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

THESIS

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Abstract

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

the *F. tularensis* LVSG phase variant is dramatically altered. Understanding the mechanism of blue to grey phase variation may lead to a way to inhibit this variation, thus making future *F. tularensis* vaccines more stable and efficacious.

Dedication

This work is dedicated to my inspiration, Nrusingh whose love and support keep me motivated and to my family for their support.

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Fields of Study

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Chapter 1

Introduction

1.1 Tularemia

Francisella tularensis was first discovered as the causative agent of "plague-like disease of rodents". A squirrel that succumbed to a plague like disease was the source of the first isolation and successful cultivation of '*Bacterium Tularense*' in Tulare County, California in 1911 by McCoy and Chapin (Chapin 1921). The first human cases were reported by Wherry and Lamb in 1914 (Wherry and Lamb 2004). The organism was part of the genus *Bacterium* and was later reclassified as *Pasturella*. The first human outbreak was reported in 1925 in Ohio involving patients with ocular infections (Nigrovic and Wingerter 2008). Dr. Edwards Francis published a comprehensive report of over 600 cases in 1928 that described the forms of disease and identified rodents, rabbits and blood feeding insects as transmission vectors (Sanford 1983). He named the disease Tularemia based on the county in which the infections occurred. Later, taxonomists determined that the bacterium causing tularemia did not fit into the previously described bacterial taxa (i.e., *Bacterium*) and had some unique properties (Keim, Johansson et al. 2007) on the basis of which, the bacteria was renamed as *Francisella tularensis* in 1947 in honor of Dr. Francis's contribution to the field (Olsufiev, Emelyanova et al. 1959). Tularemia has

also been known as rabbit fever and deertick fever and disease is more prevalent amongst hunters, trappers, cooks, landscapers, farmers, meat handlers and laboratory workers (Nigrovic and Wingerter 2008). *F. tularensis* has been characterized as a Gram negative facultative intracellular pathogen that is capable of causing a disease by manipulating innate immune system of the host. *Francisella* is understudied because of a relatively rare occurrence of outbreaks but the possibility of weaponization has been a center of focus recently and thus has renewed interest into the molecular mechanisms of virulence (Ellis, Oyston et al. 2002) and the search for a safe vaccine.

1.2 Nomenclature and Ecology

Francisella is aerobic Gram-negative coccobacilli. *Francisella*e are shown to be most closely related to proteobacteria on the basis of 16S ribosomal DNA sequencing (Forsman, Sandstrom et al. 1994). Two species *Francisella tularensis* and *F. philomiragia* and four subspecies *tularensis*, *holarctica*, *mediasiatica* and *novicida* of *Francisella* have been identified. *F. tularensis* subspecies *tularensis* is the most virulent strain that is found primarily in North America and has been designated as type A. *F. tularensis* subspecies *holarctica* has been categorized as type B strain that is less virulent and been recovered from North America and Europe. *F. tularensis* subspecies *novicida* has been isolated in North America and shares about 99.6 percent DNA homology to *tularensis* strains but is markedly less virulent and does not cause a disease in immunocompetent humans (McLendon, Apicella et al. 2006). *Francisella mediasiatica* is

found in Central Asia but is not a clinically relevant strain (Keim, Johansson et al. 2007) and thus least studied amongst all subspecies. *F. philomiragia* causes disease in fishes but has not been found to be a human pathogen (Ottem, Nylund et al. 2009).

Although no techniques have been able to find the sufficient differences to show the evolutionary differences between the subspecies of *F. tularensis*, comparison of genomes from different subspecies has revealed some general patterns in the evolution of *Francisella* subspecies (Petrosino, Xiang et al. 2006). A great amount of genomic rearrangement has been found in the genomes and the majority of these rearrangements were due to homologous recombination between the multiple copies of IS elements. There are many pseudogenes also present, in addition to multiple copies of some genes (Keim, Johansson et al. 2007).

A reservoir of *F. tularensis* has not yet been identified but *Francisella* has been isolated from water and soil environments and recovered from a wide variety of hosts including fishes, amoeba, ticks, reptiles, rodents, mammals and lagomorphs. *F. tularensis* has been found more in rabbits, ticks and squirrels while *F. holarctica* has a more aquatic habitat involving vectors like mosquito larvae, beavers and muskrat (Morner 1992). Both *F. tularensis* and *F. holarctica* are human pathogens and have been isolated from patients suggesting that they have a means of transmission from both aquatic and terrestrial environments to human. The ability of *Francisella* to infect a multitude of host species, some of them from different taxonomic orders or even classes, has made the identification of specific ecological transmission cycles a remarkable challenge.

1.3 Epidemiology

Wherry and Lamb reported the first human cases of tularemia in 1914 (Wherry and Lamb 2004) but the first extended report of over 600 case studies was published in 1928 by Edward Francis. Before this report, tularemia had been confused with other diseases. After this study, tularemia cases started being reported and by 1945, 14000 cases were reported in United States (Jellison 1950). Largest outbreak of tularemia involved 2291cases that were reported in 1939 (Sjostedt 2007). There have been several reports of tularemia cases from various parts of the world including Russia, United States, Europe and Asia but the incidents have decreased significantly over the last 90 years (Sjostedt 2007). This may be due to the increased rate of successful treatments or a decreased rate of infection. Tularemia was removed from the list of reportable diseases in 1994 due to the significant decline in the number of cases, but because of the increasing possibility of its use as a weapon, it was included again in 2000. The Island of Martha's Vineyard is considered an endemic site for tularemia due to several occurrences of pneumonic disease and two major outbreaks in 1978 (7 cases) and 2000 (11cases). The source of infection was found to be aerosol and wild life sampling trapped two rodents that were seropositive against *F. tularensis* antibodies suggesting that the natural occurring infection can occur without direct contact with an infected vector (Feldman, Stiles-Enos et al. 2003). However, most epidemiological analyses have failed to identify significant source of *F. tularensis* in the island (Feldman, Enscore et al. 2001).

1.4 Disease

1.4.1 Symptoms

The incubation period of tularemia is relatively short (3-10 days) and symptoms at the beginning are very non-specific: fever, chills, malaise and headaches (Tarnvik and Chu 2007). Rapid intracellular replication leading to high bacterial numbers and tissue necrosis are the characteristic features of the acute disease. Tularemia has several different forms of the disease that usually depend on the route of inoculation and dose of infection. Ulceroglandular tularemia is a form of the disease that comprises about 90% of the cases in Europe and is predominantly caused by *F. tularensis* subspecies *holarctica*. It is a result of the infection through skin or mucus membranes (usually vector borne or from infected animals). This form is characterized by an ulcer at the site of infection that is usually ignored by the patient, heals within a week, but bacteria trafficked to the lymph nodes can result in lymphadenopathy. Oropharyngeal or gastrointestinal forms of tularemia are rare and caused by the consumption of contaminated meat or water (Luotonen, Syrjala et al. 1986; Tarnvik and Chu 2007). Symptoms include diarrhea, vomiting and pheryngitis and regional neck lymphadenopathy. Occuloglandular tularemia cases were the first human cases reported in 1925. This is caused by the infection via the eye conjunctiva (Evans, Gregory et al. 1985; Perez-Castrillon, Bachiller-Luque et al. 2001) and the symptoms include prominent swelling of eyelids, conjunctivitis and photophobia (Tarnvik and Chu 2007). *Francisella* can survive in soil for several weeks and can easily be aerosolized making landscapers and farmers the most at risk population for inhalational tularemia. Respiratory/pneumonic tularemia is the most

serious form of tularemia. It is mainly a result of inhalation of aerosolized *F. tularensis* but any form of tularemia can cause pneumonia due to systemic spread. Infection can spread from the regional lymph nodes to other organs including spleen and liver resulting in systemic infection in any type of tularemia. When the specific route of infection is not known, the term 'Typhoidal tularemia' is used to describe the disease.

1.4.2 Diagnosis

Initial symptoms of tularemia are very non-specific and are common with other diseases making the diagnosis difficult. Most of the cases are either ignored by the patients (cutaneous, occuloglandular) or are misdiagnosed on the basis of common signs and symptoms with other diseases like flu, plague and anthrax (inhalational) and common diarrhea (gastrointestinal) (Tarnvik and Chu 2007). Commonly the diagnosis is made by culturing the organism from blood, lymph node exudates or the site of skin infections. Identifying *Francisella*-specific antibodies in the circulation is another diagnostic procedure, but generation of antibodies in the infected individual may take up to 12 days (Feldman, Stiles-Enos et al. 2003), while the therapy needs to begin quickly after the infection due to the rapid development and severity of the disease. Other detection methods using molecular techniques like PCR have been developed and are used throughout the USA in public health laboratories. Fluorescent antibody detection kits have also been developed and used but do not overcome the limitation of the time that is required for the body to accumulate detectable levels of antibody in the blood. Although

established effective treatment is available for tularemia, the need of a rapid and specific diagnostic test still remains because of the nonspecific initial symptoms of the disease.

1.4.3 Treatment

An effective treatment for tularemia is available today, as some bactericidal and bacteriostatic drugs work well against *Francisella*. Since 1940s, aminoglycosides, especially streptomycin, have been used as a drug of choice; however, it has been replaced mainly by gentamicin due to vestibular toxicity and hypersensitivity reactions among handlers (Enderlin, Morales et al. 1994). Among bacteriostatic agents, chloremphenicol was used until 1960 but now it is not used due to rare but severe hematologic reactions. Tetracyclin is the only bacteriostatic drug that is currently used, but the chances of relapse of the infection are always a cause of concern with bacteriostatic drugs (Mason, Eigelsbach et al. 1980; Enderlin, Morales et al. 1994). Doxycyclin is another drug with less severe side effects that is used mainly prophylactically. Although historically aminoglycosides have been used most frequently for treatment, less toxic and more efficient quinolones have produced some promising results for oral treatment against Type B infections (Syrjala, Schildt et al. 1991). Ciprofloxacin is a highly used drug to treat Type B tularemia. All the protection data available today for LVS against Type A challenge in human volunteers was generated in United States in 1960s as Type A strain is not found in Eurasia. Streptomycin was the drug of choice for all these vaccine studies.

1.5 Biosafety and laboratory safety

F. tularensis subspecies *tularensis* is considered the most virulent species with the airborne infectious dose being between 10-50 colony forming units according to the human volunteer studies done in 1950s and 1960s (Eigelsbach and Downs 1961; Saslaw, Eigelsbach et al. 1961; Saslaw, Eigelsbach et al. 1961). In recently reported cases from Martha's Vineyard, aerosolization of *Francisella* by mowing over the rabbit carcasses was found to be a potential cause of respiratory tularemia. A low dose of infection coupled with high lethality if untreated makes *F. tularensis* a highly infectious organism requiring special controlled laboratory environment of biosafety level -3 (BSL-3) to perform research. Organisms that are included to work in the BSL-3 conditions by the Centers for Disease Control (CDC) usually have a low infectious dose; typically have a risk for aerosol exposure and infections results in life threatening disease (http://www.cdc.gov/od/ohs/biosfty/bmbl5/bmbl5toc.htm). Before the development of these specialized laboratories, a high rate of laboratory acquired typhoidal infections were reported: 5.7 cases per 1,000 at risk employees. Use of these specialized BSL-3 laboratories has helped in reducing the cases of laboratory acquired tularemia coupled with the awareness of the risk of infections. Although tularemia cases have reduced due to the use of BSL-3 laboratories, *Francisella* researcher/clinician infections still remain high compared to other viral and bacterial agents studied in the laboratory (Pike, Sulkin et al. 1965).

1.6 Biological Warfare

Francisella is a hardy organism that can survive in soil and water environments for weeks, is infectious via the aerosol route and has a low infectious dose. These properties make it a suitable candidate for weaponization. Both U.S. and former Soviet Union had state-sponsored programs that researched and prepared *F. tularensis* as a biological weapon. Several reports have presented strong evidence for the increased incidence of tularemia during war. During the World War II, rodent populations were increased due to destruction of buildings, poor sanitary conditions and delay in harvests that lead to exposure of humans to rodents resulting in major tularemia outbreaks. The environment and parasites were left infected by these outbreaks, such that even after the mice population was controlled, there were still contaminated crops resulting in, for example 100,000 cases per annum in 1940s in the former Soviet Union (Jusatz 1952; Sjostedt 2007). Improved sanitary conditions, effective treatment and vaccination campaigns helped in reducing the number of cases subsequently and by 1997 only 100 to 400 cases were reported (Tarnvik, Priebe et al. 2004). During various civil and other wars significant numbers of cases have also been reported.

In US, virulent *F. tularensis* was being produced and loaded in warheads by 1955 at the Pine Bluff Arsenal in Pine Bluff, Arkansas. Bacterial preparations were packaged and kept rotating to keep it ready for deployment. However, the United States offensive weapon program ended after President Nixon signed the Unilateral Biological Weapons Convention in 1972 and the Pine Bluff Arsenal was decommissioned. Although the former Soviet Union signed the same treaty, BioPreparat (their biological weapons

programs) continued till 1990s. A former director of BioPreparat claimed that *F. tularensis* was genetically manipulated to increase lethality and/or decreased effectiveness of treatment or prophylaxis. He also confirmed that *F. tularensis* was used as a biological weapon by former Soviet Union in World War II.

1.7 Animal models of tularemia

F. tularensis infects a diverse variety of hosts ranging from rodents, ticks, flies, rabbits, amoeba and humans. It is almost impossible to establish an animal model that can mimic all of these host environments. The ability of *Francisellae* to survive in soil as well as aquatic environments makes it hard to establish a life cycle for this bacterium and thus makes it difficult to choose a model that can be called a 'reservoir'. Over past five decades, many models have been investigated for their suitability to determine the effectiveness of vaccines, therapeutics and/or prophylaxis. Rabbits have been of interest because they play an important role in transmission of the disease. Rabbits are very susceptible to Type A infection and the only vaccination study using formaldehyde killed bacteria produced good results with longer survival of vaccinated animals after challenge. White rats have been used as a model but have been reported to be naturally resistant to the *F. tularensis*. Guinea pigs were used in many studies but they are highly susceptible to *F. tularensis* and their response to vaccines is not known. *F. novicida* and other species of *Francisella* cause a tularemia like disease in mice, so mice are a good laboratory model to study the disease. Non-human primates have been used in the

vaccination studies as well and they respond like humans but unlike humans, are more susceptible to Type B strains than Type A (Rick Lyons and Wu 2007). Use of non-human primates is not cost effective and so limits their use in research. Human volunteers have mostly been used for vaccination studies in 1950s and 1960s by US Army. The data produced by studies on human volunteers is the most reliable and accurate on vaccines so far.

1.8 Vaccines

Only a few approved effective vaccines are in use today for intracellular bacteria like tuberculosis and typhoid despite the efforts that have been made to develop a stable vaccine over the last century. These efforts include generating vaccines that utilize whole killed bacterial preparations, specific antigens with a carrier, purified cell wall components like lipopolysaccharides and proteins, etc. All of these approaches have been tested for *F. tularensis* as well. Historically, whole killed bacterial antigen vaccine preparations have been tested in the mouse, guinea pig and rabbit models by vaccination followed by challenge by intraperitoneal, subcutaneous or intracerebral route, all failed to generate appreciable immunity in any of these models (Eigelsbach and Downs 1961). The first vaccine tested in human volunteers was called the Foshay vaccine (invented by Dr. Lee Foshay). Modifications of this vaccine prepared as phenolized gelatin hydrolysate liquid culture, phenolized synthetic liquid culture and acetone extracted

vaccine preparations were tested in laboratory workers and hunters showing only slight protection (Van Metre and Kadull 1959). Recipients of these vaccines still developed a lesion at the inoculation site but the frequency of generalized infection was reduced and a milder infection occurred. However therapeutic intervention was needed upon exposure to Type A virulent strain Schu4 (Van Metre and Kadull 1959).

The first Live-attenuated vaccine strain was tested in the US in the 1950s by Eigelsbach and McCrumb. A Turkish investigator had claimed that the live attenuated strain is a superior immunogen and works better than killed vaccines in mice and guinea pigs {reviewed in (Eigelsbach and Downs 1961)} . Several other reports also confirmed that this vaccine was prophylactically used in human in USSR {reviewed in (Eigelsbach and Downs 1961)}, however it was only tested against the Type B strain since only Type B strains are found in the USSR. In the US, a *F. tularensis* Type B attenuated strain was passaged in mice multiple times until it regained mouse virulence but was still attenuated in human. This strain was designated as *F. tularensis* subspecies *holarctica* Live Vaccine Strain or LVS. LVS is under review as an 'Investigational new drug' by FDA and is used to vaccinate at-risk populations such as laboratory and army personnel. Although the protective studies done with LVS show promising results, the unknown genetic mechanism of attenuation makes it difficult to certify it as a vaccine. Eigelsbach's studies also showed that there were two types of colonies in each culture that had different morphology, blue and grey, under oblique light settings and the grey variants lost the vaccine protective capacity. He called this phenomenon phase variation and it poses

another problem in determining the efficacy of LVS or any *F. tularensis* vaccines and their safe use in humans.

Eigelsbach, Saslaw and others have tested the efficacy of several vaccines including LVS in early studies. He used the LVS that was derived from an original culture of *F. tularensis* subspecies *holarctica* strain from Gamaleia Institute in former Soviet Union (Eigelsbach and Downs 1961). This vaccine protected against subcutaneous infection of virulent *F. tularensis* but it was not as effective against aerosol challenge (Saslaw and Carhart 1961). The US army administered a program called "Operation Whitecoat" that allowed the testing of therapeutics and prophylactics against biological warfare agents in human volunteers. These volunteers were usually prisoners, army recruits, or conscientious objectors. LVS was tested in human volunteers followed by challenge with virulent strain in 1956 where it proved to limit subcutaneous and pneumonic forms of disease. In eleven out of twelve non-vaccinated volunteers in this study, the disease was initiated by subcutaneous inoculation of 10 CFU of *F. tularensis* Schu4 (Wayne Conlan and Oyston 2007). In the same study, only two out of nineteen intradermally vaccinated volunteers showed signs of the disease upon subcutaneous challenge with same dose of Schu4 (Saslaw and Carhart 1961; Wayne Conlan and Oyston 2007). Intradermal vaccination was also protective against challenge with 10-51 CFU of Schu4 via aerosol route in 8 to10 volunteers while non-vaccinated volunteers had symptoms of disease with the same dose (Saslaw and Carhart 1961). Saslaw also determined that the killed whole bacteria vaccine was less effective than LVS. LVS was field tested in all the staff involved in the Tularemia program by vaccination by

scarification at the United States Army Medical Research Institute for Infectious Diseases (USAMRIID). Volunteers, who developed the disease after vaccination with LVS in this study, had milder disease and the recovery was rapid upon therapeutic intervention. Although the LVS has limitations to be used as a vaccine, no better vaccine has been developed or submitted for approval to FDA. The efficacy of LVS was not proved to be 100% in any human study via any route of challenge but the fact that it was able to provide some level of protection has made it the only vaccine that has been administered to laboratory and military personnel (Wayne Conlan and Oyston 2007).

