

Review

***Francisella tularensis*: unravelling the secrets of an intracellular pathogen**

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Francisella tularensis has been recognized as the causative agent of tularaemia for almost a century. Since its discovery in 1911, it has been shown to infect a wide range of hosts, including humans. As early as the 1920s it was suggested to be an intracellular pathogen, but it has proven to be an enigmatic organism, whose interaction with the host has been difficult to elucidate, and we still have a very limited understanding of the molecular mechanisms of virulence. However, the recent availability of genome sequence data and molecular tools has allowed us to start to understand the molecular basis of *F. tularensis* pathogenicity, and will facilitate the development of a vaccine to protect against infection.

Francisella tularensis

Francisella tularensis is a small pleiomorphic Gram-negative coccobacillus. It was first isolated in 1911 from ground squirrels found dying of a plague-like illness in Tulare County, CA, USA (McCoy & Chapin, 1912). Initially called *Bacterium tularense*, it was eventually allocated to a new genus named *Francisella* in honour of the man who pioneered research on the organism, Dr Edward Francis (Dorofe'ev, 1947). Modern taxonomic methods have assigned *Francisella* to the γ -subclass of *Proteobacteria* (Forsman *et al.*, 1994). Sequencing of 16S genes, and more recently whole genome analysis, indicates that the closest relatives are endosymbionts, such as *Wolbachia persica* (Forsman *et al.*, 1994; Keim *et al.*, 2007). The taxonomic status of *Francisella* is supported by an unusual fatty acid composition and the high lipid content of the cell wall (Hood, 1977). Three species are currently recognized: *F. tularensis*, *Francisella philomiragia* and *Francisella novicida*, although it has been suggested that *F. novicida* should be reclassified as a subspecies of *F. tularensis*, and this classification has been adopted in many publications, including Bergey's Manual of Systemic Bacteriology (Sjostedt, 2005). Three *F. tularensis* subspecies are recognized, *tularensis*, *holarctica* and *mediasiatica*, which differ in geographical distribution and virulence (Table 1). The subspecies *tularensis* has been divided into two clades, A.I and A.II (Farlow *et al.*, 2005; Johansson *et al.*, 2004), based on geographical distribution, disease outcome and transmission routes. However, recent isolation of *Francisella*-like organisms from fish and human infections indicates that the genus may be more diverse and widespread than previously assumed (Nylund *et al.*, 2006; Ostland *et al.*, 2006; Whipp *et al.*, 2003).

F. tularensis is a fastidious organism that requires enriched medium for growth, such as cysteine glucose blood agar or

an enriched cysteine-supplemented chocolate agar (Ellis *et al.*, 2002). A heavy inoculum will yield visible growth in 18 h at 37 °C, but the appearance of individual colonies may require 2 to 4 days of incubation. On chocolate agar, colonies are 2 to 4 mm in size, greenish-white, round, smooth and slightly mucoid, whilst on media containing whole blood there is usually a small zone of α -haemolysis surrounding colonies. *F. tularensis* does not grow well in liquid medium even when the medium is supplemented with cysteine. Fully virulent strains of *F. tularensis* must be handled at containment level 3 (Titball *et al.*, 2007). In the USA, *F. tularensis* is listed as a 'select agent' by the Centers for Disease Control and Prevention.

Tularaemia

Tularaemia is a zoonotic infection caused by *F. tularensis* (Ellis *et al.*, 2002; Sjostedt, 2007). It circulates in populations of rodents and lagomorphs, and outbreaks in humans often parallel outbreaks in animal populations. However, it is not clear whether these animal species are the true reservoir of the bacterium in the environment. A wide range of arthropod vectors have been implicated in the transmission of the pathogen causing tularaemia between mammalian hosts, including mosquitoes, ticks and deer flies (Boyce, 1975; Mörner, 1992). These vectors can also transmit the pathogen to man. In addition, *F. tularensis* can be acquired by contact with, or ingestion of, contaminated material, including food and water, and by inhalation of infectious particles. Rural populations, and especially those individuals who spend periods of time in endemic areas, such as farmers, hunters, walkers and forest workers, are most at risk of contracting tularaemia (Levesque *et al.*, 1995; Syrjala *et al.*, 1985). Outbreaks associated with contaminated water supplies can involve large numbers of cases, but usually the incidence of the

