THE CONTRIBUTION OF ONE-CARBON METABOLISM TO THE PATHOGENESIS OF *FRANCISELLA TULARENSIS*

by

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An emerging paradigm shift in bacterial pathogenesis has resulted in renewed interest in metabolism during infection. The acquisition and synthesis of metabolites by pathogens in the host represents a critical obstacle to successful colonization and infection. The value of bacterial metabolic pathways during infection remains poorly characterized for many pathogens and warrants further investigation. One aspect of this, the contribution of one-carbon metabolism to pathogenic fitness was assessed. This metabolic pathway primarily transfers single carbons and contributes to the synthesis of amino acids, DNA, and proteins. The donated carbon is typically derived from 5,10-methylenetetrahydrofolate, a central compound in one-carbon metabolism. Since the requirement of a metabolic pathway may vary among nutritionally diverse sites within a host, a systemic infection involving multiple niches would be an ideal model to assess the contribution of these pathways. *Francisella tularensis* is a gram-negative bacterium, a tier one bioterrorism agent, and the causative agent of a debilitating febrile illness known as tularemia. Since this organism is capable of establishing a systemic infection throughout the host, it provides an excellent opportunity to evaluate bacterial metabolism during infection. To evaluate this system in *F. tularensis*, mutagenesis of the two known sources of 5,10-methylenetetrahydrofolate, the glycine cleavage system and the serine hydroxymethyltransferase, was performed. Loss of either of these pathways resulted in serine auxotrophy, identifying one-carbon metabolism as the exclusive serine biosynthetic pathway of

F. tularensis. Under standard assay conditions, neither the glycine cleavage system nor the serine hydroxymethyltransferase proved to be essential for intracellular replication in the virulent *F. tularensis* subsp. *tularensis*. Despite this result, both 5,10-methylenetetrahydrofolate producing pathways contributed to pathogenesis in a murine model of pneumonic tularemia. Further, these pathways contributed to pathogenic fitness of *Francisella* to varying degrees throughout the host. Critically, a significant reduction in bacteremia was associated with the loss of either system. These studies highlight differing nutritional environments of distinct sites in the host and confirm that these sites exert variable metabolic stresses on this invasive pathogen. This work identifies one-carbon metabolism as a key bacterial metabolic pathway employed by *F. tularensis* to overcome nutritional limitation during infection.

TABLE OF CONTENTS

LIST OF FIGURES

PREFACE

This thesis would not be possible without the continuous support, guidance, and discussions provided by my parents, lab mates, and mentor. I am eternally grateful to you all.

1.0 INTRODUCTION

1.1 *FRANCISELLA TULARENSIS*

1.1.1 The Discovery of *Francisella tularensis*

While examining ground squirrels for plague in 1910, McCoy observed a plague-like disease of unknown etiology in Tulare County, California (McCoy, 1910). Further investigation of this disease suggested the causative agent was bacterial and he referred to this new organism as *Bacterium tularense* (McCoy and Chapin, 1912). This bacterium was first recognized as a pathogen of man in 1914 after being isolated from a conjunctival ulcer (Wherry and Lamb, 1914). In 1911, clinical descriptions of a human febrile illness known as "deer-fly fever" caused by the bite of the deer fly appeared in Utah (Pearse, 1911). Additional work performed by Dr. Edward Francis revealed that *Bacterium tularense* was the causative agent of deer-fly fever (Francis, 1919). Dr. Francis characterized and monitored several clinical cases of this disease which, due to the presence of the organism in the blood, he called tularemia (Francis et al., 1921). In addition to this clinical work, Francis went on to confirm the presence of the bacterium in diseased jack rabbits and demonstrated transmission of disease by deer flies, lice, bed bugs and ticks (Francis and Lake, 1922; Francis et al., 1921; Parker et al., 1924). Outside of the US, reports of a febrile illness associated with the consumption of hare meat have been noted

as early as 1837 in Japan (Ohara, 1954). Interest in these observations emerged in 1925 when Dr. Hachiro Ohara began a thorough characterization of an endemic Japanese febrile illness associated with hares (Ohara, 1925a; Ohara, 1925b). His observations, both clinically and bacteriologically were strikingly similar to those made in the United States. This relationship was solidified by Dr. Francis, who obtained clinical samples from patients infected with "Ohara's disease" and isolated *Bacterium tularense* (Francis and Moore, 1926). Due to the significant contribution of Dr. Edward Francis to the understanding of this bacterium, the nomenclature *Francisella tularensis* was proposed in 1947 (Dorofeev, 1947).

1.1.2 Taxonomy

The proposed family Francisellaceae contains a single genus, *Francisella*. The genus *Francisella* has had several nomenclatures through its history. Originally, experimental observations indicated that the etiological agent of a plague-like illness in rodents was likely bacterial and thus the temporary genus *Bacterium* was applied (McCoy and Chapin, 1912). As a result of further investigation using differing classifications (serological, pathological, bacteriological), the bacterium was subsequently referred to as *Bacillus tularense, Brucella tularensis, Coccobacterium tularense,* and most commonly, *Pasteurella tularensis* (Breed et al., 1957; Topley and Wilson; Vail, 1914). The difficulty in assigning this bacterium to an established category led to the proposal of a new genus, *Francisella,* in honor of Dr. Edward Francis (Dorofeev, 1947). Despite the initial proposal occurring in 1947, the nomenclature *Francisella* was not uniformly adopted until the mid-1970s. *Francisella* currently contains six species (*F. tularensis, F. philomiragia, F. noatunensis F. halioticida, F. hispaneinsis, F.*

guangzhouensis). A perusal of the taxonomy of this genus generates a strong sense for the diverse and globalized nature of *Francisella*.

1.1.2.1 *Francisella tularensis*

The first *Francisella* species to be described and characterized was *F. tularensis*. This nomenclature is derived from Tulare County, California, which was the location of the initial disease observations (McCoy and Chapin, 1912). This species currently contains 5 subspecies (*tularensis, holarctica, japonica, novicida, mediasiatica*), which can be distinguished biochemically, pathologically, and geographically (Johansson et al., 2004; Vogler et al., 2009).

Francisella tularensis subspecies *tularensis* is predominately found in the United States (Johansson et al., 2004). In fact, it was widely believed to be confined geographically to this region until the recovery of this subspecies from mites in Slovakia (Gurycova, 1998). Subsequent studies revealed this European *F. tularensis* subspecies *tularensis* isolate was nearly genetically identical to a widely used laboratory strain (Chaudhuri et al., 2007). Since related environmental isolates have since been recovered repeatedly, it is thought that this represents a laboratory release with the establishment of this subspecies in the local environment (Chaudhuri et al., 2007). The *tularensis* subspecies has recently been further divided into two clades (A1 and A2) each with two genotypes (A1a, A1b, A2a, and A2b) by pulsed-field gel electrophoresis (Farlow et al., 2005; Johansson et al., 2004; Kugeler et al., 2009; Staples et al., 2006). Geographically, the A1 (A-East) clade primarily occurs in the central United States while the A2 (A-West) clade occurs predominately in the Western United States (Staples et al., 2006). Clinical data from humans suggests significant differences in pathogenesis and the mortality caused by these clades. Specifically, the AI clade is more virulent than the AII clade and the A1b genotype is the most virulent genotype known (Kugeler et al., 2009). It is worth noting that the

most commonly used *F. tularensis* subsp. *tularensis* laboratory strain, Schu S4, was recently found to be an A1a genotype (Kugeler et al., 2009).

In 1959, the presence of two distinct varieties of *Francisella tularensis, F. tularensis* var. *tularensis* and *F. tularensis* var. *palaearctica* was noted (Olsufiev et al., 1959). This distinction was reinforced by additional work by Jellison in 1961, who distinguished two types, type A (now subspecies *tularensis*) and type B (now subspecies *holarctica*), based on biochemical and pathological results (Jellison, 1961). Francisella *tularensis* var. *palaearctica* was renamed later to *F. tularensis* subspecies *holarctica* to better reflect the discovery that it was not geographical bound to the Palearctic ecozone (Bell, 1965; Olsufjev, 1970; Olsufjev and Meshcheryakova, 1982). This subspecies contains two biovars which can be distinguished by erythromycin susceptibility (biovar I Ery^s, biovar II Ery^r,) (Kudelina and Olsufiev, 1980). A strain of *F. tularensis* subsp. *holarctica*, known as the live vaccine strain (LVS), is a commonly used attenuated laboratory strain. It is the descendant of a USSR vaccine strain that was originally derived by in vitro passage of a wild-type *holarctica* isolate (Eigelsbach and Downs, 1961; Tigertt, 1962).

F. tularensis subspecies *japonica* was, until recently, considered a third biovar of subspecies *holarctica*. This subspecies was described originally in 1967, is biochemically distinguishable from *holarctica*, and is isolated to the Japanese islands (Rodionova, 1967). Recent genetic analysis supports a distinction from *holarctica* and suggests it is more appropriately considered a distinct subspecies (Johansson et al., 2004; Vogler et al., 2009). Interestingly, *japonica* may also represent an evolutionary intermediate between *tularensis* and *holarctica*, as it is biochemically similar to *tularensis* but pathologically similar to *holarctica* (Johansson et al., 2004).

In 1950, an environmental water sample from Utah yielded a bacterium that caused a tularemia-like illness in animals, but, serologically, failed to identify as *F. tularensis* (Larson et al., 1955). This inexplicable result, along with follow-up biochemical, bacteriological, and immunological studies, led to this bacterium being classified as a separate species, *Francisella novicida* (Larson et al., 1955). The nomenclature of this organism as a separate species or a subspecies of *F. tularensis* has been the subject of significant debate (Busse et al., 2010; Hollis et al., 1989; Huber et al., 2010; Johansson et al., 2010; Owen et al., 1964). Ultimately, a comparison of *F. novicida* with *F. tularensis* subspecies revealed an average nucleotide identity of ~98% (Larsson et al., 2009). This degree of similarity exceeds a proposed 95% cutoff for species differentiation (Richter and Rossello-Mora, 2009). The nomenclature *F. tularensis* subspecies *novicida* has been "validly published" and will be utilized in this text (Huber et al., 2010).

F. tularensis subspecies *mediasiatica* was identified in 1966 while examining an environmental site with ongoing tularemia in Central Asia (Aikimbaev, 1966). Investigation of terrestrial animals and ticks revealed a bacterium with biochemical properties similar to subspecies *tularensis* but pathogenic properties similar to subspecies *holarctica* (Aikimbaev, 1966). This intermediate phenotype resulted in the proposal of the new subspecies, with nomenclature based on the geographic location of the isolates. The *mediasiatica* subspecies is the least studied of all subspecies, due to its rare isolation since the initial studies and overall low clinical importance. It has, however, served as an interesting tool in attempts to unravel the genetic evolution of the *Francisella tularensis* species (Champion et al., 2009).

1.1.2.2 *Francisella philomiragia*

F. philomiragia was first isolated from the liver and lungs of a dying muskrat in Utah in 1959 and subsequently from nearby environmental water samples in 1960 (Jensen et al., 1969). This bacterium was bacteriologically similar to both *Francisella* and *Yersinia* species but not identical to any known species. The nomenclature *Yersinia philomiragia* was proposed due to early DNA hybridization assays revealing a stronger relatedness to *Yersinia* species than *Francisella* species and due to the observation of mirages around the collection site (Jensen et al., 1969; Ritter and Gerloff, 1966). This classification was refuted in 1980, when follow-up studies failed to confirm any DNA relatedness between *Yersinia philomiragia* and other *Yersinia* species (Ursing et al., 1980). This species was colloquially referred to as the "Philomiragia" bacterium, until a re-examination of DNA hybridization revealed a ~40% relatedness to *Francisella* species, which along with morphological and biochemical results, prompted its inclusion in the *Francisella* genus (Hollis et al., 1989). This species currently contains no recognized subspecies.

1.1.2.3 *Francisella noatunesis*

In 2006, a causal relationship was found between a granulomatous disease in fish (*Gadus morhua* in Norway) and a bacterium that appeared morphologically and genetically similar to *F. philomiragia* (Olsen et al., 2006). A follow-up study proposed the nomenclature *F. philomiragia* subspecies *noatunesis* based on DNA hybridization assays and biochemical assays (Mikalsen et al., 2007). An independent research group, also investigating the disease, proposed the nomenclature *Francisella piscida* for an isolate of the bacterium (Ottem et al., 2007). Upon inspection, *F. piscida* was found to be indistinguishable from the *noatunesis* subspecies (Ottem et al., 2009). *F. piscida* is thus a taxonomic synonym of the earlier classification, *F.*

philomiragia subspecies *noatunesis*, and is no longer valid. The concept of a separate species, however, was deemed appropriate and in 2009 further genetic, phenotypic, and phylogenetic analysis led to the proposal of the nomenclature, *F. noatunesis* (Mikalsen and Colquhoun, 2009; Ottem et al., 2009). Studies on disease in a different fish species (*Parapristipoma trilinineatum* in Japan) revealed a bacterium that was modestly genetically distinct and phenotypically similar to *F. noatunesis* (Kamaishi et al., 2005). This species has been referred to as *F. asiatica* but a prior publication proposed the nomeclature *F. noatunesis* subspecies *orientalis* and thus has priority (Mikalsen and Colquhoun, 2009; Ottem et al., 2009). The nomenclature of these fish pathogens is currently *F. noatunesis* subspecies *noatunesis* for the Norway isolates and *F. noatunesis* subspecies *orientalis* for the Japan isolate (Colquhoun and Duodu, 2011).

1.1.2.4 *Francisella halioticida*

In 2005, a bacterium was recovered from the hemolymph of a diseased abalone (*Haliotis gigantea)* in Japan that was genetically related to *F. philomiragia* and *F. noatunesis* (Kamaishi et al., 2010). DNA-DNA hybridization assays between this isolate and *F. philomiragia* and *F. noatunesis* subspecies *noatunesis* indicated that this organism was not a subspecies and the nomenclature *F. halioticida* was proposed (Brevik et al., 2011). This species is closely related to an uncultured *Francisella* endosymbiont found in the marine ciliate *Euplotes raikovi* (Brevik et al., 2011; Schrallhammer et al., 2011).

1.1.2.5 *Francisella hispaniensis*

In 2003, a bacterium was isolated from the urine and blood of a patient in Spain suffering from acute obstructive pyelonephritis (Escudero et al., 2010). Preliminary biochemical and sequence results revealed that the causative agent of disease was potentially a new *Francisella*

species (Escudero et al., 2010). These preliminary results were confirmed by DNA-DNA hybridization with *F. tularensis* subsp. *holarctica, F. tularensis* subsp. *novicida, F. philomiragia, and F. noatunesis* and the nomenclature *Francisella hispaniensis* was proposed (Huber et al., 2010).

1.1.2.6 *Francisella guangzhouensis*

Routine investigation of air condition systems revealed a novel bacterium in water cooling towers in Guangzhou, China, in 2008 (Qu et al., 2009). Biochemical analysis, along with 16S rRNA sequencing, revealed a strong relationship to *F. philomiragia* (Qu et al., 2009). Follow-up DNA-DNA hybridization assays on this isolate revealed that it appropriately assigned to the genus *Francisella,* but distinct enough to be classified as a new species (Qu et al., 2013). The nomenclature *Francisella guangzhouensis* was proposed (Qu et al., 2013).

1.1.3 Tularemia

Infection with *Francisella tularensis* result in a disease known as tularemia (Francis et al., 1921). At the time of its discovery as a human disease, tularemia was of significance due to its ability to incapacitate a farmer for two to three months in the middle of harvest season (Francis, 1922). Clinical descriptions of disease, prior to the development of effective antibiotic interventions, were compiled by various researchers (Foshay, 1940; Francis, 1922; Francis, 1925; Francis, 1928; Pullen and Stuart, 1945; Simpson, 1928). These studies describe tularemia as a febrile illness with remarkable variability and a variety of clinical manifestations which appeared to be related to the route of exposure. Most routes of exposure require less than 50 bacteria to result in disease in humans (Saslaw et al., 1961a; Saslaw et al., 1961b). The tularemia

manifestations include ulceroglandular, oculoglandular, glandular, typhoidal, oropharyngeal, gastrointestinal, pneumonic, septicemic, and meningitic (Ellis et al., 2002; Foshay, 1937; Francis, 1928; Pullen and Stuart, 1945; Stuart and Pullen, 1945a, b).

Ulceroglandular tularemia is the most common form of the disease, accounting for around 80% of cases (Pullen and Stuart, 1945). This form typically results at the site of an insect bite or skin exposure to infected mammals (Francis, 1919, 1928; Pearse, 1911). Outbreaks of human tularemia have been directly associated with various species of biting flies, ticks, and mosquitos (Petersen et al., 2009). Furthermore, zoonotic transmission by several types of lice, fleas, and bedbugs may be important in maintaining the environmental reservoir in the over 250 animal species susceptible to tularemia (Bell, 1965; Francis, 1928). After two to six days following inoculation, the individual experiences a sudden onset of a flu-like illness (Francis, 1928). Symptoms most commonly include fever, chills, achiness and headache (Francis, 1928). This early period of the disease is associated with a transient bacteremia (Francis, 1928). Within a few days of symptoms arising, the individual experiences swelling and tenderness in the local draining lymph nodes (Francis, 1928). The site of infection then begins to develop into an inflamed papule, subsequently becoming the ulcer which is characteristic of this form of disease (Francis, 1928). Without antibiotic intervention, the flu-like symptoms will persist actively for two to three weeks and the individual experiences weakness for several months (Francis, 1928). Despite the debilitating nature of this disease, ulceroglandular tularemia rarely results in the death of healthy individuals and carries an overall mortality rate under 5% without therapeutic treatment (Pullen and Stuart, 1945).

Glandular tularemia is similar to ulceroglandular tularemia in all aspects except for the lack of an ulcer (Francis, 1928). It occurs in approximately 10% of cases (Pullen and Stuart,

1945). No clear explanation exists for why some patients manifest as glandular or ulceroglandular given similar exposure events. It is noteworthy that contraction of tularemia can occur through unbroken skin, and it is plausible that penetration of intact skin lends itself to a glandular presentation without ulceration (Francis, 1925; Francis, 1928). Some physicians have speculated that many cases classified as glandular are actually ulceroglandular and the patient has overlooked the ulcer (Evans et al., 1985). This notion is supported by the observation that the timing of ulcer formation and symptom development can vary substantially between cases (Evans et al., 1985). The overall mortality rate for glandular tularemia is also under 5% without antibiotics (Pullen and Stuart, 1945).

Oculoglandular tularemia is characterized by conjunctivitis and conjunctival ulcers (Francis, 1928). In addition to these ocular symptoms, the individual experiences painful local lymph node swelling and the flu-like symptoms described above (Francis, 1928). Acquisition of this disease is thought to be largely through accidental self-inoculation by rubbing one's eye after contact with contaminated material (Francis, 1928). The oculoglandular form comprises about 3% of all tularemia cases (Pullen and Stuart, 1945). Without treatment, this disease manifestation carries an overall mortality rate of approximately 14% (Pullen and Stuart, 1945). Whether this represents a true increase in disease severity over ulceroglandular and glandular forms or an unrepresentative small population size due to the rarity of this type remains unknown. In support of the former, some clinical cases of oculoglandular tularemia cases have involved rapid lethality (Francis, 1928).

Typhoidal tularemia differs significantly from the previously described forms. The route of transmission is unknown, no ulcers are present, and enlarged lymph nodes are absent (Francis, 1925; Francis, 1928). Despite missing these features, typhoidal tularemia still presents with the

flu-like illness symptoms described in other forms (Francis, 1925; Francis, 1928). A patient with this clinical presentation would often be initially misdiagnosed with typhoid, thus the term typhoidal tularemia (Francis, 1928). While this manifestation accounts for only 6% of natural tularemia cases, it carries a 50% mortality rate (Pullen and Stuart, 1945). It has been proposed that the lack of involvement of draining lymph nodes and dermis is detrimental and that infection at these sites promotes either a stronger immune response or locally constrains the infection until clearance by an adaptive response (Blackford and Casey, 1941).

Oropharyngeal tularemia and gastrointestinal tularemia result from the consumption of contaminated water or undercooked meat from an infected animal (Ellis et al., 2002). The symptoms and clinical course of the oropharyngeal form is very similar to ulceroglandular tularemia, except for the localization of the primary lesions to the oropharynx and the additional symptom of a sore throat in most cases (Sencan et al., 2009). In many clinical studies it is not differentiated from the ulceroglandular or glandular classification but overall appears unlikely to possess a significantly different mortality rate. This form is found more frequently in Europe than the United States due to the close association of *Francisella tularensis* subspecies *holarctica*, the predominant cause of tularemia in Europe, with fresh water (McCrumb, 1961; Tarnvik et al., 2004). Gastrointestinal tularemia is a very rare manifestation of disease and, unlike other routes of exposure, the consumption of a significant number of bacteria appears required (Hornick et al., 1966; KuoLee et al., 2007; Tulis et al., 1969). Tularemia of this variety may lead to symptoms that are uncommon in most other forms such as diarrhea and intestinal ulcerations in addition to systemic effects (Beck and Merkel, 1935). Due to the rarity of gastrointestinal tularemia, insufficient data are present to determine a mortality rate in the absence of antibiotics.

Pneumonic tularemia, also referred to as inhalational tularemia, pulmonary tularemia and respiratory tularemia, can result from either inhalation of *Francisella tularensis* or hematogenous spread associated with bacteremia (Blackford and Casey, 1941; Stuart and Pullen, 1945b). Respiratory symptoms may include chest pain, shortness of breath, cough, and pneumonia (Blackford and Casey, 1941; Stuart and Pullen, 1945b). Pulmonary tularemia can manifest in two distinct forms, a glandular form with involvement of hilar lymph nodes and a typhoidal form lacking involvement of local lymphatic tissue (Bihss and Berland, 1943). Individuals who present with localized forms of tularemia such as ulceroglandular, oculoglandular, or glandular are more likely to have involvement of hilar lymph node if they concurrently have pulmonary tularemia (Bihss and Berland, 1943). Whether this is due to a clinical difference between the routes of respiratory infection (direct inhalation or secondary hematogenous spread) or due to a variation in individual immune response is currently unknown. Early studies did not consider inhalation a likely route of tularemia exposure and thus pneumonic tularemia was typically considered a symptom of other forms, referred to as tularemic pneumonia (Stuart and Pullen, 1945b). Prior to antibiotics, individuals presenting with tularemic pneumonia had an overall mortality rate of approximately 30%, but some studies have reported almost 60% fatality (Blackford and Casey, 1941; Foshay, 1937; Stuart and Pullen, 1945b). Importantly, no conclusion can be made regarding the mortality rate of individuals after direct inhalation of tularemia. This is because a significant number of tularemic pneumonia cases were from hematogenous spread and considered to be the result of uncontrolled bacteremia prior to death (Blackford and Casey, 1941; Stuart and Pullen, 1945b). In controlled experiments, inhalational challenge of humans with *Francisella tularensis* always resulted in a febrile illness, but roentgenographic examination revealed pneumonitis in only 25% of subjects prior to the

termination of the study (McCrumb, 1961). It is thus clear that the diagnosis of pneumonia with tularemia is associated with worse prognosis. Whether this is due to pneumonia being a symptom of a failing immune response and systemic spread or indicative of an increased mortality associated with inhalation of *Francisella tularensis* remains to be clarified.

All forms of tularemia result in an early bacteremia but, in the vast majority of healthy individuals, this is a transient event (Foshay, 1937; Francis, 1928). In patients who fail to control the infection, a secondary bacteremia develops, resulting in septicemic tularemia (Foshay, 1937). The term septicemic tularemia is occasionally used as synonymous with typhoidal tularemia (Sarria et al., 2003). This is not surprising as a much larger percentage of typhoidal tularemia cases develop into septicemic tularemia than any glandular form (Foshay, 1937). This may be explained by the local immune response thought to be existing in individuals who present with lymphatic involvement (glandular forms) and this immune response also constraining systemic spread (Blackford and Casey, 1941; Foshay, 1937). Septicemic tularemia from bacteremia and the associated septic shock symptoms have been strongly implicated as the primary cause of death in human tularemia (Foshay, 1937). Patients very rarely, if ever, recover from septicemic tularemia (Beck and Merkel, 1935). In rare cases, the bacterium can be isolated from spinal fluid and tularemic meningitis is noted (Hofinger et al., 2009; Stuart and Pullen, 1945a). Meningitic tularemia is almost always fatal without appropriate antibiotic therapy and involves symptoms such as confusion, headache, stiff neck and progression into a comatose state (Hofinger et al., 2009; Stuart and Pullen, 1945a).

1.1.4 Bacteriology

Francisella tularensis is a gram-negative bacterium which does not form spores and lacks modes of motility (Francis, 1928). This organism is an obligate aerobe (Snyder et al., 1946). It is well-known for its pleomorphic morphology and can appear as a bacillus or coccus (Eigelsbach et al., 1946; Francis, 1928; Hesselbrock and Foshay, 1945). The lipid content of *Francisella* is remarkably high (51% in the cell membrane and 68% in the capsule), which is significantly higher than most other gram-negative bacteria (10-20%) (Hood, 1977). Furthermore, the cellular fatty acid profile of *Francisella* is considered a defining characteristic of this genus and is marked by an abundance of long-chain acids (C_{18} to C_{26}) and hydroxy acids (3-OH C_{16} and 3-OH C_{18}) (Hollis et al., 1989; Jantzen et al., 1979). Another defining characteristic of this bacterium is its fastidious nature, which is associated with a dramatic in vitro growth enhancement following cysteine supplementation (Francis, 1923). This phenotype has been suggested to be due to a genetic defect in sulfate assimilation (Larsson et al., 2005). The fastidious nature of *Francisella* has presented a challenge to the successful identification of, confirmation of, and cultivation from, tularemia infections (Provenza et al., 1986; Wherry and Lamb, 1914). Recent work has also challenged the supposition that this feature is universal as rare clinical isolates have been found to lack a cysteine requirement (Bernard et al., 1994).