Several protenaceous subunit vaccines have been tested but have failed to protect against virulent Schu4 strain challenge. Finding an appropriate adjuvant, the method of delivery of the antigen to the host and identifying a strong immunogen are some common problems associated with use of proteins as antigens for vaccines. Such a candidate is yet to be identified in the field of *F. tularensis.* Purified protein FopA and the lipoprotein Tul-4 are two highly immunogenic membrane proteins that have failed to generate a protective response (Wayne Conlan and Oyston 2007). Huntley et al., isolated the outer membrane proteins using a corrected method to eliminate the usual contaminants like LPS, periplasmic proteins and inner membrane proteins (Huntley, Conley et al. 2008). They used these outer membrane proteins in vaccine studies and showed that they provide up to 50% protection against *F. tularensis* Schu4 (Huntley, Conley et al. 2008). The need of characterization and further testing of proteins with correct adjuvant and most efficacious route of inoculation still remains.

O-antigen of the lipopolysaccharide (LPS) layer of the bacterium is the only antigen that has been shown to provide some degree of protection (Thomas, Titball et al. 2007). Vaccination with purified whole LPS gives protection against systemic Type B challenge but not against aerosol challenge and it does not protect against Type A systemic or aerosol challenge (Eigelsbach and Downs 1961; Fulop, Manchee et al. 1995; Thomas, Titball et al. 2007). Since some of the virulence factors have been identified, mutations in these genes have also been tested as vaccines. While mutations generated in *F. novicida* or LVS provide protection against homologous challenge, none of them have provided any protection against virulent Type A challenge (Lauriano, Barker et al. 2004; Mohapatra, Soni et al. 2007). An auxotroph of purine was also generated (by a mutation in purine biosynthetic pathway) with the same intentions in both LVS and Schu4. When tested, auxotrophic LVS provided protection against LVS challenge but auxotrophic Schu4 did not protect against homologous challenge (Pechous, McCarthy et al. 2008). With the improvement in molecular techniques, additional individual as well as combinatorial mutations need to be created in the virulent Schu4 strains that hopefully will overcome the issues with LVS and aide in construction of a successful vaccine.

1.9 Phase variation

Eigelsbach in 1951 reported two morphologically distinct colony types isolates from the Live Vaccine Strain (Eigelsbach and Downs 1961). When he saw these colonies in a dissecting microscope under obliquely transmitted light, one appeared blue and the other

appeared grey. He named them blue variant (BV) and grey variant (GV) (Eigelsbach and Downs 1961). Subsequent studies by him showed that these variants were relatively stable when streaked individually but they did revert phases occasionally. He also tested virulent Schu4 and an avirulent clinical isolate 38 in his later studies and isolated several variants including grey, non smooth (rough), non smooth with central plateau and smooth with blue ring based on opacity and color in oblique light settings (Eigelsbach, Braun et al. 1951). They were also shown to be different in growth (GV grew slower), acriflavin induced flocculation (positive in grey, negative in blue), agglutination in saline (positive in grey), acid agglutination (positive in grey) and most importantly immunogenic potency (grey less immunogenic) (Eigelsbach, Braun et al. 1951). The lethal dose for blue smooth variants of Type A was 1-10 organism administered via intraperitoneal route whereas lethal dose for non-smooth grey variants was between 10 million to 100 million organisms in mice (Eigelsbach, Braun et al. 1951). In LVS also, blue variants were more virulent and immunogenic than grey variants. On the basis of these results, he concluded that more than one colony type existed for Type A Schu4 and that variants are present in each vial of LVS. He called this phenomenon as 'phase variation' and the variant as phase variants. A comparative vaccine study was conducted by Eigelsbach in 1961 which showed that the grey variant did not protect against Schu4 challenge but blue colonies did. This became a great cause of concern with regards to potency and efficacy of LVS as a vaccine because grey variants were significantly less immunogenic and thus would decrease the potency of the vaccine.

Cowley et al., also isolated two types of variants from LVS in 1996, one was grey small slow growing colonies (called LVSG) and the other was rough (LVSR) (Cowley, Myltseva et al. 1996). Upon further characterization of these variants they found that the phase variants could revert back and that the frequency of phase variation was significantly higher in stationary phase liquid cultures, in vivo in mice and in vitro in macrophages. The most significant finding of this study was that these grey variants had immunologically different LPS than LVS and that different parts of LPS (O-antigen and Lipid A) appeared to be altered or have altered activity (Cowley, Myltseva et al. 1996). They showed that purified LPS of LVS specifically reacted with only the *F. tularensis* specific monoclonal antibodies against O-antigen whereas LPS of LVSG reacted with both *F. tularensis* and *F. novicida* specific monoclonal antibodies, suggesting that LVSG O-antigen must acquire some changes when it undergoes phase variation. They also showed that lipid A can mimic the activity of whole LPS in terms of inducing NO production in rat bone marrow derived macrophages, and that lipid A from LVSG induced more NO production than LVS lipid A. This suggested that lipid A and O-antigen both contribute to the phenotypic differences between LVS and LVSG.

In 2006, Hartley et al, identified another type of grey variant of LVS (Hartley, Taylor et al. 2006). These variant colonies were isolated by using fluorescence activated cell sorter (FACS) from the LVS culture. This grey variant lacks the O-antigen part of LPS and is thus rough and did not react with *F. novicida* specific monoclonal antibodies to O-antigen. It did not protect against Schu4 challenge and survived less well in J774.1 macrophages (Hartley, Taylor et al. 2006).

All these studies prove that phase/antigenic variation does exist and occurs spontaneously in *F. tularensis* species. It is clear that none of these grey variants induce protective immunity in the host and that virulence was often affected. Since there is not a standard foolproof technique to sort the grey variants from the vaccine lots, phase variation poses a great problem in determining the potency of a specific vaccine batch. Due to biological and immunological differences in phase variants, it is important to study the phenomenon in great detail so that the mechanism of variation could be determined. This would help in making a strain where the phase could be locked so it does not phase vary, producing a more stable and reliable vaccine.

1.10 Components of cell wall

Since Cowley et al., showed association of LPS with blue-grey phase variation in *Francisella,* it is important to understand the structure and function of the cell wall. Gram-negative bacteria are typically surrounded by an inner membrane, a thin peptidoglycan cell wall, which itself is surrounded by an outer membrane containing lipopolysaccharide. Major constituents of cell wall of the Gram-negative bacteria are glycans, lipoglycans and proteins. The outermost surface on *F. tularensis* consists of a glycocalyx, or a capsule, and LPS O-antigen. The innermost components include extracellular membrane proteins, core polysaccharide, and lipid A portion of LPS. Previously, capsule has been characterized as an electron lucent material (typical of a loose glycocalyx) surrounding bacilli that are grown in defined media (Geisbert, Jahrling

et al. 1993; Cherwonogrodzky, Knodel et al. 1994). Hood et al. compared the isolated capsular material to the cell wall contents of decapsulated bacteria and reported it to be biochemically different (Hood 1977). An ortholog of putative capsular operon in *B. anthracis* has been found in LVS and Schu4 and a mutation in the *capB* gene lead to the attenuation of *F. tularensis* subspecies *tularensis*, and this mutation also afforded protection against homologous challenge (Michell, Dean et al. 2010). Apicella et al., further characterized the capsular polysaccharides and found them to be identical to the LPS O-antigen and that mutants of LPS O-antigen genes still produced capsular O-antigen but not LPS O-antigen (Apicella, Post et al. 2010). On the basis of these observations, he called it O-antigen capsular polysaccharide (Apicella, Post et al. 2010).

LPS as mentioned above, constitutes the outer leaflet of the outer membrane and has been shown to be essential in Gram-negative bacteria. LPS plays an important role in maintaining membrane integrity and is a key factor in stimulating the immune response when detected by host pattern recognition receptors (Trent, Stead et al. 2006). Structurally, LPS can be divided in three parts; the lipid A portion of LPS that anchors it into the membrane, a polysaccharide core attached to lipid A and an oligo or polysaccharide that extends from the core to beyond the bacterial surface, also called O-antigen. Gram negative bacterial LPS has classic endotoxic properties and are typically recognized by innate immune system via Toll like receptor-4 in association with MD2, where Lipid A is the ligand to which TLR-MD2 binds. Numerous studies have shown that *F. tularensis* LPS does not elicit a strong innate immune response and this poor stimulation has been attributed to the unique LPS structure. First unique feature is that

LPS of *F. tularensis* lacks a phosphate at the 4' position on the non-reducing glucosamine backbone dimer. Secondly, lipid A is hypoacylated (tetra-acylated instead of six acyl groups) with long acyl side chains containing 16-18 carbon molecules, both factors lead to poor TLR-4/MD2 recognition (Gunn and Ernst 2007).

1.1 Structure of LPS

Lipid A- Structural analysis of the *F. tularensis* showed that both Type B and *F. novicida* have same basic lipid A structure. It consists of a β-(1,6)-linked glucosamine disaccharide backbone. Amide-linked fatty acids are attached with each glucosamine at the 2 ((18:0)-3-OH) and 2' (branched acyloxyacyl group: (18:0)-3-(16:0)) positions and ester linked fatty acids are only present at the 3 ((18:0)-3-OH) position (not the 3' position) (fig.1) (Vinogradov, Perry et al. 2002; Phillips, Schilling et al. 2004; Shaffer, Harvey et al. 2007). This acylation pattern is unusual as the inflammatory enteric bacteria that have shorter (12-14 carbons per chain) hexa-acylated lipid A species. Most of the *Francisella* strains tested have a phosphate attached to the diglucosamine backbone but in LVS, a phosphate is located on the reducing moiety at position 1. The 4' phosphate is absent due to the phosphatase activity of LpxF (Wang, Ribeiro et al. 2006) and is one of the probable causes of the reduced stimulation of TLR4 (Baldridge and Crane 1999). The addition of mannose at the 4' position can occur in *F. novicida.* Another modification at the 1' phosphate is the attachment of positively charged sugar galactosamine and glucose substituted for core region sugars at the 6' position of the non-reducing lipid A

galactosamine (Vinogradov, Perry et al. 2002; Phillips, Schilling et al. 2004; Wang, Ribeiro et al. 2006; Shaffer, Harvey et al. 2007). It was recently reported that over 95% of the LPS expressed by subspecies *novicida* is in the form of free lipid A but this finding is controversial and may not apply to all subspecies. Mass spectrometric analyses of lipid A from clinical isolates of subsp. *tularensis* and *holarctica* suggested that the common structures existed among all *F. tularensis* subspecies (Hajjar, Harvey et al. 2006).

Core- The core region of subspecies tularensis and holarctica has been studied and was found to be identical. Core is attached to the 6' position of lipid A-associated galactosamine by 2-keto-3-deoxy-D-manno-octulosonic acid (Kdo) (Vinogradov, Perry et al. 2002). Unique feature of *F. tularensis* core is that it lacks phosphate modifications and contains a single Kdo sugar. *F. novicida* is different from others in that core has addition of glucose residues attached to the β-Glc attached to the α-Man residue in the central inner core (fig.1).

O-antigen- Structural analysis performed on the O-antigen of Type A and Type B strains has revealed them to be identical whereas the O-antigen of subspecies *novicida* differs in the outside two residues of the four sugar repeating subunit (α-D-GalNAcAN and β-DQui2NAc4NAc for *F. novicida* versus β-D-Qui4NFm and β-D-QuiNAc for Type A and B) (Vinogradov and Perry 2004). The internal two carbohydrate residues (α-D-GalNAcAN- α-D-GalNAcAN) are the same in all studied strains.

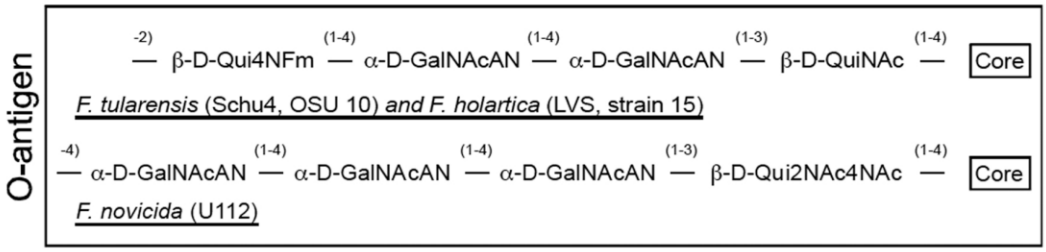
Figure 1. Structure of lipid A, core, and O-antigen molecules synthesized by *F. tularensis* subspecies *tularensis, holarctica, and novicida*.

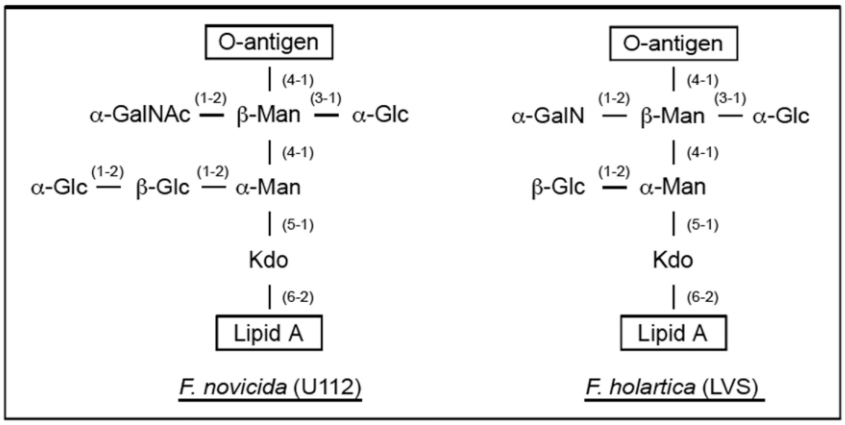
The lipid A structure consists of a β-(1,6)-linked glucosamine disaccharide with amide-linked fatty acids at the 2- and 2'-positions, and ester-linked fatty acids at the 3-position. Lipid A carbohydrate modifications include the addition of galactosamine through the 1-position phosphate, mannose at the 4'-position and glucose at the 6'-position. Lipid A molecules that have glucose in their structure would not be modified by the addition of Kdo-core-O-antigen.

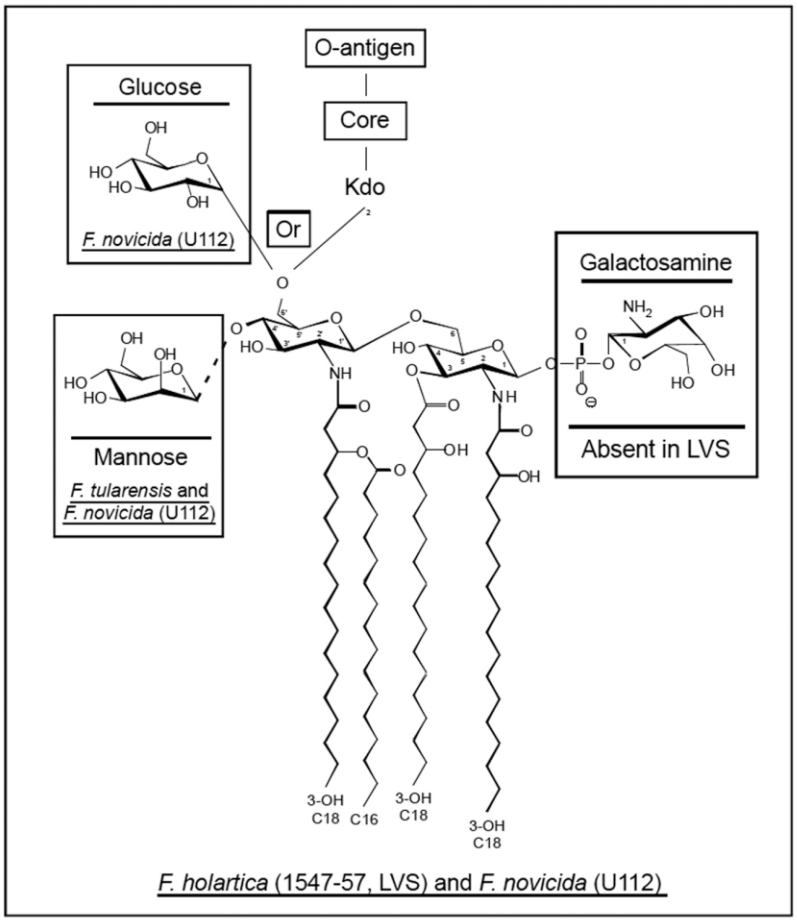
Unless noted on the structure, modifications are present in all *F. tularensis* subspecies.

Linkages of individual carbohydrate residues are shown. Core: Kdo = 2-keto-3-deoxy-Dmanno-octulosonic acid; Man = mannose; Glc = Glucose; GalNAc = N-acetylgalactosamine. O-Antigen: QuiN4Fm = 4,6-dideoxy-4-formamido-D-glucose;GalNAcAN = 2-acetamino-2-deoxy-D-galacturonamide; QuiNAc = 2-acetamino-2,6,dideoxy-D-glucose; Qui2NAc4NAc = 2,4,-diacetamino-2,4,6-trideoxy-D-glucose.

