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The *Francisella tularensis* proteome and its recognition by antibodies

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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

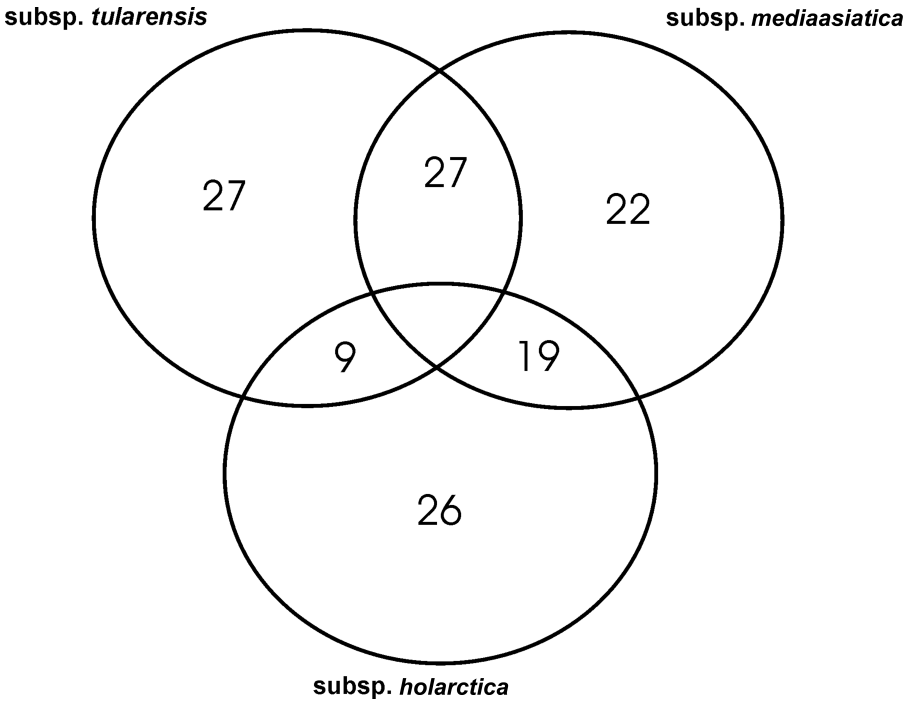

FIGURE 1 | Using 2D-PAGE coupled with mass spectrometric methods for protein identification, Hubalek et al. (2004) identified proteins that were unique to specific subspecies, or differentially expressed between them.

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).*

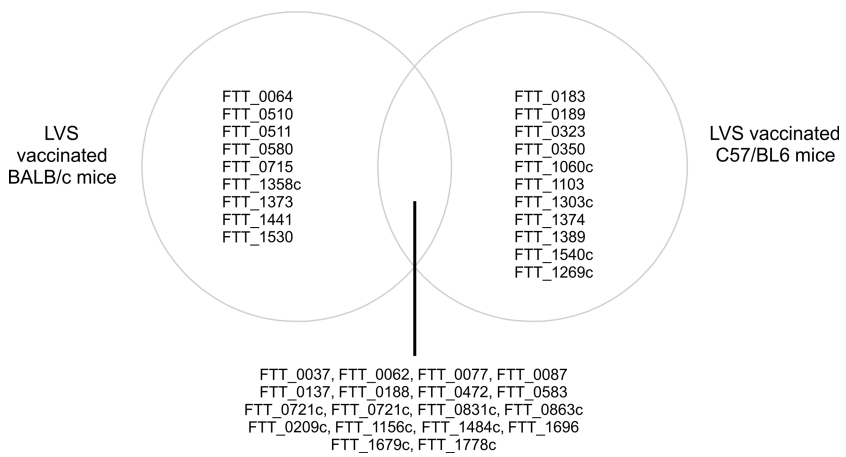

FIGURE 2 | Venn diagram showing the intersection of immunoreactive proteins observed to be reactive with sera from LVS vaccinated and protected BALB/c mice and LVS vaccinated and unprotected C57/BL6 mice (Twine et al., 2010). Left hand side lists immunoreactive proteins only observed to be reactive with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