Early studies suggested this bacterium possessed something resembling a capsule (McCoy and Chapin, 1912). This was further supported by biochemical studies confirming that capsular material was unique from the outer membrane in terms of sugar and lipid composition (Hood, 1977). Recent studies have identified a polysaccharide capsule in *Francisella tularensis* which is composed of *F. tularensis* O-antigen subunits (Apicella et al., 2010). In addition to this structure, this bacterium has also been found to possess a “capsule-like complex” which is not O-

antigen and appears to be a glycoprotein (Bandara et al., 2011). This capsule and potentially other O-antigen independent capsules have been postulated to be environmentally regulated which may delay their identification and characterization (Zarrella et al., 2011).

1.1.5 Facultative Intracellular Life Cycle

The ability of *Francisella* to invade and replicate within host cells was observed as early as 1927 (Francis, 1927). *F. tularensis* has since been found to invade and replicate successfully within a wide variety of cell types, including hepatocytes, dendritic cells, macrophages, and type II pneumocytes (Bosio and Dow, 2005; Conlan and North, 1992; Hall et al., 2007; Nutter and Myrvik, 1966). Following invasion, the bacterium escapes the phagosome and replicates in the cytoplasm (Golovliov et al., 2003). Egress of bacteria from host cells occurs by a poorly described mechanism, likely involving death of the host cell (Lai et al., 2001). In addition to a very heavily studied intracellular life cycle, *Francisella* appears to have a relevant extracellular phase during infection (Forestal et al., 2007). Despite bacteremia being a defining characteristic of tularemia and the majority of bacteria in the blood being outside cells, this extracellular stage remains poorly defined (Forestal et al., 2007; Francis et al., 1921). A significant effort has been made to elucidate the mechanisms of invasion, phagosomal escape, replication, and egress in the macrophage, a model cell-type in *F. tularensis* research (Fig. 1) (Celli and Zahrt, 2013; Chong and Celli, 2010).

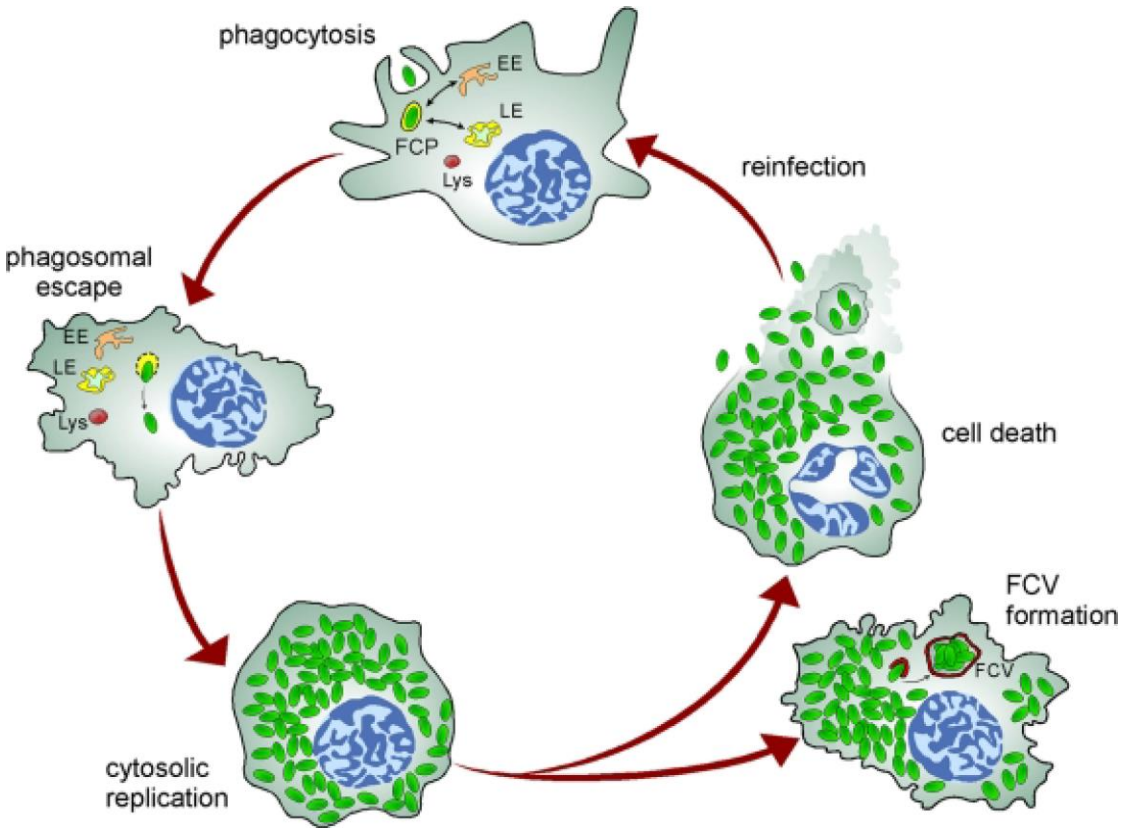

Figure 1. Intracellular life cycle of *F. tularensis* in a macrophage.

Following looping phagocytosis, *F. tularensis* (green) is located within the *Francisella*-containing phagosome (FCP). The FCP engages with early endosomes (EE) and late endosomes (LE) but not lysosomes (Lys). *F. tularensis* subsequently escapes the phagosome and replicates robustly within the cytosol. Infection ultimately results in the death of the host cell and bacterial egress occurs through a yet unknown mechanism. In murine cells, *F. tularensis* re-enters an endosomal compartment known as the *Francisella*-containing vacuole (FCV). This process requires host autophagy and the importance of this observation remains to be defined. This figure was derived from (Chong and Celli, 2010) with permission.

Invasion of the host macrophage begins with *F. tularensis* interacting with the cell surface. Engagement and uptake of opsonized bacteria is largely facilitated by interactions with scavenger receptor A, complement receptor CR3, and Fc receptor Fcγ (Balagopal et al., 2006; Clemens et al., 2005; Pierini, 2006; Schulert and Allen, 2006). Un-opsonized bacteria are primarily thought to interact with the macrophage mannose receptor (Balagopal et al., 2006;

Schulert and Allen, 2006). Following these macrophage surface interactions, *Francisella* is ingested by a novel form of looping phagocytosis (Clemens et al., 2005). This process involves actin remodeling, does not require live bacteria, and is characterized by the unique involvement of asymmetric spacious pseudopod loops (Clemens et al., 2005).

After engulfment, the loop transforms into a less spacious phagosome surrounding the bacterium (Clemens et al., 2004, 2005). The *Francisella* containing phagosome initially co-localizes with the early endosomal marker, early endosome antigen 1 (EEA1) (Clemens et al., 2004). This early endosomal marker is lost and the phagosome subsequently acquires late endosomal markers such as lysosome-associated membrane glycoprotein 1 and 2 (LAMP1, LAMP2) and CD63 (Clemens et al., 2004). The phagosome fails, however, to acquire other lysosomal markers such as cathepsin D and ultimately does not fully acidify (Clemens et al., 2004, 2009). Through an unknown mechanism, phagosomes containing *Francisella* become coated with a dense fibril coat and begin to fragment and bleb (Clemens et al., 2004). The bacteria escape the phagosome and enter the cytosol anywhere between 1 to 4 hours after phagocytosis, depending on opsinization and the methodology of measuring phagosomal integrity (Checroun et al., 2006; Clemens et al., 2004; Geier and Celli, 2011; Golovliov et al., 2003).

Once *Francisella* has successfully been released into the host cytosol, it replicates exponentially (Lai et al., 2001). This process inevitably results in bacterial egress through death of the infected host cell, although the exact mechanism of cell death appears to vary between different *Francisella* subspecies (Mariathasan et al., 2005; Wickstrum et al., 2009). In addition to these stages, *F. tularensis* has been observed re-entering the endocytic pathway after cytosolic replication. This process, occurring after proliferation and prior to cell death, results in a large

portion of intact bacteria residing in a multi-membrane, autophagy-associated compartment (Checroun et al., 2006). While the function of the compartment is unknown, this phenotype appears to be specific for murine cells and may suggest an important immunological difference between mouse and man (Akimana et al., 2010).

1.1.6 Virulence Factors

F. tularensis is a global bacterial pathogen that can infect a large repertoire of species including many protists, arthropods, and mammals (Keim et al., 2007). Given this statement, one would expect this organism to possess a significant number of potent virulence factors. Unlike many gram-negative intracellular pathogens, *F. tularensis* lacks type III and type IV secretion systems (Kostakioti et al., 2005; Larsson et al., 2005). This bacterium does, however, possess alternative virulence factors involved in immune evasion, antimicrobial peptide resistance, and the secretion of effector molecules (Apicella et al., 2010; Bandara et al., 2011; Barker et al., 2009; Forslund et al., 2010; Gil et al., 2006; Gunn and Ernst, 2007; Hager et al., 2006; Nano et al., 2004). Many facets of known virulence factors remain to be elucidated and novel virulence factors have already been discovered in the genes unique to *Francisella* (approximately 20% of annotated genes) (Brotcke et al., 2006; Fuller et al., 2008; Horzempa et al., 2008; Larsson et al., 2005; Milne et al., 2007; Napier et al., 2012). The virulence of *Francisella* is also critically regulated by a variety of environmental cues including iron concentration, temperature, nutrient levels, and host-specific cytosolic molecules such as spermine (Carlson et al., 2009; Charity et al., 2007; Horzempa et al., 2008; Milne et al., 2007).

Immune recognition of many gram-negative bacterial pathogens is facilitated by the interaction of the cellular protein Toll-like receptor 4 (TLR4) and bacterial lipopolysaccharide

(LPS) (Arbour et al., 2000; Chow et al., 1999; Hoshino et al., 1999; Poltorak et al., 1998). This interaction results in a proinflammatory signaling cascade involving the activation of the transcription factor nuclear factor-κB (NF-κB) and the release of proinflammatory cytokines such as TNF-α (Arbour et al., 2000; Chow et al., 1999; Rietschel et al., 1994). LPS is often referred to as an "endotoxin" due its ability to evoke a potent immunological response associated with significant immunopathology (Rietschel et al., 1994) (Danner et al., 1991). In contrast to most other gram-negative bacteria, the LPS of *F. tularensis* fails to interact with TLR4, does not induce a robust inflammatory response from mononuclear cells, and shows no evidence of functioning as an endotoxin in *in vivo* models (Barker et al., 2006; Hajjar et al., 2006; Sandstrom et al., 1992). These phenotypes are likely the result of the unique and atypical structure of the LPS of *F. tularensis* (Gunn and Ernst, 2007; Okan and Kasper). Several distinct features include the lack of a conserved 4'-phosphate, modification of the 1'-phosphate with galactosamine, and the presence of mostly free lipid A containing only four long acyl chains (Okan and Kasper). The removal of the 4'-phosphate and the addition of galactosamine to the 1'-phosphate of the lipid A results in the loss of negative surface charge and contributes to the intrinsic cationic peptide resistance of *Francisella* (Llewellyn et al., 2012; Wang et al., 2007). Thus far, genetic manipulation of the *F. tularensis* LPS biosynthetic pathway has failed to restore recognition by TLR4 and a definitive mechanism behind this immunologic evasion remains unknown (Kanistanon et al., 2008; Okan et al., 2013; Wang et al., 2007).

F. tularensis possesses multiple capsules that contribute to various degrees to its virulence by facilitating resistance to serum and antimicrobial peptides and shielding the surface from TLR2-mediated immune recognition (Apicella et al., 2010; Bandara et al., 2011; Clay et al., 2008; Li et al., 2007; Zarrella et al., 2011). The most well-studied capsule of *F. tularensis* is the

O-antigen capsule which is responsible for resistance to complement-mediated lysis (Apicella et al., 2010; Li et al., 2007). This capsule is similar to O-antigen capsules in other bacteria, such as *Escherichia coli* and *Salmonella enterica* and is comprised of polymers of the O-antigen subunit of lipopolysaccharide while lacking detectable lipid A and Kdo based on mass spectrometry (Apicella et al., 2010; Gibson et al., 2006; Goldman et al., 1982; Peleg et al., 2005). Complement resistance in *Francisella* involves rapid conversion of the essential complement protein C3b to C3bi, an inactive form, which prevents assembly of the membrane attack complex (MAC) and subsequent MAC mediated lysis (Ben Nasr and Klimpel, 2008; Clay et al., 2008). The mechanism by which the capsule inactivates C3b may involve the observation that factor H, a complement inhibitory protein that is involved in self-recognition, binds *Francisella* (Ben Nasr and Klimpel, 2008). While the mechanism is in need of further elucidation, it requires the O-antigen capsule and leads to opsinization of *F. tularensis*, mediating uptake by CR3 on host phagocytes (Ben Nasr and Klimpel, 2008). *Francisella* uptake into human cells utilizing CR3 has been found to facilitate immune suppression by dampening TLR2-mediated proinflammatory responses (Dai et al., 2013). In addition to immunosuppression, the capsule has also been implicated in immune evasion by shielding the availability of surface antigens from interacting with host TLRs (Zarrella et al., 2011). The capsule is also required for normal intracellular growth and prevents premature cell death in infected human macrophages, phenotypes likely the result from limiting immune stimulation (Lindemann et al., 2011). Ultimately, this O-antigen capsule is required for in vivo virulence in the murine model (Li et al., 2007). In addition to this well-studied capsule, another surface structure, the capsule-like complex (CLC), has been recently described in *F. tularensis* (Bandara et al., 2011). The CLC was found not to contain or require O-antigen, but appears to be at least partially comprised of an un-identified glycoprotein

(Bandara et al., 2011). This capsule was not required for serum resistance, intracellular growth, or prevention of host cell death but interestingly was still required for the full in vivo virulence of *F. tularensis* (Bandara et al., 2011). The contribution of the CLC and potentially other undescribed capsules has been hypothesized to be linked to general immune evasion and shielding of bacterial antigens (Zarrella et al., 2011). Further experimentation is required to substantiate these claims.

Type I secretion systems are responsible for the release of a wide variety of diverse effectors, including lipases, proteases, and toxins in a sec-system independent fashion (Kanonenberg et al., 2013). This method of secretion requires three distinct proteins, an outer membrane protein, an ABC transporter, and a membrane fusion protein (Kanonenberg et al., 2013). A protein known as TolC participates both in multidrug resistance and type I secretion as the outer membrane protein (Kanonenberg et al., 2013; Nikaido, 1996; Wandersman and Delepelaire, 1990). Mutation of a homolog of *tolC* in *F. tularensis* results in decreased intracellular replication, increased susceptibility to antibiotics and toxic compounds, and significant attenuation in a murine model (Gil et al., 2006). Interestingly, mutation in another gene, known as *ftlC*, results in comparable increased sensitivity to antibiotics and toxic compounds but does not affect intracellular replication or in vivo virulence (Gil et al., 2006). This finding led to the hypothesis that the attenuating phenotype in *tolC* mutants was linked to a defect in type I secretion rather than a loss of efflux (Gil et al., 2006). Follow-up studies demonstrated that the loss of *tolC* in *F. tularensis* results in a strain that is both proinflammatory and hyper-cytotoxic and found that *F. tularensis* subsp. *novicida* strains lacking *tolC* were defective for secretion of an uncharacterized hemolysin, an effector known to utilize the type I secretion system (Platz et al., 2010; Wandersman and Delepelaire, 1990). These studies suggest

F. tularensis utilizes unknown secreted effectors that rely on type I secretion systems to limit immune activation and contribute to in vivo pathogenesis (Gil et al., 2006; Platz et al., 2010). This attenuation may also be explained, independent of type I secretion, by recent evidence that loss of *tolC* in *E.coli* results in membrane stress and overall metabolic shutdown resulting in depletion of essential metabolites in minimal media (Dhamdhere and Zgurskaya, 2010). Thus, further work is required to confirm that type I secretion is a true contributor to the virulence of *F. tularensis*.

Initial investigations demonstrated the presence of pili-like structures on the surface of *F. tularensis* (Gil et al., 2004). Genome sequencing later revealed abundant homologs to type IV pili machinery, which might be linked to pili formation or a structurally related type II secretion system (Larsson et al., 2005). Investigation into these genes confirmed that *F. tularensis* uses type IV pili homologs to make pili, but also uncovered a functional type II secretion system (Hager et al., 2006). Distinct homologs of type IV pili were required for secretion and pili formation, allowing for these virulence attributes to be evaluated independently (Hager et al., 2006; Zogaj et al., 2008). Loss of genes required for pili formation results in a minor loss or no loss in virulence, depending on the subspecies of *F. tularensis* (Ark and Mann, 2011; Forslund et al., 2006; Forslund et al., 2010; Zogaj et al., 2008). In contrast, loss of genes required for type II secretion appears to moderately reduce virulence in all subspecies (Ark and Mann, 2011; Forslund et al., 2006; Forslund et al., 2010; Zogaj et al., 2008). Despite the apparent importance of type II secretion in all subspecies, type II secreted effectors, such as PepO, have only been characterized in *F. tularensis* subsp. *novicida* and are not all conserved in other subspecies (Forslund et al., 2010; Hager et al., 2006). Investigations into the type II secretion system of

other *F. tularensis* subspecies may reveal unappreciated secreted virulence factors (Salomonsson et al., 2011).

The completion of the *F. tularensis* genome sequence, combined with transposon mutagenesis, led to the discovery of the *F. tularensis* pathogenicity island (Gray et al., 2002; Larsson et al., 2005; Nano et al., 2004). This approximately 30kb region has a significantly lower GC content (~27%) than the average GC content of the genome (~33%) which suggests it was likely acquired by horizontal gene transfer from a lower GC content organism (Larsson et al., 2009; Nano et al., 2004). Interestingly, this pathogenicity island is present in a single copy in *F. tularensis* subsp. *novicida* but has been duplicated in all other *F. tularensis* subspecies (Larsson et al., 2009; Nano et al., 2004). This genomic cluster of 17 genes, many of which are required for phagosomal escape and virulence in animal models, was suggested to encode a secretion system with partial homology to a known type VI secretion system (T6SS) (Barker et al., 2009; de Bruin et al., 2011). Importantly, this "secretion system" of *F. tularensis* appears to be unique as many genes have relatively weak homology to their T6SS counter parts and are often missing critical functional domains (Barker et al., 2009; Broms et al., 2010). Furthermore, while investigations into the pathogenicity island have revealed novel secreted effectors required for virulence, it has also been observed that secretion was independent of the normally essential T6SS structural homologs (Barker et al., 2009; Broms et al., 2011). Some secreted effectors of the pathogenicity island were even found to be secreted in the absence of any other members of this virulence cluster (Barker et al., 2009). While many members of the *F. tularensis* pathogenicity island are required for phagosomal escape and ultimately virulence, the mechanism of action of these genes is in need of further investigation (Broms et al., 2010; de

Bruin et al., 2011). Furthermore, a role in bacterial competition has not yet been examined for this T6SS homolog.

1.1.7 *Francisella* Metabolism

The ability of *F. tularensis* to replicate exponentially in a variety of diverse cell types, at multiple physiological sites, and in a large repertoire of mammalian and arthropod species suggests that it is adapted to the host environment (Bell, 1965; Francis, 1928; Keim et al., 2007; Meibom and Charbit, 2010). Pathogens must acquire nutrients and metabolic precursors from the host during infection (Rohmer et al., 2011). Interestingly, examination of multiple *F. tularensis* genomes reveals the disruption and absence of a significant number of complete metabolic pathways, a feature likely mediated by the abundance of insertion sequence elements in the genome (Larsson et al., 2005). This genetically fragmented landscape, combined with the significant number of possible metabolism-related hypothetical genes, makes empiric evaluation of *F. tularensis* metabolism challenging (Larsson et al., 2005; Meibom and Charbit, 2010). Experimental evaluation of metabolism can be performed in vitro using a chemically defined media (CDM) known as Chamberlain's chemically defined media, which was developed specifically for *F. tularensis* cultivation (Chamberlain, 1965). While use of CDM can reveal basic nutritional requirements, understanding metabolism in the context of host-pathogen interaction, presents additional layers of complexity. Pathogens utilize host metabolic intermediates, degrade host molecules, and manipulate host metabolic machinery (Fisher et al., 2012; Lee et al., 2013; Szaszak et al., 2013). *F. tularensis* also disseminates and replicates within various organs and also has substantial extracellular phase leading to the possibility of diverse site-specific metabolic requirements (Forestal et al., 2007; Meibom and Charbit, 2010).

Ultimately, investigation into the metabolism of *F. tularensis* has revealed numerous metabolic pathways that contribute to, or are essential, to the virulence of this pathogen (Alkhuder et al., 2009, 2010; Barel et al., 2012; Chu et al., 2011; Napier et al., 2012; Pechous et al., 2006; Pechous et al., 2008; Peng and Monack, 2010; Quarry et al., 2007).

1.1.7.1 Amino Acids

Early attempts to develop a suitable chemically defined medium for various *F. tularensis* strains revealed that 7 of the 20 standard amino acids (alanine, asparagine, glutamate, glutamine, glycine, phenylalanine, and tryptophan) were not essential for in vitro growth (Nagle et al., 1960; Traub et al., 1955). The definitive requirement of most of the 13 amino acids included in all *F. tularensis* CDM remains unknown. No biosynthetic pathway for arginine, histidine, lysine, methionine, or tyrosine is annotated in the genome of *F. tularensis* (Larsson et al., 2005). Biosynthetic pathways for cysteine, isoleucine, threonine, and valine contain pseudo-genes and disruptions (Larsson et al., 2005). Intact pathways are found for serine, aspartic acid, leucine, and proline but experimental evidence regarding their functionality is lacking (Larsson et al., 2005). Interestingly, early investigators found this pathogen notoriously difficult to culture in vitro and eventually this was tied to an absolute requirement for abundant cysteine (Francis, 1923; Wherry and Lamb, 1914). Furthermore, a requirement for cysteine supplementation is typically a defining feature of *F. tularensis* isolates, but exceptions have been noted (Bernard et al., 1994). This phenotype was hypothesized to be due to a defect in adenylyl-sulfate kinase, a gene required for sulfate assimilation, discovered after examination of the sequenced genome (Larsson et al., 2005). This hypothesis is supported by the cysteine independence *of F. philomiragia* which contains an intact adenylyl-sulfate kinase as well as the fact that mutations in this gene in *Salmonella typhimurium* led to a cysteine requirement (Collins and Monty, 1975;

Hollis et al., 1989). Cysteine auxotrophy was originally thought to be compensated for by the abundance of cysteine in the skin, as ulceroglandular tularemia is the most common form of disease (Francis, 1923, 1928; Pullen and Stuart, 1945). Recent evidence, however, demonstrated that *F. tularensis* is capable of degrading a host polypeptide, glutathione, as a source of cysteine (Alkhuder et al., 2009). This activity requires a γ-glutamyltransferase and mutagenesis of that gene confirmed that it is essential for the virulence of *F. tularensis* (Alkhuder et al., 2009; Ireland et al., 2011). Tryptophan biosynthesis was also required for the full in vivo virulence of *Francisella* (Chu et al., 2011; Peng and Monack, 2010). The attenuation upon loss of tryptophan biosynthesis was found to be the result of a host protein, indoleamine 2,3-dioxygenase, which depletes intracellular tryptophan in response to immune activation (Peng and Monack, 2010). Interestingly, *F. tularensis* upregulates the host neutral amino acid transporter SLC1A5 and siRNA-mediated knockdown of this protein reduces intracellular replication (Barel et al., 2012). Future studies are needed to elucidate the pathogenic contribution of other intact amino acid biosynthetic pathways and to determine how *F. tularensis* utilizes the host environment to overcome auxotrophy for essential amino acids.

1.1.7.2 Purines and Pyrimidines

Purines and pyrimidines are the building blocks of DNA and their biosynthesis or acquisition is essential for life (O'Donovan and Neuhard, 1970). *F. tularensis* contains homologs to all required proteins for de novo synthesis of purines and pyrimidines and it is thus unsurprising that these growth factors are not required in CDM (Karlsson et al., 2000; Meibom and Charbit, 2010; Nagle et al., 1960). Disruption of de novo pyrimidine biosynthesis in *F. tularensis* leads to an auxotrophy that could be chemically complemented by uracil (Horzempa et

al., 2010a). This is not surprising as the bacterium contains functional pyrimidine salvage pathways, two of which are capable of converting uracil to uridine monophosphate and thus bypassing de novo synthesis (Larsson et al., 2005; Moffatt and Ashihara, 2002). Although loss of de novo pyrimidine synthesis dramatically attenuated intramacrophage growth, it had only a moderate effect on in vivo bacterial burden and a minor effect on in vivo survival kinetics (Horzempa et al., 2010a). This phenotype was somewhat surprising since loss of de novo pyrimidine synthesis results in attenuation in several pathogens (D'Enfert et al., 1996; Fox and Bzik, 2002; Mahan et al., 1993; Samant et al., 2008; Schwager et al., 2013). One possibility to explain this lack of attenuation is the aforementioned presence of pyrimidine salvage pathways through the utilization of host uracil or uridine. In support of this mechanism, *F. tularensis* strains lacking de novo pyrimidine synthesis appeared unable to replicate within alveolar macrophages in vivo, a cell type that has been previously suggested to contain low levels of uracil and uridine (D'Enfert et al., 1996; Horzempa et al., 2010a). Interestingly, disruption of de novo pyrimidine synthesis also has a minor effect on virulence in *Leishmania donovani* (Wilson et al., 2012). Disruption of both de novo synthetic and uracil/uridine salvage pathways, however, dramatically attenuated this pathogen (Wilson et al., 2012). Experiments are required to determine if *F. tularensis* primarily utilizes the salvage pathway in vivo or if, like *Leishmania donovani*, either pathway is largely sufficient for virulence (Wilson et al., 2012). Alternatively, *F. tularensis* could hypothetically salvage pyrimidines through degradation of host RNA and DNA, although evidence supporting this possibility is lacking (Fox and Holtman, 1968). In stark contrast to pyrimidine biosynthesis, de novo purine biosynthesis has been found to play an integral role in *F. tularensis* virulence (Pechous et al., 2006; Pechous et al., 2008; Quarry et al., 2007; Santiago et al., 2009). In fact, de novo purine biosynthesis appears fundamentally required

for intracellular replication and mutants lacking this pathway are completely attenuated (Pechous et al., 2006; Pechous et al., 2008; Quarry et al., 2007; Santiago et al., 2009). The ability to synthesize purines facilitates full virulence in several pathogens and purines have been implicated as a limiting nutrient in some host environments (Appelberg, 2006; Crawford et al., 1996; McFarland and Stocker, 1987; Samant et al., 2008). Interestingly, *F. tularensis* is lacking several enzymes that function in alternative purine salvage pathways, which likely contributes to the absolute requirement of de novo purine biosynthesis for intracellular replication (Karlsson et al., 2000). It remains to be determined if the aforementioned purine salvage pathways contribute to pathogenesis.