From Gunn *et al.*, *Ann NY Acad Sci* 1105:202 (Gunn and Ernst 2007)


O-antigen
-2) β-D-Qui4NFm (1-4) α-D-GalNAcAN (1-4) α-D-GalNAcAN (1-3) β-D-QuiNAc (1-4) Core
F. tularensis (Schu4, OSU 10) and F. holartica (LVS, strain 15)
-4) α-D-GalNAcAN (1-4) α-D-GalNAcAN (1-4) α-D-GalNAcAN (1-3) β-D-Qui2NAc4NAc (1-4) Core
F. novicida (U112)


Core
O-antigen
(4-1)
α-GalNAc (1-2) β-Man (3-1) α-Glc
(4-1)
α-Glc (1-2) β-Glc (1-2) α-Man
(5-1)
Kdo
(6-2)
Lipid A
F. novicida (U112)
O-antigen
(4-1)
α-GalN (1-2) β-Man (3-1) α-Glc
(4-1)
β-Glc (1-2) α-Man
(5-1)
Kdo
(6-2)
Lipid A
F. holartica (LVS)


Lipid A
O-antigen
Core
Kdo
Glucose
F. novicida (U112)
Or
Galactosamine
Absent in LVS
Mannose
F. tularensis and F. novicida (U112)
3-OH C18
C16
3-OH C18
3-OH C18
F. holartica (1547-57, LVS) and F. novicida (U112)

In the present study we have characterized and compared the grey variants by previously used techniques as well as some new approaches. We have extensively studied the structure of LPS in the LVSG variant and then characterized the functions of different parts of LPS. We have identified some modifications that may contribute to the phenotypic changes in the grey variants and have mutated genes controlling these modifications to examine the effects of these genes on the blue/grey phenotype. We demonstrate that grey variants exhibit multiple LPS modifications when it undergoes phase variation and these changes in LPS contribute to the LVSG phenotypes.

Chapter 2

Identification and Characterization of phase variants of *Francisella tularensis* subspecies *holarctica* Live Vaccine Strain

Introduction

Francisella tularensis is a gram negative, facultative intracellular pathogen that causes tularemia in humans and animals (Oyston, Sjostedt et al. 2004; Keim, Johansson et al. 2007; Sjostedt 2007). The host can be infected by several routes including the lungs (inhalational), skin or mucous membranes (cutaneous) or by ingestion of contaminated food or water (gastrointestinal) (Keim, Johansson et al. 2007; Sjostedt 2007). *F. tularensis* has been characterized as a category A biodefense organism by the Center for Disease Control because of its high lethality and infectivity, particularly by the aerosol route. There are two major human virulent subspecies of *F. tularensis*: *F. tularensis* subspecies *tularensis* (Type A strain) found in North America and *F. tularensis* subspecies *holarctica* (Type B strain) found in Europe, Asia as well as North America (Ellis, Oyston et al. 2002). The Type A strain is highly infectious and when inhaled, even low doses (<10 bacteria) can cause life-threatening disease in humans (2). Type B strains are considered less virulent but can still effectively cause diseases in humans. *F.*

tularensis subspecies *novicida* and *F. tularensis* subspecies *mediasiatica* are other known subspecies of *Francisella* that are considered relatively avirulent for immunocompetant humans but are capable of causing systemic infection in other mammals (Ellis, Oyston et al. 2002; Keim, Johansson et al. 2007).

There are no approved vaccines available to prevent or treat tularemia in the United States (Oyston 2009). An attenuated live vaccine strain, *F. tularensis* LVS (*Ft* LVS), was derived from a Type B isolate of the pathogen (3) and is used as a vaccine in Europe and is in clinical trials for potential approval in the US. It elicits diverse protection in humans, monkeys, guinea pigs and mice depending on the route of vaccination against systemic challenge with virulent Type A *F. tularensis* (Eigelsbach and Downs 1961). The molecular basis for the attenuation of *Ft* LVS still remains unknown, though candidate factors have been identified (Rohmer, Brittnacher et al. 2006). In 1951, Eigelsbach (Eigelsbach, Braun et al. 1951; Eigelsbach and Downs 1961) first reported colony variants of TypeA Schu4 and *Ft* LVS possessing less protective capacity. Virulent Type A Schu4 strains were isolated from human ulcers and variants were derived from these cultures as well as an avirulent strain (named 38) that was isolated by Francis in 1920 from human lymph nodes but subsequently lost virulence (Eigelsbach, Braun et al. 1951). These variants were identified on the basis of colony morphology (rough colonies and smooth colonies) and their appearance under a field microscope viewed with oblique light, where *Ft* LVS appears blue and the variant as grey. Hartley et al. also tested the spontaneous occurrence of two *F.tularensis* strains (Schu4 and HN63) and found blue to grey conversion in both (Hartley, Taylor et al.

2006). suggesting that blue to grey variation is a frequent and perhaps common occurrence in wild type strains in environment as well as the laboratory created vaccine strain FT LVS. Grey variants were reported to be less virulent with a lethal dose of $>10^7$ colony forming units (CFU) and were less immunogenic/protective in challenge studies, where they afforded minimal protection to Type A challenge (Eigelsbach, Braun et al. 1951). These variants also differentially reacted to acriflavine agglutination, and demonstrated variable stability of colony morphology upon sub-culturing (Eigelsbach, Braun et al. 1951). The observation of grey variants depended on growth conditions including culture media, size of inoculums, pH and duration of culture growth.

Grey variants were first examined molecularly by Cowley et al. (Cowley, Myltseva et al. 1996). This variant (LVSG; *Ft* LVSG) demonstrated differential survival in certain macrophage types and the LPS of this variant, which possessed a lipopolysaccharide (LPS) O-antigen, was found to possess altered anti-LPS monoclonal antibody reactivity and stimulated increased nitric oxide production in macrophages. A rough grey variant (lacking an LPS O-antigen) was also recently characterized (Hartley, Taylor et al. 2006). This variant was identified on the basis of size and opacity, grew slower, had reduced intramacrophage survival, and poorly protected against Type A *F. tularensis* challenge. These studies suggested that LPS played an important role in this phase variation phenomenon.

In the present study, we confirmed and extended the phenotypic characterization of the grey variants The grey variants primarily characterized in this study, *Ft* LVSG, possessed a full length O-antigen (as opposed to previously characterized rough grey

variants), survived less well in human and rat but not mouse macrophages, produce more nitric oxide (NO) in rat macrophages and poorly protected against *F. tularensis* SchuS4 challenge in the mouse model. It is hoped that a greater understanding of the mechanism(s) behind phase variation will lead to phase locked strains that no longer vary, thus allowing the construction of safer, more immunogenic tularemia vaccines.

Material and Methods

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

Microscopy: Choc II plates containing bacteria were visualized under oblique light settings as suggested by Robert Miller at Dynport Vaccine Company LLC, Frederick, MD and as described by Eigelsbach et al.(Eigelsbach, Braun et al. 1951). Briefly, a focused light source, concave mirror and dissecting microscope with 10X objective magnification and a transparent stage were used to visualize blue and grey variants. The concave mirror was placed horizontally tilted upward so that the light beam would hit the upper concave region and the distance between mirror and the microscope was adjusted so that the light beam would reflect on the plate sitting on the stage of the microscope. Blue and grey colonies were observed and counted using these conditions. Samples were prepared for electron microscopy from overnight (~16 hrs) grown cultures of *Ft* LVS or *Ft* LVSG in TSB containing 0.1% cysteine HCl using methods as described previously (Mohapatra, Soni et al. 2008). In brief, cells were pelleted by centrifugation, washed in PBS and fixed with 2.5% warm glutaraldehyde for 15 min. followed by fixing with a combination of 2.5% glutaraldehyde and 1% osmium tetroxide in 0.1 M sodium cacodylate (pH 7.3) for 15 min at 4°C. Staining of the cells was accomplished by using 0.25% uranyl acetate in 0.1 M sodium acetate buffer (pH 6.3) for 45 min, and viewed after further processing by transmission electron microscopy using an FEI Technai G2 Spirit microscope at 60 kV. Multiple fields (>50) were examined to determine the average size (diameter and length) and shape of bacteria.

Silver Staining : Overnight (~24 hrs) grown bacteria from Choc II agar plates were suspended in PBS at a concentration of 3×10^{10} CFU/ml as determined previously by the optical density (OD_{600}) of diluted cultures and subsequent colony counts on solid agar.

Bacteria equalized by optical density (OD_{600}) were then pelleted, frozen and lyophilized overnight to obtain ~20mg of dry cells. LPS was purified using hot phenol/water method using the standard protocol as described by Apicella et al. (Apicella, Griffiss et al. 1994). LPS was separated by 15% SDS-PAGE and silver stained as described (Clay, Soni et al. 2008). Briefly, after fixing overnight in 40% ethanol and 5% acetic acid, gels were incubated in 0.7% periodic acid in fixing solution for 7 min and subsequently washed with multiple exchanges of water. The staining solution (0.013% concentrated ammonium hydroxide, 0.02 N sodium hydroxide, and 0.67% silver nitrate (w/v) was applied with vigorous agitation for 10 min, followed by three washes (each 10 min) in water. Gels were developed using a solution containing 0.275% monohydrous citric acid (w/v) and 0.0025% formaldehyde. Upon completion, 5% acetic acid was used to stop the development.

Microarray: Microarrays were performed using the Affymetrix gene chip expression analysis system (Santa Clara, CA). The RNA was prepared according to the supplied protocol; briefly, cultures were grown in Tryptic –Soy broth with 0.1% Cysteine HCL to an optical density of 0.5 at 600nm. Cells were harvested and RNA was isolated using an RNeasy kit (Qiagen, Valencia, CA). The cDNA was then generated on these RNA templates using random primers and reverse transcriptase. cDNA was then fragmented by using DNase I and labeled at the 3' termini with terminal transferase and biotinylated GeneChip labeling reagents. These samples were then hybridized to the custom GeneChip (supplied by Battelle) that contains oligos specific for 1804 open reading frames of the *F. tularensis* Schu4 genome. Washing and staining of the chips with

streptavidin-phycoerythrin were done using the Affymetrix Fluidics Station 450. Chips were scanned using the Affymetrix Genechip Scanner 3000.

Microarray data analysis: Changes in gene expression were analyzed using the GeneSiFter microarray data analysis system. The CHP files (Affymetrix MAS 5 normalized) for all samples were loaded into GeneSiFter. A list of differentially expressed genes was generated using a filtering criterion of a 1.5-fold or greater change in expression and a *P* value of <0.05 from analysis of variance. Using the method of Hochberg and Benjamini (25a) to derive a false discovery rate estimate from the raw *P* values, and a false discovery rate of 5% was used as a cutoff to correct the data.

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

Intramacrophage survival assays: Human monocyte-derived macrophages were

isolated using standard procedure as described elsewhere (Mohapatra, Soni et al.) and obtained with informed consent from healthy donors by an OSU IRB approved protocol. 2 $x10^6$ PBMCs/well (MDMs plus lymphocytes) were plated in a 24-well plate resulting in 2 $x10^5$ MDMs/monolayer after adherence of MDMs and washing. THP-1 macrophages were induced using 10 ng/ml phorbol myristate acetate (PMA).

Intramacrophage survival assays in human monocyte-derived macrophages, THP-1 macrophages, J774.1, RAW-264, MH-S mouse macrophages and rat alveolar and bone marrow derived macrophages were performed using following procedure. Macrophages were infected with *Ft* LVS, LVSG and *F. novicida* at an MOI of 50 and incubated at 37°C in a CO_2 (5%) incubator for 2 hrs. Cells were washed and 50µg/ml gentamicin was added to each well and incubated for 30 min. Cells were washed and replenished with fresh media containing 10 µg/ml gentamicin. At various time points cells were washed and lysed with 0.1% SDS and plated on Choc II plates to enumerate the colony forming units.

Nitric Oxide Production assays:

$2X10^5$ rat macrophages (alveolar or primary) were plated in each well of a 24-well plate. Macrophages were infected with LPS or lipid A at the concentrations indicated and incubated for 1 hour at 37°C in the CO_2 incubator followed by washing of the monolayers. After 2, 12 and 24 hrs at 37°C, macrophage supernatants were removed and the Griess reaction was performed on the supernatant (Promega Griess Reaction Kit) using sodium nitrite as a standard. In the NO inhibition assays, nitric oxide synthase inhibitor, N^G-mono-methyl arginine (NMMA from Sigma) was used at a concentration of

500 μM with culture media during the infection and incubation.

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

Results

Blue-Grey phenotypic variation

F. tularensis LVS phase has been observed to vary from a blue (wild type) colony to a grey colony variant (Eigelsbach, Braun et al. 1951; Cowley, Myltseva et al. 1996). Such grey variants have been both characterized with an extended LPS O-antigen (Cowley, Myltseva et al. 1996) as well as a truncated O-antigen (Hartley, Taylor et al. 2006). Our work described here is with the *Ft* LVSG isolate (a variant with an extended O-antigen), but at times comparisons are made to *Ft* LVSGD (a variant with no O-antigen). We examined various media conditions and growth phases to determine the conditions that affected the rate of phase variation. We found that *Ft* LVSG grows slower

than *Ft* LVS, forms smaller colonies on agar surfaces, and appeared grey by eye on Choc II agar plates under oblique lighting (Figure 1A and B). The frequency of blue to grey phase variation was higher (27-31%) in liquid cultures (tryptic soy broth +0.1% Cysteine HCL) grown to stationary phase (typically 30-48 hours) and plated on solid agar. The frequency of phase variation was minimal (2-5%) for bacteria grown on plates 1-2 days and in log phase liquid cultures. We also observed that the frequency of blue to grey phase variation dramatically increased when *Ft* LVS was passed through macrophages (23-27%) or recovered from organs of infected animals (31-36%). We also observed that the frequency of forward phase variation in broth grown bacteria (blue to grey) was always higher (~30%) than frequency of reverse (grey to blue) phase variation (5-7%).

To more clearly compare *Ft* LVS to *Ft* LVSG bacteria, log phase cultures were examined by transmission electron microscopy. Comparisons of average cell size were not significantly different, but more membrane vesicles were observed in *Ft* LVSG cultures (Fig. 2.2). It is not clear what impact this increased vesiculation has on the subsequent phenotypes described for *Ft* LVSG.

Ft LVSG LPS possesses full length O-antigen

Hartley et al., reported a grey variant that did not possess a full length O-antigen (Hartley, Taylor et al. 2006). It was shown previously that the LPS of *Ft* LVS and *Ft* LVSG had a complete O-antigen and had differential reactivity to monoclonal antibodies stated to be O-antigen specific (Cowley, Myltseva et al. 1996). To further examine the LPS O-antigen, we purified LPS from *Ft* LVS, *Ft* LVSG, *F. novicida*, *Ft* LVSGD and

performed silver staining on SDS-PAGE separated samples. Consistent with previously published results, the grey variant (*Ft* LVSG) possessed an O-antigen but *Ft* LVSGD was rough (lacked O-antigen) (Fig. 2.3). We also performed the silver staining with *F. tularensis* SchuS4 and a *F. tularensis* SchuS4 small colony grey variant and the *F. tularensis* SchuS4 small colony grey variant also appeared to produce an LPS with a repeating O-antigen (data not shown in this chapter).

Microarray analysis of *Ft* LVSG shows differential expression of some stress response genes but not LPS related genes

We performed microarray assays on the *Ft* LVS and *Ft* LVSG to determine if there were any LPS related genes that were differentially expressed. We found 56 genes in *Ft* LVSG that were differentially regulated (27 upregulated and 29 downregulated with cutoff ratio set to 2) consistently in three different sets of microarrays (Table 2.1). We chose 9 genes that were upregulated in *Ft* LVSG on the basis of highest fold change over *Ft* LVS expression and performed quantitative realtime PCR (qRT-PCR) analysis on them to reconfirm the results of microarrays (Figure 2.4). qRT-PCR results were inconsistent but generally all the genes were slightly upregulated (between 2-200 fold) in Ft LVSG. Most of these genes were of unknown function and were unique to the *Francisella* genome and listed as producing hypothetical proteins. Surprisingly, neither known LPS O-antigen biosynthesis operon genes nor any of the known lipid A biosynthetic or modification genes were differentially expressed in the conditions we tested except for FTT974 and FTT420, both of which were not shown to be differentially

regulated by qRT-PCR using multiple biological and technical replicates. Constructs were created to make mutations in FTT974 and FTT420 in *Ft* LVSG using suicide plasmid (pUC19) with no success and the over expression of them in *Ft* LVS using vector pKK214 did not change the phenotype of the colonies (data not shown). These genes were not pursued further because of the insights on the involvement of the *flm* genes in lipid A structural modifications.

The *Ft* LVSG variant has a rat and human but not mouse intramacrophage survival defect

It has been shown previously by Cowley et al. (Cowley, Myltseva et al. 1996) that *Ft* LVS and *Ft* LVSG intracellular growth/survival was similar in mouse macrophages, but that differences in growth/survival could be visualized in rat bone marrow-derived macrophages. We examined the survival of *Ft* LVS and *Ft* LSVG in various macrophages including J774.1 (a mouse macrophage cell line), Raw-264 mouse macrophages cell line and MH-S (a mouse alveolar macrophage cell line) and did not find any significant differences in survival of *Ft* LVS and *Ft* LSVG (Fig.2.5 A, B and C, respectively). However, we did observe that *Ft* LVSG survived less well in rat bone marrow derived macrophages and a rat alveolar macrophage cell line (ATCC# CRL-2192) as shown in Fig. 2.6 A, B and that this inhibition of growth of *Ft* LVSG can be reversed by using the nitric oxide inhibitor NMMA (Fig. 2.7). These findings were consistent with the previous findings of Cowley et al. (Cowley, Myltseva et al. 1996). We then examined intramacrophage survival in human monocyte-derived macrophages

(MDMs) and the THP-1 macrophage–like cell line (Fig. 2.8 A). We observed that the *Ft* LVSG strain survived less well over the first 12 hrs post-infection in both cell types with macrophage cell death at later time points. The defect was most prominent for the MDMs, where the *Ft* LVSG strain demonstrated nearly a log defect in survival at 12 hrs post-infection versus the *Ft* LVS strain (Fig. 2.8 B). Thus, the *Ft* LVSG strain has an intramacrophage survival defect in rat and human but not mouse macrophages.