Table 1. Virulence and distribution of *Francisella*

Francisella species	Subspecies	Relative virulence	Distribution	
			Region	Comment
<i>F. novicida</i>		Low	Global: implied by isolates from USA and Australia	Rarely isolated
<i>F. philomiragia</i>		Low	Northern hemisphere	Rarely isolated
<i>F. tularensis</i>	<i>tularensis</i> A.I	Very high	Central and Eastern USA, California	Distribution matches that of tick vectors <i>Amblyomma americanum</i> and <i>Dermacentor variabilis</i> (Farlow <i>et al.</i> , 2005)
	<i>tularensis</i> A.II	Moderate: lower than subsp. <i>holarctica</i> (Staples <i>et al.</i> , 2006)	Western USA	Distribution matches that of vectors <i>Dermacentor andersoni</i> , <i>Chrysops discalis</i> and mountain cottontail rabbit (Farlow <i>et al.</i> , 2005)
	<i>holarctica</i>	High	Northern hemisphere, widespread	
	<i>mediasiatica</i>	High	Central Asia	

disease is low (Helvacı *et al.*, 2000; Tarnvik *et al.*, 1996). These water-associated outbreaks are mainly caused by subspecies *holarctica*; subspecies *tularensis* has never been linked to water-borne infections (Oyston *et al.*, 2004).

The type and severity of the disease is dependent on strain, dose and route of infection (Ellis *et al.*, 2002). *F. tularensis* subspecies *tularensis* and *holarctica* cause the majority of reported cases, with subspecies *tularensis* causing the more severe disease of the two. Although tularaemia can be a severely debilitating or even fatal disease, especially when caused by *F. tularensis* subspecies *tularensis*, many cases of disease caused by lower-virulence strains are undiagnosed due to the non-specific nature of the symptoms. The incubation period is 3–5 days normally (range 1–21 days), and patients develop flu-like symptoms that may be protracted and relapsing if untreated (Evans *et al.*, 1985).

Infection through the skin results in ulceroglandular tularaemia. This is the most common presentation of the disease and can arise following the bite of an infected vector or through direct contact with the flesh of infected animal (Ohara *et al.*, 1991). A lesion develops at the site of infection, often a single papule that develops into an ulcer surrounded by a zone of inflammation (Oyston *et al.*, 2004). The ulcer is relatively painless and heals within a week. Where no ulcer is reported, this is termed glandular tularaemia. Within a few days, the patient develops fever, chills, malaise, headaches and a sore throat. The local draining lymph nodes become enlarged and painful, like a bubo. Lymphadenopathy can take a significant period to resolve, even with treatment, and without treatment suppuration occurs in approximately 30% of cases (Helvacı *et al.*, 2000; Kavanaugh, 1935). Less commonly, infection can occur through the conjunctiva. This is termed oculoglandular tularaemia and arises following direct contamination of the eye (Steinemann *et al.*, 1999). The patient develops conjunctivitis in the infected eye, swollen

eyelids and a purulent secretion. Untreated, the infection can spread to the local lymph nodes, similarly to ulceroglandular tularaemia. Ingestion of infected meat or water can result in oropharyngeal or gastrointestinal tularaemia (Stewart, 1996; Tarnvik *et al.*, 1996). Ulcers, pharyngitis and swollen cervical lymph nodes develop, and a yellow-white pseudomembrane may be seen in oropharyngeal tularaemia. Gastrointestinal tularaemia can range from a mild but persistent diarrhoea to an acute fatal disease with extensive ulceration of the bowel, depending on the size of the infecting dose.