1.1.7.3 Micronutrients – Elements and Vitamins

Beyond the nutritional needs associated with protein synthesis (amino acids) and DNA/RNA synthesis (pyrimidines and purines), organisms require other micronutrients such as elements and vitamins (Mason, 2007). A critical host-pathogen metabolic competition occurs over the essential trace element, iron (Drakesmith and Prentice, 2012; Skaar, 2010). The host passively restricts the concentration of free iron, sequestering it in compounds such as transferrin, lactoferrin and heme (Drakesmith and Prentice, 2012; Ponka et al., 1998; Skaar, 2010). This process is essential in preventing free radical production and limiting systemic microbial infection (Drakesmith and Prentice, 2012; Ponka et al., 1998; Skaar, 2010). Furthermore, the host possesses active mechanisms to further deplete iron in response to infection (Drakesmith and Prentice, 2012; Flo et al., 2004). To overcome these defenses, many pathogens secrete high-affinity iron-binding proteins known as siderophores, and/or bind, take up, and degrade host iron-containing compounds (Neilands, 1995; Schmitt, 1997; Skaar, 2010).

The evolution of *Borrelia burgdorferi*, the causative agent of Lyme disease and one of the only known life forms that does not require iron, is likely the extreme result of this competition (Posey and Gherardini, 2000; Skaar, 2010). The iron metabolism of *F. tularensis* involves a high-affinity outer membrane iron transporter and a siderophore, in addition to conserved gram-negative inner membrane iron transport systems (Milne et al., 2007; Ramakrishnan et al., 2008; Ramakrishnan et al., 2012; Sullivan et al., 2006; Thomas-Charles et al., 2013). Loss of the outer-membrane iron transporter moderately decreased virulence, while loss of siderophore production had a negligible in vivo effect (Lindgren et al., 2009; Ramakrishnan et al., 2012). Siderophore production does contribute to pathogenesis, at least secondarily, as disruption of both the outer-membrane transporter and the siderophore have a synergistic effect and lead to a dramatically attenuated strain (Ramakrishnan et al., 2012). Interestingly, the siderophore of *F. tularensis* is specific for ferric iron, while the outer-membrane transporter is specific for ferrous iron (Ramakrishnan et al., 2012). Furthermore, the outer-membrane transporter is not transcriptionally regulated by iron, while the siderophore is tightly controlled by iron-responsive transcription factors (Lindgren et al., 2009; Sullivan et al., 2006). Iron-binding host proteins, such as transferrin and lactoferrin, sequester ferric iron but ferrous iron is produced when these proteins are degraded in an acidified lysosome (Breuer et al., 1995). Inhibition of lysosomal acidification attenuates intracellular replication of *F. tularensis* and this phenotype can be reversed by iron supplementation (Clemens et al., 2009) (Fortier et al., 1995). Together, these results suggest that *F. tularensis* primarily acquires ferrous iron from lysosomal degradation but, when necessary, expresses a siderophore to acquire ferric iron by chelation of iron from iron-containing host proteins.

Besides investigations into iron, relatively little work has been performed to elucidate the biosynthesis or acquisition of micronutrients by *F. tularensis*. Mutation in the major potassium transporter of *F. tularensis* did not affect in vitro intracellular replication but resulted in a significant in vivo virulence defect (Alkhuder et al., 2010). In particular, the mutant was unable to replicate within the blood (Alkhuder et al., 2010). The mechanism behind this site-specific attenuation was hypothesized to be due to low levels of potassium extracellularly (Alkhuder et al., 2010). Disruption of the synthesis of biotin (Vitamin B_7), a cofactor in multiple metabolic pathways, has been associated with phagosomal escape defects and attenuation in *F. tularensis* (Napier et al., 2012). In contrast, disruption in the synthesis of pantothenate (Vitamin B_5), the core of the essential coenzyme A cofactor, had no measurable effect on the virulence of *F. tularensis* (Miller et al., 2013). These results indicate that *F. tularensis* encounters biotin limitation in vivo, but is capable of scavenging sufficient levels of pantothenate or related compounds from the host environment through a yet unknown mechanism (Miller et al., 2013; Napier et al., 2012). Thus, it is clear that these few investigations into *Francisella* metabolism have yielded important information regarding the nutritional interactions between pathogen and host.

1.2 ONE-CARBON METABOLISM

One-carbon metabolism refers to the metabolic processes surrounding the generation and transfer of single-carbon moieties (Dev and Harvey, 1982; Locasale, 2013; Tibbetts and Appling, 2010). The central carrier of one-carbon units is tetrahydrofolate (THF), a compound derived in bacteria from pterin, 4-aminobenzoate, and glutamate (Bermingham and Derrick, 2002). Two

mechanisms, known as the serine hydroxymethyltransferase (SHMT) and the glycine cleavage system (GCS), facilitate THF methylation and transformation to 5'10-methylenetetrahydrofolate (5'10-mTHF) (Fig. 2) (Dev and Harvey, 1982; Lorio et al., 2010). The serine hydroxymethyltransferase involves the degradation of serine to glycine, while the glycine cleavage system involves the complete degradation of the glycine to carbon dioxide and ammonia (Plamann et al., 1983; Plamann and Stauffer, 1983). The absence of both pathways in *E. coli* results in a complete loss of detectable 5'10-mTHF, suggesting that these two pathways are essential in generating one-carbon folates (Dev and Harvey, 1982; Waller et al., 2010). Once acquired, 5'10-mTHF plays an essential role as a carbon donor in downstream metabolic reactions involving the generation of nucleotides and various amino acids (Locasale, 2013; Tibbetts and Appling, 2010).

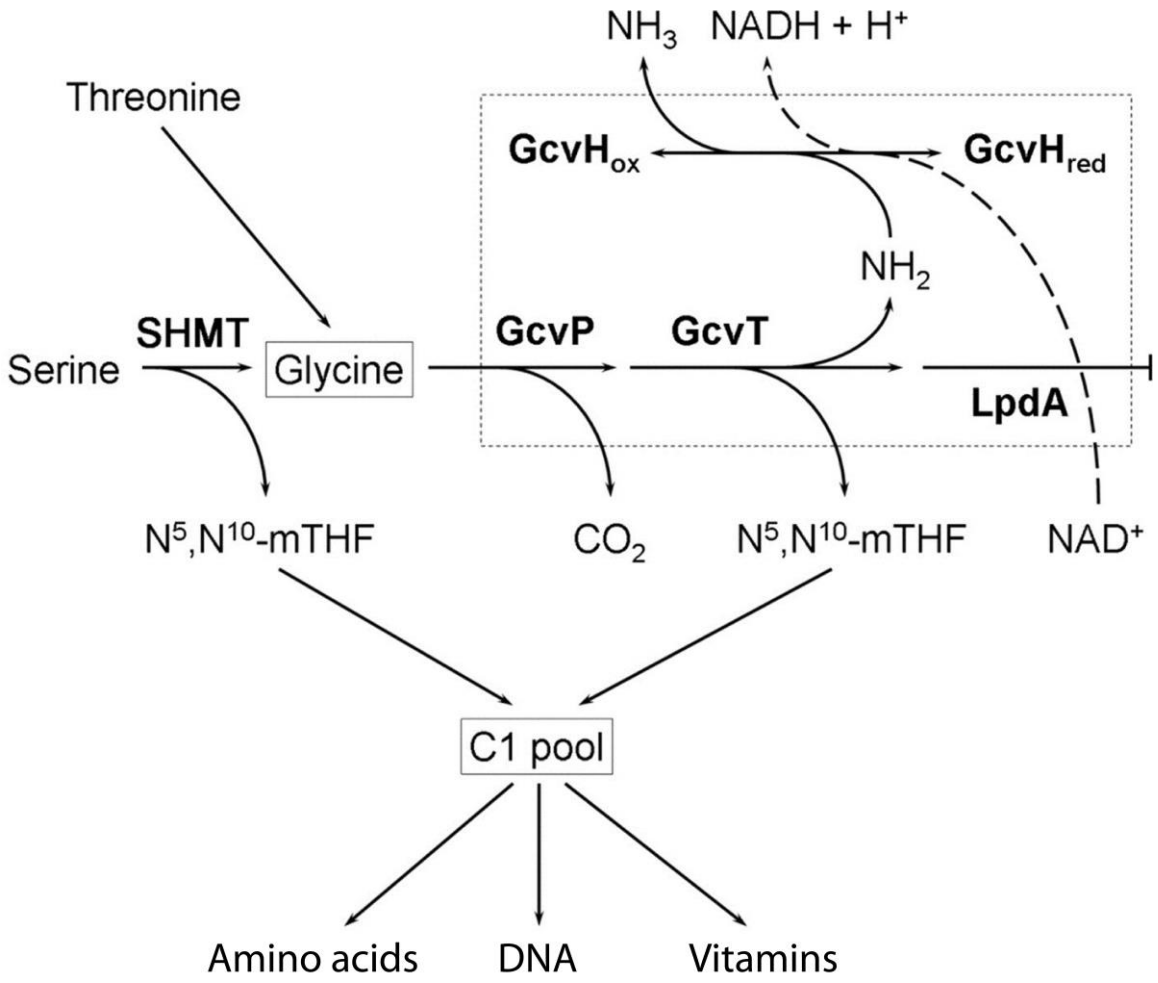

Figure 2. The generation of a single-carbon (C1) pool by the GCS and SHMT for one-carbon metabolism. 5'10-methylenetetrahydrofolate (N^5,N^{10}-mTHF) can be synthesized from either serine degradation by the serine hydroxymethyltransferase (SHMT) or from glycine degradation by the glycine cleavage system (GcvT, GcvH, GcvP, LpdA). Once generated, N^5,N^{10}-mTHF enters a single-carbon pool and is ultimately utilized for the synthesis of various amino acids, nucleosides, and vitamins. This figure is adapted from (Lorio et al., 2010) with permission.

1.2.1 5'10-methylenetetrahydrofolate (5'10-mTHF)

The production and utilization of 5'10-mTHF is the foundation of one carbon metabolism (Locasale, 2013; Tibbetts and Appling, 2010). SHMT is the main producer of the methylated co-factor in many bacteria, while the GCS provides an alternative source (Dev and Harvey, 1982).

5’10-mTHF serves to donate or capture a single carbon in numerous metabolic pathways. Primarily, donation of a single carbon from 5’10-mTHF functions in the biosynthesis of serine, methionine, purines, pantothenate, formylmethionyl-tRNA and thymidine (Guillon et al., 1992; O'Donovan and Neuhard, 1970; Ravnikar and Somerville, 1987; Webb et al., 2004; Weissbach and Brot, 1991; Zhang et al., 2008). The presence of THF as a carbon acceptor also plays a role in glycine biosynthesis (Stauffer and Brenchley, 1978). Generally, the flexibility of one-carbon metabolism and THF affords the organism a mechanism to regulate the concentration of several amino acids and synthesize nucleotides (Kikuchi et al., 2008; Stauffer and Brenchley, 1978).

One-carbon metabolism-associated enzymes can both degrade and synthesize serine (Ravnikar and Somerville, 1987; Stauffer and Brenchley, 1978). The majority of 5’10-mTHF in most microorganisms is derived from serine degradation by SHMT (Dev and Harvey, 1982). In addition, the consumption of serine by this enzyme also provides a major source of glycine (Dev and Harvey, 1982). Unsurprisingly, strains lacking SHMT activity have high glycine requirements and are sometimes referred to as glycine auxotrophs (Bogard et al., 2012; Harder and Quayle, 1971; Stauffer and Brenchley, 1978). In conditions of serine starvation, one-carbon mediated production of serine can occur and requires a functional SHMT, 5’10-mTHF, and glycine (Ravnikar and Somerville, 1987). The source of 5’10-mTHF must be from GCS-mediated glycine degradation when serine is limiting (Dev and Harvey, 1982; Ravnikar and Somerville, 1987; Waller et al., 2010). In such conditions, glycine can be acquired directly from the environment, or can be produced from threonine degradation (Ghrist and Stauffer, 1995; Newman et al., 1976). Threonine can likewise be obtained from the environment or synthesized de novo (Halpern, 1974; Wormser and Pardee, 1958). While glycine degradation by the GCS can facilitate serine biosynthesis, it also functions in avoiding toxic glycine accumulation

(Hagemann et al., 2005; Scott et al., 2008). Glycine toxicity appears to be partially linked to the ability of this amino acid to chelate divalent cations and may also involve inhibition of cell wall synthesis in bacteria (Eisenhut et al., 2007; Hammes et al., 1973). The GCS is induced by glycine, allowing this pathway to sense and limit glycine concentration (Heil et al., 2002). Thus, one-carbon metabolism is capable of circumventing serine starvation and regulating glycine concentration.

De novo production of methionine requires the donation of a methyl group, acquired from one-carbon metabolism, to homocysteine (Weissbach and Brot, 1991). Homocysteine is a non-protein amino acid that is synthesized from aspartate and cysteine (Chattopadhyay et al., 1991). The methyl group is donated primarily from 5'methyltetrahydrofolate (5'-mTHF) (Guest et al., 1964b). 5'-mTHF is generated from 5'10-mTHF by the activity of a methylenetetrahydrofolate reductase (Guest et al., 1964a). The biosynthesis of methionine is tightly regulated in many bacteria and this partially occurs through the regulation of SHMT-mediated 5'10-mTHF production (Weissbach and Brot, 1991). The methionine precursor, homocysteine, is capable of inducing SHMT while the downstream methionine product, S-adenosylmethionine is capable of repressing SHMT (Weissbach and Brot, 1991). Overall, it is clear that one-carbon metabolic reactions are important to methionine production and regulation.

Biosynthesis of most nucleotides in a *de novo* fashion requires one-carbon metabolism (Moffatt and Ashihara, 2002; O'Donovan and Neuhard, 1970; Zhang et al., 2008). Thymidine monophosphate is produced from its precursor uridine monophosphate by the action of a thymidylate synthase (O'Donovan and Neuhard, 1970; Wahba and Friedkin, 1962). This reaction requires the donation of a methyl group from 5'10-mTHF (O'Donovan and Neuhard, 1970; Wahba and Friedkin, 1962). Methyl donation is required for the synthesis of inosine

monophosphate, the precursor of both purine nucleotides (guanine monophosphate and adenine monophosphate) (Zhang et al., 2008). The source of the carbon donation in this scenario is 10-formyltetrahydrofolate (10-formylTHF) (Zhang et al., 2008). In many bacteria, 10-formylTHF is derived from 5'10-mTHF by a bi-functional enzyme, which converts 5'10-mTHF into 5,10-methenyltetrahydrofolate and subsequently into 10-formylTHF (D'Ari and Rabinowitz, 1991). SHMT is regulated by purine concentration, a feature that also highlights the importance of 5'10-mTHF in purine biosynthesis (Steiert et al., 1990a). In bacteria, 10-formylTHF is also combined with methionyl-tRNA to produce formylmethionyl-tRNA through the action of methionyl-tRNA formyltransferase (Guillon et al., 1992). Formylmethionyl-tRNA serves as the initiator tRNA for protein synthesis in bacteria and the formylation is thought to increase the affinity of initiation factors for this tRNA (Hartz et al., 1989). It is noteworthy that, in contrast to early dogma, formylation is not essential to bacterial protein synthesis and its loss may only result in a mild to moderate growth defect (Newton et al., 1999). Finally, ketopantoate, a precursor of pantothenate, also requires 5'10-mTHF to be produced (Webb et al., 2004). Therefore, 5,10-mTHF is also an essential carbon donor in nucleoside, pantothenate, and formylmethionyl-tRNA biosynthesis.

1.2.2 Serine Hydroxymethyltransferase (SHMT)

SHMT (EC 2.1.2.1) is capable of facilitating a reversible reaction, interconverting serine and glycine, which can generate or consume one-carbon moieties (Stauffer and Brenchley, 1978). 5'10-mTHF is generated from the reaction (serine + THF → glycine + 5'10-mTHF) and 5'10-mTHF is consumed by the reverse reaction (glycine + 5'10-mTHF → serine + THF)

(Locasale, 2013; Tibbetts and Appling, 2010). This metabolic enzyme is highly evolutionarily conserved and is present throughout many prokaryotic and eukaryotic species (Garrow et al., 1993; Plamann and Stauffer, 1983; Tibbetts and Appling, 2010). Bacteria typically possess a single copy of this gene, annotated as *glyA* (Plamann and Stauffer, 1983). In contrast, eukaryotes have been found to possess at least two isoforms, one present in the cytosol (cSHMT or SHMT1) and one localizing to the mitochondria (mSHMT or SHMT2) (Garrow et al., 1993; Narkewicz et al., 1996; Tibbetts and Appling, 2010). The significance of these two SHMT proteins remains to be fully clarified but evidence suggests that mSHMT is the major supplier of glycine and 5'10-mTHF from serine degradation while cSHMT may function in either alternative serine synthesis or regulatory reactions (Narkewicz et al., 1996; Tibbetts and Appling, 2010).

While compartmentalization and distinct isoforms may convey a sense of unidirectionality in eukaryotes, the regulation of bidirectionality in prokaryotic SHMT reactions remains unclear. The SHMT of prokaryotes is the primary source of one-carbon units in most bacteria and its default directive, at least in the laboratory, appears to be serine degradation and 5'10-mTHF production (Dev and Harvey, 1982; Stauffer and Brenchley, 1978). In fact, SHMT is responsible for the majority of one-carbon units in *E. coli* (Dev and Harvey, 1982). However, it is clear that, when required, this protein can facilitate serine synthesis from glycine and 5'10-mTHF consumption (Ravnikar and Somerville, 1987). The role of SHMT in serine synthesis is generally confounded by the presence of another serine biosynthetic pathway, which converts glucose to serine (Ravnikar and Somerville, 1987). This glucose to serine pathway requires the *serABC* genes and is generally regarded as the primary serine biosynthetic pathway of most bacteria (Ravnikar and Somerville, 1987; Umbarger et al., 1963). Interestingly, it has been noted that binding serine results in a confirmational change in SHMT, while binding glycine seems to

have no effect (Florio et al., 2011). This may help explain the apparent preference of the enzyme for serine degradation over serine synthesis in most settings. It is important to note however that serine degradation is not always the primary function of SHMT and in some bacteria, such as methylotrophs, this protein serves primarily to synthesize serine and assimilate single-carbon compounds (Izumi et al., 1993; Miyata et al., 1993). Overall, it is clear that the function and importance of SHMT depends both on the genetic landscape of the organism (i.e. the presence of additional serine biosynthetic pathways, the auxiliary production of one-carbon units from the GCS) and on nutrient availability.

Given the role of SHMT encoded by *glyA* in many prokaryotes as a primary source of 5'10-mTHF and the importance of 5'10-mTHF in DNA and protein synthesis, one would expect that *glyA* would be an important fitness factor for bacterial pathogens (Dev and Harvey, 1982). This gene, however, has rarely been studied in the context of virulence. One plausible explanation is that mutants in this pathway suffer from a growth defect in typical laboratory media and are either unattainable or consciously excluded. In support of this, *glyA* has been designated as essential in numerous bacterial species due to a lack of genetic disruption of this locus following transposon screens (Gallagher et al., 2007; Kobayashi et al., 2003; Simic et al., 2002). It is unlikely, however, that this metabolic pathway is truly essential in all these conditions but rather appears that these studies fail to supplement the nutritional needs of an auxotroph. Indeed, *glyA* mutants in *Escherichia coli* have been generated but require glycine supplementation (Ravnikar and Somerville, 1987; Stauffer and Brenchley, 1978). This supplementation likely promotes 5'10-mTHF production through the GCS and replaces the pool of glycine produced from *glyA*-mediated serine degradation (Dev and Harvey, 1982; Ravnikar and Somerville, 1987). Mutants in *glyA* have been investigated in the intestinal pathogen *Vibrio*

cholerae (Bogard et al., 2012). Strains lacking *glyA* required additional amino acid supplementation and were found to be attenuated in a murine intestinal model (Bogard et al., 2012). The importance of *glyA* has also been evaluated in the fish pathogen *Edwardsiella ictaluri* (Dahal et al., 2013). In this study, the contribution of *glyA* to metabolism was not evaluated but *glyA* was found to be essential for virulence in a catfish model of enteric septicemia (Dahal et al., 2013). This gene has also been linked indirectly to virulence. For example, mutants in *glyA* have reduced spore formation efficiency in *Bacillus subtilis* and regulation of *glyA* is linked to curli fiber formation in *E. coli* (Chirwa and Herrington, 2003; Dartois et al., 1997). Curli fibers are involved with biofilm formation and contribute to host cell adhesion and invasion (Barnhart and Chapman, 2006). While the role of *glyA* in many other bacterial pathogens remains unknown, these studies suggest that perturbation of this metabolic process leads to overall reduced fitness and loss of virulence.

1.2.3 Glycine Cleavage System (GCS)

In addition to serine degradation, 5'10-mTHF can also be acquired from glycine degradation (Dev and Harvey, 1982; Meedel and Pizer, 1974). While serine degradation only requires a single enzyme, SHMT, glycine degradation requires at least four proteins. These four proteins, referred to as the glycine cleavage system (GCS), are the glycine cleavage T-protein (GcvT, EC 2.1.2.10), the glycine cleavage H-protein (GcvH), the glycine cleavage P-protein (GcvP, EC 1.4.4.2), and the lipoamide dehydrogenase (LpdA, EC 1.8.1.4) (Kikuchi et al., 2008; Plamann et al., 1983) (Steiert et al., 1990b). Interestingly, the GCS is moderately evolutionarily conserved and is present in many bacterial and mammal species (Bogard et al., 2012; Kikuchi et al., 2008; Plamann et al., 1983; Stauffer et al., 1989). This auxiliary system contributes serine-

independent 5'10-mTHF pools and also plays a significant role in regulating glycine concentration in most organisms (Hagemann et al., 2005; Kikuchi et al., 2008; Scott et al., 2008; Stauffer et al., 1989). In fact, defects in the mammalian GCS are associated with hyperglycinemia and can lead to glycine encephalopathy (Kikuchi et al., 2008; Perry et al., 1975). The capacity of this system to restrict glycine concentration is likely facilitated by the fact that most GCS members are upregulated in the presence of excess glycine. (Heil et al., 2002).

In most physiologic settings, the GCS mediates a unidirectional reaction (glycine + THF $\rightarrow$ CO_2 + NH_3 + 5'10-mTHF) (Kikuchi et al., 2008). This reaction revolves around GcvH, a lipoylated protein that serves as an intermediate carrier of the methyl moiety and importantly interacts as a substrate with every protein of the GCS (Fujiwara et al., 1979; Kikuchi et al., 2008). The first step in the GCS involves the decarboxylation of glycine by GcvP (Fujiwara and Motokawa, 1983; Kikuchi et al., 2008). This releases carbon dioxide and subsequently transfers the remaining amino methyl residue to GcvH (Fujiwara and Motokawa, 1983; Kikuchi et al., 2008). This residue is attached to the lipoate of GcvH by the reduction of a disulfide bond (Fujiwara and Motokawa, 1983; Kikuchi et al., 2008). The methylamine carrying GcvH is then acted upon by GcvT (Kikuchi et al., 2008; Okamura-Ikeda et al., 1982). This next step requires THF as a co-factor, leads to the release of ammonia, and finally transfers the methyl group creating 5'10-mTHF (Kikuchi et al., 2008; Okamura-Ikeda et al., 1982). While 5'10-mTHF can be utilized in downstream metabolic reactions, the GcvH remains in an unusable reduced form (Kikuchi et al., 2008). Recycling of GcvH requires oxidization and regeneration of the disulfide bonds in the lipoate, a reaction carried out by LpdA (Kikuchi et al., 2008; Steiert et al., 1990b). It is important to note that, unlike the other members of the GCS, LpdA functions directly in

various other metabolic pathways, including multiple steps in the tricarboxylic acid cycle (Li et al., 2006). Furthermore, while *gcvT*, *gcvP*, and *gcvH* often constitute an operon, *lpdA* is typically found unlinked and elsewhere in the genome (Okamura-Ikeda et al., 1993; Steiert et al., 1990b).

As opposed to SHMT, the GCS does not provide the main source of one-carbon units and is not directly capable of amino acid biosynthesis (Dev and Harvey, 1982; Kikuchi et al., 2008). It is, however, capable of reducing glycine concentration and providing serine-independent 5'10-mTHF (Hagemann et al., 2005; Kikuchi et al., 2008; Scott et al., 2008). Importantly, SHMT-mediated serine biosynthesis from glycine utilizes this 5'10-mTHF source (Ravnikar and Somerville, 1987). Thus, the role of the GCS in bacterial fitness may be limited to nutritional environments with limiting serine or excess glycine. As mentioned previously, many bacteria possess the *serABC* pathway to synthesize serine from glucose and this may provide alternatives to GCS-dependent metabolism (Ravnikar and Somerville, 1987; Umbarger et al., 1963). Based upon this, the contribution of the GCS to virulence may vary between species and would depend on the pathogen's metabolism and specific infection niche. Mutations in *lpdA* have been studied in a wide variety of bacterial pathogens (Hallstrom et al., 2012; Herbert et al., 2003; Meibom et al., 2008; Smith et al., 2002; Venugopal et al., 2011). Although these mutant strains are almost universally attenuated, interpretation of these findings is confounded by the role of LpdA in multiple metabolic pathways (Hallstrom et al., 2012; Herbert et al., 2003; Li et al., 2006; Meibom et al., 2008; Smith et al., 2002; Venugopal et al., 2011). Although *E. coli* strains lacking *lpdA* had undetectable GCS activity, little work has been done to confirm this finding in other bacterial species, some of which possess additional lipoamide dehydrogenase enzymes (Argyrou and Blanchard, 2001; Steiert et al., 1990b). Thus, no direct conclusion can be made regarding the contribution of the GCS from these studies. Loss of GCS function alone has no

apparent effect on serine metabolism of wild-type *E. coli* (Plamann et al., 1983). Interestingly, concurrent loss of the GCS and *serABC* pathways result in a serine auxotrophy, confirming the role of GCS in an alternative SHMT-mediated serine biosynthetic pathway (Ravnikar and Somerville, 1987). Furthermore, GCS deficiency results in increased sensitivity to glycine in several organisms (Hagemann et al., 2005; Scott et al., 2008). In regards to virulence, disruption of the GCS was found to have no role in a murine intestinal infection model in *V. cholera* (Bogard et al., 2012). This study characterized the nutritional environment of the murine gut as serine rich but glycine limiting, a milieu that would not favor GCS function (Bogard et al., 2012). In contrast to *V. cholerae*, *E. ictaluri* requires a functional GCS for pathogenesis in a catfish model of enteric septicemia (Dahal et al., 2013; Karsi et al., 2009). Interestingly, the study did not examine the metabolic contribution of the GCS but did find that mutant strains had significantly increased sensitivity to serum and neutrophils (Karsi et al., 2009). While these phenotypes may explain the *in vivo* attenuation, it remains to be elucidated how GCS activity contributes to serum and neutrophil resistance in this bacterium. The GCS has been identified in *Brucella abortus* as contributing to chronic infection in a murine model (Hong et al., 2000). This study did not examine metabolism, but it is plausible that loss of the GCS may dampen the overall metabolic fitness of *B. abortus* and promote early clearance (Hong et al., 2000). The GCS has also been investigated in the protozoa, *Leishmania major* (Scott et al., 2008). In this pathogen, the GCS contributed to fitness in serine-limiting environments and was found to be attenuated in murine models of infection (Scott et al., 2008). Importantly, *L. major* lacks a functional *serABC* pathway and this reduced serine biosynthetic potential is likely to contribute to the importance of GCS in this organism (Scott et al., 2008). Overall, the GCS is not

universally required for virulence and its contribution appears to be linked to the pathogens metabolic pathways and life cycle.