Lipid A can mimic the activity of whole LPS in inducing NO production in rat macrophages

We found that *Ft* LVSG survived less well in rat bone marrow derived macrophages and rat alveolar macrophages (Fig.2.6 A, B) and that this inhibition of growth of *Ft* LVSG can be reversed by using nitric oxide inhibitor NMMA. Previous studies and reversal of growth of *Ft* LVSG in rat bone marrow derived macrophages in the presence of NMMA (nitric oxide inhibitor) indicated that nitric oxide production by macrophages is induced by *Ft* LVSG. To determine the involvement of LPS in the nitric oxide production, we isolated rat bone marrow derived macrophages and infected them with whole LPS (Fig. 2.9A) and lipid A (Fig. 2.9B) of *Ft* LVS & *Ft* LVSG, and collected supernatants that were further analyzed for nitric oxide production. The results showed that *Ft* LVSG LPS produced higher amounts of nitric oxide inside the macrophages whereas *Ft* LVS LPS produced much less nitric oxide. Interestingly, lipid A of *Ft* LVS and *Ft* LVSG could mimic this activity of whole LPS in rat macrophages and produced almost similar amounts of nitric oxide suggesting that lipid A is the biologically active

portion of LPS and that differences in lipid A were responsible for the variations in NO production by *Ft* LVS and *Ft* LVSG.

Ft LVSG and *Ft* LVS are similarly virulent in the mouse model but differentially protect against *F. tularensis* SchuS4 challenge

Grey variants have shown to be less virulent (Eigelsbach, 1961) and/or less protective as vaccines against *F. tularensis* SchuS4 challenge (Eigelsbach and Downs 1961; Cowley, Myltseva et al. 1996; Hartley, Taylor et al. 2006; Wayne Conlan and Oyston 2007) To compare the virulence of *Ft* LVS and *Ft* LVSG, BALB/c mice were infected with 100 cfu of *Ft* LVS and *Ft* LVSG intranasally and observed for survival. Both *Ft* LVS and *Ft* LVSG infected mice demonstrated 80% survival (N=10 mice; Fig. 2.10 A). The surviving mice were challenged with 1000 cfu of *F. tularensis* SchuS4 (~100-fold above the LD_{50}) intranasally four weeks post vaccination (Fig. 2.10 B). All *Ft* LVS vaccinated mice (N=8) survived the challenge whereas *Ft* LVSG vaccinated mice (N=8) could not survive the challenge and succumbed to infection within 5 days. These results suggest that *Ft* LVSG is as virulent in mice as *Ft* LVS but it does not protect against Type A challenge.

Discussion

F. tularensis LVS has been known to phase vary from a blue (i.e. wildtype) to a grey variant since the phenomenon was first described by Eigelsbach in 1951 (Eigelsbach, Braun et al. 1951). Such variation has proven to be an issue historically in vaccine production runs of *Ft* LVS (Wayne Conlan and Oyston 2007; Oyston 2009). The *Ft* LVS grey variants are problematic because they are dramatically less efficacious than the blue colony morphotypes in protection studies against the virulent *F. tularensis* Type A strain. The phenotypes associated with the grey variants are quite variable, as they have been described to give rise to different colony sizes and opacity, but the only two that have been molecularly characterized share the characteristic of LPS alteration. While this characteristic is shared, this also presents yet another difference, as the grey variant described by Hartley et al. (Hartley, Taylor et al. 2006), (similar to *Ft* LVSGD studied in this work) has been shown to lack O-antigen while *Ft* LVSG still possessed an O-antigen, albeit reduced in amount, which has altered *F. novicida*/*F. tularensis*-LPS specific monoclonal antibody reactivity patterns. Thus, grey variants are themselves variable, but an increased understanding of the mechanism(s) behind this variation would aid future tularemia vaccine production.

Clay et al. also demonstrated that the *Ft* LVSG strain was bound by complement component C3 in higher amounts than *Ft* LVS and was dramatically more susceptible to complement-mediated killing (Clay, Soni et al. 2008). It is of interest that, while susceptible to complement, *Ft* LVSG is still as virulent as *Ft* LVS in the mouse model. It is likely that the effect of complement-mediated killing is quantitative rather than

absolute and that the current mouse model is not sensitive enough to record an effect. Alternatively, the result in the mouse model may reflect a fundamental difference in complement function between mouse and man. For example, it is known that C3 in mouse serum is more labile than in human serum and this may result in different levels of C3 opsonization and regulation in mouse versus human serum.

Experiments performed in this study confirmed those of Cowley et al. (Cowley, Myltseva et al. 1996) regarding increased nitric oxide induction in rat but not mouse phagocytes by the grey strain LPS. This correlated with decreased survival of these grey variants in rat and human macrophages, which could be reversed in rat macrophages by the addition of a nitric oxide synthase inhibitor. However, experience suggests that stimulated mouse macrophages are better capable than, for e.g. human MDMs, of nitric oxide production. Thus, there is no direct correlation of strain intracellular survival with the inherent capabilities of the macrophages of the chosen animal model to produce nitric oxide, suggesting the involvement of additional factors.

Microarray results show a few differentially regulated genes of which only two genes were of interest listed as amidinotransferase family protein (FTT974) and UDP-N-acetylmuramoylalanyl-D-glutamate--2,6-diaminopimelate ligase (FTT420). Upon genomic analysis, FTT974 turned out to be a pseudogene and FTT420 did not show consistent differential expression by qRT-PCR. This is of note that the custom chips used to perform the microarrays had probes for *F. tularensis* subspecies *tularensis* as this was the only available genome sequence at the time and the array contains the genomes of several other pathogens. This may have caused some non-specific binding/unimportant

specific binding considering that there are significant differences in the genomic sequences of *Ft* LVS and virulent strain Schu4.

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

move the field closer to the ability to phase lock a wildtype strain for the development of effective and safe tularemia vaccines.

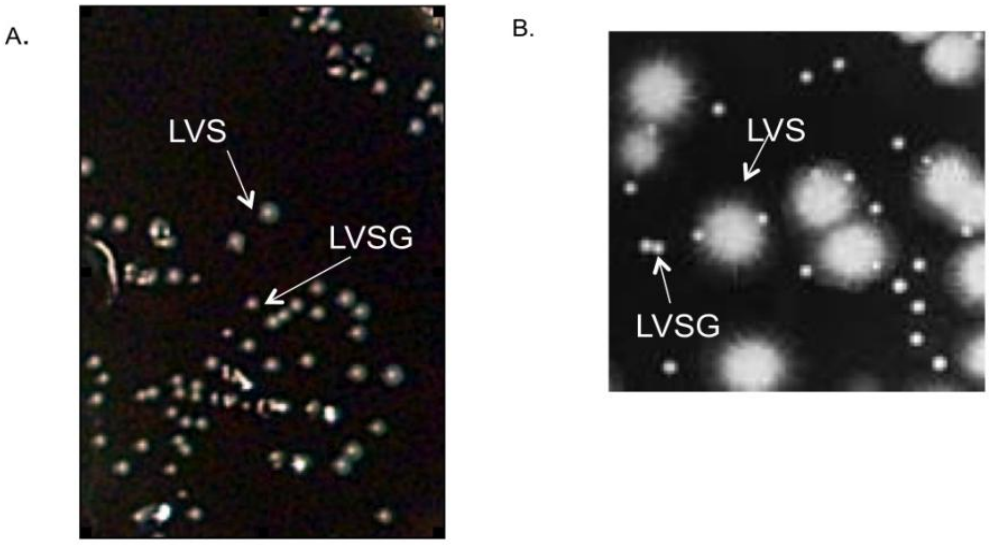


Figure 2.1: *Ft* LVS blue-grey colony and bacterial morphology;

(A) Mixed cultures of *Ft* LVS and phase variant (*Ft* LVSG) viewed on the surface of a Choc II agar plate. Note that *Ft* LVSG forms smaller colonies that *Ft* LVS. (B) Blue (LVS) and Grey (LVSG) colonies viewed under a compound light microscope by oblique lighting. The smaller colonies appeared grey.

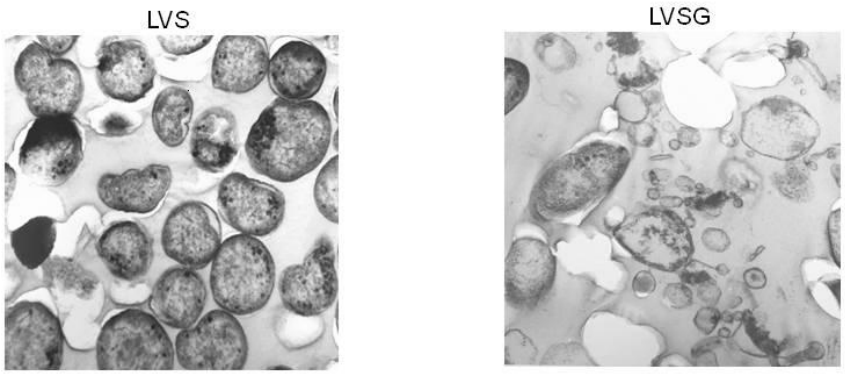


Figure 2.2: Transmission electron microscopy of *Ft* LVS and *Ft* LVSG;

While the bacterial dimensions were similar between these two strains from measuring >50 fields, the *Ft* LVSG strain formed a large amount of membrane vesicles.

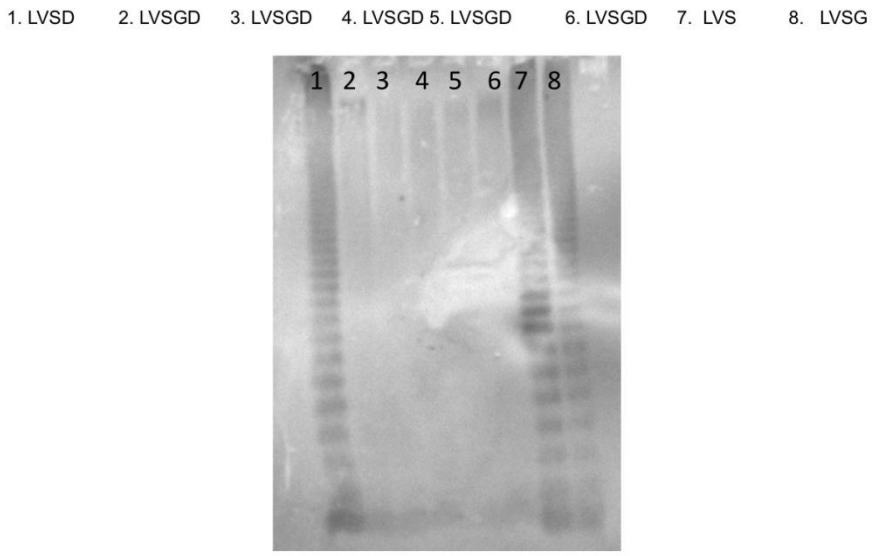


Figure 2.3: Silver stained gel of LPS purified from various strains; LPS purified from strains listed above the lanes shows rough and smooth phase variants. LVSGD do not posses O-antigen and LVSG possesses full length O-antigen.

FTT No.	Ratio	Gene name
0553	4.13	hypothetical protein
0821	3.6	conserved hypothetical membrane protein
1047	3.15	hypothetical protein
0974	2.93	amidinotransferase family protein, pseudogene
0640	2.85	Dihydroxy-acid dehydratase
0677	2.72	hypothetical protein
0420	2.47	UDP-N-acetylmuramoylalanyl-D-glutamate--2,6-diaminopimelate ligase
0639	2.45	hypothetical protein
0850	2.44	hypothetical protein

Table 2.1: Microarray analysis in LVS and LVSG; Genes listed above are upregulated in LVSG by the indicated fold change.

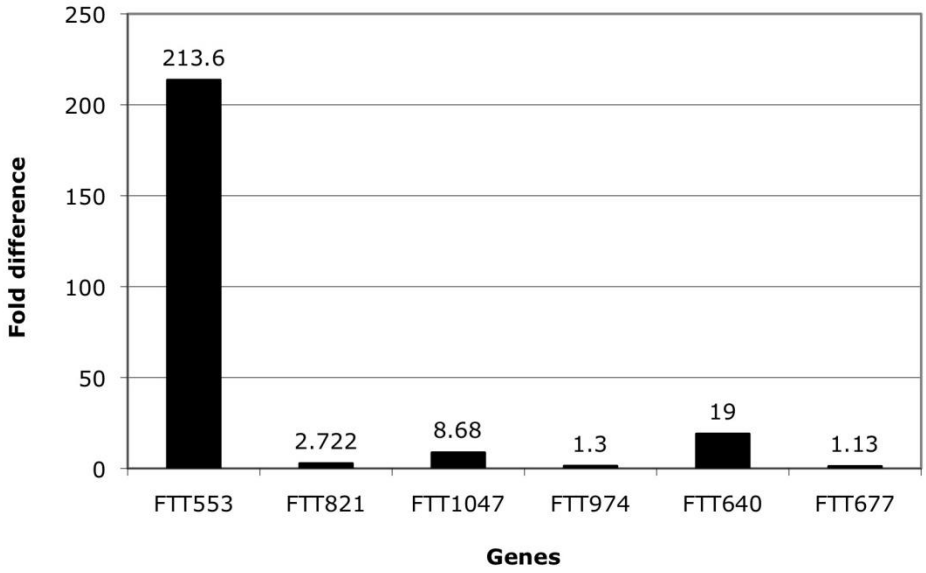


Figure 2.4: Differential expression of upregulated genes in *Ft* LVSG;

as determined by real-time PCR. RCN-Relative copy number, actual values are shown above the bars.

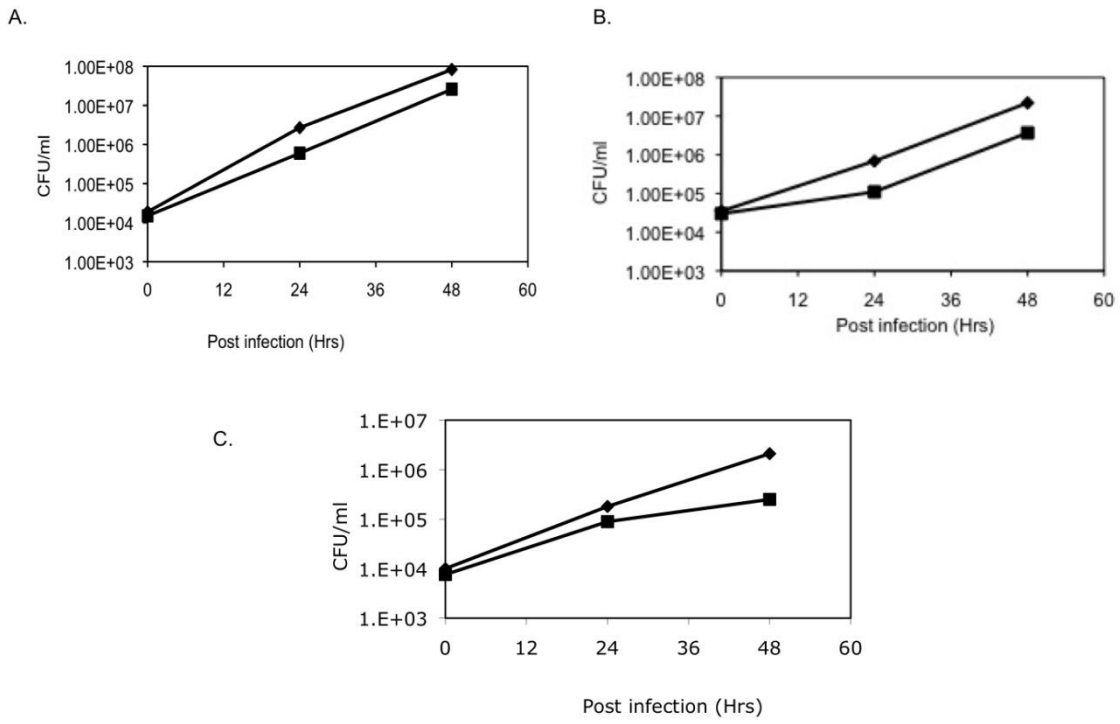


Figure 2.5: Intramacrophage survival assay of *Ft* LVS and *Ft* LVSG;

in J774.1 murine macrophages (A), RAW 264.7 macrophages (B) and MHS murine alveolar macrophages . Symbols: diamond, *Ft* LVS; square, *Ft* LVSG. The data presented is from an assay performed in triplicate, and this experiment was performed three times on separate occasions with similar results.

