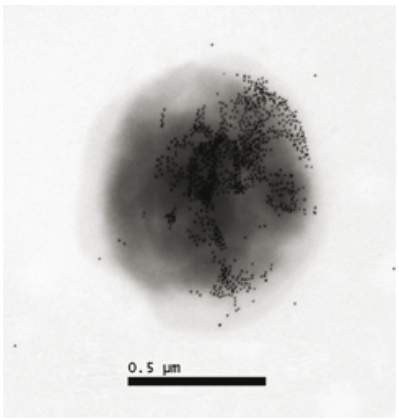

FIGURE 3 | Expression of FLAG-tagged PilA on the bacterial surface of a type B strain visualized by immunogold electron microscopy (Forslund et al., 2006). Reprinted with permission. Copyright 2006, Blackwell publishing ltd.

FIGURE 4 | Piliation of a gonococcal strain expressing *F. novicida* derived PilA visualized by immunogold electron microscopy (Salomonsson et al., 2009a). Reprinted with permission. Copyright 2009, Society for General Microbiology.

have also been demonstrated to form pilus-like structures when overexpressed in the T2S pathway (Sauvonnet et al., 2000). Hence, the Tfp-like structures seen in *N. gonorrhoeae* could be built up by pseudopilins. Importantly, the *Francisella* specific appendages in *N. gonorrhoeae* were dependent on Tfp biogenesis factors and pseudopilins does not to support genetic transformation which provides further support for the idea that PilA indeed functions as a Tfp pilin (Salomonsson et al., 2009a).

In conclusion, there are physical evidence for Tfp-like structures in *Francisella*, still, conclusive evidence as to which protein constitutes the major structural subunit has yet to be presented.

POSTTRANSLATIONAL MODIFICATION OF Tfp

In our first study of Tfp in *Francisella*, where we showed that loss of *pilA* also resulted in virulence attenuation, we also presented evidence for posttranslational modification of PilA (Forslund et al., 2006). PilA expressed by *Francisella* displayed significantly lower mobility in SDS-PAGE compared to PilA expressed by a non-glycosylating strain of *P. aeruginosa*. Recently we have shown that a gene encoding a glycosyltransferase with homology to transferases known to be required for glycosylation of Tfp in other pathogens (Aas et al., 2007; Faridmoayer et al., 2007; Qutyan et al., 2007), is required for glycosylation of PilA (unpublished results). In addition we have preliminary evidence that other pilins expressed by the different *Francisella* subspecies also are posttranslationally modified by a modification that requires the same glycosyltransferase (Näslund Salomonsson et al., unpublished). Recently published results indicate that glycosylation of proteins in *Francisella* is not limited to Tfp related proteins, but includes other surface proteins (Balonova et al., 2010). Genome information, as well as our own preliminary data, suggests that glycosylation of pilins is common to all subspecies (Brotcke et al., 2006; Weiss et al., 2007; Näslund Salomonsson et al., unpublished). It is also possible that the degree of modification at specific glycosylation sites may vary depending on growth conditions which are reflected in the ladder like appearance of PilA in Western blot analysis (Forslund et al., 2006). Posttranslational modification of Tfp has been shown to affect twitching motility and properties of the pilus by rendering the pilus fiber less hydrophobic and more stable (Smedley et al., 2005). For *P. aeruginosa* there is also a strong correlation between glycosylation of Tfp and clinical isolates, and Tfp glycosylation has been found to provide a competitive advantage in macrophage and lung infection models (Smedley et al., 2005). This opens up the exciting possibility that glycosylation of Tfp may also be of relevance for tularemia either at the level of infection or at the level of transmission.

PROSPECTS

The *F. tularensis* subspecies show great diversity with respect to virulence in humans from the highly pathogenic subsp. *tularensis* (type A) to the essentially non-pathogenic subspecies *novicida*. This is intriguing as the genome sequences have revealed that they are highly homologous with >97% identity at DNA level between the most and least virulent subspecies (Larsson et al., 2009). When it comes to the gene clusters encoding Tfp there are specific differences at genetic level, where type A strains and *F. novicida* only show significant differences in one gene, *pilA*, while type B strains have acquired non-sense mutations in several Tfp genes. These mutations are remarkably conserved in the strains analyzed so far, suggesting that loss of function of these genes has occurred in order to adapt to a specific environmental and/or host niche.

One interesting functional difference between the subspecies is that Tfp can promote secretion of a subset of proteins in *F. novicida* (Hager et al., 2006; Zogaj et al., 2008), but so far there is no evidence for Tfp-mediated secretion of proteins *in vitro* in the human pathogenic subspecies. While it remains a possibility that the *in vitro* conditions that promotes secretion may differ, it is still a possibility that evolution of the highly virulent subspecies included loss of Tfp-mediated secretion and that the key molecule in this development is the differences seen in the C-terminal part of PilA between *F. novicida* and the pathogenic subspecies.

When it comes to functional analysis and virulence, PilA has been found to be required for virulence in the human pathogenic subspecies (Forslund et al., 2006, 2010; Salomonsson et al., 2009b). In addition PilA has been shown to function as a pilin subunit when expressed in a heterologous Tfp expressing system in *N. gonorrhoeae*, both with respect to forming a Tfp filament but also in the Tfp-mediated DNA uptake assay (Salomonsson et al., 2009a). Several other pilin proteins that were evaluated in this assay were also expressed in *N. gonorrhoeae* but did not promote formation of filaments or DNA uptake.

PilA has been shown to localize to the bacterial surface and there is also evidence that export/assembly is important for the role of PilA in virulence as mutants in the assembly/secretion genes *pilC* and *pilQ*, similar to *pilA* mutants, were attenuated in a mouse infection model (Forslund et al., 2006, 2010). Several studies have also revealed the presence of Tfp-like structures in various *Francisella* strains even if no one yet have been able to identify the major subunit in pili expressed by *Francisella* (Gil et al., 2004; Chakraborty et al., 2008; Zogaj et al., 2008). The only way to validate if different subspecies express different Tfp is to use biochemical or immunological approaches to identify the main component of the Tfp filaments in different strains. However, it is fair to say that Tfp expressed by *Francisella* appear to be different in many aspects and properties compared to what is known from studies in other pathogens.

Another significant finding regarding the role of PilA in virulence is that the attenuation of *pilA* mutants in the mouse infection model for tularemia is less pronounced the more virulent the strain is (Forslund et al., 2006, 2010; Salomonsson et al., 2009b). The interpretation and significance of these results is somewhat difficult as all these studies were conducted in the mouse infection model which fails to discriminate between strains that differ significantly in virulence for humans. This makes it difficult to assess if the differences in Tfp genes between type A and type B strains also results in differences in human virulence.

Our recent finding, that PilA and also other pilin proteins are glycosylated, opens other possibilities and raises new questions regarding the role of Tfp in tularemia. Glycosylation is known to influence the properties of Tfp by lowering hydrophobicity, increasing stability and motility. In addition to its role in virulence it is possible that Tfp glycosylation is important for survival and transmission of tularemia.

We are still far from understanding the role of Tfp in virulence and transmission of tularemia, but the identified highly conserved differences strongly suggest that the different subspecies may express Tfp with different properties with respect to filament subunits as well as ability to promote motility. Future work to resolve the role of Tfp in tularemia, need to include both infection models which better reflect the human infection as well as studies on how Tfp genes affect survival and transmission in different natural environments.

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Francisella tularensis blue–gray phase variation involves structural modifications of lipopolysaccharide O-antigen, core and lipid A and affects intramacrophage survival and vaccine efficacy

Shilpa Soni[1], Robert K. Ernst[2], Artur Muszyński[3], Nrusingh P. Mohapatra[1], Malcolm B. Perry[4], Evgeny Vinogradov[4], Russell W. Carlson[3] and John S. Gunn[1]*

[1] Center for Microbial Interface Biology, Department of Molecular Virology, Immunology and Medical Genetics, and Department of Internal Medicine, Division of Infectious Diseases, The Ohio State University, Columbus, OH, USA
[2] Department of Microbial Pathogenesis, School of Dentistry, University of Maryland-Baltimore, Baltimore, MD, USA
[3] Complex Carbohydrate Research Center, University of Georgia, Athens, GA, USA
[4] Institute for Biological Sciences, National Research Council, Ottawa, ON, Canada

Edited by:
Adel M. Talaat, University of Wisconsin Madison, USA

Reviewed by:
Martin I. Voskuil, University of Colorado Denver, USA
Francis Nano, University of Victoria, Canada

***Correspondence:**
John S. Gunn, The Ohio State University, Biomedical Research Tower, Rm. 1006, 460 W. 12th Ave., Columbus, OH 43210-1214, USA.
e-mail: john.gunn@osumc.edu

Francisella tularensis is a CDC Category A biological agent and a potential bioterrorist threat. There is no licensed vaccine against tularemia in the United States. A long-standing issue with potential *Francisella* vaccines is strain phase variation to a gray form that lacks protective capability in animal models. Comparisons of the parental strain (LVS) and a gray variant (LVSG) have identified lipopolysaccharide (LPS) alterations as a primary change. The LPS of the *F. tularensis* variant strain gains reactivity to *F. novicida* anti-LPS antibodies, suggesting structural alterations to the O-antigen. However, biochemical and structural analysis of the *F. tularensis* LVSG and LVS LPS demonstrated that LVSG has less O-antigen but no major O-antigen structural alterations. Additionally, LVSG possesses structural differences in both the core and lipid A regions, the latter being decreased galactosamine modification. Recent work has identified two genes important in adding galactosamine (*flmF2* and *flmK*) to the lipid A. Quantitative real-time PCR showed reduced transcripts of both of these genes in the gray variant when compared to LVS. Loss of *flmF2* or *flmK* caused less frequent phase conversion but did not alter intramacrophage survival or colony morphology. The LVSG strain demonstrated an intramacrophage survival defect in human and rat but not mouse macrophages. Consistent with this result, the LVSG variant demonstrated little change in LD_{50} in the mouse model of infection. Furthermore, the LVSG strain lacks the protective capacity of *F. tularensis* LVS against virulent Type A challenge. These data suggest that the LPS of the *F. tularensis* LVSG phase variant is dramatically altered. Understanding the mechanism of blue to gray phase variation may lead to a way to inhibit this variation, thus making future *F. tularensis* vaccines more stable and efficacious.

Keywords: *Francisella*, LPS, phase variation, tularemia, vaccine

INTRODUCTION

Francisella tularensis is a gram-negative, facultative intracellular pathogen that causes tularemia in humans and animals (Oyston et al., 2004; Keim et al., 2007; Sjostedt, 2007). The host can be infected by several routes including the lungs (inhalational), skin, or mucous membranes (cutaneous) or by ingestion of contaminated food or water (gastrointestinal) (Keim et al., 2007; Sjostedt, 2007). *F. tularensis* has been characterized as a category A bio-defense organism by the Centers for Disease Control and Prevention because of its high lethality and infectivity, particularly by the aerosol route. There are two major human virulent subspecies of *F. tularensis*: *F. tularensis* subspecies *tularensis* (Type A strain) found in North America and *F. tularensis* subspecies *holarctica* (Type B strain) found in Europe, Asia as well as North America (Ellis et al., 2002). The Type A strain is highly infectious and when inhaled, even low doses (<10 bacteria) can cause life-threatening disease in humans (Sjostedt, 2007). Type B strains are considered less virulent but can still effectively cause diseases in humans. *F. tularensis* subspecies *novicida* (*F. novicida*) and *F. tularensis* subspecies *mediasiatica* are other known subspecies of *Francisella* that are considered relatively avirulent for immunocompetent humans but are capable of causing systemic infection in other mammals (Ellis et al., 2002; Keim et al., 2007).

There are no approved vaccines available to prevent or treat tularemia in the United States (Oyston, 2009). An attenuated live vaccine strain, *F. tularensis* LVS (*Ft* LVS), was derived from a Type B isolate of the pathogen (Oyston, 2009) and is used as a vaccine in Europe and is in clinical trials for potential approval in the US. It elicits diverse protection in humans, monkeys, guinea pigs, and mice depending on the route of vaccination against systemic challenge with virulent Type A *F. tularensis* (Eigelsbach and Downs, 1961). The molecular basis for the attenuation of *Ft* LVS still remains unknown, though candidate factors have been identified (Rohmer et al., 2006). Eigelsbach (Eigelsbach et al., 1951; Eigelsbach

and Downs, 1961) first reported colony variants of the prototypical virulent Type A SchuS4 strain and *Ft* LVS, which were identified on the basis of colony morphology (rough colonies and smooth colonies) and their appearance under a field microscope viewed with oblique light, where *Ft* LVS/SchuS4 appears blue and the variant as gray. Gray variants were reported to be less virulent with a lethal dose of $>10^7$ colony forming units (CFU) and were less immunogenic/protective in challenge studies, where they afforded minimal protection to Type A challenge (Eigelsbach et al., 1951). These variants also differentially reacted to acriflavine agglutination, and demonstrated variable stability of colony morphology upon subculturing (Eigelsbach et al., 1951). The observation of gray variants depended on growth conditions including culture media, size of inoculum, pH, and duration of culture growth. Hartley et al. (2006) also identified the spontaneous gray variants of three *F. tularensis* strains (LVS, SchuS4, and HN63), further suggesting that blue to gray variation is a frequent and perhaps common occurrence in wildtype strains in the environment.

Gray variants were first examined at the molecular level by Cowley et al. (1996). This variant (LVSG; *Ft* LVSG) demonstrated differential survival in certain macrophage types and the lipopolysaccharide (LPS) of this variant, which possessed a LPS O-antigen, was found to possess altered anti-LPS monoclonal antibody reactivity and stimulated increased nitric oxide (NO) production in macrophages. A rough gray variant (lacking an LPS O-antigen) was also recently characterized (Hartley et al., 2006). This variant was identified on the basis of size and opacity, grew slower, had reduced intramacrophage survival, and poorly protected against Type A *F. tularensis* challenge. These studies suggested that LPS played an important role in this phase variation phenomenon.

In the present study, we confirmed and extended the phenotypic characterization of the gray variants and further analyzed the LPS of one of these strains. Multiple LPS alterations were noted, including those in O-antigen, core and lipid A. The gray variant primarily characterized in this study, *Ft* LVSG, possessed a full length O-antigen (as opposed to previously characterized rough gray variants), survived less well in human and rat but not mouse macrophages and poorly protected against *F. tularensis* SchuS4 challenge in the mouse model. It is hoped that a greater understanding of the mechanism(s) behind phase variation will lead to phase locked strains that no longer vary, thus allowing the construction of safer, more immunogenic tularemia vaccines.

MATERIALS AND METHODS

STRAINS AND MEDIA

Francisella tularensis subsp. *holarctica* LVS (ATCC 29684) was obtained from Karen Elkins (Center for Biologics Research and Evaluation, U.S. Food and Drug Administration, Bethesda, MD, USA). *F. novicida* (U112) was obtained from ATCC, LVSG (a spontaneous gray phase variant) was provided by F. Nano (University of Victoria, Victoria, BC, Canada). *Ft* LVSGD (another spontaneous gray variant we found to lack O-antigen) was obtained from lot number 703-0102-080 produced at Cambrex BioSciences in Baltimore, MD, USA. The cell bank was produced from a lyophilized vial of *Ft* LVS lot NDBR-101, lot 4 (Salk produced). *F. tularensis* subsp. *tularensis* strain SchuS4, a Centers for Disease Control and Prevention clinical isolate, was provided by Rick Lyons (University of New Mexico, Albuquerque, NM, USA). For most experiments, bacteria were grown overnight (~24 h) on Choc II agar (BD Biosciences, San Jose, CA, USA) at 37°C as the frequency of phase variation was minimal in these conditions. Liquid cultures were grown overnight (~16 h) in tryptic soy broth (TSB; Difco Laboratories, Detroit, MI, USA) containing 0.1% cysteine HCl (Sigma-Aldrich, St. Louis, MO, USA) for specified times as described in the results or Figure legends.

GENETIC MANIPULATION

Constructs for the deletion of FTL1611 (*flmF2*) and FTL1609 (*flmK*) were made in pJC84 (Wehrly et al., 2009). The ~1 kb upstream region of *flmF2* was amplified using forward primer JG1823 (5′-aaacgagctcgGGGTTATGGTGACTTCTGCATC-3′) with *Sac*I restriction site at the 5′ end and reverse primer JG1824 (5′-cgcggatccCACAAATACAAAATATATTAACCTTAATTAATGCTATTATAACC-3′) with a 5′ *Bam*HI restriction site. The ~1 kb downstream region was amplified using forward primer JG1825 (5′-cgcggatccAATATTGTTTTAAGCTAATGAAT CAATACTTATTAAATTCTTAG-3′) and reverse primer JG1826 (5′-acgcgtcgacGTATTATATTTTTAGTAGCAGCTGTTGCTGTTAT-3′) with *Bam*HI and *Sal*I 5′ flanking restriction sites, respectively. Similarly, the *flmK* upstream region was amplified by JG2290 (5′-aaacgagctcgGATCTAATACTGGATACCACTCATTATC-3′) forward primer with a 5′ *Sac*I site and JG2291 (5′-cgcggatccCTTCTTTACCCTCAAATAGAAACTTATAC-3′) reverse primer with a 5′ *Bam*HI site, and the downstream region using the JG2292 (5′-cgcggatccGATTTATCAGCATTAATTACTTTGATAAGCTAAG-3′) forward primer with a 5′ *Bam*HI site and JG2293 (5′-acgcgtcgacCATAGATAAGCGTACAGTTGTTTCATG-3′) reverse primer with a 5′ *Sal*I site. Fragments were cloned in pJC84 sequentially and the construct was transformed into *Ft* LVS followed by chromosomal recombination using the procedure described by Wehrly et al. (2009). Mutants were confirmed by PCR amplification and sequencing of the deleted region.

MICROSCOPY

Choc II plates containing bacteria were visualized under oblique light settings as suggested by Robert Miller at Dynport Vaccine Company LLC, Frederick, MD, USA and as described by Eigelsbach (Eigelsbach et al., 1951). Briefly, a focused light source, concave mirror and dissecting microscope with 10× objective magnification and a transparent stage were used to visualize blue and gray variants. The concave mirror was placed horizontally tilted upward so that the light beam would hit the upper concave region and the distance between mirror and the microscope is adjusted so that the light beam would reflect on the plate sitting on the stage of the microscope. Blue and gray colonies were observed and counted using these conditions. Samples were prepared for electron microscopy from overnight (~16 h) grown cultures of *Ft* LVS or *Ft* LVSG in TSB containing 0.1% cysteine HCl using methods as described previously (Mohapatra et al., 2008). In brief, cells were pelleted by centrifugation, washed in PBS, and fixed with 2.5% warm glutaraldehyde for 15 min. followed by fixing with a combination of 2.5% glutaraldehyde and 1% osmium tetroxide in 0.1 M sodium cacodylate (pH 7.3) for 15 min at 4°C. Staining of the cells was accomplished by using 0.25% uranyl acetate in

0.1 M sodium acetate buffer (pH 6.3) for 45 min, and viewed after further processing by transmission electron microscopy using an FEI Technai G2 Spirit microscope at 60 kV. Multiple fields (>50) were examined to determine the average size (diameter and length) and shape of bacteria.

SILVER STAINING AND WESTERN BLOTTING

Overnight (~24 h) grown bacteria from Choc II agar plates were suspended in PBS at a concentration of 3×10^{10} CFU/ml as determined previously by the optical density (OD_{600}) of diluted cultures and subsequent colony counts on solid agar. Bacteria equalized by optical density (OD_{600}) were then pelleted, frozen, and lyophilized overnight to obtain ~20 mg of dry cells. LPS was purified using hot phenol/water method using the standard protocol as described by Apicella et al. (1994).

Lipopolysaccharide was separated by 15% SDS-PAGE and silver stained as described (Clay et al., 2008). Briefly, after fixing overnight in 40% ethanol and 5% acetic acid, gels were incubated in 0.7% periodic acid in fixing solution for 7 min and subsequently washed with multiple exchanges of water. The staining solution (0.013% concentrated ammonium hydroxide, 0.02 N sodium hydroxide, and 0.67% silver nitrate (w/v) was applied with vigorous agitation for 10 min, followed by three washes (each 10 min) in water. Gels were developed using a solution containing 0.275% monohydrous citric acid (w/v) and 0.0025% formaldehyde. Upon completion, 5% acetic acid was used to stop the development.

Purified LPS samples (10 μg/well) were electrophoresed on a 15% SDS-PAGE gel and transferred on nitrocellulose membrane using the Bio-Rad semi-dry transfer system. Immunoblotting was performed using either anti-*F. tularensis* LVS or *F. novicida* polyclonal sera (from infected mice) or monoclonal sera specific to the LPS of *Ft* LVS or *F. novicida*. Polyclonal sera to *Ft* LVS or *F. novicida* (1:1000 dilution), commercial *F. tularensis* FB-11 (1:1000, Abcam, Cambridge, MA, USA), *F. tularensis* LPS specific monoclonal (1:10), or *F. novicida* LPS specific monoclonal (1:10) were used as primary antibodies with alkaline phosphatase conjugated goat anti-mouse IgG (1:4000) as the secondary antibody. The *F. tularensis*-specific and *F. novicida*-specific anti-LPS monoclonal antibodies were obtained from monoclonal hybridoma cell lines (ImmunoPrecise, Victoria, BC, Canada). Blots were developed using 5-bromo-4-chloro-3-indolyl phosphate/NBT (Sigma-Aldrich) as the substrate.

O-ANTIGEN AND CORE ANALYSIS

Lipopolysaccharide was isolated using the hot phenol/water method (Apicella et al., 1994). Crude LPS was enzymatically treated to remove contaminating nucleic acids and proteins and ultracentrifuged for 18 h. The LPS pellet was collected and the carbohydrate portion of LPS was released from lipid A via 2 h mild hydrolysis with 1% acetic acid at 100°C followed by centrifugation of the lipid A at 3500×*g*. The carbohydrate fraction in the supernatant was extracted threefold with chloroform to remove any contaminating lipid A, lyophilized, re-suspended in water, filtered through nylon filter 0.2 μm prior the HPLC separation, and lyophilized again. Carbohydrates from *Ft* LVS and *Ft* LVSG of were resolved on a Superdex Peptide HPLC column with ammonium acetate used as an eluent. The eluting fractions were pooled and salts removed by repeated evaporations from de-ionized water on a rotary evaporator. The elution profiles for the *Ft* LVS and *Ft* LVSG carbohydrates were examined and Fraction 1 contained the O-polysaccharide (OPS), Fraction 2 contained slightly lower molecular weight OPS, and Fraction 3 contained the core oligosaccharides (OSs) with some possibly low molecular weight OPS repeat units. Fractions 1 and 3 were analyzed by NMR spectroscopy. Fraction 1 from both LVS and LVSG were compared to each other using 2D NMR. OSs found in Fraction 3 from LVS and LVSG were analyzed by 1D proton NMR spectroscopy.

LIPID A ANALYSIS

LPS purification and lipid A isolation

Lipopolysaccharide was isolated using the rapid small-scale isolation method for mass spectrometry analysis as described (Yi and Hackett, 2000). Briefly, 1.0 ml of Tri-Reagent (Molecular Research Center, Cincinnati, OH, USA) was added to a cell culture pellet (2–5 ml of an overnight culture), re-suspended, and incubated at room temperature for 15 min. Chloroform (200 μl) was added, vortexed, and incubated at room temperature for 15 min. Samples were centrifuged for 10 min at 12,000 rpm and the aqueous layer was removed. An aliquot of water (500 μl) was added to the lower layer and vortexed well. After 15–30 min, the sample was spun down and the aqueous layers were combined. The process was repeated two more times. The combined aqueous layers were lyophilized overnight. Lipid A was isolated after hydrolysis in 1% SDS at pH 4.5 (Caroff et al., 1988). Briefly, 500 μl of 1% SDS in 10 mM Na-acetate, pH 4.5 was added to a lyophilized sample. Samples were incubated at 100°C for 1 h and lyophilized. The dried pellets were resuspended in 100 μl of water and 1 ml of acidified ethanol (100 μl 4 N hydrochloric acid in 20 ml 95% ethanol). Samples were centrifuged at 5,000 rpm for 5 min. The lipid A pellet was further washed three times in 1 ml of 95% ethanol. The entire series of washes was repeated thrice. Finally, samples were re-suspended in 500 μl of water, frozen on dry ice, and lyophilized. Alternatively for harsher lipid A cleavage conditions, LPS samples were dissolved in water, and mixed with the same volume of 10% acetic acid to give final 5% acetic acid concentration. Samples were hydrolyzed with 5% acetic acid (100°C, 2 h, with constant stirring- the precipitate appeared after 1 h). The precipitate was collected by centrifugation at 14,000 rpm for 6 min, then re-suspended in water and lyophilized. The supernatant was stored for future chemical analyses.

MALDI-TOF mass spectrometry

MALDI-TOF mass spectrometry analysis of lipid A was performed on a Voyager spectrometer. The samples were dissolved in CH_3Cl–CH_3OH mixture (3:1) and 1 μl of each mixed with 1 μl of 0.5 M 2,5 dihydroxybenzoic acid in methanol matrix solution. Other MALDI-TOF experiments were performed using a Bruker Autoflex II MALDI-TOF mass spectrometer (Bruker Daltonics, Inc., Billerica, MA, USA). Each spectrum was an average of 200 shots. Calibration was performed with ES Tuning Mix (Agi-lent, Palo Alto, CA, USA). Spectra were recorded in both the negative-ion and positive-ion modes.

GC–MS fatty acid analysis

Fatty acids were analyzed as methyl esters. The lipid-containing fraction was dissolved in 0.5 ml of 2 M MeOH–HCl and the mixture was kept at 80°C for 18 h. After cooling down, the hydrolyzate

was mixed with 0.5 ml 50% NaCl solution and extracted with 1 ml of chloroform. The organic layer was collected and aqueous layer extracted two times more with chloroform. Combined organic layers were extracted again three times with water. Water traces were removed from collected chloroform phase by addition of anhydrous Na_2SO_4. The organic phase was then filtered through cotton filters prewashed with chloroform, concentrated under the stream of nitrogen and applied to GC–MS analyses.

Dephosphorylation of lipid A

To remove phosphate groups from lipid A, samples were treated with HF for 48 h at 4°C with constant stirring. HF was evaporated from samples under vacuum in a desiccator attached to NaOH trap for 1 h then removed with nitrogen.

Trimethylsilyl analysis of fatty acid methyl esters

To show the presence of hydroxyl groups in fatty acids of lipid A, the fatty acid methyl esters were treated with TriSil reagent for 30 min at 80°C. The samples were cooled and dried under a nitrogen stream. Derivatized samples were suspended in hexane and filtered through cotton filters. Filtrates were condensed under a nitrogen stream and analyzed by GC–MS.

Galactosamine quantification

Standards and samples were prepared using the established protocol (Kalhorn et al., 2009). Stock solutions of carbohydrate and internal standards were prepared in deionized water to a final concentration of 100 ng/ml. Serial dilution of carbohydrate standards were prepared to 0.125–25 ng/ml. Internal standards were prepared to a final concentration of 5 ng/ml. Individual samples containing a cocktail of carbohydrate standards ranging from 0.125 to 25 ng/ml in addition to the 5 ng/ml internal standard were prepared and lyophilized in glass screw top vials. After lyophilization, 100 ml water was added followed by 100 ml 2 M TFA. The standard samples were sealed with polytetrafluoroethylene (PTFE)-lined caps, vortexed briefly and heated at 90°C for 30 min for analysis, flash frozen and lyophilized. TFA-treated samples were reconstituted in 50 µl 0.2 M borate buffer, pH 8.8, and 50 µl 1.0 mg/ml derivatizing/labeling reagent (AccQ-Tag) in dry acetonitrile. The samples were vortexed and incubated at room temperature for 15–30 min after which they were dried under nitrogen stream at room temperature. Derivatized samples were reconstituted in 100 µl of distilled water, vortexed, and transferred to injection vials for analysis by GC–MS.

For analysis of lipid A samples, 10 mg of lipid A isolated from individual preparations was used. The vial was incubated at 90°C for 24 h for analysis of galactosamine. Aliquots were then frozen and lyophilized to dryness. They were then derivatized by the same procedure as the standards as described above (Kalhorn et al., 2009).

REAL-TIME PCR

RNA from log phase (0.4–0.5 optical density at 600 nm) cultures of *Ft* LVS and *Ft* LVSG was extracted using the RNeasy kit (Qiagen, Valencia, CA, USA). The quality and quantity of RNA was determined using the Experion automated electrophoresis system (Bio-Rad, Hercules, CA, USA). One microgram of total RNA was reverse transcribed to cDNA using Superscript II RNase H^- reverse transcriptase (Invitrogen, Carlsbad, CA, USA). cDNA was then normalized according to the concentration and 2 ng of the converted cDNA was used for quantitative PCR with the SYBR green PCR master mixture in the Bio-Rad iCycler apparatus (Bio-Rad, Hercules, CA, USA). All primers were designed to give 200- to 220-nucleotide amplicons with melting temperatures of 48–52°C. Relative copy numbers and expression ratios of selected genes were normalized to the expression of the housekeeping gene (*dnaK*) and calculated as described by Mohapatra et al. (2007).

INTRAMACROPHAGE SURVIVAL ASSAYS

Human monocyte-derived macrophages (MDMs) were isolated using standard procedure as described elsewhere (Mohapatra et al., 2010) and obtained with informed consent from healthy donors by an OSU IRB approved protocol. Intramacrophage survival assays in human MDMs were performed using following procedure; 2×10^6 PBMCs/well (MDMs plus lymphocytes) were plated in a 24-well plate resulting in 2×10^5 MDMs/monolayer after adherence of MDMs and washing. *Francisella* spp. were opsonized with 0.1% serum for 30 min at 37°C. Macrophages were infected with *Ft* LVS, *Ft* LVSG and *F. novicida* at an MOI of 50 and incubated at 37°C in a CO_2 (5%) incubator for 2 h. Cells were washed and 50 µg/ml gentamicin was added to each well and incubated for 30 min. Cells were washed and replenished with fresh media containing 10 µg/ml gentamicin. At various time points cells were washed and lysed with 0.1% SDS and plated on Choc II plates to enumerate the colony forming units.

MICE VIRULENCE ASSAYS

Bacteria grown overnight (~24 h) on Choc II plates were scraped and suspended, washed twice and diluted in PBS. Four- to six-week-old BALB/c mice were anesthetized and infected with ~1000 bacteria in a 20-µl volume by the intranasal route and dilutions were plated on Choc II plates to enumerate the inoculum. Mice were anesthetized and challenged with 1000 CFU of overnight (~24 h) grown *F. tularensis* subsp. *tularensis* SchuS4 intranasally 4 weeks post vaccination and observed daily for survival. These procedures were performed as described in an OSU IACUC approved protocol in an inspected and approved biosafety level 3 laboratory.

RESULTS

BLUE–GRAY PHENOTYPIC VARIATION

Francisella tularensis LVS phase has been observed to vary from a blue (wild-type) colony to a gray colony variant (Eigelsbach et al., 1951; Cowley et al., 1996). Such gray variants have been both characterized with an extended LPS O-antigen (Cowley et al., 1996) as well as a truncated O-antigen (Hartley et al., 2006). Our work described here is with the *Ft* LVSG isolate (a variant with an extended O-antigen), but at times comparisons are made to *Ft* LVSGD (a variant with no O-antigen). We examined various media conditions and growth phases to determine the conditions that affected the degree of phase variation. We found that *Ft* LVSG grows slower than *Ft* LVS, forms smaller colonies on agar surfaces, and appeared gray by eye on Choc II agar plates under oblique lighting (**Figures 1A,B**). The frequency of blue to gray phase variation was higher (27–31%) in liquid cultures (TSB + 0.1% cysteine HCL) grown to stationary phase (typically 30–48 h) and plated on solid agar. The frequency of

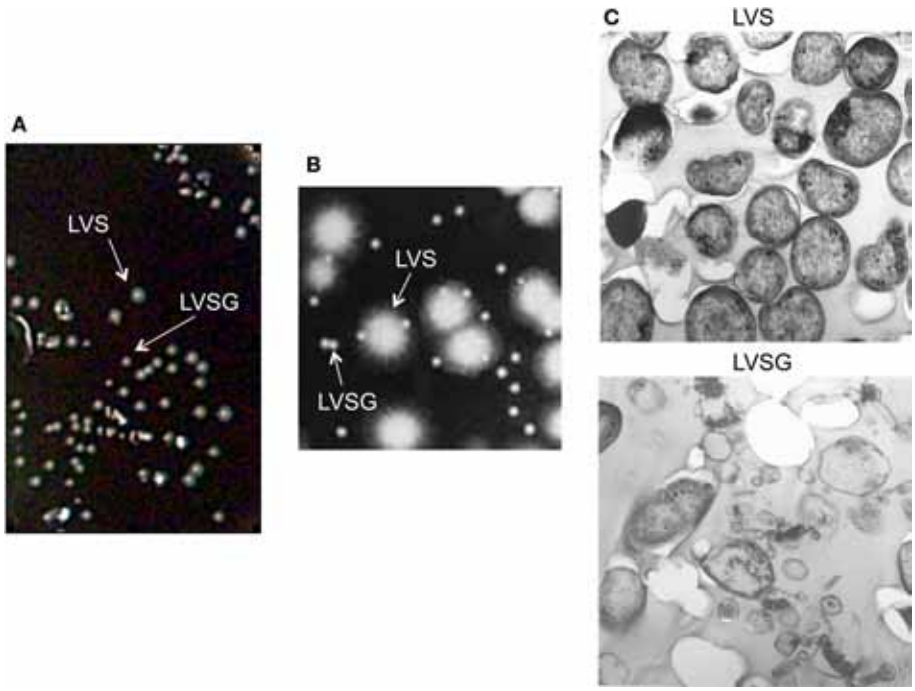

FIGURE 1 | *Ft* LVS blue–gray colony and bacterial morphology. (A) Mixed cultures of *Ft* LVS and phase variant (*Ft* LVSG) viewed on the surface of a Choc II agar plate. Note that *Ft* LVSG forms smaller colonies that *Ft* LVS. **(B)** Blue (LVS) and Gray (LVSG) colonies viewed under a compound light microscope by oblique lighting. The smaller colonies appeared gray. **(C)** Transmission electron microscopy of *Ft* LVS and *Ft* LVSG. While the bacterial dimensions were similar between these two strains from measuring >50 fields, the *Ft* LVSG strain formed a large amount of membrane vesicles.

phase variation was minimal (2–5%) for bacteria grown on plates 1–2 days and in log phase liquid cultures. We also observed that the frequency of blue to gray phase variation dramatically increased when *Ft* LVS was passed through macrophages (23–27%) or recovered from organs of infected animals (31–36%). We also observed that the frequency of forward phase variation in broth grown bacteria (blue to gray) was always higher (~30%) than frequency of reverse (gray to blue) phase variation (5–7%).

To more clearly compare *Ft* LVS to *Ft* LVSG bacteria, log phase cultures were examined by scanning electron microscopy. Comparisons of average cell size were not significantly different, but more membrane vesicles were observed in *Ft* LVSG cultures (**Figure 1C**). It is not clear what impact this increased vesiculation has on the subsequent phenotypes described for *Ft* LVSG.

FT LVSG LPS POSSESSES LESS O-ANTIGEN

It was shown previously that the LPS of *Ft* LVS and *Ft* LVSG had differential reactivity to monoclonal antibodies stated to be O-antigen specific (Cowley et al., 1996), suggesting an O-antigen antigenic switch. To further examine the LPS O-antigen and its antigenic properties, we purified LPS from *Ft* LVS, *Ft* LVSG, *F. novicida*, *Ft* LVSGD, *F. tularensis* SchuS4 and a *F. tularensis* SchuS4 small colony gray variant and performed silver staining on SDS-PAGE separated samples. Consistent with previously published results, the gray variant (*Ft* LVSG) possessed an O-antigen but *Ft* LVSGD was rough (lacked O-antigen) (**Figure 2A**). The *F. tularensis* SchuS4 small colony gray variant also appeared to produce an LPS with a repeating O-antigen.

Glycosyl composition analysis of the OSs released from the purified LPS preparations show that the *Ft* LVS and *Ft* LVSG OSs contain the same glycosyl residues, but there is a large quantitative difference, in that the *Ft* LVS OS contains much larger amounts of QuiN and Gal than what is found in the OS from the *Ft* LVSG strain (**Table 1**). The QuiN could be due to QuiNFo as NMR analysis (data not shown) shows a significant resonance at around 8 ppm, which is consistent with a formyl proton. During the preparation of trimethylsilyl (TMS) methyl glycoside, which is accompanied by N-acetylation, this formyl group would have been replaced by an acetyl group. The large difference in these components between the LPS of *Ft* LVS and *Ft* LVSG would indicate that the *Ft* LVS LPS contains much more of the QuiN/Gal-containing O-antigen chain polysaccharide than *Ft* LVSG. Thus, these data suggest that the *Ft* LVSG LPS has an O-antigen but the O-antigen contains fewer repeating units than seen in *Ft* LVS LPS or that LVSG lipid A-core is capped less frequently with O-antigen. Interestingly, even though the two variants (*Ft* LVSG and *Ft* LVSGD) have distinct LPS regarding the amount of O-antigen present (albeit both with amounts less than that of wildtype), they give rise to morphologically similar gray variants.

MONOCLONAL AND POLYCLONAL ANTIBODY REACTIVITY AND LPS STRUCTURAL ANALYSIS SUGGESTS BLUE/GRAY STRAIN LPS CORE ALTERATIONS

Western blot analysis was performed on LPS samples using commercially available anti-*F. tularensis* FB-11 (**Figure 2B**) antibodies stated to be specific to the O-antigen, as well as anti-*F. tularensis* (**Figure 2C**)

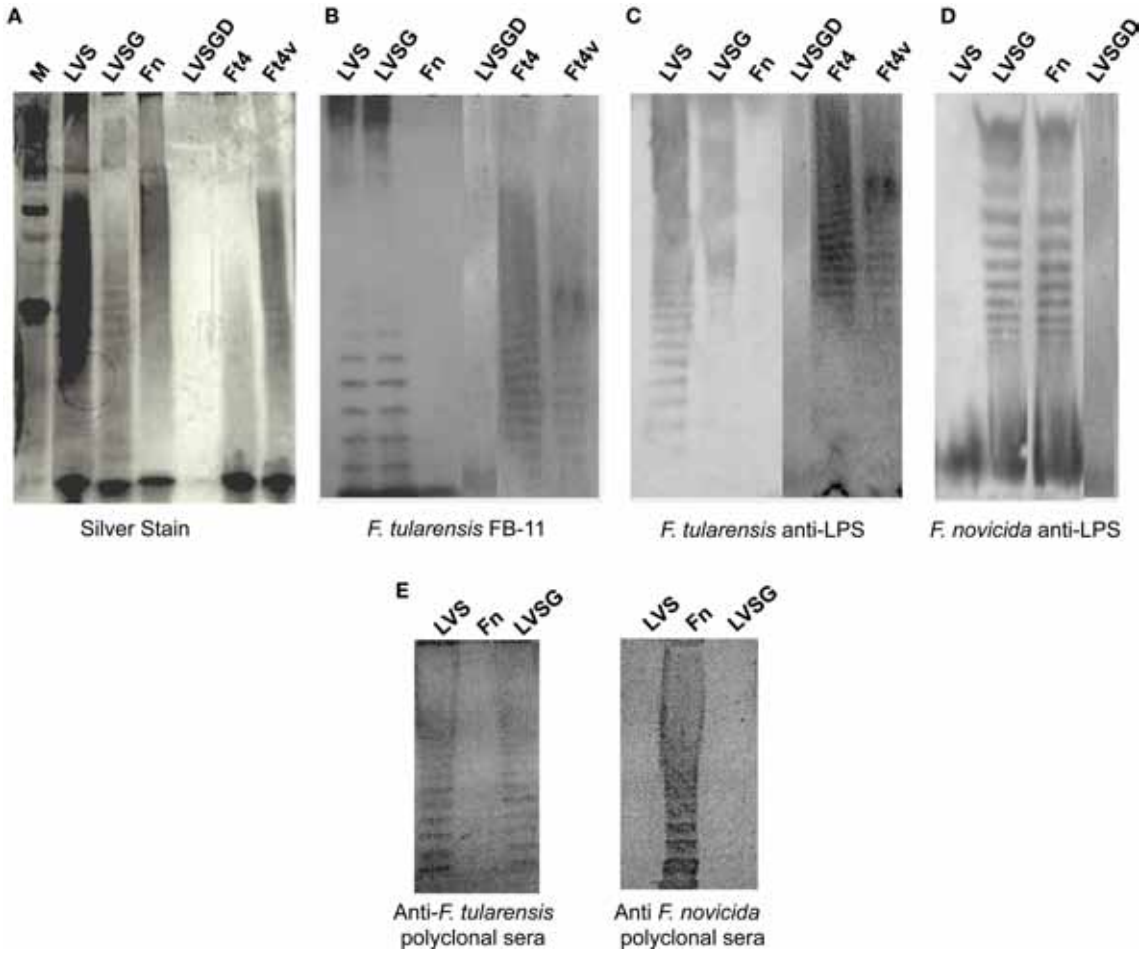

FIGURE 2 | Lipopolysaccharide analysis by Silver staining and Western blotting. (A) Silver stained gel of LPS purified from various strains listed above the lanes. **(B)** Immunoblotting of purified LPS of *Francisella* spp. with the anti-LPS O-antigen *F. tularensis* FB-11 antibody. *F. tularensis* FB-11 (1:1000) and secondary goat anti-mouse IgG (1:4000) were used. **(C)** Immunoblotting of purified LPS of *Francisella* spp. with *F. tularensis* anti-LPS specific monoclonal antibodies. *F. tularensis* IgG (1:10) and secondary goat anti-mouse IgG (1:4000) were used. **(D)** Immunoblotting of purified LPS of *Francisella* spp. with *F. novicida* anti-LPS specific monoclonal antibodies. *F. novicida* #5 IgG (1:10) and secondary goat anti-mouse IgG (1:4000) were used. **(E)** Immunoblotting of LPS with polyclonal sera specific to *Ft* LVS or to *F. novicida*. Polyclonal sera (1:1000) and secondary goat anti-mouse IgG (1:4000) were used. Lane assignments; M (Molecular weight marker), LVS (blue colonies), LVSG (gray variant), Fn (*F. novicida*), LVSGD (rough gray variant), Ft4 (*F. tularensis* SchuS4), Ft4v (*F. tularensis* SchuS4 gray variant).

Table 1 | Trimethylsilyl methyl glycoside analysis of OSs purified from the LPS of *Ft* LVS LPS and the gray variant *Ft* LVSG LPS.

OS	Man	Gal	Glc	QuiNAc*	GalNAc	Kdo
LVS	13	18	28	36	3	3
LVSG	24	5	62	0.5	7	3

**The amount of the QuiNAc could not be precisely quantified since there is no original standard available and, therefore, quantification was based on using the response factor for GlcNAc.*

Man, mannose; Gal, galactose; Glc, glucose; QuiNAc, 2-acetamino-2,6,dideoxy-D-glucose; GalNAc, N-acetyl galactosamine; Kdo, 3-deoxy-D-manno-octulosonic acid.

and anti-*F. novicida* LPS monoclonal antibodies (**Figure 2D**). The results showed that *Ft* LVS LPS reacted with both the *F. tularensis* monoclonal and FB-11 antibodies showing the typical LPS laddering, and while the laddering was not observed on *Ft* LVS LPS Western blots with the *F. novicida* specific antibodies, reactivity was observed to a low molecular weight species that is typically lipid A plus core (**Figures 2B–D**). *F. novicida* LPS did not react at all to the anti-*F. tularensis* LPS or FB-11 antibody, but strongly to a low molecular weight species (plus typical laddering) with the *F. novicida* specific monoclonal antibody. *Ft* LVSG reacted with both *F. tularensis* and the *F. novicida* anti-LPS monoclonal antibodies. These results are consistent with those of Cowley et al. (1996) with regard to O-antigen ladder reactivity, but the lipid A core region is not clearly visible on their gels. As expected, LVSGD did not demonstrate the typical O-antigen ladder due to its the lack of O-antigen (**Figures 2B–D**). The *F. tularensis* SchuS4 gray variant reacted with both anti-*F. tularensis* monoclonal antibodies and clearly possesses an O-antigen based on the observed laddering. However, the modal chain length or capping frequency of this O-antigen, like that of *Ft* LVSG, appears reduced versus *F. tularensis* SchuS4 (**Figures 2B–D**).

These LPS samples were reacted in a Western blot with anti-*F. tularensis* or anti-*F. novicida* polyclonal sera generated from infected mice (**Figure 2E**). *Ft* LVS LPS and *F. novicida* LPS only reacted with their respective antisera while *Ft* LVSG now reacted only with *F. tularensis* polyclonal sera. These results suggest that changes in the *Ft* LVSG LPS are specifically recognized by monoclonal but not polyclonal antibodies.

We next isolated and performed extensive NMR analyses on the *Ft* LVS and *Ft* LVSG OPSs to determine if any structural differences could be detected between these molecules. Crude LPS was enzymatically treated to remove contaminating nucleic acids and proteins and ultracentrifuged. The LPS pellet was collected and the carbohydrate portion of LPS was released from lipid A via mild hydrolysis. The carbohydrate fractions from *Ft* LVS and *Ft* LVSG of were resolved by HPLC. The carbohydrates eluted in three primary fractions. Fraction 1 contained the OPS, Fraction 2 contained slightly lower molecular weight OPS, and Fraction 3 contained the core OSs with some possibly low molecular weight OPS repeat units. From these results, the Fraction 1/3 ratio for *Ft* LVS LPS is 17, while it is 4.6 for LVSG (**Table 2**). These results are consistent with the above data showing that the *Ft* LVSG strain contains less OPS as reflected by the lower QuiN level during composition analysis. Fractions 1 and 3 were analyzed by NMR spectrometry. Fraction 1 from both *Ft* LVS and *Ft* LVSG were compared to each other using 2D NMR experiments – COSY, TOCSY, NOESY, and HSQC (data not shown). These results indicate that the OPS from *Ft* LVS and *Ft* LVSG have the same structures. In addition, the data are completely consistent with the structure reported for *F. tularensis* strain 15, strain SchuS4, and OSU10 (Vinogradov et al., 2002; Prior et al., 2003; Thirumalapura et al., 2005). The results clearly support the conclusion that *Ft* LVS and *Ft* LVSG have the following OPS structure as previously reported for the above *F. tularensis* strains:

-4)-α-D-Gal*p*NAcAN-(1 → 4)-α-D-Gal*p*NAcAN-(1 → 3)-β-D-Qui*p*NAc-(1 → 2)-β-D-Qui*p*4Fo-(1 →

The differential staining with the *F. tularensis* and *F. novicida* monoclonal antibodies coupled with the fact that the *F. novicida* monoclonal antibody binds to the LMW LPS and is, therefore, likely binding to the core OS, suggests that the true monoclonal antibody-tracked alteration between *Ft* LVS and *Ft* LVSG is related to the core region. Therefore, we analyzed OSs found in Fraction 3 from *Ft* LVS and *Ft* LVSG by 1D proton NMR spectroscopy. The results are shown in **Figure 3**. The spectrum of *Ft* LVS Fraction 3 indicates that a small amount of truncated OPS is still present.

Table 2 | Main fraction and yields obtained in gel filtration using Superdex peptide column.

Oligosaccharide	HPLC Fr1 (mg) (tube 18–25)	HPLC Fr2 (mg) (tube 26–28)	HPLC Fr3 (mg) (tube 29–50)
Ft LVS	7.62*	0.3	0.46**
Ft LVSG	0.65*	0.02	0.14**

**Used in 1D and 2D NMR experiments.*
***Used in 1D NMR experiments.*

We also see resonances that are consistent with the published core structure with the exception that we do not observe evidence for the core GalNAc residue. The *Ft* LVS and *Ft* LVSG proton spectra clearly differ from one another indicating that the *Ft* LVSG Fraction 3 contains different structures than found in the *Ft* LVS Fraction 3. Therefore, since the OSs in Fraction 3 would be those that would comprise the core region, as well as some possible truncated OPS, these data support the conclusion that the *Ft* LVSG has an altered core region compared to that of *Ft* LVS.

ANALYSIS OF *FT* LVSG LIPID A DEMONSTRATES A REDUCTION IN GALACTOSAMINE MODIFICATION

Cowley et al. (1996) demonstrated that *Ft* LVSG lipid A versus that of *Ft* LVS elicited increased NO induction in rat macrophages. We confirmed this finding (NO production by rat macrophages measured as nitrate by the Griess reagent system) by both *Ft* LVSG LPS and purified lipid A (data not shown). This suggested that the lipid A of *Ft* LVSG was different than that of *Ft* LVS. To explore these differences, we performed structural analyses on purified lipid A of *Ft* LVS and *Ft* LVSG. MALDI-TOF analysis in negative and positive ion mode was performed on replicate lipid A preparations.

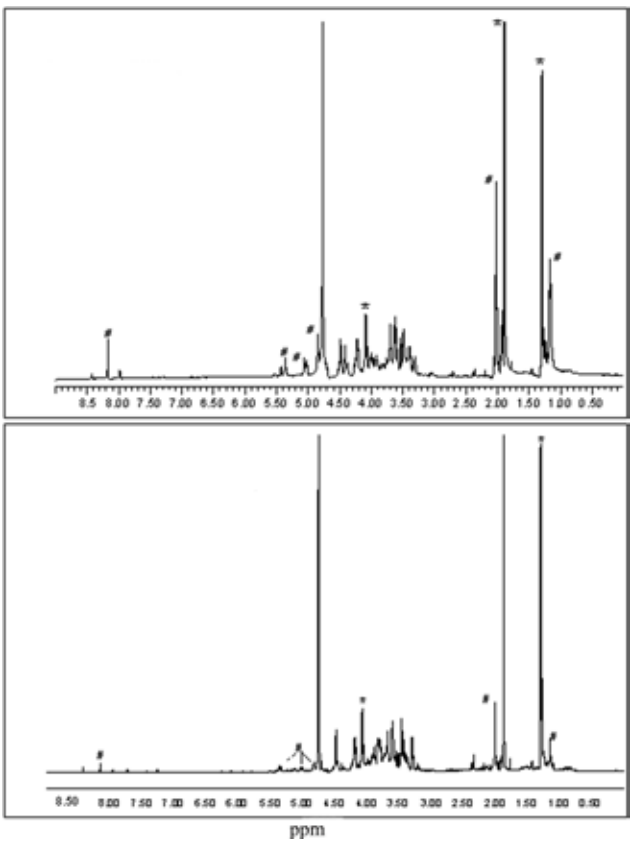

FIGURE 3 | The proton NMR spectra of the LPS core region oligosaccharides from *Ft* LVS and *Ft* LVSG. The proton NMR spectra of the oligosaccharides found in Fraction 3 from *Ft* LVS (top) and Fraction 3 from *Ft* LVSG (bottom) are shown. The resonances marked with # are likely due to OPS fragments. Those marked with * are due to contaminating acetate and lactate. Thus, non-marked resonances denote the LPS core region.

Both showed the absence of a peak at *m/z* 1666 in *Ft* LVSG that was present in *Ft* LVS. It is known that this peak represents the addition of galactosamine (161 Da) to the basic structure at *m/z* 1504 (2× GlcN, 3× C18:0 (3-OH), C16:0, P), though previous data suggested that this modification was not observed in the *Ft* LVS strain (Kanistanon et al., 2008; **Figures 4A,B**). This was the only structural alteration noted. Since the MALDI-TOF analysis is only semi-quantitative, we performed galactosamine quantitation assays to further demonstrate the reduced galactosamine modification of lipid A in *Ft* LVSG. The lipid A was derivatized and analyzed by GC–MS. *F. novicida* showed the highest degree of modification at 25% while *Ft* LVS was at 14%. Both *Ft* LVSG and *Ft* LVSGD showed reduced galactosamine modification, with 6 and 7%, respectively (**Figure 5A**).

Three genes have been identified that are responsible for galactosamine or mannose lipid A modification (Gunn and Ernst, 2007; Kanistanon et al., 2008). The transferases FlmF1 and FlmF2 are required for adding mannose or glucosamine residues, respectively, to the lipid A. The glycosyltransferase FlmK can add both mannose and galactosamine to lipid A (Gunn and Ernst, 2007; Kanistanon et al., 2008). A real-time PCR assay was performed on the genes *flmF2* and *flmK* from *Ft* LVS and *Ft* LVSG to determine if their expression was altered and might be responsible for the observed lipid A galactosamine modification alteration. Expression of both genes was found to be significantly less in *Ft* LVSG (**Figure 5B**), correlating with the reduction in galactosamine modification. Mutation of the *flmF2* gene in *Ft* LVS, which eliminates the galactosamine modification, reduced the frequency of phase variation from 30% to 5-7% in stationary phase liquid cultures. Though *F. novicida* strains carrying this mutation have been shown to affect mouse virulence and cytokine/chemokine induction in macrophages, the *Ft* LVS *flmF2* mutant demonstrated no defect in survival in macrophages of mouse (Raw, J774.1 and MH-S), rat (bone marrow derived, alveolar), or human (THP-1 and monocyte derived macrophages) origin (data not shown, see below section).

THE *FT* LVSG VARIANT HAS A RAT AND HUMAN BUT NOT MOUSE INTRAMACROPHAGE SURVIVAL DEFECT

It has been shown previously by Cowley et al. (1996) that *Ft* LVS and *Ft* LVSG intracellular growth/survival was similar in mouse macrophages, but that differences in growth/survival could be visualized in rat bone marrow-derived macrophages. We examined the survival of *Ft* LVS and *Ft* LSVG in various macrophages including J774.1 (a mouse macrophage cell line), MH-S (a mouse alveolar macrophage cell line) and mouse bone marrow-derived macrophages and did not find any significant differences in survival of *Ft* LVS and *Ft* LSVG (data not shown). However, we observed that *Ft* LVSG survived less well in rat bone marrow derived macrophages and a rat alveolar macrophage cell line (ATCC# CRL-2192) and that this inhibition of growth of *Ft* LVSG can be reversed by using the NO inhibitor NMMA (data not shown). These findings were consistent with the previous findings of Cowley et al. (1996). We

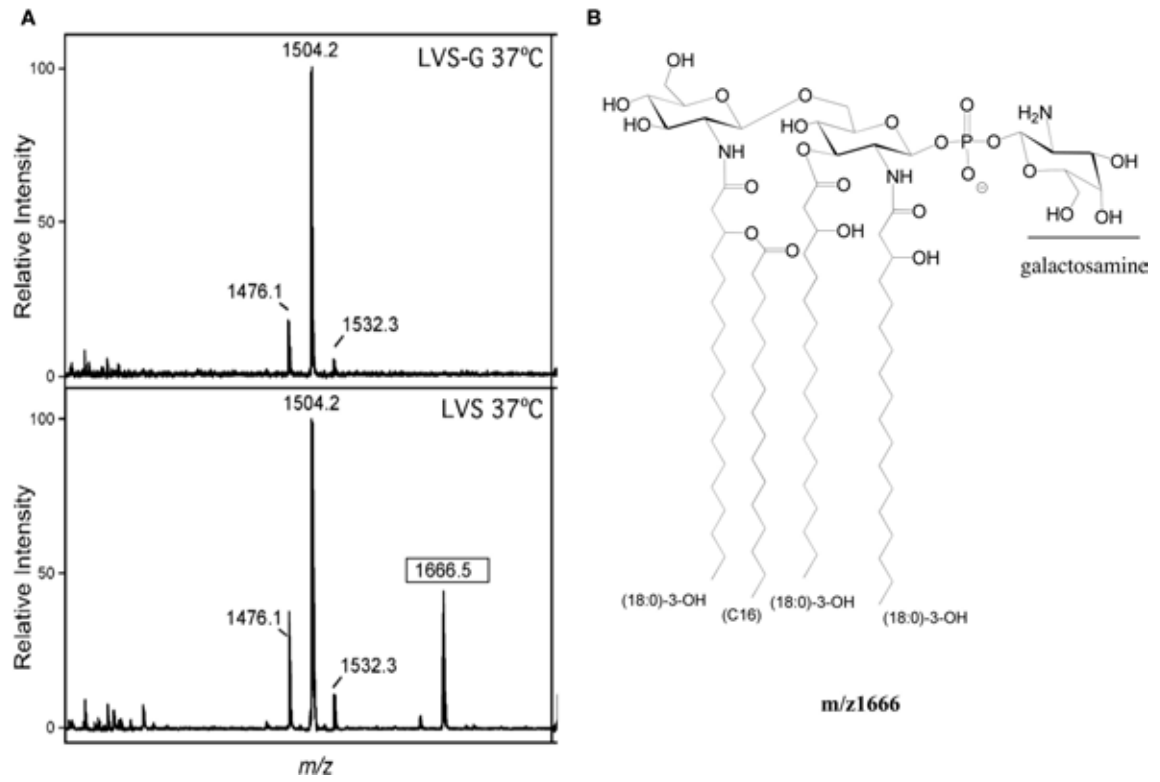

FIGURE 4 | Structural analysis of lipid A. (A) MALDI-TOF analysis of lipid A of *Ft* LVS and *Ft* LVSG. The boxed *m/z* denotes the species containing galactosamine that shows differential relative intensity between *Ft* LVS and *Ft* LVSG. **(B)** The *m/z* 1666 structure of lipid A of *Ft* LVS.

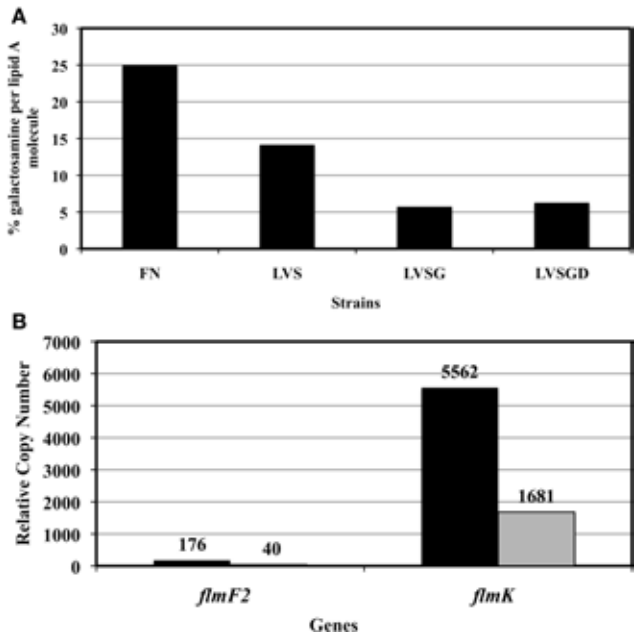

FIGURE 5 | (A) Glucosamine quantification in the lipid A samples. **(B)** Differential expression of LPS modification genes *flmF2* and *flmK* in *Ft* LVS and *Ft* LVSG as determined by real-time PCR. RCN-Relative copy number, actual values are shown above the bars.

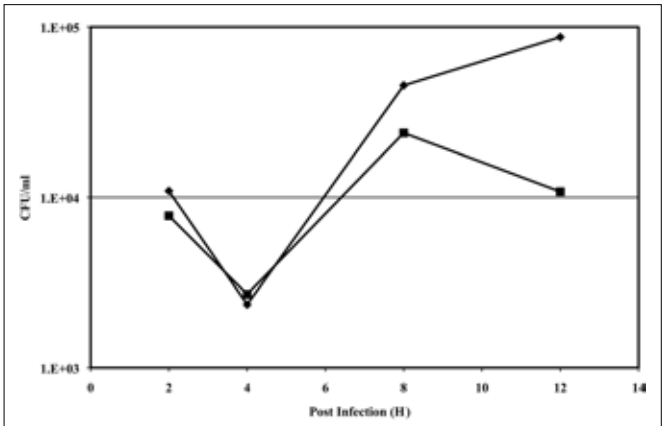

FIGURE 6 | Intramacrophage survival assay of *Ft* LVS and *Ft* LVSG in human monocyte-derived macrophages. Symbols: diamond, *Ft* LVS; square, *Ft* LVSG. The data presented is from an assay performed in triplicate with little variation between replicates (resulting in non-visible error bars), and this experiment was performed three times on separate occasions with similar results.

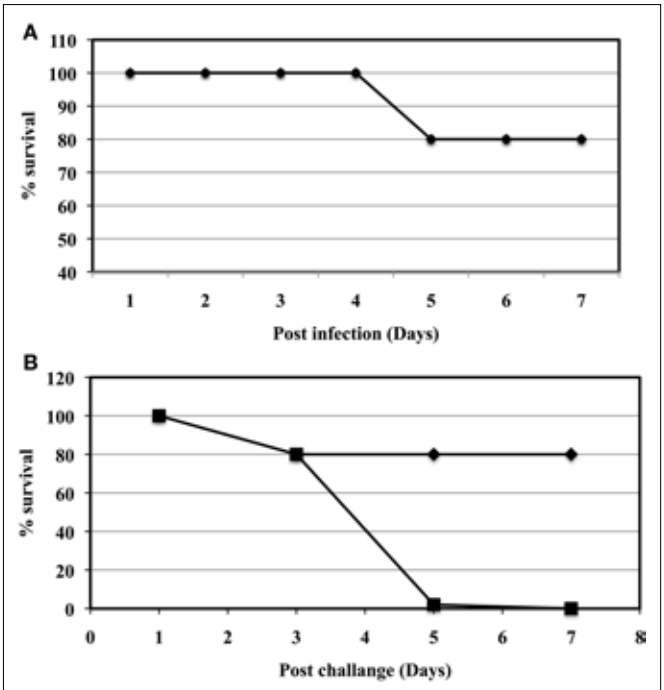

FIGURE 7 | Virulence assays in the mouse model of tularemia. (A) The virulence of *Ft* LVS and *Ft* LVSG in mice inoculated by the intranasal route. Symbols: diamond, *Ft* LVS; square, *Ft* LVSG. *N* = 10 mice per strain. **(B)** Challenge of vaccinated mice with *F. tularensis* SchuS4. Symbols: diamond, *Ft* LVS; square, *Ft* LVSG. *N* = 8 mice per strain.

then examined intramacrophage survival in human MDMs and the THP-1 macrophage-like cell line. We observed that the *Ft* LVSG strain survived less well over the first 12 h post-infection in both cell types with macrophage cell death at later time points. The defect was most prominent for the MDMs, where the *Ft* LVSG strain demonstrated nearly a log defect in survival at 12 h post-infection versus the *Ft* LVS strain (**Figure 6**). Thus, the *Ft* LVSG strain has an intramacrophage survival defect in rat and human but not mouse macrophages.

FT LVSG AND *FT* LVS ARE SIMILARLY VIRULENT IN THE MOUSE MODEL BUT DIFFERENTIALLY PROTECT AGAINST *F. TULARENSIS* SCHUS4 CHALLENGE

Gray variants have been shown to be less virulent (Eigelsbach and Downs, 1961) and/or less protective as vaccines against *F. tularensis* SchuS4 challenge (Eigelsbach and Downs, 1961; Cowley et al., 1996; Hartley et al., 2006; Conlan and Oyston, 2007) However, the specific virulence of the *Ft* LVSG strain in the mouse model of tularemia has not been determined. To compare the virulence of *Ft* LVS and *Ft* LVSG, BALB/c mice were infected with 100 CFU of *Ft* LVS and *Ft* LVSG intranasally and observed for survival. Both *Ft* LVS and *Ft* LVSG infected mice demonstrated 80% survival ($N = 10$ mice; **Figure 7A**). The surviving mice were challenged with 1000 CFU of *F. tularensis* SchuS4 (~100-fold above the LD_{50}) intranasally 4 weeks post vaccination (**Figure 7B**). All *Ft* LVS vaccinated mice ($N = 8$) survived the challenge whereas *Ft* LVSG vaccinated mice ($N = 8$) could not survive the challenge and succumbed to infection within 5 days. These results suggest that *Ft* LVSG is as virulent in mice as *Ft* LVS but it does not protect against Type A challenge.

DISCUSSION

Francisella tularensis LVS has been known to phase vary from a blue (i.e., wildtype) to a gray variant since the phenomenon was first described by Eigelsbach in 1951 (Eigelsbach et al., 1951). Such variation has proven to be an issue historically in vaccine production runs of *Ft* LVS (Conlan and Oyston, 2007; Oyston,

2009). The *Ft* LVS gray variants are problematic because they are dramatically less efficacious than the blue colony morphotypes in protection studies against the virulent *F. tularensis* Type A strain. The phenotypes associated with the gray variants are quite variable, as they have been described to give rise to different colony sizes and opacity, but the only two that have been molecularly characterized share the characteristic of LPS alteration. While this characteristic is shared, this also presents yet another difference, as the gray variant described by Hartley et al. (2006), (similar to *Ft* LVSGD studied in this work) has been shown to lack O-antigen while *Ft* LVSG still possessed an O-antigen, albeit reduced in amount, which has altered *F. novicida*/*F. tularensis*-LPS specific monoclonal antibody reactivity patterns. Thus, gray variants are themselves variable, but an increased understanding of the mechanism(s) behind this variation would aid future tularemia vaccine production.

In addition to the presence/absence of the O-antigen, we demonstrate biochemically that the *Ft* LVSG O-antigen chain carbohydrates are reduced in *Ft* LVSG versus *Ft* LVS. It is unclear if this demonstrates that the O-antigen chain length of LVSG is shorter or if the lipid A plus core is capped less efficiently with O-antigen. A reduction in *Ft* LVSG O-antigen chain carbohydrates was previously suggested in a manuscript by Clay et al. (2008) based on evidence from silver staining patterns and Western blot analysis. Clay et al. (2008) also demonstrated that the *Ft* LVSG strain was bound by complement component C3 in higher amounts than *Ft* LVS and was dramatically more susceptible to complement-mediated killing. It is of interest that, while susceptible to complement, *Ft* LVSG is still as virulent as *Ft* LVS in the mouse model. It is likely that the effect of complement-mediated killing is quantitative rather than absolute and that the current mouse model is not sensitive enough to record an effect. Alternatively, the result in the mouse model may reflect a fundamental difference in complement function between mouse and man. For example, it is known that C3 in mouse serum is more labile than in human serum and this may result in different levels of C3 opsonization and regulation in mouse versus human serum.

Both *F. tularensis* and *F. novicida* anti-LPS monoclonal antibodies reacted with LVSG, which had been interpreted to demonstrate that the O-antigen of *Ft* LVSG was altered, creating an epitope(s) reactive to both antibodies. However, closer examination of the Western blots show *F. novicida* monoclonal antibody reactivity to *Ft* LVSG LPS with low molecular weight species, likely lipid A plus core, as well as higher species containing lipid A, plus core, plus O-antigen repeats. The *F. novicida* anti-LPS monoclonal antibody reacted only with the low molecular weight species to *Ft* LVS LPS. This suggested that the *F. novicida* anti-LPS monoclonal antibody recognized a core epitope and that core, and not the O-antigen regions of *Ft* LVS and *Ft* LVSG, may differ. This was confirmed by NMR analysis of purified LPS carbohydrates, which demonstrated identity between the *Ft* LVSG and *Ft* LVS O-antigen regions while the LPS core region of *Ft* LVSG was different from that of *Ft* LVS. If, as we suspect, the *F. novicida* anti-LPS monoclonal antibody epitope is within the core region and the *Ft* LVS anti-LPS monoclonal epitope is within the O-antigen, then because of observed Western blot reactivities, both *Ft* LVS and *Ft* LVSG contain an "*F. novicida*-like" core. Thus, it is possible that *Ft* LVS makes two distinct core OSs (an *F. novicida* monoclonal antibody reactive and an *Ft* monoclonal antibody reactive), but cannot add O-antigen to the "*F. novicida*-like" core. *Ft* LVSG, on the other hand, can either produce both core types and ligate O-antigen to both, or can only make the *F. novicida* reactive core type and can add O-antigen to this core.

Regarding the mechanism of O-antigen chain length reduction/differential O-antigen ligation, these processes are typically mediated by an O-antigen ligase (e.g., RfaL), O-antigen polymerase (e.g., Wzy) and a chain length determinant (e.g., Cld). *Francisella* spp. appear to have multiple proteins with homology to RfaL, and their differential expression may account for the observed O-antigen phenotypes. BLAST searches of the *Ft* LVS genome with the RfaL locus of *Salmonella typhimurium* revealed three high scoring loci: FTL1122 (41% identity but 188 amino acids in *Ft* LVS versus 292 amino acids in *F. tularensis* SchuS4), FTL 0706 (37% identity), and FTL0598 (26% identity, called the *wzy* locus in *Ft* LVS; Prior et al., 2003). Surprisingly, BLAST searches with the *S. typhimurium* Wzy showed no strong identity to *Francisella* proteins. No Cld ortholog exists in *F. tularensis* (Prior et al., 2003; our recent BLAST searches). It is unclear if another unknown enzyme serves this function or is the O-antigen chain length is unregulated in this bacterium. Thus, it is possible that multiple or novel proteins mediate the O-antigen polymerase, ligase and length determination functions in *Francisella* and may be responsible for the core/O-antigen data described here. Further biochemical analysis is ongoing to determine the exact structural changes in the *Ft* LVSG LPS core region, as are genetic experiments to identify genes conferring *F. novicida* monoclonal antibody reactivity to *Ft* LVS.

The lipid A regions of *Ft* LVS and *Ft* LVSG were shown to be identical with the exception of a galactosamine modification, which was reduced/absent in the *Ft* LVSG variant. The galactosamine modification was not detected previously in *Ft* LVS, but was clearly evident in the *Ft* LVS strain analyzed here. Mannose is also observed as a modification of lipid A in *F. novicida* and Type A subspecies, but not Type B subspecies (the background of the *Ft* LVS strain). Consistent with these published data, mannose was not observed in our assays. While the *flmF2* (glucosamine addition) and *flmK* (both mannose and glucosamine addition) genes showed reduced transcription in the *Ft* LVSG strain consistent with the reduced lipid A modification, mutants in these genes in *Ft* LVS did not result in the small colony or gray phenotype on plates nor were the mutants defective in intramacrophage survival. Thus, these mutants did not exhibit obvious characteristics of the *Ft* LVSG gray variant. These mutants did, however, demonstrate a dramatically reduced phase variation rate to gray variants, suggesting that galactosamine modification may be involved in but is not sufficient for the gray phenotype. Further phenotypic testing (e.g., lipid A NO induction, mouse virulence) is ongoing, as is the construction of a double *flmF2 flmK* mutant. It is also possible that no expression versus reduced expression of these enzymes may have different effects, but titrated gene expression in *Francisella* is not yet a reality, so this concern will be addressed when the technology is available.

Experiments performed in this study confirmed those of Cowley et al. (1996) regarding increased NO induction in rat but not mouse phagocytes by the gray strain LPS. This correlated with decreased

survival of these gray variants in rat and human macrophages, which could be reversed in rat macrophages by the addition of a NO synthase inhibitor. However, experience suggests that stimulated mouse macrophages are better capable than, for e.g., human MDMs, of NO production. Thus, there is no direct correlation of strain intracellular survival with the inherent capabilities of the macrophages of the chosen animal model to produce NO, suggesting the involvement of additional factors.

Based on the results of Eigelsbach and colleagues (Eigelsbach et al., 1951; Eigelsbach and Downs, 1961) and Hartley et al. (2006), we were surprised that the *Ft* LVSG strain did not possess a virulence defect by the intranasal route. However, in the Hartley et al. (2006) study, mice were vaccinated and challenged by the subcutaneous route, and the Eigelsbach work (Eigelsbach et al., 1951; Eigelsbach and Downs, 1961) typically used intraperitoneal vaccination and subcutaneous challenge. Thus the route of administration may play a role in gray variant virulence. Consistent in all gray variant mouse model vaccination experiments is their reduced capacity to protect against challenge by the Type A *F. tularensis* subspecies. The mechanism behind the lack of protective capacity is not known. *Ft* LVS *flmF2* and *flmK* mutants were avirulent in the mouse model, thus the observed reduction in galactosamine modification may play a role in early clearance and the lack of development of a protective immune response (Kanistanon et al., 2008). While 1-D gel electrophoresis of whole cell lysates and fractions showed no obvious protein differences between *Ft* LVSG and *Ft* LVS, it is possible that these bacteria may possess alterations other than those observed in the LPS. Ongoing assays include microarray analysis, 2-D gel electrophoresis and other more sophisticated proteomic analysis. The continued study of gray variants of *F. tularensis* will provide important mechanistic details behind these phenotypically distinct bacteria, which will move the field closer to the ability to phase lock a wildtype strain for the development of effective and safe tularemia vaccines.

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The *capBCA* locus is required for intracellular growth of *Francisella tularensis* LVS

Jingliang Su[1], Rexford Asare[2], Jun Yang[3], Manoj Kumar Mohan Nair[3], Joseph E. Mazurkiewicz[4], Yousef Abu-Kwaik[2] and Jing-Ren Zhang[3,5]*

[1] Key Laboratory of Zoonosis of Ministry of Agriculture, College of Veterinary Medicine, China Agricultural University, Beijing, China
[2] Department of Microbiology and Immunology, College of Medicine, Louisville, KY, USA
[3] Center for Immunology and Microbial Disease, Albany Medical College, Albany, NY, USA
[4] Center for Neuropharmacology and Neuroscience, Albany Medical College, Albany, NY, USA
[5] Center for Infectious Disease Research, School of Medicine, Tsinghua University, Beijing, China

Edited by:
Anders Sjostedt, Umeå University, Sweden

Reviewed by:
Jason F. Huntley, University of Toledo, USA
Rachel Elizabeth Dean, Defence Science and Technology Laboratory, UK

***Correspondence:**
Jing-Ren Zhang, Center for Infectious Disease Research, School of Medicine, Tsinghua University, Beijing 100084, China.
e-mail: zhanglab@tsinghua.edu.cn

Francisella tularensis is the causative agent of tularemia and a category A bioterrorism agent. The molecular basis for the extreme virulence of *F. tularensis* remains unclear. Our recent study found that *capBCA*, three neighboring genes, are necessary for the infection of *F. tularensis* live vaccine strain (LVS) in a respiratory infection mouse model. We here show that the *capBCA* genes are necessary for *in vivo* growth of *F. tularensis* LVS in the lungs, spleens, and livers of BALB/c mice. Unmarked deletion of *capBCA* in type A strain Schu S4 resulted in significant attenuation in virulence although the level of the attenuation in Schu S4 was much less profound than in LVS. We further demonstrated that CapB protein is produced at a low level under the *in vitro* culture conditions, and *capB* alone is necessary for *in vivo* growth of *F. tularensis* LVS in the lungs of BALB/c mice. Finally, deletional mutations in *capB* alone or *capBCA* significantly impaired intracellular growth of *F. tularensis* LVS in cultured macrophages, thus suggesting that the *capBCA* genes are necessary for intracellular adaptation of *F. tularensis*. The requirement of this gene locus in intracellular adaption at least in part explains the significant attenuation of *F. tularensis capBCA* mutants in virulence.