1.2.4 Role of one-carbon metabolism in *F. tularensis*

To our knowledge, one-carbon metabolism has never been directly studied to assess its contribution to the pathogenesis of *Francisella*. However, there are reasons to hypothesize it has a role in tularemia. *F. tularensis* possesses homologs to all necessary proteins to facilitate acquisition of 5'10-mTHF through the GCS and SHMT (Larsson et al., 2005). As mentioned, these one-carbons are involved in downstream metabolic reactions to synthesize glycine, serine, methionine, purines, pantothenate, formylmethionyl-tRNA and thymidine (Guillon et al., 1992; O'Donovan and Neuhard, 1970; Ravnikar and Somerville, 1987; Stauffer and Brenchley, 1978; Webb et al., 2004; Weissbach and Brot, 1991; Zhang et al., 2008). Based on genetic analysis, *F. tularensis* appears to be capable of using 5'10-mTHF for all of these reactions with the exception of methionine synthesis (Larsson et al., 2005). This bacterium appears to lack several proteins required for this pathway and is likely a methionine auxotroph (Guest et al., 1964a; Larsson et al., 2005). Thus, one-carbon metabolism could be involved with glycine, serine, purine, pantothenate, formylmethionyl-tRNA, and thymidine biosynthesis in *F. tularensis*.

The requirement of one-carbon metabolic products for *Francisella* pathogenesis is uncertain. Although de novo pyrimidine biosynthesis plays only a minor role in a murine model of pneumonic tularemia, pyrimidine salvage pathways are present in the genome (Horzempa et al., 2010a; Larsson et al., 2005). This salvage mechanism could provide an alternative source of the thymidine pre-cursor, uridine monophosphate (Moffatt and Ashihara, 2002; O'Donovan and Neuhard, 1970). The conversion of uridine monophosphate to thymidine monophosphate

requires 5'10-mTHF and thus the pathogenic contribution of thymidine biosynthesis through one-carbon metabolism is not yet known (O'Donovan and Neuhard, 1970; Wahba and Friedkin, 1962). The prerequisite of formylmethionyl-tRNA for the initiation of protein synthesis in *Francisella* has not been investigated and it is not a universal requirement in all bacterial species (Guillon et al., 1992; Newton et al., 1999). Pantothenate biosynthesis does not appear to contribute to the virulence of *F. tularensis* and auxotrophic mutants had no defects in an animal model (Webb et al., 2004). On the other hand, purine biosynthesis appears essential for the intracellular replication and pathogenesis of *F. tularensis* (Pechous et al., 2006; Pechous et al., 2008; Quarry et al., 2007). Two steps in the biosynthesis of purines utilize carbon donations from one-carbon metabolism, although alternative mechanisms that use formate instead of 10-formylTHF exist (Moffatt and Ashihara, 2002; Zhang et al., 2008). *Francisella* is annotated to contain only one of the two required enzymes to circumvent the 10-formylTHF requirement and thus one-carbon metabolism is likely necessary for de novo purine biosynthesis (Larsson et al., 2005; Zhang et al., 2008). Importantly, this has not been experimentally demonstrated and the genes involved in carbon transfer for purine synthesis have not been directly investigated. It remains plausible that *F. tularensis* circumvents a 10-formylTHF requirement for purine production, possibly utilizing one of its many hypothetical genes to fulfill the absent enzymatic activity (Larsson et al., 2005). Thus, experimental evidence is lacking regarding the importance of one-carbon mediated purine, thymidine, and formylmethionyl-tRNA biosynthesis.

The importance of serine and glycine biosynthesis and degradation by one-carbon metabolism in *F. tularensis* is unknown. Although this bacterium lacks an intact threonine biosynthetic pathway, it does possess homologs to a pathway which degrades threonine to glycine (Larsson et al., 2005; Newman et al., 1976). One-carbon metabolism is thus not the only

potential glycine source in *F. tularensis*. Due to the threonine auxotrophy, conditions with limiting threonine would be expected to accentuate SHMT-mediated glycine production. One-carbon metabolism is also not likely the only source of serine for *F. tularensis* subsp. *tularensis*. This is due to the annotated presence of the glycolytic serine biosynthetic genes, *serABC* (Kanehisa and Goto, 2000; Larsson et al., 2005). Since this pathway consumes glucose and the one-carbon metabolic pathway consumes glycine, the primary serine biosynthetic pathway may vary depending on environmental nutrient conditions (Ravnikar and Somerville, 1987; Umbarger et al., 1963). Interestingly, *F. tularensis* subsp. *holarctica* does not possess an intact *serABC* pathway, due to a frame shift mutation in the gene annotated as *serB* (Champion et al., 2009; Kanehisa and Goto, 2000). This suggests that one-carbon metabolism may be the only source of serine in the *holarctica* subspecies but not in the *tularensis* subspecies. It is formally possible, however, that the two fragments of *serB* in *holarctica* remain functionally capable of facilitating serine biosynthesis. Two important caveats exist regarding the *serABC* pathway in *Francisella*. First, SerA and SerC are not always specific for serine biosynthesis and may contribute to pyridoxine (Vitamin B_6) biosynthesis (Lam and Winkler, 1990). Secondly, the annotated *serB* (FTT_0568) in *F. tularensis* subsp. *tularensis* has only limited homology (26% identity over a common 186 amino acid region) to the serine-producing *serB* of *E. coli* (Kanehisa and Goto, 2000; Larsson et al., 2005). This is particularly relevant in light of recent findings that genes annotated as *serB* may serve non-metabolic virulence roles in other bacterial pathogens (Takeuchi et al., 2013). A definitive role for the *serABC* pathway in the metabolism of *F. tularensis* subsp. *tularensis* remains to be confirmed. Based on in silico analysis of *F. tularensis* genomes, one-carbon metabolism appears to be one of two glycine sources for all *F. tularensis*

subspecies, one of two serine sources for *F. tularensis* subsp. *tularensis*, and the only serine source for *F. tularensis* subsp. *holarctica*.

Although the production of 5'10-mTHF by the GCS and SHMT has never been thoroughly investigated in *F. tularensis*, indirect evidence suggests these pathways may contribute to pathogenesis. Global transcriptional profiling on *F.* tularensis during intramacrophage growth revealed that the GCS was induced within the host cytosolic environment (Wehrly et al., 2009). Furthermore, the GCS of *F. tularensis* is induced by mammalian body temperature and is also regulated by the host specific polyamine, spermine (Carlson et al., 2009; Horzempa et al., 2008). This pattern of transcriptional regulation in response to host conditions suggests the GCS may beneficially contribute to intracellular replication. Interestingly, multiple transcriptional regulators have been described to control expression of the GCS and SHMT but clear homologs to these appear to be absent in *F. tularensis* (Heil et al., 2002; Landgraf et al., 1994; Larsson et al., 2005; Newman and Lin, 1995; Steiert et al., 1990a; Weissbach and Brot, 1991). The regulatory mechanism behind the expression of these proteins may thus be distinct from *E. coli* and this could facilitate the response to specific host cues. In support of these transcriptional profiling studies, induction of the GCS at the protein level was found to occur in *Francisella* isolated from mouse spleens (Twine et al., 2006). A role in pathogenesis was further suggested by the identification of multiple members of the GCS in an in vivo negative selection screen in *F. tularensis* subsp. *novicida* (Weiss et al., 2007). Of note, mutagenesis of *lpdA* in *F. tularensis* subsp. *holarctica* results in *in vivo* attenuation (Meibom et al., 2008). As mentioned, *lpdA* is not only required for GCS activity in *E. coli* but also functions broadly in other critical metabolic pathways (Li et al., 2006; Steiert et al., 1990b). This study did not investigate any specific metabolic defects in

strains lacking *lpdA* and did not include genetic complementation (Meibom et al., 2008). Thus, the GCS of *F. tularensis* is regulated by host environmental cues and has been indirectly implicated as contributing to pathogenesis.

In contrast to the GCS, the SHMT of *F. tularensis* does not appear to be regulated in response to host environmental signals that have been tested (Carlson et al., 2009; Horzempa et al., 2008; Wehrly et al., 2009). To our knowledge, the *glyA* homolog in *F. tularensis* has not been identified in any transcriptional profiling studies and is rarely found in virulence screens (Tempel et al., 2006). Interestingly, *glyA* has been labeled as an essential gene in *F. tularensis* subsp. *novicida* as no disruptions of this locus were identified following genome-saturating transposon mutagenesis (Gallagher et al., 2007). In contrast to this, another study identified a viable *glyA* transposon mutant in *F. tularensis* subsp. *novicida* while searching for mutants defective in intracellular growth (Tempel et al., 2006). The later study also performed an in vivo screen using various strains, including the *glyA* transposon mutant (Tempel et al., 2006). Data from this single-iteration screen revealed that two of three animals infected with this mutant survived a lethal dose (Tempel et al., 2006). This study did not attempt to assess this phenotype by statistical analysis, nor did they seek to genetically complement this gene (Tempel et al., 2006). In fact, the *glyA* mutant was excluded from further *in vivo* characterization due to the death of one animal, as it wasn't fully attenuated (Tempel et al., 2006). It remains unclear why this gene was identified as essential but a significant *in vitro* growth defect may have given rise to a false positive (Gallagher et al., 2007). Ultimately, the lack of genetic complementation, metabolic analysis, statistics, and thorough in vivo characterization leaves significant questions remaining regarding the role of *glyA* in the pathogenesis of *F. tularensis*.

1.3 STATEMENT OF THE PROBLEM

The contribution of a metabolic pathway to bacterial pathogenesis is dependent on the pathogen's niche, life cycle, and genetics (Eisenreich et al., 2010; Rohmer et al., 2011). The host-pathogen metabolic interaction is of great interest to anti-bacterial drug development, although specific targets must be identified, validated, and studied thoroughly (Zhang and Rubin, 2013). One-carbon metabolic pathways are conserved among bacterial species, including many pathogens, indicating that inhibition of these pathways may have broad therapeutic potential (de Crecy-Lagard et al., 2007; Tibbetts and Appling, 2010). Surprisingly, the contribution of bacterial one-carbon metabolism to pathogenesis is poorly described as little work has been performed to characterize this system during infection. Ultimately, this represents an unacceptable gap in current knowledge and a barrier to the development of novel antibiotics. To address this problem, the importance of one-carbon metabolism to the pathogenesis of *Francisella tularensis* has been investigated in this thesis. *F. tularensis* is an excellent model organism for this study because it is capable of infecting and replicating in a large repertoire of distinct host niches in a murine model of tularemia (Forestal et al., 2007; Hall et al., 2008; Horzempa et al., 2010a). The work detailed in this thesis has identified one-carbon metabolism as the exclusive serine biosynthetic pathway of *F. tularensis*. It has identified a niche-specific contribution of this pathway during infection, specifically a previously unrecognized role in bacteremia. Overall these studies have demonstrated that one-carbon metabolism contributes to the pathogenesis of *F. tularensis* and may represent a useful target for therapeutic intervention.

2.0 THE CONTRIBUTION OF THE GLYCINE CLEAVAGE SYSTEM TO THE PATHOGENESIS OF *FRANCISELLA TULARENSIS*

2.1 ABSTRACT

Biosynthesis and acquisition of nutrients during infection are integral to pathogenesis. Members of a metabolic pathway, the glycine cleavage system, have been identified in virulence screens of the intracellular bacterium *Francisella tularensis* but their role in pathogenesis remains unknown. This system generates 5,10-methylenetetrahydrofolate, a precursor of amino acid and DNA synthesis, from glycine degradation. To characterize this pathway, deletion of the *gcvT* homolog, an essential member of this system, was performed in attenuated and virulent *F. tularensis* strains. Deletion mutants were auxotrophic for serine but behaved similar to wild-type strains in vitro with respect to invasion, intracellular replication, and stimulation of TNF-α. Unexpectedly, the glycine cleavage system was required for the pathogenesis of virulent *F. tularensis* in vivo. Deletion of the *gcvT* homolog delayed mortality and lowered bacterial burden, particularly in the liver and bloodstream. To reconcile the divergent in vitro and in vivo phenotypes, minimal tissue culture media was employed to mimic the nutritionally limiting environment of the host. This reevaluation demonstrated that the glycine cleavage system contributes to the intracellular replication of virulent *F. tularensis* in serine limiting

environments. Thus, the glycine cleavage system is the serine biosynthetic pathway of *F. tularensis* and contributes to pathogenesis in vivo.

2.2 INTRODUCTION

Francisella tularensis is an intracellular bacterium and a formidable pathogen. It is highly infectious, requiring inhalation of only 10 to 50 bacteria to cause a febrile illness known as tularemia (Saslaw et al., 1961a). The pulmonary manifestation of the disease is fatal in up to 60% of cases without medical intervention (McCrumb, 1961). Due to these properties, there is significant concern for intentional aerosolized release and misuse of this agent in the form of bioterrorism (Oyston et al., 2004). As such, *F. tularensis* is categorized by the Centers for Disease Control and Prevention as a tier one select agent (Centers for Disease Control and Prevention (CDC), 2012).

Often referred to as a "stealth pathogen", *F. tularensis* is capable of both suppressing and avoiding the host immune response (Sjostedt, 2006). Infection with *Francisella* evokes little to no proinflammatory response in vitro and a delayed proinflammatory response in vivo (Bosio and Dow, 2005). While eluding detection, this bacterium has a complex intracellular life cycle involving invasion, phagosomal escape, cytosolic replication, and egress (Chong and Celli, 2010). Significant questions remain regarding the host pathogen interaction throughout its life cycle, but it is clear that *Francisella* is well suited for its intracellular niche. In support of this, *F. tularensis* is capable of successful infection and replication in an extensive repertoire of host cells. This repertoire ranges from immune cells such as dendritic cells, neutrophils, and macrophages to non-immune cells such as hepatocytes and type II pneumocytes (Conlan and

North, 1992; Hall et al., 2008). Thus, *Francisella* is capable of circumventing host defense systems and gaining access to the cytosolic environment.

Organisms must acquire or synthesize various metabolites in order to survive and replicate. For pathogens, metabolites and metabolic precursors must be derived from the host. *Francisella* infects a wide range of host sites including the lung, liver, spleen, and blood (Conlan et al., 2003). The bacterium must therefore be metabolically competent for these nutritionally diverse environments. In support of this, tryptophan biosynthesis in *F. tularensis* has been found to be essential in counteracting lung specific inducible tryptophan starvation involving host production of indoleamine 2,3-dioxygenase (Peng and Monack, 2010). Furthermore, the extracellular phase of this bacterium relies on a potassium uptake protein known as TrkH to grow in the potassium-limiting environment of the host's blood (Alkhuder et al., 2010). Cell type specific nutritional requirements have also been discovered as pyrimidine biosynthesis is required for replication in macrophages but not in epithelial cells (Horzempa et al., 2010a). In contrast, purine biosynthesis is important to *Francisella* intracellular replication across cell types and loss of this pathway results in a dramatic attenuation in vivo (Pechous et al., 2006). Thus, investigation into pathogen metabolism during infection has revealed important pathways contributing to *F. tularensis* pathogenesis. Broadly, these results have also added to a growing understanding of the microenvironments in host tissues and the biosynthetic and nutrient acquisition pathways that are critical for pathogens to colonize these niches.

Despite recent advances, a significant number of metabolic pathways remain uncharacterized in *F. tularensis* and their contribution to pathogenesis is unknown (Meibom and Charbit, 2010). One particular unstudied pathway, the glycine cleavage system (GCS), has a variety of noteworthy properties. This system facilitates the degradation of glycine to acquire

5,10-methylene-tetrahydrofolate, a one-carbon donor utilized in the production of serine, thymidine, and purines (Kikuchi, 1973). Therefore, this pathway is expected to contribute to pathogen fitness in host compartments where these metabolites, such as serine, are limiting. The homologs of the GCS are transcriptionally upregulated in *F. tularensis* during infection of macrophages (Wehrly et al., 2009). A member of this system, the homolog of *gcvH*, was found to be strongly induced at the protein level in *Francisella* isolated from mouse spleens (Twine et al., 2006). Furthermore, this system was identified in an in vivo negative selection screen in the related bacterium, *Francisella novicida* (Weiss et al., 2007). These data suggest that the glycine cleavage system may play an important role in the metabolic fitness of *Francisella tularensis*.

In this report, we evaluated the contribution of the glycine cleavage system to the pathogenesis of *F. tularensis.* To investigate this pathway, we deleted the *F. tularensis* homolog of a required member of this system, the glycine cleavage protein T (*gcvT*). Following mutagenesis, strains were assayed for metabolic defects, in vitro virulence phenotypes, and in vivo pathogenesis. Our results demonstrate that the homolog of the glycine cleavage system is functional, essential when serine is limited, and ultimately required for the full in vivo pathogenesis of virulent *F. tularensis.*

2.3 MATERIALS AND METHODS

2.3.1 Bacterial strains

The following reagent was obtained through BEI Resources, NIAID, NIH: *Francisella tularensis* subsp. *tularensis,* Strain SCHU S4 (FSC237), NR-643. *Francisella tularensis* subsp.

holarctica Live Vaccine Strain (LVS) was provided as a gift from Dr. Karen Elkins (U.S. Food and Drug Administration). Routine culture of *Francisella* was performed by streaking frozen bacterial stocks onto chocolate agar (GC medium base, hemoglobin, and isovitaleX). Complement and vector strains were streaked onto chocolate agar supplemented with hygromycin (200μg/mL). Bacteria were grown on plates for three days at 37 °C with 5% CO_2 and subsequently used to inoculate overnight cultures. Unless otherwise specified, overnight cultures were performed in trypticase soy broth supplemented with cysteine (TSB-C) and were shaken at 250 rpm at 37 °C. All work with Schu S4 strains was performed in BSL3 containment with approval from the Centers for Disease Control and Prevention Select Agent Program. Cloning was performed using the *E. coli* EC100D strain.

2.3.2 Generation of deletion mutants, complements, and vector controls

Deletion of the *gcvT* homolog in LVS (FTL_0477) and Schu S4 (FTT_0407) was performed by allelic replacement as described previously (Horzempa et al., 2010b). The flanks of *gcvT* share 99% identity between LVS and Schu S4, therefore we utilized a suicide vector (pJH1) containing the regions upstream and downstream of *gcvT* in LVS for deletions in both strains. The upstream flank (1,053 bp) was generated using CATGGGATCCCCGATAGTTGCTAGCGTGG as a forward primer and GATCGGTACCTGCTATATGTGATTCATAAAGAGG as a reverse primer. The downstream flank (1,057 bp) was generated using CTAGGGTACCGTCGAGCTAGTTAAACCTAAG as a forward primer and CTAGGCATGCCTCTCAAATAAGTTGGGTGTAAAGC as a reverse primer. This mutational strategy is expected to remove nucleotides (38-1026) of the *gcvT* locus, while leaving behind 37 nucleotides at the 5' end and 51 nucleotides at the 3' end of this gene.

The remaining nucleotides are expected to remain in-frame as one of the homologous nucleotides of the 5' end is part of the 6 bp restriction site linker connecting the two flanks. Loss of *gcvT* was confirmed by genomic PCR using CTAGGGATCCGCTACCAACTTTATATGCGGAAGATCC as a forward primer and CTAGGGTACCTGACCCACTCATGCGACTTTGTATAC as a reverse primer (Appendix 3). These primers are 523 bp upstream and 108 bp downstream of *gcvT*, respectively, and are expected to produce products of either 1,708 bp (wild-type locus) or 719 bp (Δ*gcvT*). Strains with deletions of the *gcvT* homolog are annotated as LVS Δ*gcvT* and Schu S4 Δ*gcvT*.

Complementation of *gcvT* was performed in cis using a modified pJH1 suicide plasmid (pMB1). pMB1 was generated by cutting pJH1 with XhoI to remove extraneous yeast genes and subsequent religation. The LVS *gcvT* (FTL_0477) was amplified by PCR with 249 bp of upstream region and 108 bp of downstream region and ligated into pGEM-T, generating pGEM*gcvT*. This was performed using GATCGGATCCCCTGGAGAGAAGATAACCGAAGAATC as a forward primer and CTAGGCATGCTGACCCACTCATGCGACTTTGTATAC as a reverse primer. Digestion of pGEM*gcvT* with BamHI and SphI (restrictions sites present in the forward and reverse primers) allowed for subcloning of this sequence into pMB1 to create pMB1*gcvT*. This procedure was repeated with a PCR amplicon containing only the 249 bp upstream region to serve as a pMB1 vector control. Generation of this amplicon was achieved by using GATCGGATCCCCTGGAGAGAAGATAACCGAAGAATC as a forward primer and CTAGGCATGCGAACTATCCACCTAAAAAATTATGCTCG as a reverse primer. The 249 bp upstream region was expected to contain the *gcvT* promoter and also served as the homologous sequence for chromosomal integration of both complement and vector control. The

suicide vectors were transferred by tri-parental mating into LVS Δ*gcvT* and Schu S4 Δ*gcvT* as described previously (Horzempa et al., 2010b). This procedure was used to generate the complementing strains (LVS Δ*gcvT*:pMB1*gcvT* and Schu S4 Δ*gcvT*:pMB1*gcvT*) and the vector control strains (LVS Δ*gcvT*:pMB1 and Schu S4 Δ*gcvT*:pMB1).

2.3.3 Growth kinetics in broth culture

LVS and Schu S4 strains were cultured overnight, pelleted, resuspended in PBS, and diluted to an OD_{600} of 0.1. Chamberlain's chemically defined media (CDM) was modified as stated in each figure and overall prepared as previously described (Chamberlain, 1965). Cultures were shaken at 37 °C for 28 hours with serial measurements of the optical density at 600 nm (OD_{600}) taken every two to four hours. OD_{600} readings were performed in a cuvette (1 cm path length) with a CO8000 Cell Density Meter (WPA) for Schu S4 strains and in a 96-well plate using an M2 plate reader (Molecular Devices) for LVS strains.

2.3.4 Generation of primary macrophages and propagation of A549 cells

Murine macrophages were derived from the bone marrow of C57BL/6J mice as described previously (Russo et al., 2011). Macrophages were propagated in Dulbecco's Modified Eagles Medium (DMEM) with 20% FBS, 25 mM HEPES, 2 mM glutaMAX, 1 mM sodium pyruvate, 1 X MEM non-essential amino acids, and 25% L-cell supernatant. L-cell supernatant was derived from L929 cells as previously described (Russo et al., 2011).

Human macrophages were differentiated from monocytes isolated from human peripheral blood mononuclear cells as previously described (Carlson et al., 2007). Briefly, buffy coats were

purchased from the New York Blood Bank. Mononuclear cells were enriched from the buffy coat using a Ficoll gradient and monocytes were isolated using an Optiprep gradient and subsequent panning for adherent cells. Following isolation, monocytes were differentiated for seven days in DMEM supplemented with 20% FBS, 10% AB human serum (Complement-Replete Gem Cell; Gemini Bio-Products), 2 mM glutaMAX, and 25 mM HEPES. After this differentiation, macrophages were used within three days.

A549 (human lung carcinoma) cells were obtained from ATCC (ATCC CCL-185) and handled per ATCC recommendations. Cells were propagated in Ham's F-12 (Kaighn's modification) with 10% FBS and 25 mM HEPES.

2.3.5 Intracellular growth assays (standard)

Cells were harvested using lidocaine (4 mg/ml)/EDTA (5 mM) in PBS for macrophages or 0.25% trypsin/1 X EDTA (Gibco) for A549 cells. Cells were transferred from culture dishes to 96-well Primaria plates (BD biosciences) at a density of $5x10^4$ cells per well. Human macrophages were placed in infection media (1% AB human serum, DMEM, 2 mM glutaMAX, and 25 mM HEPES), while murine macrophages and A549 cells were placed in their normal growth medium described above. Cells were allowed to rest at 37 °C with 5% CO_2 overnight following harvest. Bacteria from overnight cultures were pelleted, resuspended in PBS, and added to cells at an multiplicity of infection (MOI) of 500. Bacteria and cells were co-cultured for two hours to allow the bacteria to invade, after which the media was removed and replaced with HBSS containing 50 μg of gentamicin per ml. Cells were incubated with gentamicin at 37 °C with 5% CO_2 for 30 minutes to kill extracellular bacteria. Cells were then washed twice with HBSS and placed back in infection/growth media as per cell type. Two hour time points were

harvested immediately following the gentamicin treatment, while 24 hour time points were harvested after an additional 22 hour incubation at 37 °C with 5% CO_2. To enumerate CFU at each time point, cells from triplicate wells were lysed with 0.02% SDS in PBS and serially diluted in TSB-C as performed previously (Russo et al., 2011). Dilutions were plated onto chocolate agar and CFU counted after three days of growth at 37 °C with 5% CO_2.