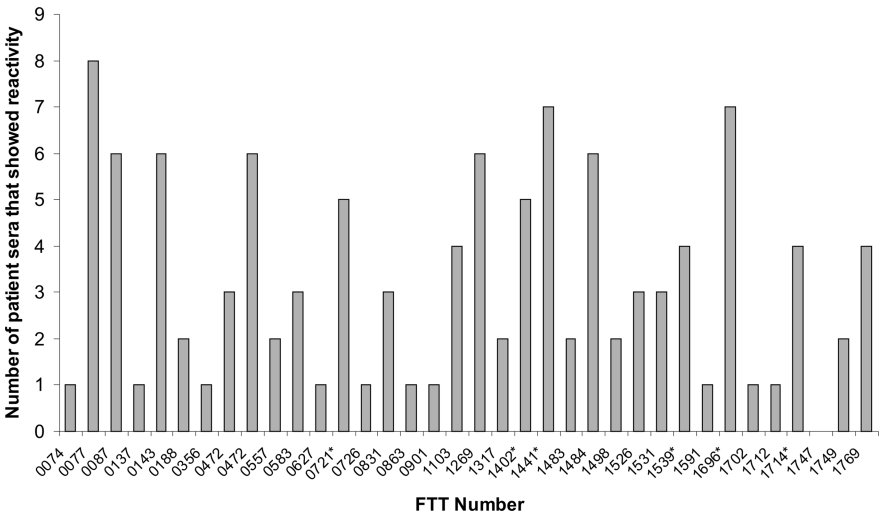

FIGURE 3 | Frequency with which immunoreactive proteins from LVS proteome were observed to react with sera from patients recovering from type B *Francisella tularensis* infections. FTT_number refers to the locus tag of the ORF within the SCHU S4 genome sequence. *Indicates that sera reacted with isoforms 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