Keywords: *Francisella tularensis*, *capBCA*, fitness, virulence, macrophage

INTRODUCTION

Francisella tularensis is a Gram-negative intracellular bacterium and causative agent of tularemia in humans and many other species (Sjostedt, 2007). There are four *F. tularensis* subspecies or biotypes: *tularensis* (type A), *holarctica* (type B), *mediasiatica*, and *novicida*. All *F. tularensis* subspecies are able to cause lethal infection in mice, but only the strains of types A and B are mostly associated with human disease (Keim et al., 2007). *F. tularensis* live vaccine strain (LVS) is a type B derivative. LVS is relatively avirulent in humans but causes a lethal infection in mice that highly resembles human tularemia (Eigelsbach and Downs, 1961; Anthony and Kongshavn, 1987). The infection can be transmitted by inhalation of *F. tularensis*-containing aerosols, bites of blood-sucking insects, handling of infected animal carcasses, and consumption of contaminated food or water. Respiratory tularemia has attracted the most attention because inhalation of as few as 10–50 live organisms of *F. tularensis* type A can cause disease and the mortality rate can be >30% by the respiratory route in the absence of antibiotic therapy (McCrumb, 1961). As a result, *F. tularensis* type A strains are listed as a category A potential agent of bioterrorism (Dennis et al., 2001).

Francisella tularensis is able to infect a range of cell types, but its primary target during infection appears to be the macrophage (Clemens and Horwitz, 2007). The pathogenic mechanisms of *F. tularensis* infection are poorly understood. The extraordinary infectivity of *F. tularensis* is correlated with its efficient uptake, survival, and replication within host cells (Clemens and Horwitz, 2007). The ability of *F. tularensis* to propagate intracellularly is enhanced by the failure of lysosomes to fuse with the phagosome (Anthony et al., 1991) and the lack of stimulation of a respiratory burst (Wilson et al., 1980; Fortier et al., 1994). *F. tularensis* appears to bind host cells by interacting with complement receptors (Clemens et al., 2005). After entry into macrophages, the spacious phagosomes are rapidly modified to a tight phagosome (Clemens et al., 2005). In the initial phase of intracellular infection, *F. tularensis* resides in the membrane-bound phagosomes of macrophages and does not appear to replicate (Golovliov et al., 2003; Clemens et al., 2004; Santic et al., 2005a). Replication occurs once the bacterium escapes into the cytosol when the phagosomal membranes are damaged (Golovliov et al., 2003; Clemens et al., 2004; Santic et al., 2005a). The mechanisms for bacterial escape into the cytosol remain to be defined. It is also unclear how *F. tularensis* disseminates from infected host cells to uninfected cells *in vivo*. It is thought that *F. tularensis*-infected macrophages are lysed by bacterium-induced apoptosis (Lai et al., 2001; Lai and Sjostedt, 2003).

The virulence determinants of *F. tularensis* are not well understood (Barker and Klose, 2007). The lipopolysaccharide (LPS) of *F. tularensis* has been extensively studied because of its unusually low toxicity *in vitro* and *in vivo* and potential as a vaccine component (Sandstrom et al., 1992; Ancuta et al., 1996). Several bacterial proteins have recently been implicated in *F. tularensis* pathogenesis. These include AcpA (Mohapatra et al., 2007), MglA/MglB (Baron and Nano, 1998), and the proteins encoded by the

iglABCD intracellular growth operon (Nano et al., 2004). Recent whole-genome screens in *F. tularensis* strains Schu S4 (Qin and Mann, 2006; Kadzhaev et al., 2009), LVS (Maier et al., 2007; Su et al., 2007; Schulert et al., 2009), and U112 (Tempel et al., 2006; Weiss et al., 2007; Kraemer et al., 2009; Ahlund et al., 2010) have identified a large number of bacterial genes that are involved in *F. tularensis* growth in cultured macrophages and host tissues. Our recent study revealed that the *capBCA* genes of *F. tularensis* strain LVS are necessary for bacterial growth in the lungs of mice (Su et al., 2007). Growth as used here refers to the bacterial load in host tissues, which reflects the net outcome of bacterial replication and survival in the context of host defense.

In this study, we sought to understand how the *capBCA* locus contributes to *F. tularensis* pathogenesis. The *in vivo* growth kinetics of LVS and the isogenic *capBCA* mutants were determined in the lungs, spleens, and livers of infected BALB/c mice at various stages of infection. Unmarked deletion of *capBCA* was generated to assess the significance of this gene locus in the virulence of type A virulent strain Schu S4. We further characterized the expression of *capB* and its impact on *F. tularensis* pathogenesis. Finally, the contribution of *capBCA* to intracellular growth of *F. tularensis* was evaluated in cultured macrophage lines. Our results suggest that the *capBCA* genes contribute to the virulence and pathogenesis of *F. tularensis* at least in part through enhancing bacterial intracellular adaptation.

MATERIALS AND METHODS

BACTERIAL STRAINS AND CHEMICAL REAGENTS

Francisella tularensis LVS and its derivatives were cultured as described previously (Su et al., 2007). When necessary, kanamycin (10 μg/ml) or hygromycin (200 μg/ml) was added in the broth and agar media for selection purposes. *F. tularensis* Schu S4, originally isolated from a human case of tularemia (Eigelsbach et al., 1951), was obtained from the U.S. Army Medical Research Institute for Infectious Diseases (Frederick, MD, USA; Malik et al., 2007). The culture media and conditions for Schu S4 strain are the same as for LVS. *Escherichia coli* strains were grown in Luria-Bertani (LB) broth or on LB agar plates in the presence or absence of ampicillin (100 μg/ml), kanamycin (50 μg/ml), and hygromycin (200 μg/ml). All ingredients for bacterial culture media and other chemicals used in this work were obtained from Sigma (St. Louis, MO, USA) unless otherwise stated.

SITE-DIRECTED MUTAGENESIS IN *F. TULARENSIS*

Unmarked in-frame deletional mutations were generated in the *capBCA* locus of strains LVS and Schu S4 by allelic replacement and counterselection as described (Su et al., 2007). All genetic manipulations of strain Schu S4 were performed with the approval of the Centers for Disease Control and Prevention (CDC) in a CDC-certified ABSL-3/BSL-3 facility at Albany Medical College. The entire coding region of the *capBCA* genes was deleted in Schu S4 using the conjugative plasmid pST937. pST937 was previously used to generate an unmarked deletion in *capBCA* of LVS (Su et al., 2007). *capB* was deleted in LVS in a similar manner with a few modifications. An 814-bp fragment downstream of *capB* was PCR amplified from LVS genomic DNA using primers Pr1073 (5′-TACGAGAATTCTATAGTTTTAAGATTAAACAGGAGAAA-3′) and Pr1074 (5′-CTTGTCTCGAGCATATTTGGATTAACCGAAG ACC-3′). This fragment was digested with *Eco*RI and *Xho*I and cloned behind the 5′ fragment of *capB* in plasmid pST933 (Su et al., 2007). The entire construct was subcloned into the *Eco*RV site of a suicide plasmid pMP590 (LoVullo et al., 2006), resulting in the plasmid pMP590::Δ*capB* or pST968. We chose pMP590 to delete *capB* because of its better amenability in DNA cloning (partially due to its smaller size relative to pomp) and transformation (electroporation instead of conjugation for pDMK). pST968 was transferred into LVS by electroporation and selected for kanamycin resistance (10 μg/ml) as described (LoVullo et al., 2006). To remove the inserted plasmid and generate desirable *capB* deletion, the kanamycin resistant transformants were streaked on chocolate agar plates containing 5% (w/v) sucrose. The sucrose-resistant colonies were further screened for the loss of kanamycin resistance. The resulting clones were examined for the *capB* deletion by PCR amplification using the flanking primers Pr896 (5′-AGCTGCACCTGAGTTATTTGAT-3′) and Pr901 (5′-AAATGCAAATGCGTCGTTA-3′). The *capB* deletion was finally confirmed by DNA sequencing in strain ST1092, one of the resultant Δ*capB* mutants. ST1092 retains the sequence encoding the nine N-terminal amino acids of CapB. The lack of the CapB protein in the Δ*capB* strains was assessed by Western blot using a mouse anti-CapB antiserum as described in western blot. The resultant strain ST1092 was selected for further characterization.

COMPLEMENTATION OF *CAPB* DELETION

The *capB* deletion in strain ST1092 was *in trans* complemented with an *E. coli–Francisella* shuttle plasmid pST1032 containing the intact *capB* gene as described previously (Su et al., 2007). *capB* in pST1032 was driven by the *Francisella groEL*. pST1032 was electroporated into strain ST1092 and selected with 200 μg/ml hygromycin, resulting in strain ST1104. Production of the CapB protein in the complemented strains was assessed by western blot using a mouse antiserum against glutathione *S* transferase (GST)-CapB as described in Section "Antibodies and Western blotting."

GST-CAPB FUSION PROTEIN EXPRESSION

A recombinant CapB was expressed as a fusion protein with the GST essentially as described (Lu et al., 2006). *capB* was amplified from the genomic DNA of strain LVS with primers Pr885 (5′-ATCCTGAATTCGGATCCATATTTTCTCCTGTTT-3′) and Pr1175 (5′-ACTAGACCCGGGAACTACTTTGGATTTTTGGTTA ATTG-3′). This fragment was cloned into the *Xma*I/*Eco*RI site of the pGEX-2T expression vector (GE Healthcare Bio-Science, Piscataway, NJ, USA) in *E. coli* strain BL-21 (DE3). The resultant *E. coli* strain was processed to produce and purify a GST fusion protein (designated GST-CapB) by affinity chromatography with the Glutathione Sepharose 4 Fast Flow resins (GE Healthcare Bio-Sciences) according to the supplier's instructions. Protein concentration was determined by the Bio-Rad Protein Assay kit (Bio-Rad, Hercules, CA, USA) and analyzed by SDS-PAGE as described (Lu et al., 2006).

ANTIBODIES AND WESTERN BLOTTING

Antiserum against the *F. tularensis* CapB was raised with the GST-CapB fusion protein as described (Zhang et al., 1997). Female BALB/c mice (6–8 weeks old) were immunized with purified

GST-CapB every 2 weeks via the subcutaneous route for a total of three immunizations. The immunogen for each immunization consisted of 25 μg GST-CapB in 100 μl sterile phosphate-buffer saline (PBS) and 100 μl alum (Rehydrogel Low Viscosity Gel; Reheis, Berkeley Heights, NJ, USA) as described (Sun et al., 2004). Western blotting of *F. tularensis* proteins was performed with an enhanced chemiluminescence (ECL) Western Blot Kit (Pierce, Rockford, IL, USA) according to the supplier's instructions. The GST-CapB antiserum and peroxidase-conjugated goat anti-mouse IgG antibody (Bio-Rad laboratories, Hercules, CA, USA) were used at a dilution of 1:1000 and 1:5000, respectively.

MOUSE INFECTIONS

Infection experiments with *F. tularensis* LVS and its derivates were carried out in BALB/c mice as described (Su et al., 2007). All animal infection experiments were in compliance with the guidelines of the Institutional Animal Care and Use Committee at Albany Medical College. To prepare the *F. tularensis* inocula, frozen stocks of LVS and the isogenic *capBCA* mutants were individually diluted in PBS based on predetermined colony forming unit (CFU) values. Each preparation was serially diluted in PBS and plated to assess the actual CFUs of each inoculum immediately prior to the mouse infection experiments. Each inoculum was intranasally inoculated into groups of mice (female, 6–8 weeks old). Infected mice were sacrificed at various time points post-infection. The lungs, liver, and spleen were aseptically removed and processed to determine the CFU levels of LVS and the mutants in each organ as described (Su et al., 2007). To determine the virulence levels of the Δ*capBCA* mutants in the LVS and Schu S4 backgrounds, groups of five mice were intranasally infected with serial dilutions of the wild type and isogenic Δ*capBCA* strains. Infected mice were monitored daily for signs of morbidity and mortality for 21 days. Infection experiments with Schu S4 and all of its derivatives were carried out as described for strain LVS with the exception that all of the experiments associated with Schu S4 were performed and contained in a CDC-certified ABSL-3/BSL-3 facility at Albany Medical College.

To test the immuno-protection of Δ*capBCA* mutants, groups of five BALB/c mice were inoculated intranasally with different dosages of ST938. Mice injected with sterile PBS were used as control. Three weeks after immunization, each mouse was challenged intranasally with 40,000 CFU of LVS. Infected mice were monitored daily for clinical signs and mortality for 21 days.

HISTOPATHOLOGY

BALB/c mice were intranasally infected with *F. tularensis* LVS or isogenic mutants as described above. Mice, along with uninfected controls, were sacrificed to excise the lungs, liver, and spleen at day 7. The organs were fixed in 10% neutral-buffered formalin, processed using standard histological methods to obtain 5-μm-thick paraffin sections, and stained with H&E as described (Baron et al., 2007).

MACROPHAGE INFECTION

The human monocytic cell line U937 (ATCC CRL-1593.2) and mouse alveolar macrophages MH-S cell line (ATCC CRL-2019) were obtained from the American Type Culture Collection (Manassas, VA, USA). U937 cells were maintained in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% fetal bovine serum at 37°C, 5% CO_2; MH-S cells were cultured in RPMI 1640 medium containing 10% (v/v) fetal bovine serum and 0.05 mM 2-mercaptoethanol at 37°C. Intracellular levels of *F. tularensis* were assessed out as described (Santic et al., 2005b). Briefly, 2.5×10^5 macrophages were cultured in 24-well plates (Hyclone, Logan, UT, USA) to approximately 80% confluency. The *F. tularensis* LVS derivatives were grown in MHB broth to OD_{600} ~ 1.2 (~3×10^9 CFUs/ml), pelleted by centrifugation, and resuspended in pre-warmed RPMI1640 to OD_{600} ~ 0.2 (~5×10^8 CFUs/ml). After brief rinse with PBS, macrophages were infected with the bacterial suspensions at a multiplicity of infection (MOI) of 100 (bacterium vs. macrophage) at 0 h. After 2 h of incubation at 37°C, the unbound bacteria were removed from the wells by washing with PBS. Extracellular bacteria were killed by incubation with the cell culture medium containing gentamicin (50 μg/ml) for 1 h at 37°C. The cells were extensively rinsed to remove residual gentamicin and then cultured without antibiotic. To monitor bacterial uptake and replication, the culture medium of infected macrophages was moved to sterile tubes to lyse the cells at 3, 24, and 48 h post-infection with pre-chilled water. The medium and lysates from each well were combined, diluted with sterile PBS, and spread onto chocolate agar plates for CFU counts. Each infection experiment was repeated at least three times.

FLUORESCENCE AND ELECTRON MICROSCOPY

For fluorescence microscopy, U937 macrophages were infected with *F. tularensis* LVS as described previously (Santic et al., 2005a). Briefly, differentiated U937 cells on 12-mm-diameter circular glass coverslips (Fisher Scientific, Pittsburgh, PA, USA) in 24-well culture plates were infected with LVS and its isogenic mutants at MOI of 10 for 1 h. To synchronize the infection, infected cells were centrifuged at $150 \times g$ for 5 min before incubation at 37°C in 5% CO_2. After 1 h of incubation, infected cells were washed three times with 1× PBS followed by 1 h of gentamicin (50 μg/ml) treatment. Cells were then fixed in 4% paraformaldehyde for 30 min. Paraformaldehyde was removed by washing the wells three times with PBS. The infected cells were permeabilized with 1% Triton-X100 for the LAMP-1 experiment. For the Cathepsin D experiments, the cells were permeabilized with acetone for 5 min at −20°C. Samples were stained with *F. tularensis* polyclonal antibody (1:4,000 dilution) and mouse monoclonal anti-LAMP-1 (H4B3; 1:500 dilution; Hybridoma Gene Bank) or anti-Cathepsin D (BD transduction; 1:500 dilution). Alexa fluor 594-conjugated anti-mouse secondary antibody (1:500 dilution) was used to stain the LAMP-1 and Cathepsin D. Alexa fluor 488-conjugated secondary antibody (1:500 dilution) was used to stain *F. tularensis.* Co-localization of bacteria with LAMP-1 and Cathepsin D was analyzed with FV1000 Olympus confocal microscope as described previously (Santic et al., 2005a,b, 2008). The bacteria associated with phagosomes and phagolysosomes were quantified by enumerating *F. tularensis* co-localized with LAMP-1/Cathepsin D out of the total bacteria in 100 infected cells from more than 10 different fields for each strain. Each experiment was repeated at least once.

For electron microscopy, U937 macrophages growing in 6-well tissue culture plates were inoculated with *F. tularensis* as described above. Twenty-four hours post-inoculation, the wells were washed with 0.1 M Na Cacodylate buffer, pH 7.3 and incubated for 1 h with

a solution containing 2.5% glutaraldehyde/4% formaldehyde in 0.1 M Na Cacodylate, pH 7.3. Following washing with Cacodylate buffer, the cells were post-fixed with 1.0% osmium tetroxide in 0.1 M Na Cacodylate buffer, pH 7.3 for 1 h. The monolayers were washed with normal saline, and scraped with a Costar cell scraper into normal saline and gently pelleted by centrifugation. The pellets were dehydrated in a graded Ethanol series and embedded in LR White medium (Polysciences, Inc.). Ultrathin sections were collected onto Formvar-coated grids, stained with uranyl acetate and lead citrate. Specimens were viewed and photographed with a JEOL 100CX transmission electron microscope using Kodak electron microscope film.

STATISTICAL ANALYSIS

A log-rank test was used to determine the level of significance for the Kaplan–Meier survival analyses. All other results were expressed as mean ± SEM and comparisons between the groups were made using Student's *t* test. Differences between control and experimental groups were considered significant at $p < 0.05$ levels.

RESULTS

IN VIVO GROWTH KINETICS OF THE LVS *capBCA* MUTANTS

In our previous study (Su et al., 2007), the LVS mutant lacking the *capBCA* genes (Δ*capBCA*) exhibited significantly impaired growth in the lungs of infected mice at day 7 post-intranasal inoculation. However, it was unclear whether the *capBCA* genes are required at the earlier and/or later stages of *F. tularensis* infection. We thus determined the *in vivo* growth kinetics of Δ*capBCA* by assessing the levels of bacterial burdens in the lungs, kidneys, and livers of infected BALB/c mice on days 1, 2, 4, 7, 14, and 21 post-intranasal inoculation.

To accurately determine the inoculation efficiency, we first determined bacterial loads in the lungs of infected mice immediately after intranasal inoculation. Mice infected with LVS or Δ*capBCA* (strain ST938) showed a similar level of bacteria (~3×10^3 CFUs) in the lungs 1 h post-intranasal inoculation of 5×0^3 CFUs (day 0; **Figure 1A**). This indicated that Δ*capBCA* was effectively inoculated into the lungs of mice at a similar efficiency as the parent strain LVS. Δ*capBCA* displayed a significant growth deficiency in the lungs throughout the entire test period (**Figure 1A**). In sharp contrast to a 15.1-fold growth of LVS in the lungs of infected mice in initial 24 h, the number of Δ*capBCA* dropped by 75% during the same infection period, indicating that the *capBCA* locus is required for bacterial growth in the initial phase of LVS infection. At days 2 and 4, both LVS and Δ*capBCA* exhibited substantial growth, but the numbers of Δ*capBCA* were significantly lower when compared with that of LVS. At day 7, the bacterial levels of both LVS and Δ*capBCA* were reduced bacterial burdens, likely due to the onset of the adaptive immunity. By 9 days post-inoculation, all of the LVS-infected mice died, whereas Δ*capBCA*-infected mice did not display any detectable sign of disease and remained disease-free throughout the 21-day infection period. Finally, Δ*capBCA* became undetectable from the lungs of the infected mice at days 14 and 21.

We also assessed the dissemination of Δ*capBCA* from lung to spleen and liver in the same BALB/c mice as described in **Figure 1A**. As early as 24 h post-inoculation, LVS were readily detected in the spleens (2,430 CFUs in average) and livers (42,840 CFUs in average), whereas Δ*capBCA* was barely detectable in these distal organs until day 4 (**Figures 1B,C**). Similar to the kinetics of bacterial burden in the lungs, the numbers of Δ*capBCA* were significantly lower than those of LVS in the spleens and livers from days 1 to 7. Δ*capBCA* became undetectable in the livers of all infected mice on days 14 and 21. Interestingly, there were low levels of Δ*capBCA* detectable in the spleens of two and one mice (out of six) at days 14 and 21, respectively (see below for additional interpretations). We thus conclude that the *capBCA* genes are necessary for *in vivo* growth of *F. tularensis* LVS (bacterial replication and resistance to host killing).

LD_{50} AND IMMUNO-PROTECTION OF LVS Δ*capBCA*

The persistent presence of Δ*capBCA* in the spleens (**Figure 1B**) raised the possibility that the mutant is capable of better priming the adaptive immunity against *F. tularensis* LVS. To test this notion, we first attempted to assess the attenuation level of Δ*capBCA* by determining the LD_{50}. Groups of BALB/c mice were intranasally infected with a wide range of bacterial doses ($4.5 \times 10^{2-7}$ CFUs). None of the infected mice showed any appreciable symptoms or died from the infection during the 6-week period of observation (data not shown), indicating the LD_{50} of the Δ*capBCA* mutant

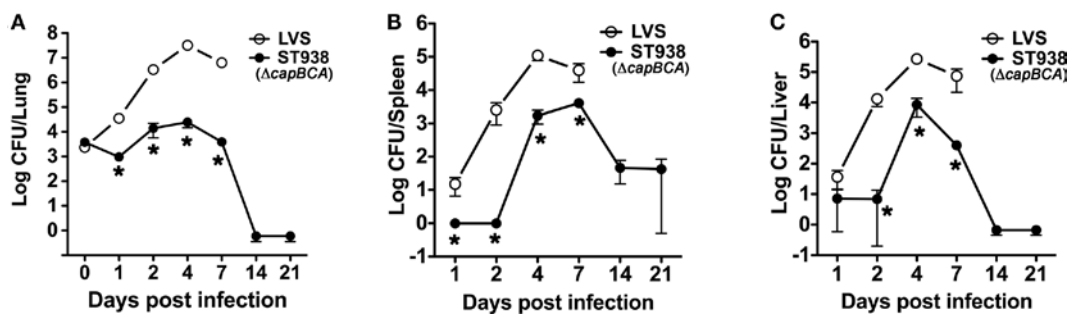

FIGURE 1 | *In vivo* growth of LVS Δ*capBCA*. (A) Bacterial burden in the lungs of BALB/c mice ($n = 5$) infected with LVS or isogenic Δ*capBCA* strain ST938 alone. Lung tissues were collected to determine the viable bacteria by plating various dilutions of tissue homogenates at days 0, 1, 2, 4, 7, 14, and 21 post-inoculation. Bars indicate the mean $\log_{10}$CFU values ± SEM. Asterisks indicate $p < 0.05$ as determined by Student's *t* test. **(B)** Bacterial burdens in the spleens of mice infected individually with LVS derivatives were determined as in **(A)**. **(C)** Bacterial burdens in the livers of mice infected individually with LVS derivatives were determined as in **(A)**.

was >4.5×10^7 CFU. LVS has an LD_{50} of ~4×10^3 CFUs in this infection model (Duckett et al., 2005); all of mice infected with ~8×10^3 CFUs of LVS died in the first 2 weeks of infection (data not shown). Therefore, LVS Δ*apBCA* is avirulent in this BALB/c mouse model.

We next tested immuno-protection of Δ*capBCA* against infection of *F. tularensis* LVS. Groups of five BALB/c mice were intranasally infected (or immunized) with ST938 in a dose range of $4.5 \times 10^{2-7}$ CFUs. The mice were intranasally challenged with 40,000 CFUs of LVS (10 times of LVS LD_{50} in this model) 3 weeks post-immunization. All of the five unimmunized mice (negative control) died within 9 days following LVS challenge (**Table 1**). Similarly, the majority (80%) of the mice immunized with 450 CFUs of ST938 succumbed to LVS challenge. In contrast, all of the mice immunized with higher doses of ST938 ($4.5 \times 10^{3-7}$ CFUs) did not show appreciable sign of illness after the challenge with 40,000 CFUs of LVS in the 4-week observation period (**Table 1**), indicating that Δ*capBCA* is able to induce strong adaptive immunity against infection of *F. tularensis* LVS even at relative low doses of immunization (e.g., 10^{3-4} CFUs). This result agrees with a recent report that an LVS Δ*capB* strain induces potent immunoprotection against LVS challenge (Jia et al., 2010). We further tested the immuno-protection potential of Δ*capBCA* against the challenges with type A virulent strain Schu S4 in a similar manner. None of the intranasal immunization doses ($4.5 \times 10^{3-7}$ CFUs of strain ST938) conferred significant protection against intranasal challenge with 100 CFUs of Schu S4 (data not shown). The sharp difference in immuno-protection of LVS Δ*capBCA* against LVS and Schu S4 demonstrate the distinct requirements for protective immunity against infections of types A and B *F. tularensis*.

IMPORTANCE OF *capBCA* IN TYPE A *F. TULARENSIS*

The gene sequence and organization of the *capBCA* locus are virtually identical between *F. tularensis* subsp. *holarctica* (type B) and subsp. *tularensis* (type A). We thus tested whether the *capBCA* genes are necessary for the virulence of *F. tularensis* type A virulent strain Schu S4. The coding sequence of the *capBCA* locus in Schu S4 was deleted by allelic replacement and counter selection as described in Section "Materials and Methods." As observed with the Δ*capBCA* mutants in LVS (Su et al., 2007), the Schu S4 Δ*capBCA* mutants did not show obvious growth defects under *in vitro* conditions (data not shown). ST965, one of the Schu S4 Δ*capBCA* mutants, was subjected to further characterization of its virulence in the lung infection mouse model. Since laboratory mice are extremely susceptible to Schu S4 infection (Malik et al., 2007), the infection doses of the Δ*capBCA* mutant were accordingly adjusted. While all of mice infected with 16 CFUs of Schu S4 died by day 9, the mortality rate of mice infected with ST965 was significantly reduced in a dose-dependent manner (**Figure 2**). In general, the attenuation of the Schu S4 Δ*capBCA* mutant was less pronounced than that of the LVS counterpart in this model. Michell et al. (2010) recently reported that deletion of *capB* in Schu S4 led to >100-fold attenuation in virulence in a subcutaneous infection model of BALB/c mice. These findings thus indicated that the *capBCA* locus is necessary for the full virulence of human virulent *F. tularensis* type A strains.

Table 1 | Protection of LVS Δ*capBCA* against lethal LVS infection.

Immunization dose (CFU)	Survived/total mice
0	0/5
4.5×10^2	1/5
4.5×10^3	5/5
4.5×10^4	5/5
4.5×10^5	5/5
4.5×10^6	5/5
4.5×10^7	5/5

CHARACTERIZATION OF *F. TULARENSIS capB*

To gain more insight into the function(s) of the *F. tularensis capBCA* locus, we further characterized *capB*. The coding sequence of *capB* in LVS was removed by allelic exchange. Three resulting strains (ST1092-4) were verified for the lack of *capB* as determined by PCR amplification (**Figure 3A**) and DNA sequencing (data not shown). Primers Pr896/Pr901 were expected to produce amplicons of 2,177 bp in LVS and 991 bp in Δ*capB* mutants ST1092-4. To determine the expression of the CapB protein, we also constructed a GST-CapB fusion protein in *E. coli* to generate an antiserum against CapB in mice. The *F. tularensis* CapB protein is predicted to contain 405 amino acids with the molecular mass of 44.9 kDa. As represented in **Figure 3B**, the antiserum reacted with a protein band of 44 kDa in LVS, which was completely absent in the isogenic Δ*capB* mutant ST1092. The missing band in ST1092 was restored *in trans* by the shuttle plasmid pST1032 (strain ST1104). We thus concluded that the antiserum specifically detected the CapB protein. The CapB protein appears to be produced at a relatively low level under *in vitro* culture conditions because it was only detected when a large amount of the total bacterial lysates (~2×10^9 CFU) was loaded in the protein gel. This was not due to a potential low titer of the antiserum because the antiserum readily detected CapB with ~2×10^8 CFUs of the complementation strain ST1104 (data not shown). Apparent overproduction of CapB in ST1104 was likely due to the strength of the *Francisella groEL* promoter and/or copy number of the

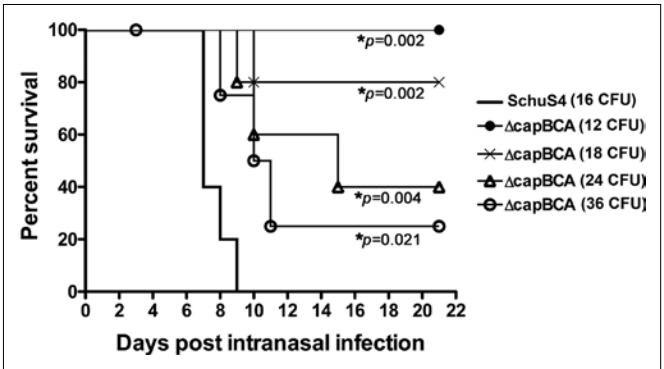

FIGURE 2 | Contribution of *capBCA* to the virulence of type A *F. tularensis*. BALB/c mice ($n = 5$) were intranasally infected with the indicated doses of Schu S4 or isogenic Δ*capBCA* mutant ST965. Survival was monitored for 21 days. The results are expressed as Kaplan–Meier curves and *p* values determined using log-rank test. The data shown are a representative of two independent experiments.

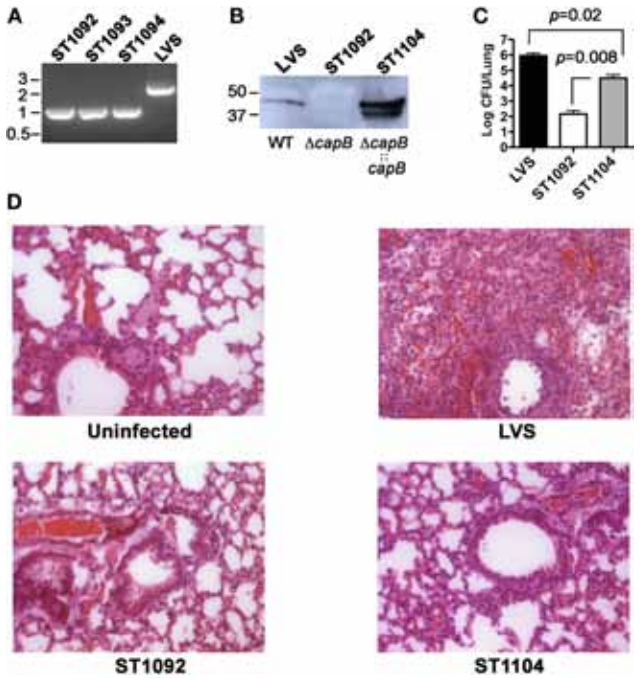

FIGURE 3 | CapB expression and its impact on *in vivo* growth of *F. tularensis*. (A) Detection of the *capB* deletion by PCR. The sequence surrounding the *capB* gene was amplified from the genomic DNA of LVS or three isogenic Δ*capB* clones (ST1092-4). The PCR products were separated in a 1% agarose gel and stained with ethidium bromide. The sizes of the DNA standards are indicated in kilobase. **(B)** Western blot detection of the *F. tularensis* CapB. Cell lysates of LVS derivatives representing ~2×10^9 CFUs were separated by SDS-PAGE, probed with the GST-CapB antiserum, and detected by an ECL method. **(C)** *In vivo* growth of the *F. tularensis* Δ*capB* mutant. BALB/c mice were infected individually with LVS (4,500 CFUs), ST1092 (5,100 CFUs), or ST1104 (4,800 CFUs) by intranasal inoculation. Bacterial levels in the lungs were assessed at day 7 as in **Figure 1**. **(D)** Lung histopathology of *F. tularensis*-infected mice. BALB/c mice were infected as in **(C)**. Lung tissues were processed for H&E staining at day 7 post-infection. Magnification = 20×.

complementation construct as reported by Charity et al. (2007). A second band detected by the antiserum in ST1104 may represent a smaller isoform of the *F. tularensis* CapB as reported for the *B. anthracis* CapB (Makino et al., 1989).

We further performed infection experiments with the Δ*capB* mutant ST1092 and isogenic complementation strain ST1104. The Δ*capB* mutant was significantly deficient in *in vivo* growth at day 7 post-intranasal inoculation compared to the parent strain LVS (**Figure 3C**; **Figure A1** in Appendix). The attenuation phenotype of ST1092 was partially *in trans* restored in complementation strain ST1104. The same *capB* complementation construct also showed partial complementation in the transposon mutant JS2512 in our previous study (Su et al., 2007). This could be due to instability of the shuttle plasmid during infection in the absence of antibiotic selection and/or inappropriate *in trans* expression level of the CapB protein. We also examined the impact of *capB* on *F. tularensis* pathogenesis by comparing histopathology of the lungs from BALB/c mice intranasally infected with either LVS or isogenic Δ*capB* mutants. In agreement with previous studies (Baron et al., 2007; Malik et al., 2007), the lungs of the LVS-infected mice had severe inflammation and tissue damage at day 7 post-infection as compared to the uninfected control (**Figure 3D**). In contrast, mice infected with ST1092 showed a relatively normal lung structure with little sign of inflammation. The lungs of mice infected with the *capB* complementation strain ST1104 exhibited a low level of tissue infiltration but the overall structure was relatively normal. Together with the experimental data with Δ*capBCA* (**Figure 1**; Su et al., 2007), these results allowed us to conclude that *capB* itself is necessary for the fitness of *F. tularensis* LVS. Our data are also consistent with a very recent study reporting that an LVS Δ*capB* mutant is severely attenuated in BALB/c mice (Jia et al., 2010).

DEFICIENCY OF THE *capBCA* MUTANTS IN INTRA-MACROPHAGE GROWTH

We sought to understand how the *capBCA* locus contributes to *in vivo* adaptation of *F. tularensis*. Since *F. tularensis* is a facultative intracellular pathogen, we tested whether the *capBCA* genes are required for intracellular growth of *F. tularensis*. We initially tested the intracellular infection of Δ*capB* and Δ*capBCA* in mouse MH-S macrophages, because this is a commonly used alveolar macrophage model derived from BALB/c mice (Mbawuike and Herscowitz, 1989; Ibrahim-Granet et al., 2003). At the end of the gentamicin treatment (hour 0), Δ*capB* and Δ*capBCA* showed similar levels of the intracellular growth as the parent strain LVS (**Figure 4A**). Because *F. tularensis* does not appear to replicate in the first 3 h post-infection (Golovliov et al., 2003; Clemens et al., 2004; Santic et al., 2005a), the result indicated that *capBCA* locus is dispensable for the initial phase of intracellular infection (bacterial adherence and uptake). By 24 and 48 h post-inoculation, the macrophages infected with Δ*capB* or Δ*capBCA* showed significantly lower CFUs (approximately 14- to 22-fold reduction) as compared to the cells infected with LVS (**Figure 4A**). Noticeably, Δ*capB* and Δ*capBCA* behaved similarly in this assay, suggesting that *capB* carries out an important function(s) in this locus. The growth defect of Δ*capB* was not due to the abnormal growth of the mutant in the culture medium because LVS and its *capBCA* derivatives showed obvious growth in the absence of macrophages under the same conditions (data not shown). The defect of Δ*capB* in intracellular growth was partially restored by *in trans* complementation (**Figure 4A**, Δ*capB::capB*). The complementation strain still exhibited significant deficiency (two- to threefold) in intracellular growth as compared to LVS, likely due to overexpression of *capB* on the complementation construct (see **Figure 3B**). We were unsuccessful in our attempts to complement the entire *capBCA* locus due to technical difficulty in cloning the three genes in *E. coli–Francisella* shuttle vectors (data not shown).

We subsequently confirmed the impairment of Δ*capB* and Δ*capBCA* in intracellular growth using human U937 macrophages. Like in MH-S cells, the intracellular growth defect of Δ*capB* and Δ*capBCA* was only observed after the initial phase of infection (**Figure 4B**, hour 0). A noticeable difference between the MH-S and U937 results is the marginal effect of *in trans* complementation construct on the intracellular growth phenotype of Δ*capB* in U937 cells (**Figure 4B**, Δ*capB::capB*). A very recent study by Jia et al. (2010) also showed that an LVS Δ*capB* mutant is significantly

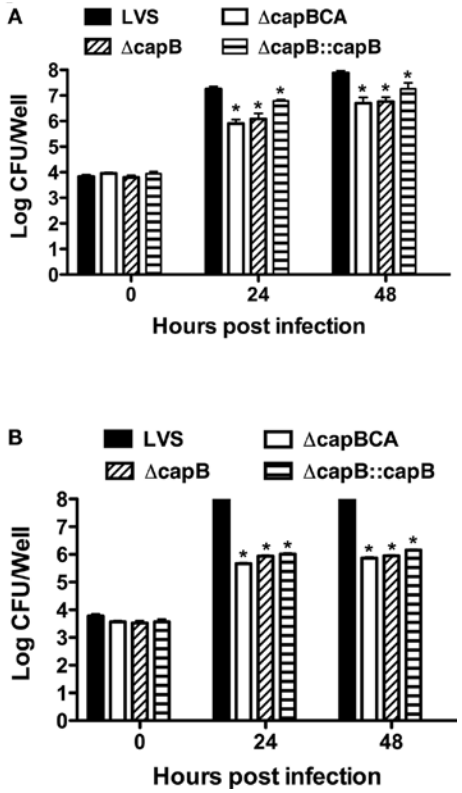

FIGURE 4 | Intracellular growth of *F. tularensis capBCA* mutants. **(A)** Intracellular growth of LVS derivatives in MH-S macrophages. Macrophages were infected at a multiplicity of infection of ~100:1 (bacterium vs. macrophage) with LVS, isogenic Δ*capBCA* (strain ST938), Δ*capB* (strain ST1092), or complemented Δ*capB* (Δ*capB*::capB, strain ST1104). Total bacteria per well were enumerated at the indicated time points. Statistic significance between LVS and its isogenic *capBCA* mutants ($p > 0.05$) was determined using Student's *t* test. **(B)** Intracellular growth of LVS derivatives in U937 macrophages. The infection experiments were carried out and analyzed as in **(A)**.

attenuated in intracellular growth in THP-1 macrophages. Together, these results demonstrate that the *capBCA* locus is required for intracellular growth of *F. tularensis* LVS.

SUB-CELLULAR LOCALIZATION OF THE *capBCA* MUTANTS IN MACROPHAGES

Previous studies indicate that *F. tularensis* transiently resides in a LAMP-1 and LAMP-2 positive phagosomes before escape into the cytosol for replication (Santic et al., 2010). The existing data have shown a correlation between escape into the cytosol and loss of LAMP-1/2 co-localization (Checroun et al., 2006; Bonquist et al., 2008; Asare and Abu Kwaik, 2010). Therefore, loss of co-localization with LAMP-1 and LAMP-2 has been used as an indicator for cytosolic localization of *F. tularensis* (Buchan et al., 2009). Based on the importance of the *capBCA* genes in *F. tularensis* intracellular growth (**Figure 4**), we reasoned that this locus might play a role in the phagosomal escape of the bacterium and/or arrest of phagosomal maturation. To test this possibility, we analyzed co-localization of Δ*capB* and Δ*capBCA* with LAMP-1 (a phagosomal marker) and Cathepsin D (a lysosomal marker) in U937 macrophages by immunofluorescence microscopy.

As exemplified in **Figures 5A,C**, only a small fraction (31%) of wild type LVS were associated with LAMP-1 and the majority of LVS bacteria were found in cytosol by 2 h post-infection, suggesting successful escape of LVS from the phagosome to cytosol. In sharp contrast, the majority of Δ*capB* (61%) and Δ*capBCA* (72%) were found to be associated with LAMP-1 during the same infection. These LAMP-1 co-localization levels are close to that of inactivated LVS (73%). Similar to the intracellular growth data (**Figure 4B**), *in trans* complementation of Δ*capB* with the wild type gene on a shuttle plasmid had marginal effect on the LAMP-1 co-localization of the mutant (**Figure 5C**). This finding thus suggested that Δ*capB* and Δ*capBCA* were severely impaired in their ability to escape from the phagosomes into cytosol.

Consistent with the co-localization of both the mutants with LAMP-1, Δ*capB* (68%) and Δ*capBCA* (73%) predominantly co-localized with Cathepsin D, a lysosomal marker, by 2 h post-infection (**Figures 5B,C**). As a positive control, only 27% of live LVS was co-localized with Cathepsin D, which is consistent with its ability to escape from the phagosome–lysosome fusion pathway. In contrast, the inactivated LVS was mostly associated with Cathepsin D (83%) in the same infection period. Similar to the result for LAMP-1 (**Figure 5A**), the *capB* complementation construct failed to restore the co-localization of Δ*capB* to the level exhibited by the wild type bacteria (**Figures 5B,C**). We subsequently confirmed these findings in a Δ*capB* mutant of *F. novicida* strain U112 (data not shown). These results strongly suggested that Δ*capB* and Δ*capBCA* are significantly impaired in their ability to arrest maturation of the phagosome.

Finally, we visualized Δ*capB* and Δ*capBCA*, and their sub-cellular localization in U937 macrophages by transmission electron microscopy. We focused our analysis on the 24-h time point because the morphology of the infected macrophages was no longer intact beyond this infection period. As represented in **Figure 6** (indicated by arrow heads), the LVS-infected cells contained numerous bacteria that appear to be free in the cytoplasm and surrounded by an electron lucent zone. However, in addition to the free bacteria, there were large numbers of partially degraded bacteria in vacuolar-like structures in the macrophages infected with Δ*capB* (strain ST1092) and Δ*capBCA* (strain ST938) as indicated by asterisks in **Figure 6**. This type of sub-cellular structures were rarely found in the LVS-infected macrophages (data not shown). The structures containing apparently degraded bacteria were also readily detectable in the *in trans* complemented Δ*capB* (strain ST1104), indicating that the complementation construct failed to restore the impairment in Δ*capB*. These bacterium-containing vacuolar-like structures are likely phagolysosomes or autophagosomes because most of them were surrounded by an identifiable membrane. Taken together, we conclude that the *capBCA* genes are required for *F. tularensis* phagosomal escape and/or arrest of phagosomal maturation.

DISCUSSION

The *F. tularensis capBCA* genes are among the 95 virulence-associated genes identified in our recent STM study (Su et al., 2007). Transposon insertions in each of the *capBCA* genes resulted in significantly impaired growth of *F. tularensis* LVS in the lungs of BALB/c mice 7 days post-intranasal inoculation. In this study, we sought to understand how this gene locus contributes to

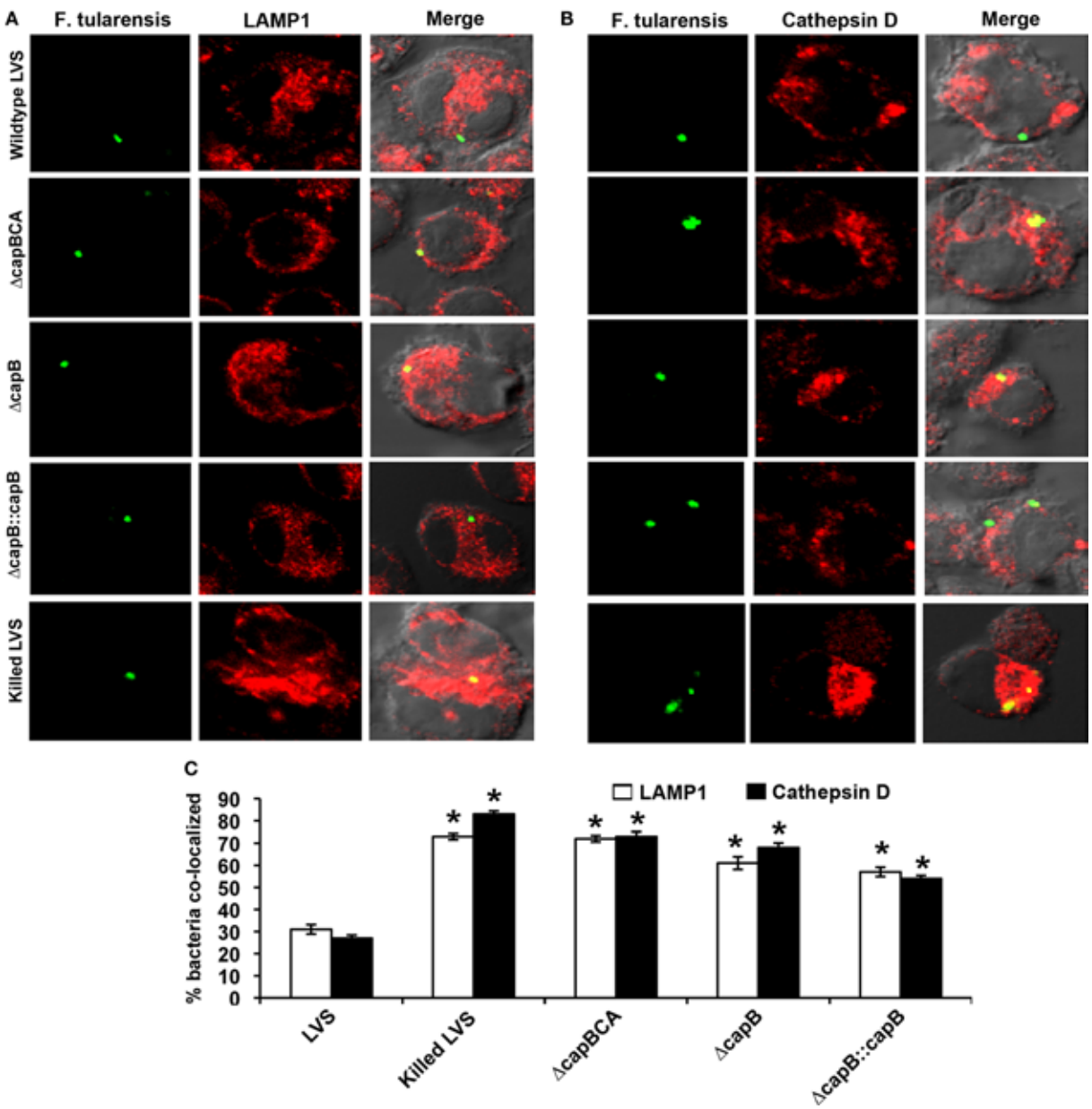

FIGURE 5 | Co-localization of *F. tularensis capBCA* mutants with phagosomal and phagolysosomal markers. (A) Representative images showing co-localization of the LVS *capBCA* mutants with the phagosomal marker LAMP-1. *F. tularensis* and LAMP-1 were stained and visualized as described in Section "Materials and Methods." **(B)** Representative images showing co-localization of the LVS *capBCA* mutants with phagolysosomal marker Cathepsin D. **(C)** Association of LVS *capBCA* mutants with LAMP-1 and Cathepsin D. The results are expressed as the percentage of bacteria co-localized with LAMP-1 or Cathepsin D out of total bacteria in 100 U937 cells. Statistic significance between LVS and its isogenic *capBCA* mutants ($p > 0.05$) was determined using Student's *t* test. The data shown are a representative of two independent experiments.

F. tularensis fitness and pathogenesis. Mouse infection experiments demonstrated that the unknown function(s) provided by the *capBCA* genes is required for the fitness and virulence of *F. tularensis* LVS and human virulent type A strain Schu S4. This finding is consistent with the recent reports that deletion mutants in *capB* of LVS (Jia et al., 2010) and Schu S4 (Michell et al., 2010) are attenuated in BALB/c mice. The experiments with the LVS *capBCA* mutants indicated that the *capBCA* genes are necessary for *F. tularensis* growth (replication and survival) in target organs at various stages of infection. The results from macrophage infection experiments suggest that the *capBCA* genes enhance *F. tularensis* fitness and thus virulence by promoting intracellular growth of the bacterium. This conclusion is supported by significant deficiency of Δ*capB* and Δ*capBCA* in intracellular growth in both MH-S and U937 macrophage models.

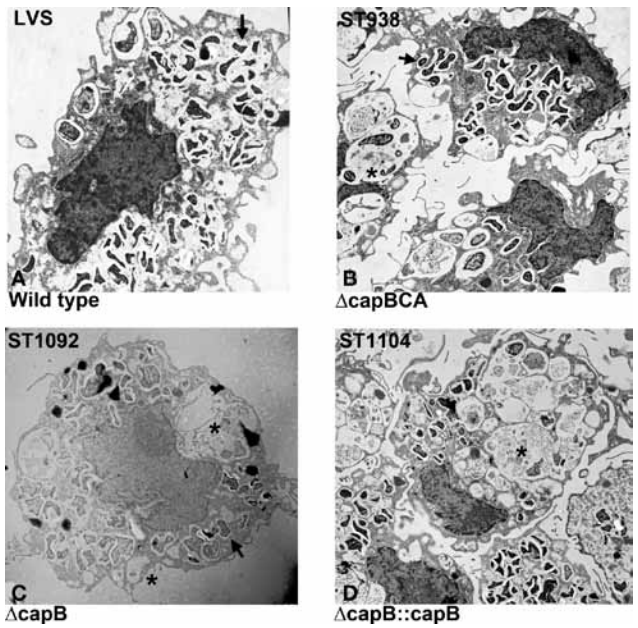

FIGURE 6 | Sub-cellular ultra-structures of U937 macrophages infected by LVS *capBCA* mutants. Electron micrographs of human macrophages inoculated with *F. tularensis* LVS **(A)**, Δ*capBCA* (ST938) **(B)**, Δ*capB* (ST1092) **(C)**, or complemented Δ*capB* (ST1104) **(D)**. Indicated are the intact bacteria (arrow heads) and vacuoles containing degraded bacteria (asterisks).

The deficiency of the *capBCA* mutants in intracellular growth lies beyond the uptake (attachment/entry) phase of intracellular infection. Specifically, the *capBCA* locus appears to enhance the intracellular growth of *F. tularensis* by promoting bacterial escape from phagosomes. This notion is consistent with multiple observations in this study. Δ*capB* and Δ*capBCA* remained predominantly co-localized with the LAMP-1 (a phagosomal marker) and Cathepsin D (a phagolysosomal marker) in U937 macrophages by 2 h post-infection. Second, degraded bacteria were abundantly observed in vacuolar-like structures of the macrophages infected with Δ*capB* and Δ*capBCA*. In this regard, the *capBCA* mutants behaved similarly to the mutants of other *F. tularensis* genes that are involved in phagosomal escape, most notably, the *iglABCD* genes located within the *Francisella* pathogenicity island (FPI; Lai et al., 2004; Nano et al., 2004; Santic et al., 2005b, 2008; de Bruin et al., 2007). However, we noticed that the *capBCA* mutants were much less impaired than the mutants of *iglC* or *mglA* (encoding a FPI regulator) in terms of intra-macrophage growth (data not shown). While the *iglC* or *mglA* mutants virtually lacked intra-macrophage growth, the *capBCA* mutants were still able to grow to a relatively high level under the same conditions (data not shown). This observation indicates that the *capBCA* and FPI genes perform distinct functions in terms of promoting intracellular adaptation of *F. tularensis*. In short, it remains to be determined whether the *capBCA* mutants are defective in one or more of the following intracellular infection stages: (i) survival/escape from the phagosomes to the cytosol, (ii) arrest of phagosomal maturation, (iii) replication in the cytosol, and/or (iv) re-infection of other cells.

It is unclear how the *capBCA* genes contribute to *F. tularensis* growth in macrophages and host tissues. *F. tularensis* CapB and CapC are homologous to the CapB and CapC proteins of *Bacillus anthracis*, the causative agent of anthrax (Koehler, 2002). CapB and CapC, together with CapA, CapD, and CapE in *B. anthracis*, are responsible for the biosynthesis of the capsule consisting of poly-γ-D-glutamic acids (PGA; Candela and Fouet, 2006). CapB, cytoplasmic protein, is the catalytic component of the *Bacillus* PGA synthesis complex (Troy, 1973). The PGA-based capsule in *B. anthracis* is a major virulence factor due to its antiphagocytic property (Koehler, 2002). Interestingly, recent genome studies have revealed homologs of the PGA biosynthetic genes in a group of highly diverse Gram-negative bacteria including *F. tularensis* and *Fusobacterium nucleatum* (Kapatral et al., 2002; Glockner et al., 2003; Ren et al., 2003; Hou et al., 2004; Larsson et al., 2005). Candela et al. (2009) recently reported the production of PGA by the *capBCA* genes of *F. nucleatum*. The *capBCA* loci of *F. tularensis* and *F. nucleatum* are highly similar in gene sequence and order. It is thus possible that the *F. tularensis capBCA* locus enhances the bacterial growth in macrophages and host tissues through PGA production. However, no PGA or capsule has been detected from *F. tularensis* in our preliminary trials (unpublished data) or previous studies (Raynaud et al., 2007; Michell et al., 2010).

The *capBCA* locus is highly conserved in genus *Francisella*. The sequences of the *capBCA* coding and intergenic regions are virtually identical among *F. tularensis* subsp. *tularensis* (type A) (Larsson et al., 2005; Beckstrom-Sternberg et al., 2007), *holarctica* (type B) (Chain et al., 2006; Petrosino et al., 2006; Barabote et al., 2009), and *novicida* (Brittnacher et al., 2007). A recent study also revealed the presence of a similar *capBCA* locus in *Francisella philomiragia* (Copeland et al., 2008). Like *F. tularensis* subsp. *novicida*, *F. philomiragia* is a water-associated bacterium and less pathogenic to humans than *F. tularensis* subsp. *tularensis* and *holarctica* (Penn, 2005). Our infection experiments indicate that the *capBCA* locus is necessary for *the in vivo* fitness and full virulence of *F. tularensis* types A and B organisms. Weiss et al. (2007) also showed that the transposon mutants in the *capB* and *capC* genes of *F. tularensis* subsp. *novicida* are attenuated in a systemic infection mouse model. Considering that mammals may not be the natural reservoirs of *F. tularensis* (Oyston and Quarry, 2005), the uncharacterized function(s) of the *capBCA* locus in *F. tularensis* and perhaps other *Francisella* species may be evolutionarily selected for a survival advantage under non-mammalian conditions.

It is noticeable that the attenuation level of the Schu S4 Δ*capBCA* mutant was significantly lower than that of the LVS counterpart in our intranasal infection model in BALB/c mice. This difference may be due to other Schu S4-specific factor(s) that might have overshadowed the impact of the *capBCA* locus in the mouse model. Laboratory mice are exceptionally susceptible to Schu S4 infection (Rick Lyons and Wu, 2007). Alternatively, the *capBCA* locus operates in a different manner at the transcriptional and/or post-transcriptional level. Lindgren et al. (2007) recently reported that a deletion in the catalase-encoding *katG* of *F. tularensis* LVS led to significant attenuation in virulence, but a similar Schu S4 Δ*katG* mutant showed no attenuation in virulence. Finally, different mouse strains and routes of infection have yielded variable outcomes with the *F. tularensis capB* mutants in terms of virulence

and immuno-protection. Michell et al. (2010) reported more than 100-fold attenuation with an Schu S4 *capB* mutant following subcutaneous inoculation of BALB/c mice, whereas no significant attenuation was observed with an independent Schu S4 *capB* mutant when BALB/c mice were infected by aerosol inoculation (Conlan et al., 2010). Further evaluation of the *capBCA* mutants in other animal models is warranted to conclusively define the contribution of this gene locus to *F. tularensis* infection in humans.

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APPENDIX

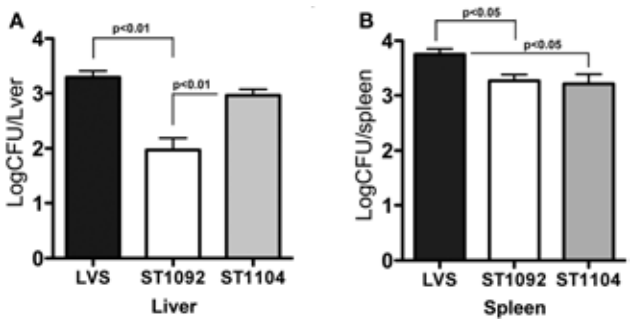

FIGURE A1 | *In vivo* growth of the *F. tularensis* Δ*capB* mutants in the livers (A) and spleens (B) of BALB/c mice. BALB/c mice were infected individually with LVS (4,500 CFUs), ST1092 (5,100 CFUs), or ST1104 (4,800 CFUs) by intranasal inoculation. Bacterial levels in the livers and spleens were assessed at day 7 as in **Figure 3**.

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Genetic manipulation of *Francisella tularensis*

Xhavit Zogaj and Karl E. Klose*

Department of Biology, South Texas Center for Emerging Infectious Diseases, University of Texas San Antonio, San Antonio, TX, USA

Edited by:
Anders Sjostedt, Umeå University, Sweden

Reviewed by:
Martin Pavelka, University of Rochester Medical Center, USA
Chandra Shekhar Bakshi, Albany Medical College, USA

****Correspondence:***
Karl E. Klose, South Texas Center for Emerging Infectious Diseases, University of Texas San Antonio, One UTSA Circle, San Antonio, TX 78249, USA.
e-mail: karl.klose@utsa.edu

Francisella tularensis is a facultative intracellular pathogen that causes the disease tularemia. *F. tularensis* subsp. *tularensis* causes the most severe disease in humans and has been classified as a Category A select agent and potential bioweapon. There is currently no vaccine approved for human use, making genetic manipulation of this organism critical to unraveling the genetic basis of pathogenesis and developing countermeasures against tularemia. The development of genetic techniques applicable to *F. tularensis* have lagged behind those routinely used for other bacteria, primarily due to lack of research and the restricted nature of the biocontainment required for studying this pathogen. However, in recent years, genetic techniques, such as transposon mutagenesis and targeted gene disruption, have been developed, that have had a dramatic impact on our understanding of the genetic basis of *F. tularensis* virulence. In this review, we describe some of the methods developed for genetic manipulation of *F. tularensis*.

Keywords: tularemia, transposon, allelic exchange, plasmid, targetron, select agent

INTRODUCTION

Francisella tularensis is the causative agent of the human disease tularemia. Various subspecies of this organism have been described, with *F. tularensis* subsp. *tularensis* (also referred to as "Type A") being the most virulent for humans. *F. tularensis* subsp. *holarctica* (also referred to as "Type B") also causes serious disease in humans, and both subsp. *tularensis* and subsp. *holarctica* have been classified as category A select agents that require biosafety level 3 (BSL-3) containment for routine laboratory culture (McLendon et al., 2006). A closely related bacterium, *F. novicida* (also referred to as *F. tularensis* subsp. *novicida*, although it is not officially classified as a *F. tularensis* subsp.), is considered avirulent for healthy humans and can be maintained under BSL-2 conditions. A live attenuated vaccine strain (LVS) derived from *F. tularensis* subsp. *holarctica* is also avirulent for humans and can also be maintained under BSL-2 conditions (Eigelsbach and Downs, 1961). *F. novicida* and LVS cause lethal diseases in mice, and share similar pathogenic mechanism(s) as the select agent designated forms of *F. tularensis*, such as intramacrophage replication (**Figure 1**). Because *F. novicida* and LVS can be grown and maintained without the requirement for restrictive BSL-3 containment, these bacteria have been invaluable for the development of genetic manipulation techniques and the dissection of virulence mechanisms of *F. tularensis*.

Initial techniques for genetic manipulation were developed in *F. novicida*, for the reasons given above, and the fact that *F. novicida* has proven to be the most amenable of all *Francisella* spp. to genetic manipulation, for reasons that are not obvious. *F. novicida* is closely related to *F. tularensis*, as has become clear through whole genome sequencing (Rohmer et al., 2007; Champion et al., 2009), yet *F. novicida* can be transformed with linear DNA fragments which integrate into the chromosome through homologous recombination (Lauriano et al., 2003), a property that does not seem to apply to *F. tularensis* subsp. Additionally, use of antibiotic resistance markers is restricted in select agent strains of *F. tularensis* (discussed below), but not *F. novicida*. Taken together, this has allowed for a wider variety of techniques to be attempted in *F. novicida* than in *F. tularensis*.

Despite these various obstacles, a number of recent advances have allowed for increasingly easier genetic manipulation in select agent forms of *F. tularensis*. Both random and directed mutagenesis can now be performed readily in *F. tularensis* subsp. *tularensis*, and several plasmids are available to facilitate complementation and expression studies. These tools are facilitating the illumination of the genetic basis of *F. tularensis* pathogenesis, and several of these will be discussed below.

ANTIBIOTIC RESISTANCE

Antibiotic resistance markers are critical for the development of genetic techniques. Genetic techniques utilizing various antibiotic resistance genes as selectable markers have been exploited in *F. novicida* (Anthony et al., 1994; Gallagher et al., 2007; Liu et al., 2007; Rodriguez et al., 2008) but the use of antibiotic resistance in *F. tularensis* subsp. (with the exception of LVS) is restricted in the US. Resistance to any antibiotic that may be used therapeutically to treat tularemia (Urich and Petersen, 2008; Hofinger et al., 2009) may not be introduced into select agent forms of *F. tularensis*, which should preclude the introduction of resistance to chloramphenicol, ciprofloxacin, levofloxacin, doxycycline, gentamicin, streptomycin, and tetracycline (Enderlin et al., 1994; Maurin et al., 2000; Antibiotic Selection Guide, 2004). This has restricted the use of antibiotic resistance markers to kanamycin, erythromycin, spectinomycin, rifampin, and hygromycin resistance in these organisms (LoVullo et al., 2006; Qin and Mann, 2006; Buchan et al., 2008; Kalivoda et al., 2010; Klose, unpublished data). These restrictions do not necessarily apply to scientists outside the US, and thus examples can be found in the published literature of other antibiotic resistance markers being introduced into, e.g., *F. tularensis* subsp. *tularensis* (Norqvist et al., 1996; Pomerantsev et al., 2001; Forslund et al., 2006; Thomas et al., 2007).

Francisella tularensis/F. novicida strains are all highly resistant to ampicillin and other penicillin derivatives, and thus these antibiotics are not used therapeutically to treat tularemia. Although two β-lactamases are encoded in the genome, only one of these, BlaB,

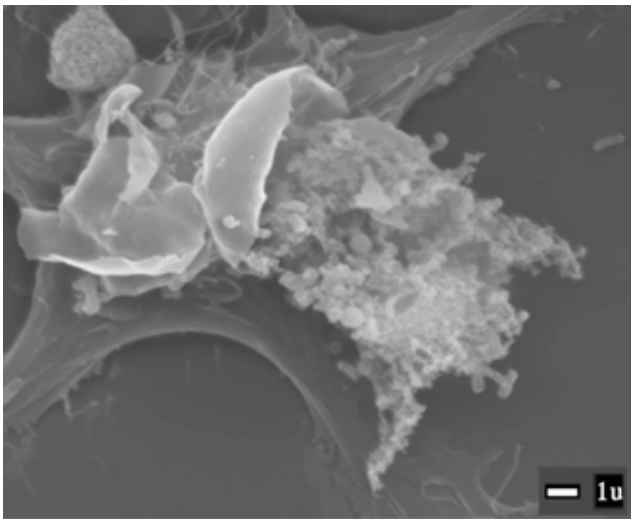

FIGURE 1 | *Francisella novicida* being released from a lysed macrophage. SEM image provided by M. Neal Guntzel and Annette R. Rogriguez.

contributes in a significant manner to the ampicillin resistance of *Francisella* strains (Lauriano et al., 2003; Bina et al., 2006; LoVullo et al., 2006). This has allowed for the use of *blaB* as a selectable marker in a *blaB*$^-$ *F. tularensis* strain, thus expanding of the use of antibiotic resistance markers in select agent strains to include ampicillin (LoVullo et al., 2006).

TRANSFORMATION

The first step to genetic manipulation is getting exogenous DNA into the bacterial cell. Host restriction/modification systems inhibit the acquisition of foreign DNA into the cell, and both *F. novicida* and *F. tularensis* subsp. *tularensis* have restriction enzymes that inhibit the acquisition of non-self DNA (Gallagher et al., 2008; Klose, unpublished data); interestingly, LVS has been reported to lack significant restriction against foreign DNA. Restriction of foreign DNA in *F. novicida* was eliminated by disruption of four different restriction enzymes (Gallagher et al., 2008), and this strain (MFN245) is ~10,000-fold enhanced for transformation with foreign DNA. Because the modification systems are intact, MFN245 can be used to transform with, e.g., plasmids, which can then be easily transformed in any *F. novicida* strain, due to the *F. novicida*-specific modifications. *F. tularensis* subsp. *tularensis* restricts DNA from *F. novicida*, indicating it has different restriction/modification systems (Klose, unpublished data), and thus transformation of MFN245 cannot be used to enhance subsequent transformation into *F. tularensis* subsp. *tularensis*.

Francisella novicida can be transformed with chromosomal DNA and linear fragments, indicating it likely has a natural competence system. The same is not true for the *F. tularensis* subsp., suggesting that natural competence was lost when *F. novicida* and *F. tularensis* diverged. There are several methods to transform *F. novicida*/*F. tularensis*. One of the most unique techniques for transformation is referred to as cryotransformation (Norqvist et al., 1996; Lauriano et al., 2003). Cryotransformation is a method that is easy to perform, and it has been shown to be effective in *F. novicida* and all *F. tularensis* subsp. The cryotransformation protocol is: (a) *F. tularensis*/*F. novicida* cells are washed and resuspended into 0.2 M $RbCl_2$; (b) DNA and cryotransformation buffer (10% Glycerol, 10 mM HEPES, pH 6.5, 0.1 M Calcium chloride, and 10 mM $RbCl_2$) are added to the cells and left on ice for 15 min; (c) the cells are then quick frozen in dry ice/ethanol bath for 5 min; (d) the cells are then thawed until at room temperature, and culture media is added and the mix is incubated for 2–3 h; (e) finally the cells are spread onto agar media with appropriate antibiotics (Liu et al., 2007; Rodriguez et al., 2008; Zogaj et al., 2008; Barker et al., 2009).

Electroporation is also commonly used for transformation into *F. novicida*/*F. tularensis*. There are reports indicating that electroporation is even more efficient than cryotransformation (LoVullo et al., 2006; Le Pihive et al., 2009). The typical protocol for electroporation of *F. novicida*/*F. tularensis* is (a) exponentially growing *Francisella* cells are centrifuged to concentrate them, (b) cells are washed three times and finally resuspended in 0.5 M sucrose, (c) DNA is mixed with cells for 10 min at RT, (d) cells are pulsed in electroporator (2.5 kV, 600 Ω, 25 μF), (e) culture media is added and the mix is incubated for 2–3 h, (f) finally the cells are spread onto agar media with appropriate antibiotics.

Chemical transformation is not commonly used in *Francisella* species, but was one of the first methods used to introduce DNA into *F. novicida* (Tyeryar and Lawton, 1969, 1970; Anthony et al., 1991). On the other hand, the direct transfer of DNA from donor bacteria into *F. novicida*/*F. tularensis* via conjugation is becoming a common technique, and has been used to introduce DNA into the various *Francisella* spp. (Golovliov et al., 2003a, Forslund et al., 2006; Mohapatra et al., 2007; Thomas et al., 2007). Transformation frequencies of approximately 1% in *F. tularensis* have been observed under optimal conditions when using *E. coli* donors to conjugate DNA (Golovliov et al., 2003a, Mohapatra et al., 2007). The conjugation procedure is simple: (a) incubate the donor *E. coli* and recipient *F. novicida*/*F. tularensis* cells together at 25°C for 18 h on non-selective agar medium (by cross-streaking or place both on filter), (b) transfer the mating mixture to selective medium and grown at 37°C to isolate desired recombinants. The selective medium typically contains antibiotic to select for the conjugated plasmid, and must also provide counter selection against the donor strain, e.g., polymyxin B (50–100 μg/ml) can be used to prevent the growth of *E. coli* without affecting the growth of *F. tularensis* (Frank and Zahrt, 2007).

PLASMIDS

Plasmids are important for the development of various reporter constructs and *in trans* complementation systems. Commonly used plasmids in other bacteria, such as those with pUC, p15a, and pSC101 origins, do not replicate in *F. novicida*/*F. tularensis*; this property has been exploited in the development of *F. tularensis* suicide plasmids, as described below. A plasmid, pFNL10, was isolated from *F. novicida* strain F6186 that can also replicate in *F. tularensis* (but is unable to replicate in, e.g., *E. coli*). Norqvist et al. modified this plasmid and added a p15a origin to allow replication in *E. coli*, resulting in the first *F. tularensis* shuttle vector, pKK202 (Norqvist et al., 1996; Frank and Zahrt, 2007). Many of the pKK202 derivatives contain tetracycline and/or chloramphenicol resistance markers, which are not allowed to be introduced into

select agent forms of *F. tularensis* in the US. In our laboratory (e.g., pKEK1140; see below) and others', these markers have been replaced with kanamycin resistance, which allows these pKK202 derivatives to be used in *F. tularensis* subsp. *tularensis* (LoVullo et al., 2006; Buchan et al., 2008).

It is difficult to transform pKK202 derivatives into several *F. tularensis* subsp., leading Maier et al. to create a new shuttle vector by first selecting for pFNL10 derivatives that would replicate better in LVS, which resulted in the deletion of a portion of the pFNL10 plasmid present in pKK202 encoding an apparent toxin–antitoxin system (ORF4–ORF5). This "modified" *Francisella ori* is the basis for the pFNLTP series of plasmids, which also contain the pUC *ori* for replication in *E. coli*, as well as kanamycin resistance for use in select agent forms of *F. tularensis*. The pFNLTP shuttle vectors have high transformation efficiency in LVS and contain multiple cloning sites (Maier et al., 2004). Additionally, these researchers identified a temperature-sensitive allele in the replicon that has become an extremely useful tool for removal of pFNL10-based plasmids from *Francisella* strains.

LoVullo et al. (2006, 2009) have constructed a series of *Francisella* shuttle vectors (pMP series) with varying portions of pFNL10. These studies established that shuttle vectors containing the *ori*, *repA*, and ORF2 regions of pFNL10, along with kanamycin resistance expressed from the *Francisella groEL* promoter, can be transformed at high efficiency into *F. tularensis* strains. Moreover, the addition of either the ORF4–ORF5 toxin–antitoxin system, or ORF3 (which increases plasmid copy number within the cell) from pFNL10 to the shuttle vector allowed for stable maintenance of the plasmid in the absence of antibiotic selection. It should be noted that the pFNLTP and pMP vectors and the pKK202 derivatives arose from the same progenitor, pFNL10, and thus belong to the same incompatibility group.

A *Francisella* shuttle vector based on the replicon of a different incompatibility group has been constructed, utilizing the origin from the *Staphylococcus aureus* pC194 plasmid, along with the pUC *ori*. This plasmid, pCU18, can be maintained in *F. novicida/F. tularensis* cells already carrying plasmids derived from pFNL10 (Rasko et al., 2007). This plasmid has not yet been adopted for widespread use in *F. novicida/F. tularensis*, and the presence of the chloramphenicol resistance marker prevents its usage in select agent forms of *F. tularensis*. However, additional shuttle vectors have recently been developed from *F. philomiragia* plasmids pF242 and pF243 (Le Pihive et al., 2009). pF242 is closely related to pC194, and can coexist in the same *F. tularensis* cells as pFNL10-based plasmids, and pF243 is closely related to pFNL10-based plasmids. Useful shuttle vectors utilizing these origins of replication have been developed for *F. tularensis* with a variety of antibiotic resistance markers (**Table 1**).

GENE COMPLEMENTATION

The pFNL10 derivative vectors discussed above have been used to achieve gene expression/complementation *in trans*. Researchers have typically either used native promoters for *in trans* expression, or frequently the *F. tularensis groE* promoter. The *groE* promoter was one of the first *Francisella* promoters identified for high-level expression within host cells (Ericsson et al., 1997), and this promoter has subsequently been utilized for expression of genes *in trans*, as well as antibiotic resistance markers (Forslund et al., 2006).

Table 1 | *Francisella tularensis* useful plasmids.

Plasmids	Attributes	Reference
pKK202	pFNL10-based plasmid; also contains p15a *ori*; Tet^R; Cm^R	Norqvist et al. (1996)
pFNLTP plasmids	pFNL10-based plasmid; also contains pUC ori; Kan^R	Maier et al. (2004)
pMP plasmids (series 1)	pFNL10-based plasmids; stable and unstable variants; also contain *colE1 ori*; Kan^R or Hyg^R	LoVullo et al. (2006)
pMP plasmids (series 2)	pFNL10-based plasmids; stable and unstable variants; increased/decreased copy number variants; also contain *colE1 ori*; Kan^R or Hyg^R	LoVullo et al. (2009)
pCU18	pC194-based plasmid; also contains pUC *ori*; Amp^R, Cm^R	Rasko et al. (2007)
pF242 plasmids	pF242-based plasmids; contain either p15a or pUC *ori*; Kan^R or Cm^R or Tet^R	Le Pihive et al. (2009)
pF243 plasmids	pF243-based plasmids; contain either p15a or pUC *ori*; Kan^R or Cm^R or Tet^R	Le Pihive et al. (2009)
pKK214	Derived from pKK202; promoter trap vector drives Cm^R; Tet^R	Kuoppa et al. (2001)
pKK214GFP/ASV	Destabilized GFP expressed from *groEL*p in pKK214, Tet^R	Abd et al. (2003)
pF242-gfp-Cm	GFP expressed from *groEL*p in pF242-based plasmid, Cm^R	Le Pihive et al. (2009)
pXB173-lux	*P. luminescens lux* operon expressed from *groEL*p in pFNL10-based plasmid, Km^R	Bina et al. (2010)
pBB107	Tn5 transposon delivery plasmid for mutagenesis of *F. tularensis*; Km^R	Buchan et al. (2008)
pHimar H3	HimarFT transposon delivery plasmid for mutagenesis of *F. tularensis*; Km^R	Maier et al. (2006)
pKEK1140	Targetron plasmid, intron expressed from *groEL*p in pFNL10-based plasmid; Km^R	Rodriguez et al. (2008)
pPV	Suicide vector for allelic replacement in *F. tularensis; sacB*; Cm^R	Golovliov et al. (2003a,b)
pMP590	Suicide vector for allelic replacement in *F. tularensis; sacB*; Kan^R	LoVullo et al. (2006)
pJC84	Suicide vector for allelic replacement in *F. tularensis; sacB*; Kan^R	Rodriguez et al. (2008), Wehrly et al. (2009)
pJH1 and pGUTS	Plasmids to facilitate allelic replacement in *F. tularensis* via I-SceI restriction	Horzempa et al. (2010)
pKEK1286 and pKEK1287	Gateway-compatible cloning vectors for bacterial 2-hybrid system	Karna et al. (2010)

Another promoter identified for high-level expression in *F. novicida in vivo* is that for *FTN1451*, which encodes a protein of unknown function (Gallagher et al., 2007); the *FTN1451* promoter has been used to drive high-level expression of antibiotic resistance markers in various *F. tularensis* strains (Liu et al., 2007). Full complementation of chromosomal mutations utilizing these promoters to drive *in trans* expression off a pFNL10 derivative plasmid has been notoriously difficult in *Francisella* strains, especially in tissue culture. This incomplete complementation is likely due to a combination of increased copy number due to the relatively high-copy number plasmid, non-native expression due to the *groE* promoter being used, and possibly instability of plasmids lacking the toxin–antitoxin system.