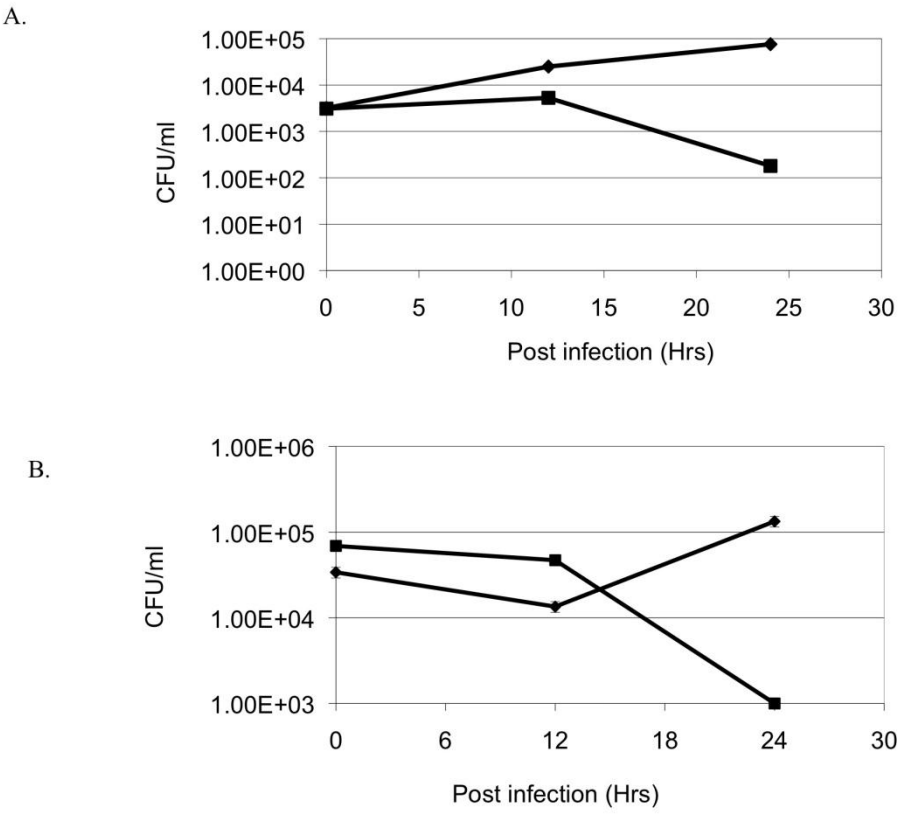
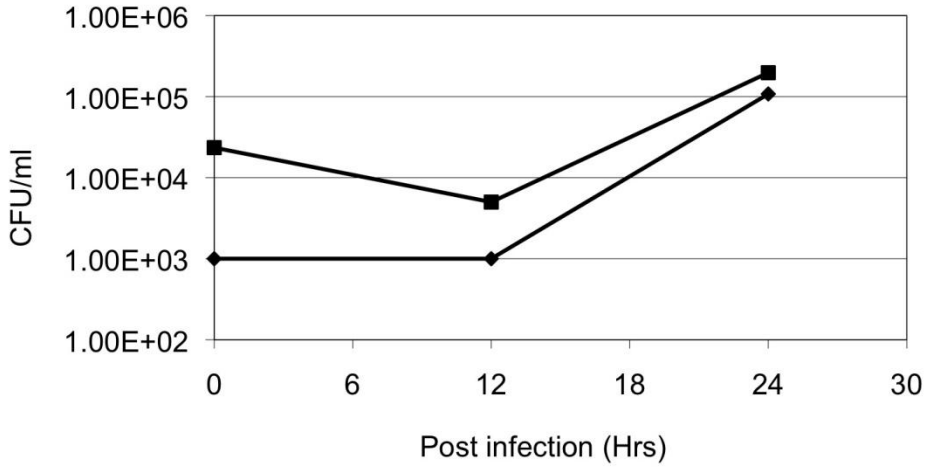


Figure 2.6: Intramacrophage survival assay of *Ft* LVS and *Ft* LVSG;

in rat primary macrophages (A), rat alveolar macrophages (B). Symbols: diamond, *Ft* LVS; square, *Ft* LVSG. The data presented is from an assay performed in triplicate, and this experiment was performed three times on separate occasions with similar results.

A.



B.

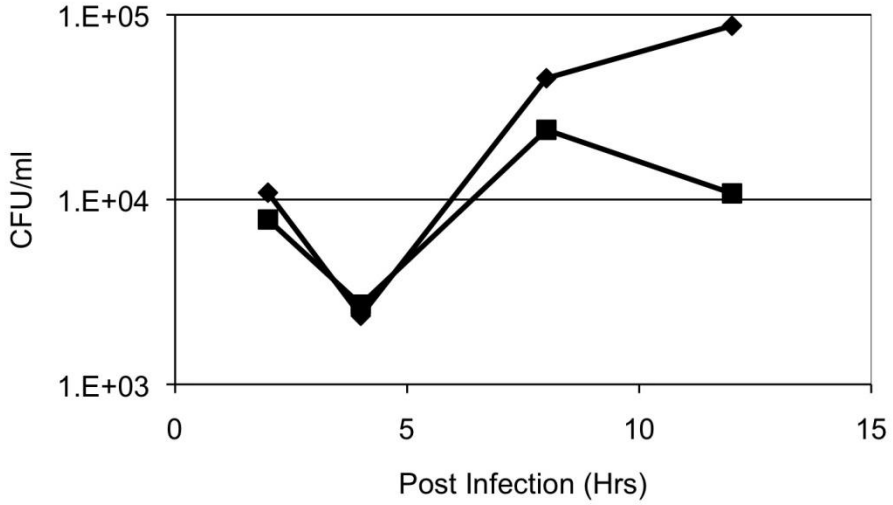


Figure 2.7: Intramacrophage survival assay of *Ft* LVS and *Ft* LVSG;

continued

Figure 2.7 continued

in PMA induced THP-1 macrophages (A), human monocyte derived macrophages (B). Symbols: diamond, *Ft* LVS; square, *Ft* LVSG. The data presented is from an assay performed in triplicate, and this experiment was performed three times on separate occasions with similar results.

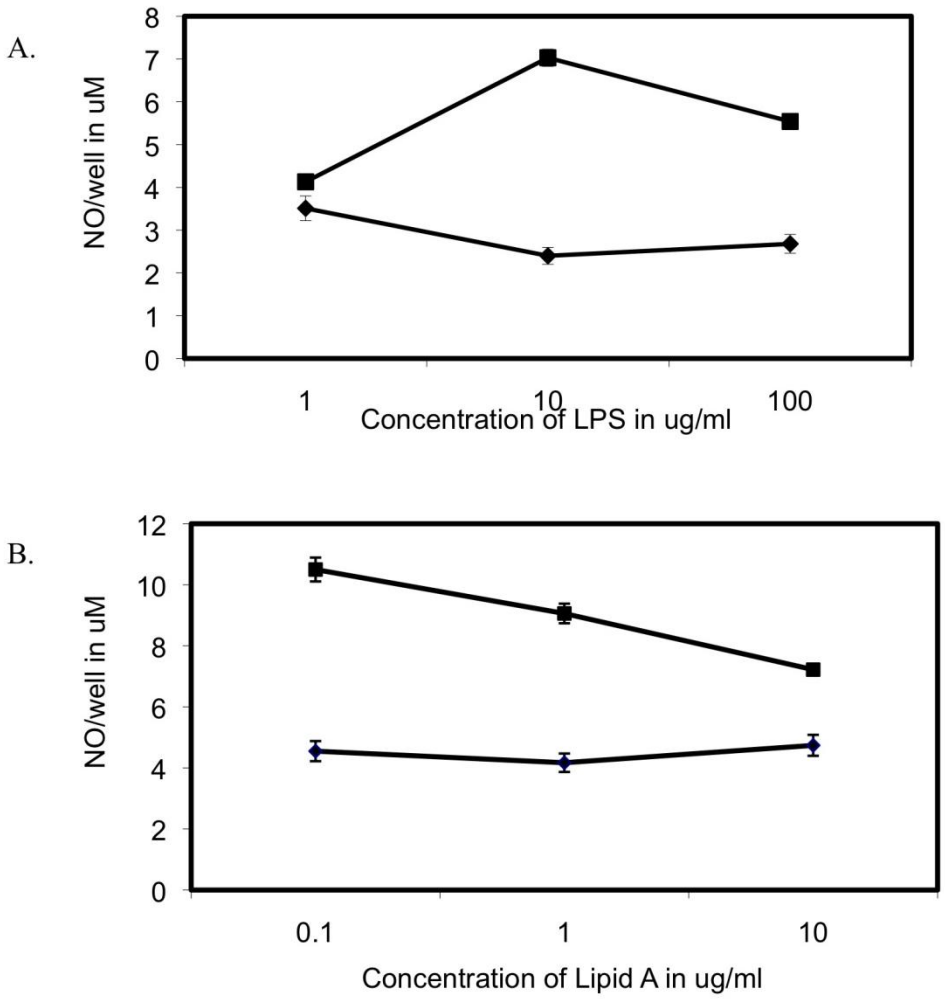


Figure 2.8: Detection of nitric oxide production, induced by different concentration of purified LPS (A) and lipid-A (B);

continued

Figure 2.8 continued

in rat bone marrow derived macrophages. Symbols: diamond, *Ft* LVS; square, *Ft* LVSG. The data presented is an average of three technical replicates from three independent experiments.

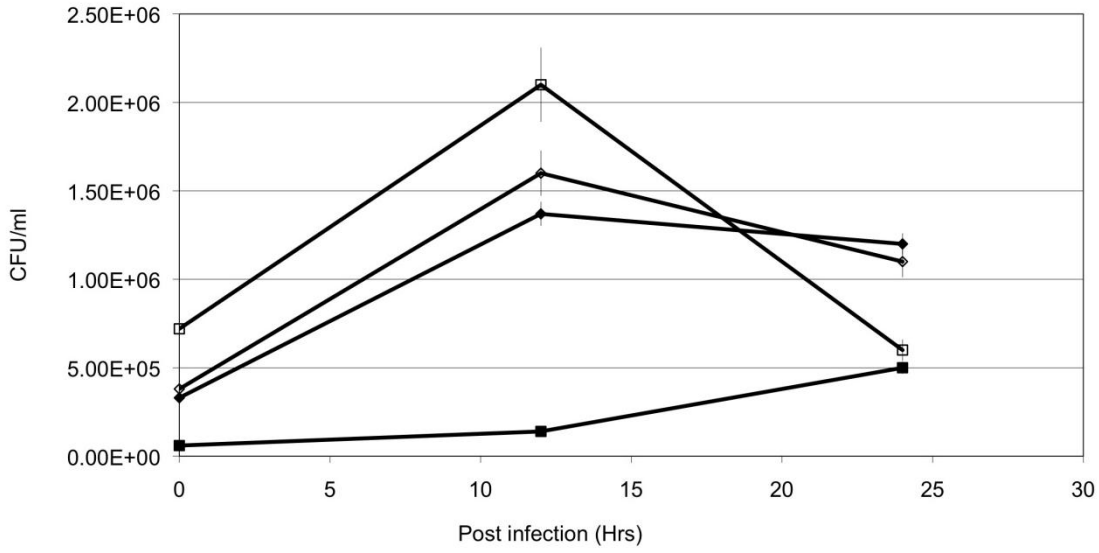


Figure 2.9: Intramacrophage survival assay of *Ft* LVS and *Ft* LVSG in presence of nitric oxide synthase inhibitor;

in rat bone marrow derived macrophages in the presence / absence of NMMA. Symbols: diamond, *Ft* LVS; square, *Ft* LVSG (Solid; absence of NMMA, open; presence of NMMA). The data presented is from an assay performed in triplicate, and this experiment was performed three times on separate occasions with similar results.

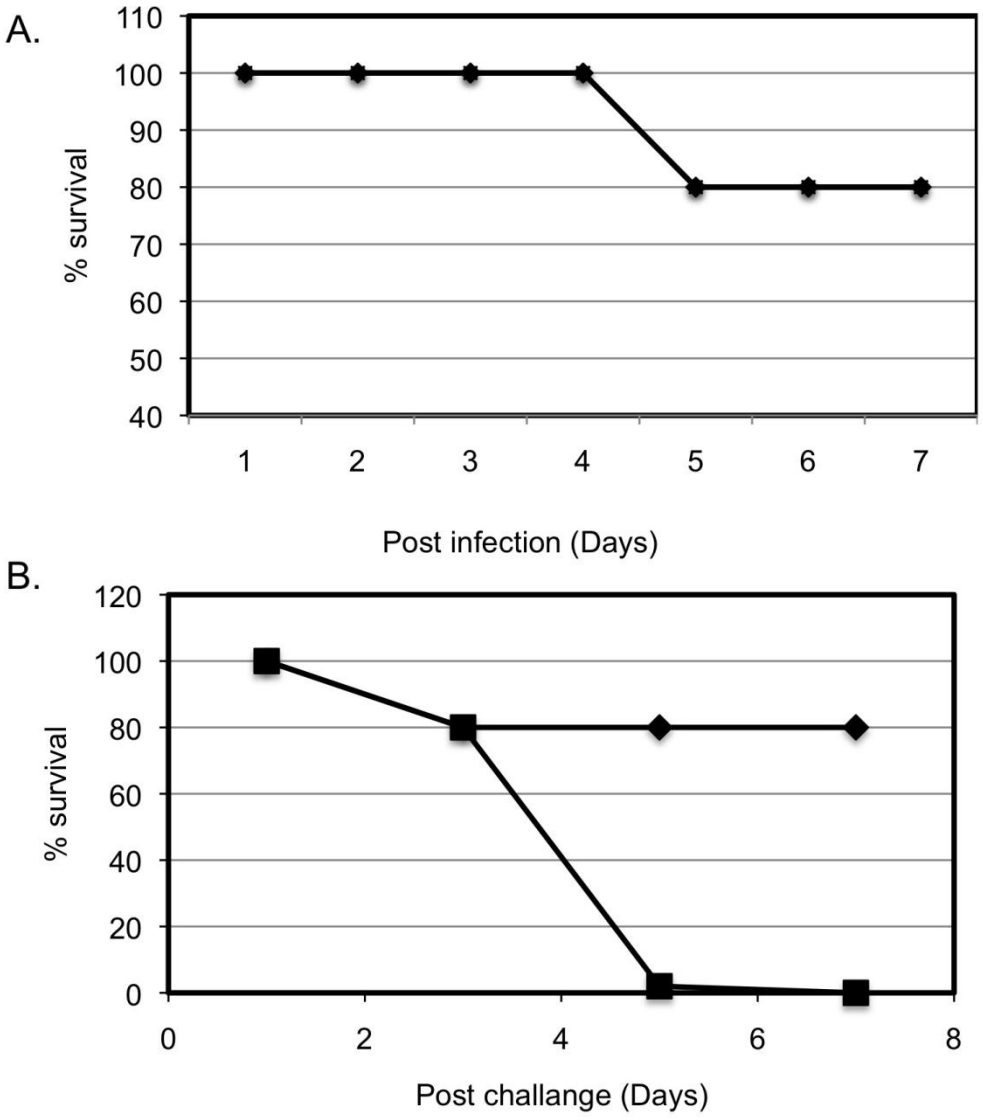


Figure 2.10: Virulence assays in the mouse model of tularemia;

Figure 2.10: Virulence assays in the mouse model of tularemia.

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

Chapter 3

Phase variants of *Francisella tularensis* subspecies *holarctica* Live Vaccine Strain have structural differences in O-antigen, core and Lipid A

Introduction

Francisella tularensis is a gram negative, facultative intracellular pathogen known to cause Flu like disease 'tularemia' in humans and animals (Oyston, Sjostedt et al. 2004; Keim, Johansson et al. 2007; Sjostedt 2007). The infection can occur by several inoculation routes in a wide variety of hosts suggesting a terrestrial as well as aquatic life for this bacterium. The localized occurrence of *F. tularensis* (endemic areas encompassing few hundred square kilometers)and capability of *F. tularensis* to persist in the environment without concomitant outbreaks of tularemia in the same geographical regions indicate the hardiness of the organism and makes the epidemiological studies difficult (Sjostedt 2007). Centers of Disease Control has categorized *F. tularensis* as a category A biodefense organism by the because of its high lethality and infectivity, particularly by the aerosol route.

An attenuated live vaccine strain, *F. tularensis* LVS (*Ft* LVS), was derived from a Type B isolate of the pathogen (3) and is used as a vaccine in Europe and is in clinical

trials for potential approval in the US. It elicits diverse protection in a range of hosts including humans, monkeys, guinea pigs and mice depending on the route of vaccination against systemic challenge with virulent Type A *F. tularensis* (Eigelsbach and Downs 1961). The molecular basis for the attenuation of *Ft* LVS is not known, though candidate factors have been identified (Rohmer, Brittnacher et al. 2006).

Several types of phase variants have been characterized that affect the potency of *Ft* LVS. In 1951, Eigelsbach (Eigelsbach, Braun et al. 1951; Eigelsbach and Downs 1961) first reported colony variants of Type A Schu4 and *Ft* LVS possessing less protective capacity. Virulent Type A Schu4 strains were isolated from human ulcers and variant were derived from these cultures as well as an avirulent strain (named 38) that was isolated by Francis in 1920 from human lymph nodes but subsequently lost virulence (Eigelsbach, Braun et al. 1951). These variants were identified on the basis of colony morphology (rough colonies and smooth colonies) and their appearance under a field microscope viewed with unique settings with oblique light, where *Ft* LVS appears blue and the variant as grey. Hartley et al., also characterized the spontaneous occurrence of two F.tularensis strains Schu4 and HN63 and found blue to grey conversion in both (Hartley, Taylor et al. 2006) suggesting that blue to grey variation is a frequent and perhaps common occurrence in wild type strains in environment as well as the laboratory created vaccine strain LVS.

Cowley et al., reported grey variants (LVSG; *Ft* LVSG), that demonstrated differential survival in different macrophage types and the LPS of this variant possessed a full length lipopolysaccharide (LPS) O-antigen (Cowley, Myltseva et al. 1996). It was

found to possess altered anti-LPS monoclonal antibody reactivity and stimulated increased nitric oxide production in macrophages. A rough grey variant (lacking an LPS O-antigen) was also recently characterized by Hartley et al. (Hartley, Taylor et al. 2006). This variant was identified on the basis of size and opacity, grew slower, had reduced intramacrophage survival, and poorly protected against Type A *F. tularensis* challenge. These studies suggested that LPS played an important role in this phase variation phenomenon.

Outermost surface on *F. tularensis* consists of a glycocalyx, or a capsule, and LPS O-antigen. The innermost components include extracellular membrane proteins, core polysaccharide and the lipid A portion of LPS. *F. tularensis* type A and type B strains produce an O-antigen capsule, composed of repeating O-antigen subunits, 2-acetamido-2,6-dideoxy-o-glucose (o-QuiNAc), 4,6-dideoxy-4-formamido-D-glucose (o-Qui4NFm), and 2-acetamido-2-deoxy-o-galacturonamide (o-GalNAcAN) (Apicella et al, 2010). This capsule has an epitopic structure distinct from the LPS as detected by monoclonal antibody 11B7. A deletion of lipid A acyltransferase mutant, *lpxL F. tularensis* and O-antigen polymerase mutant, *FTL_0706,* in *F. tularensis* LVS did not express repeating O-antigens but produced an LPS consisting of a core region plus one O-antigen subunit (Apicella, Post et al. 2010). The *lpxL* mutant expresses only a portion of the LPS core region, but still produces a capsule. The active and passive immunization studies indicate that the epitope defined by monoclonal antibody 11B7 is protective against a lethal challenge of *F. tularensis* LVS in mice and the post-immunization sera contains the O-antigen subunits of the capsule (Apicella, Post et al. 2010).