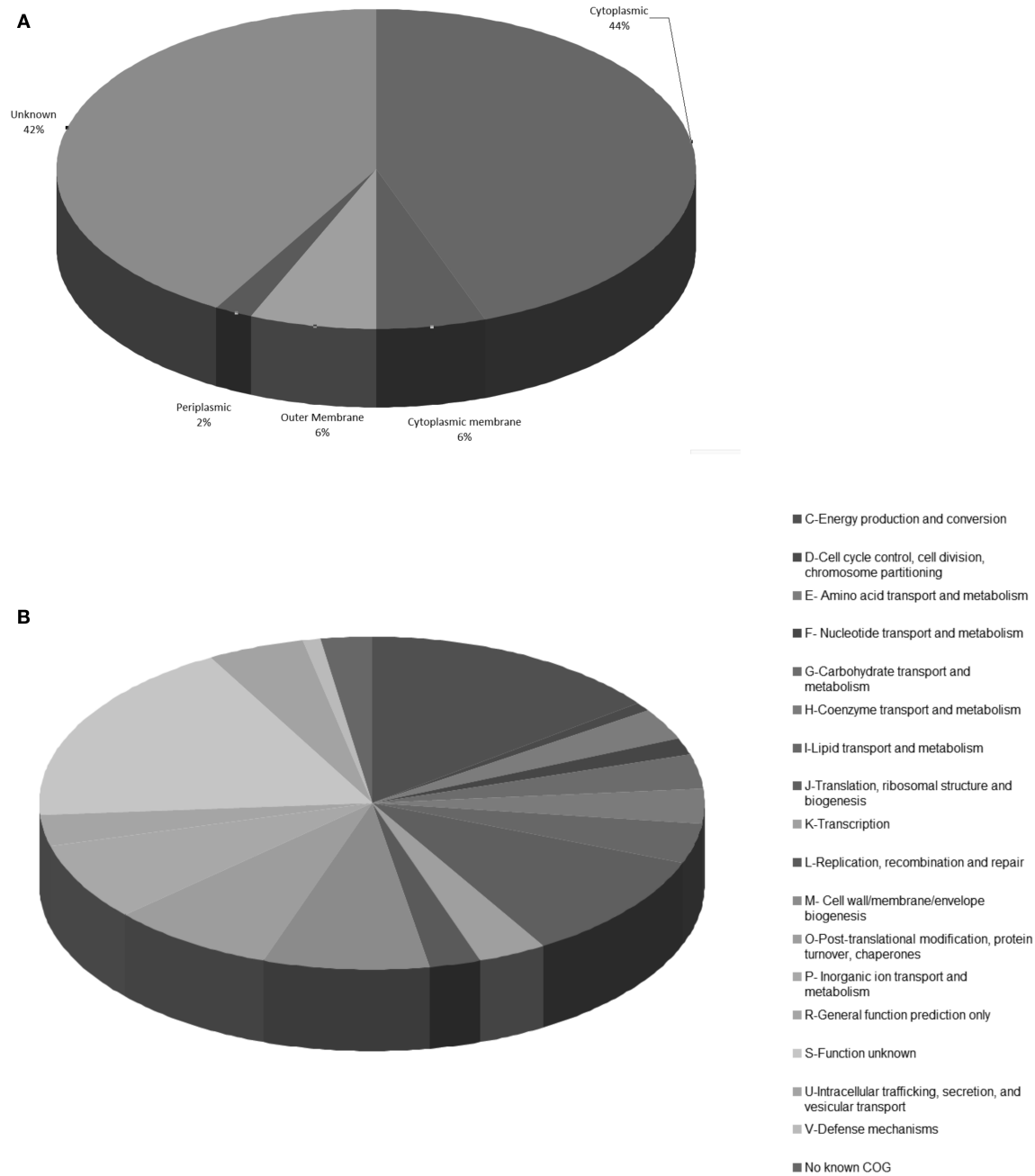

FIGURE 4 | Basic bioinformatic characterization of the repertoire of reported *Francisella* immunoreactive proteins. Graphical representation of **(A)** clusters of orthologous groups (COGs) and **(B)** predicted subcellular location using PSORT algorithm, for total reported immunoreactive proteins from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

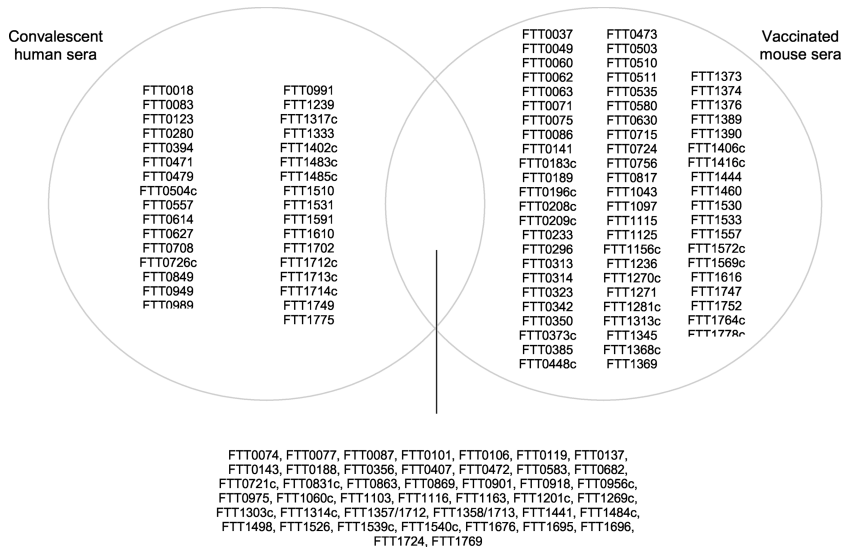

FIGURE 5 | Venn diagram showing the intersection of immunoreactive proteins observed to be reactive with sera from LVS vaccinated mice and human convalescent. From Table 3.

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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