The copy number effect of *in trans* complementation can be overcome by *in cis* complementation, by introducing the expression construct onto the *Francisella* chromosome. The first examples of complementation in *Francisella* involved *in cis* complementation, but this technique has not subsequently been utilized extensively, although there are notable examples of its use in *F. novicida*, as well as in *F. tularensis* subsp. *holarctica* (Mdluli et al., 1994; Baron and Nano, 1998; Forslund et al., 2006; de Bruin et al., 2007). A technique for *in cis* complementation in *F. novicida* has been developed that targets the FTN_1758 gene (de Bruin et al., 2007); because this gene is not found in *F. tularensis* strains this method is not applicable for use in these organisms. LoVullo et al. (2009) have recently developed two systems for *in cis* complementation in *F. tularensis*, using suicide vectors to target a Tn7 insertion site near the *glmS* gene, and to replace the *blaB* gene.

RANDOM MUTAGENESIS

Transposon-mediated random mutagenesis is an invaluable tool for the generation of populations of mutant bacteria, which can then be screened for the phenotype of interest; the mutated gene can then be easily identified by the identification of the location of the transposon. The first transposon mutants were constructed in *F. novicida,* utilizing a Tn5-based transposon to mutagenize *F. novicida* DNA that was subsequently used to transform *F. novicida*, which resulted in the identification of genes important for intramacrophage growth and virulence (Baron and Nano, 1998). While this first study was successful, the technique was cumbersome and only applicable to *F. novicida*, and the transposon was discovered to be unstably integrated (Lauriano et al., 2003).

A major milestone was achieved with the creation of the first transposon mutants in LVS, utilizing the Tn5 derivative transposon EZ::TN Kan (Kawula et al., 2004). This technique uses a Tn5-based transposon complexed with transposase, which is introduced directly into the *Francisella* cells via transformation; once the complex enters the cell, the transposase mediates random insertion of the transposon into the chromosome. The transposon insertion frequency is high and allowed for the creation of a large pool of Tn mutants in LVS (Kawula et al., 2004). A further adaptation of this technique was used to generate "tagged" transposons for a signature-tag mutagenesis study of LVS (Kawula et al., 2004; Su et al., 2007). Derivatives of the Tn5-based EZ::TN transposon have since been used to make transposon mutant libraries in *F. novicida* and *F. tularensis* subsp. *tularensis* (Qin et al., 2004; Qin and Mann, 2006; Weiss et al., 2007).

The Manoil laboratory utilized a EZ::TN-based approach to create a comprehensive transposon mutant library in *F. novicida*. Through saturation mutagenesis and high-throughput sequencing, these researchers were able to identify 396 genes that lacked Tn insertions, indicating that these are essential genes. An important observation during this effort was that the antibiotic resistance gene present in the transposon requires a *Francisella* promoter to achieve truly random insertion in non-essential genes. The resulting comprehensive library contains at least two independent Tn insertions in each of the 1,490 non-essential *F. novicida* genes. This library is a valuable resource that has been subsequently used to identify genes involved in virulence (Gallagher et al., 2007).

Variants of the EZ::TN transposon have been created that contain additional elements, including FRT sites for subsequent excision of the antibiotic resistance gene via FLP-mediated recombination (Gallagher et al., 2007), R6Kγori, a conditional origin of replication that is used to rescue the transposon after insertion into the genome (Qin et al., 2004), and outward facing T7 promoters to facilitate identification of the insertion site (Tempel et al., 2006; Weiss et al., 2007).

A further variation on Tn5-based mutagenesis was the development of a plasmid delivery system for transposon mutagenesis. The Tn5-based transposon, rather than being directly transformed as a transposon–transposase complex, is instead delivered on the plasmid pBB107, which is a shuttle vector that also contains the transposase under inducible control, as well as a temperature-sensitive pFNL10-based *ori* (Maier et al., 2004; Buchan et al., 2008); following Tn insertion into the chromosome the plasmid can be removed with elevated temperature. Variants of this transposon contain additional useful attributes, including FLP recombination sites, the R6K *ori*, and transcriptional reporters (*luxCDABE* and *lacZ*; Buchan et al., 2008).

The *Himar*-based transposons are characterized to exhibit the most random insertion of all known transposons, due to the minimal 2 bp recognition sequence (AT) utilized by this transposon. Due to the AT-rich nature of the *F. tularensis* genome, *Himar* transposons would be predicted to be particularly useful, and a *Himar1*-based transposon has been developed for use in *Francisella* (HimarFT). HimarFT was used to create transposon mutants in LVS (Maier et al., 2006), which allowed for the identification of additional virulence genes (Maier et al., 2007).

TARGETED GENE DISRUPTION

Targeted gene disruption is one of the major goals when developing genetic techniques for any organism. *F. novicida* is readily manipulated to quickly inactivate target genes in techniques that rely on double homologous recombination, primarily due to its ability to be transformed with linear DNA. However, the techniques utilizing this capability in *F. novicida* have failed to work in *F. tularensis*, as mentioned above. Still, techniques are available to specifically inactivate genes in *F. tularensis* through a two-step process, where the first homologous recombination event is selected through positive selection, and the second homologous recombination event is counter selected. An alternative technique involving inactivation by retargeted Group II introns has also been developed that works efficiently in *F. tularensis* strains.

Genes in *F. novicida* can be inactivated by transformation with linear PCR products that consist of an antibiotic resistance gene flanked by at least 500 bp of flanking homology to the gene of interest (Lauriano et al., 2003; Liu et al., 2007). Increasing the amount of flanking homology in the linear fragment increases the efficiency of mutant generation, and expression of the antibiotic resistance gene by a *Francisella* promoter is important for successful mutagenesis. Although this technique results in an antibiotic cassette inserted in the target gene, our studies suggest that the cassette does not inhibit expression of downstream genes. This technique has allowed for rapid generation of *F. novicida* mutants for the study of the genetic basis of pathogenesis.

For targeted mutagenesis in *F. tularensis*, suicide vectors have been developed to mediate the two-step chromosomal gene replacement technique. The first targeted mutant constructed in a *F. tularensis* strain was an *iglC* deletion mutant in LVS (Golovliov et al., 2003b). These researchers utilized a plasmid, pPV, with a pUC-based *ori* (which fails to function in *F. tularensis*), an antibiotic resistance marker (for selection), and the counter selectable marker *sacB* (which causes toxicity in the presence of sucrose). The two-step process consists of (a) transforming the cells with the suicide plasmid containing the deletion and/or insertion in the targeted gene, (b) selection for transformants with the antibiotic resistance gene located on the suicide plasmid (this selects for the first homologous recombination event that results in the plasmid integrating into the chromosome at the target site), (c) counter selection by growth on sucrose (this selects for second homologous recombination event that replaces chromosomal gene with the construct on plasmid). This technique has been used to modify or inactivate a number of genes in LVS (Golovliov et al., 2003a; Bakshi et al., 2006; Gil et al., 2006; LoVullo et al., 2006; Pechous et al., 2006; Ramakrishnan et al., 2008; Sen et al., 2010) and *F. tularensis* subsp. *tularensis* (Twine et al., 2005; LoVullo et al., 2006; Lindgren et al., 2007; Thomas et al., 2007; Michell et al., 2010).

This technique is a powerful tool to create chromosomal deletion mutations, which have the dual advantages of lack of polarity and lack of antibiotic resistance. Some important aspects of this technique need to be noted. The second recombination event counter selected by growth on sucrose results in the loss of the plasmid, but sucrose resistant colonies need to be screened to identify the substituted chromosomal locus, because the second recombination event can also regenerate the wildtype locus. Also, the amount of flanking homology surrounding the deletion and/or insertion is important, with 1000 bp required for efficient recombination. Finally, efficient transformation of the vector is critical, since the plasmid cannot replicate in *F. tularensis* and therefore must recombine into the chromosome immediately following transformation. This last issue can be alleviated by utilizing a vector that can be mobilized into *F. tularensis* via conjugation, which is frequently a more efficient process than transformation, and Celli and colleagues have developed a mobilizable suicide vector, pJC84, with these attributes (Wehrly et al., 2009).

Recently, a technique has been developed for *Francisella* targeted mutagenesis using a clever approach to generating a double-strand break in the chromosome that serves as a substrate for the bacterial DNA repair/recombination machinery. This method utilizes the I-SceI restriction enzyme, which has an unusually large 18 bp recognition sequence, such that the restriction site is not normally found in the chromosomal sequence. By first allowing a deletion construct in a suicide plasmid with a I-SceI site (pJH1) to integrate into the *F. tularensis* chromosome, and then transforming this merodiploid strain with a plasmid that expresses the I-SceI enzyme (pGUTS), these researchers were able to generate *F. tularensis* strains with unmarked deletions in the chromosome (Horzempa et al., 2010).

An alternate strategy for targeted gene inactivation in *F. tularensis* has been developed that utilizes group II intron insertion into the gene of interest. Group II introns form a ribonucleoprotein complex that targets the site for insertion by basepairing between the RNA and the chromosomal DNA. Thus Group II introns can be retargeted to insert at specific sites by altering the sequence of the RNA component, and this technique (referred to as Targetron) has been exploited to disrupt genes in Gram-positive and Gram-negative bacteria (Karberg et al., 2001; Frazier et al., 2003; Perutka et al., 2004; Chen et al., 2005; Yao et al., 2006; Rodriguez et al., 2008, 2009). Our laboratory has optimized this technique for use in *F. tularensis* (Rodriguez et al., 2008, 2009), which involves (a) identification of target sites in specific gene sequence via computer algorithm, (b) "retargeting" the Group II intron via PCR and subsequent cloning into Targetron plasmid (pKEK1140), (c) transformation of Targetron plasmid into *F. tularensis*, (d) screen colonies for insertion of intron into target gene, and (e) remove temperature-sensitive Targetron plasmid by growth at elevated temperature. The ribonucleoprotein complex functions independent of host recombination machinery, so the key to this technique functioning in any given organism is achieving expression of the ribonucleoprotein complex, which is accomplished by a *Francisella* promoter.

This technique has several advantages, including the continuous expression of the ribonucleoprotein complex within the cells, which leads to efficient target site insertion. Also, the intron lacks an antibiotic resistance marker, which is advantageous when working with select agent strains of *F. tularensis*. Finally, this technique allows for the inactivation of two identical genes simultaneously, which has allowed for simultaneous inactivation of both copies of the duplicated FPI genes in *F. tularensis* subsp. *tularensis*. The disadvantage of this technique is that it results in an insertion in, not a deletion of, the gene of interest, which may affect downstream gene expression.

REPORTER CONSTRUCTS

Plasmids and transposons can be engineered to serve as reporters of gene expression, and a number of these genetic constructs now exist to facilitate the study of *F. tularensis* pathogenesis. The first attempt to identify genes upregulated during *F. tularensis* growth inside cells utilized a promoter "trap" construct in which chromosomal DNA fragments were cloned in the plasmid pKK214 (Kuoppa et al., 2001), to identify promoters that drive expression of a chloramphenicol reporter gene, which led to survival in chloramphenicol-treated host cells. This initial study led to the identification of the *groE* promoter, which is still used to drive expression of various genes in *F. tularensis* constructs. Additional studies have also utilized this construct (Baron et al., 1999; Abd et al., 2003), but restrictions on antibiotic resistance prevent its usage in select agent forms of *F. tularensis* in the US. Promoter trap plasmids have also been developed for

F. tularensis that drive expression of GFP (Rasko et al., 2007). Utilizing a promoter trap plasmid that measured expression of red fluorescent protein in the heterologous host *E. coli*, the glucose-repressible promoter for the LVS gene FTL_0580 was identified and characterized, which may provide a useful tool for regulated expression in *F. tularensis* in response to glucose concentration (Horzempa et al., 2008). Transposons have also been engineered to function as reporters of gene expression in *F. tularensis*; utilizing both *lacZ* and luciferase containing transposons, Buchan et al. (2008) were able to identify iron-regulated genes.

Additional useful reporter plasmids have been created for the visualization of *F. tularensis*. The plasmid pKK214GFP-ASV was created to provide high-level GFP expression in *F. tularensis* for use in fluorescent imaging experiments; the GFP in this plasmid has been destabilized by the addition of a C-terminal tag, which prevents build-up of GFP in the cell (Abd et al., 2003). Likewise, pF242- and pF243-derived plasmids were developed that express GFP (Le Pihive et al., 2009). Finally, *Francisella* plasmids expressing the *Photorhabdus luminescens lux* operon and GFP have been developed to facilitate *in vivo* imaging of *F. tularensis* infected live animals (Bina et al., 2010).

GATEWAY CLONING

Gateway® cloning (Invitrogen) is a method that utilizes bacteriophage lambda recombination proteins to circumvent the need for traditional restriction/ligation cloning. In this method, ORFs can be easily moved from "entry" plasmids to "destination" plasmids by addition of the lambda proteins (Aguiar et al., 2004; Gao et al., 2008; Matsuyama and Yoshida, 2009). A complete Gateway® entry clone set representing all the ORFs from the *F. tularensis* subsp. *tularensis* genome has been constructed and is available through the Pathogen Functional Genomics Resource Center (PFGRC; http://pfgrc.jcvi.org/). The only thing limiting broad use of this clone set is a lack of appropriate destination vectors modified for use in *F. tularensis*. Our laboratory has recently developed a Gateway-compatible bacterial 2-hybrid system that can be used to study the interactions between proteins, and demonstrated that it can be used to detect interactions between *F. tularensis* ORFs present in the clone set (IglA and IglB; Karna et al., 2010). Hopefully more Gateway-compatible *Francisella* plasmids will be created in the future, e.g., for routine complementation, to take advantage of this tremendous resource.

PERSPECTIVE

Genetic techniques and tools for the study of *F. tularensis* have expanded dramatically in the past several years. With this new arsenal of methods, the near future should represent the "golden age" of the study of the genetic basis of *F. tularensis* pathogenesis. This will afford greater insight into why *F. tularensis* is able to cause disease and evade host immune mechanisms, and ultimately lead to novel ways to prevent and treat tularemia.

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Innate immune recognition of *Francisella tularensis*: activation of type-I interferons and the inflammasome

Jonathan Wiley Jones, Petr Broz and Denise M. Monack*

Department of Microbiology and Immunology, School of Medicine, Stanford University, Stanford, CA, USA

Edited by:
Anders Sjostedt, Umeå University, Sweden

Reviewed by:
Mikhail A. Gavrilin, Ohio State University, USA
Nelson Gekara, Umeå University, Sweden

****Correspondence:***
Denise M. Monack, Department of Microbiology and Immunology, School of Medicine, Stanford University, Stanford, CA 94305, USA.
e-mail: dmonack@stanford.edu

Francisella tularensis is an intracellular pathogen that can cause severe disease in a wide range of mammalian hosts. Primarily residing in host macrophages, *F. tularensis* escapes phagosomal degradation, and replicates in the macrophage cytosol. The macrophage uses a series of pattern recognition receptors to detect conserved microbial molecules from invading pathogens, and initiates an appropriate host response. In the cytosol, *F. tularensis* is recognized by the inflammasome, a multiprotein complex responsible for the activation of the cysteine protease caspase-1. Caspase-1 activation leads to processing and release of proinflammatory cytokines and host cell death. Here we review recent work on the molecular mechanisms of inflammasome activation by *F. tularensis*, and its consequences both *in vitro* and *in vivo*. Finally, we discuss the coordination between the inflammasome and other cytosolic host responses, and the evidence for *F. tularensis* virulence factors that suppress inflammasome activation.

Keywords: *Francisella*, inflammasome, AIM2, ASC, caspase-1, interferon, STING, interleukin-1b

FRANCISELLA TULARENSIS

Francisella tularensis is a facultative intracellular pathogen that has evolved the capacity to successfully colonize eukaryotic hosts, sometimes causing disease, even in the face of a robust immune response. The primary intracellular niche for *F. tularensis* in the mammalian host is the macrophage, though it has been shown to replicate inside dendritic cells, hepatocytes, neutrophils, and type II alveolar epithelial cells in the host (Buddingh and Womack, 1941; White et al., 1964; Long et al., 1993; McCaffrey and Allen, 2006; Hall et al., 2007). Intracellular replication is crucial to the bacterium's pathogenesis as mutants that fail to replicate intracellularly are avirulent in mice. Because *F. tularensis* is found primarily in macrophages *in vivo* and because the macrophage represents an important mediator of host defense, the macrophage serves as the most widely used *in vitro* model to study *F. tularensis* infection.

After uptake, *F. tularensis* initially resides in a membrane bound vacuole termed the Francisella-containing phagosome (FCP). The FCP acquires the early endosomal antigen 1 (EEA1) within 5 min of uptake, but this marker rapidly dissociates from the phagosome followed by the acquisition of late endosomal markers Lamp1, Lamp2, and the Rab7 GTPase within 15–30 min (Clemens et al., 2004; Santic et al., 2005b; Checroun et al., 2006). The FCP does not significantly fuse with lysosomes and though not absolutely required, acidification of the phagosome acts as a cue for *F. tularensis* to escape this compartment and enter into the host cell cytosol (Chong et al., 2008; Santic et al., 2008). Phagosomal escape is rapid, occurring within 60 min of macrophage infection. Once in the cytosol *F. tularensis* replicates to high numbers. Both phagosomal escape and intracellular replication are mediated by a locus of *F. tularensis* genes known as the *Francisella* pathogenicity island (FPI; Nano et al., 2004). FPI mutants remain in the initial phagosome, which progresses to lysosomes (Bonquist et al., 2008). FPI mutants are also avirulent *in vivo* (Brotcke et al., 2006; Weiss et al., 2007). At late stages of infection *F. tularensis* can be found again inside a membrane bound vacuole that exhibits characteristics of autophagosomes (Checroun et al., 2006). The role of autophagy in *F. tularensis* pathogenesis is unknown, but *F. tularensis* downregulates several host proteins required for the formation of autophagosomes (Butchar et al., 2008).

The intracellular lifestyle of *F. tularensis* brings it in contact with distinct environments of the macrophage, namely the surface during uptake, the initial phagosome, and the host cell cytosol. The macrophage is armed with innate immune defenses in each of these compartments that can respond to the presence of *F. tularensis*. Furthermore, there is crosstalk between signaling pathways that link sensing in one compartment to innate responses in other compartments.

At the macrophage surface, host TLRs engage *F. tularensis*. Unlike LPS from enteric bacteria, *F. tularensis* LPS is only mildly inflammatory and stimulates a low level of proinflammatory cytokine production (Hajjar et al., 2006). This likely is due to the unique structure of *F. tularensis* lipid A. Unlike the hexa-acylated lipid A from *Escherichia coli* and other gram-negative enterics, lipid A from *F. tularensis* is tetra-acylated (Raetz et al., 2009). It is thought that this altered structure makes it unrecognizable to LPS-binding protein, and therefore subverts TLR4 recognition (Barker et al., 2006; Cole et al., 2006; Hajjar et al., 2006). Consistent with this observation, TLR4 deficient mice are not more susceptible to infection with *F. tularensis* than wild-type mice (Chen et al., 2004; Collazo et al., 2006). *F. tularensis* does significantly stimulate TLR2 signaling resulting in proinflammatory cytokine production, and intracellular bacteria colocalize with TLR2 and MyD88 (Katz et al., 2006; Malik et al., 2006; Cole et al., 2007). *In vivo*, TLR2 does not seem to play a role in host protection during intradermal challenge, but is important in an intranasal model of infection (Collazo et al., 2006; Malik et al., 2006). However, Myd88 deficient mice are completely susceptible to sublethal doses of the live vaccine strain (LVS) when given intradermally (Collazo et al., 2006).

The ability of *F. tularensis* to escape the initial phagosome and reside in the macrophage cytosol is essential for the organism's pathogenesis. Paradoxically, cytosolic localization is also essential for innate immune recognition by a cytosolic surveillance system known as the inflammasome. Here we review recent work that has shed light on the molecular mechanisms that lead to cytosolic detection of *F. tularensis* in the macrophage, and host defense *in vivo*.

CASPASE-1

Some of the key regulators of inflammation are aspartate-specific cysteine proteases known as inflammatory caspases. These include caspase-1, -4, -5, -11 (which exists in rodents), and -12 (Scott and Saleh, 2007). Caspase-1, the best studied of these inflammatory caspases, is activated in intracellular complexes known as inflammasomes, which are located in the cytosol of certain immune cells and assembled in response to danger signals. Activation of caspase-1 involves autoproteolytic processing of the 45-kDa pro-caspase-1 into 20- and 10-kDa subunits (p20 and p10; Thornberry et al., 1992; Ayala et al., 1994; Wilson et al., 1994). In the cytosol, active caspase-1 processes pro-IL-1β (Black et al., 1989; Kostura et al., 1989) and pro-IL-18 (Ghayur et al., 1997) into their mature, bio-active forms that are secreted and regulate inflammation. When regulated properly, IL-1β is critical for the host response to infection, but excessive levels of Il-1β are associated with several inflammatory diseases such as rheumatoid arthritis, inflammatory bowel disease, and septic shock to name a few (Dinarello and Wolff, 1993). Clearly inflammation can be a double-edged sword but it is essential to combat infection and restore tissue homeostasis after infection (Medzhitov, 2010).

THE INFLAMMASOME RESPONDS TO INTRACELLULAR PATHOGENS AND DANGER SIGNALS

In 2002, it was discovered that a multiprotein complex termed the "inflammasome" was responsible for activating caspase-1 (Martinon et al., 2002). The inflammasome, in its simplest form, is composed of a NOD-like receptor (NLR), and the adaptor protein apoptosis associated speck-like protein containing a caspase recruitment domain (ASC). The NLRs are a family of cytosolic pattern recognition receptors (PRRs) that activate inflammatory and antimicrobial responses by sensing "danger signals" or danger-associated molecular patterns (DAMPs; Matzinger, 2002) and conserved microbial products termed pathogen associated molecular patterns (PAMPs; Tschopp et al., 2003; Inohara et al., 2005). There are 22 members of the NLR subfamily in humans and 34 in mice, which are characterized by a C-terminal leucine rich repeat (LRR) domain, a central oligomerization domain (NACHT), and an N-terminus that is either a caspase activation and recruitment domain (CARD; as in NOD1, NOD2, and the NLRC family), three baculovirus IAP repeats (BIR; as in the NAIPs), or a pyrin domain (PYD; as in the NLRP family; Kanneganti, 2010). The adaptor ASC (also known as PYCARD) has an N-terminal PYD that facilitates interactions with the PYD domain of NLRs, and a C-terminal CARD domain that recruits caspase-1 through CARD-CARD interactions (Martinon et al., 2001). Interestingly, besides its role as an adaptor, ASC is required to induce autoproteolysis of pro-caspase-1 in the inflammasome complex, a prerequirement for efficient cytokine processing (Broz et al., 2010b). LRRs are also found in the toll-like receptors (TLRs), which sense danger signals and microbial patterns on the cell surface and in endosomes (Iwasaki and Medzhitov, 2004; Takeda and Akira, 2005). Interestingly, the TLRs cannot distinguish between pathogenic and non-pathogenic microbes because they sense conserved microbial patterns that are present in both and they are located extracellularly and in endosomes, where both pathogenic and non-pathogenic microbes reside. The NLRs also sense conserved microbial patterns, but the only way these microbial patterns can reach the cytosol is if they are delivered there by disruption of cell membranes by toxins, specialized secretion systems of pathogenic microbes, and/or cytosolic pathogens. Therefore the location of the sensors and not the ligands they sense makes the NLRs specific for detecting pathogens (Brodsky and Monack, 2009).

The vast number of NLR proteins allows the inflammasome to respond to numerous pathogens and danger signals. The cytosolic DAMP or PAMP sensed determines which NLR forms the complex. An inflammasome is named after the NLR that forms it (i.e., the NLRP3 inflammasome, the NLRC4 inflammasome, etc.), and so the inflammasome's components can vary. As shown in **Figure 1**, the inflammasome has been implicated in the host response to numerous pathogens and danger signals, as well as a number of autoimmune and auto-inflammatory diseases (Brodsky and Monack, 2009; Rodrigue-Gervais and Saleh, 2010).

Conserved microbial structures, such as bacterial cell wall components are potent activators of innate immunity. Muramyl dipeptide (MDP) is a breakdown product of bacterial peptidoglycan that is recognized by NOD2 and induces transcriptional activation of proinflammatory cytokines through the adaptor RIP2. MDP is also a potent activator of the inflammasome, which results in release of mature IL-1β. Inflammasome activation by MDP involves both NOD2 and NLRP3, suggesting that NLRP3 is an additional sensor of MDP (Martinon et al., 2004; Pan et al., 2007; Marina-Garcia et al., 2008). NOD2 has also been shown to cooperate with NLRP1b in inflammasome activation in response to MDP and anthrax lethal toxin (Bruey et al., 2007; Hsu et al., 2008). These results suggest that inflammasome complexes may contain of multiple NLRs that act synergistically to activate caspase-1 in response to PAMPs.

Many bacteria employ pore-forming toxins in their pathogenic arsenal to establish infections. NLRP3 has been implicated in the inflammasome response to listeriolysin O form *Listeria monocytogenes*, α-toxin from *Staphylococcus aureus*, and aerolysin from *Aeromonas hydrophila* (Mariathasan et al., 2006; Fink et al., 2008). The precise mechanism by which NLRP3 recognizes these toxins is unknown. NLRP3 also activates the inflammasome in response to high extracellular concentrations of ATP (Mariathasan et al., 2006), various crystalline compounds (Martinon et al., 2006; Cassel et al., 2008; Dostert et al., 2008; Halle et al., 2008), as well as viral and bacterial nucleic acids (Kanneganti et al., 2006; Muruve et al., 2008). With such a diverse array of stimuli, the hypothesis is that these ligands potentiate a common terminal signal that activates NLRP3. Potassium efflux, membrane damage, and stimulation of reactive oxygen species have all been implicated as the terminal signal for NLRP3, but each has caveats. Therefore, the precise mechanism of NLRP3 activation remains a mystery.

NLRC4 was the first NLR shown to activate caspase-1 in response to bacterial infection with *Salmonella typhimurium* in a type-III secretion system (T3SS)-dependent manner (Mariathasan et al.,

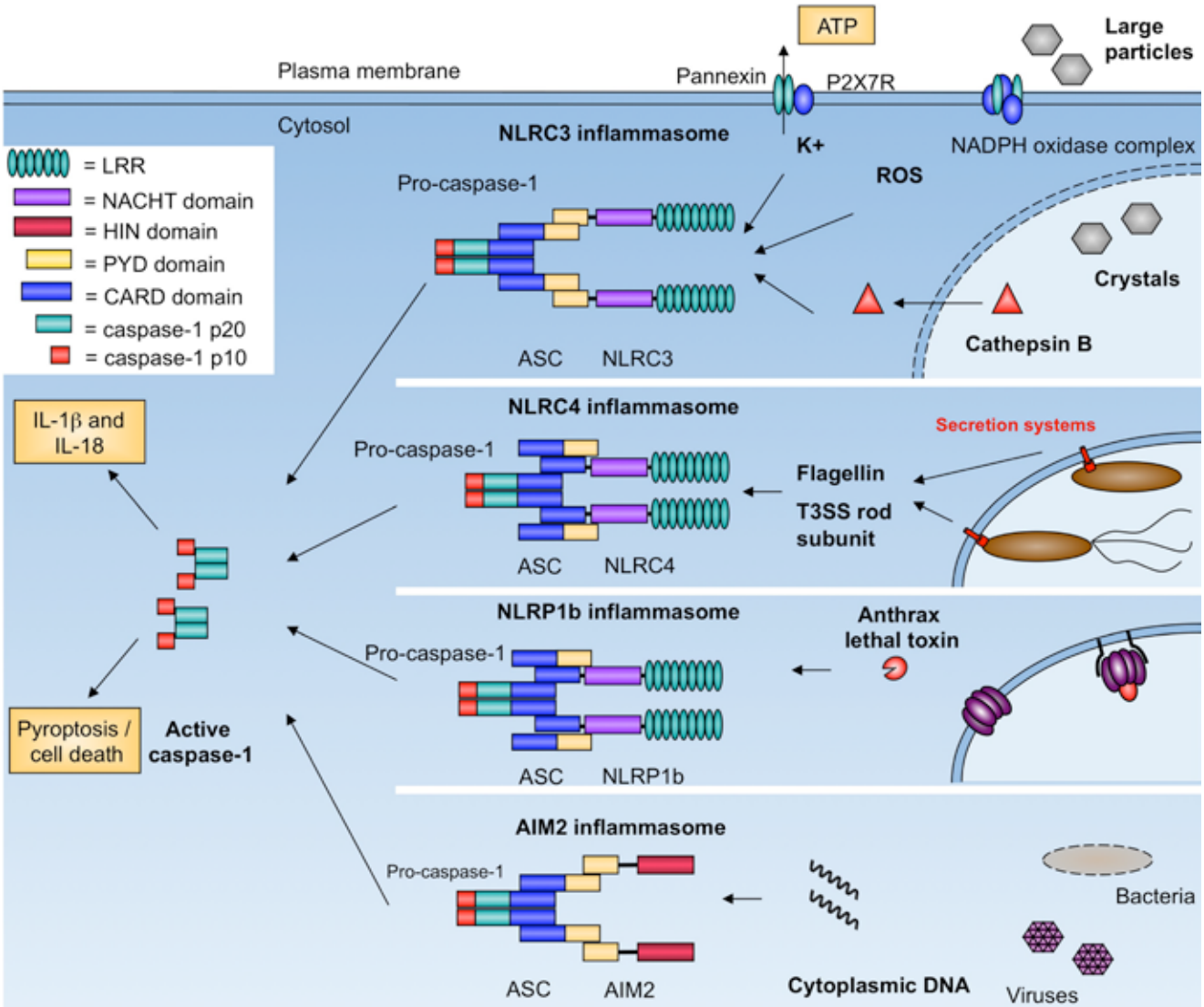

FIGURE 1 | Inflammasomes activate caspase-1 in response to pathogens and danger signals. NLRC3 responds to numerous stimuli including pore-forming toxins, extracellular ATP, and crystalline compounds. The exact mechanism of activation is unknown but may involve potassium efflux, membrane damage, and generation of reactive oxygen species. NLRC4 responds to bacterial flagellin and the T3SS rod subunit. NLRP1b responds to anthrax lethal factor. AIM2 binds cytosolic dsDNA from bacterial, viral, mammalian, and synthetic sources. Complexes are formed through homotypic interactions (LRR, leucine rich repeats; HIN, hemopoietic IFN-inducible nuclear proteins; PYD, pyrin; CARD, caspase activation and recruitment domain). All inflammasomes activate caspase-1, which leads to processing of pro-IL-1β and pro-IL-18, and host cell death.

2004). It was later revealed that NLRC4 sensed bacterial flagellin that was secreted into the host cytosol through the T3SS, likely due to the evolutional similarity of the T3SS with the flagellar biosynthesis machinery (Franchi et al., 2006; Miao et al., 2006). Detection of cytosolic flagellin was later linked to the inflammasome response to *Pseudomonas aeruginosa*, *L. monocytogenes*, and *Legionella pneumophila* (Molofsky et al., 2006; Ren et al., 2006; Miao et al., 2008; Warren et al., 2008), though in the case of *Legionella* the type-IV secretion system (T4SS) was required and Naip5 was required in addition to NLRC4 (Ren et al., 2006; Zamboni et al., 2006). NLRC4 was also implicated in recognition of *Shigella flexneri* (Suzuki et al., 2007), which encodes a T3SS but does not express flagellin (Tominaga et al., 1994), as well as non-flagellated strains of *Pseudomonas* (Sutterwala et al., 2007), suggesting that NLRC4 must recognize other ligands besides flagellin. The answer was recently provided when it was demonstrated that NLRC4 could also the rod component of the T3SS needle apparatus, which shares a sequence motif with flagellin (Miao et al., 2010). This motif is essential to NLRC4-mediated recognition and explains how NLRC4 can respond to diverse pathogens that express either a T3SS or flagellin.

Studies of NLRP1b, NLRP3, and NLRC4 have expanded our understanding of the role of the NLR family and inflammasomes in the host response to pathogens. However, transfection of syn-

thetic, bacterial, viral, or mammalian dsDNA triggers caspase-1 activation in a manner that requires ASC, but not any of the known NLRs (Muruve et al., 2008). Recently a member of the family of hemopoietic IFN-inducible nuclear proteins with a 200-amino acid motif (HIN-200), Absent in melanoma 2 (AIM2), was shown to activate the inflammasome in response to cytosolic double stranded DNA (dsDNA; Burckstummer et al., 2009; Fernandes-Alnemri et al., 2009; Hornung et al., 2009; Roberts et al., 2009). AIM2 contains a HIN domain that facilitates dsDNA binding, and a PYD domain that recruits ASC, allowing for caspase-1 activation. This was the first example of a non-NLR family protein triggering inflammasome activation. Moreover, AIM2 is interferon-inducible, establishing a link between two cytosolic innate immune responses.

FRANCISELLA TULARENSIS ACTIVATES THE AIM2 INFLAMMASOME

After *F. tularensis* escapes the phagosome, it is subject to cytosolic innate immune recognition (**Figure 2**). In murine macrophages, *F. tularensis* induced inflammasome activation was shown to be independent of known receptors, like NLRP1b, NLRC4, and NLRP3, but required the adaptor protein ASC (Mariathasan et al., 2005, 2006). In addition, inflammasome activation was also dependent on interferon signaling suggesting that an interferon-inducible inflammasome sensor, such as AIM2, might be involved in sensing cytosolic *F. tularensis* (Henry et al., 2007). Infection of primary macrophages from AIM2-deficient mice demonstrated that AIM2 was required for caspase-1 processing, release of mature IL-1β, and host cell death in response to *F. tularensis* (Fernandes-Alnemri et al., 2010; Jones et al., 2010; Rathinam et al., 2010). In addition, confocal microscopy demonstrated that cytosolic *F. tularensis* release bacterial DNA into the macrophage cytosol and that AIM2 colocalized with the bacterial DNA (Fernandes-Alnemri et al., 2010; Jones et al., 2010). Interestingly, if several lysing bacteria were observed in a single cell, their DNA colocalized with AIM2, however only one of these sites served as a nucleation point for inflammasome assembly, as visualized by the formation of the so-called ASC focus (Jones et al., 2010). This observation represented the first visualization of an endogenous inflammasome in complex with its ligand. Formation of similar ASC foci was also observed during Salmonella infections, and the focus was subsequently shown to recruit and activate caspase-1 and to serve as a major site of cytokine processing (Broz et al., 2010a).

Inflammasome activation was also shown to be critical to host defense *in vivo* as mice lacking the inflammasome components AIM2, caspase-1, or ASC have increased bacterial burden and succumb to infection much faster than wild-type mice (Mariathasan et al., 2005; Fernandes-Alnemri et al., 2010; Jones et al., 2010).

As mentioned above, *F. tularensis* activates a cytosolic surveillance pathway that is characterized by the production of type-I IFNs (Henry et al., 2007; Cole et al., 2008). The host cytosolic sensor responsible for type-I IFN production remains unknown. However, the adaptor protein stimulator of interferon genes (STING) is required for type-I IFN production in response to *F. tularensis* in murine macrophages (Jones et al., 2010). Autocrine and paracrine signaling through IFNAR leads to an increase in AIM2 protein expression in the macrophage, effectively priming the cell for recognition of cytosolic dsDNA. Although other roles for type-I IFN signaling during *F. tularensis* infection exist (Henry et al., 2010), the expression of AIM2 is sufficient for inflammasome activation irrespective of other IFN-induced genes. Type-I IFN signaling is critical to inflammasome activation in the macrophage as IFNAR-deficient macrophages do not process capsase-1, release proinflammatory cytokines, or die in response to *F. tularensis* (Henry et al., 2007).

A positive feedback loop between type-I IFN signaling and inflammasome activation in macrophages exists, but the link *in vivo* seems to be less clear. Previous reports show that mice deficient in inflammasome components are more susceptible to infection with *F. tularensis* (Mariathasan et al., 2005). However, mice deficient in type-I IFN signaling are more resistant to infection (Henry et al., 2010). Similar results were also obtained for *L. monocytogenes* infections (Henry et al., 2010). This apparent discrepancy between the *in vitro* results and the *in vivo* results is partially explained by the control of type-I IFN signaling on IL-17 production (Henry et al., 2010). Mice deficient in type-I IFN signaling produce more IL-17, which leads to a greater influx of neutrophils that can better control bacterial infections (Henry et al., 2010). A possible explanation for the *in vivo* phenotype is that Type-IFN has been shown to promote apoptosis of immune cells such as macrophages, neutrophils, and lymphocytes during *Listeria* and *Francisella* infections (O'Connell et al., 2004; Carrero et al., 2006; Navarini et al., 2006; Henry et al., 2007). Additionally, IFN-γ can restore inflammasome activation *in vivo* in an IFNAR-deficient mouse by signaling through the IFN-γ receptor, which would result in increased expression of AIM2 and subsequent inflammasome activation (our unpublished results). Thus, an interesting paradox exists, where type-I IFN is beneficial to the host *in vitro* and detrimental *in vivo* during bacterial infections.

THE INFLAMMASOME AND *F. TULARENSIS* VIRULENCE FACTORS

During a mammalian infection, *F. tularensis* must establish a replicative niche in the presence of a robust innate immune system. The inflammasome is of critical importance to mount an effective innate immune response against intracellular *F. tularensis*. Investigation into the interaction of *F. tularensis* and the inflammasome has led to the discovery of several bacterial genes that seem to suppress inflammasome activation. The first genes to be identified were *oppB* and the zinc metalloprotease *pepO* (also *FTT1209c*). Mutants in these mglA-regulated genes exhibit increased kinetics of replication in bone marrow-derived macrophages and increased macrophage cytotoxicity compared to wild-type *F. tularensis,* although it was not shown that the increased cytotoxicity of these mutants was mediated by inflammasome activation (Brotcke et al., 2006). Furthermore, both oppB and pepO showed reduced fitness in mice in intradermal competitive index experiments (Brotcke et al., 2006). However, in single infections via the intradermal route, mice infected with a *pepO* mutant had higher bacterial burdens in the spleen and, when infected by aerosol, displayed increased neutrophil influx into the lung (Hager et al., 2006). It is unclear if either *oppB* or *pepO* interacts directly with the inflammasome to suppress macrophage cytotoxicity.

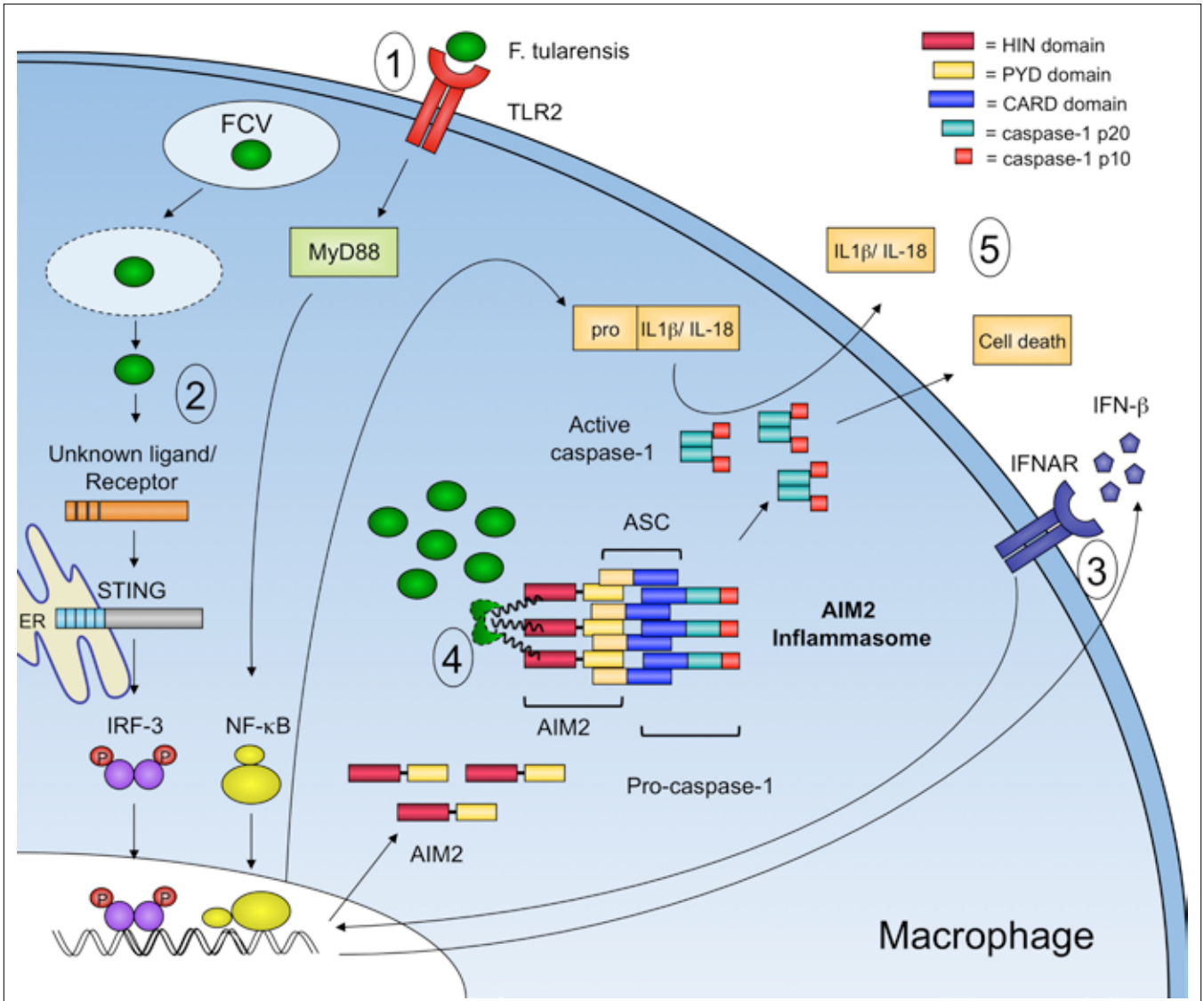

FIGURE 2 | Model of innate immune recognition of *F. tularensis* in the macrophage. Signaling events involved in the innate immune response to *F. tularensis* are highlighted. 1 – At the surface of the macrophage, TLR2 induces a MyD88-dependent transcriptional response that leads to the expression of proinflammatory cytokines including pro-IL-1β. *F. tularensis* then enters the macrophage in a membrane bound vacuole known as the FCV. 2 – Upon escape into the cytosol, an unknown receptor recognizes an unknown *F. tularensis* ligand that leads to the STING-dependent and IRF-3-dependent production of type-I IFNs. 3 – Autocrine and paracrine signaling through the type-I interferon receptor (IFNAR) leads to an increase in AIM2 protein expression. 4 – Cytosolic *F. tularensis* lyse, releasing bacterial DNA that is recognized by AIM2, which in turn recruits ASC and procaspase-1 to form an inflammasome complex. 5 – Active capsase-1 processes pro-IL-1β and pro-IL-18 into their mature forms and triggers host cell death.

Two additional *F. tularensis* genes involved in suppressing the macrophage inflammasome, *FTT0748* and *FTT0584*, were identified in a microarray-based *in vivo* negative selection screen where they displayed reduced fitness in competitive index experiments in mice. Subsequent characterization of *FTT0748* and *FTT0584* in bone marrow-derived macrophages showed that these mutants induce increased macrophage cytotoxicity and IL-1β release compared to wild-type *F. tularensis* and these phenotypes were dependent on both the ASC and caspase-1 inflammasome components, as no cell death or IL-1β release was observed in macrophages lacking ASC or caspase-1 (Weiss et al., 2007). Interestingly, and in contrast to the oppB and pepO mutants, neither *FTT0748* nor *FTT0584* displayed altered intracellular replication kinetics in bone marrow-derived macrophages. These mutants were the first to disassociate *F. tularensis* intracellular replication from inflammasome activation. *FTT0748* has homology to IclR family transcriptional regulators and *FTT0584* is a protein of unknown function. The mechanism(s) by which they suppress inflammasome activation are not known.

Investigation into the role of type I secretion systems in *F. tularensis* virulence led to the identification of TolC, an outer membrane protein that participates in multidrug resistance (Gil et al., 2006). A *tolC* mutant in the LVS strain of *F. tularensis* was hypercytotoxic to both murine bone marrow-derived macrophages as well as human monocyte-derived macrophages (huMDM) when

compared to wild-type LVS (Platz et al., 2010). The *tolC* mutant also caused higher levels of IL-1β secretion from huMDM, implicating a role for *tolC* in suppressing the inflammasome in human cells. However, the hypercytotoxicity observed in muBMDM was inflammasome independent, as hypercytotoxicity was still observed in caspase-1 deficient macrophages and was comparable to IL-1β secretion in macrophages infected with wild-type LVS (Platz et al., 2010). Unlike in human cells, the hypercytotoxicity of the *tolC* mutant seems to be the result of increased caspase-3/7 activity, resulting in cell death by apoptosis. Thus *tolC* might play a role in suppressing several different immune pathways, both inflammasome-dependent and -independent. Like FTT0748 and FTT0584, *tolC* was not required for replication in macrophages but is required for full virulence in mice (Gil et al., 2006; Platz et al., 2010).

Required for intracellular proliferation, factor A (RipA) is a cytoplasmic membrane protein conserved across *F. tularensis* subspecies that is required for intracellular replication in both macrophages and epithelial cells, and is required for virulence in a mouse model of tularemia (Fuller et al., 2008). A *ripA* deletion mutant elicits increased IL-1β and IL-18 production from bone marrow-derived macrophages relative to wild-type *F. tularensis* and induces more macrophage cell death (Huang et al., 2010). The hypercytotoxic and hyperinflammatory phenotype of the ripA mutant was abolished in BMDM deficient for ASC, caspase-1, or MyD88, suggesting that a ripA mutant could elicit higher transcriptional levels of proinflammatory genes among them pro-IL-1β, which would also explain the elevated levels of mature IL-1β. Consistently, levels of TNF-α, which is secreted independently of the inflammasome, were also elevated in infections with a ripA mutant (Fuller et al., 2008). These phenotypes were also absent in THP-1 cells treated with a caspase-1 inhibitor, Y-VAD, or expressing ShRNA against ASC (Huang et al., 2010). Furthermore, intranasal infections with the *ripA* mutant led to an increase in IL-1β secretion into the bronchial alveolar lavage fluid of mice relative to mice infected with wild-type *F. tularensis*, supporting a role for *ripA* in suppressing inflammasome activation during pneumonic tularemia.

Francisella tularensis LPS genes have also been implicated in suppression of the inflammasome. A mutant in a putative lipid II flippase, mviN, induces an increase in the kinetics of caspase-1 processing, proinflammatory cytokine secretion, and cell death in macrophages compared to wild-type *F. tularensis*. This phenotype is completely dependent on AIM2, caspase-1, and ASC (Ulland et al., 2010). The mechanism by which mviN suppresses inflammasome activation is not known but the deletion mutant exhibits a striking change in bacterial morphology, suggesting that an altered membrane may play a role in the bacteria's recognition.

Similarly, several more genes involved in LPS and capsule biosynthesis play a role in limiting macrophage cell death. These genes include *lpcC*, *manB*, *manC*, *kdtA*, *FTT1236*, *FTT1237*, and *FTT1238* (Lai et al., 2010; Lindemann et al., 2010). Mutants in these genes express a shortened LPS and are phagocytosed by macrophages at a higher frequency than wild-type bacteria. These mutants induce greater macrophage cell death, though this is incompletely explained by the higher rate of phagocytosis (Lai et al., 2010; Lindemann et al., 2010). Furthermore, the cell death pathway involved was not explored in this study, though the AIM2 inflammasome is a strong candidate.

UNANSWERED QUESTIONS AND FUTURE DIRECTIONS

The recent studies demonstrating the role of AIM2 in innate immunity to *F. tularensis* suggest that bacterial lysis in the cytosol leads to release of *F. tularensis* DNA, induction of the type-I IFN pathway, and activation of the AIM2 inflammasome. The molecular mechanism that causes bacterial lysis is unclear but several hypotheses emerge from this work. When bacteria are cultured in rich media, they exhibit a life cycle that is characterized by a lag phase of no bacterial replication, a log phase with a net increase in bacterial multiplication, a stationary phase of limited nutrient availability where replication plateaus, and a death phase with a net decrease in bacterial numbers. During this death phase many bacteria lyse, releasing their contents into the culture media. Therefore, bacterial lysis is a natural part of the bacterial life cycle and this could be the mechanism of DNA release in the host cytosol. Additionally, the macrophage phagosome is a professional microbe-killing machine, and although *F. tularensis* is well equipped to escape with its life that does not mean that it is not wounded in the battle. Studies to date on the intracellular trafficking of the *Francisella* containing vacuole suggest that the bacteria escape before acquiring markers of lysosomes or degradative enzymes (Santic et al., 2005a,b; Checroun et al., 2006). However, slight perturbations in the bacterial envelope during the vacuolar stage may be enough to induce lysis once the bacteria reach the cytosol. The large number of mutants identified in genes encoding membrane proteins and proteins involved in and LPS synthesis that result in an increased inflammasome activation in the macrophage support this hypothesis. If these mutants have an unstable outer membrane they may lyse at a higher frequency than wild-type *F. tularensis* and lead to increased cytosolic sensing by the DNA receptors. In support of this idea, recent reports show that *L. monocytogenes* lyses at a low frequency in the macrophage cytosol and induces AIM2-dependent inflammasome activation (Sauer et al., 2010). Furthermore, *L. monocytogenes* mutants that induced higher levels of inflammasome activation were shown to lyse with increased frequency compared to wild-type *L. monocytogenes*.

Yet another possible hypothesis, is the existence of antimicrobial defenses in the cytosol itself. Little is known about the cytosolic environment except that it is pH neutral. In addition, little is known about changes to this environment after macrophages are stimulated with proinflammatory cytokines such as interferons. The interferons were first described for their ability to induce and antiviral state in cells, but whether or not this includes defenses in the cytosol is unknown. However, IFN-γ treatment of primary mouse- and human-derived macrophages has been shown to restrict cytosolic growth of *F. tularensis* subsp. tularensis (Edwards et al., 2010), and INF-β induces a similar transcriptional response as IFN-γ in macrophages so we hypothesize that type-I IFNs may induce bacterial lysis in the cytosol. These hypotheses are not mutually exclusive and it is likely that multiple mechanisms contribute to bacterial lysis.

Although mainly studied as an antiviral mechanism, a number of recent reports demonstrate that type-I IFN is induced by a number of intracellular bacteria such as *Mycobaterium tuberculosis* (Weiden et al., 2000; Giacomini et al., 2001), *L. monocytogenes* (O'Riordan et al., 2002), and *Le. pneumophila* (Opitz et al., 2006). In the case of *L. monocytogenes* it was recently reported that cyclic diadenosine monophosphate (c-di-AMP) is released by the bacterium into the

macrophage cytosol during infection and can trigger the type-I IFN response (Woodward et al., 2010). The bacterial PAMP that stimulates type-IFN production in the other aforementioned bacterial infections is unknown, though in the case of *F. tularensis* infection it is suspected that a cytosolic DNA sensor could be involved (Stetson and Medzhitov, 2006; Henry et al., 2007). Similar to its effects on viral infections, type-I IFN can act in concert with other cytosolic sensing pathways like the inflammasome to trigger a protective host cell death (Coers et al., 2007; Henry et al., 2007).

The mechanism linking the type-I IFN pathway and the inflammasome is currently unknown. To better understand the molecular mechanisms of the coordination between these two pathways we need to identify the host receptors that lead to type-I IFN production. One such receptor, DAI (Takaoka et al., 2007), is either not active in macrophages, or there are redundant receptors since DAI-deficient macrophages and mice respond normally to stimulation with dsDNA (Ishii et al., 2008). However, two new cytosolic DNA sensors have recently been identified. One, LRRFIP1, mediates type-I IFN production in macrophages in response to *L. monocytogenes* and vesicular stomatitus virus (Yang et al., 2010). The other, IFI16, contains a pryin domain and an HIN domain, similar to AIM2, and mediates type-I IFN production in response to DNA and herpes simplex virus-1 (HSV-1; Unterholzner et al., 2010). In this report, the authors found that IFI16 was able to associate with STING. If LRRFIP1 and/or IFI16 were also involved in the macrophage type-I IFN response to *F. tularensis*, we would have a system to study the coordination of the IFN pathway and inflammasome pathway in a biologically relevant model.

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Francisella subverts innate immune signaling: focus on PI3K/Akt

Thomas John Cremer, Jonathan P. Butchar and Susheela Tridandapani*

Department of Internal Medicine, The Ohio State University Medical Center, Columbus, OH, USA

Edited by:
Anders Sjostedt, Umeå University, Sweden

Reviewed by:
Brian Coombes, McMaster University, Canada
John T. Belisle, Colorado State University, USA

****Correspondence:***
Susheela Tridandapani, Department of Internal Medicine, Davis Heart and Lung Research Institute, The Ohio State University Medical Center, 415, 473 West, 12th Avenue, Columbus, OH 43210, USA.
e-mail: tridandapani.2@osu.edu

Intracellular bacterial pathogens exploit host cells as a part of their lifecycle, and they do so by manipulating host cell signaling events. Many such bacteria are known to produce effector proteins that promote cell invasion, alter membrane trafficking, and disrupt signaling cascades. This review highlights recent advances in our understanding of signaling pathways involved in host cell responses to *Francisella tularensis*, a facultative Gram-negative intracellular pathogen that causes tularemia. We highlight several key pathways that are targeted by *Francisella*, with a focus on the phosphatidylinositol 3-kinase/Akt pathway. Lastly, we discuss the emerging role of microRNAs (miRs), specifically miR-155, as a key regulator of host signaling and defense.

Keywords: PI3K, Akt, SHIP, miR-155, host response, *Francisella*

INTRODUCTION

Successful intracellular pathogens must have mechanisms to usurp their hosts' normal functions and defenses, thereby creating a favorable environment. For example, *Legionella pneumophila* secrete type IV effectors that form a permissive niche (Berger et al., 1994) and *Yersinia pestis* disrupts host nuclear factor kappa B (NF-κB) signaling via the Yop J proteins (Zhou et al., 2005). However, each pathogen can use slightly – or vastly – different mechanisms to conquer the complex signaling pathways that orchestrate the host response. A better understanding of these responses and the methods by which pathogens defeat them will fuel the generation of novel therapeutics.

Francisella tularensis is a Gram-negative bacterial pathogen and is the causative agent of tularemia. The highest-virulence *F. tularensis* subspecies *tularensis* requires as few as 10 colony forming units to cause disease and death in humans (Sjostedt, 2007). Because of this it has been classified as a category A select agent by the United States Centers for Disease Control. However, there are also lower-virulence subspecies and strains, such as *F. tularensis* subspecies *novicida* and the live vaccine strains (LVS) of *F. tularensis* subspecies *holarctica*. These subspecies are used extensively for research because they lead to disease in mice that resembles human tularemia, their intracellular lifecycles are similar to that of *F. tularensis tularensis*, and they present minimal risk to humans. Since 2001 there has been a renewed interest in the study of this pathogen for biodefense purposes, and tremendous advances have been made in recent years (Oyston et al., 2004; Elkins et al., 2007; Santic et al., 2010).

One of the most interesting aspects of infection with *F. tularensis* is that there is a lack or delay of inflammatory response during the early stages of infection (Andersson et al., 2006; Bosio et al., 2007). Even though this pathogen is Gram-negative, the lipopolysaccharide (LPS) is modified in such a way that it does not activate toll-like receptor (TLR) 4 (Hajjar et al., 2006; Gunn and Ernst, 2007). Additionally, *F. tularensis* can suppress the host's ability to respond to pro-inflammatory signals (Telepnev et al., 2003, 2005; Bosio et al., 2007), thus demonstrating that it does not merely evade host cell immune responses. Indeed, genomewide studies on the effect of *F. tularensis* on human phagocytes shows a broad suppression of numerous signaling pathways including TLR signaling, autophagy, major histocompatibility complex (MHC) presentation, interferon signaling, and phosphatidylinositol 3-kinase (PI3K) signaling (Butchar et al., 2008).

BACTERIAL ENTRY INTO PHAGOCYTES

Internalization of *Francisella* by human phagocytes occurs though a unique process termed "looping phagocytosis" (Clemens and Horwitz, 2007). Optimal phagocytosis of *Francisella* requires an intact complement pathway, as internalization is dramatically reduced in the absence of complement (Lofgren et al., 1983; Balagopal et al., 2006; Clemens and Horwitz, 2007). However, the bacterium itself is resistant to complement-mediated lysis though its LPS O antigen (Clay et al., 2008). Other receptors such as the scavenger receptor (Pierini, 2006) and mannose receptor (Balagopal et al., 2006; Schulert and Allen, 2006) have been implicated in promoting bacterial uptake. Fc receptors also play a role, aiding both internalization of bacteria (Balagopal et al., 2006) and host protection (Kirimanjeswara et al., 2007).

Abbreviations: AIM-2, absent in melanoma 2; ERK, extracellular signal-regulated kinase; GSK3β, glycogen synthase kinase 3 beta; LPS, lipopolysaccharide; MHC, major histocompatibility complex; MyD88, myeloid differentiation primary response gene (88); NF-κB, nuclear factor kappa B; PI3K, phosphatidylinositol 3-kinase; PTEN, phosphatase and tensin homolog; *ripA*, required for intracellular proliferation, factor A; SHIP, SH2 domain-containing inositol 5′-phosphatase 1; SOCS3, suppressor of cytokine signaling 3; Syk, splenic tyrosine kinase; TLR, toll-like receptor.

Downstream signaling events implicated in the phagocytosis of *F. novicida* include the splenic tyrosine kinase (Syk) and extracellular signal-regulated kinase (ERK) pathways. Genetic and pharmacologic manipulation of these two kinases established that both pathways are required for macrophage phagocytosis of bacteria under heat-inactivated fetal bovine serum culture conditions (Parsa et al., 2008b). Conflicting reports exist on the role of PI3K signaling during phagocytosis. Phagocytosis of *F. tularensis* in human monocyte derived macrophages cultured in AB serum is wortmannin sensitive (Clemens and Horwitz, 2007). However, PI3K or downstream Akt signaling in murine macrophages cultured with heat-inactivated fetal bovine serum does not have any effect on bacterial internalization (Parsa et al., 2008b; Rajaram et al., 2009).

INTRACELLULAR REPLICATION

Intracellular replication requires escape from the phagosome to gain access to the cytosol. Numerous escape/replication deficient mutants of *F. novicida* have been generated (Lauriano et al., 2004; Santic et al., 2005, 2007; Mohapatra et al., 2008). Studies with these mutants have shown that *Francisella* manipulates host signaling in at least two distinct ways to promote intracellular proliferation. Firstly, *Francisella* inhibits protective responses that would promote bacterial clearance. For example, it has been shown that *F. novicida* induces suppressor of cytokine signaling 3 (SOCS3) expression to interfere with IFNγ-mediated activation of STAT1, which contributes to the intracellular killing of bacteria (Parsa et al., 2008a). Hence, although IFNγ itself is upregulated following *Francisella* infection (Butchar et al., 2007, 2008), its autocrine/paracrine activity would be minimal. Along with manipulating these downstream mediators, it was shown that *Francisella* can regulate expression of the interferon gamma receptor (IFNγR) itself. Infection with the *novicida* and *tularensis* subspecies (Butchar et al., 2008) as well as with *holarctica* (unpublished observations) leads to downregulation of the IFNγR in human monocytes, and *F. novicida* infection of RAW264.7 murine macrophages induces downregulation of the IFNγR alpha chain (Roth et al., 2009).

A second way by which *Francisella* manipulates host signaling is to activate pathways that benefit bacterial replication. Recently it was shown that host signaling through Ras, SOS2, GrB2, PKCα, and PKCβI promotes host cell survival and maintenance of the replicative niche for *F. novicida* (Al Khodor and Abu Kwaik, 2010). In another study, a forward genetics screen identified USP22, CDC27, and PI4KCA as host factors required for intracellular replication of *F. novicida* (Akimana et al., 2010). Hence, *Francisella* is capable not only of suppressing host responses, but also of manipulating the host environment to facilitate its own survival and replication. Without either of these general mechanisms, it is unlikely that *Francisella* would succeed as an intracellular pathogen.

AUTOPHAGY: RE-ENTRY INTO THE ENDOCYTIC PATHWAY

At the late stages of infection, cytosolic *F. tularensis* LVS becomes enclosed within vacuoles that resemble autophagosomes. This has been conclusively shown in murine macrophages (Checroun et al., 2006), and there is some evidence that it also occurs in human monocytic cells (Mohapatra et al., 2008). Detailed functional consequences of autophagy have yet to be elucidated, but it has been shown that human monocytes treated with an autophagy-inducing agent carried a reduced bacterial burden (Chiu et al., 2009). Hence, it is likely that autophagy is beneficial to the host and serves to promote intracellular control of bacteria by sequestering them into vacuoles after escape. It is important to note that many autophagy-related genes are downregulated during *F. novicida* and *F. tularensis* infection (Butchar et al., 2008; Cremer et al., 2009a), raising the possibility that *Francisella* is also antagonizing this means of host defense.

RECOGNITION OF *FRANCISELLA* AND TRIGGERING OF THE INFLAMMATORY RESPONSE

During the infection cycle of *Francisella* there is activation of extracellular and cytosolic pattern recognition receptors. At the cell surface and within the phagosome, TLR2/myeloid differentiation primary response gene (88) (MyD88) signaling is essential for activating NF-κB and for producing pro-inflammatory mediators (Katz et al., 2006). CD14 is also required for effective TLR signaling upon *F. tularensis* infection (Chase and Bosio, 2010). After escape from the phagosome the bacteria gain access to the cytosol and activate the inflammasome (Mariathasan et al., 2005; Gavrilin et al., 2006). Only recently were the cytosolic sensors of *F. tularensis* identified. First it was shown that pyrin was essential for *F. novicida*-induced inflammasome activation within human monocytes (Gavrilin et al., 2009). Later studies with murine macrophages led to two concurrently published reports showing that absent in melanoma 2 (AIM-2) was essential for inflammasome activation and that this was responsible for caspase-1 and IL-1β processing (Fernandes-Alnemri et al., 2010; Rathinam et al., 2010). These findings were later supported by another report (Jones et al., 2010). However, human monocytes, which are robust producers of IL-1β during *F. novicida* infection (Gavrilin et al., 2006) do not express AIM-2 (Rathinam et al., 2010). Hence, it is likely that more than one cytosolic receptor is capable of responding to *Francisella*, and that these receptors may be cell- or species-specific.

THE PI3K SIGNALING PATHWAY REGULATES HOST RESPONSES

PI3K signaling is known to regulate numerous cellular processes such as autophagy (Petiot et al., 2000), phagocytosis (Araki et al., 1996), cell survival, oxidative burst (Chen et al., 2003; Hoyal et al., 2003), and inflammatory cytokine production (Parsa et al., 2006). There are three classes of PI3K, which differ in the products that they produce and thereby drive different cellular functions. Class I PI3K convert PI(4,5)P_2 to PI(3,4,5)P_3, which then serves as a second messenger and can activate protein kinase B/Akt (Vanhaesebroeck et al., 1997). This signaling pathway can be activated by a variety of cell surface receptors such as Fc receptors (Marshall et al., 2001), TLR (Laird et al., 2009), and the insulin receptor (Lavan et al., 1992). Our laboratory has shown that PI3K (Parsa et al., 2006) and Akt (Rajaram et al., 2006) positively regulate transcriptional activity of NF-κB in response to *F. novicida* within macrophages. Furthermore, Akt promotes the production of pro-inflammatory cytokines such as TNFα, IL-6, and IL-12, while inhibiting the production of the anti-inflammatory cytokine IL-10. Studies *in vivo* have demonstrated that PI3K/Akt activation is host-protective. Compared to wild-type mice, mice expressing a macrophage-specific form of constitutively active Akt (MyrAkt) showed a survival advantage when challenged with

a lethal dose of *F. novicida* (Rajaram et al., 2006). There was also a reduced bacterial burden in the MyrAkt mice. This activated Akt was found to promote phagosome maturation, thus inhibiting bacterial escape and replication. Related to this, we found that macrophages from MyrAkt mice showed reduced Fas-induced caspase-3 death after infection. Because phagosomal escape mutants of *Francisella* do not induce Fas nor activate caspase-3 (Rajaram et al., 2009), it is likely that bacterial escape or perhaps stress from bacterial replication triggers apoptosis. Caspase-3-mediated death appears to be of major importance, as the highly virulent subspecies *F. tularensis* activates caspase-3 and not caspase-1 (Wickstrum et al., 2009). Interestingly, the virulent *F. tularensis* specifically downregulates host cell TLR2, PI3K p85α, and Akt expression (Butchar et al., 2008), which should result in suboptimal activation of the host-protective PI3K/Akt pathway. This is in line with earlier work showing that *F. tularensis* infection elicits minimal to no observable response *in vivo* during early stages of infection (Bosio et al., 2007). This ability to infect without eliciting an immune response confers a stealth-like nature to *Francisella* (Sjostedt, 2006).

A downstream target of Akt that has also been investigated within the context of *Francisella* is glycogen synthase kinase 3 beta (GSK3β). This molecule is normally active in the cell until inactivated by Akt (Sutherland et al., 1993; Sutherland and Cohen, 1994). Thus, increased Akt activity results in decreased GSK3β activity. It has been found that inhibiting GSK3β *in vivo* results in increased host survival in mice. This is consistent with the role of Akt as host-protective. However, published data on GSK3β indicates that this molecule is required for pro-inflammatory response and concurrently inhibits anti-inflammatory IL-10 production (Zhang et al., 2009). This might be expected because GSK3β has been shown to be required for NF-κB activation (Hoeflich et al., 2000). Other work, however, has shown that GSK3β can repress NF-κB activity and nuclear translocation as well as cytokine production (Vines et al., 2006; Escribano et al., 2009; Saijo et al., 2009; Beurel et al., 2010). Taken together, these results suggest that the function of GSK3β may be highly context-specific.

Although these results suggest that PI3K activity is beneficial to the host during *Francisella* infection, there is also evidence that it may be detrimental. Medina et al. (2010) reported that treatment with wortmannin, a PI3K inhibitor, led to increased ERK and p38 phosphorylation as well as enhanced TNFα and IL-6 production in murine bone marrow-derived macrophages following infection with the LVS of *Francisella*. They also found a PI3K-mediated upregulation of the MAPK inhibitor MKP-1, which likely led to much of the MAPK inhibition (Medina et al., 2010). MKP-1 is upregulated in human monocytes following infection with the *novicida*, *holarctica*, and *tularensis* subspecies of *Francisella* (Butchar et al., 2008 and unpublished observations), so may not fully explain the observed discrepancies. However, as pointed out by Medina et al. (2010), differences in subspecies and/or culture conditions of this facultative bacterium might account for this. For example, the LPS of *F. novicida* elicits a greater immune response than that of LVS (Kieffer et al., 2003). More broadly, differences in cellular context (e.g., maturation state) as well as in both strength and type of stimulus can determine whether and to what extent PI3K activity can inhibit ERK (Rommel et al., 1999; Zimmermann and Moelling, 1999; Moelling et al., 2002).

PHOSPHATASE REGULATION OF HOST RESPONSE TO *FRANCISELLA*

PIP3 levels are antagonized by the regulatory phosphatases PTEN that converts $PI(3,4,5)P_3$ back to $PI(4,5)P_2$, and SH2 domain-containing inositol 5′-phosphatase 1 (SHIP) that converts $PI(3,4,5)P_3$ to $PI(3,4)_2$. By these actions, both phosphatases negatively regulate Akt activation. Our laboratory has shown that SHIP is phosphorylated in response to *Francisella*. Consequently, SHIP represses the production of pro-inflammatory cytokines (Parsa et al., 2006), phagosome maturation, and macrophage survival (Rajaram et al., 2009). This is consistent with to role of Akt in promoting these functions within the host. We also found that SHIP expression is strongly downregulated during infection with the low-virulence *F. novicida* subspecies but not with the more virulent *F. tularensis* subspecies (Cremer et al., 2009b). This is likely a contributing factor to the lack of inflammatory response during *F. tularensis* infection (Bosio et al., 2007; Butchar et al., 2008; Cremer et al., 2009b).

Recently it has been reported that infection with the virulent *F. tularensis* SCHU S4 increases expression and activation of phosphatase and tensin homolog (PTEN), an effect brought about by the production of antioxidants from the bacterium (Melillo et al., 2010). Hence, *F. tularensis* appears to use both phosphatases – PTEN and SHIP – to dampen PI3K signaling. The idea that *F. tularensis* does so through presumably different mechanisms (upregulation of PTEN versus inhibiting downregulation of SHIP) further highlights the intricacies and complexities of this pathogen.

BACTERIAL DISRUPTION OF HOST SIGNALING

Many bacterial pathogens are known to secrete factors that disrupt host signaling proteins. Because of the numerous examples showing that microbes inhibit Akt activation (Celli et al., 2001; Wiles et al., 2008; Popova et al., 2009), components of the PI3K/Akt pathway may soon come to light as potential therapeutic targets. During the infection cycle of *Francisella* there is initial contact with the host cell and containment within a phagosome, then escape of the bacteria into the cytosol. Our laboratory has seen that there is initial phosphorylation/activation of Akt followed by decline to a more inactivated state, but infection with mutants of *Francisella* that cannot escape from phagosome results in sustained Akt activation (Rajaram et al., 2006). This is specific in the sense that the same activation – inactivation phenomenon is not seen with phosphorylation/activation of the MAPKs (ERK, JNK, and p38). Therefore, it appears that *Francisella* specifically targets the PI3K/Akt pathway.

Along with PI3K/Akt regulation, *Francisella* dampens other signaling pathways such as MAPK and the inflammasome. Recently it has been shown that *ripA* of *Francisella* inhibits MAPK activation, which subsequently inhibits the production of TLR-dependent and inflammasome-dependent pro-inflammatory cytokines (Huang et al., 2010). This bacterial factor also promotes intracellular replication, presumably though its inhibition of host response. Another recent report shows that the bacterial protein *mviN* inhibits AIM-2 inflammasome activation in murine macrophages infected with LVS (Ulland et al., 2010). Hence, *Francisella* dampens host response on many fronts, and further work will undoubtedly uncover other such mechanisms.

MicroRNA REGULATION

MicroRNAs (miRs) have been shown to be key regulators of host functions that influence viral persistence (Gottwein and Cullen, 2008). However, only a few studies address miRs within the field of bacterial pathogenesis, leaving open a potentially fruitful avenue of investigation. Early studies showed that pathogen associated molecular patterns such as LPS could induce the expression of selected miRs such as miR-155, miR-146, and miR-132 (Taganov et al., 2006; O'Connell et al., 2007). MiR-155 has been of considerable interest since it was shown that this miR is required for B-cell maturation, normal T-cell function, dendritic cell antigen presentation, and inflammatory response (Rodriguez et al., 2007; Thai et al., 2007; O'Connell et al., 2010).

MiR-155 is of relevance to *Francisella* infection because this miR negatively regulates SHIP expression, which in turn regulates Akt and multiple host responses. Our laboratory found that miR-155 expression is highly induced in monocytes/macrophages infected with the low-virulence *F. novicida*. This was also found to occur *in vivo* within 48 h in mice given a lethal dose of *F. novcidia*. The induction of this miR required TLR signaling and NF-κB activation. Genetic manipulation showed that miR-155 modulates the inflammatory response by promoting TNFα and IL-6 production (Cremer et al., 2009b). This is logical given that miR-155 represses SHIP, which represses Akt activation and the production of pro-inflammatory mediators in response to *F. novicida* (Parsa et al., 2006). Importantly, although miR-155 is highly induced upon infection with low-virulence *F. novicida* it is minimally induced with high-virulence *F. tularensis*. These results support the consensus that virulent forms of *Francisella* subvert and suppress host immune responses, as the lack of miR-155 with the virulent *F. tularensis* will result in higher levels of the inhibitory SHIP. Hence, host cell Akt activation is impaired through at least three mechanisms: downregulation of PI3K/Akt pathway members, the bacterial antioxidant system, and miR-155. A model summarizing these findings is shown in **Figure 1**.

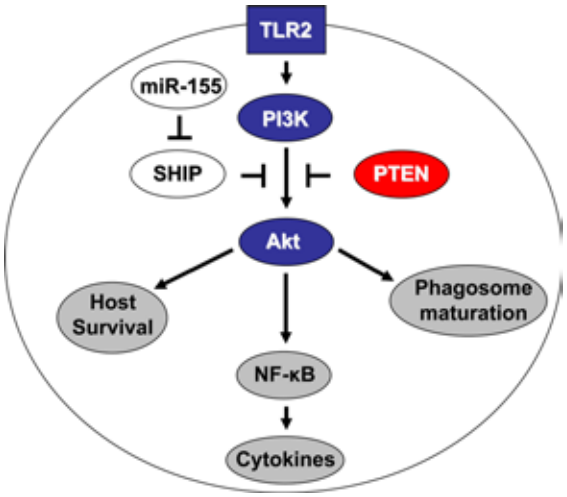

FIGURE 1 | *Francisella tularensis tularensis* targets the PI3K/Akt pathway at multiple levels. Blue represents downregulated genes, red upregulated, white unchanged, and gray the resulting cellular outcomes. TLR2 is required for full PI3K activation by *Francisella*, which in turn activates Akt. Active Akt promotes NF-κB activation and cytokine production, as well as phagosome maturation and host cell survival. However, the phosphatases SHIP and PTEN antagonize this Akt activation. SHIP expression is inhibited by miR-155, which is induced by the low-virulence but not high-virulence subspecies of *Francisella*. This lack of miR-155 induction by the high-virulence subspecies leads to maintenance of SHIP levels. The other phosphatase PTEN is upregulated and maintained in a highly active state upon infection with high-virulence *Francisella*.

CONCLUSION

This review has highlighted recent advances in our understanding of host response to *Francisella*. Research on this pathogen has been at an all time high in the recent years and great progress has been made. Not only is there interest in studying this pathogen due to its highly virulent nature, it has also become a useful tool for studying various aspects of the immune system. Here, we have discussed several critical host signaling pathways that *Francisella* manipulates in order to subvert normal pro-inflammatory responses. Importantly, the idea that miRs play key roles – as mediators of host response and as targets of *Francisella* – has begun to surface, and this may open an avenue for a new class of therapeutics. It is almost certain that even more exciting findings await us in the future, as our research tools become increasingly sophisticated and as pathogen and host continue to evolve against each other.