Inhalation of *F. tularensis* results in respiratory or pneumonic tularaemia. Respiratory tularaemia has been reported in farmers following activities such as making hay, where infectious dusts can be generated (Stewart, 1996; Syrjala *et al.*, 1985). Other high-risk activities in endemic areas are lawn-mowing and brush cutting (Feldman *et al.*, 2001; Matyas *et al.*, 2007). For example in Martha's Vineyard, MA, USA, the majority of investigated cases were respiratory, in landscapers undertaking these types of work (Matyas *et al.*, 2007). Pneumonia can also arise following haematogenous spread in other forms of tularaemia. Symptoms can be variable and depend on the virulence of the strain involved. Infection with the most highly virulent strains can have a case fatality rate of up to 30% if untreated, but antibiotic therapy reduces this to around 2% (Dennis *et al.*, 2001). Presentation can range from a mild pneumonia to an acute infection with high fever, malaise, chills, cough, delirium and pulse-temperature dissociation. Radiological examination may reveal parenchymal infiltrates, most commonly in one lobe, and hilar lymphadenopathy may be present (Tarnvik & Chu, 2007). The infectious dose required to cause disease by this route is very low (Dennis *et al.*, 2001). The high infectivity combined with the morbidity and mortality caused by *F. tularensis* led to the organism being developed as a

biological weapon by various nations, including the reported production of antibiotic-resistant strains (Dennis *et al.*, 2001; Oyston *et al.*, 2004). Since the events of September 2001 and the subsequent anthrax attacks on the USA, concern about the potential misuse of this organism has increased (Oyston *et al.*, 2004). The associated increase in funding, particularly in the USA, has resulted in an influx of researchers working on the pathogen.

Tularaemia responds well to antibiotic therapy. As described above, the mortality rate of the more acute forms of the disease is reduced significantly if the patient receives suitable antibiotics. Historically aminoglycosides have been the drugs of choice. Although clinically effective, streptomycin is rarely used now due to problems of ototoxicity and nephrotoxicity (Titball *et al.*, 2007). Similarly, although chloramphenicol has been used historically for treatment, it would be unlikely to be used as a first choice due to the possibility of irreversible effects on haematopoiesis (Titball *et al.*, 2007). Gentamicin is a suitable alternative aminoglycoside, and has been used for treatment of pneumonic tularaemia on Martha's Vineyard (Matyas *et al.*, 2007). Due to the requirement for parenteral dosing and monitoring of serum levels, aminoglycosides are now only used for the most serious cases. The tetracyclines have been associated with high relapse rates on withdrawal (Dennis *et al.*, 2001). Doxycycline is effective in the treatment of tularaemia, but should be avoided for use in young children due to possible effects on developing teeth (Tarnvik & Chu, 2007). Ciprofloxacin has been shown to be highly effective in oral therapy, and can be considered the current drug of choice for uncomplicated tularaemia (Johansson *et al.*, 2001; Syrjala *et al.*, 1991). It has shown to be effective in treating tularaemia in children, and may be suitable for use in pregnant women.

No licensed vaccine is available for prophylaxis of tularaemia. Killed cell preparations were reactogenic and of dubious efficacy (Foshay *et al.*, 1942; Foshay, 1950), although studies in humans indicated that immunization with these vaccines reduced the number of infections and considerably modified the course of the disease (Foshay *et al.*, 1942; Kadull *et al.*, 1950). The identification of the antigens responsible for induction of a protective response has been an aim of research for 50 years. To date, the only protective antigen identified is LPS. In humans the predominant antibody response is to LPS. However, animal studies have shown that while immunization with LPS isolated from a live vaccine strain (LVS) induces protection against *F. tularensis* strains of low virulence, it is less effective at providing protection against strains of *F. tularensis* subspecies *tularensis* (Conlan *et al.*, 2002; Fulop *et al.*, 1995, 2001), and LPS isolated from strain SCHU S4 was similarly unable to protect immunized mice against homologous challenge (Prior *et al.*, 2003). To date, many immunogenic proteins have been identified (Titball & Petrosino, 2007), but none capable of inducing a protective immune response. This is probably a reflection that

antibody is not sufficient to protect against tularaemia and a T-cell memory response must be induced for a vaccine to be protective (Tarnvik, 1989). A LVS was developed in the 1950s, and used extensively to vaccinate at-risk workers under 'investigational new drug' status, which resulted in a significant decrease in laboratory-acquired infections (Burke, 1977). The LVS vaccine provided good protection against an airborne challenge with 10 infectious doses of a virulent strain of *F. tularensis* subspecies *tularensis*, but only partial protection against 100 infectious doses and poor protection against 1000 infectious doses (McCrum, 1961). Although LVS appears to be effective, there have been problems with the strain, such as reversion to virulence, mixed colony morphology and variable immunogenicity, and thus the LVS strain has failed to achieve licensing for human use (Oyston *et al.*, 2004). The development of a candidate vaccine suitable for licensing is currently the focus of much research.