2.3.6 Intracellular growth assays (minimal media)

To assess the contribution of media to the intracellular replication without affecting invasion, the following alterations were made to the above protocol. Murine macrophages were harvested, infected, and underwent a gentamicin treatment as stated above. After the two hour time point, macrophages were placed in Minimal Essential Media (MEM, Gibco) with 10% FBS, 2 mM glutaMAX, and 25 mM HEPES. In some conditions MEM was further supplemented with 25 mM serine. At 24 hours post infection, cells were lysed and CFU enumerated from triplicate wells using the same method as above.

2.3.7 Detection of TNF-α

Bacteria and cells were prepared similarly to the intracellular growth assay. Cells were co-cultured with bacteria at an MOI of 10 in triplicate wells for 24 hours at 37 °C with 5% CO_2. These supernatants were collected and assessed by ELISA to determine TNF-α concentration. Human TNF-α was measured using DuoSets (R&D Systems) and murine TNF-α was measured using a matched antibody pair (eBiosciences).

2.3.8 Mouse model of pneumonic tularemia

To model pneumonic tularemia, 6-8 week old C57BL/6J mice (Jackson Laboratory) were intratracheally infected as described previously (Russo et al., 2011). Bacteria were grown overnight in TSB-C broth cultures, washed with PBS, and diluted as necessary. An oropharyngeal installation of 100 CFU was used for all strains. Over the course of disease, mice were scored using a sickness rubric and were euthanized upon achieving a predetermined score. All animal experiments were performed in ABSL-3 conditions with approval of the University of Pittsburgh Institutional Animal Care and Use Committee.

To assess bacterial burden, mice were anesthetized with ketamine (80 mg/Kg) and xylazine (8 mg/Kg) four days post infection. Blood was collected by cardiac puncture using a heparinized needle and syringe, diluted, and plated to enumerate CFU. Organs were harvested, mechanically homogenized, diluted, and plated for quantification of CFU. This procedure was described previously (Horzempa et al., 2010a).

2.4 RESULTS

2.4.1 The glycine cleavage system of *F. tularensis* is required for growth in serine limiting conditions

Previous work in *E. coli* demonstrated that the glycine cleavage system (GCS) functions in glycine-dependent serine production, a phenotype only apparent when the serine biosynthetic pathway (*serABC*) is disrupted (Ravnikar and Somerville, 1987). The *serB* gene of LVS

contains a frame shift mutation, fragmenting this gene with a stop codon. Thus, the GCS may be essential for serine biosynthesis in LVS. In order to assess the contribution of the GCS to the metabolism and pathogenesis of *F. tularensis*, the homolog of *gcvT*, a gene required for GCS activity, was deleted in the attenuated live vaccine strain (LVS, FTL_0477) and in the human pathogenic strain (Schu S4, FTT_0407). To examine the serine requirement of these strains, Chamberlain's chemically defined media (CDM) was utilized (Chamberlain, 1965). This media normally contains 3.8 mM serine and 0 mM glycine. Removal of serine from CDM limited replication of wild-type LVS (Fig. 1A). The inclusion of 25 mM glycine in serine-free CDM, however, compensated for the loss of serine and resulted in robust bacterial growth (Fig. 1B). A functional *gcvT* homolog was required for LVS growth in serine-free CDM, whether or not glycine was provided (Fig. 1A and B). LVS exhibited a diauxic growth curve in CDM (Fig. 1C), consistent with previous observations (LoVullo et al., 2012). However, LVS failed to exhibit typical diauxie in serine-free CDM supplemented with glycine (Fig. 1B). Furthermore, strains lacking *gcvT* did not exhibit diauxic growth and stopped replicating at the first plateau, demonstrating that the second phase of the diauxic growth curve required an intact GCS (Fig. 1C). Supplementing CDM with additional serine resulted in a protracted initial exponential phase in all strains, but a functional *gcvT* was still required for growth past the first plateau (Fig. 1D). These phenotypes are attributable to the presence of the *gcvT* homolog as they were restored by complementation (Fig. 1A-D). These data suggest that the diauxie of LVS is related to the source of serine and the initial growth of LVS uses exogenous serine while the second growth phase breaks down glycine to produce serine in a GCS-dependent fashion. Overall, these data demonstrate that LVS requires an intact GCS for growth in serine limiting environments.

Serine metabolism was predicted to differ in the virulent *F. tularensis* strain Schu S4. Unlike LVS, Schu S4 contains undisrupted homologs to an additional serine biosynthetic pathway (*serABC*), which prevents serine auxotrophy in the absence of GCS in *E. coli* (Kanehisa and Goto, 2000; Ravnikar and Somerville, 1987). Therefore, we expected loss of the *gcvT* homolog (FTT_0407) to have little effect on the ability of Schu S4 to grow in CDM lacking serine. Surprisingly, Schu S4 Δ*gcvT* was also a serine auxotroph and failed to grow in conditions without (Fig. 1E) and with (Fig. 1F) glycine supplementation. Schu S4 Δ*gcvT* also grew less than Schu S4 with a lower OD in stationary phase in CDM (Fig. 1G). Serine supplementation abolished differences between strains, confirming the higher serine requirement of the mutant (Fig. 1H). These mutant phenotypes are attributable to the absence of the *gcvT* homolog as they were restored by genetic complementation (Fig. 1E-H). Therefore, the GCS of Schu S4 is required for growth in serine limiting environments despite the presence of another pathway annotated for serine biosynthesis in its genome.

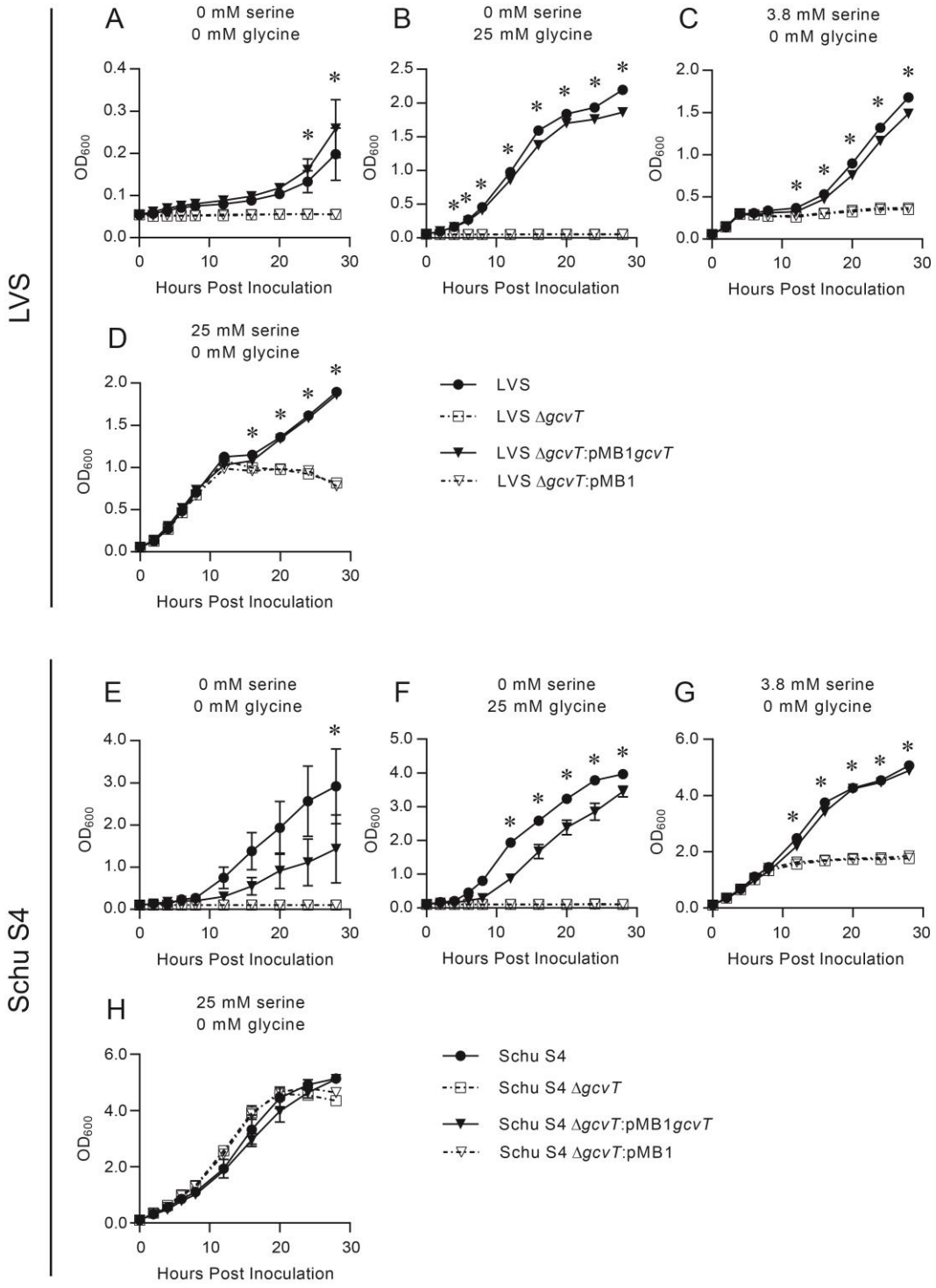

Figure 3. The glycine cleavage system of *F. tularensis* is required for glycine utilization and growth during serine limitation.

Overnight broth cultures of LVS (A-D) and Schu S4 strains (E-H) were pelleted, resuspended in PBS, and used to inoculate Chamberlain's chemically defined media (CDM) containing 0 mM serine / 0 mM glycine (A and E), 0

mM serine / 25 mM glycine (B and F), 3.8 mM serine / 0 mM glycine (C and G), or 25 mM serine / 0 mM glycine (D and H). The optical density at 600 nm (OD_{600}) of each culture was measured every two to four hours. Data are expressed as the mean ± SEM of three independent experiments except for the four hour time point of Schu S4 strains (E-H), which is from two independent experiments that were excluded from statistical analysis. ∗ denotes time points that two comparisons (wild-type vs. Δ*gcvt*, Δ*gcvT*:pMB1*gcvT* vs. Δ*gcvT*:pMB1) both achieved significance. Significance was considered to be a ($p < 0.05$) using a two-way repeated measure ANOVA followed by a Bonferroni multiple comparison correction.

2.4.2 *gcvT* homologs do not contribute to in vitro virulence phenotypes in standard tissue culture assays

Our results indicated that the *Francisella* GCS conveys a fitness advantage in specific metabolic environments (Fig. 1). To determine if this advantage would be relevant to pathogenesis, we examined the contribution of this system to several in vitro virulence-associated phenotypes. Assays with primary murine and human macrophages revealed no obvious defects in invasion or intracellular replication in LVS strains (Fig. 2A and B). Co-culture of LVS strains and either human or mouse macrophages also revealed no differences in TNF-α release (Appendix A). The loss of a *gcvT* homolog during LVS infection of non-immune cells also had no effect, as invasion and replication rates among strains were indistinguishable in A549 cells, a human lung epithelial cell line (Fig. 2C). Additionally, the GCS was not required for complement resistance in LVS, despite previous links between this system and serum sensitivity (Appendix B) (Karsi et al., 2009). Overall findings in Schu S4 were similar to LVS with no differences detected in invasion, replication, or stimulation of TNF-α production (Fig. 2D-F and Appendix A). These results demonstrate that the GCS is not required for invasion or replication and does not affect immune stimulation in standard tissue culture assays.

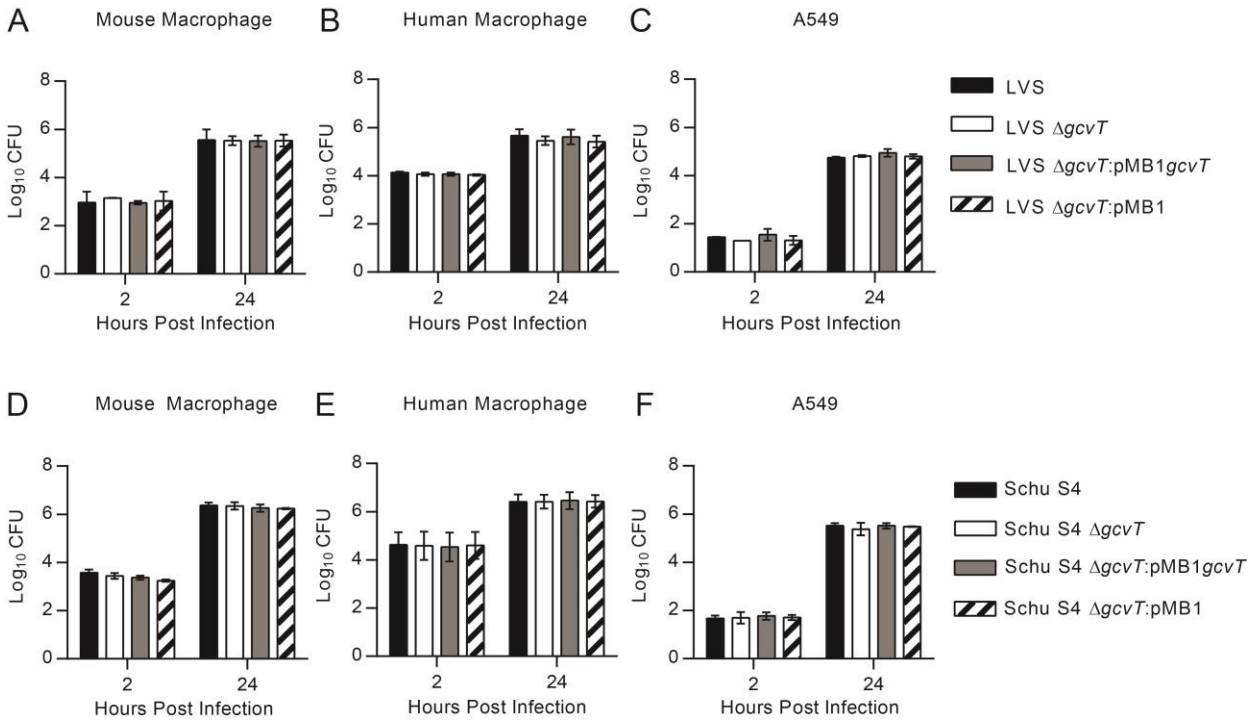

Figure 4. The glycine cleavage system does not contribute to in vitro intracellular replication using standard assay conditions.

Overnight TSB-C broth cultures of LVS (A-C) and Schu S4 strains (D-F) were pelleted, resuspended in PBS, and added to mouse macrophages (A,D), human macrophages (B,E), or A549 cells (C,F) at a MOI of 500. Bacteria were incubated with cells for two hours and subsequently subjected to gentamicin treatment as described in the Materials and Methods. At the indicated time points, cells were lysed and CFU enumerated. Data are expressed as the mean ± SEM of at least two independent experiments. Significant difference ($p < 0.05$) was not detected with any cell type or time point by one-way ANOVA followed by a Bonferroni multiple comparison correction.

2.4.3 *gcvT* contributes to the pathogenesis of virulent *Francisella tularensis* in a murine model

Thus far, our results indicated that the GCS conveyed a fitness advantage in serine limiting broth but not in macrophages or lung epithelial cells (Fig. 1 and Fig. 2). After infection of the lung, *Francisella* disseminates and replicates in a variety of physiologically diverse sites,

including the spleen, liver, and blood (Conlan et al., 2003; Horzempa et al., 2010a). Therefore, we hypothesized that the GCS of *F. tularensis* may provide a fitness advantage in specific host microenviroments, similar to the blood-specific role of the potassium uptake protein, TrkH (Alkhuder et al., 2010). As in vitro findings were similar between LVS and SchuS4 strains, we focused on the fully virulent *F. tularensis* strains in vivo using a murine model of pneumonic tularemia. Mice infected with Schu S4 and the *gcvT* complement (Schu S4 Δ*gcvT*:pMB1*gcvT*) had a median survival of 5.1 and 5.7 days, respectively (Fig. 3). In contrast, infection with Schu S4 Δ*gcvT* and the vector control (Schu S4 Δ*gcvT*:pMB1) led to a median survival of 8.7 and 7.9 days, respectively (Fig. 3). Thus, the GCS system contributes to the virulence of *F. tularensis* in vivo.

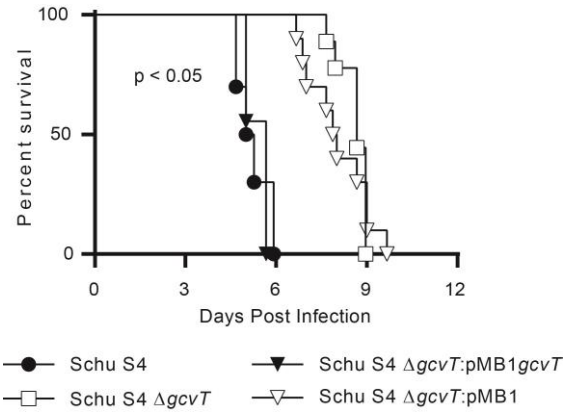

Figure 5. The glycine cleavage system contributes to the pathogenesis of virulent *F. tularensis* in a mouse model of pneumonic tularemia.

Overnight TSB-C broth cultures of Schu S4 strains were pelleted, resuspended in PBS, and intratracheally administered to anesthetized mice at 100 CFU. Mice were monitored over the course of infection and removed upon reaching a predetermined clinical sickness score. Data are a combination of four experiments, two independent experiments compared Schu S4 to Schu S4 Δ*gcvT* and two independent experiments compared Schu S4 Δ*gcvT*:pMB1*gcvT* to Schu S4 Δ*gcvT*:pMB1. Each strain had at least four mice per experiment and at least nine mice total. Statistical significance ($p < 0.05$) was assayed by a log-rank test (Mantel-Cox) and was achieved for Schu S4 vs. Schu S4 Δ*gcvT* and for Schu S4 Δ*gcvT*:pMB1*gcvT* to Schu S4 Δ*gcvT*:pMB1.

2.4.4 *gcvT* increases bacterial burden at sites of dissemination

Since the loss of *gcvT* significantly delayed mortality (Fig. 3), we hypothesized it may also alter bacterial burden. To evaluate this possibility, organs and blood were harvested four days post infection and bacterial burden was assessed. In the lungs of animals infected with Schu S4 or Schu S4 Δ*gcvT*, bacterial burdens were within one log (Fig. 4A). No significant difference was detected between the complement (Schu S4 Δ*gcvT*:pMB1*gcvT*) and vector (Schu S4 Δ*gcvT*:pMB1) at this site. In contrast, other organs had substantially fewer Schu S4 Δ*gcvT* than Schu S4: approximately 1.5 logs less in the spleen (Fig. 4B), 2.4 logs less in the liver (Fig. 4C), and 4.2 logs less in the blood (Fig. 4D). Complementation of *gcvT* significantly increased the CFU at these distal sites (Fig. 4B-D). Therefore, the *F. tularensis* glycine cleavage system contributes to bacterial burden during infection in vivo and this is particularly pronounced at sites of dissemination such as the liver and blood.

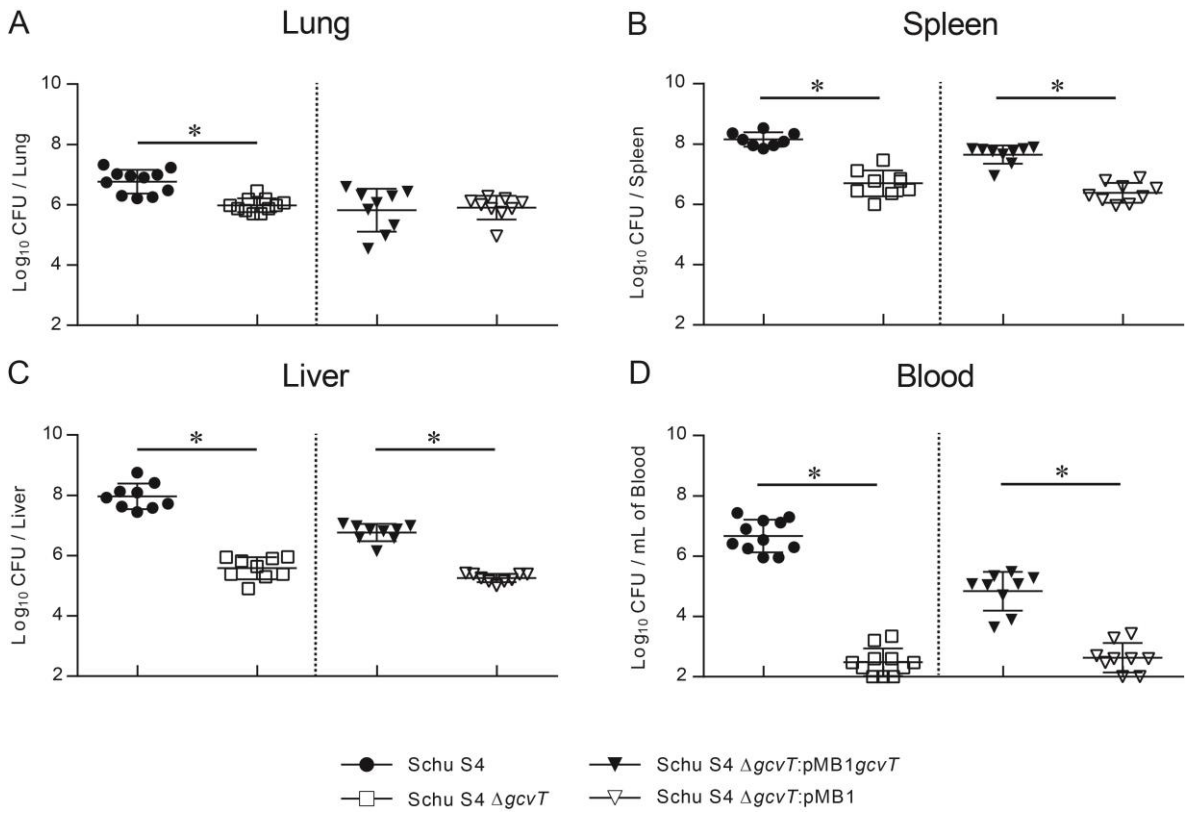

Figure 6. Schu S4 strains lacking the *gcvT* homolog have lower bacterial burdens at distal sites.

Overnight TSB-C broth cultures of Schu S4 strains were pelleted, resuspended in PBS, and intratracheally administered to anesthetized mice at 100 CFU. Four days post infection, the lung (A), spleen (B), liver (C) and blood (D) were harvested, homogenized, and plated to enumerate CFU. Data in each panel are derived from four experiments: two independent experiments compared Schu S4 to Schu S4 Δ*gcvT* and two independent experiments compared Schu S4 Δ*gcvT*:pMB1*gcvT* to Schu S4 Δ*gcvT*:pMB1. These groups of experiments are separated by a dotted line. Each symbol denotes the CFU from a single mouse. Each strain had at least four mice per experiment and at least eight mice total. A ∗ denotes statistical significance ($p < 0.05$) by two-sided t-test. The limit of detection was 50 CFU for the lung, 100 CFU for the spleen and blood, and 200 CFU for the liver.

2.4.5 *gcvT* contributes to intracellular replication in vitro in serine limiting conditions

Thus far, there is a discrepancy in which the GCS is required for the full virulence of Schu S4 in vivo, but it appears to be dispensable for the in vitro model of virulence. Importantly,

experimentation with CDM highlighted the importance of the GCS in serine limiting conditions (Fig. 1). In vitro assays will be poor indicators of in vivo attenuation for metabolic pathways if the metabolite of interest is supraphysiological. Therefore, we hypothesized that a nutritionally minimal tissue culture media, in which serine was not abundant, would unmask in vitro attenuation. Intracellular growth assays performed using a minimal media containing lower levels of serine revealed a clear intracellular growth defect in the Schu S4 strains lacking a functional *gcvT* homolog (Fig. 5). This defect was specific for intracellular replication as there was no difference in stimulation of TNF-α using minimal media (Appendix A). Intracellular growth in minimal media was restored upon complementation (Fig. 5). Furthermore, the intracellular growth defect in the Schu S4 mutant strains could be ablated by supplementation with 25 mM serine, similar to what was found in CDM (Fig. 5 and Fig. 1H). Thus, *gcvT* is required for robust intracellular replication in serine limiting tissue culture media, reconciling the in vitro and in vivo phenotypes of the glycine cleavage system in virulent *F. tularensis*.

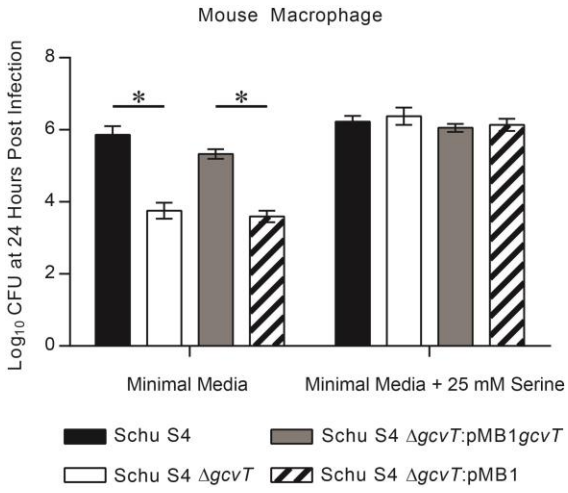

Figure 7. A functional glycine cleavage system is required for robust intracellular replication using minimal media conditions.

Overnight TSB-C broth cultures of Schu S4 strains were pelleted, resuspended in PBS, and added to mouse macrophages at an MOI of 500. Bacteria were cocultured with cells for two hours and subsequently subjected to a

gentamicin protection assay. Following the gentamicin treatment, minimal media or minimal media with 25 mM serine was added instead of standard tissue culture media. Strains were already confirmed in Fig. 2D to not have any differences in invasion (two hour time point) under the same conditions used in this assay. Cells were lysed 24 hours post infection and CFU was enumerated. Data are expressed as the mean ± SEM of three independent experiments. Significant difference ($p < 0.05$) are denoted by a ∗ and were assessed using one-way ANOVA followed by a Bonferroni multiple comparison correction.