LPS makes the outer leaflet of the outer membrane and has been shown to be essential in Gram-negative bacteria (Gunn and Ernst 2007). LPS plays an important role in maintaining membrane integrity and is a key factor in stimulating immune response when detected by host pattern recognition receptors (Coats, Berezow et al. 2010; McGettrick and O'Neill 2010). Structurally, LPS can be divided in three parts; the lipid A portion of LPS that anchors it into the membrane, a polysaccharide core attached to Lipid A and an oligo or polysaccharide that extends from the core to beyond the bacterial surface, also called O-antigen. Gram-negative bacterial LPS has classic endotoxic properties and are typically recognized by innate immune system via Toll like receptor-4 in association with MD2, where Lipid A is the ligand TLR-4 binds to. Numerous studies have shown that *F. tularensis* LPS does not elicit a strong innate immune response and this poor stimulation has been attributed to the unique LPS structure. The first unique feature is that LPS of *F. tularensis* lacks a phosphate at 4' position on the non-reducing glucosamine backbone dimmer. Secondly, Lipid A is hypoacylated (tetra-acylated instead of six acyl groups) with long acyl side chains containing 16-18 carbon molecules (Gunn and Ernst 2007).

In the present study, we confirmed and extended the characterization of the LPS of grey variants using structural approaches. The grey variant primarily characterized in this study, *Ft* LVSG, possessed a full length O-antigen (as opposed to previously characterized rough grey variants) that was identical to that of *Ft* LVS but it showed structural modification in core and lipid A regions. The greater understanding of the

mechanism(s) behind phase variation will lead to phase locked strains that no longer vary, thus allowing the construction of safer, more immunogenic tularemia vaccines.

Material and Methods

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

Genetic manipulation: Constructs for the deletion of FTL1611 (*flmF2*) and FTL1609 (*flmK*) were made in pJC84 (Wehrly, Chong et al. 2009). The ~1Kb upstream region of

flmF2 was amplified using forward primer JG1823 (5'aaacgagctcgGGGTTATGGTGACTTCTGCATC 3') with SacI restriction site at the 5' end and reverse primer JG1824 (5'cgcggatccCACAAATACAAAATATATTAACCTTAATTAATGCTATTATAACC 3') with a 5' BamHI restriction site. The ~1Kb downstream region was amplified using forward primer JG1825 (5' cgcggatccAATATTGTTTTAAGCTAATGAAT CAATACTTATTAAATTCTTAG 3') and reverse primer JG1826 (5'acgcgtcgacGTATTATATTTTTAGTAGCAGCTGTTGCTGTTAT 3') with BamHI and SalI 5' flanking restriction sites, respectively. Similarly, the *flmK* upstream region was amplified by JG2290 (5' aaacgagctcgGATCTAATAC TGGATACCACTCATTATC 3') forward primer with a 5' SacI site and JG2291 (5'cgcggatccCTTCTTTACCCTCAAATAGAAACTTATAC 3') reverse primer with a 5' BamHI site, and the downstream region using the JG2292 (5'cgcggatccGATTTATCAGCATTAATTACTTTGATAAGCTAAG 3') forward primer with a 5' BamHI site and JG2293 (5'acgcgtcgacCATAGATAAGCGTACAGTTGTTTCATG 3') reverse primer with a 5' SalI site. Fragments were cloned in pJC84 sequentially and the construct was transformed into *Ft* LVS followed by chromosomal recombination using the procedure described by Wehrly et al. (Wehrly, Chong et al. 2009). Mutants were confirmed by PCR amplification and sequencing of the deleted region.

Silver Staining: Overnight (~24 hrs) grown bacteria from Choc II agar plates were suspended in PBS at a concentration of 3×10^{10} CFU/ml as determined previously by the

optical density (OD_{600}) of diluted cultures and subsequent colony counts on solid agar. Bacteria equalized by optical density (OD_{600}) were then pelleted, frozen and lyophilized overnight to obtain ~20mg of dry cells. LPS was purified using hot phenol/water method using the standard protocol as described by Apicella et al. (Apicella, Griffiss et al. 1994). LPS was separated by 15% SDS-PAGE and silver stained as described (Clay, Soni et al. 2008). Briefly, after fixing overnight in 40% ethanol and 5% acetic acid, gels were incubated in 0.7% periodic acid in fixing solution for 7 min and subsequently washed with multiple exchanges of water. The staining solution (0.013% concentrated ammonium hydroxide, 0.02 N sodium hydroxide, and 0.67% silver nitrate (w/v) was applied with vigorous agitation for 10 min, followed by three washes (each 10 min) in water. Gels were developed using a solution containing 0.275% monohydrous citric acid (w/v) and 0.0025% formaldehyde. Upon completion, 5% acetic acid was used to stop the development.

Western Blotting: Purified LPS samples (10µg/well) were electrophoresed on a 15% SDS-PAGE gel and transferred on nitrocellulose membrane using the Bio-Rad semi-dry transfer system. Immunoblotting was performed using either anti-*F. tularensis* LVS or *F. novicida* polyclonal sera (from infected mice) or monoclonal sera specific to the LPS of *Ft* LVS or *F. novicida*. Polyclonal sera to *Ft* LVS or *F. novicida* (1:1000 dilution), commercial *F. tularensis* FB-11 (1:1000, Abcam, Cambridge, MA), *F. tularensis* LPS specific monoclonal (1:10), or *F. novicida* LPS specific monoclonal (1:10) were used as primary antibodies with alkaline phosphatase conjugated goat anti-mouse IgG (1:4000) as the secondary antibody. The *F. tularensis*-specific and *F. novicida*-specific anti-LPS

monoclonal antibodies were obtained from monoclonal hybridoma cell lines (ImmunoPrecise, Victoria, BC). Blots were developed using 5-bromo-4-chloro-3-indolyl phosphate/NBT (Sigma-Aldrich) as the substrate.

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

Lipid A analysis

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

by centrifugation at 14,000 rpm for 6 min, then re-suspended in water and lyophilized. The supernatant was stored for future chemical analyses.

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

GC-MS fatty acid analysis: Fatty acids were analyzed as methyl esters. The lipid-containing fraction was dissolved in 0.5 ml of 2M MeOH-HCl and the mixture was kept at 80°C for 18h. After cooling down, the hydrolyzate was mixed with 0.5 ml 50% NaCl solution and extracted with 1 ml of chloroform. The organic layer was collected and aqueous layer extracted two times more with chloroform. Combined organic layers were extracted again 3 times with water. Water traces were removed from collected chloroform phase by addition of anhydrous Na_2SO_4. The organic phase was then filtered through cotton filters prewashed with chloroform, concentrated under the stream of nitrogen and applied to GC-MS analyses.

Dephosphorylation of lipid A: To remove phosphate groups from lipid A, samples were treated with HF for 48 hrs at 4°C with constant stirring. HF was evaporated from samples under vacuum in a desiccator attached to NaOH trap for one hour then

removed with nitrogen.

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

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

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

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

Results

Ft LVSG LPS possesses less O-antigen

It was shown previously that the LPS of *Ft* LVS and *Ft* LVSG had differential reactivity to monoclonal antibodies stated to be O-antigen specific (Cowley, Myltseva et

al. 1996), suggesting an O-antigen antigenic switch. To further examine the LPS O-antigen and its antigenic properties, we purified LPS from *Ft* LVS, *Ft* LVSG, *F. novicida*, *Ft* LVSGD, *F. tularensis* SchuS4 and a *F. tularensis* SchuS4 small colony grey variant and performed silver staining on SDS-PAGE separated samples. Consistent with previously published results, the grey variant (*Ft* LVSG) possessed an O-antigen but *Ft* LVSGD was rough (lacked O-antigen) (Fig. 3.1 A). The *F. tularensis* SchuS4 small colony grey variant also appeared to produce an LPS with a repeating O-antigen.

Glycosyl composition analysis of the oligosaccharides (OSs) released from the purified LPS preparations show that the *Ft* LVS and *Ft* LVSG OSs contain the same glycosyl residues, but there is a large quantitative difference, in that the *Ft* LVS OS contains much larger amounts of QuiN and Gal than what is found in the OS from the *Ft* LVSG strain (Table 1). The QuiN could be due to QuiNFo as NMR analysis (data not

Table 3.1. TMS methyl glycoside analysis of OSs purified from the LPS of *Ft* LVS LPS and the grey variant *Ft* LVSG LPS.

OS	Man	Gal	Glc	QuiNAc[1]	GalNAc	Kdo
LVS	13	18	28	36	3	3
LVSG	24	5	62	0.5	7	3

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

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

shown) shows a significant resonance at around 8 ppm, which is consistent with a formyl proton. During the preparation of TMS methyl glycoside, which is accompanied by N-acetylation, this formyl group would have been replaced by an acetyl group. The large difference in these components between the LPS of *Ft* LVS and *Ft* LVSG would indicate that the *Ft* LVS LPS contains much more of the QuiN/Gal-containing O-antigen chain polysaccharide than *Ft* LVSG. Thus, these data suggest that the *Ft* LVSG LPS has an O-antigen but the O-antigen contains fewer repeating units than seen in *Ft* LVS LPS or that LVSG lipid A-core is capped less frequently with O-antigen. Interestingly, even though the two variants (*Ft* LVSG and *Ft* LVSGD) have distinct LPS regarding the amount of O-antigen present (albeit both with amounts less than that of wildtype), they give rise to morphologically similar grey variants.

Deletion of O-antigen biosynthetic genes does not affect phase variation as assessed by colony morphology

Prior et al., in 2003, demonstrated a gene cluster that was involved in O-antigen biosynthesis (Fig. 3.4A)(Prior, Prior et al. 2003). We deleted the first gene in the operon (*wbtA*) using the standard published allelic replacement technique involving suicide vector pPV in LVS. Mutants did not have an O-antigen as observed by western blotting

and silver staining (Fig. 3.4B). This mutation however did not affect the phase variation frequency, size and growth rate of the colonies or induced any other phenotypic changes in other assays suggesting no or little role of the O-antigen biosynthetic genes in phase variation. These results however do not exclude the possibility of the post synthesis modifications that may be contributing to phase variation.

Monoclonal and polyclonal antibody reactivity and LPS structural analysis suggests blue/grey strain LPS core alterations

Western blot analysis was performed on LPS samples using commercially available anti-*F. tularensis* FB-11 (Fig. 3.1B) antibodies stated to be specific to the O-antigen, as well as anti-*F. tularensis* (Fig. 3.1C) and anti-*F. novicida* LPS monoclonal antibodies (Fig. 3.1D). The results showed that *Ft* LVS LPS reacted with both the *F. tularensis* monoclonal and FB-11 antibodies showing the typical LPS laddering, and while the laddering was not observed on *Ft* LVS LPS Western blots with the *F. novicida* specific antibodies, reactivity was observed to a low molecular weight species that is typically lipid A plus core (Fig. 3.1B-D). *F. novicida* LPS did not react at all to the anti-*F. tularensis* LPS or FB-11 antibody, but strongly to a low molecular weight species (plus typical laddering) with the *F. novicida* specific monoclonal antibody. *Ft* LVSG reacted with both *F. tularensis* and the *F. novicida* anti-LPS monoclonal antibodies. These results are consistent with those of Cowley et al. (Cowley, Myltseva et al. 1996) with regard to O-antigen ladder reactivity, but the lipid A core region is not clearly visible on their gels. As expected, LVSGD did not demonstrate the typical O-antigen ladder due to it's the lack of O-antigen (Fig. 3.1B-D). The *F. tularensis* SchuS4 grey variant reacted with both

anti-*F. tularensis* monoclonal antibodies and clearly possesses an O-antigen based on the observed laddering. However, the modal chain length or capping frequency of this O-antigen, like that of *Ft* LVSG, appears reduced versus *F. tularensis* SchuS4 (Fig. 3.1B-D).

These LPS samples were reacted in a Western blot with anti-*F. tularensis* or anti-*F. novicida* polyclonal sera generated from infected mice (Fig. 3.2). *Ft* LVS LPS and *F. novicida* LPS only reacted with their respective antisera while *Ft* LVSG now reacted only with *F. tularensis* polyclonal sera. These results suggest that changes in the *Ft* LVSG LPS are specifically recognized by monoclonal but not polyclonal antibodies.

We next isolated and performed extensive NMR analyses on the *Ft* LVS and *Ft* LVSG O-polysaccharides (OPSs) to determine if any structural differences could be detected between these molecules. Crude LPS was enzymatically treated to remove contaminating nucleic acids and proteins and ultracentrifuged. The LPS pellet was collected and the carbohydrate portion of LPS was released from lipid A via mild hydrolysis. The carbohydrate fractions from *Ft* LVS and *Ft* LVSG of were resolved by HPLC. The carbohydrates eluted in three primary fractions. Fraction 1 contained the OPS, Fraction 2 contained slightly lower molecular weight OPS, and Fraction 3 contained the core oligosaccharides with some possibly low molecular weight OPS repeat units. From these results, the Fraction 1/3 ratio for *Ft* LVS LPS is 17, while it is 4.6 for LVSG (Table 2). These results are consistent with the above data showing that the *Ft*

Table 3.2. Main fraction and yields obtained in gel filtration using Superdex peptide column.

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

* used in 1D and 2D NMR experiments

** used in 1D NMR experiments

LVSG strain contains less OPS as reflected by the lower QuiN level during composition analysis. Fractions 1 and 3 were analyzed by NMR spectrometry. Fraction 1 from both *Ft* LVS and *Ft* LVSG were compared to each other using 2D NMR experiments- COSY, TOCSY, NOESY, and HSQC (data not shown). These results indicate that the OPS from *Ft* LVS and *Ft* LVSG have the same structures. In addition, the data are completely consistent with the structure reported for *F. tularensis* strain 15, strain Schu S4, and OSU10 (Vinogradov, Perry et al. 2002; Prior, Prior et al. 2003; Thirumalapura, Goad et al. 2005). The results clearly support the conclusion that *Ft* LVS and *Ft* LVSG have the following OPS structure as previously reported for the above *F. tularensis* strains:

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

The differential staining with the *F. tularensis* and *F. novicida* monoclonal

antibodies coupled with the fact that the *F. novicida* monoclonal antibody binds to the LMW LPS and is, therefore, likely binding to the core oligosaccharide, suggests that the true monoclonal antibody-tracked alteration between *Ft* LVS and *Ft* LVSG is related to the core region. Therefore, we analyzed oligosaccharides found in fraction 3 from *Ft* LVS and *Ft* LVSG by 1D proton NMR spectroscopy. The results are shown in Figure 3.3. The spectrum of *Ft* LVS fraction 3 indicates that a small amount of truncated OPS is still present. We also see resonances that are consistent with the published core structure with the exception that we do not observe evidence for the core GalNAc residue. The *Ft* LVS and *Ft* LVSG proton spectra clearly differ from one another indicating that the *Ft* LVSG fraction 3 contains different structures than found in the *Ft* LVS fraction 3. Therefore, since the oligosaccharides in fraction 3 would be those that would comprise the core region, as well as some possible truncated OPS, these data support the conclusion that the *Ft* LVSG has an altered core region compared to that of *Ft* LVS.

Analysis of *Ft* LVSG lipid A demonstrates a reduction in galactosamine modification

Cowley et al. (Cowley, Myltseva et al. 1996) demonstrated that *Ft* LVSG lipid A versus that of *Ft* LVS elicited increased nitric oxide (NO) induction in rat macrophages. We confirmed this finding (NO production by rat macrophages measured as nitrate by the Griess reagent system) by both *Ft* LVSG LPS and purified lipid A (data shown in chapter 2). This suggested that the lipid A of *Ft* LVSG was different than that of *Ft* LVS. To explore these differences, we performed structural analyses on purified lipid A of *Ft* LVS and *Ft* LVSG. MALDI-TOF analysis in negative and positive ion mode was performed on replicate lipid A preparations. Both showed the absence of a peak at *m/z* 1666 in *Ft*

LVSG that was present in *Ft* LVS. It is known that this peak represents the addition of galactosamine (161 Da) to the basic structure at *m/z* 1504 (2 x GlcN, 3x C18:0 (3-OH), C16:0, P), though previous data suggested that this modification was not observed in the *Ft* LVS strain [(Kanistanon, Hajjar et al. 2008), Fig. 3.5A, B]. This was the only structural alteration noted. Since the MALDI-TOF analysis is only semi-quantitative, we performed galactosamine quantitation assays to further demonstrate the reduced galactosamine modification of lipid A in *Ft* LVSG. The lipid A was derivatized and analyzed by GC-MS. *F. novicida* showed the highest degree of modification at 25% while *Ft* LVS was at 14%. Both *Ft* LVSG and *Ft* LVSGD showed reduced galactosamine modification, with 6% and 7%, respectively (Fig. 3.6).

Three genes have been identified that are responsible for galactosamine or mannose lipid A modification as shown in Fig. 3.7 (Gunn and Ernst 2007; Kanistanon, Hajjar et al. 2008). The transferases FlmF1 and FlmF2 are required for adding mannose or glucosamine residues, respectively, to the lipid A. The glycosyltransferase FlmK can add both mannose and galactosamine to lipid A (Gunn and Ernst 2007; Kanistanon, Hajjar et al. 2008). A real-time PCR assay was performed on the genes *flmF2* and *flmK* from *Ft* LVS and *Ft* LVSG to determine if their expression was altered and might be responsible for the observed lipid A galactosamine modification alteration. Expression of both genes was found to be significantly less in *Ft* LVSG (Fig. 3.8), correlating with the reduction in galactosamine modification. Mutation of the *flmF2* gene in *Ft* LVS, which eliminates the galactosamine modification, reduced the frequency of phase variation from 30% to 5-7% in stationary phase liquid cultures. Though *F. novicida* strains carrying this

mutation have been shown to affect mouse virulence and cytokine/chemokine induction in macrophages, the *Ft* LVS *flmF2* mutant demonstrated no defect in survival in macrophages rat (bone marrow derived, alveolar) or human (THP-1 and monocyte derived macrophages) origin (data not shown).

Discussion

In addition to the presence/absence of the O-antigen, we demonstrate biochemically that the *Ft* LVSG O-antigen chain carbohydrates are reduced in *Ft* LVSG versus *Ft* LVS. It is unclear if this demonstrates that the O-antigen chain length of LVSG is shorter or if the lipid A plus core is capped less efficiently with O-antigen. A reduction in *Ft* LVSG O-antigen chain carbohydrates was previously suggested in a manuscript by Clay et al. (Clay, Soni et al. 2008) based on evidence from silver staining patterns and Western blot analysis.