A facultative intracellular pathogen

F. tularensis can invade and multiply in a range of cell types (Anthony *et al.*, 1991; Ben Nasr *et al.*, 2006; Buddingh & Womack, 1941; Councilman & Strong, 1921; Francis, 1927; Lindemann *et al.*, 2007; Shepard, 1959), but *in vivo* its primary target appears to be the macrophage (Fortier *et al.*, 1994). Based on homology searches of the genome, *F. tularensis* does not produce any classical virulence factors, such as an exotoxin. Instead, the virulence of *F. tularensis* appears to stem from its ability to proliferate to large numbers within various host tissues and organs, thereby disrupting their normal functions and inducing a significant host inflammatory response that itself appears to contribute to the disease. The components of the organism that induce the inflammatory response are unknown, although LPS does not appear to be responsible (Ancuta *et al.*, 1996; Sandstrom *et al.*, 1992). However, differences in response observed between murine and human immune cells following exposure to LPS may indicate that the contribution of LPS to the inflammatory response may vary depending on the host (Rahhal *et al.*, 2007). The innate response to *Francisella* has been shown to be mediated by interaction of bacterial components with TLR2 (Malik *et al.*, 2006). Two lipoproteins, TUL4 and FTT1103, have been identified that interact with TLR2, which may be responsible for proinflammatory cytokine induction during infection (Thakran *et al.*, 2007). A host protein, host matrix metalloprotein 9, has also been implicated in influencing development of morbidity following infection with *Francisella* (Malik *et al.*, 2007). Host matrix metalloprotein 9, a member of the host matrix metalloproteinases family, is released by neutrophils and activated macrophages, and plays an important role in modulating leukocyte recruitment. Mice defective in production of the host matrix metalloprotein 9 were able to resolve infection, even with highly virulent subspecies *tularensis* strains, while normal expression rendered mice more susceptible to infection, with higher bacterial burdens

and more extensive histopathology, morbidity and death (Malik *et al.*, 2007).

As stated above, the LPS of *Francisella* is unique in that it does not bind to host molecules such as LPS-binding protein and TLR4 to trigger a proinflammatory response (Barker *et al.*, 2006), nor does it bind to TLR2 (Hajjar *et al.*, 2006). The lipid A from *F. novicida* and *F. tularensis* contains some unusual modifications (Phillips *et al.*, 2004), and it has been suggested that *Francisella* may possess a novel system of LPS remodelling enzymes (Wang *et al.*, 2006). The structure of the O antigen has been determined for *F. tularensis* subspecies *tularensis* and *holarctica* and *F. novicida* (Prior *et al.*, 2003; Vinogradov *et al.*, 2002, 2004). The O antigen for the two *F. tularensis* subspecies was identical, but that for *F. novicida*, while it shared many sugars with its close relatives, was structurally distinct. The structural differences seem to result in immunological differences, as the LPS did not induce cross-species protection (Shen *et al.*, 2004; Thomas *et al.*, 2007). Mutants of *F. tularensis* and *F. novicida* unable to synthesize O antigen were attenuated (Raynaud *et al.*, 2007; Thomas *et al.*, 2007), but the O antigen appeared to play a different role in the two species. The O antigen of *F. tularensis* appeared to be important for intracellular survival (Raynaud *et al.*, 2007; Thomas *et al.*, 2007), whereas the O antigen of *F. novicida* appeared to be critical for serum resistance and less important for intracellular survival (Thomas *et al.*, 2007).