2.5 DISCUSSION

Pathogens employ various mechanisms to circumvent immunity and access host metabolites. All host sites are not nutritionally equivalent; therefore pathogens encounter unique metabolic niches (Rohmer et al., 2011). These niches will be colonized and exploited by pathogens with the required metabolic pathways. In this report, we focused on a *Francisella tularensis gcvT* homolog that showed phenotypes strongly associated with the glycine cleavage system (GCS). The importance of this pathway has been suggested in screens for virulence-associated phenotypes in *Francisella* (Twine et al., 2006; Wehrly et al., 2009; Weiss et al., 2007). *Francisella* presents a unique opportunity to study this system in an acute bacterial setting because it does not have redundant serine biosynthetic pathways. Since *F. tularensis* infection results in widespread dissemination of bacteria, it also allowed examination of the role of glycine cleavage system throughout the host (Conlan et al., 2003). Our results demonstrate that the *F. tularensis* GCS is essential during serine limitation and contributes to in vivo pathogenesis.

This is the first report, to our knowledge, to demonstrate a role for the bacterial GCS in a mammalian model of acute infection (Fig. 3 and Fig. 4). This work joins a growing body of

research elucidating the role of this metabolic pathway during pathogenesis. The GCS has been previously correlated with successful persistence during chronic bacterial infection with *Brucella abortus* and has been implicated in the transition to latency in *Mycobacterium tuberculosis* (Hong et al., 2000; Wayne and Lin, 1982). Previous research demonstrated that the *Vibrio cholerae* GCS does not contribute to acute colonization of infant mouse intestines (Bogard et al., 2012). This finding is likely explained by the presence of an additional serine biosynthetic pathway, but may also indicate that the gut is not a serine-limiting environment. Surprisingly, our findings with *F. tularensis* are similar to in vitro and in vivo phenotypes in the trypanosomatid, *Leishmania major* (Scott et al., 2008). Despite differences between prokaryotes and eukaryotes, these human pathogens both lack alternative serine biosynthetic pathways and thus appear to rely on the GCS during infection. These results strongly suggest that the GCS plays a major role during serine limitation that may be obscured by other serine biosynthetic pathways. Although the metabolic contribution was not examined, the glycine cleavage system was recently found to be required for the virulence of *Edwardsiella ictaluri,* the causative agent of enteric septicemia of catfish (Dahal et al., 2013). Together, these studies highlight the importance of this metabolic pathway across biological domains and indicate knowledge of the GCS may broadly apply to diverse pathogenic agents.

The Schu S4 Δ*gcvT* strain was unexpectedly a serine auxotroph (Fig. 1E-H). Unlike LVS, Schu S4 is annotated to contain an intact *serABC* pathway, which would be predicted to function as an alternative source of serine biosynthesis (Kanehisa and Goto, 2000). The serine auxotrophy following the loss of the GCS suggested that the *serABC* pathway was either insufficiently active in in vitro assays or that it was unlinked to serine biosynthesis in *Francisella*. In support of the later, *serA* and *serC* have been previously described as

participating in alternative pyridoxine (vitamin B6) biosynthesis (Lam and Winkler, 1990). Pyridoxine biosynthesis has recently been linked to bacterial pathogenesis, although the exact pathway and role in *Francisella* virulence is currently unknown (Grubman et al., 2010). It is thus plausible that *F. tularensis* utilizes *serA* (FTT_1230) and *serC* (FTT_0560c) for other metabolic pathways besides serine biosynthesis. The gene annotated as *serB* in Schu S4, FTT_0568, contains only limited homology to the *E. coli* homolog with 26% identity over a common 186 amino acid region. A gene annotated as *serB* in *Porphyromonas gingivalis* has recently been found to play a significant role in virulence, independent of metabolism, by facilitating invasion and suppressing the innate immune response (Takeuchi et al., 2013). It is thus conceivable that *serB* may serve a non-metabolic role in *F. tularensis*. Overall, our work demonstrates that the GCS is the only source of serine biosynthesis for *F. tularensis* and that the annotated *serABC* pathway is either inadequate for, or not involved with, serine production under the conditions tested.

To our knowledge, this is the first report to reconcile the in vitro and in vivo virulence phenotypes of the GCS (Fig. 5). Our initial studies demonstrated that Schu S4 strains lacking the *gcvT* homolog, FTT_0407, were attenuated in vivo despite the absence of defects in invasion, intracellular growth, and stimulation of TNF-α in vitro. A previous attempt to resolve discrepant in vivo and in vitro phenotypes with the GCS was unsuccessful (Scott et al., 2008). The availability of serine precursors (glycine, glucose, and pyruvate) in the low serine media may have confounded this previous study. To address this possibility, we utilized a minimal media containing low levels of both serine and serine precursors. In vitro assays performed in this serine limiting MEM medium demonstrated that the GCS of virulent *F. tularensis* significantly contributes to intracellular replication (Fig. 5). While it is likely that these

phenotypes are the direct result of reduced nutrient availability, host nutritional responses, such as autophagy, may also vary between DMEM and MEM. Although the replication of wild-type Schu S4 is comparable between these conditions and *F. tularensis* is thought to circumvent autophagy, it remains formally plausible that the GCS contributes to this resistant mechanism in some fashion (Fig. 2 and 5) (Checroun et al., 2006). Regardless, these results highlight the potential for standard tissue culture media to mask relevant phenotypes of metabolic genes in vitro by providing supraphysiological levels of nutrients.

The serine auxotrophy of the Schu S4 GCS mutant suggested it could be utilized to examine tissue and fluid-specific serine availability and therefore site-specific metabolic demands on the bacterium. Schu S4 strains without *gcvT* had lower bacterial burdens in vivo, with the greatest defect in the liver and blood of infected animals (Fig. 4). *F. tularensis* replicates extracellularly in murine blood and thus encounters serum nutrient concentrations (Forestal et al., 2007). Amino acid levels present in serum are lower than those found intracellularly (Bergstrom et al., 1974; Canepa et al., 2002). Furthermore, a reduction in amino acid levels has been observed in the blood of white rats following virulent *F. tularensis* infection (Woodward et al., 1954). Based on these data, the blood represents a relatively low amino acid environment during infection. Since the GCS conveys a fitness advantage in serine limiting environments (Fig. 1), the contribution of the GCS to bacteremia by virulent *F. tularensis* is likely linked to serine limitation at this site. It is worth noting that previous work in other pathogens examining the metabolic pathways required for bacterial replication in the blood did not identify serine biosynthesis (Samant et al., 2008). These studies can be reconciled by the presence of redundant serine biosynthetic pathways, a feature that appears to be lacking in *F. tularensis* but common in other bacteria. A role for the GCS was also found during hepatic

infection (Fig. 4C). Interestingly, activity of the mammalian GCS is restricted largely to the kidney, brain, and liver (Yoshida and Kikuchi, 1973). As the liver is a major site of serine and glycine metabolism, a successful hepatic pathogen will encounter and perhaps exploit this environment. Virulent *F. tularensis* may have a need for an intact GCS to overcome serine limitation arising from active host hepatic metabolism. Alternatively or additionally, *Francisella* may utilize its bacterial GCS to access glycine pools destined for degradation by the host's GCS. These findings suggest that, in order to successfully establish hepatic infection and bacteremia, pathogens encounter and must overcome serine limitation.

In contrast to the liver and the blood, there was less than a one log difference in bacterial burden in the lungs of mice infected with Schu S4 or Schu S4 ΔgcvT (Fig. 4A). This result indicates that the GCS plays, at most, a minor role during pulmonary infection and further suggests Schu S4 has access to suitable concentrations of exogenous serine in the lung. One possible source of serine could be from host polypeptide degradation since *Francisella* has already been shown to degrade a host polypeptide, glutathione, to acquire the amino acid cysteine (Alkhuder et al., 2009). Infection with *F. tularensis* also results in degradation of the lung extracellular matrix from MMP-9, a lung matrix metalloproteinase, causing polypeptide accumulation and subsequent neutrophil recruitment (Malik et al., 2007). Compared to wild-type mice, MMP-9 deficient mice had decreased bacterial burden and increased survival after infection with *F. tularensis*, suggesting the bacterium benefits from MMP-9 mediated proteolytic cleavage (Malik et al., 2007). The negative impact of MMP-9 is not likely explained by neutrophil recruitment as depletion of neutrophils during *F. tularensis* infection does not alter bacterial burden or prolong survival (KuoLee et al., 2011). Together, these data support the notion that the lung is not a serine-limiting environment during infection and that host

polypeptide accumulation, possibly from MMP-9 cleavage, may represent a source of amino acids for pulmonary pathogens, such as *F. tularensis*.

Pathogens must meet their metabolic requirements in the host environment. Despite the critical nature of this nutritional interaction, many metabolic pathways have not been examined for their contribution to pathogenesis. To our knowledge, this study identifies the first contribution of the bacterial GCS to pathogenesis in an acute mammalian model of infection. The GCS proved to be essential in serine limiting conditions and contributed to the pathogenesis of virulent *F. tularensis.* The glycine cleavage system has now been linked to virulence in both acute and chronic infection in distantly related pathogens. Our work also suggests that pathogens encounter and must overcome low serine environments in the liver and bloodstream. This report adds to the growing body of literature regarding the environment that pathogens encounter in vivo and what pathways are utilized to cope with these metabolic stresses. Conserved metabolic pathways are attractive therapeutic targets and additional knowledge of the metabolic interaction between pathogen and host will be essential to exploit these targets (Zhang and Rubin, 2013).

3.0 THE CONTRIBUTION OF THE SERINE HYDROXYMETHYLTRANSFERASE TO THE PATHOGENESIS OF *FRANCISELLA TULARENSIS*

3.1 ABSTRACT

The availability of essential nutrients during infection is a critical aspect of the host-pathogen interaction. Pathogens must acquire or synthesize these metabolites from the diverse host environments they colonize. The serine hydroxymethyltransferase (*glyA*) is a highly conserved metabolic enzyme and may be a potential therapeutic target. This enzyme produces 5,10-methylenetetrahydrofolate, an important carbon donor in amino acid and DNA synthesis. To evaluate its contribution to pathogenesis, the *glyA* homolog was deleted in an attenuated and a virulent *F. tularensis* strain. In the attenuated strain, loss of *glyA* resulted in serine auxotrophy and an increased glycine requirement that was frequently circumvented by pseudo-reversion. The serine hydroxymethyltransferase contributed to intracellular growth in vitro, although pseudo-reversion and variations in culture medium altered the magnitude of this phenotype. In the virulent strain, deficiency in *glyA* also resulted in serine auxotrophy but no major effect on glycine metabolism or in vitro intracellular growth was observed. In a mouse model of virulent pneumonic tularemia, deletion of *glyA* resulted in a significant reduction in bacteremia but ultimately only led to a minor delay in mortality. Thus, the serine hydroxymethyltransferase of

F. tularensis is required for serine prototrophy and provides a significant fitness advantage during bacteremia, but is not essential for lethality.

3.2 INTRODUCTION

The development of resistance to antibiotics is an unavoidable fact in biomedicine (Davies and Davies, 2010; Spellberg et al., 2008). To keep pace with this evolving threat, novel therapeutics must constantly be identified, characterized, and developed (Boucher et al., 2009; Spellberg et al., 2008). Conserved and essential bacterial genes may serve as excellent targets for pharmacological intervention (Taylor and Wright, 2008). Bacterial metabolic pathways often fit the criteria of being broadly conserved but, in many cases, their requirement for in vivo pathogenesis has not been elucidated (Rohmer et al., 2011). Assessment of in vivo relevance can be challenging due to the nutritional diversity of host niches (Alteri and Mobley, 2012). Many studies of pathogen metabolism use infection models that are only representative on a single host niche and are thus limited in their applicability (Rohmer et al., 2011). An ideal candidate would contribute to fitness in various host environments and in diverse pathogens. Overall, bacterial metabolic pathways are attractive for drug development but require thorough characterization to evaluate the in vivo importance of each potential target.

Although metabolic pathways are conserved among many bacteria, some species are remarkably limited in their metabolic potential. Intracellular bacteria such as *Rickettsia prowazekii*, *Borrelia burgdorferi*, and *Mycoplasma genitalium*, all have dramatically reduced genomes and lack most biosynthetic pathways (Andersson et al., 1998; Fraser et al., 1995). The discussions of a "minimal genome" often revolve around these species and genes that persist in

this setting likely convey a strong fitness advantage (Glass et al., 2006). A gene known as the serine hydroxymethyltransferase (*glyA*) is among the few metabolic genes remaining intact in these species (Andersson et al., 1998; Fraser et al., 1995). This enzyme catalyzes the reversible degradation of serine to glycine with the transfer of the one-carbon to tetrahydrofolate (THF) to form 5'10-mTHF (Plamann and Stauffer, 1983; Stauffer and Brenchley, 1978). 5'10-mTHF can serve as a precursor for downstream one-carbon metabolic reactions for protein and DNA synthesis (Locasale, 2013). Interestingly, *glyA* is an almost universally conserved gene (de Crecy-Lagard et al., 2007). Investigations into this gene have revealed conflicting results regarding whether it is essential in bacteria (Chaudhuri et al., 2009; Gallagher et al., 2007; Glass et al., 2006; Kobayashi et al., 2003; Ravnikar and Somerville, 1987; Tempel et al., 2006). To our knowledge, mutagenesis of *glyA*, when achievable, has always resulted in a decrease in bacterial fitness during infection and significant attenuation (Bogard et al., 2012; Dahal et al., 2013). Importantly, *glyA* has not been assessed in diverse host niches and has only been investigated in limited infection models. Ultimately, it is likely that *glyA* plays a core role in bacterial physiology through its contribution to one-carbon metabolism. These findings suggest that *glyA* may be a promising conserved target, but that further investigation of the contribution of *glyA* in diverse environments is required.

Francisella tularensis is the causative agent of tularemia, a debilitating febrile illness (Francis, 1925). *F. tularensis* infects and replicates in a wide repertoire of host cell types including both immune and non-immune cells (Hall et al., 2008; Horzempa et al., 2010a). A murine model of pneumonic tularemia involves rapid disease progression and includes infection and replication in the host's lung, liver, spleen, and blood (Horzempa et al., 2010a; Schmitt et al., 2013). *F. tularensis* can thus be utilized as an excellent model to assess the role of *glyA* in a

highly pathogenic organism that encounters multiple diverse host niches. Furthermore, investigation into the *glyA* of *F. tularensis* will also contribute to our understanding of one-carbon metabolism in this pathogen.

Whether or not *glyA* contributes to *Francisella* pathogenesis is not yet clear. A transposon-site hybridization (TRASH) screen failed to identify any disruption of *glyA* and thus marked it as a candidate essential gene (Gallagher et al., 2007). In contrast, a disruption of *glyA* by a transposon was identified in a separate screen for mutants with defects in intramacrophage replication (Tempel et al., 2006). Interestingly, this study performed a small pilot experiment and suggested that the *glyA* transposon mutant may be partially attenuated in vivo (Tempel et al., 2006). This study did not, however, attempt to perform any metabolic characterization of the mutant nor did they confirm the stability of the disruption in vivo (Tempel et al., 2006). Furthermore, no genetic complementation was attempted to confirm the lack of a polar effect, a frequent concern with *Francisella* transposon mutants (Maier et al., 2007; Tempel et al., 2006). Thus, the contribution of *glyA* to *F. tularensis* metabolism and pathogenesis remains uncertain and requires further investigation.

To evaluate *glyA* in *F. tularensis*, mutagenesis was performed in both the attenuated *F. tularensis* subsp. *holarctica* LVS and the virulent *F. tularensis* subsp. *tularensis* Schu S4 strain. Following deletion of *glyA*, strains were assessed for defects in metabolism, in vitro virulence, and in vivo pathogenesis. During characterization of *glyA* in LVS, a frequent pseudo-revertant was observed and characterized. Our results demonstrate that the *glyA* homolog is not essential to *F. tularensis* but that it is required for serine biosynthesis. Furthermore, our findings suggest that loss of *glyA* results in a modest in vivo attenuation coinciding with significantly reduced bacteremia.

3.3 MATERIALS AND METHODS

3.3.1 Routine culture and source of bacterial strains

The following reagent was obtained through BEI Resources, NIAID, NIH: *Francisella tularensis* subsp. *tularensis*, Strain SCHU S4 (FSC237), NR-643. *Francisella tularensis* subsp. *holarctica* Live Vaccine Strain (LVS) was a gift from Dr. Karen Elkins (U.S. Food and Drug Administration). Chocolate agar (GC medium base, hemoglobin, and isovitaleX) was used for routine culture of *F. tularensis* from frozen bacterial stocks. Complement and vector strains were streaked onto chocolate agar supplemented with hygromycin (200 μg/mL). Plated bacteria were grown for approximately three days at 37 °C with 5% CO_2 before being used to inoculate overnight cultures. Broth cultures were shaken at 250 rpm at 37 °C for indicated durations. Schu S4 strains were utilized under BSL3 containment with approval from the Centers for Disease Control and Prevention Select Agent Program. The *E. coli* EC100D strain was utilized for standard cloning.

3.3.2 Mutagenesis of *Francisella* and generation of complements and vector controls

Allelic replacement was used to delete the *glyA* homolog of LVS (FTL_0703) and Schu S4 (FTT_1241) as described previously (Horzempa et al., 2010b). Since the flanks of *glyA* in these strains share 99% identity, a single suicide vector (pJH1) containing these regions from LVS was utilized to delete *glyA* in both strains. The upstream flank (1,103 bp) was generated using CTAGGGATCCGCTTTCAATTTACTAGAAGAGTAGC as a forward primer and CTAGGGTACCAGCATCAAAAATCTCTTTGTCGG as a reverse primer. The downstream

flank (1,037 bp) was generated using GATCGGTACCTGTGATAAGCTCCCTGTTTACAAG as a forward primer and CTAGGCATGCCGTTAAATTCAATGGCAGTAGCG as a reverse primer. This mutational strategy is expected to remove nucleotides (58-1227) of the *glyA* locus, while leaving behind 57 nucleotides at the 5' end and 27 nucleotides at the 3' end of this gene. Absence of *glyA* was confirmed by genomic PCR using GCATGGATCCCCAAATCCGGAAATTCTTGTATTAG as a forward primer and GCATGGTACCGATAACATTAGAAATAAAAGGATTTTGGTG as a reverse primer (Appendix 3). These primers are 539 bp upstream and 118 bp downstream of *glyA*, respectively, and are expected to produce products of either 1,911 bp (wild-type locus) or 741 bp (Δ*glyA*). Deletion mutants are annotated as LVS Δ*glyA* and Schu S4 Δ*glyA*.

To create the vector for *glyA* complementation, an XhoI digestion of the suicide vector pJH1 was performed to remove extraneous yeast genes from this plasmid (Horzempa et al., 2010b). Following religation, this modified pJH1 plasmid (pMB1) was employed to complement *glyA* in cis. As the *glyA* homolog was highly conserved (99% nucleotide identity) between subspecies, the same complementing construct with *glyA* from LVS was used for Schu S4 as well. To create this construct, the LVS *glyA* (FTL_0703) was amplified by PCR with the ~257 bp upstream region and 119 bp downstream region and ligated into pGEM-T. This was achieved using CTAGGGATCCGCTCTTTACTTAATTCAGTCAAAGATCTACC as a forward primer and CTAGGCATGCTGATAACATTAGAAATAAAAGGATTTTGGTG as a reverse primer. The 257 bp upstream sequence was expected to contain the *glyA* promoter and will also facilitate targeting of chromosomal integration to this homologous region. The PCR product was subsequently subcloned from this pGEM-T vector into the suicide plasmid pMB1 via restriction enzyme (BamHI and SphI) digest. These restriction sites were added on the forward and reverse

primers. This protocol was repeated with a PCR amplicon containing only the upstream region as a pMB1 vector control. To generate this amplicon, CTAGGGATCCGCTCTTTACTTAATTCAGTCAAAGATCTACC was used as the forward primer and CTAGGCATGCATTTGGTAGTCTCCTGATATTTTTTAGG was used as the reverse primer. Tri-parental mating was used to mobilize these suicide vectors into strains containing *glyA* deletions as described previously (Horzempa et al., 2010b). The complementing strains are referred to as (LVS Δ*glyA*:pMB1*glyA* and Schu S4 Δ*glyA*:pMB1*glyA*) and the vector control strain (LVS Δ*glyA*:pMB1). An attempt to create Schu S4 Δ*glyA*:pMB1 was unsuccessful.

3.3.3 In vitro broth culture assays

F. tularensis strains harvested from chocolate agar plates were used to inoculate either tryptic soy broth with supplemental cysteine (TSB-C) or brain heart infusion broth set to pH 6.8 (BHI). These cultures were pelleted and resuspended in either Chamberlain's chemically defined media (CDM) lacking serine or PBS. CDM was modified as indicated in each experiment but generally prepared as previously described (Chamberlain, 1965). Cultures were shaken (250 rpm) at 37 °C for the stated durations prior to measurements of optical density (OD_{600}). OD_{600} readings were measured in a cuvette (1 cm path length) using a CO8000 Cell Density Meter (WPA) for Schu S4 strains. The OD_{600} of LVS strains was determined in either a cuvette (single end-point experiments) or 96-well plate (serial measurement time course experiments) using a M2 plate reader (Molecular Devices). TSB-C was sterilized by routine autoclaving while BHI and CDM were sterilized through filtration (0.22 μm).

3.3.4 In vitro agar plate assays and imaging

Bacterial growth on solid media was assessed with either chocolate agar plates or modified CDM agar plates. Modified CDM agar plates were made by combining modified CDM (0 mM serine, 50 mM glycine) and ultrapure bacteriological agar (USB). When indicated, chocolate agar plates were supplemented with 5 mM serine and 50 mM glycine. Plates were incubated at 37 °C with 5% CO_2 until adequate growth was achieved (typically three days post inoculation). For experiments using modified CDM agar, bacteria were patched from chocolate agar plates. Under no circumstances was visible growth obtained on modified CDM agar plates for any strains except wild-type (LVS) and complementing strains (LVS Δ*glyA*:pMB1*glyA*, LVS Δ*gcvT*:pMB1*gcvT*). For experiments using chocolate agar plates, strains were streaked directly from frozen stocks. All images were captured using a Samsung Sch-I510 camera.

3.3.5 Generation of primary macrophages

Bone marrow was harvested from C57BL/6J mice and used to derive primary mouse macrophages as described previously (Russo et al., 2011). Murine cells were cultured in Dulbecco's Modified Eagles Medium (DMEM) with 20% FBS, 25 mM HEPES, 2 mM glutaMAX, 1 mM sodium pyruvate, 1 X MEM non-essential amino acids, and 25% L-cell supernatant. L-cell supernatant (from L929 cells) serves as a source of growth factors and was generated as previously described (Russo et al., 2011).

Human monocytes, purified from human peripheral blood mononuclear cells, were differentiated into macrophages as previously described (Carlson et al., 2007). Briefly, mononuclear cells were obtained from a buffy coat (New York Blood Bank) using gradient

centrifugation with Ficoll (GE Healthcare). From this population, monocytes were further isolated using an Optiprep (Sigma-Aldrich) gradient and panning for adherence in tissue culture dishes. Finally, macrophages were obtained through the differentiation of monocytes after seven days in DMEM supplemented with 20% FBS, 10% AB human serum (Complement-Replete Gem Cell; Gemini Bio-Products), 2 mM glutaMAX, and 25 mM HEPES. Cells were used within three days of their differentiation.

3.3.6 Measurement of invasion and intramacrophage replication

After propagation or differentiation of macrophages, cells were harvested using lidocaine (4 mg/ml)/EDTA (5 mM) in PBS. Cells were seeded at a density of $5x10^4$ cells per well in a 96-well Primaria plates (BD biosciences). Once in the 96-well plate, mouse macrophages were allowed to rest overnight (37 °C with 5% CO_2) prior to infection in either a rich culture media (DMEM with 20% FBS, 25 mM HEPES, 2 mM glutaMAX, 1 mM sodium pyruvate, 1 X MEM non-essential amino acids) or a minimal culture media (Minimal Essential Media (MEM, Gibco) with 10% FBS, 2 mM glutaMAX, and 25 mM HEPES). Human macrophages were placed in infection media (1% AB human serum, DMEM, 2 mM glutaMAX, and 25 mM HEPES) and infected the same day as harvest. Overnight cultures of *F. tularensis* strains were pelleted, resuspended in the appropriate culture media, and added to cells at a multiplicity of infection (MOI) of 500. Bacteria were incubated with cells for a two hour invasion period. In order to eliminate extracellular bacteria after this period, a gentamicin treatment was performed. This treatment lasted 30 minutes at 37 °C with 5% CO_2 and involved the replacement of culture media with HBSS containing 50 μg of gentamicin per ml. After the treatment, gentamicin was removed by gently washing the cells twice with HBSS and their respective culture media was

subsequently replenished. Cells were lysed with 0.02% SDS in PBS to release intracellular bacteria at the indicated time points. This lysate was serially diluted in TSB-C and plated as drips onto chocolate agar. Colony-forming units (CFU) were determined by visual inspection of plates after three days of growth at 37 °C with 5% CO_2. Experiments were routinely performed using triplicate wells per condition.