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Exploitation of host cell biology and evasion of immunity by *Francisella tularensis*

Rexford Asare and Yousef Abu Kwaik*

Department of Microbiology and Immunology, School of Medicine, University of Louisville, Louisville, KY, USA

Edited by:
Anders Sjostedt, Umeå University, Sweden

Reviewed by:
Tonyia Eaves-Pyles, University of Texas Medical Branch, USA
Susheela Tridandapani, Ohio State University, USA

***Correspondence:**
Yousef Abu Kwaik, Department of Microbiology and Immunology, School of Medicine, University of Louisville, Room 412A, Louisville, KY 40202, USA.
e-mail: abukwaik@louisville.edu

Francisella tularensis is an intracellular bacterium that infects humans and many small mammals. During infection, *F. tularensis* replicates predominantly in macrophages but also proliferate in other cell types. Entry into host cells is mediate by various receptors. Complement-opsonized *F. tularensis* enters into macrophages by looping phagocytosis. Uptake is mediated in part by Syk, which may activate actin rearrangement in the phagocytic cup resulting in the engulfment of *F. tularensis* in a lipid raft rich phagosome. Inside the host cells, *F. tularensis* resides transiently in an acidified late endosome-like compartment before disruption of the phagosomal membrane and escape into the cytosol, where bacterial proliferation occurs. Modulation of phagosome biogenesis and escape into the cytosol is mediated by the *Francisella* pathogenicity island-encoded type VI-like secretion system. Whilst inside the phagosome, *F. tularensis* temporarily induce proinflammatory cytokines in PI3K/Akt-dependent manner, which is counteracted by the induction of SHIP that negatively regulates PI3K/Akt activation and promotes bacterial escape into the cytosol. Interestingly, *F. tularensis* subverts CD4 T cells-mediated killing by inhibiting antigen presentation by activated macrophages through ubiquitin-dependent degradation of MHC II molecules on activated macrophages. In the cytosol, *F. tularensis* is recognized by the host cell inflammasome, which is down-regulated by *F. tularensis* that also inhibits caspase-1 and ASC activity. During late stages of intracellular proliferation, caspase-3 is activated but apoptosis is delayed through activation of NF-κB and Ras, which ensures cell viability.

Keywords: tularemia, ASC, caspase, apoptosis, Ras, Akt, SHIP

INFECTION BY *FRANCISELLA TULARENSIS*

Tularemia is a zoonotic disease caused by *Francisella tularensis*, a facultative intracellular pathogen that infects a broad range of small mammals and humans (Ellis et al., 2002; Pechous et al., 2009; Santic et al., 2010a). Four subspecies of *F. tularensis* have been identified to date (Keim et al., 2007; Nigrovic and Wingerter, 2008; Santic et al., 2009) and they share about 97% genomic identity (Champion et al., 2009; Larsson et al., 2009). These are subspecies *tularensis*, *holarctica*, *mediasiatica*, and *novicida*. Disease in humans is mostly caused by subspecies *tularensis* and *holarctica*. Subspecies *tularensis* is found in North America and is the most virulent, causing the most severe form of tularemia. In contrast subspecies *holarctica* is distributed throughout the northern hemisphere and causes a mild form of tularemia (Santic et al., 2006). Subspecies *novicida* does not cause disease in humans but causes a disease in mice that is similar to the disease in humans.

Francisella tularensis is transmitted to humans through inhalation of contaminated aerosol or ingestion of contaminated food and water, a bite by an arthropod vector, or direct contact with infected animals through skin abrasions (Ellis et al., 2002). Clinical presentation of disease depends on the route of infection and include pneumonic tularemia, oropharyngeal tularemia, and glandular or ulceroglandular tularemia (Ellis et al., 2002). Occasionally, *F. tularensis* can also infect the eye resulting in oculoglandular tularemia (Harrell and Whitaker, 1985). Ulceroglandular tularemia is characterized by an ulcer at the infected site with swelling of the regional lymph node. Glandular tularemia is similar to ulceroglandular but without the ulcer. In oropharyngeal tularemia the ulcer occurs in the mouth with swelling of the lymph nodes around the neck region. Irrespective of the route of infection the bacteria ultimately enter the blood stream, causing typhoidal tularemia, which leads to septicemia (Oyston et al., 2004; Nigrovic and Wingerter, 2008). Symptoms of the typhoidal tularemia include headache, fever, chills, nausea, diarrhea, and myalgia (Oyston et al., 2004; Nigrovic and Wingerter, 2008). Due to the high morbidity and mortality rate, the ease of dissemination and the fact that inhalation of as few as 10 organisms of subspecies *tularensis* can cause disease, *F. tularensis* has been classified by the CDC as a category A select agent.

Once inside the mammalian host, *F. tularensis* enters and replicates in macrophages (Anthony et al., 1991; Conlan and North, 1992; Fortier et al., 1994). However, there is increasing evidence that the organism can infect other cell types including neutrophils, dendritic cells, hepatocytes, and lung epithelial cells (Pechous et al., 2009). During infection, bacteria migrate from the initial site of infection to the liver and spleen where they replicate (Eigelsbach et al., 1962; Conlan et al., 2003). Although it has been shown that *F. tularensis* exhibit extracellular phase during *in vivo* infection in mice (Forestal et al., 2007; Yu et al., 2008), there is no data demonstrating extracellular growth during human or animal infection.

Available data indicate that intracellular trafficking of *F. tularensis* is similar in macrophages, neutrophils, epithelial cells, and *Drosophila melanogaster* S2 cells suggesting trafficking might be similar in all cell types (Golovliov et al., 2003a; Clemens et al., 2004; Santic et al., 2005a; McCaffrey and Allen, 2006; Craven et al., 2008; Santic et al., 2009).

F. tularensis enters into host cells through binding to surface receptors. This results in the uptake of the bacterium in a spacious loop by a mechanism referred to as looping phagocytosis (Clemens et al., 2005). Uptake by neutrophils and dendritic cells is dependent on opsonization (Lofgren et al., 1983; Ben Nasr et al., 2006) whereas entry into macrophages is through both opsonin dependent and independent mechanisms (Clemens et al., 2005; Balagopal et al., 2006; Pierini, 2006; Schulert and Allen, 2006; Barel et al., 2008). Inside the host cell, the bacteria reside transiently in a phagosome before escaping into the cytosol (**Figure 1**; Golovliov et al., 2003b; Clemens et al., 2004; Santic et al., 2005a,b; Checroun et al., 2006; Santic et al., 2007; Bonquist et al., 2008; Santic et al., 2008; Qin et al., 2009). Escape is preceded by modification of the phagosome to an acidified late endosome-like compartment (Fortier et al., 1995; Chong et al., 2008; Santic et al., 2008). Within this acidified compartment *F. tularensis* activates virulence genes that allow it to disrupt the phagosome membrane and escape into the cytosol (Chong et al., 2008; Santic et al., 2008).

Once inside the cytosol, the bacteria is recognized by the host cell inflammasome resulting in the cleavage of IL-1 and IL-18 (**Figure 1**; Mariathasan et al., 2005; Gavrilin et al., 2006; Henry et al., 2007; Fernandes-Alnemri et al., 2010; Jones et al., 2010). Similarly, there is activation of caspase-3 through both the extrinsic and intrinsic pathways between 6 and 12 post-infection. Although caspases are activated early during infection (Lai and Sjostedt, 2003; Mariathasan et al., 2005; Santic et al., 2010b), *F. tularensis* is able to delay death of the cells for its survival and replication by activating NF-κB and Ras both of which stimulate cells survival (Al-Khodor and Abu Kwaik, 2010; Santic et al., 2010b). During late stages of infection of mouse macrophages, *F. tularensis* is taken up in an autophagy-like compartment (Checroun et al., 2006). However, this re-entry of the *F. tularensis* into the endosomal–lysosomal pathway through autophagy does not occur in human macrophages, and therefore is not relevant to infection of humans (Akimana et al., 2010). Toward the end of the infectious cycle, the induction of apoptosis allows the bacteria to disrupt the cytoplasmic membrane and escape the spent cell to begin new infectious cycle (**Figure 1**).

ENTRY INTO AND REPLICATION WITHIN HOST CELLS

Francisella tularensis enters primary macrophages through both opsonin dependent and independent mechanisms. Complement-opsonized bacteria enter macrophages either through complement receptor 3 (CR3) or the scavenger receptor A (SRA)(Clemens et al., 2005; Pierini, 2006). Antibody-opsonized *F. tularensis* enters macrophages through FC gamma receptor (Balagopal et al., 2006) in contrast to unopsonized bacteria that enter macrophages through binding to the mannose receptor and surface nucleolin (Balagopal et al., 2006; Schulert and Allen, 2006; Barel et al., 2008). It has also been shown that opsonization of *F. tularensis* with lung collectin surfactant protein A (SP-A) enhance bacterial uptake by primary macrophages but the host cell receptor is not known (Balagopal et al., 2006). Similarly, the bacterial ligand for mannose receptor

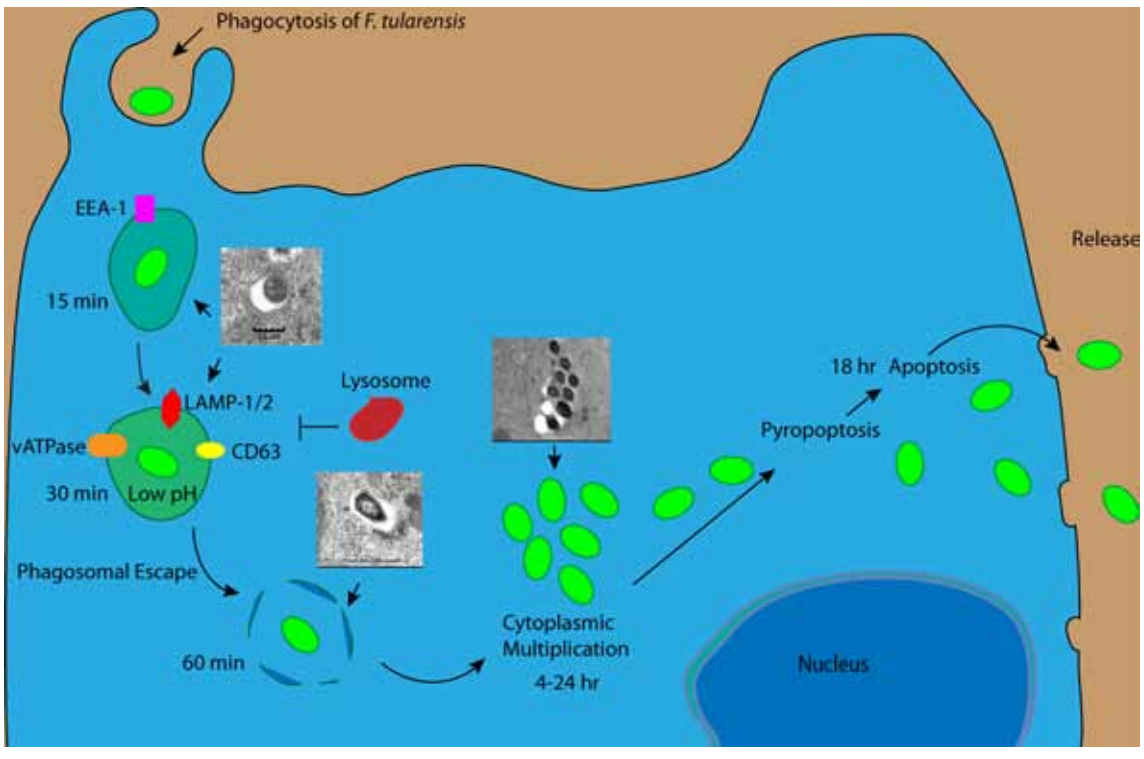

FIGURE 1 | Intracellular trafficking of *Francisella tularensis* within macrophages. *F. tularensis* enters macrophages using different receptors and resides transiently in the FCP, which acquires EE1 followed Lamp-1, Lamp-2, and Rab7 but excludes Cathepsin D. Within 30 min of infection the FCP acquires vATPase enabling *F. tularensis* to acidify the FCP and escape into the cytosol. Within the cytosol, *F. tularensis* activates caspase-1 and caspase-3 but delays pyropoptosis and apoptosis and maintain cell viability till late stages of infection when the bacteria exit the spent cell.

has not been identified. Elongation factor E2 is expressed on the surface of *F. tularensis* and bind to surface nucleolin expressed on the surface of macrophages (Barel et al., 2008). It is however not known which of these receptors are used predominantly *in vivo*.

When opsonized *F. tularensis* binds macrophages, it is engulfed in a unique asymmetric spacious pseudopod loops (Clemens et al., 2005). This unique mechanism of uptake has been shown to be dependent on intact CR3 and complement factor 3 (Clemens et al., 2005). Syk is important for Fcγ-mediated phagocytosis in macrophages and neutrophils (Greenberg et al., 1994; Raeder et al., 1999). Activation of Syk results in the activation of MAP kinase (ERKs) through Protein kinase C (PKC) leading to actin polymerization and induction of phagocytosis (Cox et al., 1996; Raeder et al., 1999). Syk has been shown to be important for the uptake of *F. tularensis* but the upstream receptor required for activation of Syk has not been identified (Parsa et al., 2008). Activation of Syk leads to subsequent activation of the Erk pathway but the direct binding partner of Syk is yet to be identified (**Figure 2**; Parsa et al., 2008). Interestingly, actin microfilament has been shown to be important for this process (Clemens et al., 2005).

In contrast to other intracellular bacteria such as *Salmonella typhimurium*, which requires PI3K to form the phagocytic cup, the uptake of *F. tularensis* is not affected by inhibition of the PI3K pathway (Parsa et al., 2006, 2008). This is consistent with a different mechanism used by *F. tularensis* to enter into host cells (Clemens et al., 2005). In addition to actin microfilament, the entry of *F. tularensis* into macrophages has been shown to be dependent on cholesterol-rich lipid domains known as lipid rafts since lipid rafts-associated components such as cholesterol and caveolin-1 are incorporated into the *Francisella*-containing phagosome (FCP) membrane upon its biogenesis from the macrophage plasma membrane (Tamilselvam and Daefler, 2008). The recruitment of lipid rafts to the FCP may act as a platform for linking the entry process of *F. tularensis* at the cell membrane to the cytoskeleton and the intracellular signaling pathways (Tamilselvam and Daefler, 2008).

To date, at least 268 gene products have been identified, that are important for replication of *F. tularensis* within mammalian cells (**Table 1**; Anthony et al., 1994; Baron and Nano, 1998;

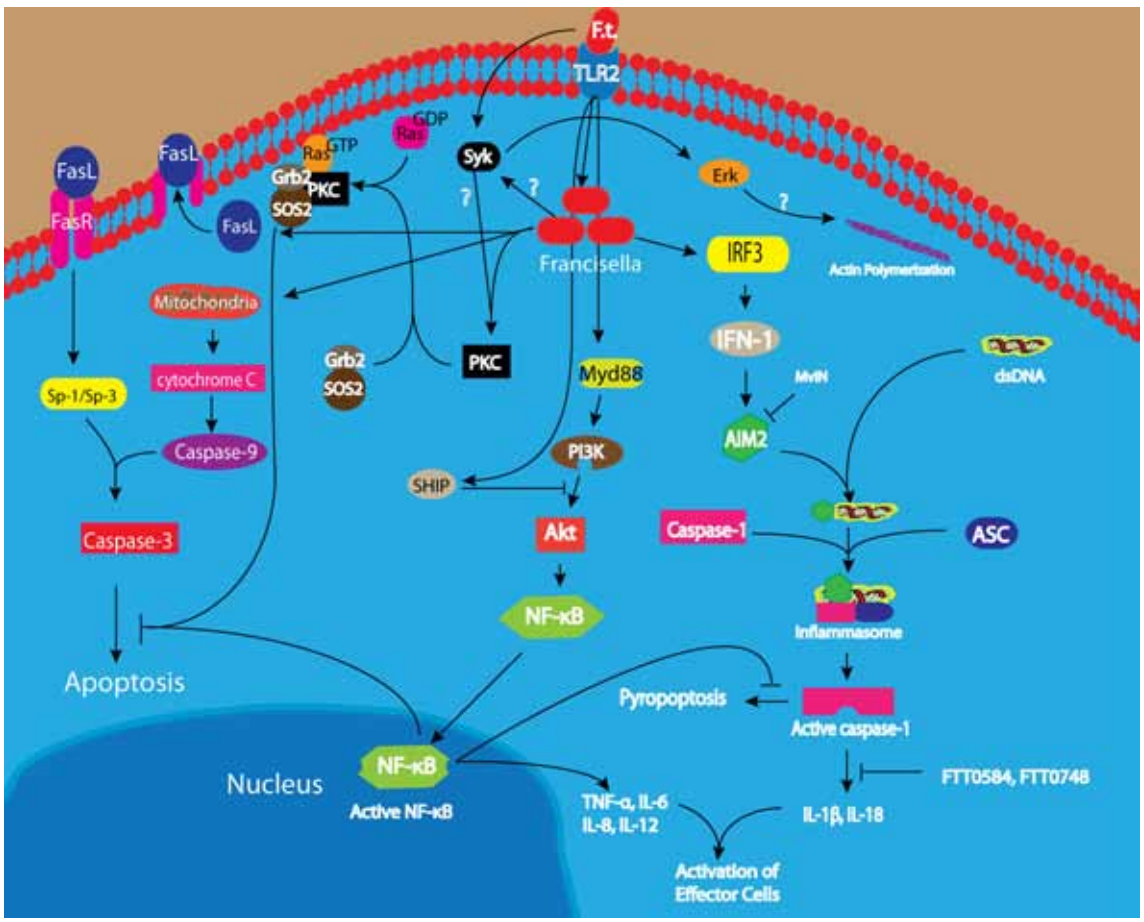

FIGURE 2 | Entry into and evasion of host cell innate immune response by *Francisella tularensis*. Phagocytosis of *F. tularensis* by macrophages is mediated by Syk-dependent activation of Erk, which likely triggers actin polymerization at the phagocytic cup. In addition, there is TLR2 dependent activation of Akt leading to induction of proinflammatory cytokines and phagosomal maturation. Akt activation is counteracted by SHIP activation, but the balance between the two opposing process is tilted toward escape of *F. tularensis* into the cytosol. Within the cytosol, *F. tularensis* activates both caspase-1 and caspase-3 but it is able to delay induction of apoptosis and pyropoptosis through Ras and NF-κB dependent anti-apoptotic mechanisms as well as AIM2-dependent inhibition of caspase-1 activation.

Table 1 | List of intracellular growth defective mutants.

PROTEINS OF UNKNOWN FUNCTION		
FTN_0027		Conserved protein of unknown function
FTN_0041		Protein of unknown function
FTN_0109		Protein of unknown function
FTN_0132		Protein of unknown function
FTN_0149		Conserved protein of unknown function
FTN_0275		Conserved protein of unknown function
FTN_0290		Protein of unknown function
FTN_0297		Conserved protein of unknown function
FTN_0428		Protein of unknown function
FTN_0444		Membrane protein of unknown function
FTN_0477		Conserved protein of unknown function
FTN_0788		Conserved protein of unknown function
FTN_0855		Protein of unknown function
FTN_0915		Conserved protein of unknown function
FTN_0925		Protein of unknown function
FTN_0930		Protein of unknown function
FTN_0933		Protein of unknown function
FTN_0977		Conserved protein of unknown function
FTN_1170		Conserved protein of unknown function
FTN_1172		Conserved protein of unknown function
FTN_1175		Membrane protein of unknown function
FTN_1256		Membrane protein of unknown function
FTN_1343		Conserved protein of unknown function
FTN_1367		Protein of unknown function
FTN_1457		Protein of unknown function
FTN_1542		Conserved protein of unknown function
FTN_1624		Conserved protein of unknown function
FTN_1696		Protein of unknown function
FTN_1713		Protein of unknown function
FTN_1764		Protein of unknown function
HYPOTHETICAL PROTEINS		
FTL_0439		Hypothetical outer membrane protein
FTL_0544		Hypothetical protein; polyphosphate kinase
FTL_0706		Hypothetical membrane protein; LPS biosynthesis
FTL_0886		Conserved hypothetical protein YleA; possible tRNA-i (6)A37 methylthiotransferase
FTL_1096		Hypothetical lipoprotein; ABC transporter and potential disulfide bond formation
FTL_1414		Hypothetical protein; possible capsule-related protein
FTN_0030		Hypothetical membrane protein
FTN_0038		Hypothetical protein
FTN_0169		Conserved hypothetical membrane protein
FTN_0336		Hypothetical protein
FTN_0384		Conserved hypothetical protein
FTN_0403		Hypothetical membrane protein
FTN_0534		Conserved hypothetical membrane protein
FTN_0556		Hypothetical protein
FTN_0696		Hypothetical membrane protein
FTN_0709		Hypothetical protein
FTN_0727		Hypothetical membrane protein
FTN_0759		Conserved hypothetical protein
FTN_0792		Hypothetical protein
FTN_0847		Conserved hypothetical protein
FTN_0888		Hypothetical membrane protein
FTN_0895		Hypothetical protein
FTN_1098		Conserved hypothetical membrane protein
FTN_1156		Hypothetical protein
FTN_1349		Hypothetical protein
FTN_1395		Conserved hypothetical protein
FTN_1406		Conserved hypothetical membrane protein
FTN_1612		Hypothetical protein
FTN_1656		Conserved hypothetical protein
FTN_1686		Hypothetical membrane protein
FTN_1736		Hypothetical protein
FTT1103		Conserved hypothetical lipoprotein
FTT1236		Hypothetical protein
FTT1244c	yfiO	Conserved hypothetical lipoprotein
FPI PROTEINS		
FTN_1309	pdpA	Protein of unknown function
FTN_1310	icmF	Conserved protein of unknown function
FTN_1311	iglE	Protein of unknown function
FTN_1312	vgrG	Conserved hypothetical protein
FTN_1313	iglF	Hypothetical protein
FTN_1314	iglG	Conserved hypothetical protein
FTN_1315	iglH	Protein of unknown function
FTN_1316	dotU	Conserved protein of unknown function
FTN_1317	iglI	Protein of unknown function
FTN_1318	iglJ	Hypothetical protein
FTN_1321	iglD	Intracellular growth locus, subunit D
FTN_1322	iglC	Intracellular growth locus, subunit C
FTN_1323	iglB	Intracellular growth locus protein B
FTN_1324	iglA	Intracellular growth locus A
FTN_1325	pdpD	Protein of unknown function
METABOLIC PROTEINS		
FTL_0028	pryB	Aspartate carbamoyltransferase
FTL_0029	carB	Carbamoyl-phosphate synthase large chain
FTL_0030	carA	Carbamoyl-phosphate synthase small chain
FTL_0483	*glgB*	Glycogen branching enzyme, GlgB; polysaccharide metabolism
FTL_0592	*wbtA*	dTDP-glucose 4,6-dehydratase, WbtA, O-antigen polysaccharide biosynthesis
FTL_0594	*wbtC*	UDP-glucose-4-epimerase, WbtC, O-antigen polysaccharide biosynthesis
FTL_0606	*wbtM*	dTDP-glucose 4,6-dehydratase, WbtM, O-antigen polysaccharide biosynthesis
FTL_0766	*ggt*	Gamma-glutamyl transpeptidase; amino acid, arachidonic acid, and glutathione
FTL_0789	*aspC2*	Aspartate aminotransferase; amino acid biosynthesis
FTL_1262		Chorismate family binding protein; aromatic amino acid, and folate biosynthesis
FTL_1415	*capC*	Capsule biosynthesis protein CapC
FTL_1416	*capB*	Capsule biosynthesis protein CapB
FTN_0020	carB	Carbamoyl-phosphate synthase large chain
FTN_0035	pyrF	Orotidine-5-phosphate decarboxylase

(Continued)

Table 1 | Continued

FTN_0036	pyrD	Dihydroorotate oxidase
FTN_0063	ilvE	Branched-chain amino acid aminotransferase protein (class IV)
FTN_0090	acpA	Acid phosphatase
FTN_0111	ribH	Riboflavin synthase beta-chain
FTN_0113	ribC	Riboflavin synthase alpha chain
FTN_0125	ackA	Propionate kinase 2/acetate kinase A
FTN_0178	purA	Adenylosuccinate synthetase
FTN_0199	cyoE	Heme O synthase
FTN_0211	pcp	Pyrrolidone carboxylate peptidase
FTN_0218	nfnB	Dihydropteridine reductase
FTN_0319		Amino acid–polyamine–organocation family protein
FTN_0343		Aminotransferase
FTN_0358		tRNA-methylthiotransferase MiaB protein
FTN_0420		SAICAR synthetase/ phosphoribosylamine-glycine ligase
FTN_0483		Bifunctional NMN adenylyltransferase/nudix hydrolase
FTN_0496	Slt	Soluble lytic murein transglycosylase
FTN_0504		Lysine decarboxylase
FTN_0507	gcvP1	Glycine cleavage system P protein, subunit 1
FTN_0511		Shikimate 5-dehydrogenase
FTN_0524	Asd	Aspartate semialdehyde dehydrogenase
FTN_0527	thrC	Threonine synthase
FTN_0545		Glycosyl transferase, group 2
FTN_0567		tRNA synthetase class II (D, K, and N)
FTN_0588		Asparaginase
FTN_0593	sucD	Succinyl-CoA synthetase, alpha subunit
FTN_0598		tRNA-dihydrouridine synthase
FTN_0633	katG	Peroxidase/catalase
FTN_0692	nadA	Quinolinate synthetase A
FTN_0695	Add	Deoxyadenosine deaminase/adenosine deaminase
FTN_0746	Alr	Alanine racemase
FTN_0806		Glycosyl hydrolase family 3
FTN_0811	birA	Biotin—acetyl-CoA-carboxylase ligase
FTN_0822		*p*-Aminobenzoate synthase component I
FTN_0840	mdaB	NADPH-quinone reductase (modulator of drug activity B)
FTN_0877	cls	Cardiolipin synthetase
FTN_0928	cysD	Sulfate adenylyltransferase subunit 2
FTN_0954		Histidine acid phosphatase
FTN_0957		Short chain dehydrogenase
FTN_0965		Metal-dependent exopeptidase
FTN_0983		Bifunctional protein: glutaredoxin 3/ribonucleotide reductase beta subunit
FTN_0988	prmA	50S ribosomal protein L11, methyltransferase
FTN_0995	hslV	ATP-dependent protease HslVU, peptidase subunit
FTN_1018		Aldolase/adducin class II family protein
FTN_1046	wzb	Low molecular weight (LMW) phosphotyrosine protein phosphatase
FTN_1061		Acid phosphatase, HAD superfamily protein
FTN_1121	phrB	Deoxyribodipyrimidine photolyase
FTN_1131	putA	Bifunctional proline dehydrogenase, pyrroline-5-carboxylate dehydrogenase
FTN_1135	aroB	3-Dehydroquinate synthetase
FTN_1222	kpsF	Phosphosugar isomerase
FTN_1231	gloA	Lactoylglutathione lyase
FTN_1233		Haloacid dehalogenase-like hydrolase
FTN_1234	queA	S-adenosylmethionine: tRNA ribosyltransferase-isomerase
FTN_1333	tktA	Transketolase I
FTN_1415		Thioredoxin
FTN_1417	manB	Phosphomannomutase
FTN_1421	wbtH	Glutamine amidotransferase/asparagine synthase
FTN_1428	wbtO	Transferase
FTN_1494	aceE	Pyruvate dehydrogenase complex, E1 component, pyruvate dehydrogenase
FTN_1523		Amino acid–polyamine–organocation family protein
FTN_1553	nudH	dGTP pyrophosphohydrolase
FTN_1557		Oxidoreductase iron/ascorbate family protein
FTN_1584	glpD	Glycerol-3-phosphate dehydrogenase
FTN_1585	glpK	Glycerol kinase
FTN_1597	prfC	Peptide chain release factor 3
FTN_1619	appC	Cytochrome bd-II terminal oxidase subunit I
FTN_1620	appB	Cytochrome bd-II terminal oxidase subunit II
FTN_1621		Predicted NAD/FAD-dependent oxidoreductase
FTN_1655	rluC	Ribosomal large subunit pseudouridine synthase C
FTN_1701		Glutamate decarboxylase
FTN_1767	rbsK	Ribokinase, pfkB family
FTN_1777	trpG	Anthranilate synthase component II
FTT0203c	purH	Bifunctional purine biosynthesis protein
FTT0204	purA	Adenylosuccinate synthetase
FTT0435	Ctu	Citrulline ureidase
FTT0588	aroA	3-Phosphoshikimate 1-carboxyvinyl transferase
FTT1234		Choloylglycine hydrolase family protein
FTT1665	purL	Aspartate carbamoyltransferase
FTT1720c		Phosphoribosylformylglycinamidine synthase
FTT1721c	purF	(Amidophosphoribosyltransferase)2
FTT1762c		Acetyltransferase protein
FTL_1071	guaA	GMP synthase (glutamine-hydrolyzing)
FTL_1478	guaB	Inosine-5-monophosphate dehydrogenase
TRANSPORTER PROTEINS		
FTL_0304		Na+/H+ antiporter; regulation of pH
FTL_0837	*metIQ*	D-Methionine transport protein (ABC transporter), MetIQ
FTL_0838	*metN*	D-Methionine transport protein (ABC transporter), MetN
FTL_1583	*xasA*	Glutamate–aminobutyric acid antiporter, XasA; amino acid transport
FTL_1806		Major facilitator superfamily transporter
FTN_0008		10 TMS drug/metabolite exporter protein
FTN_0018	sdaC	Serine permease

(Continued)

Table 1 | Continued

FTN_0141		ABC transporter, ATP-binding protein
FTN_0299	putP	Proline: Na+ symporter
FTN_0619		Pseudogene: nicotinamide ribonucleoside (NR) uptake permease (PnuC) family protein
FTN_0624		Serine permease
FTN_0636	glpT	Glycerol-3-phosphate transporter
FTN_0687	galP1	Galactose-proton symporter, major facilitator superfamily (MFS) transport protein
FTN_0728		Predicted Co/Zn/Cd cation transporter
FTN_0739	potG	ATP-binding cassette putrescine uptake system, ATP-binding protein
FTN_0799	emrE	Putative membrane transporter of cations and cationic drugs, multidrug resistance protein
FTN_0800		ArsB arsenite/antimonite exporter
FTN_0848		Amino acid antiporter
FTN_0885		Proton-dependent oligopeptide transporter (POT) family protein, di-, or tripeptide: H+ symporter
FTN_0997		Proton-dependent oligopeptide transporter (POT) family protein, di-, or tripeptide: H+ symporter
FTN_1215	kpsC	Capsule polysaccharide export protein KpsC
FTN_1344		Major facilitator superfamily (MFS) transport protein
FTN_1368	feoA	Fe_2 transport system protein A
FTN_1441		Sugar porter (SP) family protein
FTN_1581		Small conductance mechanosensitive ion channel (MscS) family protein
FTN_1593	oppA	ABC-type oligopeptide transport system, periplasmic component
FTN_1611		Major facilitator superfamily (MFS) transport protein
FTN_1711	tyrP	Tyrosine permease
FTN_1716	kdpC	Potassium-transporting ATPase C chain
FTN_1716	kdpC	Potassium-transporting ATPase C chain
FTN_1733		Nicotinamide ribonucleoside (NR) uptake permease (PnuC) family protein
FTT0056c		Major facilitator super family (MFS) transport protein
FTT0129		Major facilitator super family (MFS) transport protein
DNA MODIFYING		
FTL_0878		DNA/RNA endonuclease family
FTN_0133		Ribonuclease II family protein
FTN_0287		Type I restriction-modification system, subunit R (restriction)
FTN_0577	mutL	DNA mismatch repair enzyme with ATPase activity
FTN_0680	uvrC	Excinuclease ABC, subunit C
FTN_0710		Type I restriction-modification system, subunit R (restriction)
FTN_0838	xthA	Exodeoxyribonuclease III
FTN_1017		Pseudogene: DNA-3-methyladenine glycosylase
FTN_1027	ruvC	Holliday junction endodeoxyribonuclease
FTN_1073		DNA/RNA endonuclease G
FTN_1154		Type I restriction–modification system, subunit S
FTN_1176	uvrB	Excinuclease ABC, subunit B
FTN_1197	recR	RecFOR complex, RecR component
FTN_1293	rnhB	Ribonuclease HII
FTN_1357	recB	ATP-dependent exoDNase_subunit
FTN_1487		Restriction endonuclease
TRANSCRIPTION/TRANSLATION		
FTL_1542	migR	Macrophage intracellular growth regulator
FTL_1606	sspA	Stringent starvation protein A/regulator of transcription
FTL_1914	ripA	Required for intracellular proliferation, factor A
FTN_0480	fevR	*Francisella* effector of virulence regulation
FTN_0567		tRNA synthetase class II (D, K, and N)
FTN_0598		tRNA-dihydrouridine synthase
FTN_1290	mglA	Macrophage growth locus, protein A
FTN_1291	mglB	Macrophage growth locus, subunit B
FTN_1412		DNA-directed RNA polymerase subunit
FTL_0552		Transcriptional response regulator
CELL DIVISION		
FTN_0162	ftsQ	Cell division protein FtsQ
FTN_0330	minD	Septum formation inhibitor-activating ATPase
FTN_0331	minC	Septum formation inhibitor-activating ATPase
TYPE IV PILIN		
FTN_0415	pilA	Type IV pili, pilus assembly protein
FTN_1137	pilQ	Type IV pili secretin component
FTN_1139	pilO	Type IV pili glycosylation protein
OTHERS		
FTL_0094	clpB	ClpB protein
FTL_1670	*dsbB*	Disulfide bond formation protein, DsbB
FTN_0107	lepA	GTP-binding protein LepA
FTN_0155		Competence protein
FTN_0182		ATP-binding cassette (ABC) superfamily protein
FTN_0286		Transposase
FTN_0338		MutT/nudix family protein
FTN_0465		Sua5/YciO/YrdC family protein
FTN_0646	cscK	ROK family protein
FTN_0672	secA	Preprotein translocase, subunit A (ATPase, RNA helicase)
FTN_0768	tspO	Tryptophan-rich sensory protein
FTN_0985		DJ-1/PfpI family protein
FTN_1002	blaA	Beta-lactamase class A
FTN_1031	ftnA	Ferric iron binding protein, ferritin-like
FTN_1034	rnfB	Iron–sulfur cluster-binding protein
FTN_1058	tig	Trigger factor (TF) protein
FTN_1064		PhoH family protein, putative ATPase
FTN_1145	era	GTP-binding protein
FTN_1217		ATP-binding cassette (ABC) superfamily protein
FTN_1240		BolA family protein
FTN_1241		DedA family protein
FTN_1263	comL	Competence lipoprotein ComL
FTN_1355		Regulatory factor, Bvg accessory factor family
FTN_1518	relA	GDP pyrophosphokinase/GTP pyrophosphokinase
FTT0029c	figA	*Francisella* iron regulated gene A
FTT0918		
FTL_0380	sodC	Superoxide dismutase (Cu–Zn) precusor
FTL_0439	fupA/B	Siderophore biosynthesis

Gray et al., 2002; Golovliov et al., 2003a; Nano et al., 2004; Santic et al., 2005b, 2007; Twine et al., 2005; Deng et al., 2006; Pechous et al., 2006, 2008; Tempel et al., 2006; Charity et al., 2007; de Bruin et al., 2007; Maier et al., 2007; Mohapatra et al., 2007a,b, 2008; Raynaud et al., 2007; Bonquist et al., 2008; Brotcke and Monack, 2008; Fuller et al., 2008; Meibom et al., 2008; Sammons-Jackson et al., 2008; Alkhuder et al., 2009; Buchan et al., 2009; Dean et al., 2009; Mahawar et al., 2009; Santiago et al., 2009; Schulert et al., 2009; Ahlund et al., 2010; Asare and Abu Kwaik, 2010; Jia et al., 2010; Sen et al., 2010). These include the *Francisella* pathogenicity Island (FPI) proteins IglA, IglB, IglC, IglD, pdpA, pdpB, pdpD and their regulators, MglA, SspA, FevR, MigR, RipA, PigR, and PmrA (Baron and Nano, 1998; Gray et al., 2002; Golovliov et al., 2003a; Charity et al., 2007, 2009; de Bruin et al., 2007; Mohapatra et al., 2007b; Bonquist et al., 2008; Brotcke and Monack, 2008; Fuller et al., 2008; Buchan et al., 2009). The FPI is composed of 17 genes and recent mutagenesis experiments have shown that most of the genes are important for survival within the host cell (Golovliov et al., 2003a; de Bruin et al., 2007; Barker et al., 2009; Broms et al., 2009; Schmerk et al., 2009). Some of the gene products on the FPI form a type VI-like secretion system through which effector proteins are injected into the host cell cytosol to modulate biogenesis of the FCP and to enable the bacterium to disrupt the phagosome membrane and escape into the cytosol (Golovliov et al., 2003a; Santic et al., 2007; Barker et al., 2009; Broms et al., 2009; Schmerk et al., 2009). MglA, SspA, and PmrA bind cooperatively with RNA polymerase to regulate a large number of genes including those of the FPI (Brotcke et al., 2006; Charity et al., 2007; Mohapatra et al., 2007b; Bell et al., 2010) that are important for survival within the host cell. This regulation is mediated in part by FevR which is important for escape and replication within the cytosol (Brotcke and Monack, 2008). FevR is also independently regulated by MigR (Buchan et al., 2009) indicating that FevR plays a central role in the regulation of virulence in *F. tularensis*. Independent of FevR, MglA, and SspA also regulate virulence genes through cooperative interaction with PigR and the alamone ppGpp (Charity et al., 2009). Whereas most of the genes regulated by the different pathways are common, there are subsets of genes that are regulated independently by the different pathways (Brotcke et al., 2006; Charity et al., 2007; Mohapatra et al., 2007b). A large number of these gene products and most of the proteins that are necessary for escape and replication are hypothetical proteins. It is conceivable to speculate that some of these gene products constitute effector proteins that are secreted by the type VI secretion-like system. Also important for intracellular replication are genes involved in the transport of metabolic intermediates and different metabolic pathways including amino acid metabolism, nucleotide metabolism, and carbohydrate metabolism (Pechous et al., 2006; Alkhuder et al., 2009; Mahawar et al., 2009; Schulert et al., 2009; Asare and Abu Kwaik, 2010). The large number of metabolic genes that is required for replication indicates that the FCP is replete of nutrients and *F. tularensis* require *de novo* synthesis in order to survive and replicate within the host cells. Once in the cytosol where nutrient is readily available *F. tularensis* may acquire nutrients through the importation of metabolic intermediates from the host cell cytosol. This may explain why mutations in a large number of metabolic intermediate transporters block bacterial escape into the cytosol (**Table 1**; Qin and Mann, 2006; Maier et al., 2007; Asare and Abu Kwaik, 2010).

MODULATION OF PHAGOSOME BIOGENESIS

Phagosomal maturation involves sequential interaction between the nascent phagosome and the endocytic and lysosomal vesicles resulting in the conversion of the phagosome to a phagolysosome within which the bacterium or a particle is degraded (Duclos and Desjardins, 2000; Hackstadt, 2000; Kahn et al., 2002). After biogenesis from the plasma membrane, the nascent phagosome fuses with vesicles from the early endosome in a process that is regulated by Rab5 GTPase and the downstream effector early endosomal antigen 1 (EEA1). This is followed by interaction with the late endosome that is controlled by Rab7 GTPase. The late endosome-like phagosome becomes acidified through acquisition of the vacuolar ATPase, which pumps protons into the lumen of the phagosome resulting in acidification of the lumen. The acidified phagosome subsequently fuses to the lysosomes to form a phagolysosome, which is very rich in acid hydrolases. Within this compartment the microbe or particle is degraded (Duclos and Desjardins, 2000; Hackstadt, 2000; Kahn et al., 2002; **Figure 1**). The maturation process is very rapid and is completed within 15–30 min of phagosome biogenesis from the plasma membrane (Duclos and Desjardins, 2000; Hackstadt, 2000; Kahn et al., 2002).

Different intracellular pathogens have evolved different mechanisms to subvert the default endocytic maturation to create permissive niches that allow intracellular replication (Duclos and Desjardins, 2000; Hackstadt, 2000; Kahn et al., 2002). The strategies include (i) Arrest of phagosome maturation at a distinct stage in the endosomal–lysosomal degradation pathway, as occurs in infection with *Legionella pneumophila*; (ii) Survival and replication within an acidic environment of a mature phagolysosomes, as exemplified by *Coxiella burnetii*; and (iii) Replication within the cytosol after degradation of the phagosomal membrane, as occurs in infection with *Listeria monocytogenes* and *Shigella flexneri* (Duclos and Desjardins, 2000; Hackstadt, 2000; Kahn et al., 2002). Although the endocytic maturation stage of phagosome harboring vacuolar pathogens has been classified into early or late endosome, maturation of phagosome harboring intracellular pathogens is aberrant and is not a classical full maturation of any of the defined endocytic stages (Santic et al., 2010a). For example, the *Mycobacterium tuberculosis* phagosome acquires Rab5 but lacks several downstream effectors of Rab5 that are present on mature early endosome. It also acquires procathepsin D, which is the immature form of the lysosomal enzyme cathepsin D (Sturgill-Koszycki et al., 1996; Derre and Isberg, 2004). Therefore, it might be more accurate to classify phagosome of intracellular vacuolar pathogens as early endosome-like or late endosome-like phagosome (Santic et al., 2010a).

The FCP transiently acquires the EEA1 followed by the acquisition of the late endosomal markers, Lamp1/2, Cd63, and Rab7 as well as the vacuolar ATPase, which acidifies the phagosome (**Figure 1**; Golovliov et al., 2003b; Clemens et al., 2004; Santic et al., 2005a,b, 2007, 2008; Checroun et al., 2006; Bonquist et al., 2008; Qin et al., 2009). The FCP does not however co-localize with the lysosomal acid hydrolase cathepsin D and the fluid face marker, lysotraker

(**Figure 1**; Chong et al., 2008; Santic et al., 2008). Within 30–60 min, the bacterium disrupts the phagosomal membrane and escapes into the host cell cytosol (**Figure 1**; Chong et al., 2008; Santic et al., 2008). Acidification of the vacuole is important for the ability of the bacteria to escape into the cytosol, since inhibition of the vATPase results in a delay in bacterial escape into the cytosol (Chong et al., 2008; Santic et al., 2008). *F. tularensis* has been shown to escape into the cytosol in different cell types including macrophages and neutrophils (**Figure 1**; Golovliov et al., 2003a; Clemens et al., 2004; Santic et al., 2005a; McCaffrey and Allen, 2006). It has not been determined if *F. tularensis* arrest phagosome maturation before escaping into cytosol or if the bacterium manages to escape before the phagosome fuses to the lysosome. However, trafficking of the *migR* and *fevR* mutants of *F. tularensis* in macrophages suggests that there is arrest of phagosome biogenesis prior to bacterial escape into the cytosol (Buchan et al., 2009). Comparison of trafficking of the *migR* and *fevR* mutants showed that whereas the *fevR* mutant is trapped in a LAMP1-positive compartment, the phagosome containing the *migR* mutant matures into a phagolysosome enriched in LAMP1 and cathepsin D (Buchan et al., 2009). Conversely, data from studies of *F. tularensis* trafficking in neutrophils suggest that a fraction of the phagosome of wild-type bacteria that are unable to escape end up in a phagolysosome. This may suggest that *F. tularensis* does not inhibit phagosome maturation in neutrophils but rather escape into the cytosol before the phagosome matures into a phagolysosome (McCaffrey and Allen, 2006). Interestingly, arrest in phagosome biogenesis and rapid escape of *F. tularensis* into the cytosol is also exhibited in arthropod vector-derived cells, indicating exploitation of conserved eukaryotic processes by *F. tularensis* to infect and proliferate within arthropod and mammalian cells (Santic et al., 2009).

ESCAPE INTO THE CYTOSOL

Like other intracellular pathogens, *F. tularensis* must overcome the host innate immune response to successfully colonize the intracellular niche. Since the primary host defense is centered on the antimicrobial properties of the phagosome, *F. tularensis* like other cytosolic bacteria escapes from the phagosome into the cytosol where it replicates (Goebel and Kuhn, 2000; Golovliov et al., 2003a; Clemens et al., 2004; Santic et al., 2005a; McCaffrey and Allen, 2006; Ray et al., 2009). In order to escape into the cytosol, the FCP transiently acquires the vacuolar ATPase, which acidifies the phagosome followed by rapid escape of *F. tularensis* to the cytosol (**Figure 1**; Golovliov et al., 2003b; Clemens et al., 2004; Santic et al., 2005a,b, 2007, 2008; Checroun et al., 2006; Bonquist et al., 2008; Qin et al., 2009). The acidification is important since inhibition of the vATPase by bafilomycin A delays escape of the bacterium into the host cell cytosol indicating that there is a factor involved in disruption of the phagosome that is expressed or activated at acidic pH. Between 15 and 30 min of residence in the phagosome in human macrophages, the bacteria begin to escape into the cytosol (**Figure 1**; Santic et al., 2010a). It is within the cytosol that the bacteria replicate (**Figure 1**). The mechanism by which the bacterium escapes into the cytosol is not well understood.

Unlike *L. monocytogenes*, a large number of genes have been shown to be important for escape of *F. tularensis* into the host cell cytosol (**Table 2**; Golovliov et al., 2003a; Santic et al., 2005b;

Table 2 | List of escape defective mutants.

PROTEINS OF UNKNOWN FUNCTION		
FTN_0027		Conserved protein of unknown function
FTN_0109		Protein of unknown function
FTN_0149		Conserved protein of unknown function
FTN_0297		Conserved protein of unknown function
FTN_0444		Membrane protein of unknown function
FTN_0788		Conserved protein of unknown function
FTN_0855		Protein of unknown function
FTN_0915		Conserved protein of unknown function
FTN_0925		Protein of unknown function
FTN_0930		Protein of unknown function
FTN_0933		Protein of unknown function
FTN_0977		Conserved protein of unknown function
FTN_1175		Membrane protein of unknown function
FTN_1256		Membrane protein of unknown function
FTN_1343		Conserved protein of unknown function
FTN_1624		Conserved protein of unknown function
FTN_1764		Protein of unknown function
HYPOTHETICAL PROTEINS		
FTN_0030		Hypothetical membrane protein
FTN_0038		Hypothetical protein
FTN_0096		Conserved hypothetical membrane protein
FTN_0403		Hypothetical membrane protein
FTN_0727		Hypothetical membrane protein
FTN_0792		Hypothetical protein
FTN_0847		Conserved hypothetical protein
FTN_1098		Conserved hypothetical membrane protein
FTN_1349		Hypothetical protein
FTN_1395		Conserved hypothetical protein
FTN_1406		Conserved hypothetical membrane protein
FTN_1612		Hypothetical protein
FTN_1686		Hypothetical membrane protein
FTT1103		Conserved hypothetical lipoprotein
FPI PROTEINS		
FTN_1309	pdpA	Protein of unknown function
FTN_1313	vgrG	
FTN_1317	iglI	Intracellular growth locus, subunit I
FTN_1322	iglC	Intracellular growth locus, subunit C
FTN_1323	iglB	Intracellular growth locus protein B
FTN_1324	iglA	Intracellular growth locus A
FTN_1325	pdpD	Protein of unknown function
METABOLIC PROTEINS		
FTN_0019	pyrB	Aspartate carbamoyltransferase
FTN_0063	ilvE	Branched-chain amino acid aminotransferase protein (class IV)
FTN_0090		Acid phosphatase
FTN_0125	ackA	Propionate kinase 2/acetate kinase A
FTN_0483		Bifunctional NMN adenylyltransferase/Nudix hydrolase
FTN_0504		Lysine decarboxylase
FTN_0511		Shikimate 5-dehydrogenase
FTN_0524	asd	Aspartate semialdehyde dehydrogenase

(Continued)

Table 2 | Continued

FTN_0527	thrC	Threonine synthase
FTN_0545		Glycosyl transferase, group 2
FTN_0692	nadA	Quinolinate synthetase A
FTN_0746	alr	Alanine racemase
FTN_0811	birA	Biotin–acetyl-CoA-carboxylase ligase
FTN_0822		*p*-Aminobenzoate synthase component I
FTN_0840	mdaB	NADPH-quinone reductase (modulator of drug activity B)
FTN_0877	cls	Cardiolipin synthetase
FTN_0954		Histidine acid phosphatase
FTN_0965		Metal-dependent exopeptidase
FTN_0983		Bifunctional protein: glutaredoxin 3/ribonucleotide reductase beta subunit
FTN_0988	prmA	50S ribosomal protein L11, methyltransferase
FTN_1061		Acid phosphatase, HAD superfamily protein
FTN_1222	kpsF	Phosphosugar isomerase
FTN_1231	gloA	Lactoylglutathione lyase
FTN_1234	queA	S-adenosylmethionine: tRNA ribosyltransferase-isomerase
FTN_1333	tktA	Transketolase I
FTN_1376		
FTN_1418	manC	
FTN_1428	wbtO	Transferase
FTN_1494	aceE	Pyruvate dehydrogenase complex, E1 component, pyruvate dehydrogenase
FTN_1553	nudH	dGTP pyrophosphohydrolase
FTN_1597	prfC	Peptide chain release factor 3
FTN_1621		Predicted NAD/FAD-dependent oxidoreductase
FTN_1655	rluC	Ribosomal large subunit pseudouridine synthase C
TRANSPORTER PROTEINS		
FTN_0624		Serine permease
FTN_0728		Predicted Co/Zn/Cd cation transporter
FTN_0997		Proton-dependent oligopeptide transporter (POT) family protein, di-, or tripeptide: H+ symporter
FTN_1344		Major facilitator superfamily (MFS) transport protein
FTN_1611		Major facilitator superfamily (MFS) transport protein
FTN_1711	tyrP	Tyrosine permease
DNA MODIFICATION		
FTN_0133		Ribonuclease II family protein
FTN_0680	uvrC	Excinuclease ABC, subunit C
FTN_0710		Type I restriction–modification system, subunit R (restriction)
FTN_1027	ruvC	Holliday junction endodeoxyribonuclease
FTN_1073		DNA/RNA endonuclease G
FTN_1154		Type I restriction–modification system, subunit S
TRANSCRIPTION/TRANSLATION		
FTN_1290	mglA	Macrophage growth locus, protein A
FTL_1542	migR	Macrophage intracellular growth regulator
FTL_1914	ripA	Required for intracellular proliferation, factor A
FTN_0480	fevR	*Francisella* effector of virulence regulation
TYPE IV PILIN		
FTN_1137	pilQ	Type IV pili secretin component
FTN_1139	pilO	Type IV pili glycosylation protein
OTHERS		
FTN_0286		Transposase
FTN_0646	cscK	ROK family protein
FTN_0768	tspO	Tryptophan-rich sensory protein
FTN_1034	rnfB	Iron–sulfur cluster-binding protein
FTN_1145	era	GTP-binding protein
FTN_1241		DedA family protein
FTN_1263	comL	Competence lipoprotein ComL
FTN_1453		Two-component regulator, sensor histidine kinase
FTN_1518	relA	GDP pyrophosphokinase/GTP pyrophosphokinase

Qin and Mann, 2006; Mohapatra et al., 2008; Barker et al., 2009; Broms et al., 2009; Buchan et al., 2009; Schmerk et al., 2009; Schulert et al., 2009; Asare and Abu Kwaik, 2010). Recent mutagenesis experiments have shown that most of the genes of the FPI that form the type VI-like secretion system, affect escape of the bacterium into the cytosol and subsequent replication (Golovliov et al., 2003a; de Bruin et al., 2007; Barker et al., 2009; Broms et al., 2009; Schmerk et al., 2009). In contrast, IglD has been shown to be important for replication of the bacteria within the cytosol without any effect on phagosomal escape of the bacterium (Santic et al., 2007). The FPI protein VgrG has been shown to be a component of the secretary system as well as a substrate of the system (Barker et al., 2009). Unlike VgrG, IglI is a substrate of the type VI secretion system with no effect on the secretion apparatus. Both genes are important for escape of *F. tularensis* into the host cell cytosol but the mechanism of action has not been elucidated (Barker et al., 2009). The FPI proteins IglA, IglC, and pdpA are also required for escape of *F. tularensis* into the host cells cytosol but it has not been determined if they are secreted substrates or component of the type VI-like secretion apparatus.

Mutations in MglA and FevR negatively affect the ability of *F. tularensis* to escape from the phagosome into the cytosol (Santic et al., 2005b; Bonquist et al., 2008; Buchan et al., 2009). In contrast, MigR mutant behaves similar to the IglD mutant, which escapes but is unable to replicate within the cytosol indicating that MigR regulate genes that are important for replication within the cytosol (Santic et al., 2007; Buchan et al., 2009). Other genes that play critical roles in the escape of bacteria into the host cell cytosol include genes involved in DNA modification, transcription and translation, type II secretion, metabolic genes as well as genes involved in the transport of nutrients (Schulert et al., 2009; Asare and Abu Kwaik, 2010).

Although hemolytic activity has been observed in *F. tularensis* subspecies *novicida* and *F. philomiragia* (Lai et al., 2003), no hemolysin homolog has been identified in all the sequenced *Francisella* genome to date including those of *novicida* and *philomiragia*. There are between four and eight acid phosphatases in the *Francisella* genome depending on the subspecies. There are eight acid Phosphatases in the subspecies *novicida* genome, four (AcpA, AcpB, AcpC, and HAP) of which are also found in the virulent subspecies *tularensis* genome (Mohapatra et al., 2008). AcpA has

been shown to possess lipase activity (Mohapatra et al., 2007a), but all three Acp molecules are predicted to possess phosphoric ester hydrolase activity. Independent studies have shown that mutations in AcpA, AcpC, and HAP result in delay or inhibition of escape into the cytosol and reduced replication within human macrophages (Mohapatra et al., 2007a; Asare and Abu Kwaik, 2010), and that combined deletion of AcpA, AcpB, AcpC, and HAP results in complete inhibition of phagosomal escape and replication of subspecies *novicida* in the cytosol (Mohapatra et al., 2008). However, there is contradictory data on the role of these acid phosphatases in escape and intracellular replication. For example, Baron et al. (1999) have shown that AcpA in subspecies *novicida* is not important for replication within mouse macrophages. The difference between the role of AcpA in various subspecies may be due to the difference in the macrophages used, since trafficking of *F. tularensis* has been shown to be slightly different in mouse and human macrophages (Clemens et al., 2004; Checroun et al., 2006). Similarly, Child et al. (2010) have shown that combined deletion of AcpA-C does not affect the phagosomal escape or replication of the virulent subspecies *tularensis* within human macrophages. This indicates that there may be subtle differences in the mechanisms used by the different subspecies to escape into the host cell cytosol. There are numerous genes identified to be important for escape of *F. tularensis* that are designated as hypothetical proteins or proteins with unknown functions (Asare and Abu Kwaik, 2010). Some of these may be potential substrates for the type VI-like secretion system and identifying and characterizing them will help us to understand how *F. tularensis* modulates biogenesis of its phagosome and escape into the cytosol.

MODULATION OF INFLAMMATORY RESPONSE TO INFECTION BY *F. TULARENSIS*

The transcription factor NF-κB is involved in the regulation of inflammation by activating the induction of different proinflammatory cytokines (Lawrence, 2009). NF-κB represents a family of homo and heterodimer transcription factors, and the p65/p50 heterodimer is the most predominant active complex in mammalian cells (Burstein and Duckett, 2003). In resting cells, NF-κB proteins are predominantly sequestered in the cytoplasm by the NF-κB inhibitory proteins (IκBs; Karin and Ben-Neriah, 2000). The IκB kinase mediates phosphorylation of IκBs, followed by ubiquitination and proteasomal degradation, which is crucial to the activation and nuclear translocation of NF-κB (Karin and Ben-Neriah, 2000).

Early during infection when *F. tularensis* is localized within the phagosome, it activates the inflammatory response in macrophages by inducing the secretion of TNF-α in TLR-2 dependent manner (**Figure 2**; Telepnev et al., 2005; Katz et al., 2006). Induction of TNF-α secretion is mediated by the PI3K/Akt pathway, which also leads to activation of NF-κB (Telepnev et al., 2003; Katz et al., 2006; Parsa et al., 2006; Rajaram et al., 2006). Activation of NF-κB results in the induction and secretion of proinflammatory cytokines that restrict the escape of *F. tularensis* from the phagosome into the cytosol and promotes fusion of the FCP with the lysosome (**Figure 2**; Rajaram et al., 2009). Concomitant with escape into the cytosol, *F. tularensis* down-regulates NF-κB activation and TNF-α, IL-6, IL-8, and IL-12 secretion within 5 h post-infection, since the *IglC* mutant which is unable to escape into the cytosol, does not down-regulate TNF-α, IL-6, IL-8, and IL-12 secretion (**Figure 2**; Telepnev et al., 2003, 2005).

The activation of PI3K/Akt pathway is negatively regulated by the Src homology 2 (SH2) domain-containing inositol-5′-phosphatase (SHIP) protein, since deficiency in SHIP expression results in enhanced Akt activation and NF-κB-driven transcription of proinflammatory cytokines, which promote fusion of the FCP with the Lysosome (**Figure 2**; Parsa et al., 2006; Rajaram et al., 2009). Conversely, over expression of SHIP leads to a decrease in NF-κB activation (Parsa et al., 2006). It is unknown how the delicate balance of Akt and SHIP activation is tilted toward SHIP promoted escape of *F. tularensis* into the cytosol. It will be interesting to determine how *F. tularensis* activates SHIP and the relations between SHIP activation and the disruption of the phagosome membrane that allow *F. tularensis* to escape into the cytosol (**Figure 2**). Once inside the cytosol *F. tularensis* induces Sp-1/Sp-3 dependent Fas expression that results in activation of caspase-3 and host cell death (Rajaram et al., 2009).

Cytosolic localization of *F. tularensis* in mouse macrophages results in type I interferon (IFN-I) and AIM2 dependent activation of the inflammasome (**Figure 2**; Mariathasan et al., 2005; Gavrilin et al., 2006; Henry et al., 2007; Fernandes-Alnemri et al., 2010; Jones et al., 2010). Cytosolic bacteria induce IRF-3 dependent activation of IFN-I, which in turn increases the expression of AIM2 (**Figure 2**; Jones et al., 2010). AIM2 recognize *F. tularensis* (Fernandes-Alnemri et al., 2010), likely through binding to dsDNA from the bacteria since *F. tularensis* DNA co-localizes with AIM2 (Jones et al., 2010). Upon binding to *F. tularensis* DNA, AIM2 forms a complex with the adapter protein ASC and caspase-1 known as the inflammasome (Mariathasan et al., 2005; Fernandes-Alnemri et al., 2010; Jones et al., 2010). Inflammasome activation by *F. tularensis* results in the formation of pyropoptosome, which is critical for innate host defense and leads to pyropoptotic death of infected cells and the concomitant release of the proinflammatory cytokines IL-1β and IL-18 (**Figure 2**; Mariathasan et al., 2005; Gavrilin et al., 2006). The *F. tularensis* lipid/polysaccharide (MOP) transporter protein, MviN, which is homologous to the *E. coli* putative lipid II flippase, has recently been shown to suppress the induction of AIM2 (Ulland et al., 2010), since a mutation in the gene results in increase induction of AIM2 inflammasome-dependent IL-1β secretion and cytotoxicity in macrophages (Ulland et al., 2010). In addition to MviN, two other genes FTT_0584, with no characterized orthologs, and FTT_0748, which is homologous to the IclR family of transcriptional regulators, have been shown to suppress caspase-1 and ASC dependent secretion of IL-1β, since mutations in these genes resulted in hyper secretion of IL-1β (Weiss et al., 2007). Since AIM2 is not present in human macrophages, inflammasome mediators are likely to be different from the one described for mouse macrophages.

ACTIVATION AND CONTROL OF HOST CELL APOPTOSIS

Between 6 and 12 h post-infection, *F. tularensis* induce caspase-3 activation within the host cells, which culminate in the induction of apoptosis (Lai and Sjostedt, 2003; Santic et al., 2010b). *F. tularensis* LVS induces apoptosis in the J774A.1 murine macrophage cell

line through a pathway partly resembling the intrinsic apoptotic pathway (Lai and Sjostedt, 2003). The induction of apoptosis involves the release of mitochondrial cytochrome C into the cytosol with concomitant activation of caspase-9 and caspase-3 but not caspase-1, caspase-8, Bcl-2, or Bid (Lai and Sjostedt, 2003). In contrast, another study has shown that *F. tularensis* induces Sp-1/Sp-3 activation of Fas in RAW 264.7 murine macrophage cells line, which results in activation of caspase-3, suggesting that *F. tularensis* induce apoptosis through the extrinsic pathway (**Figures 1 and 2**; Rajaram et al., 2009). Interestingly, infection of murine macrophages by *F. tularensis* has been shown to induce apoptotic cell death through down-regulation of activation of p38 MAPK compared to uninfected cells, but the mechanism of induction is yet to be defined (Hrstka et al., 2005). Although caspase-3 activation occurs early during infection in non-activated macrophages, it is not until about 18 h post-infection before there is induction of apoptosis, which is likely due to triggering anti-apoptotic processes (**Figures 1 and 2**; Lai and Sjostedt, 2003; Al-Khodor and Abu Kwaik, 2010; Santic et al., 2010b).

HOST FACTORS REQUIRED INTRACELLULAR GROWTH OF *F. TULARENSIS*

NF-κB plays a crucial role in regulation of apoptosis by triggering expression of various anti-apoptotic genes (Burstein and Duckett, 2003). We have shown that in order to maintain viability of the infected cell and allow *F. tularensis* to survive, there is simultaneous activation of caspases and NF-κB creating a delicate balance between them to maintain cell viability that is necessary for proliferation of the bacterium. Activation of NF-κB involves IκB kinase-mediated phosphorylation of IκBs, followed by ubiquitination and proteasomal degradation (Karin and Ben-Neriah, 2000). Interestingly, in activated macrophages, *F. tularensis* elicit ubiquitin-dependent MHC class II down-regulation and degradation, thus compromising antigen presentation by macrophages to CD4 T cells (Wilson et al., 2009). It is not surprising that two ubiquitin proteins, the ubiquitin hydrolase USP22, and the ubiquitin ligase CDC27 has been shown to be important for replication of *F. tularensis* in human macrophages (Akimana et al., 2010).

It has been shown that *F. tularensis* triggers activation of Ras through the recruitment of PKCα and PKCβ-I to the SOS2/GrB2 complex (**Figure 2**; Al-Khodor and Abu Kwaik, 2010). Silencing of SOS2, GrB2, PKCα, and PKCβ-1 is associated with rapid early activation of caspase-3 but does not affect phosphorylation of Akt or Erk (Al-Khodor and Abu Kwaik, 2010). This indicates that *F. tularensis* utilizes two independent mechanisms to modulate caspase-3 activity in order to survive inside host cells till the terminal stages of infection when induction of apoptosis leads to cell lysis and release of bacteria to the extracellular milieu. The bacterial factor necessary for the activation of NF-κB and Ras are yet to be identified.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

Upon infection with *F. tularensis*, the host cells employ a myriad of arsenals to try to limit proliferation of the bacteria. The host cells activate signaling pathways to try to restrict escape of *F. tularensis* into the cytosol. Once the bacteria escape into the cytosol, a new arsenal is put into motion by the host cells through activation of caspase-1 and caspase-3, geared toward pyropoptosis and apoptosis of the infected cells. Concomitantly, there is activation of NF-κB geared toward triggering pro-survival signals and the induction of proinflammatory cytokines. Intuitively, *F. tularensis* has devised different strategies to counteract the innate host defense mechanisms. These include inhibition of components of the host defense mechanism and hijacking the cells own defense system and other signaling pathways through bacterial effectors that are likely exported through a type VI-like secretion system. For example, *F. tularensis* co-opts the host cell NF-κB transcription factor, which is used to activate proinflammatory cytokines, to induce the expression of anti-apoptotic genes to maintain cell viability. Similarly, *F. tularensis* co-opts the host cell Ras signaling pathway to inhibit caspase-3-induced apoptosis. Finally, *F. tularensis* utilizes the host cell ubiquitin-dependent proteasome degradation system to degrade MHC class II molecules on activated macrophages to inhibit antigen presentation to effector T cells.

Many virulence factors have been identified that are required for bacterial escape and replication within the cytosol. Although some of these are involved in known pathways, majority of these have no known functions. The roles of some of these factors in the virulence mechanisms exhibited by *F. tularensis* are beginning to be defined but the functions are largely unknown. Cytosolic *F. tularensis* activates PKC leading to activation of Ras and inhibition of apoptosis. MigR regulates genes that are important of phagosome biogenesis. Identifying and characterizing MigR-regulated genes will lead to an understanding of how *F. tularensis* arrest phagosome maturation. Delineating how MviN, FTT0584, and FTT0748 inhibit caspase-1 activity will shed light on how *F. tularensis* modulate pyropoptosis and proinflammatory cytokine induction. Since NF-κB is required for both cytokine induction and inhibition of apoptosis, its activation must be tightly controlled. It will be interesting to identify the bacterial factors important for activation of Ras and diversion of NF-κB to the expression of anti-apoptotic effectors. It is not known how *F. tularensis* tilts the balance of power between Akt and SHIP toward SHIP activation and bacterial escape and the mechanism by which this is achieved. Unlike *L. monocytogenes*, no hemolysin-like molecule have been identified in *F. tularensis* and there is contradictory data on role of AcpA in escape, which may be partly due to the studies being done using different species of *Francisella* and different sources of macrophages. It will be interesting to know if SHIP does not only inhibit cytokine activation but also activate a host cell factor that leads to disruption of the phagosome membrane and escape of the bacteria. It will be interesting to determine how *F. tularensis* modulate the ubiquitin ligase in activated macrophages leading to degradation of MHC II molecules and evasion of adaptive immunity. There is little doubt that *F. tularensis* employs various strategies to modulate cellular processes that have evolved to degrade invading microbes in addition to evasion of various innate and adaptive immune processes to inflict disease in the mammalian host. It is just as interesting to uncover the molecular and cellular bases of the interaction of *F. tularensis* with the arthropod vector and its role in pathogenic evolution and infection of the mammalian host. It is likely that the pathogen exploits conserved eukaryotic processes to infect evolutionarily distant hosts as well as processes unique to the infection of mammals.

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Immunity to *Francisella*

Siobhán C. Cowley* and Karen L. Elkins

Center for Biologics Evaluation and Research, U.S. Food and Drug Administration, Bethesda, MD, USA

Edited by:
Anders Sjostedt,
Umeå University, Sweden

Reviewed by:
Dennis Metzger,
Albany Medical College, USA
Shabaana Khader,
University of Pittsburgh, USA

****Correspondence:***
Siobhán C. Cowley, Laboratory of Mycobacterial Diseases and Cellular Immunology, Division of Bacterial, Parasitic and Allergenic Products/ Office of Vaccines Research and Review/Center for Biologics Evaluation and Research, U.S. Food and Drug Administration, HFM-431, 29 Lincoln Drive, Room 516, Bethesda, MD 20892, USA.
e-mail: siobhan.cowley@fda.hhs.gov

In recent years, studies on the intracellular pathogen *Francisella tularensis* have greatly intensified, generating a wealth of new information on the interaction of this organism with the immune system. Here we review the basic elements of the innate and adaptive immune responses that contribute to protective immunity against *Francisella* species, with special emphasis on new data that has emerged in the last 5 years. Most studies have utilized the mouse model of infection, although there has been an expansion of work on human cells and other new animal models. In mice, basic immune parameters that operate in defense against other intracellular pathogen infections, such as interferon gamma, TNF-α, and reactive nitrogen intermediates, are central for control of *Francisella* infection. However, new important immune mediators have been revealed, including IL-17A, Toll-like receptor 2, and the inflammasome. Further, a variety of cell types in addition to macrophages are now recognized to support *Francisella* growth, including epithelial cells and dendritic cells. $CD4^+$ and $CD8^+$ T cells are clearly important for control of primary infection and vaccine-induced protection, but new T cell subpopulations and the mechanisms employed by T cells are only beginning to be defined. A significant role for B cells and specific antibodies has been established, although their contribution varies greatly between bacterial strains of lower and higher virulence. Overall, recent data profile a pathogen that is adept at subverting host immune responses, but susceptible to many elements of the immune system's antimicrobial arsenal.

Keywords: *Francisella*, immunity, innate, adaptive, lymphocyte, cytokine

INTRODUCTION AND OVERVIEW

Although *Francisella tularensis* is highly infectious and readily establishes disease at low doses in both humans and animals, it has long been recognized that human tularemia victims rarely if ever suffer a second episode of disease. The collective human infection experience therefore strongly suggests that natural infection engenders strong immune responses that are usually protective. The older literature contains a wealth of information on the pathogenesis and host response to *Francisella* drawn from studies in both humans and animals, including vaccination and challenge studies in humans that would be difficult if not impossible to replicate today. These studies have been recently reviewed extensively elsewhere (Conlan and Oyston, 2007; Elkins et al., 2007). But the history of studies on *Francisella* includes its development as a biowarfare pathogen (Dennis et al., 2001); as a result, the recent heightened interest in biodefense has produced a flood of new and exciting data, particularly on respiratory infection and mucosal immune responses to this pathogen. The upsurge in studies is impressive: from 1900 to 2005, a search of PubMed for "Tularemia or *Francisella*" yielded a total of 2921 references, but 858 citations from 2006 to 2011. Thus this review will focus primarily on developments in about the last 5 years.

HUMAN *IN VIVO* IMMUNE RESPONSES TO *FRANCISELLA* INFECTION AND VACCINATION

IMPORTANT COMPONENTS OF HUMAN IMMUNE RESPONSES REVEALED BY *FRANCISELLA* INFECTION

The most recent studies of the epidemiology of infection with *F. tularensis* subsp. *tularensis*, as well as subsp. *holarctica*, clearly indicate differences in virulence among clades of Type A *F. tularensis* subsp. *tularensis*. These are now denoted A1a, A1b, and A2, in addition to Type B *F. tularensis* subsp. *holarctica* (Kugeler et al., 2009). Type A1 infections of humans tend to have a fulminant course with a high mortality rate, while Type A2 and Type B infections are rarely if ever lethal in humans (Staples et al., 2006; Kugeler et al., 2008). In studies in the U.S., differences in the attack rates of immunocompromised people for the various clades and subspecies have been described; 11 of 108 (10%) of Type B infections, 6 of 133 (6%) of A1 infections (both subtypes), and none of 68 A2 infections were diagnosed in people with an underlying immunocompromising condition, including medical conditions as diverse as end stage renal disease (Staples et al., 2006) and chronic granulomatous disease (CGD; Maranan et al., 1997). Nonetheless, the collective evidence suggests that the differences in virulence are largely due to intrinsic properties of the bacterial strains, and not directly related to host gender, susceptibility, genetics, or otherwise failed immune responses (Kugeler et al., 2009).

Because infection with *Francisella* is relatively infrequent in nature, informative examples of infection of people with primary or acquired immunodeficiencies subjects are rare. The handful of such cases prior to 2006, including infection of AIDS patients with reduced $CD4^+$ T cell counts, has been reviewed elsewhere (Elkins et al., 2007), and thus will not be repeated here. More recently, an interesting case involving a 58-year-old man with refractory rheumatoid arthritis was described. The patient had been treated for about a year with methotrexate and an anti-TNF-α therapeutic (Humira®, adalimumab), when he presented with fever, a leg wound, enlarged lymph nodes, and eventually skin fistula. Tuberculosis was suspected initially; the lesion was surgically removed, and found

to contain necrotic epithelioid granulomas. Surprisingly, both serology and PCR of a biopsied lymph node diagnosed *F. tularensis* infection (Konstantinou et al., 2009). It is tempting to speculate that TNF-α deficiency, provided in this case by drug treatment, increased susceptibility to *Francisella* infection, similar to observations in animal models (Cowley et al., 2008) and in mycobacteria infections of humans (Gardam et al., 2003).

Of note, although only about 20 cases of human infection with *F. philomiragia* have been reported in the literature, disease caused by this species is usually associated with immune defects. These include corticosteroid treatment, CGD, and near-drowning episodes (Hollis et al., 1989; Sicherer et al., 1997). The association between *Francisella* and CGD, in which neutrophils fail to produce fully functional NADPH oxidase and thus reactive oxygen radicals, obviously suggests a role for these mediators in human resistance to infection.

The experimental literature now frequently refers to the immunosuppressive nature of infection with virulent *Francisella* (the topic of another chapter in this issue), but naturally infected patients as well as vaccines eventually develop robust and readily measurable T and B cell responses to the bacterium. These responses have been studied for many years, particularly in regions such as Scandinavia which have appreciable amounts of disease. An outbreak of ulceroglandular tularemia in Sweden in 2003–2004 provided a unique opportunity to obtain peripheral blood cells from patients within days of infection, and perform large scale transcriptional profiling of human gene expression by microarray (Andersson et al., 2006a). Within 2–3 days, there was clear evidence for increased expression of genes regulated by interferon gamma (IFN-γ) and related to apoptosis, but also indications of down-regulation of many genes related to both innate and adaptive immune responses. Thus infection in humans may be characterized by both appropriate and subversive types of host reactions.

The major means to diagnose tularemia is still based on four-fold increase of serum antibodies to the bacterium between acute and convalescent sera. Robust specific IgM, IgG, and IgA serum antibodies, much of them directed against *Francisella* lipopolysaccharide (LPS), can be detected roughly simultaneously about 6–10 days after the onset of symptoms, or about 2 weeks after infection. Antibody responses peak between 1 and 2 months after infection, and persist for about a decade before diminishing (Koskela and Salminen, 1985). Large scale efforts using 2-D gel blotting (Janovska et al., 2007) or protein chips with a library of *Francisella* proteins (Sundaresh et al., 2007), reacted with patient sera, are beginning to identify and catalog the bacterial proteins recognized. No obvious immunodominant B cell epitopes have been revealed, however, and as a result panels of antigens have been proposed for diagnostic purposes (Sundaresh et al., 2007).

Within about 2 weeks after infection, *ex vivo* production of typical Th1-type cytokines such as IFN-γ, TNF-α, and IL-2 by $CD4^+$ and $CD8^+$ T cells is readily detectable in human peripheral blood lymphocytes (PBLs) obtained from tularemia patients (Koskela and Herva, 1980; Surcel et al., 1991). Unlike antibody responses, however, human $CD4^+$ and $CD8^+$ T cell PBL responses persist for as long as 30 years after documented infection (Ericsson et al., 1994). The development of more sensitive assays has facilitated even earlier detection of cytokines such as IFN-γ, which may be produced by both innate immune cells and nascent specific T cells. As a result, recent biological assays being developed for diagnosis have been based on *ex vivo* stimulation of whole peripheral blood leukocytes with bacteria, followed by assessment of IFN-γ secretion (Eliasson et al., 2008).

A curious, and still unexplained, feature of natural infection is the large expansion of phosphoantigen-responsive Vγ9/Vδ2 T cells in peripheral blood within the first 7 days after onset of symptoms in tularemia patients (Poquet et al., 1998). This intriguing phenomenon, which can result in as many as 30% of all $CD3^+$ cells in human peripheral blood being Vγ9/Vδ2 T cells, is discussed in more detail below.