F. tularensis has a novel invasion strategy to enter host macrophages whereby it induces the macrophage to produce asymmetric spacious pseudopod loops (Clemens *et al.*, 2005). This uptake is dependent on serum with complement activity, and host cell receptors for the complement factor C3, and appears to involve bacterial surface polysaccharides. Once inside the cell, the bacterium arrests maturation of the phagosome at a late endosomal-like stage. Within 15–30 min, the phagosome is transiently acidified, and this acidification is essential for the subsequent escape of *F. tularensis* into the cytosol of the macrophage (Santic *et al.*, 2008). The phagosome appears structurally unusual, appearing to acquire a dense fibrillar coat (Clemens *et al.*, 2004). Eventually, the phagosomal membrane is degraded and the bacteria escape into the cytoplasm by 30–60 min after phagocytosis, and multiply to high levels in the cytoplasm (Checroun *et al.*, 2006; Santic *et al.*, 2007). Subsequently, it has been reported that the bacteria appear to re-enter the endocytic pathway by inducing an autophagy-mediated process, to reside in large double-membrane bound vacuoles, containing clusters of bacterial cells in each vacuole (Checroun *et al.*, 2006), although this observation remains to be confirmed. Bacterial release is thought to occur following *Francisella*-induced apoptosis (Lai *et al.*, 2001) and pyroptosis (Mariathasan *et al.*, 2005). Pyroptosis is a newly described pathway of proinflammatory cell death (Fink & Cookson, 2005) that results in the release of bacteria from within the infected cell. However, the final stages of the intracellular cycle are not well understood.

The acid phosphatase AcpA has been shown to play a key role in intracellular survival of *Francisella*, by inhibiting the respiratory burst generated by macrophages (Reilly *et al.*, 1996). Deletion of *acpA* resulted in a mutant that was more susceptible than wild-type to killing by human macrophages and had decreased phosphatase activity associated with membrane fractions (Mohapatra *et al.*, 2007). Additionally, the mutant showed a decreased ability to escape from the phagosome (Mohapatra *et al.*, 2007). Several theories have been proposed to explain this observation (Mohapatra *et al.*, 2007), one possibility being membrane disruption by phospholipase activity, which has been reported for AcpA in addition to its acid phosphatase activity (Reilly *et al.*, 1996). Multiple acid phosphatases have been identified in the genome sequence of *Francisella*, but the contribution of each to the observed inhibition of the respiratory burst has not been determined. *Francisella* has also been shown to enter neutrophils without triggering the respiratory burst, by an unknown mechanism that inhibits NADPH oxidase assembly (McCaffrey & Allen, 2006), and then escapes the phagosome to survive in the neutrophil cytoplasm. Thus *Francisella* belongs to a very small group of microbial pathogens able to avoid killing by neutrophils, and the importance of this observation to pathogenesis has yet to be determined.

The intracellular life cycle of *F. tularensis* is complex, and the genes involved with all stages have yet to be elucidated. The large *Francisella* pathogenicity island (FPI) was originally identified when inactivation of genes by random transposon mutagenesis resulted in an intracellular growth defect (Gray *et al.*, 2002). For this reason the region was initially named the intracellular growth locus. The FPI contains 19 genes, including *iglABCD* and *pdpABCD*, which have been shown to be essential for virulence (Nano *et al.*, 2004). While *F. novicida* contains only a single copy of the FPI, subspecies *tularensis* and *holarctica* both possess two copies (Larsson *et al.*, 2005). This may be one reason for the lower virulence of *F. novicida*. The functions of the proteins encoded by the FPI are currently the focus of much research. Bioinformatic analysis revealed the FPI to encode a putative type VI secretion system, similar to the systems involved in virulence of *Pseudomonas aeruginosa* (Mougous *et al.*, 2006) and *Vibrio cholerae* (Pukatzki *et al.*, 2006). The effectors secreted by the type VI secretion apparatus have yet to be determined. However, the IglABC proteins have received much attention previously due to their contribution to intracellular pathogenesis. IglA and IglB appear to associate in the bacterial cytoplasm (de Bruin *et al.*, 2007) and are suggested to be involved in secretion. IglC is a 23 kDa protein with no homologues identified to date. *Francisella*-induced apoptosis is dependent on IglC (Lai *et al.*, 2004), and IglC appears to downregulate Toll-like receptor signalling to modulate the immune response (Telepnev *et al.*, 2003). Genes within the FPI are regulated by MglA, which shows high similarity to SspA of *Escherichia coli*, a stringent response transcriptional regulator. Similarly to SspA, MglA interacts with