3.3.7 Indirect threonine dehydrogenase assay

To assess the threonine dehydrogenase (TDH) activity of *Francisella* cultures, an enzymatic assay was adapted with modifications from previously described protocols (Newman et al., 1976; Simic et al., 2002). Overnight cultures of LVS strains were washed with PBS, standardized by OD_{600}, and resuspended in Tris-HCl (250 mM, pH 8.4). Bacterial samples were chilled on ice prior to and during mechanical disruption by sonication (30 second burst on setting 8 of a Microson XL sonicator followed by 30 seconds of rest on ice, repeated 5 times). Lysates were clarified by centrifugation (7000 x *g*) for 20 minutes at ~ 4 °C. The supernatant was removed, held on ice, and utilized henceforth as a crude *F. tularensis* protein lysate. This crude protein preparation was diluted with Tris-HCl (250 mM, pH 8.4) and set to a volume of 625 μL prior to 125 μL of DL-threonine (1 M, pH 8.4) being added. This mixture was equilibrated for five minutes at 37 °C prior to the addition of 50 μL nicotinamide adenine dinucleotide (NAD, 100 mM). NAD is an essential co-factor of TDH and initiates the reaction, allowing this enzyme to convert the excess threonine to 2-amino-3-oxobutyrate (Newman et al., 1976). The absence of excess coenzyme A prevents significant processing of 2-amino-3-oxobutyrate (Newman et al., 1976). This reaction was incubated at 37 °C for 30 minutes before it was stopped with the addition of 200 μL 25% trichloroacetic acid. The sample was subsequently deproteinated by

centrifugation (3000 x g) for 10 minutes. This supernatant was collected and presumably contains quantities of 2-amino-3-oxobutyrate proportional to the TDH activity of the bacterial culture. While 2-amino-3-oxobutyrate is not readily measurable, it can be converted to aminoacetone, a quantifiable compound (Newman et al., 1976; Simic et al., 2002). To achieve this, samples (500 μL) were mixed with 500 μL sodium acetate buffer (2 M, pH4.6), 50 μL sodium hydroxide (2.5 M), and 25 μL acetylacetone. This mixture is boiled for 10 minutes before being allowed to cool to room temperature. This procedure results in the production of aminoacetone from the decarboxylation of 2-amino-3-oxobutyrate (Simic et al., 2002). To determine the relative aminoacetone levels in these samples, they were mixed with 1 mL modified Ehrlich's reagent (0.2 g of 4-(Dimethylamino)benzaldehyde dissolved in 8.4 mL glacial acetic acid then 1.6 mL 70% perchloric acid was added). This reaction results in a colorimetric shift depending on the quantity of aminoacetone reacting with the Ehrlich's reagent (Newman et al., 1976; Simic et al., 2002). After a 15 minute incubation at room temperature, this colorimetric readout was measured at 553 nM and appears visually as a red hue to an otherwise clear solution.

3.3.8 Mouse model of pneumonic tularemia

Intratrachaeal infection of 6-8 week old C57BL/6J mice (Jackson Laboratory) with Schu S4 is an established murine model of pneumonic tularemia (Russo et al., 2011). To utilize this model with our strains, bacteria were grown overnight in TSB-C broth cultures, washed with PBS, and diluted as necessary. Mice were infected with approximately 100 CFU of each strain via oropharyngeal installation. Animals were monitored after infection and scored using a sickness rubric (Russo et al., 2011). Upon reaching a predetermined sickness score, mice were

euthanized and the mortality recorded. Approval from the University of Pittsburgh Institutional Animal Care and Use Committee was obtained for all animal experiments. These studies were performed under ABSL-3 conditions at the University of Pittsburgh.

Bacterial burden was assessed using a previously described protocol (Horzempa et al., 2010a). Briefly, ketamine (80mg/kg) and xylazine (8mg/kg) were used to anesthetize animals four days post infection. A heparinized needle and syringe was utilized to collect blood via a cardiac puncture, while other organs (lung, liver, and spleen) were harvested and homogenized. Once harvested, blood and organ homogenates were diluted and plated as drips onto chocolate agar. After approximately three days of growth, CFU was enumerated by visual inspection.

3.4 RESULTS

3.4.1 The serine hydroxymethyltransferase of *F. tularensis* is not essential but is required for serine prototrophy and growth in limiting glycine

Previous work suggested that *F. tularensis* possesses a single pathway for serine biosynthesis and that the glycine cleavage system (GCS) is required for production of this amino acid (Fig. 1). In *Escherichia coli*, serine biosynthesis from glycine also requires the presence of *glyA*, encoding the serine hydroxymethyltransferase which catalyzes the formation of serine from glycine and 5'10-mTHF (Ravnikar and Somerville, 1987). To determine if *glyA* was essential and to assess its role in *F. tularensis* metabolism, the homolog of *glyA* was deleted in the attenuated live vaccine strain (LVS, FTL_0703). Analysis of the metabolic features of this strain was performed using Chamberlain's chemically defined media (CDM) (Chamberlain, 1965).

The resultant strain (LVS Δ*glyA*) was unable to grow in the absence of serine and, unlike wild-type LVS, was unable to utilize glycine to overcome serine limitation (Fig. 6A). This auxotrophy is similar to that seen in strains lacking an intact GCS (LVS Δ*gcvT*) (Figure 6A). In contrast to LVS Δ*gcvT*, however, LVS Δ*glyA* grew poorly in standard CDM (3.8 mM serine, 0 mM glycine) (Fig. 6B). Furthermore, supplementation of CDM with serine failed to restore the growth of LVS Δ*glyA* to wild-type levels (Fig. 6C). In addition to serine biosynthesis, the serine hydroxymethyltransferase of *E. coli* also serves as a major source of glycine from serine degradation (Pizer, 1965). This led to the hypothesis that loss of *glyA* in *Francisella* may result in a requirement for both glycine and serine. In support of this, supplementation of CDM with glycine and serine led to near wild-type levels of LVS Δ*glyA* growth (Fig. 6C). To confirm that these phenotypes were due to the loss of *glyA*, an in cis complement (LVS Δ*glyA*:pMB1*glyA*) and in cis vector (LVS Δ*glyA*:pMB1) were generated. Modified CDM agar plates (0 mM serine, 50 mM glycine) supported the growth of only LVS and LVS Δ*glyA*:pMB1*glyA* (Fig. 6D). This matched results performed with the GCS-associated strains, which served as a positive control for serine auxotrophy (Fig. 6D). These results suggest that the serine hydroxymethyltransferase is not essential to the viability of *F. tularensis* but is required for serine biosynthesis and contributes significantly to glycine biosynthesis in LVS.

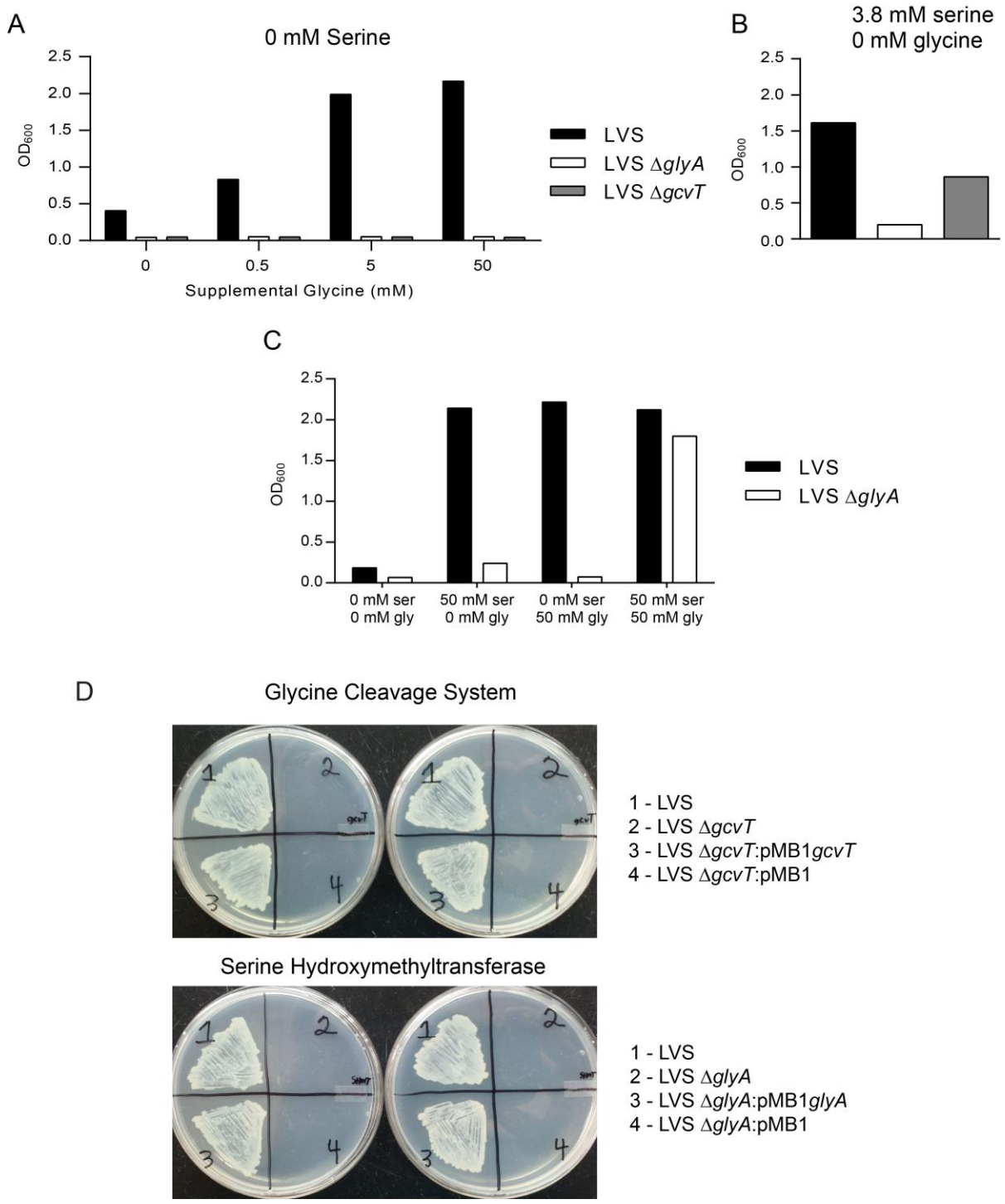

Figure 8. The serine hydroxymethyltransferase of *F. tularensis* is not essential but is required for serine prototrophy and growth in limiting glycine.

(A-C) Overnight TSB-C broth cultures of LVS strains were pelleted and resuspended in either serine-free Chamberlain's chemically defined media (CDM) (A and B) or in PBS (C). These suspensions were used to

inoculate serine-free CDM with varying glycine concentrations (A), standard CDM containing 3.8 mM serine and 0 mM glycine (B), and CDM with all combinations of 0 mM or 50 mM serine and glycine (C). The starting OD_{600} was set at 0.05 and the data represent the measured OD_{600} approximately 20 hours post inoculation. These data were obtained from a single experiment. (D) LVS strains were streaked out onto chocolate agar plates and grown for approximately three days. Bacteria from these plates were subcultured onto CDM agar plates containing 0 mM serine and 50 mM glycine. Images were obtained using a Samsung Sch-I510 camera after several days of growth. Side by side images are duplicate experiments performed concurrently.

3.4.2 The discovery of two distinct variants of LVS Δ*glyA*

While loss of *glyA* was not lethal to *F. tularensis*, LVS Δ*glyA* had reduced growth on chocolate agar (data not shown). During characterization of this strain, it became apparent that the initial stock of LVS Δ*glyA* contained colonies of variable size (data not shown). To investigate and determine the significance of this observation, stocks were made of an isolated large colony and an isolated small colony. The large variant (LV) shall be referred to as LVS Δ*glyA* LV and the small variant (SV) as LVS Δ*glyA* SV. For size reference, LVS was streaked onto chocolate agar and grew as expected (Fig. 7A). LVS Δ*glyA* SV exhibited reduced growth on chocolate agar, observed as smaller colony size, while LVS Δ*glyA* LV grew similar to wild-type (Fig. 7B). Interestingly, large colonies were found at low frequency in LVS Δ*glyA* SV (Fig. 7B and data not shown). LV stocks demonstrated a stable large phenotype while, despite further attempts at isolation, large colonies consistently emerged from SV stocks (data not shown). To determine if the poor growth of LVS Δ*glyA* SV was due to nutrient limitation, chocolate agar was supplemented with 50 mM glycine and 5 mM serine. After amino acid supplementation, LVS Δ*glyA* SV was indistinguishable from LVS Δ*glyA* LV (Fig. 7B). The

characterization of LVS Δ*glyA* has thus revealed two distinct variants. These data suggest that large variants emerge from small variants and may have altered metabolism to facilitate growth in the absence of *glyA*. Based on these findings, we hypothesized that the large variant of LVS Δ*glyA* is a suppressor and the synonymous term "pseudo-revertant" will be utilized in this text to describe this event.

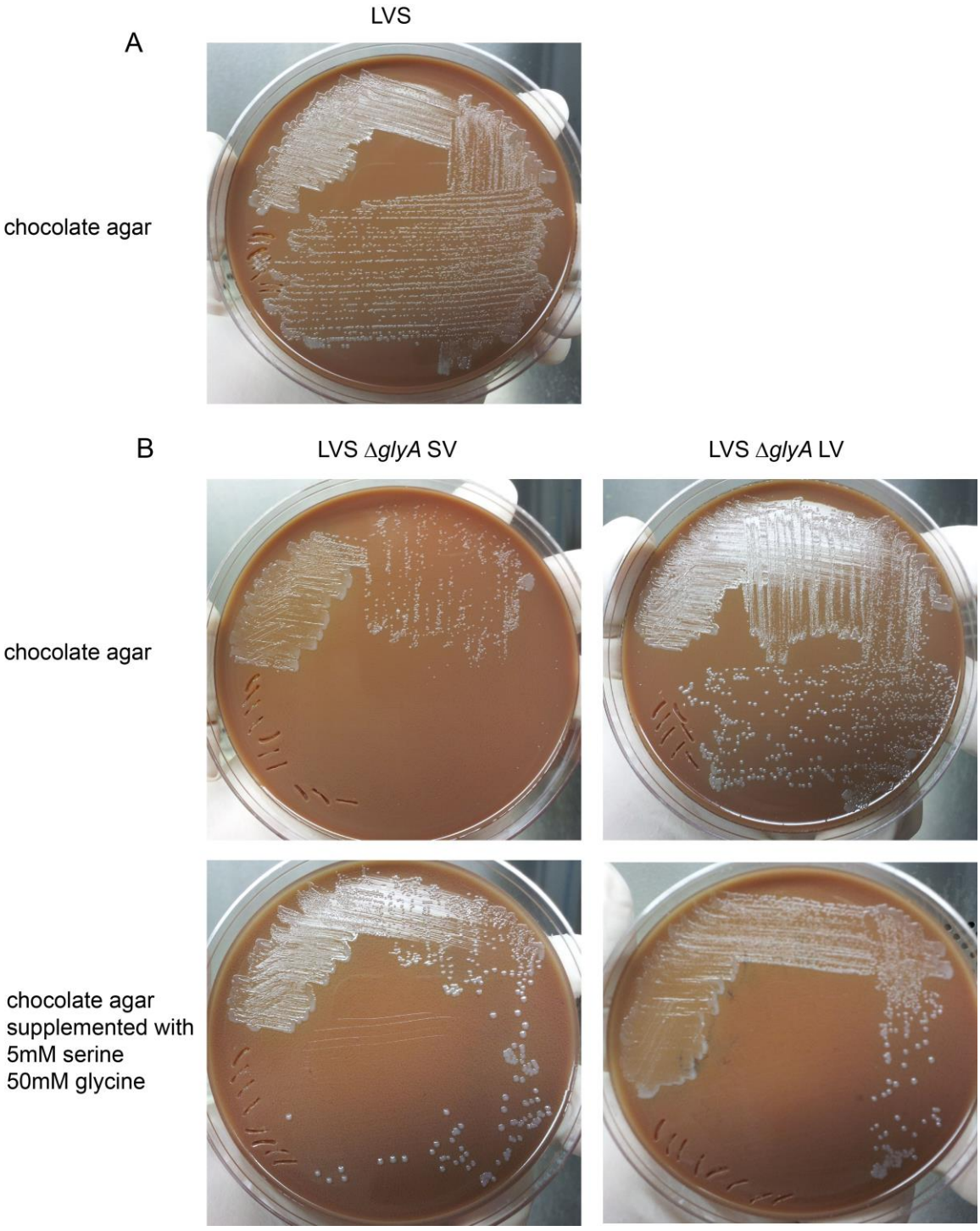

Figure 9. Two distinct variants of LVS Δ*glyA* exist.

(A) LVS stocks were streaked onto chocolate agar. (B) Stocks of the LVS Δ*glyA* small variant (SV) (left column) and the LVS Δ*glyA* large variant (LV) (right column) were streaked onto either chocolate agar (top row) or

chocolate agar supplemented with 5 mM serine and 50 mM glycine (bottom row). All images were collected using a Samsung Sch-I510 camera and are from a single experiment after approximately three days of growth.

3.4.3 The large variant is a pseudo-revertant that maintains serine auxotrophy despite a reduced glycine requirement

Since the growth defect of the small variant could be complemented by glycine and serine, we hypothesized that the large variant may have altered requirements for these amino acids. Although *glyA* is required for glycine-mediated serine production in *E. coli* and our initial results supported this, the genome of *F. tularensis* does possess annotated homologs of *serABC*, a glycolytic serine biosynthetic pathway (Fig. 6) (Larsson et al., 2005; Ravnikar and Somerville, 1987). While previous work suggested this system does not contribute to serine biosynthesis, the LVS Δ*glyA* LV may have activated this alterative pathway to overcome serine auxotrophy. To investigate the possibility that the large variants were no longer serine auxotrophs, strains were plated on modified CDM agar (0 mM serine, 50 mM glycine). Only wild-type LVS and the complement LVS Δ*glyA*:pMB1*glyA* were capable of growth under these conditions, suggesting that the large variant was still a serine auxotroph (Fig. 8A). All strains grew well in a rich media, brain heart infusion broth, although LVS Δ*glyA* SV maintained a minor growth defect (Fig. 8B). Results from standard CDM broth (3.8 mM serine, 0 mM glycine) revealed a severe growth defect for LVS Δ*glyA* SV (Fig. 8C). In contrast, LVS and LVS Δ*glyA*:pMB1*glyA* grew well in CDM with typical growth kinetics (Fig. 8C). Interestingly, LVS Δ*glyA* LV and LVS Δ*glyA*:pMB1 had very similar patterns of growth in CDM, matching LVS levels after a prolonged lag phase (Fig. 8C). The addition of 5 mM glycine to CDM increased the growth of all strains and also reduced the lag phase of LVS Δ*glyA* LV and LVS Δ*glyA*:pMB1 (Fig. 8D).

Furthermore, all strains were indistinguishable after 24 hours except for LVS Δ*glyA* SV, which exhibited a moderate growth defect (Fig. 8D). Finally, the serine auxotrophy of all strains except LVS and LVS Δ*glyA*:pMB1*glyA* was confirmed using modified CDM broth (0 mM serine, 5 mM glycine) (Fig. 8E). Overall, these results indicate that the large variant is a pseudo-revertant which greatly reduces the glycine requirement of LVS Δ*glyA* SV while maintaining serine auxotrophy. Furthermore, these results suggest that the stock of the vector control, LVS Δ*glyA*:pMB1, is also a pseudo-revertant. This is supported by the wild-type size of this strain's colonies on solid media (data not shown).

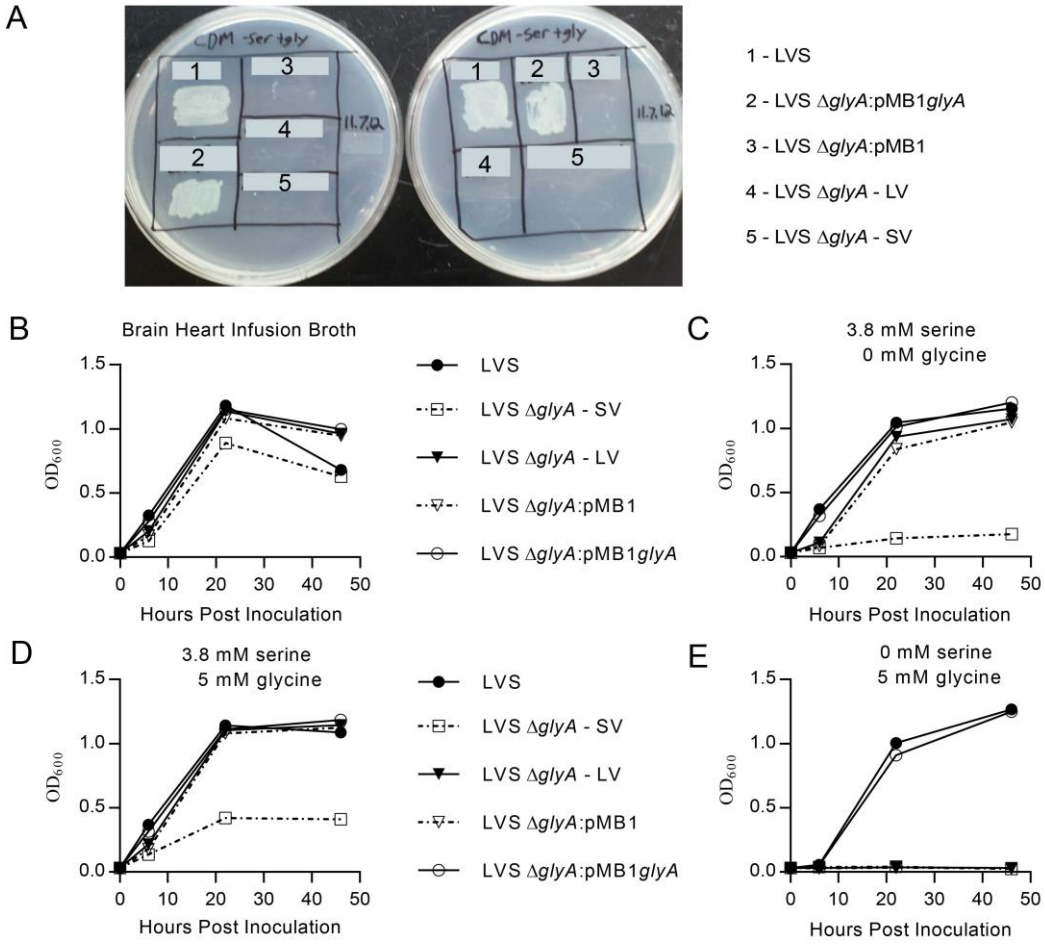

Figure 10. The large variant is a pseudo-revertant that maintains serine auxotrophy despite a reduced glycine requirement.

(A) After approximately three days of growth on chocolate agar, LVS strains were subcultured onto CDM agar plates containing 0 mM serine and 50 mM glycine. A Samsung Sch-I510 camera was used to image the plates after approximately two days of growth. Side by side images are duplicate experiments performed concurrently. Colored boxes were added to cover up colloquial strain designations and provide contrast for numeral assignments linked to proper nomenclature. (B-E) Overnight BHI cultures of LVS strains were pelleted, resuspended in PBS, and used to inoculate BHI (B), CDM (3.8 mM serine, 0 mM glycine) (C), CDM with glycine (3.8 mM serine, 5 mM glycine) (D), and serine-free CDM with glycine (0 mM serine, 5 mM glycine) (E). Cultures were initially set to an OD_{600} of 0.03 and were monitored for OD_{600} changes at the indicated time points. Data are from a single experiment.

3.4.4 Large and small variants of LVS Δ*glyA* have distinct intramacrophage growth phenotypes

While the two variants of LVS Δ*glyA* were similar in their serine auxotrophy, they were readily distinguishable by their glycine requirement (Fig. 8). We next sought to evaluate the importance of *glyA* and these variants to the intracellular growth of *F. tularensis*. Primary mouse macrophages were infected and maintained using either a "nutrient rich" Dulbecco's modified Eagle medium (DMEM) or a "nutrient limiting" minimal essential media (MEM). No significant difference in invasion after two hours was detected between LVS, LVS Δ*glyA* SV, and LVS Δ*glyA* LV in either condition (Fig. 9A and 9B). In macrophages cultured in DMEM, LVS grew approximately three logs while LVS Δ*glyA* LV grew approximately two logs and LVS Δ*glyA* SV grew only one log in 24 hours (Fig. 9A). These results were consistent with the enhanced fitness of the large variant on chocolate agar, brain heart infusion broth, and CDM broth (Fig. 7 and Fig. 8). In contrast to results using DMEM, infection of macrophages cultured in MEM yielded unexpected results. LVS was only modestly more proficient at intracellular growth than LVS Δ*glyA* SV and LVS Δ*glyA* LV failed to increase in CFU over 24 hours (Fig. 9B). To extend

these results to human cells, primary human macrophages were employed. LVS Δ*glyA* SV failed to invade as proficiently as other strains (Fig. 9C). Since the human macrophage model contains fresh human serum, the sensitivity of these strains to complement was tested. LVS and both variants of LVS Δ*glyA* were equally resistant to complement, indicating that the reduced invasion is not the result of complement mediated killing (Appendix B). Similar to invasion, all strains grew well within humans macrophages except for LVS Δ*glyA* SV, which failed to increase in CFU over 24 hours (Fig. 9C). This result also confirmed that the vector control, LVS Δ*glyA*:pMB1, behaved similar to the large variant (Fig. 9C). These data indicate that while the loss of *glyA* limits the intramacrophage growth of LVS, the pseudo-revertant is capable of overcoming a significant portion of this defect. Furthermore, the altered metabolism of this pseudo-revertant appears to require a nutritionally rich media and may be otherwise detrimental.