HUMAN IMMUNE RESPONSES REVEALED BY VACCINATION AGAINST *FRANCISELLA*

As noted above, and discussed in detail in another chapter in this issue, there is a long history of using various live attenuated strains of *Francisella* as vaccines, particularly in the former Soviet Union (Sändstrom, 1994). As a reference point for further discussion of immune responses generally, here we include a brief summary of human immune responses to the LVS strain. Usually administered by scarification, LVS has been studied in western countries and particularly in the U.S. These studies have mostly used investigational lots of LVS produced in the 1970s by the Department of the Army; more recently, LVS was re-derived from the original lots, and newly manufactured under modern GMP conditions (El Sahly et al., 2009). Microarray studies of gene changes in PBLs from five volunteers obtained between 1 and 14 days after vaccination with LVS indicated a robust up-regulation of expression of pro-inflammatory mediators and genes involved in dendritic cell (DC) function, which peaked within 2 days (Fuller et al., 2006, 2007). Similar to natural infection, humans vaccinated with LVS develop specific IgM, IgA, and IgG antibodies in serum about 2 weeks after vaccination that persist for at least 1.5 years (Waag et al., 1995; El Sahly et al., 2009). However, as noted at the outset, anti-*Francisella* serum antibodies titers in vaccinated individuals have not been predictive of protection against virulent tularemia, and vaccination with killed bacteria (that elicit anti-*Francisella* antibodies but no detectable cell-mediated immune responses) has provided little or no benefits in human studies (Francis and Felton, 1942; Foshay, 1950; Overholt et al., 1961; Saslaw et al., 1961a,b; Hambleton et al., 1974; Burke, 1977; Tärnvik, 1989). In addition to serum antibodies, stimulated PBLs, or enriched $CD4^+$ and $CD8^+$ T cells, obtained from volunteers 2–4 weeks or more after LVS vaccination proliferated and produced typical Th1-type cytokines, especially IFN-γ, *ex vivo* (Waag et al., 1995; El Sahly et al., 2009). In one study, the responding cells from both LVS vaccines and patients after natural infection were characterized as traditional CD4 and CD8 memory T cells, mostly with an effector memory phenotype ($CD45RA^{-/+}$, $CD62^-$; Salerno-Goncalves et al., 2009).

ANIMAL MODELS OF HUMAN IMMUNITY TO *FRANCISELLA*

Detailed consideration of animal models of *Francisella* infection is well beyond the scope of this article, and a comprehensive recent review is available elsewhere (Lyons and Wu, 2007). Nonetheless, because the bulk of data on immune responses is currently being generated using animal models of infection, here we include brief summaries of recent developments as they relate to modeling the immune responses of humans (for summary, see **Table 1**).

Table 1 | Characteristics of human *Francisella* infections compared to current experimental animal models.

Strain	Human infection/vaccination	Experimental animal models				
		Non-mammalian		Mammalian[+]		
		Insect (*Drosophila*)	Fish (zebrafish)	Mice (C57BL/6J)	Rats (Fisher 344)	Marmosets (common)
Type A1a/b *Ft*	Aggressive infection, highly virulent, can be lethal	Not tested	Not tested	$LD_{50} < 10$, all routes tested	LD_{50} IT 5×10^2	LD_{50} by aerosol <10
Type A2 *Ft*	Causes morbidity, but rarely lethal	Not tested	Not tested	$LD_{50} < 10$, all routes tested	Not tested	Not tested
Type B *Ft*	Causes morbidity, but rarely lethal	Not tested	Not tested	$LD_{50} < 10$, all routes tested	LD_{50} IT 10^5, IP < 10	Lethal natural infections observed
LVS	Attenuated Type B; skin or respiratory inoculation establishes productive infection, vaccinates*	Productive infection; intra/extracellular replication	Not tested	LD_{50} 10^6 ID, 10^3 IN, <10 IP or IV;vaccinates when sublethal*	LD_{50} IT 10^5, IP >10^8; vaccinates when sublethal*	Not tested
F. novicida	Infections rare, never lethal; some association with immunodeficiencies	Productive infection; intra/extra cellular replication	Not tested	LD_{50} 10^3 ID#, <10 IP, IV, or IN	LD_{50} IT 5×10^6	Not tested
F. philomiragia	Infections rare, never lethal; associated with CGD, other immunodeficiencies	Not tested	Not tested	Not tested	Not tested	Not tested
Francisella spp. from fish	No known human pathogenicity	Not tested	Infects; ↑ cytokine mRNA	Not tested	Not tested	Not tested

**Stimulates production of pro-inflammatory cytokines, serum anti-Francisella antibodies, Th1 T cells, and Th1-related cytokines; extent of protection against secondary challenge infection varies with route of vaccination, route of challenge, and challenge strain.*

[+]All LD_{50}s are approximate and expressed in terms of CFU.

#Survival of primary and secondary infections are heavily B cell dependent, unlike other strains.

For references, see corresponding text.

NON-MAMMALIAN INFECTION MODELS

Two groups have explored insect models of *Francisella* infection. Both LVS (Vonkavaara et al., 2008) and *F. novicida* (Ahlund et al., 2010; Moule et al., 2010) productively infect and replicate in *Drosophila melanogaster*, both by infection of phagocytic fly hemocytes and by extracellular replication. Both efforts focused on the ability of the model to identify bacterial virulence factors; mutations in bacterial genes important to virulence in mouse models, notably pathogenicity island genes including *mglA* and others regulated by *mglA*, clearly contributed to virulence in flies. Given that deer flies are vectors of *Francisella* infection, however, it remains to be revealed whether the fly host biology discovered is applicable only to the vector, or also helpful in modeling human responses.

Initial efforts to establish *Francisella* infection of zebrafish, another genetically tractable system that has a more complex immune system than *Drosophila*, have recently been reported as well. In the last 3–4 years, new *Francisella* spp. have been isolated from diseased fish, both wild and cultivated. The zebrafish study used one of these strains, and demonstrated productive experimental infection followed by up-regulated expression of IL-1β, IFN-γ, and TNF-α, analogous to mammalian pro-inflammatory responses. There was no increase, and perhaps transient down-regulation, of iNOS, however (Vojtech et al., 2009).

MAMMALIAN INFECTION MODELS

For all the obvious reasons, the majority of immunological studies of *Francisella* have used mouse models. The available data indicate that mice are a reasonable model of human immune responses, at least at a first approximation, but less satisfying as a model for pathogenicity. Inbred laboratory strains are readily susceptible to infection with all *Francisella* isolates tested to date; bacteria disseminate to the same target organs of the reticuloendothelial system, and infected tissues develop granulomatous pathologies that appear roughly comparable to lesions described in tissues of infected people. There is a major discrepancy between humans and mice in virulence and lethality, however. In mice, the LVS strain establishes a sublethal vaccinating infection when administered via skin inoculation, but kills mice at low doses when introduced by other routes, including intravenous (IV), intramuscular, or intraperitoneal (IP), and is intermediate for respiratory infections (Elkins et al., 2003). Importantly, infection with both Type A and Type B *F. tularensis*, as well as *F. novicida*, kills mice within a week with essentially any dose and when introduced by any route of infection. In contrast, as noted above, human Type B infections in particular are rarely lethal (Staples et al., 2006; Kugeler et al., 2008). Human *F. novicida* infections are quite rare, and when detected are sometimes associated with immunocompromised individuals (Hollis et al., 1989; Whipp et al., 2003; Leelaporn et al., 2008). To date, there is only one report of *Francisella* infection of HLA-DR4 transgenic mice, with the goal of uncovering antigens recognized by human T cells (Yu et al., 2010); but as expected, these mice were equally susceptible to intranasal LVS infection as wild type mice. It remains to be determined whether "humanized" mice created by engraftment of human stem cells exhibit susceptibilities that better approximate human infection profiles.

Over the years, other small animal models for *Francisella* infection have been developed using rats, guinea pigs, hamsters, voles, and rabbits. Several recent reports, coupled with older literature, indicate that infection of Fisher 344 rats with different *Francisella* strains may better approximate the phenotype of human infections that these other models. Both Fisher 344 and Lewis rats are much more resistant to *F. novicida* infection than mice, due at least in part to rapid production of nitric oxide from macrophages following recognition of the *F. novicida* LPS chemotype (Cowley et al., 1997; Ray et al., 2010). In a direct comparison, the intratracheal (IT) LD_{50} of Fisher 344 rats was recently reported to be 5×10^2 for Type A *F. tularensis* subsp. *tularensis* (SchuS4); 1×10^5 for Type B *F. tularensis* subsp. *holarctica* (OR960246); 5×10^6 for *F. novicida*; and greater than 10^7 for LVS (Ray et al., 2010). Of note, however, Fisher 344 rats were highly susceptible to IP infection with a different Type B *F. tularensis* subsp. *holarctica* strain (FSC108), although still quite resistant to IP LVS infection (Raymond and Conlan, 2009). Nonetheless, overall the hierarchy of susceptibility of rats appears to generally reflect human *Francisella* infections, and provide a more satisfying profile than that of mice. Equally important, Fisher 344 rats vaccinated IT, intradermally (ID), or subcutaneously (ID) with ~10^7 LVS survived IT challenge with at least 100 LD_{50}s of challenge with Type A *F. tularensis* subsp. *tularensis* (SchuS4; Wu et al., 2009; Ray et al., 2010). Thus this rat strain appears to provide both a useful model for both infection and immunological studies for further analyses.

Despite the practical appeal of small animal models, studies using non-human primates remain important not only for basic studies of host–pathogen interactions but for testing of drugs, vaccines, and therapeutics. Historically, monkeys have been considered to be even more susceptible than humans to *Francisella* infection. Outbreaks of tularemia in various species of monkeys in both zoo and experimental colonies have been reported repeatedly (Splettstoesser et al., 2007). There is an extensive older literature using monkeys, particularly Rhesus monkeys, for both natural history and vaccination studies (Lyons and Wu, 2007; Kugeler et al., 2008). Most recently, interest in product development has spurred renewed efforts to establish non-human primate models using species currently available for experimental studies. Marmosets (*Callithrix jacchus*) suffer lethal infection with as few as 10 CFU of Type A *F. tularensis* subsp. *tularensis* (SchuS4) administered by aerosol, with pathology that appears similar to that of humans. Infected marmosets further exhibited production of pro-inflammatory cytokines, as well as increased numbers of the major lymphoid and myeloid cell subpopulations in lungs and blood (Nelson et al., 2009, 2010). Similarly, the profile of aerosol infection of African green monkeys (*Chlorocebus aethiops*) given ~700 CFU of SchuS4 was described recently (Twenhafel et al., 2009), and studies in cynomolgus monkeys as well as comparisons between species are underway (Wilder and Gelhaus, 2009). While it is premature to draw conclusions about the relative strengths and weaknesses of each of these approaches, further studies will no doubt provide data that informs the value of different non-human primate models for both pathogenesis and immunological studies.

IN VIVO IMMUNE RESPONSES TO *FRANCISELLA NOVICIDA* AND MUTANTS BASED ON *F. NOVICIDA*

Although a comprehensive discussion of the relative virtues of conducting experimental studies using *F. tularensis* strains versus *F. novicida* strains is outside of the topic of this review, some general comments regarding immunological responses are pertinent here. For many years, only *F. novicida* was amenable to genetic manipulation; efforts to transform *F. tularensis* strains with transposons or develop mutants via allelic exchange using traditional techniques either failed, or were of such low efficiency as to be impractical. Following recent concerted efforts to develop better genetic tools for manipulation of *F. tularensis*, this situation has changed greatly. A number of transposon banks and deletion mutants of *F. tularensis* Type A, Type B, and LVS strains have been developed in the last ~5 years. Nonetheless, manipulation of *F. novicida* remains considerably easier; further, this strain is exempt from Select Agent registration in the U.S., and generally used under BSL-2 laboratory conditions. Thus *F. novicida* remains popular for genetic studies, and mutants have frequently been used for *in vivo* infection studies, particularly those seeking virulence factors. However, when reading this literature, it is important to note that *F. novicida*'s name has been written as both *F. novicida* and later *F. tularensis* subsp. *novicida*, and the appropriate current designation remains controversial (Busse et al., 2010; Huber et al., 2010; Johansson et al., 2010).

Francisella tularensis subsp. *tularensis* and *holarctica* clearly have strong genetic homologies with *F. novicida*, and findings regarding pathogenesis and cell biology made using *F. novicida* and its mutants have often been applicable to the biology of *F. tularensis*. However, there are important biological differences between *F. novicida* and *F. tularensis* strains that appear particularly problematic for immunological studies. As discussed above, the virulence of *F. novicida* in both humans and various animal models is quite distinct from that of the *F. tularensis* subsp. In mice given *F. novicida* intranasally, the cell tropism in lungs is noticeably different from that for LVS and SchuS4. The latter two strains preferentially infect alveolar macrophages and later expand in macrophages, DCs, and neutrophils; in contrast, *F. novicida* starts in neutrophils and alveolar macrophages, and then expands in neutrophils while macrophages and DCs are lesser targets (Hall et al., 2008). Importantly, *F. novicida* expresses a structurally distinct chemotype of LPS that is more pro-inflammatory in mice than the dominant LPS chemotype expressed by *F. tularensis* strains (Cowley et al., 1996; Kieffer et al., 2003; Gunn and Ernst, 2007). Although not yet examined directly, the LPS bioactivity may contribute to the observed sepsis-like syndrome that follows intranasal infection of mice with *F. novicida* (Mares et al., 2008). Each chemotype of LPS also appears to play distinct roles in virulence of the respective bacteria and in contributing to protection in mouse models (Thomas et al., 2007).

Studies of immune responses to *F. novicida* in mice consistently reveal a prominent role for B cells and antibodies that is considerably more dramatic than LVS or fully virulent *Francisella* strains. The LD_{50} of *F. novicida* administered to inbred mice ID is about 10^3. The ID LD_{50} of *F. novicida* administered to B cell knockout mice is less than 5×10^1, and those that do survive vaccination are severely compromised compared to wild type mice for survival of secondary lethal IP challenge with LVS (Chou and Elkins, manuscript in preparation). Similarly, mice vaccinated IN with attenuated mutants in *iglB* or *iglC* of *F. novicida* have large amounts of both IgG_1 and IgG_{2a} serum anti-*Francisella* antibodies, which adoptively transfer protection against *F. novicida* challenge to recipients in the absence of primed T cells (Pammit et al., 2006; Powell et al., 2008). For the *iglC* mutant of *F. novicida*, vaccinated mice depleted of $CD4^+$ T cells at the time of challenge with *F. novicida* survived, and thus protection depended on the presence of antibodies but not effector T cells (Powell et al., 2008). Collectively, the mechanistic data to date paints a picture that is quite distinct from studies using LVS as a vaccine or challenge with virulent *Francisella*.

SYSTEMIC VERSUS MUCOSAL IMMUNITY TO *F. TULARENSIS*

Although *Francisella* infection can be initiated via multiple routes, historically the majority of studies in the *Francisella* murine model have focused on ID or SC exposure to the pathogen. In particular, ID infection with LVS has been utilized because this route approximates the most likely method of vaccination in humans (scarification or SC inoculation), and also conveniently allows for mechanistic studies of immunity after a sublethal infection in mice. More recent studies have focused on respiratory infections, given the interest in biodefense applications. Mice are much more susceptible to *Francisella* infections initiated via pulmonary routes as compared to the ID/SC route. For LVS, the IN LD_{50} is ~10^2–10^4 bacteria, whereas infection via the ID/SC route exhibits an LD_{50} of ~10^6–10^7 bacteria. For the more virulent Type A and Type B *F. tularensis* strains, both routes of primary infection are rapidly lethal, although protection against secondary respiratory challenge is much more difficult to achieve than protection against ID/SC challenge (Chen et al., 2003). Indeed, to date reasonable protection against virulent Type A respiratory challenge has only been achieved following mucosal (but not parenteral) LVS vaccination (Conlan et al., 2005; Wu et al., 2005; KuoLee et al., 2007). These observations recall theories of the "compartmentalization" of the mucosal immune system (Gill et al., 2010). In support of this concept, respiratory vaccination is not the only mucosal immunization route that is protective against respiratory challenge: LVS immunization via the oral route also results in survival of Type A pulmonary challenge, whereas ID/SC immunization does not (KuoLee et al., 2007). The immune mechanisms that are uniquely induced by mucosal – but not parenteral – vaccination remain to be identified. Recent data indicates that important cytokines such as IL-17A are preferentially produced in LVS-infected lungs following respiratory – but not parenteral – infection (Woolard et al., 2008; Cowley et al., 2010). Thus certain immune mediators may be of greater importance depending on the initial tissue encountered during vaccination and/or challenge; this possibility remains an interesting avenue of future investigation.

As noted above and in detail in another article in this issue, one theme that has emerged in recent years is the ability of *Francisella* to initially suppress or avoid induction of early immune responses following primary respiratory infection. Although multiple cell functions are clearly suppressed by both LVS and Type A *F. tularensis*, virulent Type A strains execute a broader range of immunosuppression that likely contributes to their increased virulence (Bosio et al., 2007). Further, some immunosuppressive functions – such

as prostaglandin-E2 (PGE2) production or reduced levels of CD14 – may be operative primarily in certain tissues such as the lung (Woolard et al., 2008). Thus, both the route of inoculation and the strain of *Francisella* can have a widely different impact on the quantity and quality of immune responses measured. Differences in these factors can often make it difficult to draw comparisons between different studies.

MEDIATORS OF INNATE IMMUNE RESPONSES

COMPLEMENT

Recent studies documenting that *F. tularensis* and *F. novicida* can survive extracellularly in whole blood *in vitro* and *in vivo* during mouse infections (Forestal et al., 2007; Yu et al., 2008) have generated new perspectives on the role of extracellular mediators of immunity during infection. *F. tularensis* is clearly resistant to the bactericidal effects of sera from a variety of species, a feature that was initially associated with cell surface carbohydrate structures described as a capsule (Hood, 1977). More recently, however, studies using *Francisella* strains with targeted mutations in LPS biosynthesis genes have demonstrated that complement resistance is critically dependant upon LPS O antigen (Thomas et al., 2007; Clay et al., 2008). Although resistant to its bactericidal effects, *Francisella* clearly binds by complement components: complement-derived opsonins and complement receptors enhance phagocytic uptake of *F. tularensis* by a variety of cell types, including human and mouse monocytes and macrophages (Clemens et al., 2005; Pierini, 2006; Schulert and Allen, 2006), and human monocyte-derived DCs (Ben Nasr et al., 2006; Ben Nasr and Klimpel, 2008). Indeed, C3 complement components – but not the lethal C5b-C9 membrane attack complex – were shown to be deposited on the cell surface of *F. tularensis* after incubation in human sera. These C3-derived opsonins enhanced phagocytic uptake by human DCs via a process that promoted intracellular survival, bacterial growth, and DC death (Ben Nasr et al., 2006; Ben Nasr and Klimpel, 2008). This strategy of resistance to complement killing, and the use of complement opsonins to gain entry into an intracellular niche that supports bacterial growth, is likely an important virulence determinant of *Francisella*. In support of this hypothesis, *Francisella* O antigen mutants are attenuated in the mouse model of infection (Thomas et al., 2007).

PATTERN-RECOGNITION RECEPTORS AND EARLY CYTOKINE PRODUCTION

Host cells express a variety of germline-encoded "pattern-recognition receptors" (PRRs), which recognize a number of evolutionarily conserved molecular patterns expressed only by pathogens. PRRs include the membrane-anchored Toll-like receptors (TLRs) and the cytosolic NOD-like receptors (NLRs). Because *Francisella* initially resides in a membrane-bound phagosome prior to escape into the cytosol, the bacterium has the potential to interact with both membrane and cytosolic PRRs. Indeed, recent evidence has shown that bacterial DNA engaged the cytoplasmic NLR sensor "absent in melanoma 2" (AIM2) within *Francisella*-infected macrophages, a process that was necessary to initiate inflammasome assembly, caspase-1 activation, and IL-1β release (Jones et al., 2010; Rathinam et al., 2010; Ulland et al., 2010). Since the role of the inflammasome in *Francisella* infection is reviewed in detail in another article in this issue, here we focus our discussion on the current knowledge of the contribution of TLRs to the initiation of *in vivo* host responses to *Francisella*.

Current *in vitro* and *in vivo* evidence indicates that TLR2 and MyD88 are critical mediators of inflammatory responses to *Francisella*. *In vitro* studies have demonstrated that TLR2 is required for murine DCs to activate NF-KB, produce TNF-α, and up-regulate maturation markers in response to LVS (Katz et al., 2006). Similarly, TLR2 was required for induction of a range of pro-inflammatory cytokine genes (e.g., TNF-α, IL-1β, KC, p40, RANTES, IFN-γ, IL-6, IFN-β, MCP-1, and iNOS) by peritoneal macrophages in response to LVS (Cole et al., 2007; Abplanalp et al., 2009). Full signaling via TLR2 in LVS-infected macrophages required the adaptor molecules MyD88 and TIRAP (Cole et al., 2010). Consistent with the membrane-bound nature of TLR2, LVS co-localized intracellularly within a TLR2/MyD88-containing phagosome (Cole et al., 2007). Interestingly, an LVS mutant that cannot escape the phagosome induced greatly increased expression of TLR2-dependent pro-inflammatory genes (such as TNF-α), and decreased expression of genes that rely on cytosolic recognition of bacteria (such as IFN-β; Cole et al., 2008). These data underscore the existence of the two separate arms of pathogen detection and early cytokine responses – those cytokines whose production is initiated solely via TLR signaling (TNF-α, IL-6), and those cytokines that require both TLR and NLR signaling (IFN-β, IL-1β).

In vitro data are supported by numerous *in vivo* studies that demonstrate increased susceptibility of TLR2 and MyD88 KO mice to LVS infection (Collazo et al., 2006; Malik et al., 2006; Abplanalp et al., 2009). Intranasal LVS infection of TLR2 KO mice resulted in increased mortality and decreased survival times as compared to their WT counterparts, accompanied by higher bacterial organ burdens and lower levels of TNF-α and IL-6 in the lungs (Malik et al., 2006; Abplanalp et al., 2009). Although TLR2 KO mice were significantly more susceptible to LVS infection than wild type mice via both the IN and ID routes, mice were consistently able to survive lower doses of LVS. This was in stark contrast to MyD88 KO mice, which were exquisitely susceptible to even the smallest doses of LVS delivered via both routes. This indicates a role for MyD88 in LVS infection that extends beyond its function as an adaptor for TLR2 signaling (Collazo et al., 2006; Abplanalp et al., 2009). Other MyD88-dependent molecules that have been tested in the LVS infection model include IL-18, IL-1R, TLR4, TLR1, TLR6, and TLR9 (for summary, see **Table 2**). Although most have not been exhaustively studied, mice singly deficient for these molecules did not exhibit notably increased susceptibility to LVS infection as compared to their WT counterparts (Collazo et al., 2006; Abplanalp et al., 2009). Similarly, TLR2/9 double KO mice readily survived doses of up to 10^5 LVS given ID (Chou and Elkins, manuscript in preparation). Further studies in mice deficient in multiple MyD88-dependent receptors would be needed to rule out the possibility of redundancy and/or compensatory effects.

Investigations of the bacterial ligands responsible for TLR recognition of *Francisella* indicate that LVS expresses lipoproteins that can activate HeLa cells transfected with either TLR2/TLR1 or TLR2/TLR6 heterodimers. Specifically, the lipoproteins Tul4 and FTT1103 stimulated activity via the TLR2/1 heterodimer, and induce expression of a panel of chemokines in both human peripheral blood

Table 2 | The contribution of cytokine receptors, toll-like receptors, and other effector components of immune responses to *in vivo* murine infection with *Francisella tularensis.**

Component	Effect of depletion/knockout§		Cellular source(s)‡	
	1° infection	2° infection	Innate	Acquired (T cells)
CYTOKINE RECEPTORS				
IL-4R	Slightly more susceptible at high dose (3×10^3 IN)	N/A	MØs	N/A
IL-4Rα	Less susceptible to lethal dose (10^4 IP)	N/A	N/A	N/A
IFNαR1	Less susceptible† (ID)	N/A	N/A	N/A
IL-1R	No change (ID)	Survive lethal 10^6 IP challenge	N/A	N/A
TNFR1/2	Lethal at low doses (10^2 aerosol and 20 ID)	N/A	MØs	$CD4^+$, $CD8^+$
TOLL-LIKE RECEPTOR-RELATED				
TLR2	More susceptible at high/moderate doses (4×10^3 IN and 4×10^4 ID)	Survive lethal 10^6 IP challenge	MØs, DCs	N/A
MyD88	Lethal at all doses (IN and ID)	N/A	MØs, DCs	N/A
TLR4	No change (IN and ID)	Survive lethal 10^6 IP challenge	MØs, DCs	N/A
TLR9	No change (ID)	Survive lethal 10^6 IP challenge	N/A	N/A
TLR6	No change (IN)	N/A	MØs, DCs	N/A
TLR1	No change (IN)	N/A	N/A	N/A
OTHER				
STAT1	Lethal at high dose† (10^3 aerosol)	N/A	MØs	N/A
iNOS	Lethal at all doses (ID and aerosol)	N/A	MØs	N/A
$p47^{phox}$	Sublethal at low doses; lethal at moderate doses (LD_{50} 4×10^3 ID)	N/A	MØs	N/A
MMP-9	Less susceptible (IN)	N/A	N/A	N/A

**Data presented are for LVS unless otherwise noted.*
†Data for F. novicida infection.
§Data presented describe changes in organ CFUs or survival differences as compared to WT mice. All bacterial doses are expressed in terms of CFU.
‡Only the cell types for which there is direct or indirect evidence of expression during Francisella infection (in vitro or in vivo) are listed.
N/A, data not available; ID, intradermal; IN, intranasal; IP, intraperitoneal; MØs, macrophages; DCs, dendritic cells.
For references, see corresponding text.

mononuclear cell (PBMC) and mouse bone marrow-derived DCs (Thakran et al., 2008). A role for TLR6 in recognition of LVS was similarly demonstrated by the inability of bone marrow-derived DCs harvested from TLR6 KO mice to produce TNF-α in response to LVS (Katz et al., 2006). Conversely, in other studies, macrophages from TLR6 KO mice infected with LVS expressed higher levels of TNF-α, IL-6, and MCP-1 than their WT counterparts, and, as mentioned above, TLR6 KO mice exhibit no increased susceptibility to LVS infection (Abplanalp et al., 2009). Thus, it is possible that in some circumstances TLR6 and TLR1 are redundant in their abilities to recognize LVS ligands in concert with TLR2.

Bacterial LPS is usually one of the first Gram negative pathogen-associated molecular patterns to be detected by the immune system, specifically targeted by the PRR TLR4. However, studies of *Francisella* LPS over the years have found it to be biologically inactive, unable to induce production of pro-inflammatory cytokines from all cell types tested. Subsequent studies have shown that *Francisella* LPS is not recognized by either human or murine TLR4 or TLR2, and further cannot act as an antagonist to block binding of *Salmonella* LPS to TLR4 (Duenas et al., 2006; Hajjar et al., 2006). Elucidation of the structure of *Francisella* LPS demonstrated that it is uniquely tetra-acylated and monophosphorylated, both structural properties that can reduce the endotoxicity of LPS (Gunn and Ernst, 2007). Indeed, the only biological activity ascribed to *F. tularensis* LPS (Ft-LPS) thus far is its ability to bind and stimulate rapid antibody production by the "innate" B lymphocyte subset B1a cells, and this function was clearly TLR4-independent (Cole et al., 2009); see below. Although *Francisella* LPS clearly has little if any TLR4-dependent stimulatory activities (Duenas et al., 2006), one recent study revealed that the *F. tularensis* heat shock protein DnaK can initiate cytokine production by DCs through TLR4 *in vitro* (Ashtekar et al., 2008). Despite the potential for *Francisella* to stimulate TLR4 through non-LPS ligands, TLR4-deficient mice do not exhibit increased susceptibility to *Francisella* infection, indicating that this TLR is not critical for *in vivo* defense (Chen et al., 2004, 2005b; Collazo et al., 2006).

More recently, the downstream effects of TLR2/MyD88 signaling have been studied in more detail. LVS infection of bone marrow-derived macrophages (BMDMs) induced TLR2-dependent splicing of mRNA for XBP-1, a transcription factor necessary for production of TNF-α and IL-6 mRNA. The importance of XBP-1 in resistance to LVS infection was confirmed by the presence of higher levels of

LVS in the organs of XBP-1-deficient mice as compared to WT mice after aerosol infection (Martinon et al., 2010). In a different study, *F. novicida* infection of human PBMCs and murine macrophages resulted in up-regulation of the micro-RNA miR-155 in a manner that was dependent on TLR2 and MyD88. This micro-RNA blocks translation of SHIP, a negative regulator of the PI3K/Akt pathway (Cremer et al., 2009). The net effect of the release of Akt from inhibition was an increase in pro-inflammatory gene expression. Indeed, mice that express a constitutively active form of Akt survive an otherwise lethal *F. novicida* infection (Rajaram et al., 2009). Interestingly, experiments using the virulent Type A *F. tularensis* SchuS4 indicated that SchuS4 had the opposite effect on miR-155 induction, namely down-regulating the pro-inflammatory cytokine response. These results thus reveal one mechanism by which virulent *Francisella* may inhibit early immune responses (Cremer et al., 2009). These data are also in agreement with Affymetrix microarray and Western blot analyses of human PBMCs that indicated down-regulation of the PI3K/Akt pathway and TLR2 after SchuS4, but not *F. novicida*, infection (Butchar et al., 2008a; Melillo et al., 2010).

CYTOKINES AND CHEMOKINES IN PRIMARY INFECTION WITH *FRANCISELLA*

Cytokine production during *Francisella* infection is a very active area of research, and our understanding of the important cytokine and chemokine mediators continues to evolve (for an overview, refer to **Figure 1**). Similar to other intracellular pathogens, rapid production of pro-inflammatory and Th1-type cytokines is critical for initial control of *Francisella* infection in all settings examined to date. However, as noted earlier, there is a striking lack of crucial pro-inflammatory cytokines during the first 48 h of murine pulmonary *Francisella* infection. It is not until after the first 48–72 h of murine infection that key cytokines and chemokines become readily detectable. During virulent *F. tularensis* respiratory infection of mice, mRNA levels of essential antimicrobial cytokines such as IFN-γ and TNF-α rose in the lungs between days 2 and 4 (Andersson et al., 2006b), and serum/distal organ levels of pro-inflammatory mediators such as RANTES, IL-6, and IL-1β became detectable on days 3–4 (Conlan et al., 2008). However, after 2 days of unrestricted bacterial growth, key organs such as the lungs and liver harbor extremely high bacterial burdens. Thus the relatively late up-regulation of antimicrobial host immune mechanisms appears to be too late to prevent death. Indeed, a number of studies support the hypothesis that augmenting production of pro-inflammatory cytokines very early during infection can be beneficial. For example, mice administered either a synthetic TLR4 agonist, the TLR3 agonist poly I:C, or recombinant IL-12 shortly before inhalation of *Francisella* exhibited diminished organ bacterial burdens, and enhanced survival (Duckett et al., 2005; Lembo et al., 2008; Pyles et al., 2010).

The widespread up-regulation of multiple cytokines and chemokines by day 3 after murine *F. novicida* pulmonary infection resembles a "cytokine storm" that is associated with severe sepsis – a condition characterized by excessive production of pro-inflammatory cytokines that culminates in capillary leakage, tissue injury, and organ failure. One mediator of severe sepsis, the nuclear DNA-binding protein HMGB-1, was strongly up-regulated and localized extracellularly in mouse lungs by day 3 after *F. novicida* intranasal infection (Mares et al., 2008). Thus damage-associated molecular pattern molecules such as HMGB-1 are shed from dying host cells, and may be responsible for lethal severe sepsis. This striking outcome was age-dependent: older mice given a pulmonary *F. novicida* infection had significantly increased survival and an associated reduction in development of hypercytokinemia and cell death (Mares et al., 2008). Whether a similar intriguing phenomenon is responsible for mortality during LVS or virulent *F. tularensis* infection *in vivo* remains to be determined, but macrophages infected *in vitro* with SchuS4 also release HMGB-1 (Mares et al., 2008).

Consistent with the ability of virulent *F. tularensis* strains to initially suppress – and then ultimately overwhelm – the murine immune response, most studies examining the role of various cytokines and chemokines in this infection model have shown that mice deficient for such key cytokines as IFN-γ, TNF-α, lymphotoxin-α, and iNOS exhibit the same extreme susceptibility to infection via the pulmonary and parenteral routes as fully immunocompetent mice (Chen et al., 2004; Zhang et al., 2008). In contrast, the lower virulence of LVS allows for the determination of a spectrum of susceptibility for the different cytokines and chemokines that contribute to immunity. Thus far, the only cytokine-deficient mice that are exquisitely susceptible to all doses of LVS delivered via any route are those that lack either of the canonical Th1 cytokines, IFN-γ or TNF-α (for summary, see **Table 3**; Elkins et al., 2007). Mice deficient for either cytokine usually die within a week after inoculation, suggesting that early innate immune cell production of IFN-γ and TNF-α is critical for survival. A recent study of the cell types that produce IFN-γ after primary sublethal LVS ID infection revealed that a wide variety of liver and spleen innate immune cells produce IFN-γ during the first 7 days after infection, including NK cells, neutrophils, DCs, and cells that match the staining profile of "NK DCs" (De Pascalis et al., 2008). Given that both IFN-γ and TNF-α are important for macrophage production of RNI, and that RNI are effective mediators of inhibition of LVS intramacrophage growth *in vitro*, it is likely that induction of iNOS-derived products is one critical early role for these cytokines *in vivo* (Anthony et al., 1992; Lindgren et al., 2004, 2005). Indeed, iNOS-deficient mice die following sublethal LVS infections initiated via a variety of routes (Lindgren et al., 2004), although are not as dramatically impaired as IFN-γ or TNF-α knockout mice. Although the cells that produce TNF-α in response to ID LVS infection have not yet been systematically identified, the role of TNF-α during *in vivo* primary ID LVS infection clearly includes induction of reactive nitrogen species (RNS; Cowley et al., 2008). Further, membrane TNF-α is sufficient to partially mediate resistance to primary LVS ID infection, since mice that express only the membrane-bound form of TNF-α (and not the soluble form) exhibited an intermediate level of susceptibility to LVS infection, as well as intermediate levels of RNS (Cowley et al., 2008).

In contrast to the prototypic Th1 cytokines, Th2 mediators have not been examined in much detail during *in vivo* infection. Mice treated with anti-IL-4 antibodies and then infected with LVS ID exhibited an ID LD_{50} that was comparable to wild type mice, if not higher (Leiby et al., 1992), while IL-4 knockout mice were found to be only slightly more susceptible to IN LVS pulmonary infection (Ketavarapu et al., 2008), and IL-10 knockout mice were considerably more susceptible to IN LVS pulmonary infection

Table 3 | The contribution of cytokines and chemokines to *in vivo* murine infection with *Francisella tularensis.**

Component	Effect of depletion/knockout[§]		Cellular source(s)[‡]	
	1° infection	2° infection	Innate	Acquired (T cells)
IFN-γ	Lethal at all doses (ID, IV, and IN)	Sublethal at some challenge doses (2×10^6 IV and ~10^2 IP)	MØs, DCs, NK cells, "NK DCs"	$CD4^+$, $CD8^+$, and DN
TNF-α	Lethal at all doses (ID, IV, and IN); memTNF survive only low doses (~10^2 ID)	Sublethal at lower challenge doses (9×10^3 IV); memTNF survive high lethal 5×10^5 IP challenge	MØs, DCs	$CD4^+$, $CD8^+$
IL-17A	Sublethal at moderate doses (10^4 ID and 10^2 IN); lethal at high doses (~10^3 IN and IT)	N/A	γδ T cells	$CD4^+$, $CD8^+$, and DN
IL-18	No change (ID)	Survive high lethal 10^6 IP challenge	N/A	N/A
IL-4	No change (ID)	N/A	Mast cells	N/A
IL-10	More susceptible at high dose (10^3 IN)	N/A	N/A	N/A
IL-12p35	Sublethal at moderate doses, infection cleared (~10^6 ID); lethal at high doses (~10^3 IN and IT)	Survive high lethal 5×10^6 IP challenge	MØs, DCs	N/A
IL-12p40	Sublethal at moderate doses, chronic infection (~10^6 ID); lethal at high doses (~10^3 IN and IT)	Survive high lethal 10^6 IP challenge	MØs, DCs	N/A
IL-23p19	Lethal at high doses (~10^3 IT)	N/A	N/A	N/A
IL-22	No change (IT)	N/A	N/A	N/A
Mig/CXCR3	No change (ID)	Survive high lethal IP challenge	N/A	N/A
CX3CR1	No change (IN)	N/A	N/A	N/A

**Data presented are for LVS unless otherwise noted.*
[§]Data presented are for survival (or organ CFUs) as compared to WT mice.
[‡]Only the cell types for which there is direct or indirect evidence of expression during Francisella infection (in vitro or in vivo) are listed.
N/A, data not available; IN, intranasal; IT, intratracheal; ID, intradermal; IV, intravenous; IP, intraperitoneal; MTD, mean time to death; MØs, macrophages; DCs, dendritic cells.
For references, see corresponding text.

(Metzger et al., 2007). Interestingly, as discussed in more detail below, IL-4 receptor α chain knockout mice were less susceptible to lethal IP LVS challenges (Shirey et al., 2008).

Unlike IFN-γ and TNF-α, the role for IL-12 in primary *in vivo* LVS infection is more nuanced. IL-12 is a heterodimer that consists of two distinct proteins, the p35 and p40 subunits. In addition to constituting one component of IL-12, the p40 subunit can also pair with another protein, denoted p19, to produce the IL-23 heterodimer. Thus, mice deficient for p35 lack IL-12, whereas mice deficient for p40 lack both IL-12 and IL-23. Although both p35- and p40-deficient mice can survive sublethal LVS ID infection, they are both clearly compromised. Whereas 35-deficient mice exhibit higher bacterial organ burdens and cleared the infection more slowly than WT mice, p40 KO mice were unable to clear ID LVS infection, exhibiting chronic high liver and spleen bacterial numbers (Elkins et al., 2002). Both types of LVS-infected knockout mice exhibit reduced levels of serum IFN-γ (Elkins et al., 2002), a finding that is consistent with the role of IL-12 in positive feedback regulation of IFN-γ production by T cells and NK cells. Interestingly, p40-deficient mice exhibited a greater defect in IFN-γ-production than their p35 counterparts, indicating that IL-23 may have an additional role in inducing IFN-γ production (Elkins et al., 2002). Indeed, recent studies found that IL-23 produced by *Francisella*-infected human monocytes induced NK cell production of IFN-γ, indicating that both IL-23 and IL-12 can positively regulate IFN-γ production (Butchar et al., 2007, 2008b). The unique phenotype of p40 knockout mice indicates that p40 – and by extension, IL-23 – has an as-yet unidentified role in the clearance of sublethal murine LVS ID infection. Whether a similar phenotype occurs in mice given LVS infection via other routes awaits comprehensive characterization: mice deficient for p35, p40, p19, and/or their associated receptors were more susceptible to pulmonary LVS infection, but the studies to date only used doses approaching the LD_{50} (Duckett et al., 2005; Lin et al., 2009).

IL-17A has an unexpected and critical role in primary LVS pulmonary murine infection. First detectable in mouse lungs by day 3 after infection, by days 6–7 the IL-17A-producing T cells identified in mouse lungs included $CD4^+$ T cells, $CD8^+$ T cells, double negative (DN) T cells, and γ/δ T cells (Lin et al., 2009; Cowley et al., 2010; Markel et al., 2010). The role of IL-17A in LVS pulmonary infection appears to be multi-fold; IL-17A stimulates LVS-infected DCs to up-regulate production of IL-12 and IFN-γ *in vitro*, and stimulates IFN-γ production by ovalbumin-specific transgenic T cells. Thus IL-17A appears poised to augment early IFN-γ production and aid in polarization of Th1 cells (Lin et al., 2009). However, IL-17A also has a role in the later stages of *in vivo* infection (days 10–21), when T cell-mediated immunity is critical for clearance of the infection: IL-17A knockout mice given a sublethal LVS pulmonary infection exhibit significantly increased bacterial organ burdens at these late time points (Cowley et al., 2010). Higher numbers of IFN-γ-producing $CD4^+$ and DN T cells were present in the lungs of IL-17A KO mice than their WT counterparts at these later time points, indicating that although IL-17A has a role in inducing early Th1 immunity, IFN-γ-producing T cells are capable of responding to the infection at later time points. Importantly, the discovery that IL-17A can act in concert with IFN-γ to inhibit LVS intracellular growth in macrophages and alveolar type II epithelial

cells (ATII) *in vitro* indicates that IL-17A can be a potent effector cytokine with more than just regulatory properties (Lin et al., 2009; Cowley et al., 2010).

IL-17A is perhaps best known for its ability to recruit neutrophils to the site of infection. In LVS pulmonary infection, IL-17A-deficient mice exhibited decreased levels of lung G-CSF that was accompanied by a reduction in the proportion of lung neutrophils at early time points (days 4 and 6) after infection (Lin et al., 2009; Cowley et al., 2010). Interestingly, type 1 Interferon receptor knockout mice exhibit increased resistance to ID *F. novicida* infection, a phenomenon that was associated with the ability of Type I IFNs to down-regulate the number of IL-17A$^+$ γ/δ T cells and diminished recruitment of neutrophils to infected spleens (Henry et al., 2010). A similar Type I IFN-mediated negative regulation of γ/δ T cell IL-17A production was also observed in mice infected IN with SchuS4, as well as mice given IV *L. monocytogenes* infection. Thus, low levels of IL-17A production by γ/δ T cells during *Francisella* infection may be a consequence of negative regulation effected by Type I IFNs. This outcome was at least partially attributed to the ability of type I IFNs to induce IL-27 production by *Francisella*-infected macrophages (Henry et al., 2010).

The role of chemokines in host responses to *Francisella* infection is only beginning to be elucidated. Potent mediators of cell trafficking, chemokines are responsible for drawing critical cell types to the site of infection. Indeed, multiple cell types have been shown to produce chemokines in response to *Francisella* infection, including DCs, endothelial cells, alveolar type II cells, and macrophages. Despite the abundant production of many chemokines in response to *Francisella* infection, only a few have been directly examined to date. CCR2 knockout mice, which lack responses to MCP-1/3/5 group of macrophage chemotactic proteins, are quite susceptible to ID LVS infection compared to knockout mice, and fail to exhibit increases in numbers of responding lymphoid and myeloid cells typically found in the spleens of LVS-infected wild type mice (Meierovics and Elkins, manuscript in preparation). However, mice lacking Mig or its receptor CXCR3 did not exhibit increased susceptibility to primary sublethal ID or secondary lethal IP LVS challenges (Park et al., 2002). Similarly, mice deficient for the chemokine receptor CX3CR1 were not more susceptible to IN LVS infection, although they did exhibit modest but significant dysregulation in recruitment of monocytes, neutrophils, and DCs to the lungs (Hall et al., 2009). Chemokines and/or their receptors are functionally redundant, so future studies utilizing mice that are multiply deficient for these factors will no doubt be needed to better define the critical roles of chemokines during *in vivo* *Francisella* infection.

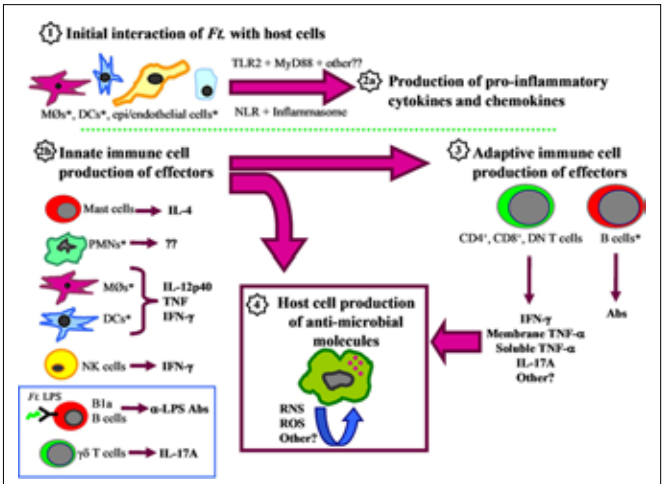

FIGURE 1 | Components of murine innate and adaptive immune responses to *Francisella*. (1) The initial interaction of *Francisella* with host cells, such as macrophages, dendritic cells, epithelial cells, and endothelial cells, stimulates production of pro-inflammatory cytokines and chemokines (2a) in a manner that is dependent upon MyD88, TLR2, and other unidentified receptors that signal through MyD88. Bacterial DNA engagement of the NOD-like receptor (NLR) AIM2 may also be critical for inflammasome assembly and release of IL-1β. Simultaneously, important innate immune cells recruited to the area of infection produce effector cytokines such as IL-12p40, TNF-α, IFN-γ, and IL-17A (2b) that influence T cell development (3), and induce host cell production of antimicrobial molecules (4). In addition to the classic TH1-type cytokines, other mediators include mast cell production of IL-4, which can directly inhibit *Francisella* intramacrophage growth, and B-1a B cell production of anti-LPS antibodies that limit intraperitoneal infection. PMNs are essential for survival of *Francisella* infections initiated via some routes, but fail to eradicate intracellular organisms *in vitro*, so their contribution to infection remains unclear. After several days, activation and expansion of *Francisella*-specific T cells and B cells occurs (3). αβ T cells are essential for clearance of primary infection, and produce effector cytokines such as IL-17A and IFN-γ, as well as the membrane-bound and soluble forms of TNF-α. These factors presumably amplify and extend activation of infected host cells to limit *Francisella* intracellular growth through production of reactive oxygen and nitrogen intermediates, as well as other unidentified antimicrobials (4). Asterisks (*) indicate host cells that have been shown to harbor intracellular *Francisella*. The blue box indicates cell types that are neither fully innate nor adaptive, based on classical definitions.

INNATE IMMUNE RESPONSES FOLLOWING INTERACTIONS OF *FRANCISELLA* WITH HOST CELLS

MACROPHAGES AND DENDRITIC CELLS

The virulence of *Francisella* has long been associated with its ability to exploit host phagocytic cells to support its own growth. In particular, the ability of macrophages and DCs from a variety of different host tissues to act as a replicative niche, as well as provide antimicrobial effector functions, has been intensively studied. Resident peritoneal macrophages from rats, mice, and guinea pigs, as well as human peripheral blood monocytes and monocyte-derived DCs, support *Francisella* growth when infected *in vitro* (Anthony et al., 1991; Ben Nasr et al., 2006; Katz et al., 2006; Chase et al., 2009; Chase and Bosio, 2010). Further, *Francisella* can grow *in vitro* in murine phagocytes harvested from a variety of different tissues, including elicited peritoneal macrophages, alveolar macrophages, BMDMs, bone marrow-derived DCs, and alveolar DCs (Anthony et al., 1991; Polsinelli et al., 1994; Bosio and Elkins, 2001; Bosio et al., 2007). In the absence of exogenous factors that induce activation, intracellular bacterial growth continues unrestricted until death of the host cell; at high multiplicities of LVS infection, this process results in caspase 3-dependent apoptosis of macrophages *in vitro* (Lai et al., 2001; Lai and Sjostedt, 2003). Although this phenomenon has not been demonstrated *in vivo* with LVS, virulent Type A *Francisella* induced widespread caspase 3-dependent apoptosis of macrophages in the organs of mice infected IN. Thus unrestricted growth of *Francisella* can induce apoptotic death of infected host cells *in vivo* as well as *in vitro* (Wickstrum et al., 2009).

Similar to many other intracellular pathogens, treatment of infected macrophages *ex vivo* with IFN-γ inhibits *Francisella* growth. In quiescent murine and human macrophages, *Francisella* survives in these cells by rapidly escaping the phagosome to replicate in the cytosol, thereby circumventing phagosome–lysosome fusion and the associated bactericidal effects. LVS phagosomal escape was partially diminished in IFN-γ-treated murine peritoneal exudate cells (PECs; Lindgren et al., 2004). Similarly, control of *F. novicida* growth by IFN-γ-activated human monocyte-derived macrophages was attributed to its inability to disrupt phagosome–lysosome fusion in the activated cells (Santic et al., 2005). These data suggest that prevention of efficient escape from the phagosome is one mechanism by which IFN-γ inhibits *Francisella* intracellular growth. However, more recent studies examining LVS and SchuS4 growth in murine BMDMs and the murine macrophage-like cell line J774.1 demonstrated that IFN-γ-activation did not disrupt phagosomal escape (Bonquist et al., 2008; Edwards et al., 2010). These discrepancies are likely to be related to the different *Francisella* subsp. and host cells used for the different studies; regardless, although disruption of phagosomal escape is one potential mechanism by which IFN-γ-activation could diminish *Francisella* intracellular growth, it is certainly not the only mechanism at work.

The generation of reactive oxygen species (ROS) and RNS are two well-known mechanisms elicited by IFN-γ that can inhibit the growth of intracellular pathogens. Several studies have focused on defining the role of these two classic mechanisms in the control of *Francisella* growth both *in vitro* and *in vivo*. Optimal IFN-γ-induced inhibition of LVS growth in murine PECs required the combined action of Phox and iNOS, and was dependent upon the generation of peroxynitrite (Lindgren et al., 2005). These data are supported by the increased susceptibility of mice deficient for either iNOS or the phagocyte oxidase $p47^{phox}$ to ID LVS infection as compared to WT mice (Lindgren et al., 2004). In contrast, killing of virulent SchuS4 by IFN-γ-activated murine PECs was only partially dependent on iNOS, and correspondingly, SchuS4 was very resistant to killing by H_2O_2 and peroxynitrite compared to LVS (Lindgren et al., 2007). Further, a study using murine $gp91^{phox}$/iNOS double-deficient BMDMs determined that control of SchuS4 growth by IFN-γ was independent of ROS and RNS, iron sequestration, and tryptophan depletion by indoleamine 2,3-dioxygenase (Edwards et al., 2010). Therefore, the IFN-γ-elicited bactericidal mechanism(s) involved in inhibition of SchuS4 growth in activated macrophages remain to be fully elucidated, while the less virulent LVS is more susceptible to the well-known bactericidal effects of ROS and RNS.

Interestingly, although LVS-infected murine macrophages initially produce pro-inflammatory cytokines *in vitro*, after several hours they begin to exhibit an "alternatively activated" phenotype – a condition characterized by mitigation of the pro-inflammatory response (including iNOS expression) and up-regulation of anti-inflammatory cytokines such as IL-4, IL-13, and TGF-β (Shirey et al., 2008). Differentiation of LVS-infected macrophages into the alternatively activated phenotype required TLR2, IL-4, and IL-13. These alternatively activated macrophages are evident in the peritoneum upon *in vivo* IP LVS infection, and mice deficient for the IL-4 receptor α chain (used by both IL-4 and IL-13 for signaling) exhibited increased survival following a normally lethal IP LVS infection. This increased resistance was associated with prolonged pro-inflammatory cytokine production and reduced expression of alternative activation markers (Shirey et al., 2008). Similarly, lethal pulmonary *F. novicida* infection resulted in the development of alternatively activated macrophages in mouse lungs (Mares et al., 2010). Alternative macrophage activation in the *F. novicida* model was exacerbated by the accumulation of dead cell debris *in vitro* and the poor ability of *F. novicida*-infected macrophages to perform efferocytosis (the uptake and clearing of dead cells; Mares et al., 2010). Thus, it appears that during lethal *Francisella* infections, alternative activation of macrophages diminishes pro-inflammatory cytokine production and the generation of macrophage effector mechanisms, and may contribute to the progression of lethal disease.

Using several different *F. tularensis* strains in the mouse model of pulmonary infection, studies have demonstrated that both DCs and macrophages in the lung were infected within 1 h after inoculation; these remained the predominant infected cell types by 24 h after infection (Bosio and Dow, 2005; Hall et al., 2008). Indeed, *Francisella* grows unrestricted within both human and mouse DCs *in vitro* until the host cells die (Ben Nasr et al., 2006; Bosio et al., 2007). *In vitro* and *in vivo* evidence indicates that *Francisella*-infected human and mouse DCs are actively immunosuppressed by the bacterium. Both are impaired in the ability to produce pro-inflammatory cytokines, and are refractory to stimulation with potent immunomodulators such as *E. coli* LPS (Bosio et al., 2007; Chase et al., 2009). This immunosuppression has been attributed to a variety of factors, including the ability of SchuS4 to increase production of the immunosuppressive cytokine TGF-β (an indicator of alternative macrophage activation, as described above), as well as the absence of CD14 expression by pulmonary DCs (Bosio et al., 2007; Chase and Bosio, 2010). IN administration of soluble CD14 to SchuS4-infected mice increased lung cytokine production, and reduced SchuS4 replication in the lungs and dissemination to the spleen (Chase and Bosio, 2010). Thus CD14 increased the capacity of DCs to "detect" SchuS4 infection, although paradoxically CD14 is not normally abundant in pulmonary tissues and thus its paucity may contribute to SchuS4 immune evasion. Interestingly, recent data has shown that DCs not only serve as a silent replicative niche for *Francisella* after respiratory infection, but they also transport the pathogen to the mediastinal lymph nodes, and therefore may play a prominent role in early pathogen dissemination (Bar-Haim et al., 2008).

NON-PHAGOCYTIC CELLS

In recent years the spectrum of cell types infected by *Francisella* has broadened to include non-phagocytic cells, such as kidney epithelial cells, ATII, hepatocytes, and fibroblasts. The contribution of non-phagocytic cells to *Francisella* virulence was revealed by a recent study using a Δ*pyrF* SchuS4 mutant. Although this mutant grew less vigorously than WT bacteria in macrophages, it exhibited close to WT levels of growth in some non-macrophage cell types, and retained full virulence in the murine model of IT infection (Horzempa et al., 2010). Although not conclusive, this study suggests that *Francisella* growth in non-macrophage cell types contributes substantially to the virulence of the organism.

Alveolar epithelial cells, which form the interface between the outside environment and the host lung interior, are well positioned to interact with *Francisella* very early during respiratory infection.

TEM micrographs showed LVS in contact with ATII cells in the airways of mice 2 h after an IN infection (Gentry et al., 2007). *In vitro* cultures of primary human ATII cells stimulated with SchuS4 and LVS produced high levels of IL-8, MCP-1, GRO-α, and GM-CSF (Gentry et al., 2007). Further, these conditioned culture supernatants induced transmigration of PMNs and DCs through cultured primary human pulmonary microvasculature endothelial cells (HVECs), a cell type that lines blood vessels and is found in close juxtaposition to ATII cells *in vivo* (Gentry et al., 2007). Studies of the direct interaction of *Francisella* with HVECs showed that LVS could be internalized – but did not replicate – in these cells, inducing a blunted pro-inflammatory response and transmigration of PMNs with a suppressed phenotype (Forestal et al., 2003; Moreland et al., 2009; Bublitz et al., 2010). It is not yet known whether PMNs that transmigrate across the endothelial layer in response to chemokines produced by *Francisella*-infected ATII cells are also functionally inhibited. Regardless, it is clear that, in addition to providing an early replicative niche for *Francisella*, ATII cells have the potential to play a vital role in initiation of inflammatory immune responses and recruitment of key immune cells.

The mechanisms exploited by *Francisella* for uptake and growth in these non-macrophage cell types – as well as the immune mechanisms that ultimately control this growth – are only beginning to be understood. Thus far it is clear that LVS uses host processes for invasion of murine ATII cells, and – similar to its growth in macrophages – escapes the initial phagosome to replicate in the cytoplasm (Craven et al., 2008). LVS infection of the human ATII cell line A549 resulted in up-regulation of the antimicrobial β-defensin molecule hBD-2, but not hBD-3, the β-defensin that had the most potent anti-*Francisella* activity in a cell-free system (Han et al., 2008). Therefore, *Francisella* avoids eliciting detrimental antimicrobial mechanisms in resting ATII cells. However, the combined action of recombinant IFN-γ and IL-17A limited LVS growth in a murine ATII cell line *in vitro* (Cowley et al., 2010), and thus ATII cells can be activated to produce antimicrobial activity against *Francisella*.

NEUTROPHILS

Another important cell type that responds early to *Francisella* infection is the neutrophil. Neutrophils are key innate immune cells that use toxic ROS, cationic peptides, and degradative enzymes to kill ingested pathogens. In particular, the multicomponent enzyme NADPH oxidase, which catalyzes the conversion of molecular oxygen to superoxide anions, is a primary antimicrobial weapon in the neutrophil arsenal. Interestingly, although LVS and SchuS4 were readily phagocytosed by human neutrophils *in vitro*, they inhibited NADPH oxidase assembly and the associated production of ROS (McCaffrey and Allen, 2006; McCaffrey et al., 2010). Further, instead of being killed by neutrophils, *Francisella* escaped from the phagosome and persisted in the neutrophil cytosol (McCaffrey and Allen, 2006). Thus, instead of killing *Francisella*, neutrophils appear to provide a safe – if not replicative – niche for the organism. Nonetheless, in mice the *in vivo* importance of neutrophils in defense against systemic *Francisella* infection is clear: neutrophil-depleted mice are highly susceptible to otherwise sublethal parenteral LVS infections, succumbing quickly to an overwhelming disseminated infection (Sjostedt et al., 1994; Elkins et al., 1996; Conlan et al., 2002a). The inability of neutrophils to kill intracellular *Francisella* suggests that their critical contribution to early survival of systemic infection may lie in their ability to secrete cytokines and chemokines that recruit other important effector cells to the site of infection.

In contrast, the effect of neutrophil depletion on pulmonary LVS infection was much less striking than that noted for parenterally infected mice, with only a minor increase in bacterial burdens in the livers when using a dose just above the LD_{50} (Conlan et al., 2002a). Regardless, neutrophils are clearly actively recruited to *Francisella*-infected lungs: recent studies show that neutrophils constitute as much as 50% of the *Francisella*-infected cells in the lungs of mice 3 days after pulmonary LVS and SchuS4 infection (Hall et al., 2008), and depend at least partially on IL-17A for their early recruitment to the lungs (Lin et al., 2009; Cowley et al., 2010). However, excessive recruitment of neutrophils to the lung appears to contribute to *Francisella* pathogenesis: mice deficient for matrix metalloproteinase 9 – which generates neutrophil chemoattractants via cleavage of the extracellular matrix – exhibited reduced neutrophil numbers in the lungs after LVS pulmonary infection, and an associated reduction in bacterial burden that was accompanied by increased survival (Malik et al., 2007). Thus, the role of neutrophils in *Francisella* infection is likely to be a fine balance between aiding in control of infection and exacerbation of pathology.

MAST CELLS

Mast cells are classically known for their involvement in allergic conditions associated with type I hypersensitivity reactions (Geha, 2003). However, recent data has revealed an unexpected and intriguing role for mast cells and IL-4 in control of respiratory *Francisella* infection. Mast cell-deficient mice were much more susceptible to pulmonary LVS infection than WT mice, exhibiting diminished production of IL-4 in the lungs (Ketavarapu et al., 2008). Further, IL-4-deficient mice were slightly more susceptible to LVS pulmonary infection. *In vitro*, mast cells inhibited LVS intramacrophage growth in a manner that was dependent on IL-4 (Ketavarapu et al., 2008). Overall, these data suggest that mast cells are capable of IL-4-dependent inhibition of LVS growth in macrophages, a phenomenon that was associated with increased macrophage cellular ATP levels and co-localization of LVS with acidified organelles (Rodriguez et al., 2010).

NK CELLS

Numerous studies have shown that NK cell-deficient mice are not more susceptible to IN or ID LVS infection than fully immunocompetent mice (Lopez et al., 2004; Duckett et al., 2005; Bokhari et al., 2008), although clear conclusions from these studies are hampered by the inability to fully deplete mice of NK cells *in vivo*. Despite this limitation, it is clear that NK cells are important producers of IFN-γ during primary LVS infection initiated via both the ID and IN routes. During the first 96 h after an IN LVS infection, a subset of $CD11b^+DX5^+NK1.1^+$ cells were primarily responsible for IFN-γ production in the lungs and livers (Lopez et al., 2004; Bokhari et al., 2008), while $NK1.1^+$ cells were a large proportion of the IFN-γ producers in the spleens and livers of mice for the first 5–7 days after an ID LVS infection (De Pascalis et al., 2008). The $NK1.1^+$ cell types that produce IFN-γ in response to LVS infection were quite heterogeneous, and could be sub-divided into populations phenotypically reminiscent of NK T cells and "NK DCs" (Bokhari et al., 2008; De Pascalis et al., 2008).

Further studies investigating the role of NK cell production of IFN-γ in *Francisella* infection have revealed some of its downstream impacts. For example, human monocyte production of IL-23 in responses to *Francisella* infection induced NK cells to produce IFN-γ; this IFN-γ subsequently up-regulated monocyte production of IL-23 and IL-12p70, establishing NK cells at the center of a positive feedback loop for IL-23 and IFN-γ production (Butchar et al., 2007, 2008b). In addition, NK cell production of IFN-γ was critical for hepatic granuloma formation during IN LVS infection; depletion of NK cells – although not lethal to the mice – resulted in "leaky" granulomas that poorly contained the infection (Bokhari et al., 2008).

Overall, although NK1.1$^+$ cells clearly respond vigorously to early *Francisella* infection, much of their contribution identified thus far relates to IFN-γ-production; the role of the lytic activities of NK cells has not been studied directly. Since NK1.1$^+$ cells are not the only innate immune cells capable of producing IFN-γ during these early critical time points (other cell types identified included DCs, PMNs, and macrophages; De Pascalis et al., 2008), it is likely that compensation by these other cells explains the dispensability of NK cells during survival of primary sublethal LVS infection. Indeed, mice depleted of NK cells during primary IN LVS infection exhibited only a 50% decrease in serum IFN-γ as compared to their WT counterparts (Bokhari et al., 2008).

ELEMENTS OF THE ADAPTIVE IMMUNE RESPONSE

Elucidation of the mechanisms involved in protection against intracellular pathogens is critical for successful vaccine development. Adaptive immune responses typically develop over a longer time frame than that of innate immunity, first requiring activation and clonal expansion of antigen-specific B and T cells. The resulting memory T and B cells respond rapidly to a second exposure to their cognate antigen, thus forming the basis of vaccine-induced immunity. For many intracellular pathogens, cellular immune responses have received the greatest attention; organisms are sequestered within cells and relatively inaccessible to antibodies, and as discussed above, serum antibody levels often do not correlate with protection. Thus T cell mechanisms including cytokine production and cytotoxicity are logical candidates for effecting pathogen eradication. However, recent data demonstrating significant extracellular phases for *Francisella in vivo* (Forestal et al., 2007; Yu et al., 2008), as well as a clear contribution of B cell-mediated functions to protection against secondary infections with *Francisella* strains of lower virulence, are leading to a new appreciation for how a combination of both B and T cell responses contribute to protective immunity against *Francisella* (for an overview, refer to **Figure 1**).

B LYMPHOCYTES AND ANTIBODIES

As discussed above, while specific antibodies are clearly made during *Francisella* infection and following vaccination, their contribution to protection may be relatively limited, at least in isolation. Murine studies have used both passive transfer of immune sera and/or purified antibodies, as well as genetically deficient mice, to elucidate the role of B cells and antibodies in resistance to *Francisella* infection. Older studies demonstrated that immune serum transfer to naive animals does not confer protection against the highly virulent Type A *Francisella* strains (Thorpe and Marcus, 1967), although transfer of anti-LVS or anti-*Francisella* LPS antibodies conferred partial protection against LVS and the less virulent Type B *Francisella* challenges (Fortier et al., 1991; Fulop et al., 1995, 2001; Conlan et al., 2002b; Stenmark et al., 2003; Kirimanjeswara et al., 2007; Lavine et al., 2007). Similarly, efforts to stimulate protective immunity in mice through vaccination with *Francisella* LPS or its protein-conjugated derivatives have only met with success following challenge with LVS or the less virulent Type B *Francisella* (Fulop et al., 1995; Conlan et al., 2002b, 2003; Kieffer et al., 2003; Cole et al., 2009).

Although administration of inactivated preparations of *Francisella* or its components have historically been unable to confer protective immunity to virulent *Francisella* challenge, recent studies have challenged this dogma; further, some of these strategies are antibody-dependent. Vaccination with inactivated *Francisella* preparations augmented by co-administration with immunostimulatory compounds can induce antibody-dependent protection against challenge with the less virulent LVS. Mice given an IN vaccination of killed LVS and IL-12 survived a subsequent lethal IN LVS challenge in an IgA-dependent manner (Baron et al., 2007). Similarly, mice administered heat-killed LVS IP alongside an IL-12 expressing viral vector survived a high dose lethal IP LVS challenge in a manner that was mediated by antibodies (Lavine et al., 2007). The ability of IL-12 administration to augment humoral immunity and protect against virulent *Francisella* challenge remains to be explored. Further, mice vaccinated several times IP with *Francisella* outer membrane proteins or ethanol-inactivated LVS were significantly protected against subsequent IN Type A *F. tularensis* challenge (~40–50% survival; Huntley et al., 2008). Intramuscular immunization of mice with killed LVS in conjunction with immunostimulatory complexes (ISCOMs) admixed with CpG protected 40% of mice against a low dose SC challenge with SchuS4 (Eyles et al., 2008). Further, intranasal immunization of mice with inactivated LVS and the mucosal adjuvant cholera toxin B subunit resulted in substantial protection against lethal IN LVS challenge, and partial protection against IN SchuS4 challenge (Bitsaktsis et al., 2009). However, in this case the presence of a mucosal adjuvant augmented Th1-type responses, such that the protection mediated against LVS challenge was fully operative in BKO mice; thus, the observed protection was not actually mediated by B cells or antibodies. Overall, it appears that antibody-mediated immune responses generated by immunization with live or killed LVS or antigenic *Francisella* preparations are, at best, partially protective against virulent strains of *Francisella*, and that T cell functions are necessary to achieve optimal protection. Interestingly, however, antisera from mice immunized IN with virulent SchuS4 and subsequently rescued by levofloxacin treatment was able to protect ~90% of naive mice against a lethal SchuS4 IN challenge (Klimpel et al., 2008).

A recent study reveals an interesting mechanism by which virulent *Francisella* may evade the protective effects of anti-*Francisella* antibodies. The highly virulent Type A strain SchuS4 – but not LVS – can directly bind plasmin, a host serine protease that degrades opsonizing antibodies, thus inhibiting antibody-mediated uptake of SchuS4 by macrophages (Crane et al., 2009). Importantly, antibody-opsonized SchuS4 elicited increased production of TNF-α and IL-6 from macrophages, an effect that was reduced by the addition of plasmin. Thus, the ability of SchuS4 to bind plasmin likely contributes to

its capacity to evade the host antibodies response. Further, the *in vivo* protective effects provided by adoptively transferred immune serum against IN LVS challenge were dependent upon the Fcγ receptor and alveolar macrophages, indicating a role for opsonophagocytosis in antibody-mediated protection to LVS (Kirimanjeswara et al., 2007). Control of LVS growth by IFN-γ-treated alveolar macrophages was significantly greater when the bacteria had been serum opsonized prior to uptake (Kirimanjeswara et al., 2007). Since LVS does not bind plasmin, it is subject to opsonophagocytosis by macrophages that are stimulated to increase their production of pro-inflammatory mediators and control intracellular growth more potently. These phenomena may at least partially explain the aforementioned ability of adoptively transferred sera to protect against LVS, but not SchuS4, challenges.

A novel role for "innate immune B cells" in immunity to *Francisella* has emerged in the last several years. Called B-1 lymphocytes, these cells are primarily located in the pleural cavity, intestinal mucosa, and the spleen of mice, and rapidly produce antibodies against T-independent antigens. In the *Francisella* murine infection model, these cells mediate a very rapid antibody response within 2–3 days of LVS or Ft-LPS vaccination. The B-1a B cell response and resulting antibody secretion provides substantial protection against moderate lethal LVS challenges administered IP, and was not dependent on the presence of T cells (Dreisbach et al., 2000; Cole et al., 2009). In the Ft-LPS-vaccinated mice, B1a cells proliferated in the spleen, and differentiated into plasma cells that produced Ft-LPS-specific antibodies that were detectable in the sera by days 3–4 after immunization. This protection did not depend on TLR4 and was not elicited by LVS lipid A, indicating that the epitope recognized by B1a cells is LVS O antigen. Whether these rapid B1a protective responses provide immunity to *Francisella* challenge via other routes of infection remains to be determined. Nonetheless, the discovery that the resultant anti-Ft-LPS antibodies were detectable at low levels in the serum for months after immunization suggests that they have the capacity to contribute to longer-term immune responses, in addition to rapid immunity.

It is important to note that the contributions of B cells to primary and secondary *Francisella* infections may be quite different. B cell-deficient mice (BKO) given a primary ID LVS infection controlled bacterial growth with kinetics similar to WT mice with a minimal impact on overall susceptibility, and similarly, BKO mice administered a primary low dose aerosol LVS infection also readily survived (Elkins et al., 1999; Chen et al., 2004). In contrast, LVS-vaccinated BKO mice were significantly more susceptible than their WT counterparts to IP LVS secondary challenge, in a manner that could be readily rescued by transfer of immune splenic B cells but not immune sera (Elkins et al., 1999). Thus, in addition to revealing a more significant role for B cells in secondary as compared to primary infection, these results further suggest that part but not all of the contribution of B cells to protective immunity during secondary LVS challenge is antibody-mediated. The role of other functions of B cells, such as production of cytokines and chemokines and antigen presentation, remains to be fully explored. Interestingly, recent evidence indicates that *Francisella* can grow inside B cells, ultimately inducing apoptosis of the infected cells (Krocova et al., 2008). Thus the potential for *Francisella* to subvert B cell functions from within remains an interesting avenue for future research.

T LYMPHOCYTES: T CELL SUBPOPULATIONS

Although B cells and their products have significant roles in protective immunity to *Francisella*, optimal protection to *Francisella* infection clearly requires T cell-mediated immunity. Mice deficient in T cells (such as *nu/nu* or α/β TCR-deficient mice) initially control a primary sublethal parenteral LVS infection, but the mice maintain high bacterial organ burdens and ultimately succumb after approximately a month. Both $CD4^+$ and $CD8^+$ T cells are sufficient to resolve this infection, as mice depleted of either population individually clear the bacteria from the tissues. In contrast, mice simultaneously depleted of both $CD4^+$ and $CD8^+$ T cells survive – but do not clear – LVS infection, instead developing a long-term chronic infection for many months that is characterized by steady levels of bacteria in the organs of the reticuloendothelial system. Control of LVS infection in these depleted mice has been attributed to an unusual $CD4^-CD8^-NK1.1^-TCR\alpha\beta^+Thy1.2^+$ "DN" T cell population. Enriched populations of these cells potently control LVS intramacrophage growth *in vitro* (Cowley et al., 2005). Thus, a variety of T cells contribute to control of primary parenteral LVS infection in mice, although only $CD4^+$ and $CD8^+$ αβ T cells have the ability to fully resolve the infection.

The role of T cells in the control and clearance of murine primary sublethal respiratory LVS infection is considerably less well studied than parenteral infection. Mice simultaneously depleted of $CD4^+$ and $CD8^+$ T cells and infected with LVS IN also develop a long-term chronic infection, indicating that one or both of these T cell subsets are necessary for clearance of pulmonary infections (Cowley et al., 2010). However, the requirement for the individual T cell subsets, or αβ TCR^+ T cells as a group, has not been systematically studied in pulmonary LVS infection. Since fully immunocompetent mice die following both parenteral and respiratory primary infections with virulent *Francisella*, it is not surprising that infected mice deficient in T cells die at the same time (Chen et al., 2004; Wu et al., 2005). Interestingly, lethal murine pulmonary infection with virulent type A *F. tularensis* has been shown to result in thymic atrophy and loss of $CD4^+CD8^+$ thymocytes (Chen et al., 2005a), whereas mice given a lethal pulmonary *F. novicida* infection exhibited a profound depletion of αβ T cells in their lungs that was associated with apoptosis (Sharma et al., 2009). Overall, these data suggest that primary lethal pulmonary *Francisella* infections share the ability to undermine the development and/or function of T cell-mediated immune responses.

T LYMPHOCYTES: EFFECTOR MECHANISMS

Efforts to determine the T cell mechanisms that contribute to defense against *Francisella* infection have revealed a small number of key cytokines that are produced by responding T cells. $CD4^+$, $CD8^+$, and DN T cells in the lungs of mice given a primary sublethal LVS pulmonary infection produce IFN-γ and IL-17A (Woolard et al., 2008; Cowley et al., 2010). Correspondingly, mice deficient in either IFN-γ or IL-17A exhibit increased susceptibility to primary LVS pulmonary and ID infections (Lin et al., 2009; Cowley et al., 2010; Markel et al., 2010). However, since both cytokines are also produced by innate immune cells (see above), it is difficult to determine the relative importance of production of these factors by T cells versus that of other cell types during primary *in vivo* infection. Greater success on this front has been achieved through the use of

an *in vitro* co-culture system, which directly measures the ability of LVS-immune T cells to control LVS intramacrophage growth. Using this system, it is apparent that IFN-γ and TNF-α contribute to the ability of all three T cell subsets to control LVS intramacrophage growth. However, the different T cell subsets utilize these two factors differentially: LVS-immune $CD4^+$ T cells exhibited the greatest reliance on IFN-γ to control LVS growth, while LVS-immune $CD8^+$ and DN T cells displayed a greater degree of IFN-γ-independence (Cowley and Elkins, 2003). In contrast, LVS-immune $CD4^+$ T cells did not require TNF-α to control LVS intramacrophage growth, while $CD8^+$ T cells were almost completely reliant on TNF-α production (Cowley et al., 2007). Further, LVS-immune $CD8^+$ T cells were capable of utilizing the membrane-bound form of TNF-α, in the absence of soluble TNF-α, to execute full control of LVS growth *in vitro* (Cowley et al., 2007).

Secondary immune responses to LVS vaccination in mice have typically been studied by administering either IP, IV, or pulmonary *Francisella* challenges at doses that are lethal for a naive mouse. For secondary LVS IP challenges, all three αβ T cell subsets – $CD4^+$, $CD8^+$, and DN T cells – are sufficient individually for survival and clearance of an LVS infection. However, mice with only DN T cells (i.e., simultaneously depleted of both $CD4^+$ and $CD8^+$ T cells) eradicated the infection more slowly. In contrast, full resistance to virulent *F. tularensis* pulmonary secondary challenges of vaccinated mice requires both $CD4^+$ and $CD8^+$ T cells, as depletion of either T cell subset significantly reduces survival (Conlan et al., 2005; Wu et al., 2005; Bakshi et al., 2008).