RNA polymerase, but whereas SspA expression is induced at stationary phase MglA expression is maximal during lag and exponential phases of growth (Baron & Nano, 1999; Brotcke *et al.*, 2006). MglA expression is also increased during the early stages of macrophage infection, as would be predicted for a regulator of the genes of the intracellular growth locus (Baron & Nano, 1999). Inactivation of MglA results in a significant intracellular growth defect and attenuation (Baron & Nano, 1998; Lauriano *et al.*, 2004). Transcriptional analysis indicated that a wide range of genes is regulated by MglA, including many outside of the FPI (Brotcke *et al.*, 2006), such as the protease PepO and a putative β -glucosidase BglX.

In addition to the putative type VI secretion system carried by the FPI, *Francisella* has also been shown to secrete virulence factors by a type II secretion system. The type II secretion involves genes also involved in expression of type IV pili (Hager *et al.*, 2006). These surface structures have been shown to play an important role in virulence for a range of Gram-negative pathogens (Craig *et al.*, 2004). Several genes in the pili operons show extensive homology with type II secretion genes, and some pathogens, such as *P. aeruginosa*, may possess multiple type II secretion systems in addition to type IV pili (Stover *et al.*, 2000). Deletion of genes involved in type IV pili biosynthesis is attenuating for subspecies *holarctica* (Forslund *et al.*, 2006). Spontaneous recombination between direct repeats in the genome of a subspecies *holarctica* strain designated FSC074 led to the loss of *pilA* from the *pilAEV* cluster, resulting in loss of pili production and attenuation in mice challenged by the subcutaneous route of infection (Forslund *et al.*, 2006). Inactivation of *pil* genes has different effects on virulence depending on the gene inactivated: subspecies *holarctica pilC* or *pilQ* mutants retain virulence, while the isogenic *pilA* mutant is attenuated (Forsberg & Guina, 2007; Forslund *et al.*, 2006). Unexpectedly, a *F. novicida pilC* mutant was more virulent than wild-type when tested in mice (Hager *et al.*, 2006). The increased virulence appeared to be due to the effect of the mutation not on type IV pilus expression, but rather on the secretion of effector proteins. In contrast to the more virulent subspecies that do not appear to secrete proteins (Lee *et al.*, 2006) *in vitro*, *F. novicida* secretes at least seven proteins detectable in culture supernatants, including chitinases, chitin binding protein, PepO and BglX (Hager *et al.*, 2006). The increased virulence of the *F. novicida pilC* mutant was linked to a decrease in PepO secretion (Hager *et al.*, 2006). PepO has homology with proteins involved with cleavage of proendothelin. The endothelin thus produced is a potent vasoconstrictor. Thus PepO secretion would result in localized vasoconstriction and therefore limit dissemination of *F. novicida* from the local site of infection, but abrogating PepO secretion by mutating the *pil* genes of the secretion machinery would increase dissemination, and thus virulence. The genome sequences of subspecies *holarctica* and *tularensis* revealed that *pepO* has been mutated so it is no longer expressed by these

strains, and this appears to have been a key step in the evolution to high virulence in mammalian hosts.

A putative type I secretion system has been proposed for *F. tularensis*, although this remains to be proven. *F. tularensis* possesses two orthologues of the *E. coli* TolC protein, TolC and FtlC (Gil *et al.*, 2006). In *E. coli*, TolC is the outer membrane channel component used by type I secretion systems and multidrug efflux pumps. Inactivation of *tolC* and *ftlC* increased sensitivity to a range of toxic compounds, indicating a role in efflux, but only the *tolC* mutant was attenuated (Gil *et al.*, 2006). This may reflect differences in substrates between TolC and FtlC efflux pumps, or it may indicate that *F. tularensis* possesses a type I secretion system, even though the other components of type I secretion have not been identified by homology as yet.