Both *F. tularensis* and *F. novicida* anti-LPS monoclonal antibodies reacted with LVSG, which had been interpreted to demonstrate that the O-antigen of *Ft* LVSG was altered, creating an epitope(s) reactive to both antibodies. However, closer examination of the Western blots show *F. novicida* monoclonal antibody reactivity to *Ft* LVSG LPS with low molecular weight species, likely lipid A plus core, as well as higher species containing lipid A, plus core, plus O-antigen repeats. The *F. novicida* anti-LPS monoclonal antibody reacted only with the low molecular weight species to *Ft* LVS LPS. This suggested that the *F. novicida* anti-LPS monoclonal antibody recognized a core epitope and that core, and not the O-antigen regions of *Ft* LVS and *Ft* LVSG, may differ.

This was confirmed by NMR analysis of purified LPS carbohydrates, which demonstrated identity between the *Ft* LVSG and *Ft* LVS O-antigen regions while the LPS core region of *Ft* LVSG was different from that of *Ft* LVS. If, as we suspect, the *F. novicida* anti-LPS monoclonal antibody epitope is within the core region and the *Ft* LVS anti-LPS monoclonal epitope is within the O-antigen, then because of observed Western blot reactivities, both *Ft* LVS and *Ft* LVSG contain an "*F. novicida*-like" core. Thus, it is possible that *Ft* LVS makes two distinct core oligosaccharides (an *F. novicida* monoclonal antibody reactive and an *Ft* monoclonal antibody reactive), but cannot add O-antigen to the "*F. novicida*-like" core. *Ft* LVSG, on the other hand, can either produce both core types and ligate O-antigen to both, or can only make the *F. novicida* reactive core type and can add O-antigen to this core.

Regarding the mechanism of O-antigen chain length reduction/differential O-antigen ligation, these processes are typically mediated by an O-antigen ligase (e.g. RfaL), O-antigen polymerase (e.g. Wzy) and a chain length determinant (e.g. Cld). *Francisella* spp. appear to have multiple proteins with homology to RfaL, and their differential expression may account for the observed O-antigen phenotypes. BLAST searches of the *Ft* LVS genome with the RfaL locus of *Salmonella typhimurium* revealed three high scoring loci: FTL1122 (41% identity but 188 amino acids in *Ft* LVS versus 292 amino acids in *F. tularensis* SchuS4), FTL 0706 (37% identity), and FTL0598 (26% identity, called the *wzy* locus in *Ft* LVS (Prior, Prior et al. 2003)). Surprisingly, BLAST searches with the *S. typhimurium* Wzy showed no strong identity to *Francisella* proteins. No Cld ortholog exists in *F. tularensis* ((Prior, Prior et al. 2003), our recent BLAST

searches). It is unclear if another unknown enzyme serves this function or is the O-antigen chain length is unregulated in this bacterium. Thus, it is possible that multiple or novel proteins mediate the O-antigen polymerase, ligase and length determination functions in *Francisella* and may be responsible for the core/O-antigen data described here. Further biochemical analysis is ongoing to determine the exact structural changes in the *Ft* LVSG LPS core region, as are genetic experiments to identify genes conferring *F. novicida* monoclonal antibody reactivity to *Ft* LVS.

The lipid A regions of *Ft* LVS and *Ft* LVSG were shown to be identical with the exception of a galactosamine modification, which was reduced/absent in the *Ft* LVSG variant. The galactosamine modification was not detected previously in *Ft* LVS, but was clearly evident in the *Ft* LVS strain analyzed here. Mannose is also observed as a modification of lipid A in *F. novicida* and Type A subspecies, but not Type B subspecies (the background of the *Ft* LVS strain). Consistent with these published data, mannose was not observed in our assays. While the *flmF2* (glucosamine addition) and *flmK* (both mannose and glucosamine addition) genes showed reduced transcription in the *Ft* LVSG strain consistent with the reduced lipid A modification, mutants in these genes in *Ft* LVS did not result in the small colony or grey phenotype on plates nor were the mutants defective in intramacrophage survival. Thus, these mutants did not exhibit obvious characteristics of the *Ft* LVSG grey variant. These mutants did, however, demonstrate a dramatically reduced phase variation rate to grey variants, suggesting that galactosamine modification may be involved in but is not sufficient for the grey phenotype. Further phenotypic testing (e.g. lipid A nitric oxide induction, mouse virulence) is ongoing, as is

the construction of a double *flmF2 flmK* mutant. It is also possible that no expression versus reduced expression of these enzymes may have different effects, but titrated gene expression in *Francisella* is not yet a reality, so this concern will be addressed when the technology is available.

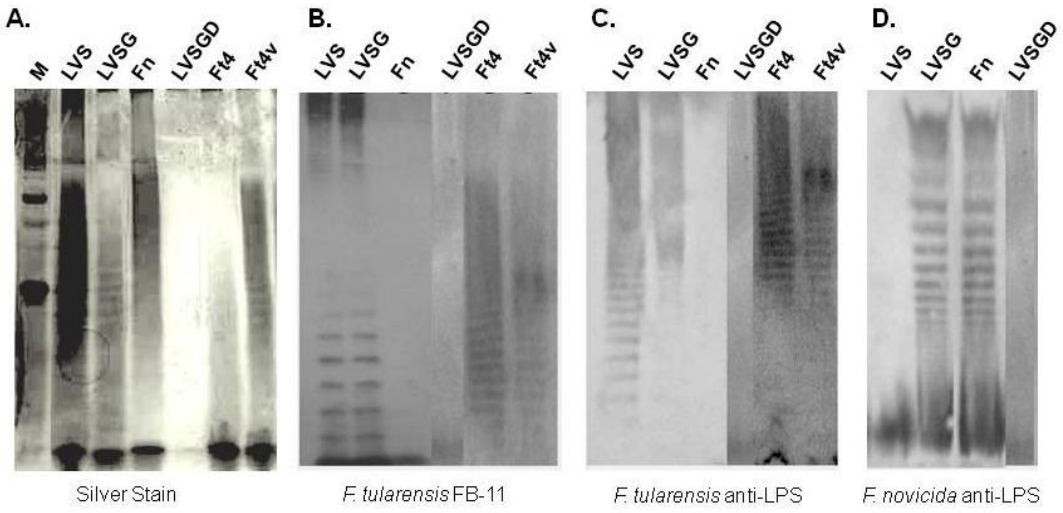


Fig.3.1: LPS analysis by Silver staining and Western blotting;

(A) Silver stained gel of LPS purified from various strains listed above the lanes. (B) Immunoblotting of purified LPS of *Francisella* spp. with the anti-LPS O-antigen *F. tularensis* FB-11 antibody. *F. tularensis* FB-11 (1:1000) and secondary goat anti-mouse IgG (1:4000) were used. (C) Immunoblotting of purified LPS of *Francisella* spp. with *F. tularensis* anti-LPS specific monoclonal antibodies. *F. tularensis* IgG (1:10) and secondary goat anti-mouse IgG (1:4000) were used. (D) Immunoblotting of purified LPS of *Francisella* spp. with *F. novicida* anti-LPS specific monoclonal antibodies. *F. novicida* #5 IgG (1:10) and secondary goat anti-mouse IgG (1:4000) were used. continued

Figure 3.1 continued

Lane assignments; M (Molecular weight marker), LVS (blue colonies), LVSG (grey variant), Fn (*F. novicida)*, LVSGD (rough grey variant), Ft4 (*F. tularensis* SchuS4), Ft4v (*F. tularensis* SchuS4 grey variant).

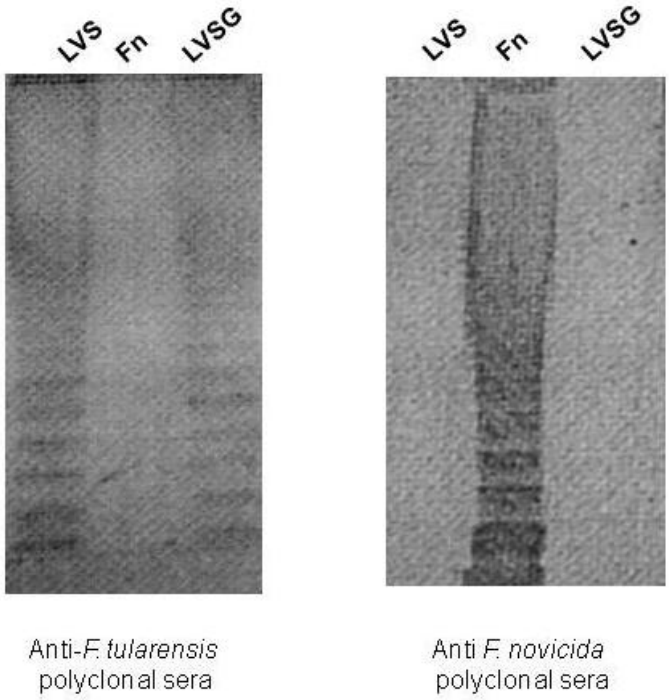


Fig. 3.2: Immunoblotting of LPS with polyclonal sera specific to *Ft* LVS or to *F. novicida*;

Polyclonal sera (1:1000) and secondary goat anti-mouse IgG (1:4000) were used. Lane assignments; LVS (blue colonies), Fn (*F. novicida)*, LVSG (grey variant).

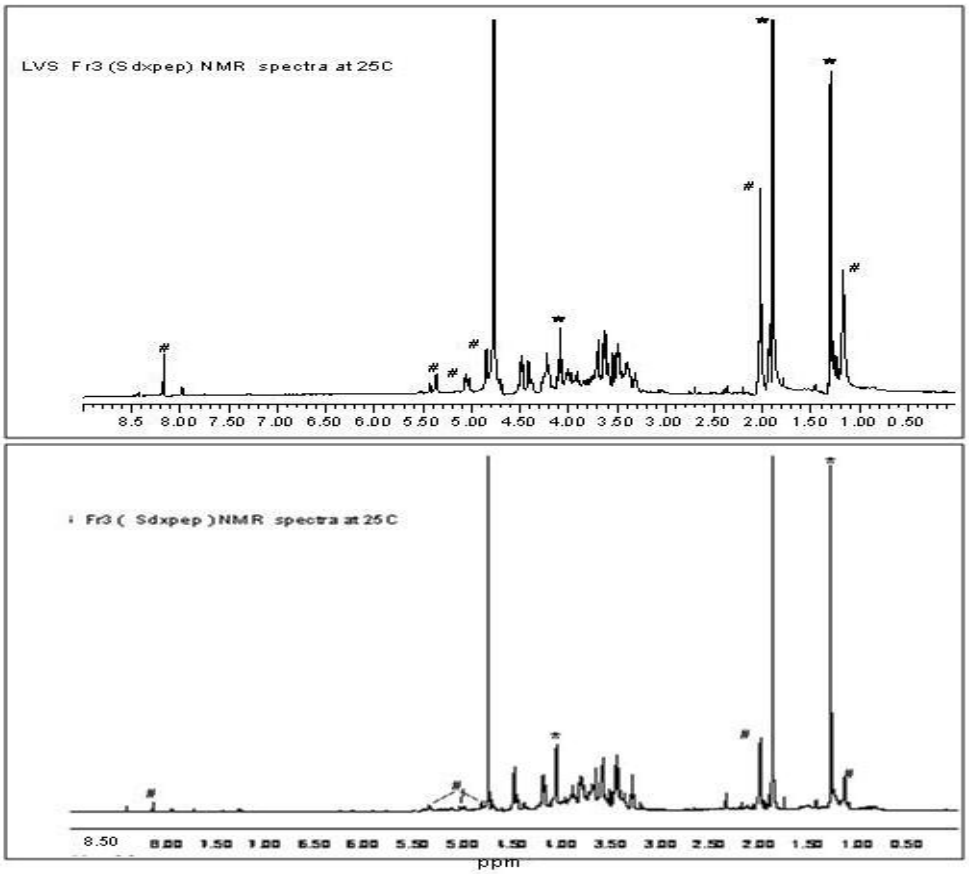


Fig. 3.3: The proton NMR spectra of the LPS core region oligosaccharides from *Ft* LVS and *Ft* LVSG;

The proton NMR spectra of the oligosaccharides found in fraction 3 from *Ft* LVS (top)

Continued

Figure 3.3 continued

and fraction 3 from *Ft* LVSG (bottom) are shown. The resonances marked with # are likely due to OPS fragments. Those marked with * are due to contaminating acetate and lactate. Thus, non-marked resonances denote the LPS core region.

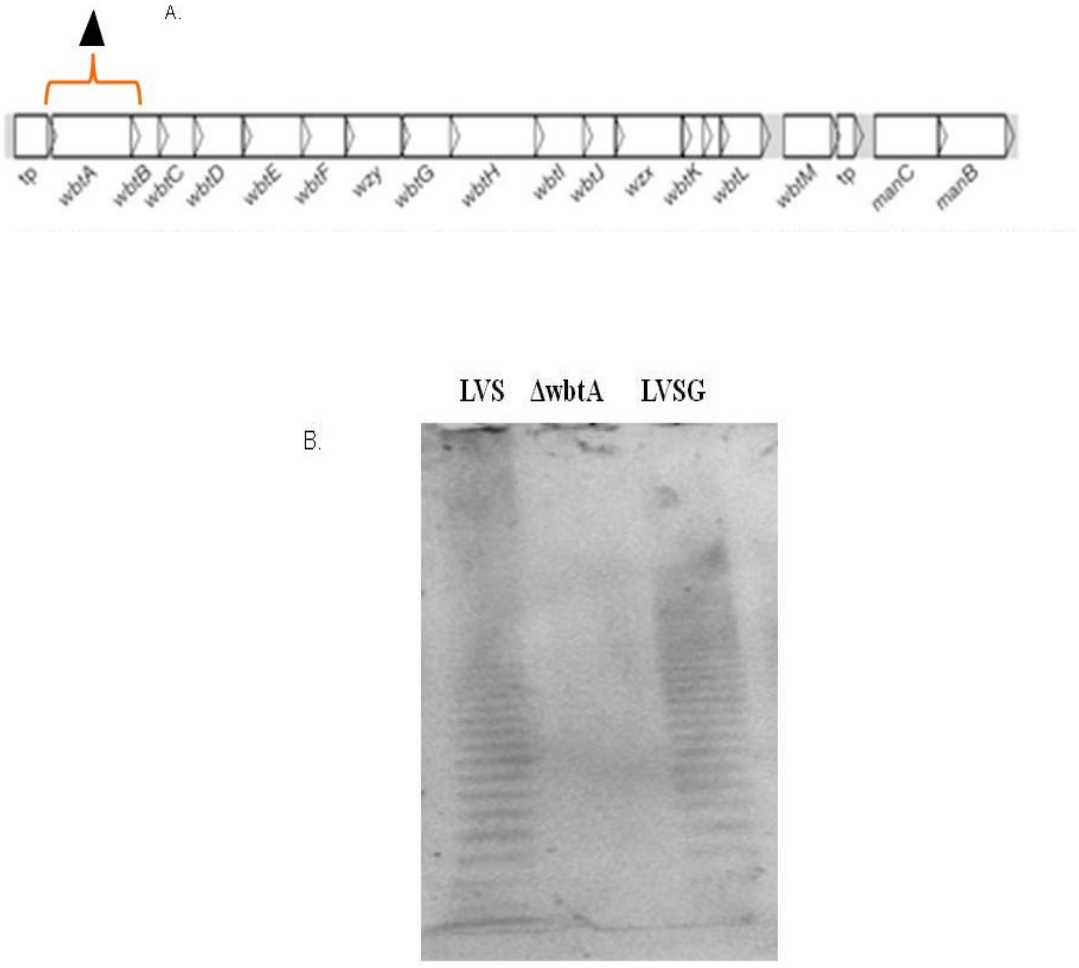


Fig. 3.4: (A). O-antigen biosynthetic gene cluster of *F. tularensis* subsp. *Holarctica;*

continued

Figure 3.4 continued

Genetic arrangement of O-antigen biosynthetic genes (taken from Prior et al. (2003); J Med Microbiol: 52, 845-851). First gene in the operon (*wbtA*) was mutated.

(B). Silver stained gel of LPS purified from *Ft* strains and *wbtA* mutant;

Comparison of LPS from wild type and mutant strain by silver staining shows rough LPS in mutant confirming the mutation.