Elucidation of immune T cell effector mechanisms that promote survival of secondary challenges remains an ongoing area of active investigation. Such information is likely to be critical for derivation of protective correlates of immunity. Mice administered neutralizing anti-TNF-α antibodies during lethal IV LVS secondary challenge were highly susceptible to the infection, exhibiting bacterial burdens that exceeded those of mice similarly treated with anti-IFN-γ antibodies (Sjostedt et al., 1996). However, mice genetically engineered to produce only the membrane-bound form of TNF-α (and not the soluble form), were resistant to a high lethal secondary IP LVS challenge and generated similar numbers of CD44hi-responding T cells in their spleens. These data suggest that membrane TNF-α is sufficient for the generation of activated responding memory T cells (Cowley et al., 2007). In contrast, mice treated with anti-IFN-γ Abs at the time of secondary IP LVS challenges were only able to survive the lowest challenge doses, and T cells harvested from vaccinated IFN-γ-deficient mice failed to control LVS intramacrophage growth *in vitro*, indicating that IFN-γ is required for successful priming of LVS-immune T cells (Elkins et al., 2010). In addition to clear roles for IFN-γ and TNF-α, one recent study found that vaccine efficacy against SchuS4 challenge best correlated with pulmonary levels of IL-17 (Shen et al., 2010). Further, antibody neutralization of IL-17A during *in vivo* secondary challenge slightly enhanced SchuS4 growth in mouse lungs, indicating that IL-17A may participate in vaccine-induced immunity to *Francisella* (Shen et al., 2010).

Classic cytotoxic T cell mechanisms do not appear to play a significant role in survival of primary or secondary LVS infections in mice, and similarly do not contribute to the ability of LVS-immune murine T cells to control LVS intramacrophage growth *in vitro* (Cowley, manuscript in preparation). However, the recent finding that *Francisella* is susceptible to killing by granulysin, a lytic antimicrobial peptide component of human – but not mouse – cytolytic granules, suggests that granule cytotoxicity may be an effective part of the human T and NK cell arsenal that cannot be assessed in the mouse model (Endsley et al., 2009).

The study of T cell responses during *Francisella* infection has been hampered by a paucity of information on the precise epitopes recognized by antigen-specific T cells, and a corresponding lack of tools such as tetramers to probe T cell biology. Efforts to identify protein epitopes recognized by T cells during *Francisella* infection have only recently begun to come to fruition. To date, T cell hybridomas, bioinformatics, proteomics, and "immunoinformatics" have been applied to identify several *Francisella* T cell epitopes in both mice and humans (McMurry et al., 2007; Valentino and Frelinger, 2009; Valentino et al., 2009; Yu et al., 2010). One murine $CD4^+$ T cell epitope has been identified as amino acids 86–99 of the lipoprotein Tul4 (Valentino et al., 2009). Constituting as much as 20% of the responding splenic $CD4^+$ T cells at the height of an ID LVS infection, this epitope appears to be immunodominant in C57Bl/6 mice (Valentino et al., 2009). Other murine studies have identified the LVS proteins GroEl, KatG, and bacterioferritin (Bfr) as immunostimulatory during *in vitro* recall assays with splenocytes harvested from LVS-vaccinated BALB/c mice, although the exact epitopes present in these proteins have yet to be identified (Lee et al., 2006). The identification of human epitopes has been approached largely through bioinformatics analyses to select candidate promiscuous or supertype $CD4^+$ and $CD8^+$ epitopes in the SchuS4 genome; this effort yielded a large number of candidate epitopes that induced low levels of IFN-γ production by PBMCs from former tularemia patients (McMurry et al., 2007). Immunization with a pool of 13 of these epitopes provided some protective immunity against a two or five LD_{50} IT LVS challenge in HLA class II (DRB1*0401) transgenic mice (Gregory et al., 2009), but this effort utilized a difficult vaccination regimen. Further studies to identify alternate vehicles or routes of vaccination will no doubt be necessary to develop a peptide-based vaccination approach that is feasible for humans.

Despite apparently productive interactions between T cells and *Francisella*-infected macrophages *in vitro*, infected macrophages also have the capacity to directly inhibit T cell responses *in vitro*. PGE2 production by BMDMs infected with a high multiplicity of infection of LVS inhibited proliferation of T cell hybridomas specific for SIINFEKL and hen egg lysozyme antigens, skewing the T cell responses from IL-2 to IL-5 production (Woolard et al., 2007). In addition, macrophage production of a PGE2-dependent factor modulated antigen presentation by LVS-infected macrophages via ubiquitinization and degradation of macrophage MHC class II molecules (Wilson et al., 2009). Finally, *F. novicida*-infected macrophages inhibited expression of the IFN-γ receptor and diminished the ability of ovalbumin-specific T cells to produce IL-2 in response to their cognate antigen (Roth et al., 2009). The *in vivo* consequences of these phenomena remain to be fully explored, although inhibition of the PGE-2-producing enzyme cyclooxygenase in mice during LVS pulmonary infection increased the number of IFN-γ-producing T cells and decreased the bacterial burden in the lungs. Thus lung PGE2 production may have a significant immunosuppressive impact *in vivo*.

Similar to murine *Francisella* infections and as discussed above, human CD4+ and CD8+ T cell responses have been demonstrated. PBMCs from LVS vaccines and naturally infected tularemia patients produce typical Th1-type cytokines such as IL-2, IFN-γ, and TNF-α upon *ex vivo* re-stimulation with LVS or its antigens (Karttunen et al., 1991; Surcel et al., 1991; Salerno-Goncalves et al., 2009). Further, memory CD4+ and CD8+ T cells in re-stimulated PBMCs from LVS-vaccinated volunteers also produce the Th17 cytokines IL-17 and IL-22 (Paranavitana et al., 2010). One recent study further investigated the phenotype of LVS-induced recall responses in the PBMCs of tularemia patients and LVS vaccines, revealing that CD4+ and CD8+ T cell responders (as defined by IFN-γ-production and/or proliferation) largely exhibited an effector memory phenotype, and expressed cell surface markers that were indicative of cytolytic potential and the ability to home to both mucosal and non-mucosal sites (Salerno-Goncalves et al., 2009). These effector memory cells were long-lived and in some cases detected greater than 25 years after primary pneumonic tularemia.

In addition to CD4+ and CD8+ T cell responses, it is interesting to note that γ/δ T cells respond vigorously to *Francisella* infection in humans. The Vγ9/Vδ2 γδ T cell subset is known for its ability to specifically respond to pathogen-produced non-peptidic phosphoesters known collectively as "phosphoantigens." In tularemia patients, the Vγ9/Vδ2 subset expanded to constitute as much as 30.5% of all CD3+ cells in human peripheral blood within the first 7 days after the onset of symptoms (Poquet et al., 1998). These cells remained elevated for at least a month, and persisted in some individuals for as long as a year after infection (Kroca et al., 2000). Acellular extracts from a variety of *Francisella* strains, including LVS, contained phosphoantigens that activated Vγ9/Vδ2 T cell expansion and effector functions in human PBMCs. Curiously, despite the existence of activating phosphoantigens in LVS extracts, LVS vaccines do not exhibit the same Vγ9/Vδ2 T cell subset expansion as tularemia patients. Since mice do not express a γδ T cell receptor that is homologous to Vγ9/Vδ2, it has not been possible to further explore this phenomenon in the murine model of infection. To date, only a small number of studies have addressed the role of γδ T cells in murine tularemia. Mice deficient for the γδ T cell receptor did not exhibit notable increased susceptibility to primary sublethal ID or IN LVS infection, and only a small but detectable increase in susceptibility was evident upon secondary IP challenge (Yee et al., 1996; Markel et al., 2010). However, studies using the primary sublethal pulmonary infection model revealed that these cells respond in the lungs of LVS-infected mice by producing IL-17A (Lin et al., 2009; Markel et al., 2010). Thus, overall it appears that any responses provided by γδ T cells in the murine model are not essential for survival of LVS infection.

CONCLUSIONS AND PERSPECTIVES

In the last 5 years, abundant new findings have added to an already rich literature regarding *Francisella* pathogenesis and immunity. Of necessity, some of it has been more descriptive than hypothesis-driven, in reporting new genetic systems, results of genomic mining, advances in establishing and characterizing novel animal models, and in cataloging responding host cell types and mediators. But the tools developed are now poised to have payoffs not only for understanding of human infection with *Francisella per se*, but for insights into the biology of intracellular pathogens in general. As an experimental model, *Francisella* is unique in establishing productive infection in a huge variety of insects and mammals by many routes. Between this convenient biology and the plethora of experimental options now available, the field is well poised to perform detailed mechanistic studies. To do so, immunologists, long biased toward studies in mice, may need to make room in their thinking and their animal colonies for species with advantages in modeling human infection, such as rats. The *Francisella* models seem to be particularly attractive for tackling some of the current themes regarding immunity to pathogens: comparing similarities and differences in systemic or mucosal immunity; understanding the tension between pathogen-induced immunosuppression and host immunoresponsiveness; appreciating the ongoing interplay between rapid innate reactions and later specific immunity; and getting past a tendency to think of adaptive immune responses as either antibody-centric or T cell dominated, when instead both may complement, augment, and regulate each other. Collectively, we expect studies on immunity to *Francisella* to continue to be exciting, novel, and perhaps pivotal in achieving a critical public health goal: the search for predictive correlates of protective immunity to intracellular pathogens.

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The *Francisella tularensis* proteome and its recognition by antibodies

Sara L. N. Kilmury and Susan M. Twine*

Institute for Biological Sciences, National Research Council Canada, Ottawa, ON, Canada

Edited by:
Anders Sjostedt, Umeå University, Sweden

Reviewed by:
Barbara Mann, University of Virginia, USA
Jiri Stulik, University of Defence, Czech Republic

****Correspondence:***
Susan M. Twine, Institute for Biological Sciences, National Research Council Canada, 100 Sussex Drive, Ottawa, ON, Canada K1A 0R6.
e-mail: susan.twine@nrc-cnrc.gc.ca

Francisella tularensis is the causative agent of a spectrum of diseases collectively known as tularemia. The extreme virulence of the pathogen in humans, combined with the low infectious dose and the ease of dissemination by aerosol have led to concerns about its abuse as a bioweapon. Until recently, nothing was known about the virulence mechanisms and even now, there is still a relatively poor understanding of pathogen virulence. Completion of increasing numbers of *Francisella* genome sequences, combined with comparative genomics and proteomics studies, are contributing to the knowledge in this area. Tularemia may be treated with antibiotics, but there is currently no licensed vaccine. An attenuated strain, the Live Vaccine Strain (LVS) has been used to vaccinate military and at risk laboratory personnel, but safety concerns mean that it is unlikely to be licensed by the FDA for general use. Little is known about the protective immunity induced by vaccination with LVS, in humans or animal models. Immunoproteomics studies with sera from infected humans or vaccinated mouse strains, are being used in gel-based or proteome microarray approaches to give insight into the humoral immune response. In addition, these data have the potential to be exploited in the identification of new diagnostic or protective antigens, the design of next generation live vaccine strains, and the development of subunit vaccines. Herein, we briefly review the current knowledge from *Francisella* comparative proteomics studies and then focus upon the findings from immunoproteomics approaches.

Keywords: *Francisella tularensis*, LVS, immunoproteomics, proteome microarray, 2D-PAGE, antibody, vaccine, mouse vaccination

INTRODUCTION

The intracellular pathogen, *Francisella tularensis*, is the etiological agent of tularemia in humans and animals. It is increasingly being isolated in the United States and several European countries (Eliasson et al., 2006; Vorou et al., 2007), although humans are an accidental host. The genus *Francisella* contains the species *F. tularensis*, *F. novicida*, and *F. philomiragia*. *F. tularensis* is further subdivided into three subspecies: *F. tularensis* subspecies *tularensis* (type A), *F. tularensis* subspecies *holarctica* (type B), and *F. tularensis* subspecies *mediaasiatica* (Sjostedt, 2001). Strains of subspecies *tularensis* and *holarctica* are primarily responsible for human disease, whilst *F. philomiragia* and *F. novicida* are avirulent in healthy humans. *F. tularensis* subsp. *mediaasiatica* is a restricted entity with unique biochemical characteristics that has only been isolated in Kazakhstan and Turkmenistan in Central Asia and exhibits virulence in rabbits similar to Type B strains. In recent years, *F. tularensis* has gained significant attention as one of six organisms designated as high priority agents that could be exploited as agents of bioterror (category A pathogens) by the US Center for Disease Control and Prevention. Combined, the low infectious dose and ease of dissemination of type A *F. tularensis* have made it a threat to both military personnel and civilians alike (Dennis et al., 2001).

There is currently no licensed vaccine available in North America, although an attenuated type B strain, known as the Live Vaccine Strain (LVS), has been used to vaccinate military personnel and laboratory workers. This strain was derived from a Soviet strain in the 1960s and was not rationally attenuated, leading to concerns regarding residual virulence. In addition, the lack of knowledge of the attenuating mutations and mechanisms of protection means that LVS is unlikely to be licensed for use in the general population in the near future. Despite these concerns, LVS remains the gold standard against which new tularemia vaccine candidates will be judged. LVS is also currently the only tularemia vaccine candidate to have been evaluated and shown to be effective in humans, and after the terrorist attacks of 2001, there has been renewed interest in improving the manufacturing and testing of LVS (Pasetti et al., 2008). In addition, LVS remains virulent in animals, making it an attractive surrogate for virulent strains of the pathogen, which require use of specialized containment facilities.

Despite the recent surge of interest in *F. tularensis*, there remain many unknowns, for example in the mechanisms of pathogen virulence or the host immune response. The prevailing dogma is that humoral immunity plays a critical role in defense against extracellular pathogens, whilst cell-mediated immunity is more important for clearance of intracellular pathogens, such as *F. tularensis*. It is unclear whether this is true for *Francisella*, and whether the roles of the immune system are different for type A and B strains. Recent studies have confirmed the role of cell-mediated immunity in protection against tularemia, and in addition the importance of humoral immunity is also now recognized (reviewed in Kirimanjeswara et al., 2008). Many laboratories have reported that a robust anti-*Francisella* antibody response is generated in humans within 2 weeks of LVS vaccination or actual infection (Koskela and Herva, 1982; Koskela and Salminen, 1985; Tarnvik, 1989;

Waag et al., 1995; Janovska et al., 2007b), but the role of these antibodies in protective immunity remains unclear. A review of host immunity toward *F. tularensis* described the current knowledge in more depth and suggests that a synergy between humoral and cell-mediated immunity is required to induce effective protection (Kirimanjeswara et al., 2008). If either LVS is to be licensed, or next generation tularemia vaccines are to be successfully developed, there needs to be an understanding of the immune mechanisms in the host that need to be activated to induce protective immunity. This in turn requires a fundamental understanding of the mechanisms of virulence of *F. tularensis*.

For many years knowledge of *Francisella* virulence factors has been lacking, with no classical bacterial virulence factors having been identified. Despite a marked increase in the intensity of research surrounding *Francisella*, there is still not a complete explanation of how the pathogen can disseminate and proliferate so readily in the mammalian host. The completion of increasing numbers of *Francisella* genome sequences, commencing with that of strain SCHU S4 (Karlsson et al., 2000; Prior et al., 2001; Larsson et al., 2005), has propelled comparative *Francisella* genomics (Samrakandi et al., 2004; Rohmer et al., 2006, 2007; Champion et al., 2009) and proteomics studies (Hubalek et al., 2004; Twine et al., 2005a; Pavkova et al., 2006) toward identification of putative virulence genes and proteins. Genome comparisons (reviewed in Titball and Petrosino, 2007) have aided in the mapping of *Francisella* evolution and adaptation to different environmental niches (Karlsson et al., 2000; Prior et al., 2001; Larsson et al., 2005), whilst comparisons of the proteomes of *in vitro* grown strains of *Francisella* differing in virulence have revealed both similarities and differences in the profile of expressed proteins (Hubalek et al., 2004; Twine et al., 2005a; Pavkova et al., 2006). Such differences may shed light upon the molecular mechanisms of the marked virulence differences between the subspecies.

Proteomics studies have also extended to the characterization of the repertoire of proteins reactive with sera from convalescent or vaccinated subjects. The broad study of large sets of proteins involved in the host immune response has been termed "immunoproteomics," and provides information regarding *Francisella* immunodominant antigens. This is increasing knowledge of *Francisella* proteins that stimulate the immune system, which can then be used in the development of subunit vaccines and diagnostics, in addition to determining potential correlates of protection. Given the relative rarity of human cases of tularemia, many of the studies of the host response to vaccination or infection with tularemia have been carried out in animal models of tularemia. The murine model of tularemia is widely used (animal models of tularemia were recently reviewed in Rick and Wu, 2007). Animal studies in the past and more recently have used rabbits (Larson, 1946; Bell et al., 1955; Skrodzki, 1961), rats (Downs and Coriell, 1947; Downs et al., 1949; Raymond and Conlan, 2009; Wu et al., 2009; Ray et al., 2010), guinea pigs (Bell et al., 1955; Eigelsbach and Downs, 1961; Eigelsbach et al., 1961), and non-human primates (Eigelsbach et al., 1962; Nelson et al., 2010).

In this article we briefly give an overview of comparative studies of *Francisella* proteomes, approaches used to study the immunoproteome of *Francisella*, and the characteristics of the reported immunoreactive proteins.

THE *FRANCISELLA* PROTEOME

COMPARATIVE PROTEOMICS OF *FRANCISELLA*

Comparative genomics studies and molecular typing methods provide evidence that *F. novicida*, could be the common ancestor of *F. tularensis* subspecies *holarctica*, subspecies *mediaasiatica* and subspecies *tularensis* (Svensson et al., 2005). Type A strains have recently been further divided into type AI and AII, based largely on their geographical distribution (Svensson et al., 2005; Staples et al., 2006) and have been shown to differ in virulence in the mouse model of tularemia (Twine et al., 2006c; Molins et al., 2010). Genomic comparisons have allowed advances in explaining the differing virulence and infectivity of the four *F. tularensis* subspecies, but are insufficient to offer a complete understanding. Thus, proteomics studies have also been carried out, comparing the *in vitro* protein expression profiles of all four subspecies in hopes of addressing the differences in virulence that genomic studies alone, cannot (Hubalek et al., 2004; Pavkova et al., 2006).

The results of such studies have shown that very few differences exist at the proteome level between strains within the same subspecies, but a much greater number of differences were observed in proteome maps comparing the subspecies (Hubalek et al., 2004). The authors concluded that the proteomes of strains from subspecies *tularensis* and *mediaasiatica* showed greater similarity to one another than to the proteomes of strains from subspecies *holarctica* (Hubalek et al., 2004; Pavkova et al., 2006). The variations observed included differences in protein abundance, as well as the apparent presence and absence of specific proteins. Charge variants of certain proteins may account for some of the detected differences in protein abundance between subspecies, but it is also thought that the amount and method of gene expression and regulation may vary between subspecies of *F. tularensis*.

Species *novicida* and *mediaasiatica* are essentially avirulent in humans, therefore, of particular interest are proteins that are uniquely expressed, or up-regulated in both subspecies *tularensis* and *holarctica*, but not in *novicida* or *mediaasiatica*. Such differences in the proteomes of these subspecies may provide insight into the mechanisms used by virulent subspecies of *F. tularensis*. Using two-dimensional electrophoresis (2DE), Hubalek et al. (2004) compared the proteomes of representative strains of subspecies *tularensis*, *holarctica*, and *mediaasiatica* and identified 27 proteins that were either unique to, or produced in at least a twofold greater abundance in subspecies *tularensis*. These proteins are listed in **Table 1**, and the similarities and differences summarized in **Figure 1**. Seventeen of these proteins, included in **Table 1**, were found to have charge or mass variants present in the less virulent subspecies, possibly as a result of strain specific amino acid substitutions, or differences in the post-translational modification of the proteins in question (Hubalek et al., 2004). An additional nine proteins; signal recognition particle receptor FtsY (FTT_0120), ribosomal protein L10 (FTT_0142), 50S ribosomal protein L23 (FTT_0327), B-lactamase precursor (FTT_0611/0681), thymidylate synthase (FTT_1229), fructose bis-phosphate aldolase (FTT_1365), phosphoglycerate kinase (FTT_1367), transketolase I (FTT_1369), ClpB protein (FTT_1769), also shown in **Table 1**, were reported to be produced with at least twofold greater abundance in subspecies *tularensis* when compared to either *mediaasiatica* or *holarctica*. Heat shock protein ClpB has been shown to play a vital role in the multiplication

Table 1 | Proteins observed at higher levels, or only at detectable levels in subspecies *tularensis*.

Accession number	Protein name	Profile in subsp. *tularensis*	Immunoreactive in convalescent/ immune sera	Reference
FTT_0018	Secretion protein	Unique	Human	Pavkova et al. (2006)
FTT_0075	Succinate dehydrogenase iron-sulfur subunit	Charge variant	Mouse	Hubalek et al. (2004)
FTT_0120	Signal recognition particle receptor FtsY	Increased expression	No	Hubalek et al. (2004)
FTT_0142	Ribosomal protein L10	Increased expression	No	Hubalek et al. (2004)
FTT_0316	Ribosome recycling-factor	Charge variant	No	Hubalek et al. (2004)
FTT_0327	50S ribosomal protein L23	Increased expression	No	Hubalek et al. (2004)
FTT_0336	50S ribosomal protein L24	Charge variant	No	Hubalek et al. (2004)
FTT_0371	Conserved hypothetical protein	Unique	No	Hubalek et al. (2004)
FTT_0373c	Nucleoside diphosphate kinase	Charge variant		Hubalek et al. (2004)
FTT_0389	NAD-specific glutamate dehydrogenase	Increased expression	No	Pavkova et al. (2006)
FTT_0435	Putative carbon–nitrogen hydrolase	Unique	No	Hubalek et al. (2004)
FTT_0607	4-Hydroxy-3-methylbut-2-en-1-yl diphosphate synthase	Unique	No	Hubalek et al. (2004)
FTT_0611/0681	B-lactamase precursor	Increased expression	No	Hubalek et al. (2004)
FTT_0613	15.7 kDa putative exported protein	Unique	No	Hubalek et al. (2004)
FTT_0896	Phosphoribosylaminoimidazole carboxylase, catalytic subunit	Charge variant	No	Hubalek et al. (2004)
FTT_0903	Hypothetical protein	Unique	No	Pavkova et al. (2006)
FTT_1043	FKBP-type peptidyl-prolyl *cis–trans* isomerase family protein	Increased expression	Mouse	Pavkova et al. (2006)
FTT_1157c	Type IV pili lipoprotein (PilP)	Unique	No	Hubalek et al. (2004), Pavkova et al. (2006)
FTT_1181c	γ-Glutamyltranspeptidase	Charge variant	No	Hubalek et al. (2004)
FTT_1229	Thymidylate synthase	Increased expression	No	Hubalek et al. (2004)
FTT_1241	Serine hydroxymethyltransferase	Charge variant	No	Hubalek et al. (2004)
FTT_1260	Hypothetical protein	Unique	No	Pavkova et al. (2006)
FTT_1346/1701	Hypothetical protein	Increased expression	No	Pavkova et al. (2006)
FTT_1357c	Intracellular growth locus, subunit C	Charge variant	Mouse	Hubalek et al. (2004)
FTT_1365	Fructose bis-phosphate aldolase	Increased expression	No	Hubalek et al. (2004)
FTT_1367	Phosphoglycerate kinase	Increased expression	No	Hubalek et al. (2004)
FTT_1369	Transketolase I	Increased expression	Yes	Hubalek et al. (2004)
FTT_1377	3-Oxoacyl-[acyl-carrier-protein] synthase II	Charge variant	No	Hubalek et al. (2004)
FTT_1539c	Hypothetical protein FTT__1539c	Charge variant	Mouse, human	Hubalek et al. (2004)
FTT_1651	Conserved hypothetical protein	Unique	No	Pavkova et al. (2006)
FTT_1666	3-Hydroxyisobutyrate dehydrogenase	Unique	No	Pavkova et al. (2006)
FTT_1674	Riboflavin synthase, β subunit	Charge variant	No	Hubalek et al. (2004)
FTT_1769	ClpB protein	Increased expression	Human	Hubalek et al. (2004)
FTT_1794	Heat shock protein	Charge variant	No	Hubalek et al. (2004)

of *Francisella* in target organs during infection, and thus the overall virulence and infectivity of the bacteria (Meibom et al., 2008). Finally, three proteins reported in this study were only detected in 2DE of subspecies *tularensis*; FTT_0607, 4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase, FTT_0435, a carbon–nitrogen hydrolase and FTT_1157, a type IV pili lipoprotein. Type IV pilin proteins, such as PilA have been shown to play a significant role as virulence factors of *Francisella* (Forslund et al., 2006). The loss of the *pilA* gene also represents one of the major events that has led to the marked attenuation of the type B LVS strain (Salomonsson et al., 2009). While the pilA gene is present in *F. novicida*, and in pathogenic strains of subspecies *holarctica*, it does not appear that it functions as a component of a functional type IV pilin protein, but rather serves as a secretion system (Hager et al., 2006).

A small number of proteins that were expressed at detectable levels only in subspecies *tularensis*, or appear to be expressed in greater amounts in this subspecies have also been found to be immunoreactive in immune or convalescent sera, as highlighted in **Table 1**. These include the putative virulence factor IglC, which is expressed as a unique charge variant in subspecies *tularensis*, as

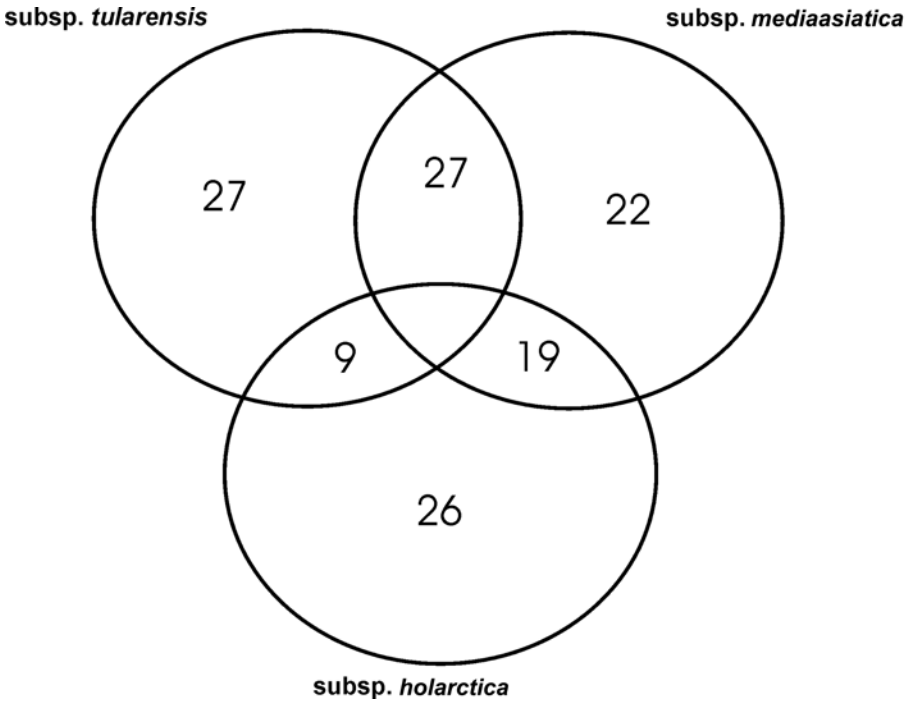

FIGURE 1 | Using 2D-PAGE coupled with mass spectrometric methods for protein identification, Hubalek et al. (2004) identified proteins that were unique to specific subspecies, or differentially expressed between them.

well as an FKBP-type peptidyl-prolyl *cis–trans* isomerase family protein (FTT_1043). Whilst it has not been conclusively shown to be true in *Francisella*, homologs of the latter protein in other intracellular pathogens such as *Legionella*, *Neisseria*, *Chlamydia*, and *Trypanosomes* play a key role in virulence and ensuring the uptake of these pathogens by host cells (Hacker et al., 1993; Ludwig et al., 1994; Moro et al., 1995; Leuzzi et al., 2005).

Also using gel-based comparative proteomics, the proteome of an attenuated, spontaneous mutant of SCHU S4, was compared with the parent strain, SCHU S4 (Twine et al., 2005a). The attenuated strain, denoted FSC043, was found to be avirulent in mice and outperformed LVS as a live vaccine strain (Twine et al., 2005a). The study determined that the strain had undergone a recombination event, resulting in the expression of a fusion protein, comprising the N-terminal half of the hypothetical protein FTT_0918 and the C-terminal half of hypothetical FTT_0919 as a single protein (Twine et al., 2005a). A similar defect, resulting in the expression of a homologous protein in LVS was also observed (Twine et al., 2005a), and has been shown to be a key cause of the attenuation of LVS in mice (Salomonsson et al., 2009). Early studies did not determine the role of the protein, FTT_0918, but show the utility of the corresponding deletion mutant as a rationally attenuated live vaccine strain in the mouse model of tularemia (Twine et al., 2005a). Since then, there have been several studies investigating the role of these proteins, which have no homology to any other proteins listed in the NCBInr. They have been proposed to be a novel family of potentially membrane associated proteins and involved in iron uptake and regulation (Lindgren et al., 2009). One study has denoted *FTT_0918* as *fupA*, and the hybrid gene in LVS as *fupA/B*, with *fupAB* having a significant role in the siderophore-mediated iron uptake mechanism of LVS (Sen et al., 2010). The functionality of the fusion protein in the attenuated SCHU S4 strain has not been determined. However, genomics studies alone would have been unable to predict the expression of the fusion protein. This information has since been exploited in tularemia vaccine development for the construction of rationally attenuated live vaccine strains (Kadzhaev et al., 2009).

In order to work toward more complete proteome coverage, many complementary approaches need to be undertaken, including subfractionation of the proteome into, for example, the membrane subproteome or glycoproteome.

THE *FRANCISELLA* MEMBRANE PROTEOME

Membrane proteins, particularly those exposed to the extracellular environment, can play important roles in the initial infection stages and the overall virulence and survival of the bacteria within a host (Haake et al., 2000). Membrane proteins have also been viewed as having the potential to act as effective subunit vaccine candidates given the frequency with which they are able to promote an immune response in a host cell (Sjostedt et al., 1992a; Huntley et al., 2007). Thus, efforts have been placed on identifying and characterizing *Francisella*'s membrane proteins (Pavkova et al., 2005, 2006; Melillo et al., 2006; Huntley et al., 2007). Early studies aimed at characterizing outer membrane proteins (OMPs) from *F. tularensis* studies carried out with LVS used bulk membrane extraction techniques, including sonication of cells followed by ultracentrifugation and/or detergent extraction. These studies did identify OMPs, and assessed their potential as subunit vaccines (Sandstrom et al., 1987; Surcel

et al., 1989; Ericsson et al., 1994a; Fulop et al., 1996), but also suffered low level contamination with periplasmic and/or cytoplasmic components (Sandstrom et al., 1987; Surcel et al., 1989; Ericsson et al., 1994a; Fulop et al., 1996). More recent studies have used sodium carbonate enrichment of membrane proteins, in which the addition of sodium carbonate preferentially causes membranes to form open membrane sheets, whilst cytoplasmic and peripheral membrane proteins are released in soluble form (Fujiki et al., 1982). Combined with gel-based proteomics approaches, two studies attempted to characterize the membrane enriched fraction of LVS (Pavkova et al., 2005; Twine et al., 2005b). Studies identified numerous candidate OMPS, although using the PSORT1b algorithm as a predictor of subcellular protein location, fewer than 10 proteins were predicted to be OMPs. One group then went further to identify immunogenic OMPs, using sera from LVS vaccinated mice. Of the 36 identified immunoreactive proteins, PSORTb analyses showed that the majority of the proteins identified in this study were more closely associated with the cytoplasm and non-membranous regions of the cell, thereby reflecting the relatively low specificity of the sodium carbonate enrichment for membrane proteins (Havlasova et al., 2005).

Using density gradient centrifugation, OMPs from LVS and SCHU S4 were purified, leading to the identification of 12 OMPs, which are listed in **Table 2** (Huntley et al., 2007). Interestingly, some of the proteins in the enriched native OMP preparation were then shown to be immunogenic with *F. tularensis* infected C3H/HeN mice, with antibodies produced toward KatG, PilQ, GroEL, ATP synthase, OmpA, FopA, and Tul4-A (Huntley et al., 2007).

Putative outer membrane or cell surface exposed proteins have been identified in *Francisella* as possibly playing a role in the adherence of the bacteria to human lung cells. Using surface biotinylation and protein purification by the use of immobilized streptavidin beads, one study identified seven putative surface exposed proteins; Chaperone protein DnaK (FTT_1269), chaperone protein GroEL (FTT_1696), hypothetical membrane protein (FTT_0119), Outer membrane associated protein FopA (FTT_0583), intracellular growth locus, subunit A (FTT_1714), conserved hypothetical lipoprotein (FTT_1347), and hypothetical protein (FTT_1441), also identified as bacterioferritin in LVS (Melillo et al., 2006). Of particular interest to this study was hypothetical membrane protein FTT_0119, also annotated as FsaP in LVS. When expressed in a non-adhering *Escherichia coli* strain, this protein enabled *E. coli* to adhere to A549 human lung epithelial cells. Furthermore, it has been shown that FsaP is present on the cell surface of *F. tularensis* subsp. *holarctica*, which readily adheres to this same cell line. However, it is absent from the cell surface of *F. novicida*, which is unable to effectively adhere to A549 cells. Combined with the observed increased expression this protein *in vivo* (Twine et al., 2006a), and the production of antibodies in response to FsaP during tularemia infection, FTT_0119 could become a point of interest in exploring the pathogenesis of *F. tularensis* (Melillo et al., 2006).

Table 2 | *Francisella tularensis* LVS outer membrane proteins, isolated using density gradient centrifugation.

Accession number	Protein name	Reference
FTT_0842	Peptidoglycan-associated lipoprotein	Huntley et al. (2007)
FTT_1095c	Hypothetical protein	Huntley et al. (2007)
FTT_1724c	Outer membrane protein tolC precursor	Huntley et al. (2007)
FTT_1156c	Type IV pilin multimeric outer membrane protein	Huntley et al. (2007)
FTT_1258	Outer membrane efflux protein	Huntley et al. (2007)
FTT_1573c	Outer membrane protein	Huntley et al. (2007)
FTT_0583	Outer membrane associated protein	Huntley et al. (2007)
FTT_1043	FKBP-type peptidyl-prolyl *cis–trans* isomerase family protein	Huntley et al. (2007)
FTT_0918	Hypothetical protein	Huntley et al. (2007)
FTT_0919	Hypothetical protein	Huntley et al. (2007)
FTL_0439	YapH-LVS	Huntley et al. (2007)
FTT_0729	ABC transporter, membrane protein	Janovska et al. (2007a)

THE *FRANCISELLA* GLYCOPROTEOME

The long prevailing dogma that bacteria cannot modify proteins with carbohydrate moieties is being met with an increasing amount of evidence to the contrary. Discovery and characterization of bacterial glycoproteins presents many challenges, including the often unknown nature of the modifying carbohydrate moiety. Many studies focus upon characterization of a single purified protein whilst others are attempting to conduct more encompassing studies of the entire bacterial glycoproteome. Preliminary work strongly suggests that *F. tularensis* strains modify multiple proteins with unknown glycan moieties, although the covalent attachment of carbohydrate moieties to specific proteins has yet to be demonstrated.

Often the first indication that a protein is modified by glycan is aberrant migration on 1D- or 2D-PAGE. A type IV pilin protein, pilA (FTT_0890) was shown using 2DE to migrate to 18 kDa; a higher molecular weight than that predicted by the *pilA* gene sequence (14 kDa), suggesting post-translational modification (Forslund et al., 2006). Additionally, *pilA* from *Francisella* was cloned into a strain of *P. aeruginosa* that is known not to glycosylate type IV pilin proteins. *Francisella* PilA was expressed, but further analysis showed that the approximate molecular weight was 14 kDa; close to the mass predicted from the translated *pilA* gene sequence. Evidence of homologous pilin protein glycosylation in other intracellular, bacterial pathogens such as *Neisseria* and some strains of *P. aeruginosa* (Stimson et al., 1995; Banerjee and Ghosh, 2003) also supports the hypothesis that this protein may be glycosylated in *Francisella*.

Another study, exploited glycoproteomics approaches developed and optimized for the detection of eukaryotic glycans. Balonova et al. (2010) reported the identification of 31 putative *Francisella* glycoproteins. Detection and enrichment of putative glycoproteins was carried out using an in-gel glycostaining kit, and a lectin based glycan differentiation. A total of 11 proteins were observed to be reactive with the gel-based glycostain, which relies upon carbohydrate diol groups, which are oxidized to aldehydes, and subsequently form a stable hydrozone in reaction to a fluorescently labeled hydrazide. Lectin affinity resulted in the enrichment and identification of 20 putative glycoproteins, although none have to date been conclusively reported as glycoproteins and the role of protein glycosylation in *Francisella* pathogenesis is unknown.

THE *FRANCISELLA IN VIVO* PROTEOME

The capacity of *F. tularensis* to cause disease appears to be a reflection of its ability to multiply intracellularly and damage various host organs rather than its ability to produce any specific toxins. This requires *F. tularensis* to subvert or otherwise avoid a variety of host defenses that possess the potential to kill it. In particular, *F. tularensis* multiplies extensively in macrophages *in vitro* and *in vivo* (Golovliov et al., 1997; Conlan et al., 2002). Studies have been carried out which attempt to mimic one or more of the stress responses to which *F. tularensis* may be exposed during proliferation within the host. Changes in the bacterial proteome in response to exposure to hydrogen peroxide, hoping to mimic the an oxidative stress response showed elevated levels of chaperonins such as GroEL, DnaK and stress response proteins such as ClpB, SodB (Ericsson et al., 1994b; Lenco et al., 2005; Twine et al., 2006a). Virulence factors, such as the pathogenicity island protein, IglC were also identified in some of these studies. Other studies have grown strains under conditions of iron restriction (Lenco et al., 2007), and manipulated growth temperatures (Lenco et al., 2009) hoping to mimic conditions to which the pathogen will be exposed within the host environment. Such *in vitro* studies offer a facile means of dissecting the response of the bacterial proteome to individual stress conditions, and some groups are working toward developing *in vitro* growth conditions that more closely mimic the host environment (Hazlett et al., 2008). Others have studied bacteria during intracellular growth in a macrophage model of tularemia and identified induced proteins (Golovliov et al., 1997; Kovarova et al., 2002). However, these studies still cannot necessarily accurately represent the myriad of stimuli to which the bacterium is exposed in the host environment. Attempting to study the proteome of bacteria growing within host tissues is extremely challenging, with limited reports of proteomes of pathogens isolated from host tissues (Becker et al., 2006; Twine et al., 2006a). An immunomagnetic separation approach was used to rapidly isolate bacteria from spleen tissues of mice infected with type A *F. tularensis* strain FSC033. In total, 78 proteins were shown to be differentially expressed when compared to the proteomes of the strain grown in liquid media. Of the proteins increased in expression some, such as IglC and chaperonins are also observed at higher levels in bacteria exposed to oxidative stress conditions. There were, however, a small number of proteins that were only observed at detectable levels in bacteria isolated from spleen tissues, including a cobalamin synthesis protein, universal stress protein and glycine cleavage system protein. These proteins are likely to be factors required for intracellular adaptation of the bacterium, rather than virulence factors. This study was the first attempt to determine the repertoire of proteins expressed by *F. tularensis* during proliferation in the hostile host environment, yet only provides a snapshot of the bacterial proteome, toward the latter stages of tularemia. Of greater interest, but also much more challenging, would be the isolation and proteome characterization of bacteria at the site of infection, during dissemination to host organs.

FRANCISELLA VIRULENCE FACTORS

Also included in comparative proteomics studies, has been the analysis of the *Francisella* pathogenicity island (FPI); a highly conserved 16–19 gene cluster found in each of the four *Francisella* subspecies. In all subspecies the FPI is present in two identical copies and has been shown to code for several putative virulence factors. Within the FPI are 16 genes that have been found to be highly conserved across each of the four subspecies, leaving an additional 2–3 genes that display greater variability. These variable genes; *pmcA* and *pdpD* in *F. novicida*, may be absent from subspecies such as *holarctica* or present but interrupted or abbreviated, as in subspecies *tularensis*. However, the levels at which the FPI gene products are produced can vary between subspecies. PigA (FTT_1701/1346), for example, is present in a two- to sixfold greater abundance in SCHU S4, a subspecies *tularensis* strain, than in other subspecies (Pavkova et al., 2006). It has also been suggested however, that virulence factors coded for by the FPI can be regulated by genes not contained in the FPI itself. MglA, for example, has been found to be a major regulator of several virulence factors encoded both within and outside of the FPI (Lauriano et al., 2004).

The *igl*ABCD operon is one of the most highly studied regions within the FPI with IglC being among the first of the FPI proteins to be elucidated. In a study by Golovliov et al. (1997), protein induction of *F. tularensis* during growth in macrophages was explored. Using protein radiolabeling coupled with 1D- and 2D-PAGE, a novel 23 kDa protein, later identified as IglC, was determined to be significantly induced during growth in macrophages and was sequenced using Edman degradation (Golovliov et al., 1997). Here, the gene products represent putative virulence factors, and their absence renders *Francisella* either avirulent or extremely attenuated (Golovliov et al., 2003b; Nix et al., 2006). Without IglA, *F. tularensis* has been shown to be entirely avirulent in a chicken embryo model, due to its inability to grow within macrophages (Nix et al., 2006). This growth has been found to be an intrinsic part of the pathogenicity of *F. tularensis* (Nix et al., 2006). Additional research has speculated that IglA associates with IglB in the cytoplasm, as they have been found to co-precipitate and insertions into the *igl*B gene results in the loss of detection of IglA as well (de Bruin et al., 2007). Such observations have led to the suggestion that the removal of either IglA or IglB may allow for the intracellular degradation of the other (de Bruin et al., 2007).

Modifications to IglC and its expression also result in a mutant that is severely hindered in its ability to grow in macrophages (Golovliov et al., 2003a). IglD too, is essential for intracellular replication in both mouse and monocyte derived macrophage studies (Santic et al., 2005). Where growth is possible, deletions of the *iglC* gene in LVS, for example, often resulted in a mutant unable to escape from phagosomes and it was therefore unable to effectively disseminate from the original site of infection (Telepnev et al., 2005). Regardless of the subspecies in which IglC was removed, mutants exhibited avirulence in the models they were tested in Golovliov et al. (2003a). It does not appear, however, that *iglC* plays a significant role in regulating protein expression during oxidative stress (Lenco et al., 2005). A Δ*iglC* LVS mutant showed very similar changes in protein expression to that of LVS when in the presence of hydrogen peroxide; namely the upregulation of approximately 10 proteins. The only significant difference between protein expression of LVS and Δ*iglC* in the presence of hydrogen peroxide was the increased expression of AhpC/TSA family protein in the Δ*iglC* mutant (Lenco et al., 2005).

The FPI itself contains several putative virulence factors, many of which may be common to all four *Francisella* subspecies, but it is not the only region of the genome that contains potential virulence factors.

The list of proteins with a potential role in pathogenesis is extensive. Despite the apparent diversity amongst these proteins though, there are several main classes into which these factors can be divided, including the FPI-encoded virulence factors that have already been discussed. Often, possible virulence factors are surface associated and may be part of a capsule, the lipopolysaccharide (LPS) or having to do with a type IV pili system. Proteins, such as the pilin subunit have been shown in *Francisella* strains, to have a role in host-cell recognition, virulence (Forslund et al., 2006, 2010), protein secretion (Hager et al., 2006), and adherence to host cells (Chakraborty et al., 2008). FTT_0918, a hypothetical protein present in both subsp. *tularensis* and in the LVS strain of *holarctica*, is thought to be membrane associated (Huntley et al., 2007). Transcriptional regulators such as MglA have also been shown to have an essential role in the virulence and pathogenesis of *Francisella* (Lauriano et al., 2004). *Francisella* also possesses large numbers of hypothetical proteins and lipoproteins, such as conserved hypothetical protein FTT_1103, that have been implicated as virulence factors (Qin et al., 2009). The virulence factors described here were elucidated through various proteomics studies and only begin to unravel *Francisella*'s mechanisms of virulence.

APPROACHES TO STUDY REACTIVITY OF *FRANCISELLA* PROTEINS WITH ANTIBODIES

Scientists have been studying the antibody response to vaccination or infection with *Francisella* for many years. This has been accomplished by methods such as agglutination (Engelfried and Spear, 1966), ELISA (Carlsson et al., 1979), and 1D-Western blotting (Bevanger et al., 1988). Early work was often unable to definitively identify the immunoreactive proteins, but more recently 2D-Western blotting combined with protein identification by mass spectrometry has been exploited. The term "immunoproteomics" has been coined to describe such studies. A newer approach, proteome microarray technology, prints recombinant proteins on glass slides to allow high throughput screening of immune sera. Each approach is outlined briefly here, including a discussion of the strengths and weaknesses of each.

WESTERN BLOTTING

The 2D-Western blotting approach, combines 2D separation of the antigen with Western blotting. The antigen used in these studies is usually a bacterial cell lysate, or subproteome fraction (e.g., membrane) of *in vitro* grown bacteria. 2D-PAGE resolves the majority of bacterial proteins to a single protein spot, and retains the native protein processing and post-translational modifications. Proteins are transferred to nitrocellulose or PVDF membrane, and probed with primary sera and conjugated secondary antibody, as per traditional Western blotting (protocol is outlined in Gallagher et al., 2008). Proteins may be stained after transfer to a membrane, and the captured image used to align regions of immunoreactivity with areas of protein staining. Excising the identified immunoreactive proteins from a second protein stained 2D-PAGE, and subsequent digestion with trypsin allows identification of proteins using mass spectrometry based techniques (e.g., MS/MS).

2D-Western blotting is one of the most accessible immunoproteomics approaches, that can be carried out in any laboratory equipped with gel-based electrophoresis and electroblotting equipment. It has, therefore, been adopted in many immunoproteomics studies. Despite its wide use, the approach has some disadvantages, which are largely related to limitations of gel-based 2D protein separations, including difficulties in resolving very large, small, hydrophobic or basic proteins. The analysis is also limited to those proteins expressed by bacteria under *in vitro* growth conditions, which may not be representative of the proteome expressed by the pathogen when proliferating in the host environment. 2D-Western blotting using bacteria isolated from host tissues has been carried out in tularemia proteomics work (Twine et al., 2006a), however the limited amount of antigen isolated makes this challenging to do with more than a few serum samples. In addition, the abundance of expressed proteins, whatever the growth conditions, can vary from a few copy numbers per cell to millions, therefore the observed intensity of immunoreactivity may not be representative of the immunogenicity of the protein, but its abundance. The relatively low throughput of 2D-Western blotting and mass spectrometry can be a bottleneck in immunoproteomics studies. Large format 2D gels offer superior proteome resolution, but the complete 2D-Western blotting experiment typically takes 4–5 days to perform. Experiments can be multiplexed, depending upon the resources of the lab, but this is still a relatively slow and laborious process. Despite the limitations of this approach, it remains by far the most accessible approach for most laboratories.

IMMUNOPROTEOMICS USING A PROTEIN MICROARRAY

The bacterial proteome microarray has been described in a number of reports, including two studies conducted with *Francisella* immune sera (Eyles et al., 2007; Sundaresh et al., 2007). The construction of the bacterial proteome microarray consists of three steps; a single-round PCR to amplify each open reading frame (ORF), followed by *in vivo* recombination cloning and *in vitro* protein expression and microarray printing (Eyles et al., 2007). Specifically, the PCR primers are designed with a gene specific portion, and an adaptor sequence. The adaptor sequences flank the amplified gene and are homologous to portions of the cloning vector (pXT7). The PCR products are cloned into the expression vector by *in vivo* homologous recombination using competent *E. coli* DH5α cells. The resulting clone also harbors an ATG start codon, polyhistidine and hemagglutinin tags. Proteins are expressed using commercially available *in vitro* transcription/translation kits and the resulting proteins printed directly onto nitrocellulose coated glass slides. This method allows the entire proteome of *F. tularensis* SCHU S4, comprising 1741 ORFs onto a single slide (Eyles et al., 2007). The chips are then treated in a manner analogous to traditional Western blotting; the chips are incubated with blocking buffer, then primary immune sera, washed, incubated with a conjugated secondary antibody, with detection of the fluorescent conjugate performed by a microarray scanner. It is reported that upwards of 800 sera could be process in a single experiment.

The proteome microarray has many advantages compared with 2D-Western blotting approach. Firstly, the antigens are presumably present at equal concentrations in contrast with gel-based approaches, where the concentration of each protein is dependent upon expression levels during *in vitro* bacterial growth. In addition, if desired the entire theoretical proteome of the organism can be interrogated. Proteome microarrays also offer multiplexing, high throughput, and reduced serum volume requirements (2 μl versus ~50–100 μl for large 2D-Western blot). However, the proteins printed on the chip likely do not harbor native post-translational modifications, and may not be post-translationally processed in the same manner as native *Francisella* proteins, a drawback common to most recombinant protein expression systems. Other disadvantages include the cost and feasibility; many labs which are equipped to perform standard proteomic experiments are not able to fabricate proteome microarrays, and analysis, at present, is limited to specialist laboratories.

IMMUNOPROTEOMICS OF *FRANCISELLA TULARENSIS*

In the following sections, we will review the immunoproteomics studies reported in the literature to date, including characteristics of the identified immunoreactive proteins and a brief comparison of the proteins identified using gel-based and proteome microarray studies.

IMMUNOPROTEOMICS IN THE MURINE MODEL OF TULAREMIA

The majority of our recent knowledge on the pathogenesis of *F. tularensis* infection has been derived from studies of mice infected with either the attenuated live vaccine strain (LVS) or virulent strains of *F. tularensis* by the intradermal (i.d.) or respiratory route (Golovliov et al., 1996; Conlan et al., 2003; Elkins et al., 2003; Wu et al., 2005) and more recently oral route (Kuolee et al., 2007). Studies have included mice of various genetic backgrounds, including immunodeficient mice (Chen et al., 2004), mouse strains deficient in Toll-like receptor 4 (TLR4) signaling (Macela et al., 1996). In the following sections, we review the current knowledge of *Francisella* immunoproteomics, carried out using the murine model of tularemia.

Successful versus unsuccessful vaccination of mice

TLR4, the signal transducing element of the LPS receptor complex, is thought to play an important role in innate immunity against Gram-negative bacteria (Underhill, 2004). TLR4-defective (TLR4d) mice (C3H/HeJ) are reported to be more susceptible to subcutaneous challenge with LVS than wild type (TLR4+/+) mice (C3H/HeN). The LD50 of the pathogen was 100-fold lower for C3H/HeJ mice compared with that for C3H/HeN mice (Macela et al., 1996). A follow-up study used gel-based immunoproteomics to compare the repertoire of immunoreactive proteins generated by C3H/HeN and C3H/HeJ mice in response to infection with LVS (Havlasova et al., 2005). The immunoreactive proteins identified in this study are summarized in **Table 3**. Of particular interest, this study monitored the immunoblotting patterns of sera drawn from mice over a 28-day period post-LVS infection, and reported that sera from infected C3H/HeJ had higher antibody titers, compared with C3H/HeN mice, up to 21 days post-infection. Despite this observation, the antibody patterns observed for each mouse strain were directed toward an almost identical subset of antigens (Havlasova et al., 2005). This study suggests, that in these mouse strains, protective immunity may not be dependent upon antibodies.

Another study exploited the differing susceptibility of four mouse strains to protective vaccination with an LVS strain derived from ATCC LVS, in order determine whether a subset of immunoreactive proteins would be predictive of protective vaccination. LVS inoculated intradermally elicits a similar sub-lethal infection in the skin, liver, and spleen of both BALB/c and C57BL/6 mice that persists for approximately 2 weeks (Chen et al., 2003). However, whereas this infection renders BALB/c and CH3/HeN mice immune to a subsequent systemic challenge with a virulent type A strain of *F. tularensis*, it fails to protect C57BL/6 mouse strains from a 100-fold smaller challenge (Chen et al., 2003). The repertoire of proteins reactive only with sera from mouse strains that were successfully vaccinated with LVS (BALB/c, CH3/HeN) were compared with immunoreactive proteins from strains that cannot be successfully vaccinated with LVS (C57BL/6, DBA). The difference in protective immunity of these mouse strains was hoped to reveal specific antigenic markers of protective immunity. The mouse strains successfully vaccinated with LVS, harbored antibodies toward a small set of proteins, that were not reactive with immune sera from vaccinated but unprotected mice, but no overall conclusion regarding patterns of protective immunoreactivity were drawn (Twine et al., 2006b; **Table 3**).

A more recent study used an almost identical approach of exploiting the varying ability of LVS vaccination to protect BALB/c and C57/BL6 mouse strains against type A challenge with LVS. This study differed in the LVS preparation, using a new lot of LVS, denoted lot 17. This LVS vaccine lot was produced in compliance with Current Good Manufacturing Practice (CGMP) guidelines and this new vaccine formulation was evaluated in a recent Phase 1 clinical study in humans (El Sahly et al., 2009). When compared to the previous work (Twine et al., 2006b), there are distinct differences in the observed immunoreactive proteins, which could potentially be attributed to the vaccinating strain of LVS. However, this study, identified nine proteins that were reactive with sera from lot 17 LVS vaccinated BALB/c mice, that were not observed to be reactive with sera from unsuccessfully vaccinated C57/BL6 mice (Twine et al., 2010). The proteins are listed in **Table 3** and in addition, **Figure 2** shows the similarities and differences in the profile of immunoreactive proteins identified in this study. The majority of the nine proteins have been observed to be reactive with sera from other murine or human tularemia studies and could have potential as markers of successful vaccination.

Vaccination of mice with killed LVS

During the 1920s and 1930s there was a significant effort to develop killed *F. tularensis* vaccines, due to the reduced intrinsic safety and liability concerns, compared with live vaccines. Studies with heat or formalin killed *Francisella* strains showed that vaccines made using these methods are generally ineffective (Foshay et al., 1942; Kadull et al., 1950). However, recent work showed that LVS killed by irradiation, and administered to mice in combination with either alum, and immunostimulating complexes (ISCOMs) or CpG afforded some protection against challenge with virulent type B or A strains (Eyles et al., 2007). In addition, the authors used

Table 3 | Proteins which are recognized by sera from convalescent humans or from LVS vaccinated mice.

Locus tag	Protein name	Gene	Reactivity sera[1]	Screening method[2]	PSORT[3]	Reference
FTT_0018	Secretion protein		Human	Proteome microarray	Cytoplasmic membrane	Sundaresh et al. (2007)
FTT_0037	NADH dehydrogenase I G subunit		Mouse	2D-Western blot	Unknown	Twine et al. (2010)
FTT_0049	N utilization substance protein A	nusA	Mouse	2D-Western blot	Cytoplasmic	Twine et al. (2006a)
FTT_0060	ATP synthase B chain	atpF	Mouse	Proteome microarray	Cytoplasmic	Eyles et al. (2007)
FTT_0062	ATP synthase alpha chain	atpA	Mouse	2D-Western blot	Unknown	Twine et al. (2006b, 2010)
FTT_0064	ATP synthase beta chain		Mouse	2D-Western blot	Cytoplasmic	Huntley et al. (2007), Twine et al. (2010)
FTT_0071	Citrate synthase	gltA	Mouse	2D-Western blot	Cytoplasmic	Havlasova et al. (2005)
FTT_0074	Succinate dehydrogenase catalytic and NAD flavoprotein subunit	shdA	Human, presumed type B; mouse	2D-Western blot	Unknown	Twine et al. (2006b), Janovska et al. (2007a)
FTT_0075	Succinate dehydrogenase iron-sulfur subunit	sdhB	Mouse	2D-Western blot	Cytoplasmic	Twine et al. (2006b)
FTT_0077	Dihydrolipoamide succinyltransferase component of 2-oxoglutarate dehydrogenase complex	sucB	Human, type A; human, presumed type B; mouse	Proteome microarray/2D-Western blot	Cytoplasmic	Twine et al. (2006b), Eyles et al. (2007), Janovska et al. (2007a,b), Sundaresh et al. (2007), Twine et al. (2010)
FTT_0083	Hypothetical membrane protein		Human, presumed type B	Proteome microarray	Unknown	Sundaresh et al. (2007)
FTT_0086	Hypothetical protein	–	Mouse	2D-Western blot	Unknown	Twine et al. (2006b)
FTT_0087	Aconitate hydratase		Human, type A; human, presumed type B; mouse	2D-Western blot	Cytoplasmic	Janovska et al. (2007a,b), Twine et al. (2010)
FTT_0101	Conserved membrane hypothetical protein	–	Human, presumed type B; mouse	Proteome microarray	Cytoplasmic membrane	Eyles et al. (2007), Sundaresh et al. (2007)
FTT_0106	Efflux protein, RND family, MFP subunit	–	Human, presumed type B; mouse	Proteome microarray	Cytoplasmic membrane	Eyles et al. (2007), Sundaresh et al. (2007)
FTT_0119	Hypothetical membrane protein	–	Human, presumed type B; mouse	2D-Western blot/ proteome microarray	Unknown	Eyles et al. (2007), Sundaresh et al. (2007), Twine et al. (2006a)
FTT_0123	Pseudogene		Human, presumed type B	Proteome microarray	Cyt mem	Sundaresh et al. (2007)
FTT_0137	Elongation factor Tu	tufA	Human, presumed type B; mouse	2D-Western blot	Cytoplasmic	Havlasova et al. (2002, 2005), Twine et al. (2006a,b, 2010), Janovska et al. (2007a)
FTT_0141	50S ribosomal protein L1	rplA	Mouse	2D-Western Blot	Unknown	Twine et al. (2006b)
FTT_0143	50S ribosomal protein L7/L12	rplL	Human, presumed type B; mouse	2D-Western blot	Unknown	Havlasova et al. (2002, 2005), Twine et al. (2006a,b), Janovska et al. (2007a)
FTT_0183c	30S ribosomal protein S1	rpsA	Mouse	2D-Western blot	Cytoplasmic	Twine et al. (2006b, 2010)
FTT_0188	Cell division protein	ftsZ	Human, presumed type B; mouse	2D-Western blot	Cytoplasmic	Twine et al. (2006b, 2010), Janovska et al. (2007a)
FTT_0189	UDP-3-*O*-[3-hydroxymyristoyl]		Mouse	2D-Western Blot	Unknown	Twine et al. (2010)
FTT_0194	Conserved hypothetical membrane protein		Human, presumed type B	Proteome microarray	Cyt mem	Sundaresh et al. (2007)
FTT_0196c	Glutamine synthetase	glnA	Mouse	Proteome microarray	Cytoplasmic	Eyles et al. (2007)
FTT_0208c	ABC transporter, ATP-binding protein		Mouse	2D-Western blot	Unknown	Twine et al. (2006b)
FTT_0209c	Periplasmic solute binding family protein	–	Mouse	2D-Western blot	Unknown	Twine et al. (2006a,b, 2010)
FTT_0233	Inner-membrane protein	yidC	Mouse	Proteome microarray	Cytoplasmic membrane	Eyles et al. (2007)

(Continued)

Table 3 | Continued

Locus tag	Protein name	Gene	Reactivity sera[1]	Screening method[2]	PSORT[3]	Reference
FTT_0280	Major facilitator superfamily (MFS) transport protein		Human, presumed type B	Proteome microarray	Cyt mem	Sundaresh et al. (2007)
FTT_0296	Pyrrolidone-carboxylate peptidase	pcp	Mouse	2D-Western blot/ proteome microarray	Unknown	Havlasova et al. (2005), Eyles et al. (2007)
FTT_0313	30S ribosomal protein S2	rpsB	Mouse	2D-Western blot	Unknown	Twine et al. (2006b)
FTT_0314	Protein chain elongation factor EF-Ts	tsf	Mouse	2D-Western blot/ proteome microarray	Cytoplasmic	Havlasova et al. (2005), Twine et al. (2006a,b), Eyles et al. (2007)
FTT_0323	Elongation factor G	fusA	Mouse	2D-Western blot	Cytoplasmic	Twine et al. (2006b, 2010)
FTT_0342	30S ribosomal protein S5	rpsE	Mouse	2D-Western blot	Unknown	Twine et al. (2006b)
FTT_0350	DNA-directed RNA polymerase	rpoA1	Mouse	2D-Western blot	Cytoplasmic	Twine et al. (2006a, 2010)
FTT_0356	Heat shock protein	htpG	Human, presumed type B; mouse	2D-Western blot	Cytoplasmic	Havlasova et al. (2005), Twine et al. (2006b), Janovska et al. (2007a)
FTT_0373c	Nucleoside diphosphate kinase	ndk	Mouse	2D-Western blot	Cytoplasmic	Havlasova et al. (2005), Twine et al. (2006b)
FTT_0385	Hypothetical protein	–	Mouse	Proteome microarray	Unknown	Eyles et al. (2007)
FTT_0394	Hypothetical protein		Human, presumed type B	2D-Western blot	Unknown	Havlasova et al. (2002)
FTT_0407	Glycine cleavage complex protein T	gcvT	Human, presumed type B; mouse	2D-Western blot	Cytoplasmic	Havlasova et al. (2002, 2005)
FTT_0448c	Glutaminyl-tRNA synthetase	glnS	Mouse	2D-Western blot	Cytoplasmic	Twine et al. (2006b)
FTT_0471	3-Dehydroquinate dehydratase	aroD	Human, presumed type B	2D-Western blot	Cytoplasmic	Havlasova et al. (2002)
FTT_0472	Acetyl-CoA carboxylase, biotin carboxyl carrier protein subunit	accB	Human, presumed type B; mouse	2D-Western blot/ proteome microarray	Unknown	Havlasova et al. (2002), Twine et al. (2006b, 2010), Eyles et al. (2007), Janovska et al. (2007a), Sundaresh et al. (2007)
FTT_0473	Acetyl-CoA carboxylase, biotin carboxylase subunit	accC	Mouse	2D-Western blot	Cytoplasmic	Havlasova et al. (2005)
FTT_0479	PerM family		Human, presumed type B	Proteome microarray	Cytoplasmic membrane	Sundaresh et al. (2007)
FTT_0503	Succinyl-CoA synthetase	sucD	Mouse	2D-Western blot	Cytoplasmic	Havlasova et al. (2005), Twine et al. (2006b)
FTT_0504c	Succinyl-CoA synthetase subunit beta		Human type A	2D-Western blot	Cytoplasmic	Janovska et al. (2007b)
FTT_0510	DNA gyrase subunit B		Mouse	2D-Western blot	Cytoplasmic	Twine et al. (2010)
FTT_0511	Pyridoxine/pyridoxal 5-phosphate biosynthesis protein	–	Mouse	2D-Western blot	Cytoplasmic	Twine et al. (2006a, 2010)
FTT_0535c	Malate dehydrogenase	mdh	Mouse	2D-Western blot	Cytoplasmic	Havlasova et al. (2005)
FTT_0557	lemA-like protein		Human, presumed type B	2D-Western blot	Unknown	Janovska et al. (2007a)
FTT_0580	Hypothetical protein		Mouse	2D-Western blot	Cytoplasmic	Twine et al. (2010)
FTT_0583	Outer membrane associated protein	fopA	Human, presumed type B; mouse	2D-Western blot/ proteome microarray	Outer membrane	Havlasova et al. (2005), Twine et al. (2006a,b, 2010), Eyles et al. (2007), Huntley et al. (2007), Janovska et al. (2007a)
FTT_0614	Apolipoprotein *N*-acyltransferase		Human, presumed type B	Proteome microarray	Cytoplasmic mem	Sundaresh et al. (2007)
FTT_0627	DNA binding protein	hupB	Human, presumed type B	2D-Western blot	Unknown	Havlasova et al. (2002), Janovska et al. (2007a)

(Continued)

Table 3 | Continued

Locus tag	Protein name	Gene	Reactivity sera[1]	Screening method[2]	PSORT[3]	Reference
FTT_0630	Host factor I for bacteriophage Q beta replication	hfq	Mouse	2D-Western blot/ proteome microarray	Cytoplasmic	Havlasova et al. (2005), Eyles et al. (2007)
FTT_0682	Hypothetical protein	–	Human, presumed type B; mouse	Proteome microarray	Periplasmic	Eyles et al. (2007), Sundaresh et al. (2007)
FTT_0708	Major facilitator superfamily (MFS) transport protein		Human, presumed type B	Proteome microarray	Cytoplasmic membrane	Sundaresh et al. (2007)
FTT_0715	Chitinase family protein – inner membrane		Mouse	2D-Western blot	Unknown	Havlasova et al. (2005), Twine et al. (2006b, 2010)
FTT_0721c	Peroxidase/catalase	katG	Human type A; human, presumed type B; mouse	2D-Western blot	Periplasmic space/outer membrane	Havlasova et al. (2005), Twine et al. (2006a,b, 2010), Huntley et al. (2007), Janovska et al. (2007a,b
FTT_0724	Part of pseudogene dacB1	–	Mouse	Proteome microarray	–	Eyles et al. (2007)
FTT_0726c	Glycerophosphoryl diester phosphodiesterase family protein		Human, presumed type B	2D-Western blot	Unknown	Janovska et al. (2007a)
FTT_0756	Cation-efflux family protein	–	Mouse	Proteome microarray	Cytoplasmic membrane	Eyles et al. (2007)
FTT_0817	Threonyl-tRNA synthetase	thrS	Mouse	2D-Western blot	Cytoplasmic	Twine et al. (2006b)
FTT_0831c	OmpA family protein	–	Human, presumed type B; mouse	Proteome microarray/2D-Western blot	Unknown	Eyles et al. (2007), Huntley et al. (2007), Janovska et al. (2007a), Twine et al. (2010)
FTT_0849	Bile acid symporter family protein		Human, presumed type B	Proteome microarray	Cytoplasmic mem	Sundaresh et al. (2007)
FTT_0863	LemA-like protein	–	Human, presumed type B; mouse	2D-Western blot/ proteome microarray	Cytoplasmic	Twine et al. (2006b, 2010), Eyles et al. (2007), Janovska et al. (2007a), Sundaresh et al. (2007)
FTT_0869	Hypothetical protein	–	Human, presumed type B; mouse	Proteome microarray	Unknown	Eyles et al. (2007), Sundaresh et al. (2007)
FTT_0901	Conserved hypothetical lipoprotein (17 kDa major membrane protein precursor)	lpnA	Human, presumed type B; mouse	2D-Western blot/ proteome microarray	Unknown	Havlasova et al. (2002), Eyles et al. (2007), Huntley et al. (2007), Janovska et al. (2007a)
FTT_0918	Hypothetical protein	–	Human type A; mouse	2D-Western blot	Outer membrane	Twine et al. (2006a, Janovska et al. (2007b)
FTT_0949	Pseudogene		Human, presumed type B	Proteome microarray	Unknown	Sundaresh et al. (2007)
FTT_0956c	Hypothetical membrane protein	–	Human, presumed type B; mouse	Proteome microarray	Unknown	Eyles et al. (2007), Sundaresh et al. (2007)
FTT_0975	Hypothetical protein	–	Human, presumed type B; mouse	2D-Western blot/ proteome microarray	Unknown	Havlasova et al. (2005), Eyles et al. (2007), Sundaresh et al. (2007)
FTT_0989	Hypothetical protein	–	Human, presumed type B	Proteome microarray	Unknown	Sundaresh et al. (2007)
FTT_0991	Hypothetical lipoprotein		Human, presumed type B	Proteome microarray	Unknown	Sundaresh et al. (2007)
FTT_1043	FKBP-type peptidyl-prolyl *cis–trans* isomerase family protein	–	Mouse	2D-Western blot	Outer Membrane	Twine et al. (2006b)
FTT_1060c	50S ribosomal protein L9	rplI	Human, presumed type B; mouse	2D-Western blot	Cytoplasmic	Havlasova et al. (2002, 2005), Twine et al. (2006b, 2010)
FTT_1097	Hypothetical protein	–	Mouse	Proteome microarray	Unknown	Eyles et al. (2007)

(Continued)

Table 3 | Continued

Locus tag	Protein name	Gene	Reactivity sera[1]	Screening method[2]	PSORT[3]	Reference
FTT_1103	Conserved hypothetical lipoprotein	–	Human, presumed type B; mouse	2D-Western blot/ proteome microarray	Unknown	Twine et al. (2006b, 2010), Eyles et al. (2007), Janovska et al. (2007a)
FTT_1115	Preprotein translocase, subunit D, membrane protein	secD	Mouse	Proteome microarray	Cytoplasmic membrane	Eyles et al. (2007)
FTT_1116	Preprotein translocase family protein	yajC	Human, presumed type B; mouse	Proteome microarray	Unknown	Eyles et al. (2007), Sundaresh et al. (2007)
FTT_1125	D-methionine binding transport protein, ABC transporter, membrane and periplasmic protein	metIQ	Mouse	Proteome microarray	Cytoplasmic membrane	Eyles et al. (2007)
FTT_1156c	Type IV pilin multimeric outer membrane		Mouse	2D-Western Blot	Outer membrane	Huntley et al. (2007), Twine et al. (2010)
FTT_1163	Hypothetical membrane protein	–	Human, presumed type B; mouse	Proteome microarray	Cytoplasmic membrane	Eyles et al. (2007), Sundaresh et al. (2007)
FTT_1201c	Oxidoreductase	–	Human, presumed type B; mouse	2D-Western blot	Cytoplasmic	Havlasova et al. (2002, 2005)
FTT_1236	Hypothetical protein		Mouse	Proteome microarray	Unknown	Eyles et al. (2007)
FTT_1239	Hypothetical membrane protein		Human, presumed type B	Proteome microarray	Cyt mem	Sundaresh et al. (2007)
FTT_1269c	Chaperone protein (heat shock protein family 70 protein)	dnaK	Human type A; human, presumed type B; mouse	2D-Western blot/ proteome microarray	Periplasmic space	Havlasova et al. (2002, 2005), Twine et al. (2006a,b, 2010), Eyles et al. (2007), Janovska et al. (2007a,b), Sundaresh et al. (2007)
FTT_1270c	Chaperone protein	grpE	Mouse	2D-Western blot	Cytoplasmic	Havlasova et al. (2005)
FTT_1271	Membrane-bound lytic murein transglycosylase A	mltA	Mouse	Proteome microarray	Unknown	Eyles et al. (2007)
FTT_1281c	Sigma 54 modulation protein	yhbH	Mouse	2D-Western blot	Cytoplasmic	Havlasova et al. (2005)
FTT_1303c	Hypothetical protein		Mouse; human, presumed type B	2D-Western blot/ proteome microarray	Unknown	Sundaresh et al. (2007), Twine et al. (2010)
FTT_1313c	Transcriptional elongation factor	greA	Mouse	2D-Western blot	Cytoplasmic	Twine et al. (2006b)
FTT_1314c	Type IV pili fiber building block protein		Human, presumed type B; mouse	Proteome microarray	Unknown	Eyles et al. (2007), Sundaresh et al. (2007)
FTT_1317c	Inosine-5′-monophosphate dehydrogenase		Human, presumed type B	2D-Western blot	Unknown	Janovska et al. (2007a)
FTT_1333	Hypothetical protein		Human, presumed type B	Proteome microarray	Unknown	Sundaresh et al. (2007)
FTT_1345	Hypothetical protein	pdpB	Mouse	2D-Western blot	Outer Membrane	Havlasova et al. (2002)
FTT_1357/1712	Intracellular growth locus, subunit C	iglC	Human, presumed type B; mouse	2D-Western blot	Unknown	Havlasova et al. (2002, 2005), Twine et al. (2006a)
FTT_1358/1713	Intracellular growth locus, subunit B	iglB	Human, type A; mouse	2D-Western blot/ proteome microarray	Unknown	Eyles et al. (2007), Twine et al. (2010)
FTT_1368c	Glyceraldehyde-3-phosphate dehydrogenase	gapA	Mouse	2D-Western blot	Cytoplasmic	Havlasova et al. (2005)
FTT_1369	Transketolase	tktA	Mouse	2D-Western blot	Unknown	
FTT_1373	3-Oxoacyl-[acyl-carrier-protein] synthase III		Mouse	2D-Western blot	Unknown	Twine et al. (2010)
FTT_1374	Malonyl CoA acyl carrier protein	fabD	Mouse	2D-Western blot	Cytoplasmic	Havlasova et al. (2005), Twine et al. (2010)
FTT_1376	Acyl carrier protein	acpP	Mouse	2D-Western blot	Cytoplasmic	Twine et al. (2006b)

(Continued)

Table 3 | Continued

Locus tag	Protein name	Gene	Reactivity sera[1]	Screening method[2]	PSORT[3]	Reference
FTT_1389	3-Methyl-2-oxobutanoate hydroxymethyl-transferase		Mouse	2D-Western blot	Unknown	Twine et al. (2010)
FTT_1390	Pantoate-beta-alanine ligase	panC	Mouse	2D-Western blot	Cytoplasmic	Twine et al. (2006b)
FTT_1402c	Hypothetical protein		Human, presumed type B	2D-Western blot	Unknown	Janovska et al. (2007a)
FTT_1406c	Hypothetical protein	–	Mouse	Proteome microarray	Unknown	Eyles et al. (2007)
FTT_1416c	Hypothetical lipoprotein	–	Mouse	Proteome microarray	Unknown	Eyles et al. (2007)
FTT_1441	Hypothetical protein	–	Human, presumed type B; mouse	2D-Western blot	Cytoplasmic	Havlasova et al. (2005), Janovska et al. (2007a), Twine et al. (2010)
FTT_1444	Exopolyphosphatase	ppx	Mouse	Proteome microarray	Cytoplasmic membrane	Eyles et al. (2007)
FTT_1460	UDP-glucose/GDP mannose dehydrogenase	wbtE	Mouse	2D-Western blot	Cytoplasmic	Twine et al. (2006b)
FTT_1483c	Dihydrolipoamide dehydrogenase		Human, presumed type B	2D-Western blot	Cytoplasmic	Janovska et al. (2007a)
FTT_1484c	Pyruvate dehydrogenase, E2 component	aceF	Human, type A; human, presumed type B; mouse	2D-Western blot/ proteome microarray	Cytoplasmic membrane	Eyles et al. (2007), Janovska et al. (2007a,b), Sundaresh et al. (2007), Twine et al. (2010)
FTT_1485c	Pyruvate dehydrogenase, subunit E1		Human, type A	2D-Western blot	Cytoplasmic	Janovska et al. (2007b)
FTT_1498	Acetyl-coenzyme A carboxylase carboxyl transferase subunit alpha		Human, presumed type B; mouse	2D-Western blot	Unknown	Janovska et al. (2007a), Havlasova et al. (2005)
FTT_1510	Aromatic amino acid transporter of the HAAAP family		Human, presumed type B	Proteome microarray	Cytoplasmic membrane	Sundaresh et al. (2007)
FTT_1526	Isocitrate dehydrogenase	idh	Human, presumed type B; mouse	2D-Western blot/ proteome microarray	Unknown	Havlasova et al. (2005), Eyles et al. (2007), Janovska et al. (2007a)
FTT_1530	Fusion product of 3-hydroxyacyl-CoA dehydrogenase and acyl-CoA-binding protein	fadB/ acbP	Mouse	2D-Western blot/ proteome microarray	Cytoplasmic	Eyles et al. (2007), Twine et al. (2010)
FTT_1531	3-Ketoacyl-CoA thiolase		Human, presumed type B	2D-Western blot	Cytoplasmic	Janovska et al. (2007a)
FTT_1533	Part of pseudogene of a sugar transport protein	–	Mouse	Proteome microarray	–	Eyles et al. (2007)
FTT_1539c	Hypothetical protein	–	Human, presumed type B; mouse	2D-Western blot/ proteome microarray	Unknown	Havlasova et al. (2005), Twine et al. (2006b), Eyles et al. (2007), Janovska et al. (2007a)
FTT_1540c	Hypothetical protein	–	Human, presumed type B; mouse	2D-Western blot/ proteome microarray	Unknown	Eyles et al. (2007), Sundaresh et al. (2007), Twine et al. (2010)
FTT_1557c	Two component response regulator	–	Mouse	2D-Western blot	Cytoplasmic	Havlasova et al. (2005)
FTT_1572c	Outer membrane protein OmpH	oomph	Mouse	2D-Western blot/ proteome microarray	Unknown	Havlasova et al. (2005), Eyles et al. (2007)
FTT_1591	Lipoprotein		Human, presumed type B	2D-Western blot	Unknown	Janovska et al. (2007a)
FTT_1569c	UDP-*N*-acetylglucosamine acyltransferase	lpxA	Mouse	2D-Western blot	Cytoplasmic	Twine et al. (2006b)
FTT_1610	Pseudogene		Human, presumed type B	Proteome microarray	Unknown	Sundaresh et al. (2007)
FTT_1616	Cysteinyl tRNA synthetase	cysS	Mouse	2D-Western blot	Unknown	Twine et al. (2006b)
FTT_1676	Hypothetical membrane protein	–	Human, type A; human, presumed type B; mouse	2D-Western blot/ proteome microarray	Unknown	Eyles et al. (2007), Janovska et al. (2007a,b)

(Continued)

Table 3 | Continued

Locus tag	Protein name	Gene	Reactivity sera[1]	Screening method[2]	PSORT[3]	Reference
FTT_1695	Chaperone protein, groES	groES	Human, presumed type B; mouse	2D-Western blot	Cytoplasmic	Havlasova et al. (2002, 2005)
FTT_1696	Chaperone protein, groEL	groEL	Human, presumed type B; mouse	2D-Western blot/ proteome microarray	Cytoplasmic	Havlasova et al. (2002, 2005), Twine et al. (2006a,b, 2010), Eyles et al. (2007), Huntley et al. (2007), Janovska et al. (2007a), Sundaresh et al. (2007)
FTT_1702	Hypothetical protein		Human, presumed type B	2D-Western blot	Unknown	Janovska et al. (2007a)
FTT_1712c	Intracellular growth locus, subunit C	iglC	Human, presumed type B	2D-Western blot	Unknown	Janovska et al. (2007a)
FTT_1713c	Intracellular growth locus, subunit B	iglB	Human, type A	2D-Western blot	Unknown	Janovska et al. (2007b)
FTT_1714c	Intracellular growth locus, subunit A	iglA	Human, presumed type B	2D-Western blot	Cytoplasmic	Janovska et al. (2007a)
FTT_1724	Outer membrane protein tolC precursor	tolC	Human, presumed type B; mouse	Proteome microarray	Outer Membrane	Eyles et al. (2007), Sundaresh et al. (2007)
FTT_1747	Outer membrane protein	–	Mouse	2D-Western blot/ proteome microarray	Unknown	Twine et al. (2006b), Eyles et al. (2007)
FTT_1749	Preprotein translocase, subunit B, chaperonin protein		Human, presumed type B	2D-Western blot	Unknown	Janovska et al. (2007a)
FTT_1752	Single stranded binding protein	ssb	Mouse	2D-Western blot	Unknown	Havlasova et al. (2005)
FTT_1764c	Ferredoxin		Mouse	2D-Western blot	Cytoplasmic	Twine et al. (2006b)
FTT_1769	ClpB	clpB	Human, presumed type B; mouse	2D-Western blot	Inner membrane	Havlasova et al. (2005), Janovska et al. (2007a), Twine et al. (2010)
FTT_1775c	Voltage-gated ClC-type chloride channel clcA	clcA	Human, presumed type B	Proteome microarray	Cytoplasmic membrane	Sundaresh et al. (2007)
FTT_1778c	Hypothetical membrane protein	–	Mouse	2D-Western blot/ proteome microarray	Unknown	Twine et al. (2006b, 2010), Eyles et al. (2007)

[1]Whether protein was reactive with sera from human infected with type A or type B strains of F. tularensis, or vaccinated mice.
[2]Method by which protein immunoreactivity was detected.
[3]Predicted protein subcellular location, using PSORT1b algorithm (Nakai and Horton, 1999; Gardy et al., 2003; Rey et al., 2005).