Iron acquisition is recognized as an important virulence trait of many intracellular pathogens, but little was known until recently about how *Francisella* acquires iron from the mammalian host. Transcriptional analysis of bacteria grown in media with low iron concentrations revealed an iron-responsive operon involved in siderophore production (Deng *et al.*, 2006; Milne *et al.*, 2007). Inactivation of a gene involved in siderophore production resulted in an intracellular growth defect in *F. novicida*, but not in the subspecies *holarctica* strain LVS (Deng *et al.*, 2006). However, the siderophore production of the wild-type LVS strain was significantly reduced compared to that of *F. novicida*, which suggests that mutations elsewhere in the genome were affecting siderophore activity in LVS. The iron-regulated operon also included the siderophore receptor required for uptake of the siderophore-iron complex (Milne *et al.*, 2007). Inactivation of the gene encoding the receptor resulted in attenuation of *F. novicida* in mice, indicating that iron acquisition is a virulence trait in *Francisella* similarly to other intracellular pathogens.

A significant proportion of *F. tularensis* genes are annotated as hypothetical proteins with unknown function, and elucidation of their roles will help in understanding this enigmatic pathogen. Several whole genome sequences are now available for different strains and subspecies of *F. tularensis* (Table 2). Comparison of the subspecies *tularensis* strain SCHU S4 with subspecies *holarctica* strains LVS and OSU18 revealed that, despite high nucleotide identity between the strains, significant rearrangements were observed between the two subspecies (Petrosino *et al.*, 2006). There were 51 syntenic blocks rearranged between SCHU S4 and OSU18 genomes. The rearrangements were due to homologous recombination between the numerous copies of insertion sequence elements around the genome, and multiple copies of rRNA sequences. Despite the repeated DNA sequences being present in subspecies *holarctica*, no rearrangements were found between the minimally passaged isolate OSU18 and LVS, a highly passaged and attenuated strain (Petrosino *et al.*, 2006). The reason for this genomic stability in subspecies *holarctica* is not known. Detailed

Table 2. *Francisella* genome sequencing projects listed on the National Centre for Biotechnology Information database

Sequenced strain	Length (nt)	Sequencing centre	Comment
<i>F. tularensis</i> subsp. <i>tularensis</i> SCHU S4	1 892 819	Swedish Defence Research Agency	Fully virulent, A.I clade, laboratory passaged
<i>F. tularensis</i> subsp. <i>holarctica</i> OSU18	1 895 727	Baylor College of Medicine	Minimally passaged
<i>F. tularensis</i> subsp. <i>tularensis</i> FSC198	1 892 616	University of Birmingham	European isolate
<i>F. tularensis</i> subsp. <i>holarctica</i> LVS	1 895 994	Lawrence Livermore National Laboratory	Attenuated, highly passaged
<i>F. novicida</i> U112	1 910 031	University of Washington	
<i>F. tularensis</i> subsp. <i>holarctica</i> FTA	1 890 909	US Department of Energy Joint Genome Institute	
<i>F. tularensis</i> subsp. <i>tularensis</i> WY96-3418	1 898 476	Translational Genomics Research Institute	A.II clade, CDC collection, isolated from a finger wound in 1996 (Beckstrom-Sternberg <i>et al.</i> , 2007)
Unfinished genomes			
<i>F. tularensis</i> subsp. <i>holarctica</i> FSC200	1 790 358	University of Washington	Minimally passaged European isolate
<i>F. tularensis</i> subsp. <i>tularensis</i> FSC33	1 844 205	Broad Institute Genome Sequencing Platform	Fully virulent strain, isolated from a squirrel in Georgia, USA (Johansson <i>et al.</i> , 2000)
<i>F. novicida</i> GA99-3548	1 845 491	Broad Institute Genome Sequencing Platform	
<i>F. novicida</i> GA99-3549	1 897 440	Broad Institute Genome Sequencing Platform	

CDC, Centers for Disease Control and Prevention.

comparison of the two subspecies *holarctica* strains revealed that LVS and OSU18 are very nearly identical. Considering that LVS was derived in Russia from a European subspecies *holarctica* strain and OSU18 was isolated over three decades later in Oklahoma, USA, this high level of identity is striking, but typing has suggested that subspecies *holarctica* strains show little genetic diversity worldwide (Johansson *et al.*, 2004). It appears, therefore, that the relative attenuation of LVS is due to small sequence variations, insertions and deletions. Comparison of the LVS with the genomes of minimally passaged *holarctica* strains identified, for example, mutations in genes encoding the type IV pili cluster discussed above, a tyrosine phosphoprotein, a multi-drug efflux pump and a glycosyltransferase (Petrosino *et al.*, 2006; Rohmer *et al.*, 2006). Individually these may not be responsible for attenuation of LVS, but could each contribute subtly and in part to the phenotype.