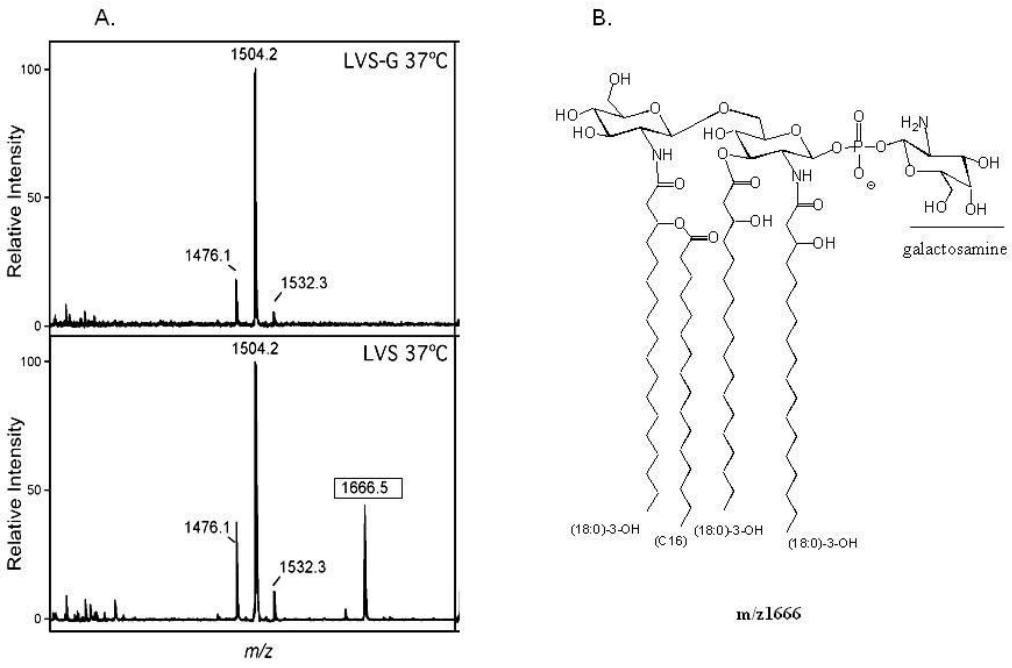


Fig.3.5: Structural analysis of lipid A;

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

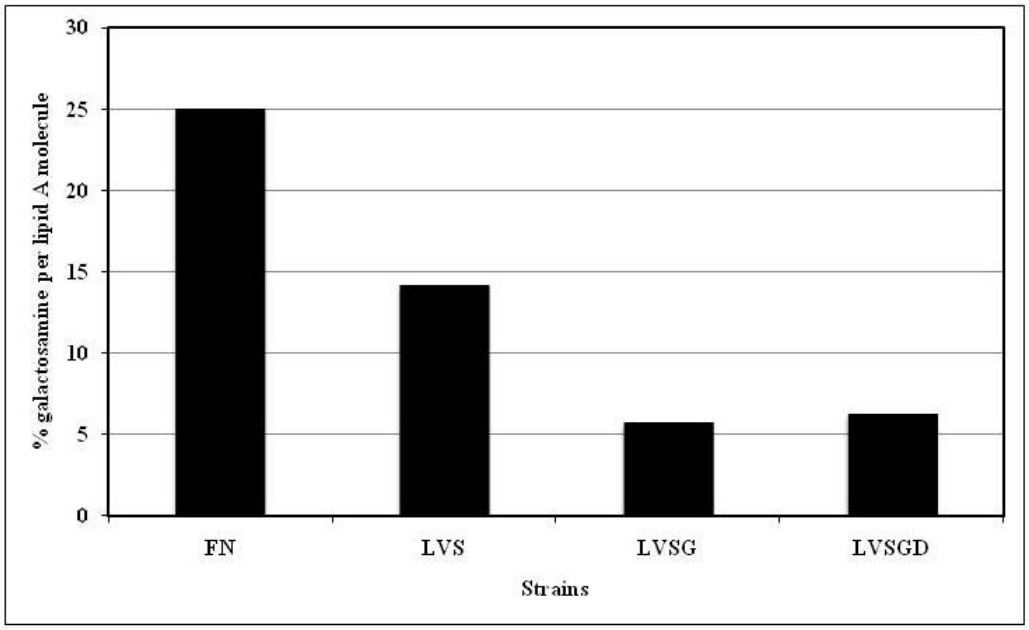


Fig. 3.6: Galactosamine quantification in the lipid A samples;

Quantification of galactosamine in lipid A samples purified from LVS and LVSG shows less amount of sugar in phase variants.

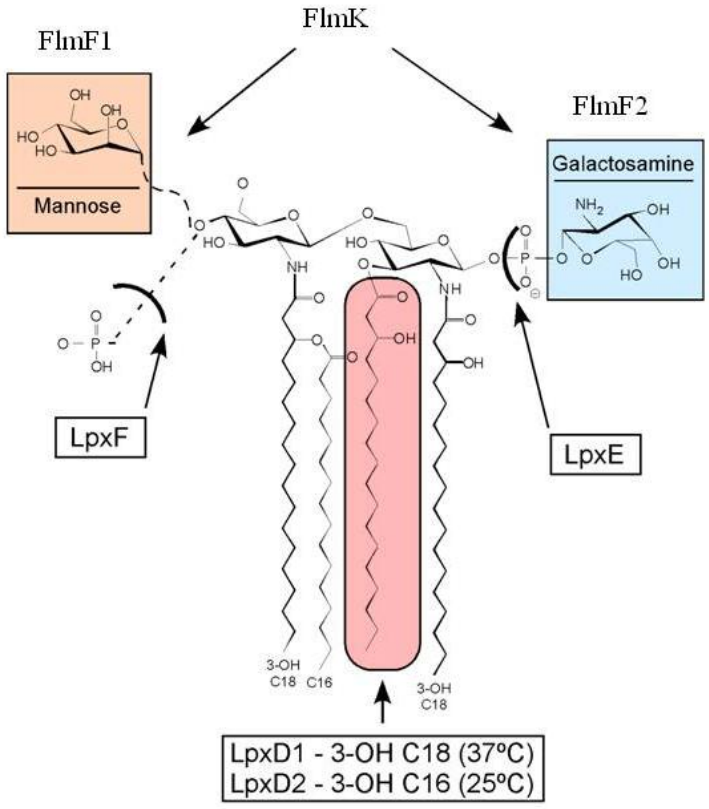


Fig. 3.7: Enzymes required for the synthesis of *F. novicida* lipid-A;

Identification of genes responsible for production of enzymes required for addition of sugar modifications to lipid A. Taken from (Gunn et al,. Ann N Y Acad Sci. 2007 Jun;1105:202-18).

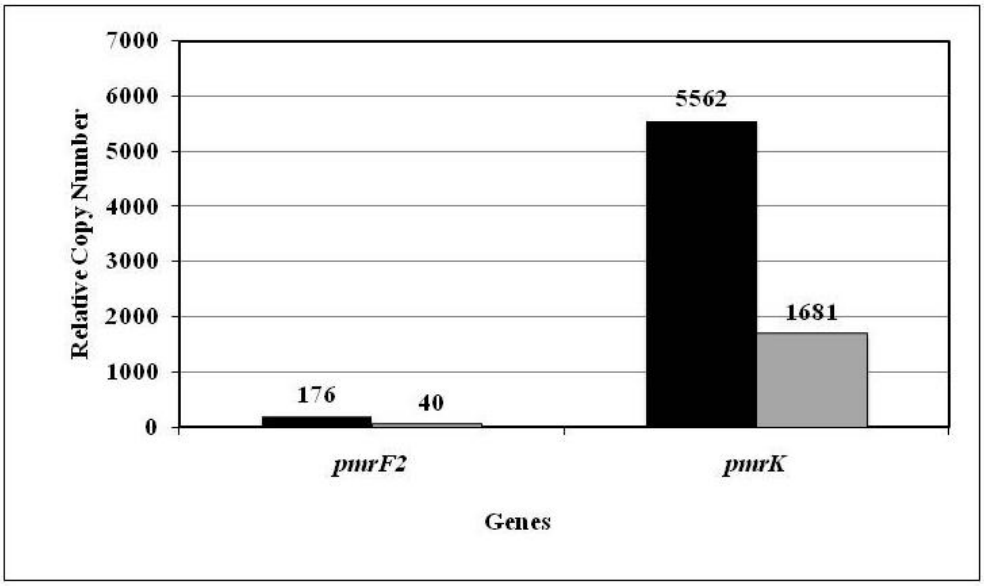


Fig. 3.8: Differential expression of LPS modification genes *flmF2* and *flmK* in *Ft* LVS and *Ft* LVSG;

as determined by real-time PCR. RCN-Relative copy number, actual values are shown above the bars.

Chapter 4

Discussion

F. tularensis is one of the deadliest known human pathogens. Inhalation of as few as 10 bacilli can result in tularemia that is characterized by the systemic dissemination of bacteria, multiorgan failure, and death within days of the initial infection. The potential use of weaponized antibiotic-resistant strains of *F. tularensis* represents a grave risk to public safety in this age of bioterrorism (Dennis, Inglesby et al. 2001). *F. tularensis* can infect a wide variety of hosts and several routes of inoculation have been identified. It is a hardy organism that can survive in the soil and water environments for extended period of time. The severity of disease depends upon the dose and route of infection. Tularemia has several forms of disease including inhalational, gastrointestinal, and cutaneous. All the forms of disease have very non-specific initial symptoms that are common for some other diseases. Although standardized successful treatment regimens are available to control the active *Francisella* infections, all the above-mentioned properties, the historical use of the organism in war and the lack of an approved vaccines available to protect at-risk populations make it a dangerous pathogen.

Several vaccines have been tested historically including the live killed vaccine called the Foshay vaccine with no or little success in complete Type A protective

efficiency. The only available vaccine, *FT* LVS, is under review as an investigational new drug by FDA and although the protective studies done with *Ft* LVS show promising results, the unknown basis of attenuation presents a problem in certifying it as a vaccine. *Ft* LVS is derived from a Type B strain, which is still virulent in humans and so *Ft* LVS still carries a risk of reversion which would result in active infections. The other potential problem was a phenomenon called phase variation, observed first by Eigelsbach in 1951 (Eigelsbach, Braun et al. 1951). He demonstrated that *Ft* LVS phase vary from a blue (i.e. wildtype) to a grey variant that possessed many phenotypically different properties. Such variation has proven to be an issue historically in vaccine production runs of *Ft* LVS (Wayne Conlan and Oyston 2007; Oyston 2009). The *Ft* LVS grey variants are a cause of concern because they are dramatically less efficacious than the blue colony morphotypes in protection studies against the virulent *F. tularensis* Type A strain. An increased understanding of the mechanism(s) behind this variation would aid future tularemia vaccine production.

Originally Eigelsbach had described several different types of but the only two have been molecularly characterized, one by Cowley et al. in 1996 and second by Hartley et al. in 2006. Both of these variants were shown to have LPS alterations. The grey variant described by Hartley et al. (Hartley, Taylor et al. 2006), (similar to *Ft* LVSGD studied in this work) has been shown to lack O-antigen while *Ft* LVSG still possessed an O-antigen which has altered *F. novicida* and *F. tularensis*-LPS specific monoclonal antibody reactivity patterns. These findings led us focus on the molecular and structural differences that LPS of different phase variants may possess/undergo.

We performed extensive biochemical analyses on the O-antigen and found that the *Ft* LVSG O-antigen chain carbohydrates are reduced in *Ft* LVSG versus *Ft* LVS. We were unable to conclude if the O-antigen chain length of *Ft* LVSG is shorter or if the lipid A plus core is capped less efficiently with O-antigen. The processes of O-antigen chain length reduction/differential O-antigen ligation, are typically mediated by an O-antigen ligase (RfaL / WaaL), O-antigen polymerase (Wzy) and a chain length determinant (Wzz). *Francisella* spp. appear to have multiple proteins with homology to RfaL, but orthologs of Wzy and Wzz were not found. BLAST searches of the *Ft* LVS genome with the RfaL locus of *Salmonella typhimurium* revealed three high scoring loci: FTL1122, FTL 0706 and FTL0598 and their differential expression may account for the observed O-antigen phenotypes.

Both *F. tularensis* and *F. novicida* anti-LPS monoclonal antibodies reacted with *Ft* LVSG, which had been interpreted to demonstrate that the O-antigen of *Ft* LVSG was altered. Prior et al., identified an O-antigen biosynthetic gene clusture and mutation in the first gene of this operon made the strain rough (no O-antigen) and provided attenuation. We created a *wbtA* mutant in *Ft* LVS and this mutation did not seem to affect the colony morphology or frequency of phase variation suggesting that the O-antigen addition is not directly responsible for blue /grey variation. Closer examination of the Western blots show *F. novicida* monoclonal antibody reactivity to (low molecular weight species) of *Ft* LVSG LPS with likely lipid A plus core, as well as higher species containing lipid A, plus core, plus O-antigen repeats. The *F. novicida* anti-LPS monoclonal antibody reacted only with the low molecular weight species to *Ft* LVS LPS. This suggested that the *F.*

novicida anti-LPS monoclonal antibody recognized a core epitope and that core, and not the O-antigen regions of *Ft* LVS and *Ft* LVSG, may differ. We confirmed this by NMR analysis of purified LPS carbohydrates, which demonstrated identity between the *Ft* LVSG and *Ft* LVS O-antigen regions while the LPS core region of *Ft* LVSG was different from that of *Ft* LVS. If, as we suspect, the *F. novicida* anti-LPS monoclonal antibody epitope is within the core region and the *Ft* LVS anti-LPS monoclonal epitope is within the O-antigen, then because of observed Western blot reactivities, both *Ft* LVS and *Ft* LVSG contain an "*F. novicida*-like" core. Thus, it is possible that *Ft* LVS makes two distinct core oligosaccharides (an *F. novicida* monoclonal antibody reactive and an *Ft* monoclonal antibody reactive), but cannot add O-antigen to the "*F. novicida*-like" core. *Ft* LVSG, on the other hand, can either produce both core types and ligate O-antigen to both, or can only make the *F. novicida* reactive core type and can add O-antigen to this core. These findings, for the first time show the involvement of core in the differential reactivity of *Francisella* LPS to the monoclonal antibodies. As mentioned above, there are several enzymes that are responsible for the determining the length and their regulation may account for the different types of core. It is unclear if another unknown enzyme serves the function of Cld or the O-antigen chain length is unregulated in this bacterium. Thus, it is possible that multiple or novel proteins mediate the O-antigen polymerase, ligase and length determination function in *Francisella* that may be responsible for the core/O-antigen data described here. Further biochemical analysis is ongoing to determine the exact structural changes in the *Ft* LVSG LPS core region, as are

genetic experiments to identify genes conferring *F. novicida* monoclonal antibody reactivity to *Ft* LVS.

While the variant described by Cowley et al. (*Ft* LVSG) was deficient in intracellular survival by inducing more nitric oxide in rat macrophages, there is no data available about its in vivo virulence/ survival. Unlike all other known grey variants, we found *Ft* LVSG to be as virulent as *Ft* LVS in mice, but like other grey variants, it did not protect against the Type A Schu4 challenge. We performed invasion assays in human monocyte derived macrophages and observed that uptake of the bacteria was enhanced when opsonized with 0.1% autologous serum. Clay et al., also demonstrated that the *Ft* LVSG strain was bound by complement component C3 in higher amounts than *Ft* LVS and was dramatically more susceptible to complement-mediated killing. These results suggest that while the complement binding helps the uptake of the bacteria in macrophages and FT LVSG is complement susceptible, *Ft* LVSG is able to evade the complement mediated killing in host. There are several factors that could be contributing to this phenotype including the inherent differences between mouse and human complement functions. For example, C3 in mouse serum is more labile than in human serum and this may affect the degree of opsonization in human versus mice serum. Clay et al suggested that O-antigen inhibits binding of C3, providing resistance against complement mediated killing. Because grey variants have less O-antigen, they are more susceptible to complement mediated killing. Our structural analysis showed that there is a possibility that O- antigen chain length is shorter or the other possibility is that lipid A plus core is capped less efficiently. In the latter case, there would still be availability of

O-antigen to bind the C3 component and thus providing resistance to the bacteria. It is also possible that effect of complement mediated killing is quantitative not absolute and the current mouse model is not able to sense an effect. Confirmation of the LPS phenotypes then prompted us to focus our efforts on LPS and perform more detailed biochemical and genetic analyses.

Upon the extensive lipid A analysis, we found that the it was identical in *Ft* LVS and *Ft* LVSG with the exception of a galactosamine modification. This modification was reduced/absent in the *Ft* LVSG variant. The galactosamine modification was not detected previously in *Ft* LVS, but was clearly evident in the *Ft* LVS strain analyzed here. Previously, mannose is also observed as a modification of lipid A in *F. novicida* and Type A subspecies, but it was not found in Type B subspecies (the background of the *Ft* LVS strain). The genes responsible to add these sugar modifications have been identified previously by Kanistanon et al (Kanistanon, Hajjar et al. 2008). Consistent with these published data, mannose was not observed in our assays. The *flmF2* (galactosamine addition) and *flmK* (both mannose and galactosamine addition) genes showed reduced transcription in the *Ft* LVSG strain consistent with the reduced lipid A modification observed by the MALDI-TOF analysis. Mutation in the *flmF2* (galactosamine addition) gene in LVS demonstrate a dramatically reduced phase variation frequency to grey variants, suggesting that galactosamine modification may be involved in the grey phenotype. This mutation however, did not result in the small colony or grey phenotype on plates nor was the mutant defective in intramacrophage survival suggesting that this modification is not sufficient for the grey phenotype. The fact that *flmK* can still add the

galactosamine and could be compensating in the *flmF2* mutant for the protein levels presents a possible explanation for the partial phenotype. Since these *flmF2* mutants did not exhibit obvious characteristics of the *Ft* LVSG grey variant, construction and characterization of combined mutations of *flmF2* and *flmK* genes would greatly enhance the study although all the unsuccessful attempts of making this double mutant indicate that these mutations could be lethal and these modifications must be essential. Further phenotypic testing (e.g. lipid A nitric oxide induction, mouse virulence) is ongoing, as is the construction of a double *flmF2*, *flmK* mutant. It is also possible that no expression versus reduced expression of these enzymes may have different effects, but titrated gene expression in *Francisella* is not yet a reality, so this concern should be addressed when the technology is available.

Based on the previous reports, it was surprising that the *Ft* LVSG strain did not possess a virulence defect by the intranasal route. However, It has been established that the size of inoculums and the route of infection affect the form and severity of tularemia. In the Hartley et al. (Hartley, Taylor et al. 2006) study, mice were vaccinated and challenged by the subcutaneous route, and the Eigelsbach work (Eigelsbach, Braun et al. 1951; Eigelsbach and Downs 1961) typically used intraperitoneal vaccination and subcutaneous challenge. Thus the route of administration may play a role in grey variant virulence. Consistent in all grey variant mouse model vaccination experiments is their reduced capacity to protect against challenge by the Type A *F. tularensis* subspecies. The mechanism behind the lack of protective capacity is not known. *Ft* LVS *flmF2* and *flmK* mutants were avirulent in the mouse model, thus the observed reduction in

galactosamine modification may play a role in early clearance and the lack of development of a protective immune response (Kanistanon, Hajjar et al. 2008). While 1-D gel electrophoresis of whole cell lysates and fractions showed no obvious protein differences between *Ft* LVSG and *Ft* LVS, it is possible that these bacteria may possess alterations other than those observed in the LPS. Ongoing assays include microarray analysis, 2-D gel electrophoresis and other more sophisticated proteomic analysis. This study showed the LPS defects in core and lipid A of the grey variants contributing to the phenotype. There may be other changes however that may not be associate with the LPS and/or may be playing a secondary role. The continued study of grey variants of *F. tularensis* will provide important mechanistic details behind these phenotypically distinct bacteria, which will move the field closer to the ability to phase lock a wildtype strain for the development of effective and safe tularemia vaccines. The present study is a step forward in defining the roles of specific structural modifications in the O-antigen, core and lipid A that contribute to the grey phenotype.

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