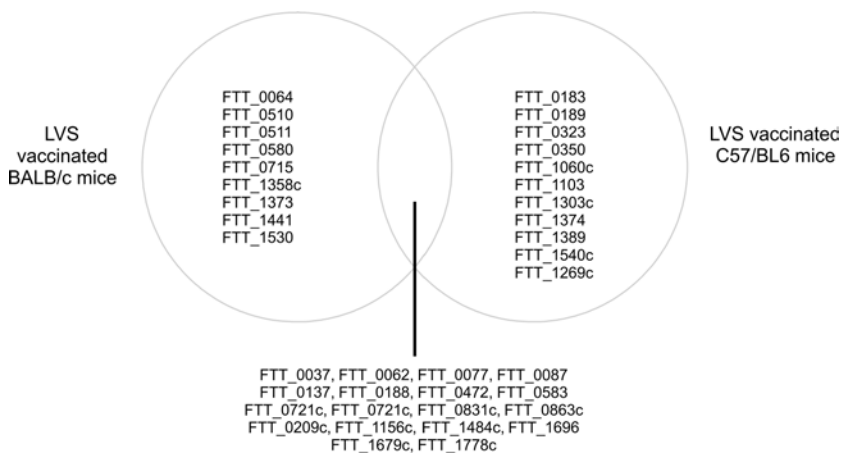

FIGURE 2 | Venn diagram showing the intersection of immunoreactive proteins observed to be reactive with sera from LVS vaccinated and protected BALB/c mice and LVS vaccinated and unprotected C57/BL6 mice (Twine et al., 2010). Left hand side lists immunoreactive proteins only observed to be reactive with sera from LVS vaccinated BALB/c mice, right hand side shows proteins only reactive with sera from C57/BL6 mice.

proteome microarray to determine the repertoire of antibodies generated in response to vaccination of mice with killed LVS and adjuvants. Overall, the murine antibody response was limited to a small subset of antigens within the total SCHU S4 proteome, 48 in total (**Table 3**). Of these, 11 of the top 12 immunoreactive proteins had previously been identified from studies using 2D-Western blotting approach. Interestingly, the study observed few differences in the profile of immunoreactive proteins generated in response to vaccination of mice with (1) viable LVS or (2) killed LVS combined with ISCOMs or (3) killed LVS combined with ISCOMs and CpG (Eyles et al., 2007; **Table 3**). This suggests that combining killed LVS with adjuvant may stimulate the humoral immune system similarly to live LVS. Killed LVS without any adjuvant produced a profile similar to killed LVS in alum or CpG, but the antibody titers were lower and reactive against just eight antigens. Overall, the data presented showed that killed LVS with a Th-1 promoting adjuvant (e.g., ISCOMS or CpG) confers protection against ID challenge with virulent type B strains and some protection against SCHU S4 challenge. Comparing the immunoreactive profiles of sera from mice vaccinated with the various preparations, it is interesting to note that the proteins FTT_1296, and FTT_0119 were reactive with sera from mice immunized with viable LVS, and killed LVS combined with ISCOMS or ISCOMS and CPG, perhaps suggesting that these antigens might be indicative of successful protection against challenge with virulent type B strains. In addition, some antigens, for example FTT_1696, and FTT_1484 were reactive in all live and killed LVS preparations and are likely not predictive of protective immunity in this case (Eyles et al., 2007).

Combined, the murine immunoproteomics studies have raised the possibility of using seroconversion to a particular subset of antigens of LVS as surrogates of or correlates of protection. The most challenging task in this regard is demonstrating that findings in mice are applicable to humans.

IMMUNOPROTEOMICS OF HUMAN TULAREMIA

The relative rarity of natural cases of type A tularemia have until recently, hampered the investigation of the humoral immune response in humans, including immunoproteomic studies. At the time of writing, only two studies describe the reactivity of human sera after type A infections, the first using a 2D-Western blotting approach (Janovska et al., 2007b) and the second, using a proteome microarray to screen a large number of sera (Sundaresh et al., 2007). The first study screened sera from a laboratory worker accidentally infected with type A strain SCHU S4 (Janovska et al., 2007b). The subject suffered oculoglandular tularemia, which recurred 17 months after the original infection. Sera collected 2, 5, and 16 years after infection, were used to screen a membrane enriched subproteome of LVS, and 10 immunoreactive proteins were identified (**Table 3**). Interestingly, the intensity of immunoreactivity toward these proteins showed no apparent decline over the three serum samples screened, with the exception of a loss of reactivity toward the hypothetical protein FTT_0918, which was only observed to be reactive with sera collected 2 years post-infection (Janovska et al., 2007b). This is in contrast with studies of patients who have recovered from type B infections. In such studies, a decline in overall antibody titers has been observed in the 25-year period post-infection (Ericsson et al., 1994a). Overall, only 3 of the 10 immunoreactive proteins observed in the study of the infected laboratory worker; intracellular growth locus, subunit B (FTT_1713c), pyruvate dehydrogenase, E1 component (FTT_1485c), and Succinyl-CoA synthetase subunit beta (FTT_0504c), have not thus far been observed to be reactive with sera from vaccinated mice, or convalescent sera from type B infected individuals.

The *Francisella* proteome microarray was used to screen sera from patients recovering from either type A or B *Francisella* infections contracted in North America (Sundaresh et al., 2007). Of the 46 sera from infected individuals, 10 were from confirmed cases of type A tularemia and 5 from confirmed type B tularemia. The immunodominant proteins were reported in this study (**Table 3**), and of the top 10 antigens, many have also been reported in immunoproteomics studies carried out with sera from LVS infected mice. In addition, a sufficient number of sera were screened, that a comparison of the repertoire of proteins reactive with sera from patients recovering from infection with different subtypes of *Francisella* and different routes of infection could be carried out. For example, authors noted that the protein FTT_1484 was more reactive with sera from patients suffering with ulceroglandular tularemia, compared with the sera from patients with the pneumonic form of the disease. Also, the protein FTT_0975 is more reactive with sera from type B infected individuals compared to type A infected individuals, although the authors note that a rigorous statistical assessment of these observations was outside the scope of their study.

Immunoproteomics was used to survey the repertoire of immunoreactive proteins with sera of human type B convalescents (Janovska et al., 2007a). This study described the reactivity of serum collected from nine tularemia patients with a membrane enriched fraction of LVS, and the proteome of LVS grown under oxidative stress conditions. Details of the type of tularemia, or the time after diagnosis that sera were drawn were not provided, presumably due to patient confidentiality. The immunoproteomics profiles of patient sera showed marked heterogeneity, with a limited number of commonly reactive proteins observed. This variability in profile of immunoreactive proteins has also been noted with human patient sera from other diseases, such as *Helicobacter pylori* (Kimmel et al., 2000; Krah et al., 2004). Of the 35 immunoreactive proteins identified (**Table 3**), a small proportion were reactive with the majority of sera screened, as illustrated in **Figure 3**; the antigens pyruvate dehydrogenase E2 component (FTT_1484), dihydrolipoamide succinyltransferase component of 2-oxoglutarate dehydrogenase component (FTT_077), chaperonin protein GroEL (FTT_1696), acetyl-CoA carboxylase (FTT_0472), hypothetical protein (FTT_1441) and 50S ribosomal protein L7/L12 (FTT_0143) were reactive with six or more of the sera screened. Of note, although the protein FTT_0077 was reactive with eight of the nine patient sera screened, no single protein was observed to be reactive with all of the patient sera in this study.

CHARACTERISTICS OF THE TOTAL REPORTED REPERTOIRE OF IMMUNOREACTIVE PROTEINS

Table 3 summarizes the antigenic proteins reported in recent immunoproteomics studies. Commonalities in the identified proteins across various studies can be noted and will be reviewed here, but comprehensive comparisons of the immunoproteomics data are hampered by distinct differences in the studies, including the immunoproteomics approach used, the host organism, the bacterial

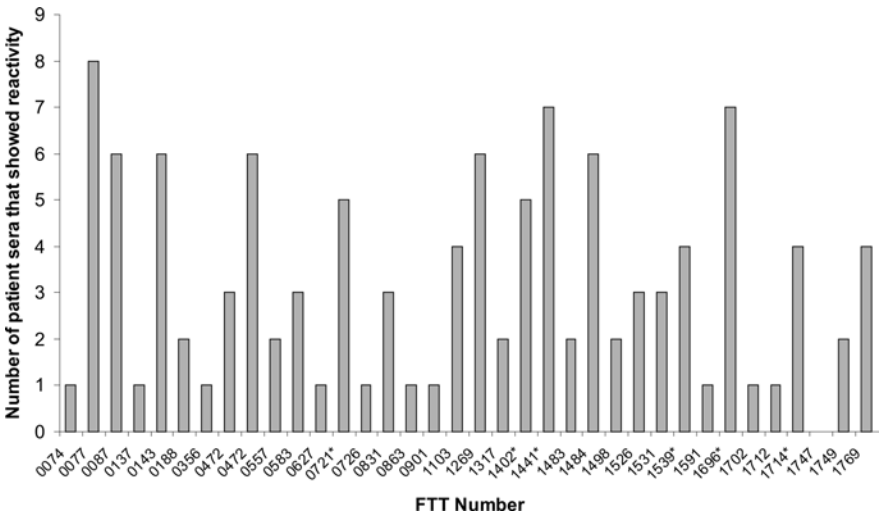

FIGURE 3 | Frequency with which immunoreactive proteins from LVS proteome were observed to react with sera from patients recovering from type B *Francisella tularensis* infections. FTT_number refers to the locus tag of the ORF within the SCHU S4 genome sequence. *Indicates that sera reacted with isoforms of the same protein, shown is the highest observed frequency of reactivity. These data were originally reported by and tabulated in Havlasova et al. (2002).

strain, source of strain, or treatment of the strain used for either infection or immunization, and the bacterial strain or subproteome used as the antigen. In total, 143 antigenic proteins were identified in 11 separate studies. Thirteen of these proteins were reported to be immunoreactive in half of the studies (FTT_1696, FTT_1269c, FTT_1696, FTT_0077, FTT_0137, FTT_0472, FTT_0583, FTT_0721c, FTT_0143, FTT_1103, FTT_0863, FTT_1484c). Of these proteins, FTT_2369c, FTT_1696, FTT_0583, FTT_0721c, and FTT_1103 were also noted to be components of LVS and SCHU S4 outer membrane preparations (Huntley et al., 2007).

Examining the immunoreactive proteins by Clusters of Orthologous groups, antigenic proteins are observed to belong to a diverse array of functional categories (**Figure 4**), but proteins involved in energy production, chaperonins and proteins of unknown function were highly represented. Using the PSORT1b algorithm as a predictor of protein localization within the bacterial cell (Nakai and Horton, 1999; Gardy et al., 2003; Rey et al., 2005), the vast majority of the identified immunoreactive proteins were predicted to be cytoplasmic (44%) or of unknown location (42%; **Figure 4**). This is in contrast with the results from proteome microarray studies alone, where half of the identified proteins were classified as membrane or surface associated (Eyles et al., 2007). This is likely a reflection of the number of immunoproteomics studies carried out using gel-based techniques, which are well documented to underrepresent very large or hydrophobic membrane proteins. The high proportion of proteins that have no predicted location reflects the high number of hypothetical proteins within the *Francisella* proteome, which have little or no homology to known proteins.

Although underrepresented in the entire dataset, membrane proteins are of some interest in immunoproteomics studies. Exposed on the outer bacterial surface, OMPs are generally involved in pathogen evasion of the host, intracellular survival and immune evasion. Characterization of LVS outer membrane proteins, listed 12 OMPs (Huntley et al., 2008). Of these, 10 were observed to be immunoreactive with sera from either murine or human immunoproteomics studies. This LVS outer membrane preparation has been reported to show potential as an acellular vaccine; vaccination of mice with LVS native OMPs resulted in 50% survival rate over 20 days post-challenge with SCHU S4 (Huntley et al., 2008). Also observed was a three log reduction in bacterial burdens in the liver and spleen compared with sham vaccinated animals. This clearly suggests a link between OMPs and protective immunity.

Of the OMPs identified by a proteomics approach (Huntley et al., 2008), some have been identified previously, with demonstrated immunoreactivity. For example, the first presumed *F. tularensis* OMP was a 43-kDa protein identified by probing lithium chloride extracts of bacteria with antisera collected from individuals involved in an outbreak of tularemia in Norway (Ericsson et al., 1994a). This protein was named FopA, for *F*rancisella outer membrane *p*rotein. Two separate vaccine trials demonstrated that FopA was not protective against type A *F. tularensis* or LVS challenge, despite its induction of antibodies (Fulop et al., 1995, 1996). FopA was observed to be immunoreactive in many of the immunoproteomics studies described herein.

The second reported presumed *F. tularensis* OMP was a 17-kDa T lymphocyte-reactive protein originally identified from an *N*-lauroylsarcosinate-insoluble protein preparation; this 17-kDa polypeptide was later named TUL4 (Sjostedt et al., 1990). TUL4 is conserved in *F. tularensis* and *F. novicida* strains, whereas an immunologically related protein is present in *F. philomiragia*. Additional studies confirmed the ability of TUL4 to stimulate lymphocyte proliferation, primarily CD4 T cells, and noted marked production of interleukin-2 (IL-2) and gamma interferon (IFN-γ) in response to TUL4 (Sjostedt et al., 1992a,b; Golovliov et al., 1995; Valentino et al., 2009). Initial [^{3}H]palmitate radiolabeling and detergent extraction of *F. tularensis* suggested that TUL4 was an integral membrane lipoprotein (LP; Sjostedt et al., 1991). Bacterial lipoproteins have an inflammatory capacity and ability to stimulate cells of the

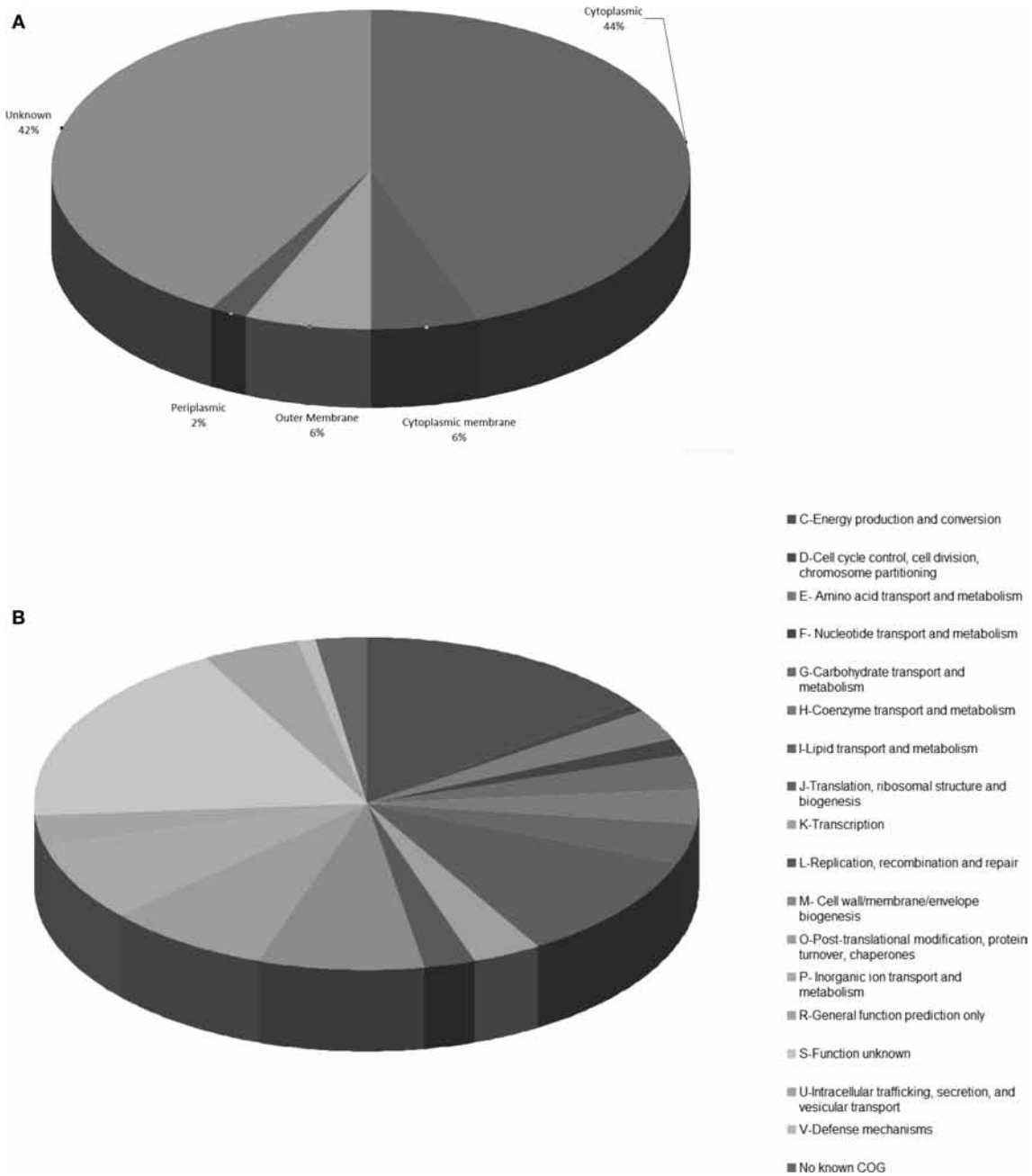

FIGURE 4 | Basic bioinformatic characterization of the repertoire of reported *Francisella* immunoreactive proteins. Graphical representation of **(A)** clusters of orthologous groups (COGs) and **(B)** predicted subcellular location using PSORT algorithm, for total reported immunoreactive proteins from *Francisella* immunoproteomics studies. Taken from the reported immunoreactive proteins listed in **Table 3**.

innate immune system via TLR2 (Thakran et al., 2008). Although the lipoprotein TUL4 is not essential for virulence of *F. tularensis* (Forestal et al., 2008), amino acids 86–99 of the protein were identified as an immunodominant epitope of CD4T cells in B6 mice (Valentino et al., 2009). A recent study showed that the hypothetical lipoprotein, denoted FTT_1416c or FTL0645 of LVS, was enriched in a sarkosyl membrane extraction. Expression of the equivalent recombinant protein in *E. coli* produced a lipoprotein that was able to activate TLR2 and induce an immunogenic response in mice (Parra et al., 2009). The lipoprotein was also shown to be immunoreactive with immune sera from mice in other work.

Chaperonin proteins feature frequently in tularemia immunoproteomics studies. The chaperonin protein DnaK and GroEL (Cpn60) were reactive with the vast majority of sera screened. Chaperonins, also known as heat shock proteins are among the most highly conserved protein families in nature and are expressed in

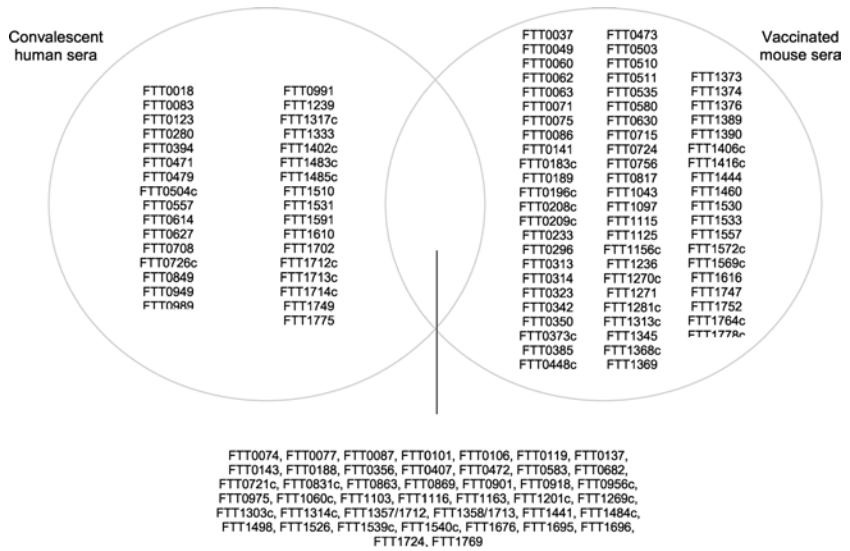

FIGURE 5 | Venn diagram showing the intersection of immunoreactive proteins observed to be reactive with sera from LVS vaccinated mice and human convalescent. From Table 3.

both prokaryotes and eukaryotes (Ranford and Henderson, 2002). These proteins facilitate the non-covalent assembly of proteins and when cells are exposed to stress conditions, such as temperature or pH, chaperones protect cellular proteins from aggregation and also promote refolding (Stewart et al., 2004). Chaperonins have also been observed to be activators of the innate immune system (Kol et al., 1999; Wallin et al., 2002), in addition to stimulating monocytes, macrophages and dendritic cells to produce nitric oxide and a variety of cytokines (Kol et al., 1999; Wallin et al., 2002). Although most heat shock proteins are widely accepted to be cellular proteins some, for example GroEL can also be released from cells, and stimulate a immune response (Henderson et al., 2006). Other studies have reported that GroEL and DnaK of *Mycobacterium tuberculosis* and *F. tularensis* are located either on the cell surface (Hickey et al., 2009) or partition with membrane protein subproteome (Huntley et al., 2007). GroEL of *F. tularensis*, has been observed at increased levels under conditions of stress (Ericsson et al., 1994b; Twine et al., 2006a) and has been detected in the cytosol of host cells infected with the bacterium (Lee et al., 2006). It has been suggested that the predominant source of *Francisella* chaperonins in the host are derived from bacteria that are either extracellular in the blood (Forestal et al., 2007) or lungs (Bosio et al., 2007) or from bacteria that fail to proliferate in the host environment. GroEL has also been reported to have an inflammatory role, stimulating macrophages via TLR4, but not human endothelial cells (Noah et al., 2010). In addition, GroEL and LPS derived from LVS acted together to elicit a synergistic response in macrophages (Noah et al., 2010). Similarly, another *Francisella* heat shock protein, DnaK, has been reported to mediate activation of dendritic cells via TLR4 (Ashtekar et al., 2008).

Heat shock proteins (Hsps) have been reported to be dominant antigens for the host immune response to various pathogens, and have been attempted to be used in subunit vaccines against some pathogens (Khan et al., 2009). The potential of Hsps to elicit both cell-mediated and humoral immune responses even in the absence of exogenous adjuvants (Lowrie et al., 1997; Harmala et al., 2002), their requirement in small quantities and ability to elicit memory T cell response, make them attractive vaccine candidates against infectious disease.

As noted in this article, the relative rarity of naturally occurring human tularemia and the ethical issues with human LVS vaccination and challenge studies, means that much of our recent knowledge stems from the murine model of tularemia. A significant challenge in this regard, is determining whether findings in mice are applicable to humans. Reviewing the complement of identified immunoreactive proteins listed in **Table 3** and also in **Figure 5**, shows the overlap between antigens reactive with both murine and human sera. This shows that the 43 antigens have been observed to be reactive with LVS vaccinated murine and human convalescent sera.

CONCLUSION

There continues to be a significant focus upon the development of efficacious, safe and licensable tularemia vaccines. Acellular or subunit vaccines carry far less risk than attenuated live vaccines, but there are limited reports of success with subunit tularemia vaccine development. The lack of knowledge of both *Francisella* virulence factors, protective antigens and the complex nature of immunity to *Francisella* is inhibiting vaccine development. Further studies of the humoral immune response, especially those in other animal models of tularemia that will bridge the differences between mice and humans, will be required to determine those antigens that can be predicted to be protective in humans. This will aid greatly in the development of subunit vaccines and selection of antigens for incorporation into diagnostic tests.

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Francisella–arthropod vector interaction and its role in patho-adaptation to infect mammals

Christine Akimana*[1] *and Yousef Abu Kwaik*[1,2]

[1] *Department of Microbiology and Immunology, College of Medicine, University of Louisville, Louisville, KY, USA*
[2] *Department of Biology, University of Louisville, Louisville, KY, USA*

Edited by:
Anders Sjostedt, Umeå University, Sweden

Reviewed by:
Karsten R. O. Hazlett, Albany Medical College, USA
Karsten Hueffer, University of Alaska Fairbanks, USA

***Correspondence:**
Yousef Abu Kwaik, Department of Microbiology and Immunology, School of Medicine, University of Louisville, 412 Building A, Louisville, KY 40292, USA.
e-mail: abukwaik@louisville.edu

Francisella tularensis is a Gram-negative, intracellular, zoonotic bacterium, and is the causative agent of tularemia with a broad host range. Arthropods such as ticks, mosquitoes, and flies maintain *F. tularensis* in nature by transmitting the bacteria among small mammals. While the tick is largely believed to be a biological vector of *F. tularensis*, transmission by mosquitoes and flies is largely believed to be mechanical on the mouthpart through interrupted feedings. However, the mechanism of infection of the vectors by *F. tularensis* is not well understood. Since *F. tularensis* has not been localized in the salivary gland of the primary human biting ticks, it is thought that bacterial transmission by ticks is through mechanical inoculation of tick feces containing *F. tularensis* into the skin wound. *Drosophila melanogaster* is an established good arthropod model for arthropod vectors of tularemia, where *F. tularensis* infects hemocytes, and is found in hemolymph, as seen in ticks. In addition, phagosome biogenesis and robust intracellular proliferation of *F. tularensis* in arthropod-derived cells are similar to that in mammalian macrophages. Furthermore, bacterial factors required for infectivity of mammals are often required for infectivity of the fly by *F. tularensis*. Several host factors that contribute to *F. tularensis* intracellular pathogenesis in *D. melanogaster* have been identified, and *F. tularensis* targets some of the evolutionarily conserved eukaryotic processes to enable intracellular survival and proliferation in evolutionarily distant hosts.

Keywords: arthropod, vector, tularemia, virulence factor, *Drosophila*, *F. tularensis*

FRANCISELLA TULARENSIS – AN ETIOLOGICAL AGENT OF THE ARTHROPOD-BORNE TULAREMIA

Francisella tularensis is a Gram-negative, intracellular, zoonotic bacterium, and is the causative agent of tularemia (Ellis et al., 2002; Santic et al., 2010). The transmission of *F. tularensis* to humans is mediated by the bites of arthropods, such as ticks, flies, and mosquitoes, by inhalation, or by handling or ingesting contaminated meat or water (**Figure 1**; Ellis et al., 2002; Oyston et al., 2004; Santic et al., 2010). *F. tularensis* is among the most infectious pathogens known. The infective dose in humans is as low as 10 bacteria when injected subcutaneously and 25 bacteria when given as an aerosol (McCrumb, 1961; Saslaw and Carlisle, 1961). Since this bacterium is highly infectious, easily disseminated, and acquired via multiple routes, *F. tularensis* is one of the six pathogens classified by the CDC as a category A select agent (Dennis et al., 2001; Oyston et al., 2004; Santic et al., 2006).

There are four recognized subspecies of *F. tularensis*: *tularensis*, *holarctica*, *mediasiatica, and novicida* (Forsman et al., 1994; Keim et al., 2007; Nigrovic and Wingerter, 2008). The four subspecies share about 97% genomic identity (Champion et al., 2009; Larsson et al., 2009). However, classification of *novicida* as a subspecies is still a matter of debate. Two subspecies of *F. tularensis* cause most human tularemia infections: subspecies *tularensis*, also known as type A, and subspecies *holarctica*, referred to as type B. The subspecies *tularensis* is the most virulent of *F. tularensis* ssp. for humans, whereas the subspecies *holarctica* causes milder infections and lower mortality rates in humans (Nigrovic and Wingerter, 2008). Both *tularensis* and the *holarctica* subspecies require level 3 bio-containment (Oyston et al., 2004; Keim et al., 2007; Nigrovic and Wingerter, 2008). The subspecies *novicida* and *holarctica*-derived LVS strain are often used to study the pathogenesis by *F. tularensis*, since they are attenuated in humans, but cause disease in animal models similar to the virulent subspecies (Santic et al., 2010). In addition, both of these attenuated species replicate intracellularly within human and mouse macrophages, an important step in the disease process in mammals (Oyston et al., 2004; Santic et al., 2010).

Arthropods carry disease causing agents and present a major problem worldwide as vectors of human diseases (Kay and Kemp, 1994). Ticks and flies are common arthropod vectors of *F. tularensis* transmission in the US (Keim et al., 2007). The Type A strain of *F. tularensis* is commonly transmitted by ticks and by tabanid flies, whereas the Type B strain is commonly transmitted by ticks, tabanid flies, and by blood-feeding mosquitoes (**Figure 1**; Keim et al., 2007; Nigrovic and Wingerter, 2008).

One major preventive measure to avoid tularemia, as any other arthropod-borne disease, is to use chemical repellants and pesticides in endemic regions (Nigrovic and Wingerter, 2008). However, potential resistance to pesticides and chemical repellant, contamination of food and the environment are major concerns associated with the usage of such pesticides (Kay and Kemp, 1994). Thus, it is desirable to develop alternative effective preventive measures (Kay and Kemp, 1994). One such measure would be to develop

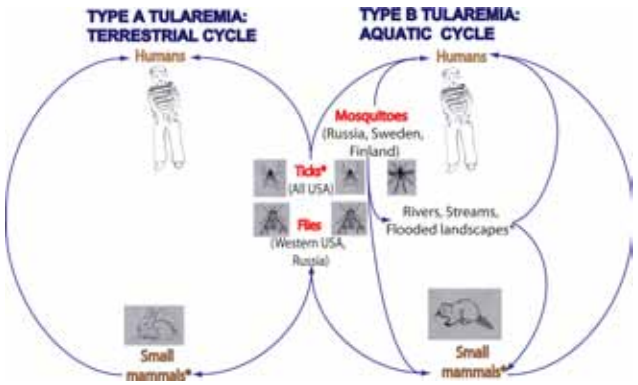

FIGURE 1 | The role that arthropods play in the transmission of *F. tularensis* type A and B. Arthropods maintain *F. tularensis* infections in nature by transmitting *F. tularensis* between small mammals, such as rabbits and beavers in order to maintain the reservoir. Type A strain of *F. tularensis* is commonly transmitted by ticks and by tabanid flies, whereas Type B strain is commonly transmitted by ticks, blood-feeding mosquitoes, and by tabanid flies. In USA, bites by ticks are the predominant mode of tularemia transmission. Transmission by biting flies is observed in western regions of the USA and in Russia. Transmission by mosquitoes is observed in the northern countries of Sweden, Finland, and Russia. Classification of hosts of *F. tularensis* based on the subspecies of *F. tularensis* associated with them indicates that there are two cycles of *F. tularensis*: terrestrial and aquatic. Type A has a terrestrial cycle with the main reservoirs being cottontail rabbits and ticks. Type B has mainly a water-borne cycle with semi-aquatic rodents as reservoirs of infection, such as muskrats and beaver in North America, and ground voles in the former Soviet Union. Type B infections have also been associated with rivers, streams, and temporarily flooded landscapes. Asterisk (*) indicates reservoirs of *F. tularensis* infections.

a tularemia vaccine (Nigrovic and Wingerter, 2008). Promising novel strategies are being developed to reduce microbial transmission by arthropod vectors. One example is illustrated in the study by McMeniman et al. (2009), who used *Wolbachia* infection to shorten the life span of the populations of mosquito *Aedes aegypti*. Shortening the lifespan of these mosquitoes results in fewer cases of mosquito-borne dengue fever illnesses in the human population.

Alternatively, reducing the transmission of vector-borne illness to humans can be achieved indirectly, by including in a vaccine formulation antigens that are important for the successful infection of the pathogen. For instance, the outer membrane lipoprotein A (OSPA) of *Borrelia burgdorferi* is up regulated and expressed in the tick but not in the mammalian host. However, an OSPA vaccine was shown to have 79% efficacy in a phase III human trial and was an FDA-approved vaccine from 1998 until 2002 (Earnhart et al., 2007). Recently a study has shown that antiserum against salp15, a tick salivary antigen, is protective, and enhances protection of OSPA and OSPC (another *B. burgdorferi* surface antigen) antiserum in a murine model of Lyme disease (Dai et al., 2009). Therefore, virulence determinants associated with *F. tularensis*–arthropod vectors might be important in developing vaccine antigens and/or therapeutic measures. Other studies have shown that vaccination against components of the saliva of arthropods or against antigens expressed in the gut of arthropods protected the host from infection and decreased the viability of the arthropod (Titus et al., 2006). In the context of vaccine development, a multi-subunit vaccine that targets *F. tularensis* itself, as well as components of the arthropod vector, might be worth exploring to control bacterial transmission to humans. This review focuses on *Francisella*–arthropod interactions, while other reviews related to other aspects of *Francisella* biology, genetics, physiology, and pathogenesis are included in this special topic issue (Broms et al., 2010; Chong and Celli, 2010; Meibom and Charbit, 2010; Asare and Abu Kwaik, 2011; Bosio, 2011; Cremer et al., 2011; Dai et al., 2011; Jones et al., 2011; Kilmury and Twine, 2011; Telford and Goethert, 2011; Zogaj and Klose, 2011).

EPIDEMIOLOGY OF ARTHROPOD-TRANSMITTED TULAREMIA

Tularemia outbreaks are usually rare and sporadic, and occur as an epidemic both in humans and in animals (Morner, 1992). Workers at increased risk for acquiring tularemia include laboratory workers, landscapers, farmers, veterinarians, hunters, trappers, cooks, and meat handlers (Nigrovic and Wingerter, 2008). There was a recent outbreak of pneumonic tularemia that occurred on Martha's Vineyard (MA, USA) during the summer of 2000, and 11 out of 15 confirmed cases of *F. tularensis* infection had pneumonia (Feldman et al., 2001). Although the cause of this outbreak was pronounced to be an incident of aerosolized *F. tularensis* caused by a lawnmower running over the carcass of an infected rabbit (Nigrovic and Wingerter, 2008), the origin of the infections was traced back to transmission by ticks (Keim et al., 2007). Genetic data indicates that the *F. tularensis* genotype from the landscape worker who contracted fatal Type A tularemia on Martha's Vineyard was a perfect MLVA genotype match for the *F. tularensis* genotypes obtained from ticks collected in the Squibnocket area on Martha's Vineyard, where he previously worked (Keim et al., 2007).

Arthropods, especially ticks, play a significant role in maintaining *F. tularensis* infections in nature, often by transmitting *F. tularensis* between small mammals, such as rabbits and other lagomorphs in order to maintain the reservoir (**Figure 1**; Francis, 1927; Morner, 1992; Keim et al., 2007; Petersen et al., 2009). Transmission of *F. tularensis* in nature has been documented in other less prevalent arthropod vectors, including fleas, lice, midges, and bedbugs (Hopla, 1974; Petersen et al., 2009). Geographic differences have been observed for the arthropod vectors transmitting *F. tularensis* (Keim et al., 2007; Petersen et al., 2009). These differences are linked to the geographic location and abundance of their host species (Petersen et al., 2009), usually small mammals. In the USA, Sweden, Finland, and Russia, arthropod bites, especially by ticks, are a common mode of tularemia transmission to humans (Petersen et al., 2009; **Figure 1**). Transmission, especially by the deer fly, *Chrysops discalis*, and by horse flies has been documented in western regions of the USA and Russia (**Figure 1**). In the Western USA, both deer flies and ticks are considered important vectors, whereas in the Eastern USA, only ticks are considered significant vectors (Petersen et al., 2009). In the USA, tick bites are the predominant mode of transmission (Petersen et al., 2009). The three tick species that are most important for human transmission include *Dermacentor andersoni*, *D. variabilis*, and *A. americanum*. *D. variabilis* and *A. americanum* are the two tick species found in regions of the USA reporting the highest incidence of tick-borne tularemia (Arkansas, Missouri, Oklahoma; Petersen et al., 2009). These two tick species have a high affinity for humans, which likely contributes to their success as vectors of tularemia (Parola and Raoult, 2001). In the

northern countries of Sweden, Finland, and Russia, mosquitoes have been identified as the major vector transmitting tularemia to humans. In central Europe, contact with infected animals and ingestion of contaminated food or water are the more common modes of transmission in this region rather than arthropod transmission (Hubalek et al., 1996; Tarnvik et al., 2004; Petersen et al., 2009).

Common hosts associated with *F. tularensis* are rodents, ground squirrels, wild rabbits, semi-aquatic rodents, hares, ticks, tabanid flies, and mosquitoes (reviewed in Nigrovic and Wingerter, 2008). However, *F. tularensis* is found to be associated with numerous animals, including birds, fish, amphibians, arthropods, and protozoa (Morner, 1992). Hosts that are susceptible to *F. tularensis* infections include 190 mammals, 88 invertebrates, 23 birds, and 3 amphibians (Keim et al., 2007). Classification of hosts of *F. tularensis,* based on the subspecies of *F. tularensis* associated with them, indicates that there are two cycles of *F. tularensis*: terrestrial and aquatic (**Figure 1**; Morner, 1992). *F. tularensis* ssp. *tularensis* has a terrestrial cycle with the main reservoirs being cottontail rabbits and ticks. Arthropods such as ticks and flies are the most important vectors in this cycle (Morner, 1992; Nigrovic and Wingerter, 2008). *F. tularensis* ssp. *holarctica* or type B mainly has a water-borne cycle with semi-aquatic rodents as reservoirs of infection, such as muskrats and beaver in North America, and ground voles in the former Soviet Union (Morner, 1992). As part of this water-borne cycle, mosquitoes have been reported as significant vectors of tularemia in Sweden and Finland (Petersen and Schriefer, 2005; Nigrovic and Wingerter, 2008).

At a cellular level, *F. tularensis* has been reported to infect and replicate in macrophages of a broad range of mammals, as well as a plethora of other cell types, including fibroblasts, endothelial cells, hepatocytes, and muscle cells (Penn, 2005). Some studies with arthropods show restricted proliferation by *F. tularensis* in the natural arthropod hosts, the ticks, mosquitoes, and flies (reviewed in Petersen et al., 2009). In the fruit fly, a model of the fly arthropod vector for *F. tularensis*, the bacteria infect hemocytes (macrophage-like cells), other tissue, and are found in the hemolymph (Vonkavaara et al., 2008; Santic et al., 2009; Moule et al., 2010). A similar observation of infection that spread in diverse arthropod tissues was found in at least some species of ticks, such as *D. andersoni*, a natural host and vector of tularemia (Francis, 1927). Therefore, more studies are needed to decipher the infection process in the arthropod hosts. Overall, *F. tularensis* infects a plethora of host species, and arthropod-borne transmission plays an important role in the infectious life cycle of *F. tularensis* and subsequent pathogenesis in mammalian hosts. Therefore, understanding the interaction of *F. tularensis* with the arthropod vector at the molecular, cellular, and organismal level will advance our understanding of tularemia and transmission of *F. tularensis*.

PATHOPHYSIOLOGY OF INFECTION WITH *F. TULARENSIS*

After infection of humans with *F. tularensis*, the incubation period is usually 3–6 days (Nigrovic and Wingerter, 2008), which is immediately followed by the onset of the disease (Oyston et al., 2004). Clinical manifestation of tularemia has been classified in two general groups. The ulceroglandular form is associated with systemic symptoms, and is often accompanied by a painful maculopapular lesion at the entry site. The typhoidal form is a severe form of tularemia without the skin or lymph node symptoms, but with gastrointestinal and pulmonary symptoms. The ulceroglandular form is more common and found in approximately 75% of patients, whereas the typhoidal form appears in approximately 25% of patients (Nigrovic and Wingerter, 2008). Although the mortality rate decreases significantly once an effective antibiotic is administered, the mortality rate for untreated pneumonia associated with tularemia can be as high as 60% (Nigrovic and Wingerter, 2008). Pneumonic tularemia occurs in approximately 30% of ulceroglandular tularemia and 80% of typhoidal tularemia (Nigrovic and Wingerter, 2008). Both ulceroglandular and typhoidal tularemia are associated with arthropod transmission of infection, but ulceroglandular tularemia is the most common form associated with an arthropod bite (Petersen et al., 2009). After successful infection, *F. tularensis* multiplies at the initial site of infection, and then spreads to the regional lymph nodes, liver, and spleen (Oyston et al., 2004; Santic et al., 2006). In small mammals such as guinea pigs, death is observed 3–5 days after infection due to *F. tularensis*-infected tick bites (Parker et al., 1924; Francis, 1927).

Ticks are established biological vectors of tularemia, as they are responsible for supporting *F. tularensis* infections in nature, facilitated by their lengthy lifecycle, which is about 2 years (Petersen et al., 2009). The study by Francis in 1927 established the tick *D. andersoni* as a biological host of *F. tularensis*. *D. andersoni* harbors *F. tularensis* in its feces, epithelial cells of the digestive tract and Malpighian tubules, as well as the coelomic fluid (Francis, 1927). Studies have been performed on ticks after taking a blood meal from *F. tularensis*-infected guinea pigs. The ticks were incubated for 30 days after the blood meal, dissected, and pathological analysis were conducted microscopically. Anatomical changes observed included the distention of the epithelial cells of the rectal sac, intestines, and Malpighian tubes. Invaded cells are swollen, and contain large numbers of *F. tularensis,* which are located in the protoplasm. Occasionally, *F. tularensis* multiplied in the gut wall, cells were swollen, and then ruptured, releasing their contents in a mass, which explains the recovery of *F. tularensis* from feces of ticks. Other studies have confirmed the localization of *F. tularensis* in the gut, in the hemolymph, and in excrements of ticks (Vyrostekova, 1993; Petersen et al., 2009). Surprisingly, *F. tularensis* was not localized in the salivary gland of the tick, suggesting that the transmission of *F. tularensis* by the tick *D. andersoni* was mechanically mediated through *F. tularensis*-containing feces directly into the skin wound (Francis, 1927). To date, *F. tularensis* infection has never been documented in the salivary glands of the primary human biting ticks (Petersen et al., 2009). In addition, in the bed bug as well, *F. tularensis* is not isolated from the salivary glands (Francis, 1927). However, one study reported that *F. tularensis* was localized in the salivary glands of the species *D. marginatus*, a non-primary biting tick (Hopla, 1974). Although, the transmission rate of *F. tularensis* to mammalian host by the adult tick is high and of a significant concern, the nymphal stage of this arthropod is not a significant vector of tularemia. A recent study compared the transmission rates among nymphal *D. variabilis* infected as larvae with wild-type strains of A1b, A2, and type B. As expected, *D. variabilis* larvae were able to acquire, maintain, and transstadially transmit *F. tularensis*. Significant replication of the bacteria also occurred in infected nymphs. However, transmission

of *F. tularensis* to Swiss Webster mice was not observed with A1b, and low rates were observed with A2 (8.0%) and type B (13.5%) strains (Reese et al., 2010).

Biting arthropods vectors insert their piercing mouthparts in the host skin, lacerate the skin, and then inject their anticoagulant-containing saliva to prevent blood clot (Atkins, 1978). Biological vectors allow the pathogen to multiply or develop before being transferred to another host, whereas mechanical vectors transmit pathogens to susceptible host without the development of the pathogen, by for instance transferring the pathogen on feet or mouth of the arthropod (Gray and Banerjee, 1999). *F. tularensis* transmission by mosquitoes and flies is not well understood, but it is believed to be mechanical, on the mouthpart through interrupted feedings. An infected biting fly in nature can transmit tularemia only up to 4 days following its initial infection. In a laboratory setting, *F. tularensis* is consistently recovered from deer flies for up to 5 days, but no longer than 14 days (Petersen et al., 2009). Similar to deer flies, the mosquito is not believed to support multiplication of *F. tularensis* (Triebenbach et al., 2010). A recent study indicated that *Francisella* DNA was detected in 30% of >2,500 mosquitoes captured in Alaska (Triebenbach et al., 2010). However, *F. tularensis* was not transstadially transmitted in mosquitoes tested. Furthermore, although adult female *Anopheles gambiae* and *Ae. aegypti* retained detectable levels of *Francisella* DNA for 3 days, *F. tularensis* was not transmitted to the mammalian host by these mosquitoes (Triebenbach et al., 2010). Thus, the absence of *F. tularensis* in the salivary glands of several arthropods makes a non-biting insect, such as *Drosophila melanogaster*, a more anatomically and physiologically relevant model of an arthropod vector of tularemia, which could be used to elucidate mechanisms of transmission by arthropod vectors of *F. tularensis* (Petersen et al., 2009). In *D. melanogaster*, after pricking (septic injury by needle) and introduction of *F. tularensis* in the hemolymph, bacteria were observed in the head, legs, and wings veins (Vonkavaara et al., 2008). Intracellular bacteria were localized in the cardia, at the invagination of the esophagus, and in hemocytes. Interestingly, when infection was attempted by oral route, *F. tularensis* survived in the gastric system for only 24 h after feeding, however the bacteria were cleared thereafter (Vonkavaara et al., 2008).

DROSOPHILA MELANOGASTER IS A TRACTABLE ARTHROPOD MODEL FOR TULAREMIA

Drosophila melanogaster has been used as a model in almost every aspect of eukaryotic biology, and we understand more about the biology of this insect than almost any other multicellular organism (Boutros and Perrimon, 2000; Rubin and Lewis, 2000). This knowledge stems from Thomas Morgan's decision in early 1900 to use *D. melanogaster* as a model to study genetics (Rubin and Lewis, 2000). Interestingly, most biological processes are remarkably similar between flies and vertebrates, such as humans. For instance, sequence searches with 289 human cancer-related genes reveal that 61% of those genes have orthologs in *D. melanogaster* (Rubin et al., 2000). Conducting biological studies in *Drosophila* has allowed major scientific milestones in many fields, including microbial pathogenesis (Cherry and Silverman, 2006). *Drosophila* has been established as a useful model to dissect microbial pathogenesis of some important pathogens, such as *Pseudomonas aeruginosa*, *Mycobacterium marinum*, and *Listeria monocytogenes*, which successfully infect adult fruit flies (review in Cherry and Silverman, 2006). Thus, *D. melanogaster* is a general attractive model system for microbial pathogenesis. In addition, the signaling pathways regulating innate mammalian immune response are evolutionarily conserved and have similar function in insect immunity (Hoffmann et al., 1999). For instance, in *D. melanogaster* and in mammals, Toll family receptors (Hoffmann et al., 1999; Anderson, 2000) trigger host innate immune responses that are highly conserved. This conservation makes flies particularly useful for investigation of fundamental biological processes of great relevance to microbial pathogenesis. Furthermore, flies are inexpensive and grow quickly, and many studies have used forward and reverse genetics in *Drosophila*, which allowed the identification and characterization of many aspects of biological processes that are conserved through evolution.

Drosophila melanogaster is emerging as an attractive arthropod model of infection by *F. tularensis* and has facilitated the dissection of many processes of *F. tularensis* pathogenesis. Recent studies have used various arthropods as general models, as well as arthropod vector models of tularemia (Aperis et al., 2007; Read et al., 2008; Vonkavaara et al., 2008; Santic et al., 2009; Ahlund et al., 2010; Akimana et al., 2010; Asare et al., 2010; Moule et al., 2010) For example, the *Drosophila*-derived cell lines and the sualB cell line from *An. gambiae* have been used as models to study intracellular replication of *F. tularensis* (Read et al., 2008; Vonkavaara et al., 2008; Santic et al., 2009; Ahlund et al., 2010; Akimana et al., 2010; Asare et al., 2010). Recent studies have also shown that adult flies could be used as a model system to study *Francisella* pathogenesis (Vonkavaara et al., 2008; Santic et al., 2009). *D. melanogaster* is especially an attractive model system to study the pathogenesis of *F. tularensis* because arthropods are vectors for transmission of tularemia between mammals. This makes the *Drosophila* model system particularly useful for studying both general *F. tularensis* host–pathogen interactions and arthropod vector-specific factors.

BACTERIAL VIRULENCE FACTORS IN THE ARTHROPOD MODEL OF TULAREMIA

To successfully establish a niche in a susceptible host, pathogens use virulence factors to invade, colonize, and survive within the host. After uptake by cells, *F. tularensis* escapes from the phagosome and propagates in the cytosol (Golovliov et al., 2003; Clemens et al., 2004; Santic et al., 2005b, 2008; McCaffrey and Allen, 2006; Chong et al., 2008). Multiplication results in cell death and release of bacteria (Lai et al., 2001), allowing them to spread to regional lymph nodes and to colonize the spleen, liver, and lung (Tempel et al., 2006). A substantial proportion of the bacterial burden can persist extracellularly in the bloodstream (Forestal et al., 2007; Yu et al., 2008). The virulence factors that are well studied and known to play a role in *F. tularensis* pathogenesis are involved in lipopolysaccharide biosynthesis or intracellular survival. Most research interest has been on a 30-kb genomic region called the *Francisella* pathogenicity island (FPI), which has been shown to be required for intracellular replication of *F. tularensis* within macrophages (Baron and Nano, 1998; Santic et al., 2005b; Bonquist et al., 2008; Schmerk et al., 2009), and which encodes a putative type VI-like secretion system (Nano and Schmerk, 2007; Filloux et al., 2008; Barker et al., 2009).

Studies with pathogenic bacteria in the fly have shown that virulence factors that function in the vertebrate hosts of these pathogens are often required for the pathogen to survive in the fly. Intramacrophage proliferation is essential for *F. tularensis* pathogenesis. Similar to macrophages, replication of *F. tularensis* in S2 and SualB cells is dependent on *MglA, MglB, IglA, IglC, IglD, PdpA,* and *PdpB*, which are components or regulators of the FPI (Read et al., 2008; Vonkavaara et al., 2008; Santic et al., 2009). In addition, trafficking and robust intracellular proliferation of *F. tularensis* ssp. *novicida* in *D. melanogaster*-derived S2 cells are similar to trafficking and proliferation in mammalian macrophages (Santic et al., 2009). Within both host cells, *F. tularensis* transiently occupies a late endosome-like phagosome, followed by rapid bacterial escape into the cytosol, where the bacteria proliferate robustly (Golovliov et al., 2003; Clemens et al., 2004; Santic et al., 2005a,b, 2007, 2008; Checroun et al., 2006; Bonquist et al., 2008; Chong et al., 2008; Qin et al., 2009; Wehrly et al., 2009). This may suggest that some common mechanisms are utilized by *F. tularensis* to modulate phagosome biogenesis, escape into the cytosol, and to proliferate within mammalian and arthropod-derived cells.

All studies of proliferation of *F. tularensis* in adult flies indicate that this bacterium grows to high levels within flies and causes a lethal infection (Vonkavaara et al., 2008; Santic et al., 2009; Ahlund et al., 2010; Asare et al., 2010; Moule et al., 2010). *F. tularensis* kills the fly with a median time to death of 5–12.9 days post-infection, depending on the number of CFUs injected and the strain of *F. tularensis* used. Extremely high bacterial levels are observed within the fly due to bacteria growing extracellularly (Vonkavaara et al., 2008; Moule et al., 2010). Therefore, screening *F. tularensis* strains for lethality to *D. melanogaster* is likely to be an effective approach to identify important bacterial factors involved in arthropod–*Francisella* interaction. Consistent with this idea is an observation by Ahlund et al. (2010) that there is a significant correlation between fly survival and bacterial proliferation within mammalian cells. Genome-wide screens were conducted to identify factors required for intracellular proliferation within *Drosophila*-derived cells, and for *in vivo* growth and survival within the fly (**Table 1**). It has been shown that ~400 genes, representing 22% of the bacterial genome, are required for intracellular proliferation of *F. tularensis* within *D. melanogaster*-derived S2 cells (Asare and Abu Kwaik, 2010). Interestingly, many genes are required for intracellular proliferation in both *Drosophila*-derived S2 cells and human macrophages (Asare and Abu Kwaik, 2010; Moule et al., 2010). Among 149 *F. tularensis* ssp. *novicida* mutants attenuated in the fly, 41 of these mutants (28%) had previously been shown to be attenuated in the mouse model (Weiss et al., 2007a). Among ~250 *F. tularensis* ssp. *novicida* mutants that are attenuated in mice, 49 (20%) of them are attenuated in flies (Ahlund et al., 2010). Interestingly, among 168 mutants defective for intracellular growth in S2 cells, 80 are attenuated for lethality to *D. melanogaster* adult flies (Asare et al., 2010), indicating that >50% of genes required for intracellular proliferation in S2 derived cells play a crucial role in survival of the fly.

Overall, *F. tularensis* grows in large numbers in *D. melanogaster* resulting in lethality, similar to mammals (Vonkavaara et al., 2008; Santic et al., 2009; Ahlund et al., 2010; Asare et al., 2010; Moule et al., 2010). In addition, contrasting studies in flies to those in mammalian models (Ahlund et al., 2010; Asare et al., 2010; Moule et al., 2010) indicates that *F. tularensis* might have acquired some of the mechanisms to proliferate within mammalian cells through patho-adaptation to the arthropod host. Some of the virulence factors that have been possibly acquired through patho-adaptation in insect hosts include most genes of the FPI. However, additional distinct molecular mechanisms are also required for proliferation within both evolutionarily distant hosts, as numerous factors important for infectivity of *D. melanogaster* are not required for infectivity of mammalian hosts and *vice versa* (Ahlund et al., 2010; Asare et al., 2010).

HOST VIRULENCE DETERMINANTS FOR INTRACELLULAR PROLIFERATION OF *F. TULARENSIS* IN THE ARTHROPOD-DERIVED CELLS

To reduce transmission and morbidity associated with arthropod-borne tularemia, not only bacterial factors are important, but also arthropod host factors can be used to develop therapeutic measures against *F. tularensis*. For example, it has been shown in Lyme disease that a tick antigen Salp15, a salivary gland protein, can be a protective immunogen to some degree, and can be used to enhance the potency of a bacterial vaccine antigen OSPA (Dai et al., 2009).

Like other intracellular bacterial pathogens, *F. tularensis* has evolved varying strategies to avoid being attacked by the host macrophages (Aderem and Underhill, 1999). Within mammalian and arthropod-derived cells, *F. tularensis* escapes the acidified late endosome-like phagosome to reach the host cell cytosol, where replication occurs (Santic et al., 2009). Therefore, it is reasonable to assume that *F. tularensis* targets evolutionarily conserved eukaryotic factors for intracellular survival and growth. Some of the strategies to evade the host defense efforts by *F. tularensis* involve its ability to modulate the host cellular and molecular machinery. While several bacterial determinants that facilitate intracellular infection by *F. tularensis* have been characterized (Asare et al., 2010; Moule et al., 2010), such as genes of the FPI, less is known about the host factors that are exploited or subverted by *F. tularensis*.

Some of the immune system processes are known to be manipulated by *F. tularensis* to avoid being attacked by the host. For instance, *F. tularensis* ssp. *novicida* delays inflammasome activation (reviewed in Weiss et al., 2007b). However, until recently there has been no comprehensive genome-wide analysis that has been conducted to identify all host genes that are important for *F. tularensis* infection. Since until recently it has been difficult to conduct extensive genetic manipulation in the mammalian hosts, many investigators have used *D. melanogaster* to model microbial diseases (Cherry, 2008). The genetic tractability of *Drosophila* has enabled the identification of host-encoded factors that affect the pathogen–host interaction at both the cellular and molecular levels in many pathogens, such as *L. monocytogenes*, *M. marinum*, and *Legionella pneumophila* (Dionne et al., 2003; Cheng et al., 2005; Dorer et al., 2006). It has also been shown that infection of *D. melanogaster* cells by intracellular bacterial pathogens is similar to infection of mammalian host cells. Thus, it is likely that the intracellular infection requires conserved host factors in mammals and arthropods.

In contrast to many other pathogens for which *D. melanogaster* has been used to identify host factors required for the pathogen–host interaction (Cherry, 2008), *F. tularensis* is naturally transmitted

Table 1 | A combined list of genes essential for *F. tularensis* lethality to adult fruit flies.

Gene loci (U112)	Gene product	Gene	Ahlund et al. (2010)	Asare et al. (2010)	Moule et al. (2010)
FTN_0019	Aspartate carbamoyltransferase	pyrB	×		
FTN_0020	Carbamoyl-phosphate synthase large chain	carB	×		
FTN_0021	Carbamoyl-phosphate synthase small chain	carA	×		
FTN_0024	Dihydroorotase	pyrC			×
FTN_0030	Hypothetical membrane protein			×	
FTN_0035	**Orotidine-5-phosphate decarboxylase**	**pyrF**	**×**		**×**
FTN_0036	**Dihydroorotate dehydrogenase**	**pyrD**	**×**		**×**
FTN_0038	Hypothetical protein			×	
FTN_0051	Conserved protein of unknown function			×	
FTN_0052	Protein of unknown function			×	
FTN_0063	**Branched-chain amino acid aminotransferase protein (class IV)**	**ilvE**		*****	**×**
FTN_0066	Ferrous iron transport protein B	feoB	×		
FTN_0068	Oligoribonuclease	orn			×
FTN_0077	Protein of unknown function			×	
FTN_0078	Shikimate 5-dehydrogenase	aroE1			×
FTN_0090	Acid phosphatase (precursor)	acpA			×
FTN_0096	Conserved hypothetical membrane protein		**×**		
FTN_0097	Hydroxy/aromatic amino acid permease (HAAAP) family protein			×	
FTN_0101	Transcription regulator				×
FTN_0107	GTP-binding protein LepA	lepA		×	
FTN_0109	**Protein of unknown function**		**×**	**×**	
FTN_0111	Riboflavin synthase beta-chain	ribH		×	
FTN_0113	Riboflavin synthase alpha chain	ribC	×		
FTN_0115	**Overlaps Na+/H+ antiporter NHAP, fragment**			**×**	**×**
FTN_0124	Single-strand DNA binding protein	ssb			×
FTN_0141	ABC transporter, ATP-binding protein			×	
FTN_0162	Cell division protein FtsQ	ftsQ		×	
FTN_0190	Major facilitator superfamily (MFS) transport protein, fragment				×
FTN_0192	Cytochrome ***d*** terminal oxidase, polypeptide subunit II	cydB			×
FTN_0207	Protein of unknown function containing a von Willebrand factor type A (vWA) domain			×	
FTN_0214	Valyl-tRNA synthetase	valS			×
FTN_0217	L-lactate dehydrogenase	lldD			×
FTN_0266	Chaperone Hsp90, heat shock protein HtpG	htpG		×	
FTN_0275	Hypothetical protein				×
FTN_0330	Septum formation inhibitor-activating ATPase	minD	×	×	
FTN_0331	Septum formation inhibitor	minC	×		
FTN_0337	Fumarate hydratase, class I	fumA			×
FTN_0338	MutT/nudix family protein			×	
FTN_0344	Aspartate:alanine antiporter				×
FTN_0346	OmpA family protein				×
FTN_0384	Conserved hypothetical protein			×	
FTN_0392	Transcriptional regulator, LysR family				×
FTN_0404	Peptide methionine sulfoxide reductase	msrB			×
FTN_0409	Alcohol dehydrogenase class III, pseudogene				×
FTN_0412	DNA repair protein recN				×
FTN_0416	Lipid A 1-phosphatase	lpxE			×
FTN_0429	Hypothetical protein				×
FTN_0439	Protein of unknown function			×	

(Continued)

Table 1 | Continued

Gene loci (U112)	Gene product	Gene	Ahlund et al. (2010)	Asare et al. (2010)	Moule et al. (2010)
FTN_0482	Protein of unknown function			×	
FTN_0493	5-Methylthioadenosine/S-adenosylhomocysteine nucleosidase	mtn			×
FTN_0494	Hypothetical membrane protein				×
FTN_0495	BNR/Asp-box repeat protein				×
FTN_0496	Soluble lytic murein transglycosylase	slt	×		
FTN_0504	Lysine decarboxylase			×	
FTN_0507	Glycine cleavage system P protein, subunit 1	gcvP1		×	
FTN_0513	1,4-α-Glucan branching enzyme	glgB	×		
FTN_0516	Glycogen synthase	glgA		×	
FTN_0525	Bifunctional aspartokinase/homoserine dehydrogenase I, pseudogene	thrA			×
FTN_0526	Homoserine kinase (pseudogene)	thrB			×
FTN_0538	Conserved hypothetical membrane protein				×
FTN_0545	Glycosyl transferase, group 2			×	
FTN_0546	Dolichyl-phosphate-mannose-protein mannosyltransferase family protein				×
FTN_0549	Stringent starvation protein A	sspA	×		
FTN_0554	tRNA/rRNA methyltransferase	yibK			×
FTN_0567	tRNA synthetase class II (D, K and N)			×	
FTN_0577	DNA mismatch repair enzyme with ATPase activity	mutL		×	
FTN_0588	Asparaginase			×	
FTN_0593	Succinyl-CoA synthetase, alpha subunit	sucD		×	
FTN_0599	Conserved hypothetical protein				×
FTN_0599	Protein of unknown function			×	
FTN_0600	DNA gyrase subunit B	gyrB			×
FTN_0603	Formamidopyrimidine-DNA glycosylase	mutM			×
FTN_0623	2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase	ispD			×
FTN_0627	Chitinase, glycosyl hydrolase family 18	chiA		×	
FTN_0649	FAD-binding family protein, pseudogene				×
FTN_0651	Cytidine deaminase	cdd		×	
FTN_0652	Uridine phosphorylase	udp			×
FTN_0653	tRNA-(ms(2)io(6)a)-hydroxylase	miaE			×
FTN_0655	Methylase				×
FTN_0660	Cytosol aminopeptidase	pepA			×
FTN_0664	Type IV pili fiber building block protein				×
FTN_0666	Excinuclease ABC, subunit A	uvrA			×
FTN_0667	Major facilitator superfamily (MFS) transport protein	yieO			×
FTN_0672	Preprotein translocase, subunit A	secA	×		
FTN_0673	DNA-3-methyladenine glycosylase I (pseudogene)	tag			×
FTN_0692	Quinolinate sythetase A	nadA		×	
FTN_0696	Hypothetical membrane protein			×	
FTN_0728	Predicted Co/Zn/Cd cation transporter			×	
FTN_0732	Hypothetical protein			×	
FTN_0741	Proton-dependent oligopeptide transporter (POT) family protein, di- or tripeptide:H+ symporter			×	
FTN_0750	L-Serine dehydratase 1	sdaA			×
FTN_0759	Conserved hypothetical protein			×	
FTN_0760	Conserved hypothetical protein				×
FTN_0768	Tryptophan-rich sensory protein	tspO		×	
FTN_0770	Major facilitator superfamily (MFS) transport protein,	bcr1			×

(Continued)

Table 1 | Continued

Gene loci (U112)	Gene product	Gene	Ahlund et al. (2010)	Asare et al. (2010)	Moule et al. (2010)
	pseudogene				
FTN_0772	Conserved protein of unknown function		×		
FTN_0773	4Fe–4S ferredoxin (electron transport) family protein, pseudogene	yjeS			×
FTN_0774	Conserved hypothetical protein				×
FTN_0781	Transaldolase	talA			×
FTN_0783	Isochorismatase hydrolase family protein				×
FTN_0790	Recombination associated protein	rdgC			×
FTN_0791	Protein of unknown function			×	
FTN_0806	Glycosyl hydrolase family 3		×		
FTN_0810	ROK family protein			×	
FTN_0824	Major facilitator superfamily (MFS) transport protein, pseudogene				×
FTN_0826	Aldo/keto reductase				×
FTN_0838	Exodeoxyribonuclease III	xthA			×
FTN_0840	**Modulator of drug activity B**	**mdaB**		**×**	**×**
FTN_0848	**Amino acid antiporter**		**×**		**×**
FTN_0855	Protein of unknown function			×	
FTN_0861	**Type IV pili fiber building block protein**	**pilA**		**×**	**×**
FTN_0875	Major facilitator superfamily (MFS) transport protein				×
FTN_0877	Cardiolipin synthetase	cls		×	
FTN_0885	Proton-dependent oligopeptide transporter (POT) family protein	yhiP			×
FTN_0886	Hypothetical membrane protein				×
FTN_0887	Hypothetical protein				×
FTN_0888	Hypothetical membrane protein				×
FTN_0889	Helix-turn-helix family protein				×
FTN_0891	Holliday junction DNA helicase, subunit B	ruvB			×
FTN_0898	Amino acid permease				×
FTN_0900	Protein of unknown function with predicted hydrolase and phosphorylase activity			×	
FTN_0921	FKBP-type peptidyl-prolyl ***cis***-trans isomerase				×
FTN_0928	Sulfate adenylyltransferase subunit 2	cysD		×	
FTN_0949	50S ribosomal protein L9	rplI		×	
FTN_0954	Histidine acid phosphatase			×	
FTN_0959	Oxidative stress transcriptional regulator	oxyR			×
FTN_0972	Hypothetical protein				×
FTN_0975	Hypothetical protein				×
FTN_0976	ThiF family protein, pseudogene				×
FTN_0978	Ubiquinone biosynthesis protein				×
FTN_0982	Glutaredoxin 1	grxA			×
FTN_0984	ABC transporter, ATP-binding protein			×	
FTN_0997	Proton-dependent oligopeptide transporter (POT) family protein, di- or tripeptide:H+ symporter			×	
FTN_1006	Transporter-associated protein, HlyC/CorC family			×	
FTN_1014	Nicotinamide mononucleotide transport (NMT) family protein				×
FTN_1016	Hypothetical protein				×
FTN_1026	Major facilitator superfamily (MFS) transport protein, pseudogene				×
FTN_1027	**Holliday junction endodeoxyribonuclease**	**ruvC**		**×**	**×**
FTN_1034	Iron-sulfur cluster-binding protein	rnfB		×	

(Continued)

Table 1 | Continued

Gene loci (U112)	Gene product	Gene	Ahlund et al. (2010)	Asare et al. (2010)	Moule et al. (2010)
FTN_1038	Conserved hypothetical membrane protein				×
FTN_1058	Trigger factor (TF) protein	tig	×		
FTN_1066	Metal ion transporter protein				×
FTN_1073	DNA/RNA endonuclease G			×	
FTN_1091	3-Phosphoshikimate 1-carboxyvinyltransferase	aroA			×
FTN_1099	Transcriptional regulator, LysR family				×
FTN_1115	Type IV pili nucleotide binding protein, ABC transporter, ATP-binding protein	pilB			×
FTN_1116	Type IV pili polytopic inner membrane protein	pilC			×
FTN_1135	3-Dehydroquinate synthetase	aroB		×	
FTN_1137	**Type IV pilin multimeric outer membrane protein**	**pilQ**		**×**	**×**
FTN_1168	Exodeoxyribonuclease VII large subunit	xseA			×
FTN_1170	Conserved protein of unknown function			×	
FTN_1171	Conserved hypothetical lipoprotein				×
FTN_1174	Glutamate racemase	murI		×	
FTN_1176	Excinuclease ABC, subunit B	uvrB			×
FTN_1179	Transcriptional regulator, LysR family				×
FTN_1186	**Metallopeptidase family M13 protein, pseudogene**			**×**	**×**
FTN_1196	Conserved hypothetical UPF0133 protein	ybaB			×
FTN_1198	Guanosine-3,5-bis(diphosphate) 3-pyrophosphohydrolase/(p)ppGpp synthase	spoT			×
FTN_1214	Glycosyl transferase, family 2				×
FTN_1215	Capsule polysaccharide export protein KpsC	kpsC		×	
FTN_1220	Bacterial sugar transferase family protein				×
FTN_1223	Conserved hypothetical membrane protein			×	
FTN_1240	BolA family protein		×		
FTN_1241	DedA family protein			×	
FTN_1243	DNA repair protein recO	recO			×
FTN_1257	Membrane protein of unknown function				×
FTN_1261	Protein of unknown function			×	
FTN_1268	Mycobacterial cell entry (mce) related family protein				×
FTN_1275	Drug:H+ antiporter-1 (DHA2) family protein			×	
FTN_1276	Membrane fusion protein				×
FTN_1278	NH(3)-dependent NAD(+) synthetase	nadE			×
FTN_1282	LysR transcriptional regulator family protein				×
FTN_1290	Macrophage growth locus, subunit A	mglA			×
FTN_1291	Macrophage growth locus, subunit B	mglB			×
FTN_1300	LysR transcriptional regulator family protein				×
FTN_1309	Protein of unknown function	pdpA	×		
FTN_1310	**Conserved hypothetical protein; conserved hypothetical protein**	**pdpB; pdpB1**	**×**		**×**
FTN_1311	Protein of unknown function	iglEa	×		
FTN_1312	Conserved hypothetical protein	vgrGa	×		
FTN_1313	Hypothetical protein	iglFa	×		
FTN_1314	Conserved hypothetical protein	iglGa	×		
FTN_1315	Protein of unknown function	iglHa	×		
FTN_1316	Conserved protein of unknown function	dotUa	×		
FTN_1317	Protein of unknown function	iglIa	×		
FTN_1318	Hypothetical protein	iglJa	×		

(Continued)

Table 1 | Continued

Gene loci (U112)	Gene product	Gene	Ahlund et al. (2010)	Asare et al. (2010)	Moule et al. (2010)
FTN_1319	**Conserved hypothetical protein; conserved hypothetical protein**	**pdpC**		×	×
FTN_1321	**Intracellular growth locus, subunit D; subunit D**	**iglD; iglD1**	×	×	×
FTN_1322	**Intracellular growth locus, subunit C; subunit C1**	**iglC; iglC1**	×	*	×
FTN_1323	Conserved protein of unknown function	iglB	×		
FTN_1324	Conserved protein of unknown function	iglA	×		
FTN_1343	Conserved protein of unknown function			×	
FTN_1357	**ATP-dependent exoDNAse (exonuclease V) beta subunit**	**recB**	×		×
FTN_1359	Exodeoxyribonuclease V gamma chain	recC			×
FTN_1368	Fe2+ transport system protein A	feoA	×		
FTN_1372	Protein of unknown function			×	
FTN_1376	Disulfide bond formation protein, DsbB family			×	
FTN_1386	Protein of unknown function			×	
FTN_1406	Conserved hypothetical membrane protein			x	
FTN_1409	**Major facilitator superfamily (MFS) transport protein, pseudogene**			×	×
FTN_1412	DNA-directed RNA polymerase e subunit		x		
FTN_1417	**Phosphomannomutase**	**manB**	×		×
FTN_1428	Transferase	wbtO		×	
FTN_1439	3-Ketoacyl-CoA thiolase	fadA			×
FTN_1441	**Sugar transport protein, pseudogene**			×	×
FTN_1448	Protein of unknown function			×	
FTN_1452	Two-component response regulator				×
FTN_1457	Protein of unknown function			×	
FTN_1465	Two-component response regulator	pmrA			×
FTN_1488	Prophage maintenance system killer protein (DOC)			×	
FTN_1491	Adenine specific DNA methylase			×	
FTN_1494	Pyruvate dehydrogenase complex, E1 component, pyruvate dehydrogenase	aceE		×	
FTN_1501	Monovalent cation:proton antiporter-1				×
FTN_1513	Site-specific recombinase	xerC			×
FTN_1518	GDP pyrophosphokinase/GTP pyrophosphokinase	relA		×	
FTN_1530	Diaminopimelate decarboxylase	lysA			×
FTN_1534	Conserved protein of unknown function			×	
FTN_1542	Conserved protein of unknown function			×	
FTN_1549	Drug:H+ antiporter-1 (DHA1) family protein			×	
FTN_1552	Acid phosphatase, PAP2 family			×	
FTN_1557	Oxidoreductase iron/ascorbate family protein				×
FTN_1580	Helicase				×
FTN_1582	Hypothetical membrane protein				×
FTN_1584	Glycerol-3-phosphate dehydrogenase	glpD		×	
FTN_1593	ABC-type oligopeptide transport system, periplasmic component	oppA		×	
FTN_1595	Signal recognition particle receptor FtsY	ftsY			×
FTN_1599	Nucleoside permease NUP family protein	nupC			×
FTN_1600	Nucleoside permease NUP family protein	nupC1			×
FTN_1608	Disulfide bond formation protein	dsbB	×		
FTN_1611	Major facilitator superfamily (MFS) transport protein	–		×	

(Continued)

Table 1 | Continued

Gene loci (U112)	Gene product	Gene	Ahlund et al. (2010)	Asare et al. (2010)	Moule et al. (2010)
FTN_1612	Hypothetical protein			×	
FTN_1617	Sensor histidine kinase	qsec			×
FTN_1618	Conserved hypothetical protein				×
FTN_1621	Predicted NAD/FAD-dependent oxidoreductase			×	
FTN_1630	Preprotein translocase, subunit G, membrane protein	secG			×
FTN_1654	Major facilitator superfamily (MFS) transport protein				×
FTN_1657	Major facilitator superfamily (MFS) transport protein				×
FTN_1658	Histidyl-tRNA synthetase	hisS			×
FTN_1678	NADH dehydrogenase I, C subunit	nuoC		×	
FTN_1682	Conserved hypothetical protein				×
FTN_1683	Drug:H+ antiporter-1 (DHA1) family protein				×
FTN_1685	Drug:H+ antiporter-1 (DHA1) family protein			×	
FTN_1692	Secretion protein				×
FTN_1704	Protein-L-isoaspartate O-methyltransferase	pcm			×
FTN_1714	Transcriptional regulatory protein, pseudogene	kdpE			×
FTN_1715	Two-component sensor protein	kdpD			×
FTN_1716	Potassium-transporting ATPase C chain	kdpC			×
FTN_1718	Potassium-transporting ATPase, A chain, pseudogene	kdpA			×
FTN_1719	NAD-dependent formate dehydrogenase, fragment				×
FTN_1733	Conserved hypothetical membrane, pseudogene				×
FTN_1743	Chaperone ClpB	clpB	×		
FTN_1745	Phosphoribosylglycinamide formyltransferase 2	purT			×
FTN_1750	Acyltransferase				×
FTN_1753	Oxidase-like protein, pseudogene				×
FTN_1762	ABC transporter, ATP-binding protein	yjjK			×
FTN_1763	Major facilitator superfamily (MFS) transport protein				×
FTN_1776	Anthranilate synthase component II, pseudogene	trpG1			×

*An × marks a screen, where the gene was identified to be essential for lethality to flies, whereas * marks a gene not essential for lethality to flies, but the gene is important for reduction of bacterial load in the indicated screen (mentioned because it was found in at least one other screen). Genes found in multiple screens are shown in a bold font.*

to mammalian hosts by arthropod vectors. While many pathogens can only be transmitted by a single specific arthropod vector species, *F. tularensis* is associated with various arthropods ranging from ticks to multiple species of mosquitoes to biting flies such as deer flies. This makes the *D. melanogaster* model system particularly useful for studying both general *F. tularensis* host–pathogen interaction and insect-specific factors. Thus, we can expect that *F. tularensis* targets many insect specific factors that *D. melanogaster* is likely to harbor.