Comparison of virulent and attenuated strains identified another locus of interest. An empirically attenuated mutant of SCHU S4 possessed a deletion spanning two adjacent genes, *FTT0918* and *FTT0919*, resulting in a novel ORF that encodes a hybrid protein consisting of the N terminus of the protein encoded by the former gene and the C terminus of the protein encoded by the latter gene (Twine *et al.*, 2005). The attenuated subspecies *holarctica* strain LVS possesses a similar deletion. Isogenic deletion of *FTT0918*, but not *FTT0919*, severely attenuated SCHU S4. Combined restoration of *FTT0918* and *pilA* genes in LVS by complementation resulted in a fully virulent phenotype,

indicating that these two mutations contribute significantly to the observed attenuation of LVS. The function of *FTT0918* is not known. However, although it has no homology to any other protein in the database, it was identified as being one member of a protein family along with *FTT0919*, *FTT0025*, *FTT0267* and *FTT0602* (Larsson *et al.*, 2005).

With the availability of genome sequence data, the main impediment to understanding the molecular basis of *Francisella* virulence was the paucity of molecular tools with which to manipulate the pathogen, particularly for global mutagenesis approaches. However, shuttle plasmids (Bina *et al.*, 2006; LoVullo *et al.*, 2006; Maier *et al.*, 2004; Rasko *et al.*, 2007), transposons (Gallagher *et al.*, 2007; Kawula *et al.*, 2004; Maier *et al.*, 2006; Weiss *et al.*, 2007) and allelic replacement methodologies (Golovliov *et al.*, 2003; Lauriano *et al.*, 2003; Twine *et al.*, 2005) have all recently been developed. Global mutagenesis of *F. tularensis* subspecies *tularensis* strain SCHU S4 (Qin & Mann, 2006), subspecies *holarctica* strain LVS (Maier *et al.*, 2007; Su *et al.*, 2007) and *F. novicida* (Gallagher *et al.*, 2007; Tempel *et al.*, 2006; Weiss *et al.*, 2007) has resulted in the identification of genes essential for growth *in vitro* (Gallagher *et al.*, 2007), for intracellular survival in hepatic cells (Qin & Mann, 2006) or macrophages (Maier *et al.*, 2007; Tempel *et al.*, 2006) and *in vivo* (Su *et al.*, 2007; Weiss *et al.*, 2007). The genes identified as important to the intracellular pathogenesis of *Francisella* could be targets for novel antimicrobial development (Gallagher *et al.*, 2007) or a novel attenuated vaccine. In addition these studies have

provided information about the mechanisms of pathogenesis employed by the organism. For example, one of these global mutagenesis studies identified that bacteria injected subcutaneously passed through a bottleneck before systemic dissemination occurred (Weiss *et al.*, 2007). This bottleneck was not apparent when the mice were challenged by the intranasal route (Su *et al.*, 2007) or intraperitoneally (Weiss *et al.*, 2007). Similar bottlenecks have been reported for enteric pathogens invading from the gut (Meccas *et al.*, 2001). The nature of the bottleneck is not known, and shows that we still have much to learn about the life of the pathogen *in vivo*.

Conclusion

Until recently, our understanding of the molecular mechanisms of virulence of *Francisella* was significantly impaired by a lack of molecular tools and information. As indicated above, multiple genome sequences are becoming available, but low levels of homology between *Francisella* genes and those of other pathogens have resulted in as many questions being raised as answered. New molecular tools are being developed, and our ability to manipulate the pathogen is improving. Several studies now report global approaches to identifying genes involved in virulence and particularly genes involved in intracellular multiplication. As such, we are getting closer to addressing key issues such as the need for an effective licensable vaccine. It appears we are still some way from understanding the pathogenesis of the organism however.

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