A recent study has used a genome-wide RNAi screen to identify host factors that contribute to intracellular proliferation of *F. tularensis* within *D. melanogaster*-derived cells. In this screen at least 186 host factors have been shown to be required for intracellular bacterial proliferation (Akimana et al., 2010). The discovery of these genes initiated studies to uncover host processes that are likely important in the arthropod vector. The predominant functional category of the host factors identified in the screen are involved in signal transduction, indicating that *F. tularensis* modulate many host signaling molecules for its own advantage (Hrstka et al., 2005; Al-Khodor and Abu Kwaik, 2010). Silencing mammalian homolog of the factors identified in the *D. melanogaster* RNAi screen shows that four conserved factors are also required for replication of *F. tularensis* in human cells (**Table 2**): the Ras/Rho guanyl-nucleotide exchange factor activity SOS2, the PI4 kinase PI4KCA, the ubiquitin hydrolase USP22, and the ubiquitin ligase CDC27 (Akimana et al., 2010; Al-Khodor and Abu Kwaik, 2010). Furthermore, one of these evolutionally conserved factors, the CDC27 ubiquitin ligase, is required for evading lysosomal fusion and for bacterial escape into the cytosol (Akimana et al., 2010).

The SOS2 mammalian host factor and its arthropod homolog sos has been shown to be important for proliferation of *F. tularensis* in S2 cells and human cells. Intracellular *F. tularensis* ssp. *novicida* triggers temporal and early activation of Ras through the SOS2/ GrB2/ PKCα/PKCβI, and that this signaling cascade is essential for intracellular bacterial proliferation within the cytosol, and associated with down-regulation of early caspase-3 activation, which promotes survival of the infected cells (Al-Khodor and Abu Kwaik, 2010). Thus, using *D. melanogaster* as a model, host factors important for *F. tularensis* intracellular proliferation in the arthropod host have been identified, and some are conserved in mammalian cells (**Table 2**).

Table 2 | List of evolutionally conserved host factors involved in intracellular trafficking of *F. tularensis* in both *D. melanogaster* and human cells.

Category	Description	*Drosophila Melanogaster* gene	Human homolog gene
Cell cycle	Mitosis	cdc27	CDC27*[a]
Proteolysis	Ubiquitin thiolesterase activity	not	USP22[a]
Signal transduction	Ras/Rho guanyl-nucleotide exchange factor activity	sos	SOS2[b]
Signal transduction	1-Phosphatidylinositol 4-kinase activity	CG10260	PI4KCA[a]

**Indicates that this gene is also involved in escape of F. tularensis in HEK293T cells. [a]Indicates from a study by Akimana et al. (2010), and [b]indicates from a study by Al-Khodor and Abu Kwaik (2010).*

ENVIRONMENTAL FACTORS RELEVANT TO ARTHROPOD-MAMMALIAN ADAPTATION

Although this review focuses largely on the genes required for arthropod and mammalian infection, other important studies identified some environmental factors that are relevant to arthropod–mammalian transition of *F. tularensis*. Horzempa et al. (2008) has examined the impact of arthropod-like versus mammalian-like temperatures, 26°C versus 37°C, respectively on gene regulation of *F. tularensis*. Interestingly, they found that the FPI genes *pdpC, iglC,* and *iglD* were down-regulated at 26°C (Horzempa et al., 2008), yet these genes are required for *F. tularensis* survival in *D. melanogaster* as shown in **Table 1** (Asare et al., 2010; Moule et al., 2010). However, *pdpC*, which is significantly down-regulated in arthropod-like temperature, is dispensable for infection of Sua1B mosquito-derived cells (Read et al., 2008; Vonkavaara et al., 2008; Santic et al., 2009). It will be interesting to test whether *pdpC* has a similar role in *F. tularensis* as the role of OspC in *B. burgdorferi*; i.e., requirement for initial mammalian infection (Schwan et al., 1995; Tilly et al., 2006). Alternatively, FTL_1581, a hypothetical lipoprotein induced by mammalian temperature (Horzempa et al., 2008) could have a similar role as OspC. In addition, *F. tularensis* ssp. *novicida* has been shown to alter its outer membrane at 25 versus 37°C by differentially modifying the lipid A component of the lipopolysaccharide, but this modification does not alter the virulence of *F. tularensis* (Shaffer et al., 2007). Another interesting environment factor is that spermine and spermidine are novel triggers to alert *F. tularensis* of its eukaryotic host environment (Carlson et al., 2009). All these differences in mammalian-like versus arthropod-like conditions observed reveal bits of patho-adaptation by *F. tularensis* in arthropods and human that still needs to be elucidated. However, it is important to note that the temperature is only one variable between the environments of the two hosts and that the actual composition of the environments and the host–microbe interaction within these distinct hosts are much more complex than just the temperature variable.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

Arthropod-borne transmission of *F. tularensis* is responsible for maintaining tularemia in nature and is of significant concern worldwide. So far, there are many unanswered questions pertaining to *F. tularensis*–arthropod vector interaction and its role in patho-adaptation to infect mammals. The study of arthropod vectors–*F. tularensis* interaction or comparing these studies to mammalian studies helps us understand patho-adaption aspect of this bacterium in its diverse hosts.

Outbreaks of *F. tularensis* are connected to the arthropod transmission. Thus, it is desirable to develop strategies to reduce arthropod vector transmission of tularemia. Francis showed that the transmission of *F. tularensis* occurs through the tick feces rather than through the salivary gland, unlike other blood-feeding arthropods, such as Lyme disease transmitting ticks. Although ticks transmit *F. tularensis* transovarial, one other possibility is transmission of this pathogen from one tick developmental stage to the other through feces, which is a frequent method of transmission in small mammals and birds.

While mammals such as guinea pigs, mice, and humans are very susceptible to *F. tularensis* infections, arthropod vectors that are natural host of *F. tularensis* are able to limit the severity of infection by *F. tularensis*. It will be interesting to identify factors underlining the difference in theses two evolutionary distant hosts. Many bacterial factors are required for intracellular proliferation within both arthropod-derived and human-derived cells. In addition, many eukaryotic host factors conserved in arthropods and mammals are required for intracellular proliferation of *F. tularensis* within the two evolutionarily distant hosts. Therefore, it is likely that patho-adaptation of *F. tularensis* in the arthropod vector has allowed this bacterium to successfully infect the human host.

Many studies to date have utilized *D. melanogaster* as a general model and have shown that it is a tractable genetic arthropod vector model of tularemia. A unique advantage of using *D. melanogaster* as a model of *F. tularensis* is that *F. tularensis* infections are transmitted to mammalian hosts by at least three established arthropod vectors: ticks, biting flies, and mosquitoes, whereas in almost all other arthropod-borne diseases, only one arthropod vector is solely responsible for transmitting the disease. Studies utilizing the well studied and genetically tractable model *D. melanogaster*, are likely to help us understand the arthropod host, since *F. tularensis* likely uses similar virulence strategies to infect its diverse arthropods hosts. However, additional studies are needed to fully establish *D. melanogaster* as a vector model to decipher *F. tularensis*–arthropod vector interaction.

As shown in **Table 1**, three large-scale screens using *F. tularensis* transposon insertion mutants have led to the rapid identification of 250 different genes required for *F. tularensis in vivo* infection of *D. melanogaster*. Overall, there is a poor overlap between hits identified in these studies. The FPI genes *iglB, iglC, iglD,* and *mglA* have been previously identified to be required for *F. tularensis* infection of the *D. melanogaster* and were expected to become hits in all theses screens, but only *iglC* and *iglD* were identifies by all the screens. These results are not surprising since an inherent problem of large-scale screens is the presence of false positive and false negative hits. In addition, transposon mutants might not exhibit a loss of function phenotype. These results suggest that this overwhelming amount of

data need to be analyzed by first looking at overlapping information from these studies; and also suggest that even non-overlapping data is essential and should be further analyzed as well.

Interestingly, studies with the insect model *D. melanogaster* have shown that the FPI genes are equally important in the arthropod models. Furthermore, a large number of bacterial factors are required for proliferation within both *D. melanogaster* and mammalian cells. Since the tick is a major vector of *F. tularensis* infections, future studies should determine the role of these factors in the tick.

To reduce transmission and morbidity associated with arthropod-borne tularemia, not only bacterial factors are important, but also arthropod host factors may be used to develop therapeutic measures against *F. tularensis*. Recent microbial pathogenesis studies are uncovering more about bacterial effectors that modulate important host processes. Numerous *Drosophila* genes that are essential for *F. tularensis* infection have been identified. To further confirm the role of arthropod host genes play in the pathogenesis by *F. tularensis*, one could study and screen for host genes important for *F. tularensis* virulence *in vivo* in the arthropod hosts by using *D. melanogaster* mutants defective in host genes essential for *F. tularensis* virulence. It has also been shown that *F. tularensis* targets some evolutionarily conserved host factors for intracellular survival and growth. Determining whether other *D. melanogaster* genes have mammalian homologs, and whether these homologs are also involved in intracellular infection or other biological function, will be at the crux of our understanding of bacteria–arthropod interaction and its role in patho-adaptation to infect mammals. In the future, bioinformatics studies should facilitate the dissection of biochemical pathways that are important for *F. tularensis* infection by using both bacterial and host genes shown to date to be essential for *F. tularensis* in the arthropod host. The accumulated knowledge of vector–*F. tularensis* interactions will ultimately allow the development of strategies to prevent and treat tularemia.

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Intra-vacuolar proliferation of *F. novicida* within *H. vermiformis*

Marina Santic[1]*†, Mateja Ozanic[1†], Vildana Semic[1], Gordana Pavokovic[1], Valentina Mrvcic[1] and Yousef Abu Kwaik[2]

[1] *Department of Microbiology and Parasitology, Medical Faculty, University of Rijeka, Rijeka, Croatia*
[2] *Department of Microbiology and Immunology, College of Medicine, University of Louisville, Louisville, KY, USA*

Edited by:
Anders Sjostedt, Umeå University, Sweden

Reviewed by:
Sahar H. El-Etr, Lawrence Livermore National Laboratory, USA
Svenja Stöven, Umeå University, Sweden

****Correspondence:***
Marina Santic, Department of Microbiology and Parasitology, Medical Faculty, University of Rijeka, HR-51000 Rijeka, Croatia.
e-mail: marina@medri.hr

†Marina Santic and Mateja Ozanic have contributed equally to the work.

Francisella tularensis is a gram negative facultative intracellular bacterium that causes the zoonotic disease tularemia. Free-living amebae, such as *Acanthamoeba* and *Hartmannella*, are environmental hosts of several intracellular pathogens. Epidemiology of *F. tularensis* in various parts of the world is associated with water-borne transmission, which includes mosquitoes and amebae as the potential host reservoirs of the bacteria in water resources. *In vitro* studies showed intracellular replication of *F. tularensis* within *A. castellanii* cells. Whether ameba is a biological reservoir for *Francisella* in the environment is not known. We used *Hartmannella vermiformis* as an amebal model system to study the intracellular life of *F. novicida*. For the first time we show that *F. novicida* survives and replicates within *H. vermiformis*. The *iglC* mutant strain of *F. novicida* is defective for survival and replication not only within *A. castellanii* but also in *H. vermiformis* cells. In contrast to mammalian cells, where bacteria replicate in the cytosol, *F. novicida* resides and replicates within membrane-bound vacuoles within the trophozoites of *H. vermiformis*. In contrast to the transient residence of *F. novicida* within acidic vacuoles prior to escaping to the cytosol of mammalian cells, *F. novicida* does not reside transiently or permanently in an acidic compartment within *H. vermiformis* when examined 30 min after initiation of the infection. We conclude that *F. tularensis* does not replicate within acidified vacuoles and does not escape into the cytosol of *H. vermiformis*. The *Francisella* pathogenicity island locus *iglC* is essential for intra-vacuolar proliferation of *F. novicida* within *H. vermiformis*. Our data show a distinct intracellular lifestyle for *F. novicida* within *H. vermiformis* compared to mammalian cells.

Keywords: *Francisella novicida, Hartmannella vermiformis,* vacuolar replication, LysoTracker, *iglC*

INTRODUCTION

Francisella tularensis is a gram negative, facultative intracellular bacterium that causes the zoonotic disease tularemia in humans and animals, and various recent reviews in this special topic issue have discussed various aspects of *Francisella* (Chong and Celli, 2010; Meibom and Charbit, 2010; Akimana and Abu Kwaik, 2011; Asare and Abu Kwaik, 2011; Bosio, 2011; Bröms et al., 2011; Cremer et al., 2011; Dai et al., 2011; Gavrilin and Wewers, 2011; Jones et al., 2011; Zogaj and Klose, 2011). Tularemia is a zoonotic disease of the northern hemisphere. Humans acquire infection by exposure to infected arthropod vectors, or by handling, ingesting, or inhaling infectious materials. *F. tularensis* has been isolated from over 250 animal species, including fish, birds, amphibians, rabbits, squirrels, hares, voles, ticks, and flies (Santic et al., 2010; Akimana and Abu Kwaik, 2011). Three closely related subspecies of *F. tularensis* have been identified: *tularensis*, *holarctica*, and *mediasiatica* (Forsman et al., 1994). Recently *F. novicida* has been accepted as new species (Sjöstedt, 2005). It has been suggested that *holarctica* ssp. has a strong association with water-borne disease (Greco et al., 1987; Thelaus et al., 2009; Broman et al., 2011). An *in vitro* study showed that *F. tularensis* subsp. *holarctica* can survive and grow within *Acanthamoeba castellanii* (Abd et al., 2003). In addition, *F. tularensis* subsp. *holarctica* was found within amebal cysts, suggesting potential for long-term survival and an important environmental reservoir for tularemia. The isolation of the bacterium from a water eco-system, as well as from natural spring water (Thelaus et al., 2009; Willke et al., 2009; Broman et al., 2011), supports the hypothesis that protozoa may serve as a reservoir for *F. tularensis* in nature (Morner, 1992; Thelaus et al., 2009; Broman et al., 2011). Very little is known about the *F. tularensis*–ameba interaction.

It has been shown that within mammalian and arthropod-derived cells, the *Francisella* containing phagosome (FCP) transiently matures to an acidified late endosomal stage with limited fusion to lysosomes, followed by rapid bacterial escape into the host cell cytosol (Clemens et al., 2004; Chong et al., 2008; Santic et al., 2008, 2009; Asare and Abu Kwaik, 2011). The FCP is acidified by the vATPase proton pump within 15–30 min of phagosome biogenesis, which is essential for subsequent rapid disruption of the FCP and escape of *F. tularensis* into the host cell cytosol, where the bacterium replicates (Chong et al., 2008; Santic et al., 2008; Chong and Celli, 2010; Asare and Abu Kwaik, 2011; Bröms et al., 2011; Dai et al., 2011). Inhibition of the vATPase proton pump causes a significant delay in phagosomal escape and blocks bacterial proliferation (Chong et al., 2008; Santic et al., 2008; Chong and Celli, 2010; Asare and Abu Kwaik, 2011; Bröms et al., 2011), indicating a major role for acidification of the FCP in rapid bacterial escape into the cytosol and subsequent replication (Chong et al., 2008; Santic et al., 2008; Chong and Celli, 2010; Asare and Abu Kwaik, 2011; Bröms et al., 2011).

A gene cluster, the *Francisella* pathogenicity island (FPI), that regulates phagosomal escape and intracellular survival of *F. tularensis* within macrophages, has been identified (Nano et al., 2004; Nano and Schmerk, 2007; Meibom et al., 2009). It has been suggested to encode a type VI-like secretion system (de Bruin et al., 2007; Nano and Schmerk, 2007; Bingle et al., 2008; Ludu et al., 2008; Barker et al., 2009; Bröms et al., 2011). It has also been shown that IglC is essential for avoiding lysosomal fusion (Santic et al., 2005b; Bonquist et al., 2008) and for bacterial escape into the host cytosol (Lindgren et al., 2004; Santic et al., 2005a) in macrophages. In addition, the *iglC* mutation diminishes intracellular replication in *A. castellanii* (Lauriano et al., 2004).

Free-living amebae such as *Acanthamoeba* and *Hartmannella* are environmental hosts of several intracellular pathogens such as *Legionella*, *Chlamydia*, and *Mycobacterium* (Amann et al., 1997; Abu Kwaik et al., 1998; Steinert et al., 1998; Molmeret et al., 2005). It has been shown that legionellae interact with their protozoan hosts and mammalian cells in a similar way (Harb et al., 2000). Since the host reservoir of *F. tularensis* in water systems is not known, we used *H. vermiformis*, which is the most predominant non-pathogenic ameba in water resources, as an amebal model system to study the intracellular life of *F. novicida*. Our data indicate that *F. novicida* survives within *H. vermiformis* and that the bacteria do not escape into the cytoplasm, which is very distinct from the lifestyle of *F. novicida* within mammalian cells. The *iglC* bi-cistronic locus plays an important role in intra-vacuolar replication in *H. vermiformis*.

MATERIALS AND METHODS

BACTERIA AND PROTOZOAN STRAINS AND MEDIA

The wild type (wt) *F. novicida* strain U112 and it isogenic *iglC* mutant were grown on buffered-charcoal yeast extract (BCYE) agar plates and have been described previously (Santic et al., 2005b). Construction of ΔiglC::ermC has been described previously (Lauriano et al., 2003). The *iglD* gene was not affected. The tetracycline-resistant plasmid pKK214, encoding green fluorescent protein (GFP), was introduced into *F. novicida* (Abd et al., 2003).

Acanthamoeba castellanii and *H. vermiformis* were obtained from the American Type Culture Collection, 30234 and 1034, respectively. The amebae were grown in medium 30234 and 1034 at 25°C, as described elsewhere (Pedersen et al., 2001; Viswanathan et al., 2002).

INFECTION AND INTRACELLULAR SURVIVAL ASSAY IN AMEBAL CELLS

Infection of protozoan strains with *F. novicida* has been described previously (Abu Kwaik, 1996; El-Etr et al., 2009). Briefly, triplicate cultures of protozoan strains were seeded into 96-well plates at 1×10^5 amebal cells/well and allowed to adhere for a few hours at 25°C. The amebae were washed and infected with *F. novicida* at a multiplicity of infection (MOI) of 10. After coincubation for 15 min, the cells were washed once and incubated with 100 μg gentamicin/ml for 1 h at 37°C and 5% CO_2, followed by gentamicin treatment at the end of all time points examined. The amebae were then washed once to remove gentamicin and lysed with Triton-X100 (0.1%) for 10 min. The number of *F. novicida* in each well was determined by plating serial dilutions on BCYE agar plates.

CONFOCAL LASER SCANNING AND ELECTRON MICROSCOPY

For confocal microscopy, acidification of the *Francisella* containing vacuoles (FCVs) was determined using the lysosomotropic agent LysoTracker Red DND-99 (Molecular Probes). *H. vermiformis* cells were grown on glass cover slips in 24-well plates and then used for subsequent invasion assays with live or heat-killed bacteria. Briefly, triplicate cultures of protozoan strains were seeded into plates at 1×10^5 amebal cells/well and allowed to adhere for a few hours at 25°C. The amebae were washed and infected with *F. novicida* at a MOI of 10. After coincubation for 15 min, the cells were washed once and incubated with 100 μg gentamicin/ml for 1 h at 37°C and 5% CO_2. Thirty minutes prior to the time point, the amebal cells were washed and incubated with 1 ml of 1 μM LysoTracker, washed three times with PBS, fixed with 4% paraformaldehyde, and then mounted on glass slides for confocal microscopy analysis. All confocal microscopy analyses were performed on one hundred infected amebal cells from three different cover slips, for each time point in each experiment, and all experiments were performed three times. The analysis of colocalization has been performed on individual optical sections. The quantification was performed manually on a FV1000 Olympus confocal microscope. The images shown in the figures are stacks of 15 one-micron-thick Z-series sections.

For electron microscopy, triplicate cultures of protozoan strains were seeded into 12-well plates at 1×10^5 amebal cells/well and allowed to adhere for a few hours at 25°C. The amebae were washed and infected with *F. novicida* at a MOI of 10. After coincubation for 15 min, the cells were washed once and incubated with 100 μg gentamicin/ml for 1 h at 37°C and 5% CO_2. At several time intervals, the infected and uninfected monolayers were fixed for transmission electron microscopy with glutaraldehyde, post fixed with OsO_4 in Sorenson's buffer (pH 7.4), dehydrated with ethanol, and embedded in Epon (Miller-Stephenson), as described previously (Santic et al., 2005b). The sections were stained with lead citrate and uranyl acetate and examined with a Phillips Morgany transmission electron microscopy.

RESULTS

INTRACELLULAR REPLICATION OF *F. NOVICIDA* WITHIN *A. CASTELLANII* AND *H. VERMIFORMIS*

Previous studies have shown that *F. tularensis* subsp. *holarctica and tularensis*, and *F. novicida* survive and replicate in *A. castellanii* (Abd et al., 2003; Greub and Raoult, 2004; Lauriano et al., 2004; Hazlett et al., 2008; El-Etr et al., 2009). Since *H. vermiformis* is the most predominant non-pathogenic ameba in water supplies, we determined the ability of *F. novicida* to replicate within *H. vermiformis* and compared that to *A. castellanii* during early and late stages of infection. In addition, it has been shown that *iglC* is required for growth in macrophages and *Acanthamoeba* (Abd et al., 2003; Greub and Raoult, 2004; Lauriano et al., 2004; Hazlett et al., 2008; El-Etr et al., 2009), but there is no evidence about the intracellular replication of *F. novicida* within *H. vermiformis*.

The amebal cells (1×10^5 amebal cells/well) were infected with *F. novicida* and/or the *iglC* mutant at a MOI of 10 for 15 min, followed by treatment with gentamicin for 1 h to kill extracellular bacteria,

followed by further incubation. The time at the end of 15 min of infection was considered T_0. At different time points after infection (2, 24, 48, and 72 h), the amebal cells were lysed and bacteria were grown on agar plates to determine the number of colony forming units (CFU).

The data showed that *F. novicida* exhibited robust replication and bacterial numbers increased by 10,000-fold by ~48 h after infection within *A. castellanii* cells (**Figure 1A**). We also determined the ability of *F. novicida* to replicate within *H. vermiformis*. *F. novicida* replicated in *H. vermiformis* cells at a much faster rate than in *A. castellanii* cells, and the bacterial number increased by 10,000-fold within 24 h after infection (**Figure 1B**). The *iglC* mutant was unable to multiply in *A. castellanii* or *H. vermiformis*, and their viability decreased dramatically by 48 h post-infection (**Figures 1A,B**). Only around 10% of cells were lysed by 48 h after infection (data not shown). The above data showed that *F. novicida* survives and replicates intracellularly within *A. castellanii* and *H. vermiformis*, and that the *iglC* bi-cistronic locus is necessary for intracellular growth and survival within *H. vermiformis*.

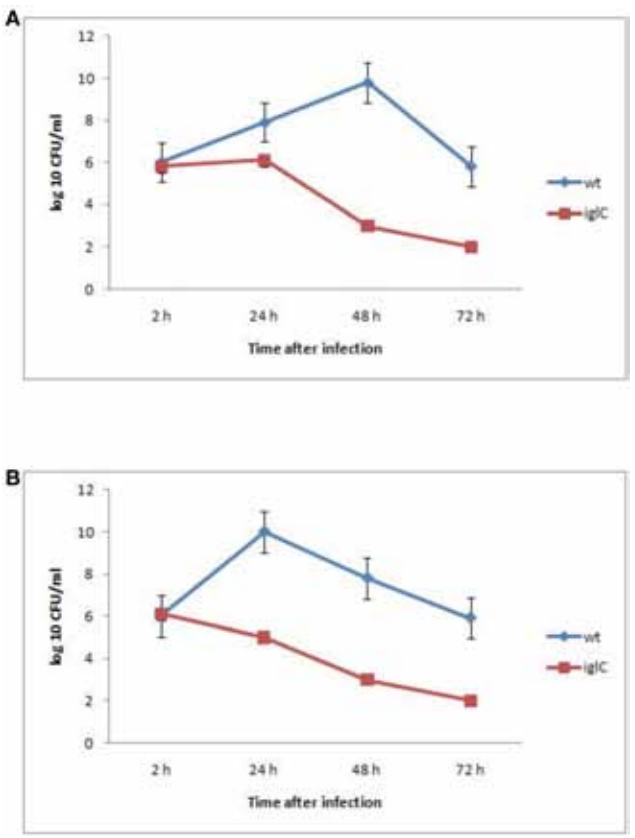

FIGURE 1 | Intracellular growth of *F. novicida* and its isogenic mutant *iglC* in *A. castellanii* (A) and in *H. vermiformis* (B). The cells were infected for 15 min, followed by gentamicin treatment, and determination of the number of intracellular bacteria at the indicated time points. The error bars represent standard deviations of triplicate samples and results shown are representative of three independent experiments.

F. TULARENSIS REPLICATES IN VACUOLES WITHIN *H. VERMIFORMIS* CELLS

It has been shown that the FCP matures to a late-endosome-like-phagosome prior to bacterial escape into the cytosol of macrophages, where bacterial proliferation occurs (Clemens et al., 2004; Santic et al., 2005b, 2010; Chong and Celli, 2010; Asare and Abu Kwaik, 2011; Bröms et al., 2011). The process of phagosomal disruption is rapid and occurs within 30 min. of infection in mammalian cells (Chong et al., 2008; Santic et al., 2008; Chong and Celli, 2010; Asare and Abu Kwaik, 2011; Bröms et al., 2011). The *iglC* locus is essential for bacterial escape into the cytosol of macrophages (Lindgren et al., 2004; Santic et al., 2005b; Asare and Abu Kwaik, 2011). Therefore, we examined at the ultra-structural level the intracellular infection of *H. vermiformis* with *F. novicida*.

Up to 2 h after infection by *F. novicida*, the bacteria were localized in intact vacuoles within *H. vermiformis* (**Figures 2A,B**), and efficient phagocytosis of bacteria by the amebae was observed. Almost 95% of the vacuoles harboring *F. novicida* had intact vacuolar membranes. By 6 h after infection, the bacteria were still enclosed in intact vacuoles within amebal cells, and still 90% of *H. vermiformis* harboring the bacteria had intact vacuolar membranes (**Figures 2A,B**). There was clear evidence that by 6 h bacterial replication had been initiated, since only one bacterium per vacuole was detected at 2 h while two to six bacteria per vacuole were detected by 6 h post-infection. During all time points examined more than 90% of bacteria were intra-vacuolar. Only 10% of the intracellular bacteria were localized in the cytosol.

By 24 h after infection ~90% of the vacuoles were intact with a clear and distinct vacuolar membrane (**Figures 2A,B**). Similar results to intra-vacuolar localization of the wt strain have been also observed for the *iglC* mutant. The mutant resides in intact vacuoles within *H. vermiformis* cells at all time points after infection (**Figures 2A,B**). However, the *iglC* mutant failed to replicate within the vacuole. In addition, *H. vermiformis* did not differentiate into cysts during the time points examined. There have been just a few cysts containing multiplying bacteria.

We conclude that in contrast to mammalian cells, where bacteria do not replicate in the vacuole but escape into the cytosol where they replicate, *F. novicida* does not escape into the cytosol, but replicates within the vacuoles in *H. vermiformis*.

F. TULARENSIS DOES NOT RESIDE IN AN ACIDIC COMPARTMENT WITHIN *H. VERMIFORMIS*

Previous data in human macrophages showed that within the first 15 min after infection, ~90% of the FCPs acquire the lysosomotropic dye LysoTracker, which concentrates in acidic compartments (Chong et al., 2008; Santic et al., 2008; Chong and Celli, 2010; Asare and Abu Kwaik, 2011; Bröms et al., 2011). Colocalization of phagosomes harboring the *iglC* mutant with the LysoTracker dye was persistent, which is consistent with fusion to the lysosomes and failure of the *iglC* mutant to escape into the macrophage cytosol (Lindgren et al., 2004; Santic et al., 2005b; Chong and Celli, 2010; Asare and Abu Kwaik, 2011; Bröms et al., 2011). The acquired lysosomotropic dye is gradually lost by 30–60 min post-infection, which coincides with bacterial escape into the cytosol of human monocyte derived macrophages (hMDMs; Chong et al., 2008; Santic et al., 2008;

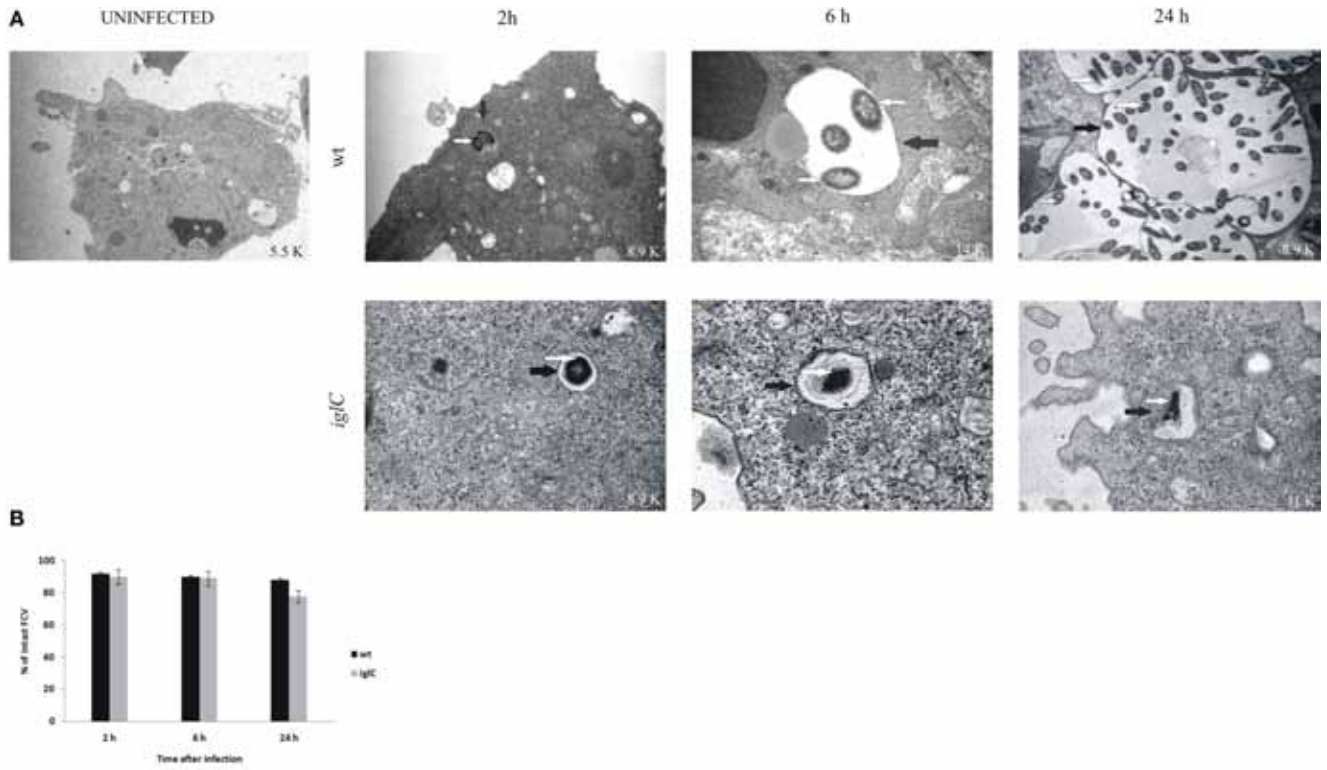

FIGURE 2 | *Francisella novicida* replicates in vacuoles of *H. vermiformis*. **(A)** Representative electron micrographs of *H. vermiformis* infected with wt *F. tularensis* subsp. *novicida* or the *iglC* mutant at 2, 6, and 24 h after infection. Thin black arrows show intact vacuolar membranes and white arrows show bacteria. **(B)** Quantitative analysis of the integrity of vacuolar membranes containing wt *F. novicida* or *iglC* in *H. vermiformis* cells. The percentage of disrupted vacuoles harboring bacteria at different time points.

Chong and Celli, 2010; Asare and Abu Kwaik, 2011; Bröms et al., 2011). This transient acidification is essential for subsequent bacterial escape and replication in the macrophage cytosol (Chong et al., 2008; Santic et al., 2008; Chong and Celli, 2010; Asare and Abu Kwaik, 2011; Bröms et al., 2011). Based on our ultra-structural studies, we did not observe dramatic changes between 1 and 2 h after infection and 6 and 12 h after infection; therefore we monitored the acidification of the vacuoles at 30 min, 1, and 12 h by using the lysosomotropic agent LysoTracker Red DND-99, which concentrates in acidified vesicles and compartments.

Our results showed that 90% of the wt bacteria in FCVs did not co-localize with the LysoTracker Red DND-99 dye at 30 min, 1, or 12 h post-infection (**Figures 3A,B**). In contrast, many of the *iglC* mutant-containing vacuoles acquired the LysoTracker Red DND-99 dye (~55% colocalization) at 12 h after infection (**Figures 3A,B**). We conclude that *F. tularensis* does not reside transiently or permanently in acidic compartments within *H. vermiformis*, which is distinct from mammalian cells.

DISCUSSION

Epidemiology of *F. tularensis* in various parts of the world is associated with water-borne transmission, which includes mosquitoes and amebae as the potential host reservoirs of the bacteria in water resources (Thelaus et al., 2009; Chong and Celli, 2010; Akimana and Abu Kwaik, 2011; Asare and Abu Kwaik, 2011; Bosio, 2011; Broman et al., 2011; Bröms et al., 2011; Dai et al., 2011; Gavrilin and Wewers, 2011; Jones et al., 2011; Zogaj and Klose, 2011). However, the main aquatic reservoir of the bacterium is still not known, but likely includes mosquitoes and amebae. Recently, it has been shown that *F. novicida* and LVS utilize *A. castellanii* as a natural reservoir (Abd et al., 2003; Hazlett et al., 2008; El-Etr et al., 2009). In addition, *F. tularensis* LVS and *F. novicida* survive in *A. castellanii* for weeks of infection (Abd et al., 2003; Hazlett et al., 2008; El-Etr et al., 2009). Surprisingly, *F. novicida* multiplied to a much higher degree in *H. vermiformis* in comparison to what has been found in *A. castellanii* (Abd et al., 2003; Hazlett et al., 2008; El-Etr et al., 2009). Another explanation is that we used rich 30234 medium compared to the El-Etr et al. (2009) study, where they used High Salt buffer, which does not support *F. novicida* replication. At 24 h after infection there were 10^{10} CFU/ml in *H. vermiformis*. In addition, there was a sudden drop in bacterial numbers, probably due to lysis by amebae. El-Etr et al. (2009) showed that by 30 min post-infection, the bacteria were in spacious vacuoles and continued to replicate until 24 h after infection. They suggested that spacious vacuoles are not lysosomal in nature, and that enclosure within these vacuoles may provide a survival advantage to *F. novicida*, SCHU S4 and other virulent strains of *F. tularensis*. However, it was not examined whether the bacteria replicated within vacuoles, and whether

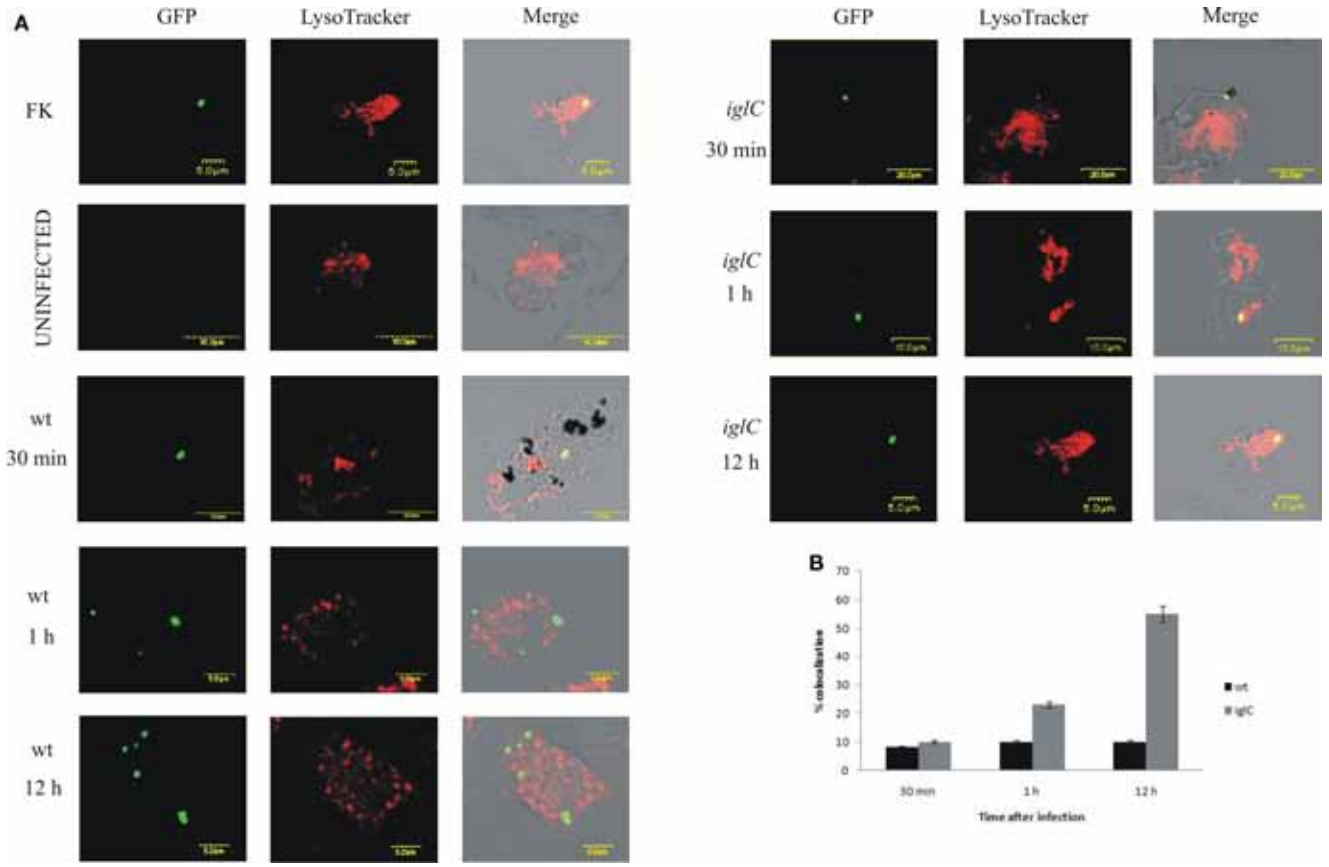

FIGURE 3 | *Francisella novicida* does not reside in acidic compartments within *H. vermiformis* cells. (A) Representative confocal microscopy images of colocalization of FCVs with the LysoTracker dye by the GFP-expressing wt *F. novicida* and *iglC* at 30 min, 1, and 12 h post-infection is shown. Uninfected cells were used as a negative control, while formalin killed bacteria served as a positive control. The images are representatives of 100 infected cells examined from three different cover slips. The results shown are representative of three independent experiments. **(B)** Quantification of colocalization of the LysoTracker DND-99 dye with the FCVs of the bacterium at 30 min, 1, and 12 h post-infection is shown. The results shown are representative of three independent experiments, and error bars represent standard deviations of triplicate samples.

formation of vacuoles was necessary for efficient replication (El-Etr et al., 2009). The long-term survival of pathogenic *F. tularensis* in amebae is dependent on induction of amebal cyst formation. In comparison to *A. castellanii*, *H. vermiformis* did not differentiate into cysts. There have been just a few cysts containing multiplying bacteria, which is very different from *A. castellanii*. The same culture conditions have been used as in the El-Etr et al. (2009) study. It is possible that the encystation process is delayed in *H. vermiformis* in comparison to *A. castellanii* infected with *F. novicida*.

Lauriano et al. (2004) showed that *F. tularensis mglA* and *iglC* mutant strains are not only defective for survival and replication within the macrophage-like cell line, but also within *A. castellanii*. In the present study we examined the interaction of *F. novicida* with *H. vermiformis* and the role of the FPI gene *iglC* in this interaction. The results show, for the first time, that *F. novicida* can survive and grow within *H. vermiformis*. The bacteria replicate and grow in vacuolar structures inside the trophozoites of *H. vermiformis*. Our ultra-structural studies showed that the vacuoles are tight and intact at 2, 6, and 24 h after infection. The *iglC* locus plays an important role in survival and replication of bacteria within *H. vermiformis* cells.

Other previous studies have shown that within mammalian cells, *F. tularensis* resides in acidic vacuoles before escaping to the cytosol where it replicates (Chong et al., 2008; Santic et al., 2008; Chong and Celli, 2010; Asare and Abu Kwaik, 2011; Bröms et al., 2011). Recently El-Etr et al. (2009) have shown that ~40% of the FCVs harboring *F. novicida* co-localized with the acidic dye, LysoTracker Red, in *A. castellanii* vacuoles at 2 h after infection. However, our results in *H. vermiformis* show that only 10% of the FCVs harboring *F. novicida* co-localize with the acidic dye at 30 min, 1, and 12 h after infection. The effect of the lysotracker was examined in uninfected cells as well as in *H. vermiformis* cells with formalin killed bacteria. In both cases *H. vermiformis* cells were in the trophozoite stage, and the lysotracker did not affect the physiology of the amebae. Whether the lysotracker affects cyst formation or vice versa has not been examined in this study. El-Etr et al. (2009) also showed that there was little difference between colocalization of the FCVs with LysoTracker Red for the different *Francisella* subsp. (*holarctica*-derived LVS and *novicida*). Our data show that many of the *iglC* mutant-containing FCVs acquired the LysoTracker Red DND-99 dye at a late stage of infection

of *H. vermiformis*, which likely coincided with loss of bacterial viability (**Figure 1**). Similar observations have been described in macrophages (Santic et al., 2005b). Our results clearly show that *F. novicida* does not reside transiently or permanently in acidic compartments within *H. vermiformis* cells after 30 min of initiation of the infection.

CONCLUSION

There are major differences in the life style of *F. tularensis* within various protozoa and macrophages. The bacteria multiply to a higher degree in *H. vermiformis* in comparison to *A. castellanii* cells (Abd et al., 2003; Hazlett et al., 2008; El-Etr et al., 2009). The formation of cysts is not significant in *H. vermiformis* cells compared to *Acanthamoeba* (Abd et al., 2003; Hazlett et al., 2008; El-Etr et al., 2009). Our results showed intra-vacuolar replication of *F. novicida* within *H. vermiformis*. This is very different from mammalian cells, where cytosolic location of bacteria is a key component in productive intracellular replication. In *H. vermiformis* the bacteria do not escape from the vacuole into the cytosol to replicate. In contrast, they are enclosed in vacuoles. Despite the major differences between the intracellular lifestyles within *H. vermiformis* and macrophages, the IglC protein is essential for bacterial proliferation in both hosts. It is clear that this protein is essential for intra-vacuolar proliferation in amebae, but is also essential for evasion of lysosomal fusion/and or bacterial escape into the cytosol in macrophages. It is likely that these two events in the evolutionarily distant host cells are due to a secondary effect of the biological function of IglC, which still remains to be determined.

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Toward an understanding of the perpetuation of the agent of tularemia

Sam R. Telford III* and Heidi K. Goethert

Division of Infectious Diseases, Cummings School of Veterinary Medicine, Tufts University, North Grafton, MA, USA

Edited by:
Anders Sjostedt, Umeå University, Sweden

Reviewed by:
Raquel Escudero, Institutes of Health Carlos III – National Center of Microbiology, Spain
Jeannine Petersen, Centers for Disease Control and Prevention, USA

****Correspondence:***
Sam R. Telford III, Division of Infectious Diseases, Cummings School of Veterinary Medicine, Tufts University, 200 Westboro Road, North Grafton, MA 01536, USA.
e-mail: sam.telford@tufts.edu

The epidemiology of tularemia has influenced, perhaps incorrectly skewed, our views on the ecology of the agent of tularemia. In particular, the central role of lagomorphs needs to be reexamined. Diverse observations, some incidental, and some that are more generally reproducible, have not been synthesized so that the critical elements of the perpetuation of *Francisella tularensis* can be identified. Developing a quantitative model of the basic reproduction number of *F. tularensis* may require separate treatments for Type A and Type B given the fundamental differences in their ecology.

Keywords: tularemia, perpetuation, basic reproduction number, ecology

INTRODUCTION

The ecology of tularemia is represented by a literature that reflects the diversity of this complex zoonosis. Like the literature of many other infections, the sheer volume of observations makes it difficult to organize and synthesize sets of working hypotheses for how the causative agent exists in nature. An organized understanding of tularemia ecology serves as the basis for developing public health interventions and to predict or explain changes in incidence or distribution. In addition, knowing how the agent is currently maintained in nature provides information that helps us to reconstruct its evolutionary history. We review herein features of tularemia ecology that are particularly critical and suggest lacunae that hinder us from distinguishing major themes from variations on themes. The reader is referred to excellent reviews of the subject by Hopla (1974), Friend (2006), and Petersen et al. (2009) for significantly more detail on the breadth and diversity of the ecological literature.

GENERAL COMMENTS ON THE ECOLOGY OF INFECTIOUS AGENTS

We use some basic terms and concepts in the population biology of infectious agents that help to organize our interpretation of the existing literature. Maintenance refers to the life cycle of the agent: how one infection gives rise to at least one other infection. Perpetuation is maintenance over larger periods of time. Perpetuation of zoonotic infections may involve a vector, an intermediary in the life cycle that imparts directionality to the agent. Hematophagous arthropods are vectors because they require blood and thus directly transport an infectious agent to a relevant host. In contrast, scalars may also maintain an agent, but there is no directionality; copepods containing *Dracunculus medinensis* nematodes, for example, are passively imbibed with water. Vectors may support biological transmission of an agent, a process that involves replication or developmental changes. Mechanical transmission is contaminative; no replication or change is implied.

Vectorial capacity (Spielman and Rossignol, 1984) refers to the sum of vector traits that ensures that the basic reproduction number (BRN) of an infection exceeds unity and comprises competence (ability to support replication and effectively deliver the agent) as well as factors such as abundance, longevity, and narrowness of host range. A mosquito that requires a large dose of pathogen and rarely passes it during feeding has poor vector competence and thus might not contribute much to BRN, but even a highly competent vector (agent replicates well and is readily ejected during feeding) can have poor vectorial capacity if it feeds only on an animal that is a "dead end" host for the pathogen. The central question in the ecology of infectious agents is to describe how an agent ensures that BRN > 1 (Anderson and May, 1981); BRN < 1 implies unstable transmission and extinction. Quantitative modeling of BRN helps to synthesize diverse field observations and rank the contributions of factors and influences.

Basic reproduction number models may be developed from simple flow charts representing the presumed life cycle of the infectious agent; boxes can be outcomes (e.g., number of non-immune hosts) and arrows are processes (e.g., infection). Such compartmental models (**Figure 1**) can then be quantified by expressing the transitions between boxes as differential equations. We could adopt for the purposes of constructing a general tularemia model conditions similar to that for pasteurellosis in mice (Anderson and May, 1979). The agents of tularemia and pasteurellosis are categorized as microparasites: they are physically small with short generation times; they have a high replicative rate within a host; they tend to induce immunity to reinfection in survivors; and, the duration of infection is short relative to host lifespan. In addition, these infections may modify the demography of their host. If for the purposes of constructing a model, we

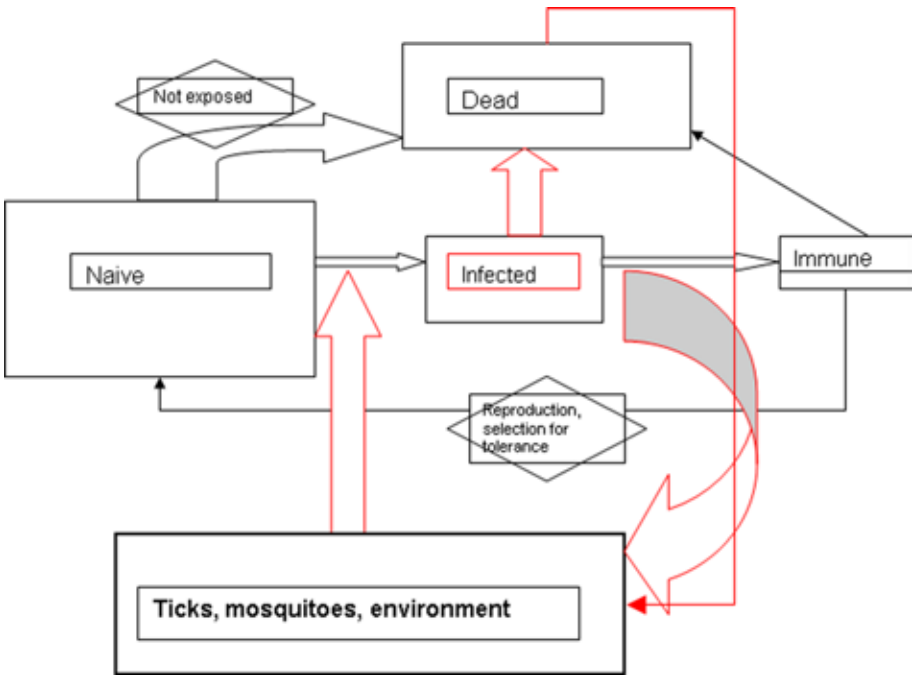

FIGURE 1 | Flow chart ("compartmental model") for general model of *F. tularensis* perpetuation. Rectangles represent hosts; size of rectangle might represent magnitude of host population. Red lines or arrows represent path taken by *F. tularensis*. Mode of transmission is a black box which comprises multiple factors. No time scale is implied in this simple model.

assume that the only mode of tularemia transmission is direct (no vectors; transmission by contact with, inhalation of or ingestion of saliva, excreta, or blood), then model development may proceed as outlined in Anderson and May (1979). The model then distils down to critical variables such as absolute number of susceptibles (non-infected); number that are infected; number immune; the natural mortality rate of the host population; the rate of introduction of susceptibles (immigration, birth); and a measure of the acquisition of infection (contact rate of susceptibles with infected). Even at this rudimentary level of discussion, we can see how difficult it would be to have an empirical basis for the model: we still debate the identity of the reservoir host for *Francisella tularensis* – or whether the reservoir might be environmental – and even if we assumed a specific animal, would we have data on its demography, behavior (contact between individuals), or prevalence of infection? However, by constructing such models, we can prioritize the field observations required for us to refine and validate the models, which summarize our current understanding of the life cycle. Resources should be expended in describing the circumstances of acquiring infection over determining the number of infected hosts, for example, because in the model the process drives the outcome.

THE ROLE OF RABBITS IN TYPE A ECOLOGY: CONFLATION WITH EPIDEMIOLOGIC RISK

> *Tularemia is a specific infectious disease due to Bacterium tularense and is transmitted from rodents to man by the bite of an infected bloodsucking insect or by the handling and dissecting of infected rodents by market men or laboratory workers.* (Francis, 1922)

The first written account of tularemia in the US was in 1907 noting signs and symptoms compatible with tularemia in Native Americans who had handled jackrabbits (Barnes, 1928). A "plague-like" disease in ground squirrels was identified in 1909 during animal surveillance in California but microscopic observation of tissue sections demonstrated organisms inconsistent with the characteristic "safety pin" morphology of plague bacilli (McCoy, 1911). The bacterium was quickly cultivated and it was apparent that a new entity had been discovered (McCoy and Chapin, 1912). Although Pearse (1911) first described "deer fly fever" in Utah residents bitten by tabanid flies, Francis (1922) demonstrated its identity with the ground squirrel disease, and proposed the name "tularemia." He provided definitive evidence by isolating the agent from fly bitten humans, from jackrabbits, and ground squirrels. Francis also provided experimental evidence for transmission of tularemia by the bites of deerflies, lice, and bugs (Francis, 1922). Investigations of the Bitterroot Valley Rocky Mountain spotted fever epidemic in the 1920s isolated *F. tularensis* from the main RMSF vector, *Dermacentor andersoni* (Parker et al., 1924). Other human biting ticks (*Dermacentor variabilis*, *Amblyomma americanum*) were soon documented as vectors (Philip and Jellison, 1934; Parker, 1934). Therefore, within 20 years of its discovery as an infection of rodents in California, the most important aspects of the epidemiology (factors relating to human risk) of tularemia in the US had been described, as summarized by Francis' pithy statement, but taken together, the sum of knowledge would not allow quantitative modeling of BRN (ecology) for *F. tularensis* in any site. It is not clear that 80 years later that we have sufficient information to do so.

Francis (1937) noted that >90% of the >6000 tularemia case reports that he had compiled from 1924 to 1935 were associated with exposure to cottontail rabbits or hares, and this analysis surely helped to formally develop tularemia's reputation as "rabbit fever." It is possible that the strong rabbit association was due to an active market for rabbit meat in the north central states where there was a tradition of rabbit hunting (Yeatter and Thompson, 1952). This rabbit association, interestingly, obscured the fact that in the south central US, tick exposure accounted for 70% of all cases (Brooks and Buchanan, 1970) during the 1960s. Tularemia incidence in the US started to diminish in the 1960s (Boyce, 1975), perhaps as a result of a loss in popularity of rabbits as food and of hunting in general. It seems unlikely that the force of transmission of the agent diminished in nature during this time. The tick vectors (*D. andersoni*, *D. variabilis*, and *A. americanum*) for tick-transmitted tularemia in the US are the same as those for RMSF, which increased in incidence during the 1960s and 1970s (Childs and Paddock, 2002).

Russia and Japan had concurrently "discovered" tularemia (Francis, 1934; Pollitzer, 1967). Episodes of morbidity and mortality in hares were associated with an increase in the number of human cases of "yato-byo" and the disease could be acquired by skin contact with hare tissues (Ohara, 1926). Apparently, 90% of all Japanese tularemia cases were associated with exposure to the hare *Lepus brachyurus* (Toyoshima and Ohara, 1967). Thus, in North America and Japan, during the very first decades of epidemiologic investigations of the disease, lagomorphs (rabbits and hares) were the main focus of attention. Researchers in the former Soviet Union were extremely active in investigations of tularemia, producing 1300 publications from 1928 to 1960 (Pavlovsky, 1966), and were the first to describe at least six perpetuation scenarios centered around habitat types (floodplain–swamp; meadow–field; forest; steppe; piedmont–river; and desert–floodplain); lagomorphs were requisites for none. Paradoxically, a hypothesis for the evolution of *F. tularensis* by Russia's most prominent tularemia researcher (Olsufiev, 1963) focused on associations with lagomorphs, mainly based on their great degree of "susceptibility and sensitivity" to infection as well as a scenario for the zoogeography of the steppes, which were thought by the former USSR workers to have been a pivotal habitat for tularemia.

Differences in distribution, ecology, biochemistry, and virulence led to the seminal classification of tularemia into distinct types (Olsufiev et al., 1959). Type A organisms (now known as *F. tularensis tularensis*) are prevalent in North America but not in Eurasia, are frequently transmitted by ticks, and may cause severe disease. Type B (*F. tularensis holarctica*) causes episodic outbreaks (epizootics) in beavers, muskrats, and arvicoline rodents in either North America or Eurasia, may be isolated from water or soil, and may cause a milder disease (Jellison et al., 1942; Parker et al., 1951). These eco-epidemiological paradigms retain tremendous utility and argue for modeling BRN separately.

The perception that tularemia was due to lagomorphs was largely the influence of Francis himself and also of William Jellison of the Rocky Mountain Laboratories, who compiled and interpreted the existing literature on tularemia biology in a seminal monograph (Jellison, 1974). Jellison argued that human risk and geographic distribution of North American tularemia was strongly associated with cottontail rabbits (Jellison and Parker, 1945; Jellison et al., 1961). Cottontail rabbits are very susceptible to infection by Type A, dying within 7 days, and are large enough animals to attract attention when there is an epizootic, making them good sentinels for transmission activity. Furthermore, because of their value as food, their populations were a focus of attention by local residents and by state game management divisions: 25,000,000 rabbits were killed annually with a value of $5,000,000 during the 1920s (Henderson and Craig, 1932).

Whether cottontail rabbits are required for Type A BRN > 1 remains unproven and requires further study. It may be that the question has been considered resolved due to conflating the requirements for maintenance with the proximal determinants for human exposure. Of course, human exposure (the subject of epidemiology) may provide clues to the mode of perpetuation (ecology) but this is not axiomatic. Zoonotic infections may exist in sites with no implied human risk in the absence of an effective epidemiological "bridge." The classical theory of natural nidality (Pavlovsky, 1966) posits that most zoonotic agents exist in longstanding foci that comprise optimal physical (weather, geology) and biological (fauna, flora) associations and that humans only become aware of their existence when they intrude. Accordingly, rabbits and hares may only be the epidemiological bridge and are not necessarily an element of natural focality.

The concept of rabbits as central to Type A ecology was bolstered by the identification of the rabbit tick, *Haemaphysalis leporispalustris*, as an effective enzootic vector (Parker 1934). These ticks are widely distributed across North America and their feeding is focused primarily on lagomorphs. Narrowness of host range (bites focused on relevant hosts) greatly contributes to vectorial capacity and BRN (Spielman and Rossignol, 1984). These ticks transmit *F. tularensis* and pass the agent by inheritance (transovarial transmission; Parker 1934); presumed Type A isolates were made from field collected *H. leporispalustris* (Philip and Parker, 1938). Rabbits may be infested by hundreds of these ticks and all stages may infest a rabbit simultaneously, thereby providing an opportunity for non-systemic (co-feeding) transmission (Randolph et al., 1996). Thus, rabbits and rabbit ticks could serve to powerfully maintain Type A. In addition, because subadults (larvae and nymphs) of this tick will infest ground-inhabiting birds, including those that migrate, the agent could be readily transported. Rabbit ticks range from Alaska to Argentina, reflecting such transport. This fact begs the question: why has *F. tularensis* not been detected in Latin America south of Mexico, particularly given the presence of rabbits throughout South America?

The central importance of rabbits and their ticks in Type A ecology is not supported by recent studies on Martha's Vineyard (MV). This is the only site in the US which has endemic primary (inhalational) pneumonic tularemia (Matyas et al., 2007); of more than 100 tularemia cases reported from MV from 2000 to 2010, nearly two-thirds have been pneumonic. Case control studies demonstrate that landscapers are the major risk group and use of lawnmowers or leaf blowers are the main risk factors, suggesting environmental contamination. Exposure to ticks or rabbits was not associated with risk (Feldman et al., 2001) other than for one case who mowed over a rabbit. Landscapers there insist that they rarely mow over animal carcasses because they visually inspect properties prior to their activities to reduce

hazard due to rocks and other debris. The nature of the fomites that served to infect these case-patients remains undescribed but unseen remnants of animal carcasses, animal feces, urine-soaked soil, ticks, tick feces, fleas, and contaminated water are possible sources. Why MV alone reports numerous pneumonic tularemia cases when tularemia is more prevalent in the south central US where lawnmowers are certainly used remains enigmatic. It is possible that the heavily salt-spray influenced landscape of the ocean facing southern edge of MV is more conducive to the agent remaining viable for a longer duration than elsewhere in the US (Berrada and Telford, in press).

Ecological studies, extended from Lyme disease surveillance starting in 1994, quickly suggested that dog ticks (*D. variabilis*) were important to Type A perpetuation (Goethert et al., 2004; Matyas et al., 2007; Goethert and Telford, 2009). Infection has been found in MV dog ticks every year to date, comprising a large degree of genetic heterogeneity (Goethert et al., 2009). Rabbits were indeed infected but virtually disappeared on MV, probably due to tularemia mortality; their disappearance did not influence the force of transmission of Type A, which continued to remain prevalent in dog ticks. Intensive studies of cottontail rabbits had been undertaken on Nantucket Island, which is visible from the eastern portion of MV. Rabbits attained densities of 15–20 per hectare and were heavily infested by *H. leporispalustris* (Telford and Spielman, 1989; Goethert and Telford, 2003). Evidence of tularemia had never been detected in more than 200 rabbits sampled on Nantucket, even though five human cases had been identified from 1990 to 2005. One of these cases was definitively associated with rabbits: a worker who had helped his colleague move a rabbit that had been mutilated by a lawnmower developed pneumonic tularemia (Goethert and Telford, 2005). This event demonstrated that even though Type A had been introduced at least once to Nantucket, despite the presence of dense rabbits and heavy *H. leporispalustris* infestations, the agent did not perpetuate. No mass die-offs of rabbits were noted during the year when the lawn mowing incident occurred, nor did active surveillance for rabbit carcasses by landscapers yield any evidence of mortality due to tularemia. There is one important difference in the ecology of Nantucket relative to MV: Nantucket lacks appreciable numbers of dog ticks (indeed, the tick may now be extirpated from that island due to the recent widespread use of topical anti-ectoparasiticides on dogs) because it does not have their reproductive hosts, skunks, raccoons, foxes, or coyotes. Although one exception to the rule of "rabbits maintain tularemia" does not necessarily invalidate the rule, we note that MV is one of few sites where longitudinal ecological studies have been undertaken and thus where incidental findings can be distinguished from general findings.

We suspect that rabbits are not necessarily critical to the BRN of Type A, or if they are, it is a function of local conditions. This dependence on local conditions, in fact, is the challenge of developing a quantitative model for the BRN of tularemia: should there be a general model, or should we approach the subject as did the researchers of the former USSR, focusing on independent natural foci? At one extreme, the 4 genotypes/subclades of Type A and 11 of Type B (Kugeler et al., 2009; Vogler et al., 2009) might each require a specific BRN model. But, a general model would have to assume that all elements for the ecology of Type A would apply to Type B and *vice versa*, not necessarily a good assumption.

Although our hypothesis is that dog ticks are critical to BRN on MV, experimentally infected as well as naturally infected ticks die more quickly than do those that are not (Reese et al., 2010; Goethert and Telford, submitted). A negative effect on fitness cannot be an adaptive strategy that would ensure BRN > 1. These findings stand in contrast to our empirical observation of infected ticks each year, and suggest that there may be factors that mitigate the negative fitness of infection in nature.

TYPE B ECOLOGY: DRIVEN BY WATER OR BY RODENTS?

Tularemia in Eurasia and non-rabbit or tick associated infection in North America seem to have a strong environmental basis, acquired from agricultural activities such as hay threshing; from water contaminated by muskrats or water voles; or during the trapping of furbearers (Pavlovsky, 1966; Syrjala et al., 1985; Reintjes et al., 2002; Allue et al., 2008). In addition, transmission of the pathogen could occur via contamination of foodstuffs by urine or fecal material from infectious rodents (Karpoff and Antonoff, 1936; Parker et al., 1951). Experimental studies with voles suggested the possibility that some *F. tularensis*-infected animals developed a chronic nephritis and bacteriuria that could serve as a protracted source of environmental contamination (Bell and Stewart, 1975). Of particular interest was the suggestion that voles became partially immune due to low level infection resulting from cannibalism of tularemic carcasses and that this immunity allowed survival of the vole during subsequent infection, increasing the probability for shedding in the excreta. (Cannibalism of moribund cagemates is well known as a mode of transmission for Type A in the laboratory, Owen and Buker, 1956, and could be a complementary factor in perpetuation.) This suggestion of orally induced immunity has not been explored further but if confirmed could be a critical factor for the BRN of Type B, particularly in the context of environmental persistence.

The role of vectors in Type B perpetuation remains to be fully described. Ticks may be infected by Type B and are said to be the "reservoir" (Pavlovsky, 1966). In the former Soviet Union, 17 species of ixodid ticks have been found to be naturally infected (Balashov, 1972), presumably by Type B inasmuch as Type A is virtually restricted to North America. In addition, Type B is well known to be tick-transmitted in North America and both types may be present in ticks in the same site (Markowitz et al., 1985). Human cases certainly result from tick exposure but this mode of transmission is less common than exposure to furbearers or contaminated water (Pavlovsky, 1966). *Dermacentor marginatus* and *Dermacentor reticulatus* appear to be the main vectors there as well as into central Europe. As with Type A, Type B-infected *D. marginatus* or *D. variabilis* die more rapidly than do uninfected ticks (Petrov, 1960; Reese et al., 2010), which again raises the question of whether a non-adaptive trait may be associated with stable BRN.

Mosquitoes are strongly implicated as vectors in Sweden, given that tularemic ulcers are most frequently found on the upper back, neck, and ears of case-patients (Eliasson and Back, 2007), where mosquitoes are more likely to feed. In addition, the agent has been isolated from mosquitoes there (Olin, 1942) and recent studies provide evidence for mosquito larvae acquiring infection from water, perhaps by the ingestion of predatory protozoa (Mathisen et al., 2009). GIS studies of the Orebro endemic area in Sweden

demonstrate that there is temporal–spatial association of incidence with mosquito breeding (Svensson et al., 2009). More analyses of the contribution of mosquitoes to BRN are needed. One recent study reported a third of mosquito pools to be infected in Alaska (Triebenbach et al., 2010) when tested by PCR, but this finding was at odds with the epidemiological evidence as well as with the difficulty of finding infection in animals. Older studies in Alaska failed to isolate *F. tularensis* from mosquitoes (Hopla, 1974) and thus it is not clear what the PCR findings represent; it should be noted that the assay that was used might also detect *Francisella novicida*. Future mosquito surveys should always attempt to confirm PCR findings with a complementary assay such as culture or even indirect immunofluorescence using monoclonal antibodies. At the very least, multiple gene targets should corroborate the findings. Definitive evidence for biological transmission by naturally infected mosquitoes might be provided by the use of deliberately placed sentinel mice but given the difficulties of animal experimentation in Sweden, not a likely approach. The recent suggestion that infectious agents may be detected by assaying sugar sources probed by mosquitoes (Hall-Mendelin et al., 2010) may be an effective alternative approach to demonstrating that naturally infected mosquitoes can transmit.

Given that mechanical transmission causes infection and thus BRN > 1, it might be considered academic to determine whether biological transmission occurs. But, the duration of mosquito infectivity would differ depending on whether it was mechanical or biological transmission, thereby impacting the magnitude of BRN. In addition, the possibility that mosquito larvae may acquire infection from water might greatly enhance BRN if those larvae became adult females that transmitted. Whether there is a main mode of perpetuation (e.g., ticks and rodents) with ancillary cycles (spillover into a water cycle, mechanical transmission by mosquitoes), whether it is the other way around (perpetuation within water and spillover into rodents and their ectoparasites) or whether there may be multiple parallel cycles in sites where there are ticks would be difficult to answer with field studies but might be facilitated by mathematical modeling of BRN. Such questions have more than just academic interest: if ticks and rodents drive the ecology of Type B, then intervention could be considered to reduce risk, for example, by rodent or tick control. If water drives the ecology, then risk reduction would have to focus on personal protection (e.g., with vaccination) given the difficulty of manipulating water ecosystems.

WHY IS TULAREMIA RELATIVELY RARE?

Both Type A and Type B are highly infectious and may be transmitted mechanically on the mouthparts of various hematophagous arthropods, or by contact with body or tissue fluids through abraded and even intact skin, and by ingestion, in addition to true biological (vector) transmission. Aerogenic infection was also very quickly noted by laboratory workers. Such a wide spectrum of modes of exposure and great infectivity helps to explain the wide range of kinds of animals known to be exposed or infected (Burroughs et al., 1945; Friend, 2006). It is likely that most of these animals do not serve as amplifying hosts ("reservoirs") that increase the BRN of the agent of tularemia, but are incidental "dead end" hosts. Many of the reports of an animal serving as host simply document exposure (seroreactivity) in a limited sample which does not allow inference about whether the exposure might be common over many sampling periods or among many sites. If an animal contributes significantly to BRN, it should do so for successive generations and in more than one site. This is the rationale for undertaking longitudinal ecologic studies: to determine whether an observation is incidental or is a generality.

The reason it is important to determine whether there is a main theme for perpetuation (one important reservoir host such as a lagomorph) as opposed to many themes (almost anything can serve as a reservoir) is that an ecological paradox otherwise exists: if virtually any hematophagous arthropod and vertebrate could maintain infection, then tularemia should be readily perpetuated and extremely common across the Holarctic. Thorough search of any site should document the presence of *F. tularensis*. From an epidemiological standpoint, tularemia is only moderately common, with global incidence in the range of 100–1000 cases annually (Paddock and Telford, in press). Tick surveys in known endemic sites generally record prevalence of *F. tularensis* infection in the range of 0.1–5% (e.g., Green, 1931; Hopla, 1960; Hubalek and Halouzka, 1997; Goethert et al., 2004) which is similar to that for tick borne encephalitis (TBE) virus in *I. persulcatus* complex ticks (Gresikova and Nosek, 1967; Korenberg, 1994; Schafer et al., 1999). TBE is considered to be a common tick borne infection, with 1000–10,000 new cases each year (Paddock and Telford, in press). TBE virus has a Palearctic distribution similar to that of tularemia. *F. tularensis* has been detected in *I. ricinus*, the main European vector of TBE. With the potential for transmission by mosquitoes, in addition to tick transmission and environmental exposure, tularemia risk (human incidence) over the Palearctic should approach or exceed that of TBE.

The question "why is tularemia not more common?" becomes even more vexing given the possibility of environmental persistence, that is, perpetuation that may be independent of vertebrate hosts. Surface water and sediment yielded indisputable DNA sequence evidence of contamination with Type B in Swedish endemic sites, even in years with little epidemiological activity (Broman et al., 2010). Infected carcasses contaminated water for 10 days and contaminated water stored in the cold infected animals after 2 weeks. Naturally contaminated mud remained infectious as long as 10 weeks (Parker et al., 1951). Experimental microcosm experiments demonstrated that *F. tularensis* in silt could infect animals for 2 months. About half of rodents immersed in contaminated water became infected with exposure to as few as 100–1000 cfu/mL (Pavlovsky, 1966), which appears to be large dose but the spleen alone of a mouse dying of tularemia may have 10^{10} cfu (Molins et al., 2010) and it would not take many carcasses to contaminate a contained body of water. Indeed, many have speculated that environmental persistence depends on continual contamination by dead animals. However, water invertebrates such as shrimp or snails could retain viable organisms for 20 days (Mironchuk and Mazepa, 2002), and indeed, invertebrates were first described as contributing to *F. tularensis* survival within water by former USSR research (Pavlovsky, 1966). More recent reports (Anda et al., 2001) of crayfish infection suggest that additional surveys using modern methods are warranted. Even more interesting is the hypothesis

that free-living amebae serve as hosts (Berdal et al., 1996; Abd et al., 2003; El-Etr et al., 2009), which would allow for even greater duration of persistence and even the possibility of amplification in the absence of vertebrates. In terms of BRN, environmental persistence within aquatic invertebrates or protozoa would have implications for ensuring that BRN > 1 when vertebrates are scarce but water contamination could serve to greatly increase BRN and trigger an epizootic or outbreak.

PRIORITIES FOR TULAREMIA ECOLOGY RESEARCH

The ecology of tularemia is sufficiently complex that limited resources for research should be targeted to the most relevant questions. Developing quantitative models of the BRN for tularemia, probably separately for Type A and Type B, would appear to be a priority because such models provide structure for observations. Critical life cycle breakpoints and influential variables can be hypothesized *a priori* and tested by field observations. Surveys should be undertaken to identify useful study sites where the agent is reliably detected and longitudinal observations (on a scale of years) may be undertaken. Cooperative agreements should be developed among researchers and long term funding jointly sought to support such studies. We have outlined some of the more interesting lacunae in our knowledge of tularemia ecology, and additional information for all of these would greatly enhance iterative refinements of BRN quantitative models in the future.

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