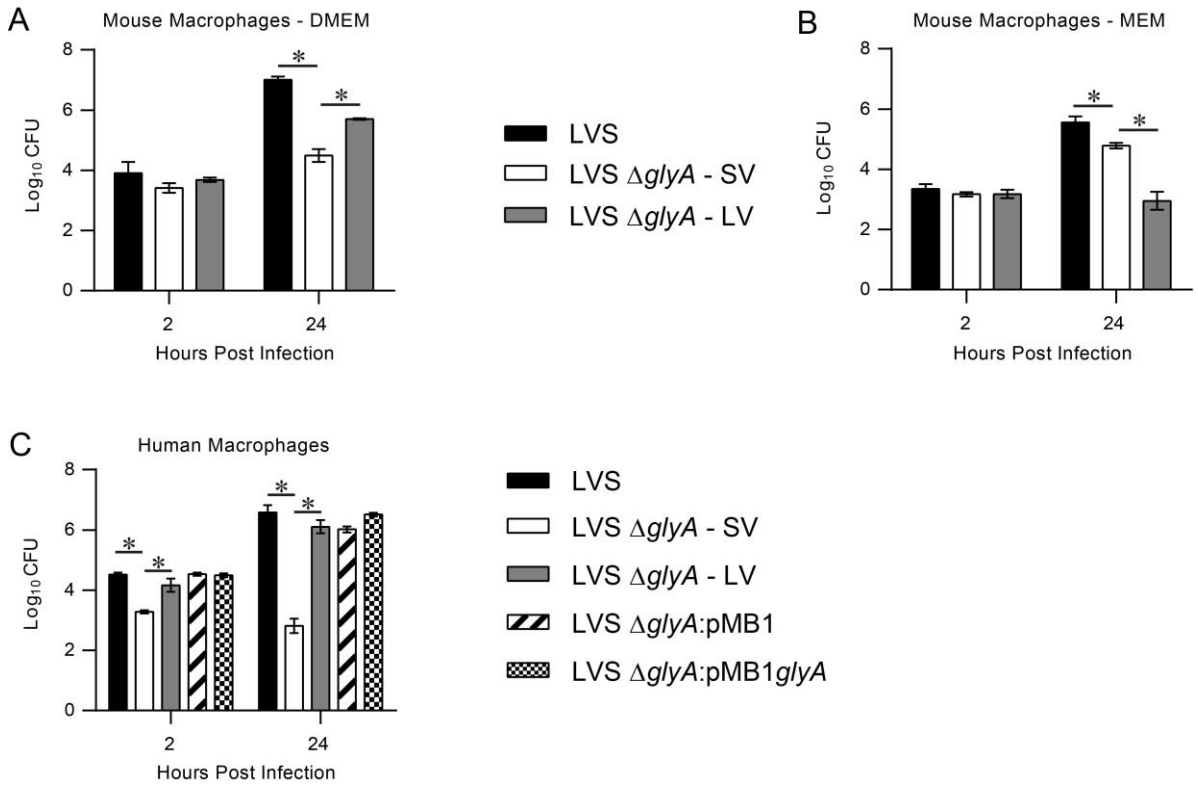

Figure 11. Large and small variants of LVS Δ*glyA* have distinct intramacrophage growth phenotypes.

(A-B) Mouse macrophages were harvested and placed in either rich media (A) (DMEM with 20% FBS, 25 mM HEPES, 2 mM glutaMAX, 1 mM sodium pyruvate, 1 X MEM non-essential amino acids) or minimal media (B) (MEM with 10% FBS, 25 mM HEPES, 2 mM glutaMAX). (C) Human macrophages were harvested and placed in human infection media (DMEM, 1% AB human serum, 2 mM glutaMAX, and 25 mM HEPES). Overnight TSB-C broth cultures of LVS strains were pelleted, resuspended in either rich media (A), minimal media (B), or human infection media (C) and added to cells at an MOI of 500. Bacteria were incubated with cells for two hours before a gentamicin treatment as described in the Materials and Methods. Cells were lysed and the CFU enumerated at the indicated time points. Data represent the mean ± SD value of triplicate wells from one experiment. Statistical significance ($p < 0.05$) for each time point was determined using a one-way ANOVA followed by a Bonferroni multiple comparison correction. A * is used to denote time points where statistical significance was achieved between LVS and LVS Δ*glyA* SV or LVS Δ*glyA* SV and LVS Δ*glyA* LV.

3.4.5 The activity of the threonine dehydrogenase is not dramatically different in the large variant

Pseudo-reversion is not an uncommon event when manipulating bacterial metabolism and may involve overexpression of related enzymes or pathways (Patrick et al., 2007). Interestingly, manipulation of serine biosynthesis and *glyA* in *E. coli* also leads to identifiable pseudo-revertants (Fraser and Newman, 1975; Ravnikar and Somerville, 1987). Several of these pseudo-revertants were found to have increased activity of an enzyme known as the threonine dehydrogenase (TDH) (Fraser and Newman, 1975). This enzyme is essential in the production of glycine from threonine and functionally may suppress the glycine requirement of *glyA* mutants (Newman et al., 1976). In theory, increased TDH activity could be the result of a variety of factors ranging from protein mutations to altered transcriptional regulation. To assess enzymatic activity of TDH, a colorimetric assay can be utilized to quantify aminoacetone levels

from bacterial lysates (Boylan and Dekker, 1981; Newman et al., 1976). Aminoacetone can be produced by decarboxylation of 2-amino-3-oxobutyrate, the immediate product of the TDH, and thus serves as an indirect but reliable measure of the TDH activity in a sample (Boylan and Dekker, 1981; Newman et al., 1976). In *E. coli*, supplementation of culture media with glycine (a downstream TDH product) and threonine (the TDH substrate) do not alter TDH activity but leucine is a potent inducer of TDH activity through transcriptional regulation (Newman et al., 1976). TDH activity was readily detected in CDM-grown LVS lysates but was reduced in lysates from LVS grown in CDM with supplemental 50 mM glycine or 50 mM threonine (Fig. 10A). Furthermore, the addition of 50 mM leucine to CDM failed to increase the relative level of TDH activity (Fig. 10A). Boiled lysate from CDM grown LVS failed to produce any detectable TDH activity, establishing the baseline of this assay and requirement for enzymatic activity in the lysate (Fig. 10A). Some pseudo-revertants of the *glyA* mutation in *E. coli* possess constitutively high levels of TDH activity that circumvent transcriptional regulation (Newman et al., 1976). We thus hypothesized that LVS Δ*glyA* LV may possess higher levels of TDH activity than LVS or be resistant to a reduction in activity following growth in glycine. In contrast to this expectation, LVS Δ*glyA* LV did not possess higher levels of TDH activity in CDM nor was this strain resistant to glycine inhibition of TDH activity (Fig. 10B). TDH activity following growth in BHI was lower in LVS Δ*glyA* LV than LVS Δ*glyA* SV, but both strains were below LVS levels (Fig. 10C). Previously, LVS Δ*glyA* SV was found to possess only a small growth defect in BHI and a moderate to severe defect in CDM with or without supplemental glycine (Fig. 8B-D). Comparison of these strains from lysates grown in either CDM or CDM with 5 mM glycine indicated that, in these conditions, LVS Δ*glyA* LV had slightly higher TDH activity than LVS Δ*glyA* SV (Fig. 10D). Interestingly, both variants had lower levels of TDH activity in CDM

when compared to wild-type LVS (Fig. 10D). Additionally, 5 mM glycine failed to inhibit TDH activity in either LVS Δ*glyA* LV or LVS Δ*glyA* SV (Fig. 10D). Finally, examination of lysates from *F. tularensis* strains grown on chocolate agar revealed slightly higher TDH activity in LVS and LVS Δ*glyA* LV when compared to LVS Δ*glyA* SV (Fig. 10E). Thus, the activity of the TDH was not dramatically different between the LVS Δ*glyA* variants but did appear to be slightly higher in the large variant when cultured in conditions where this strain has fitness advantage over the small variant.

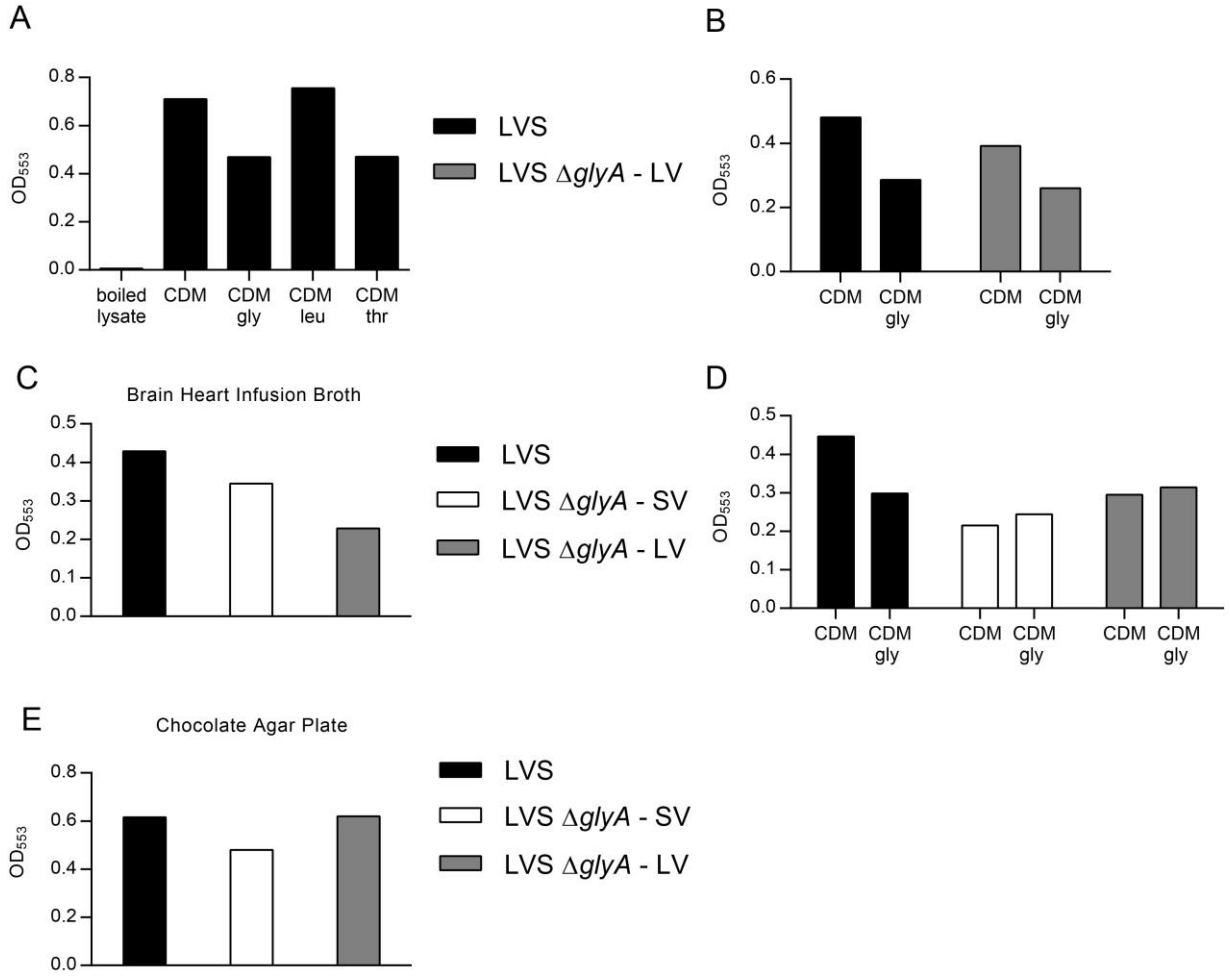

Figure 12. The activity of the threonine dehydrogenase is not dramatically different in the large variant.

(A-D) LVS strains were harvested from overnight cultures of (A) CDM or CDM with 50 mM glycine, leucine, or threonine, (B) CDM or CDM with 50 mM glycine, (C) BHI, and (D) CDM or CDM with 5 mM glycine. (E) LVS

strains were harvested directly off of a chocolate agar plate. Bacteria were harvested from these conditions, washed with PBS, and a crude Francisella protein lysate was prepared by sonication as described in the Materials and Methods. Samples were standardized by OD_{600}. Boiled lysate refers to an LVS protein lysate from a CDM overnight culture that was boiled for 10 minutes to inactivate enzymes. These lysates were utilized in an indirect threonine dehydrogenase assay, which culminated in the relative comparison, colormetrically (OD_{553}), of aminoacetone levels. Aminoacetone is derived proportionally from 2-amino-3-oxobutyrate, the product of the threonine dehydrogenase. This procedure is described in further detail in the Materials and Methods. Data are derived from a single assay.

3.4.6 Mutation of the *glyA* homolog in the *F. tularensis* subsp. *tularensis* Schu S4 strain results in serine auxotrophy but no glycine requirement

To determine if these findings in LVS would translate to *F. tularensis* subsp. *tularensis*, deletion of the *glyA* homolog was performed in the fully virulent Schu S4 strain (Schu S4, FTT_1241). Similar to LVS Δ*glyA* strains and the Schu S4 GCS mutant, Schu S4 Δ*glyA* was a serine auxotroph and could not utilize glycine for serine biosynthesis (Fig. 11A). Interestingly, Schu S4 did not require supplemental glycine for respectable levels of growth without serine, although a dose response could still be observed (Fig. 11A). This was in contrast to LVS and may highlight a metabolic difference between these subspecies (Fig. 6A). Of interest, streaking Schu S4 Δ*glyA* on chocolate agar resulted in colonies that were similar in size to wild-type and no variants could be readily distinguished (data not shown). In addition to chocolate agar, no apparent difference could be detected in CDM broth between Schu S4 and Schu S4 Δ*glyA* (Fig. 11B). An in cis complement (Schu S4 Δ*glyA*:pMB1*glyA*) was capable of restoring serine prototrophy (Fig. 11C). These data suggest that the serine hydroxymethyltransferase is required for serine biosynthesis in both LVS and Schu S4. Furthermore, these findings suggest that our

initial stock of Schu S4 Δ*glyA* appears to mimic, at least with respect to serine and glycine metabolism, the large variant of LVS Δ*glyA*.

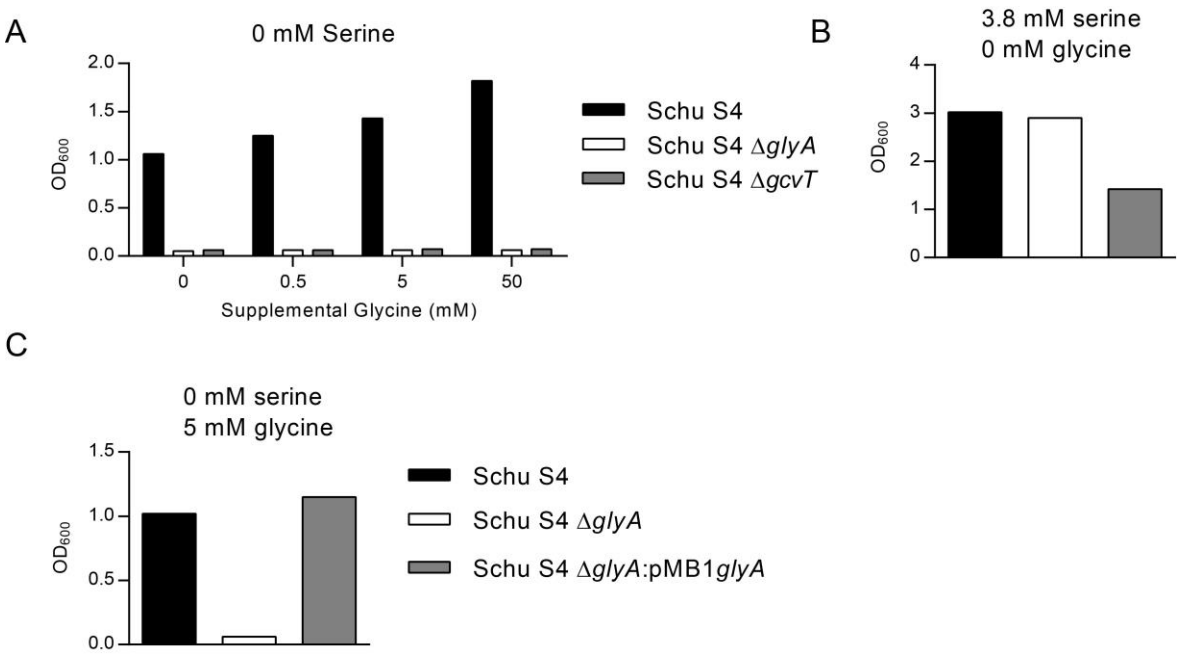

Figure 13. Mutation of the *glyA* homolog in the *F. tularensis* subsp. *tularensis* Schu S4 strain results in serine auxotrophy but no glycine requirement.

Overnight TSB-C cultures of Schu S4 strains were pelleted, resuspended in serine-free CDM and used to inoculate (A) serine-free CDM with various glycine concentrations, (B) standard CDM (3.8 mM serine, 0 mM glycine), or (C) Serine-free CDM with glycine (0 mM serine, 5 mM glycine). All cultures were set to an initial OD_{600} of 0.05 and were measured after approximately 16 hours (A and B) or 19 hours (C) of growth. These data are a single replicate and are derived from a single culture.

3.4.7 The loss of *glyA* in Schu S4 has almost no effect on intramacrophage replication

Due to the similarities between LVS Δ*glyA* LV and Schu S4 Δ*glyA*, we were particularly interested in evaluating the intramacrophage growth phenotypes of the Schu S4 mutant. LVS Δ*glyA* LV is attenuated for intramacrophage replication in murine cells, particularly when the

assay was performed in minimal nutrient conditions (MEM) (Fig. 9). Unexpectedly, Schu S4 Δ*glyA* grew proficiently within mouse macrophages and was similar to Schu S4 in terms of growth and invasion in both DMEM and MEM culture conditions (Fig. 12A and B). The serine hydroxymethyltransferase is thus not required for efficient intracellular replication of Schu S4, regardless of exogenous nutrient availability. Additionally, this result suggests serine biosynthesis is dispensable for intramacrophage growth of Schu S4. Finally, these data phenotypically distinguish the Schu S4 Δ*glyA* strain from both LVS Δ*glyA* variants and suggest underlying metabolic differences between LVS and Schu S4.

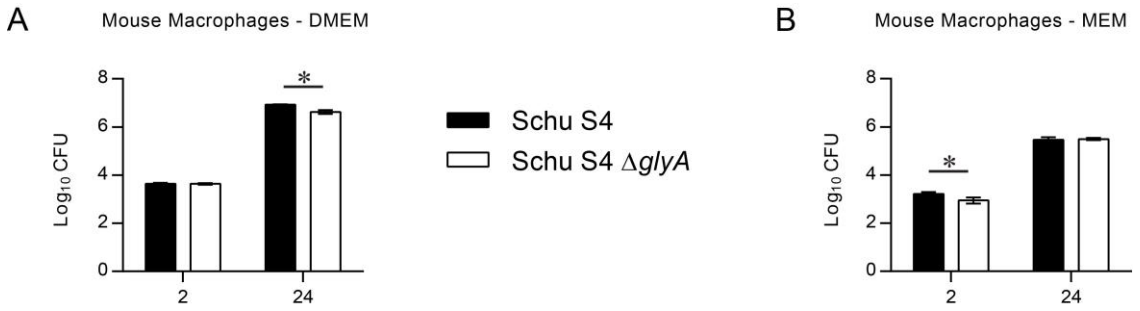

Figure 14. The loss of *glyA* in Schu S4 has almost no effect on intramacrophage replication.

Mouse macrophages were harvested and placed in either rich media (A) (DMEM with 20% FBS, 25 mM HEPES, 2 mM glutaMAX, 1 mM sodium pyruvate, 1 X MEM non-essential amino acids) or minimal media (B) (MEM with 10% FBS, 25 mM HEPES, 2 mM glutaMAX). Overnight TSB-C broth cultures of Schu S4 strains were pelleted, resuspended in either rich media (A) or minimal media (B) and added to cells at an MOI of 500. Bacteria were incubated with cells for two hours before a gentamicin treatment as described in the Materials and Methods. Cells were lysed and the CFU enumerated at the indicated time points. Data represent the mean ± SD value of triplicate wells from one experiment. Statistical significance ($p < 0.05$) for each time point was determined using a two-tailed Student's t-test. A * is used to denote time points where statistical significance was achieved.

3.4.8 Loss of *glyA* results in a modest attenuation in the virulence of Schu S4

The loss of *glyA* in Schu S4 led to serine auxotrophy but had no measurable effects on growth on chocolate agar, in CDM broth, or within murine macrophages (Fig. 11 and 12). These results suggest that the serine hydroxymethyltransferase may be dispensable to *Francisella* pathogenesis. To evaluate the contribution of *glyA* to the virulence of *F. tularensis*, we employed a murine model of pneumonic tularemia. Using this model, infection with Schu S4 led to mortality with a median survival time of approximately six days (Fig. 13). Interestingly, murine mortality following infection with Schu S4 Δ*glyA* was statistically significantly delayed, albeit by a modest one day (Fig. 13). The minor role of *glyA* in the pathogenesis of Schu S4 was nonetheless surprising and defied in vitro results.

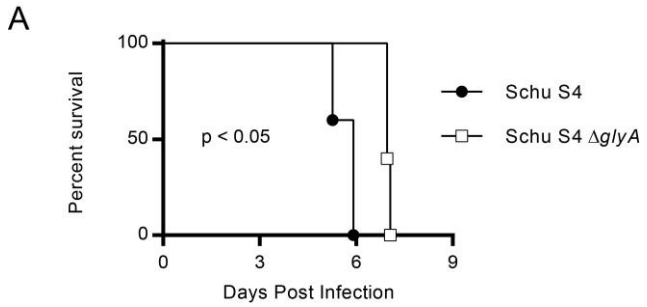

Figure 15. Loss of *glyA* results in a modest attenuation in the virulence of Schu S4.

Overnight TSB-C cultures of Schu S4 strains were pelleted, resuspended in PBS, and used to infect mice. Approximately 100 CFU was intratracheally administered per anesthetized mouse. Following infection, mice were monitored and removed upon reaching a predetermined clinical sickness score. Data represent a single experiment with five mice per group. Statistical significance ($p < 0.05$) was determined by a log-rank test (Mantel-Cox). Data for the wild-type Schu S4 strain are also represented within (Fig. 3) as these experiments were performed concurrently.

3.4.9 Deletion of the serine hydroxymethyltransferase decreases bacterial burden, especially at sites of dissemination

Since Schu S4 Δ*glyA* was a serine auxotroph, we hypothesized that this strain may be at a disadvantage in particular host niches where serine may be limiting. To evaluate this hypothesis, bacterial burden was assessed at multiple host sites four days after infection with Schu S4 or Schu S4 Δ*glyA*. Overall, loss of *glyA* reduced bacterial burden following infection, however the magnitude of this phenotype varied per site (Fig. 14). The initial site of infection, the lung, was also the site with the smallest difference in burden as wild-type and mutant strains were less than one log apart (Fig. 14). Examination of bacterial numbers present in the spleen and liver revealed that Schu S4 reached levels approximately one and a half logs higher than Schu S4 Δ*glyA* (Fig. 14B and 14C). The largest difference in bacterial burden, however, was clearly in the blood of the animals as Schu S4 Δ*glyA* burden was over three logs lower than Schu S4 (Fig. 14D). These data are consistent with the survival data and suggest that a functional *glyA* modestly enhances the virulence of *F. tularensis*. Furthermore, the presence of *glyA* appears to be of great benefit in the blood, of moderate benefit in the liver and spleen, and of minor benefit in the lung.

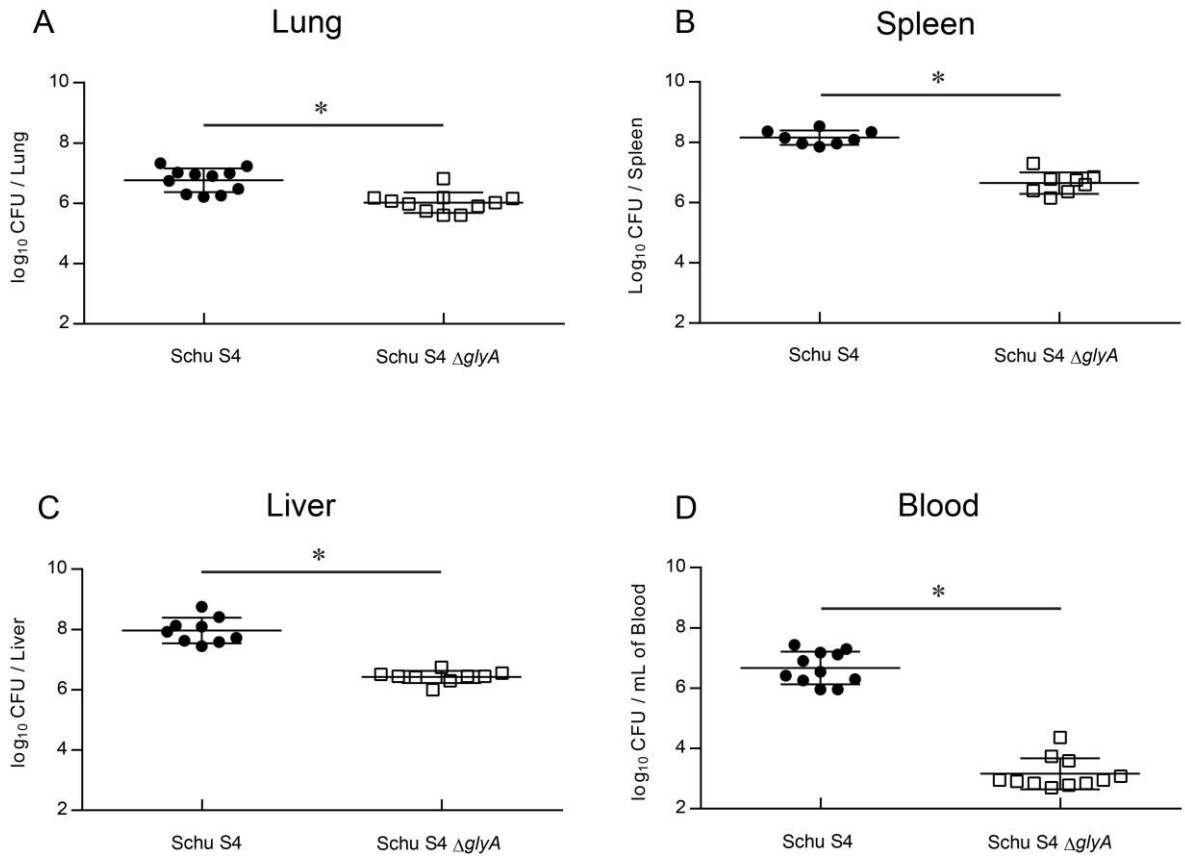

Figure 16. A functional serine hydroxymethyltransferase leads to increased bacterial burden, especially at sites of dissemination.

Overnight TSB-C cultures of Schu S4 strains were pelleted, resuspended in PBS, and used to infect mice. Approximately 100 CFU was intratracheally administered per anesthetized mouse. Four days post infection, the lung (A), spleen (B), liver (C), and blood (D) were harvested, homogenized, and plated to enumerate CFU. Data are the combination of two independent experiments with at least four mice per group per experiment. Each symbol represents the CFU of a single mouse. The line represents the mean ± SD of the combined data set. Statistical significance ($p < 0.05$) was determined using a two-tailed Student's t-test. A * is used to denote host sites where statistical significance was achieved. Data for the wild-type Schu S4 strain are also represented within (Fig. 4) as these experiments were performed concurrently.

3.5 DISCUSSION

Pathogen metabolism varies accordingly with unique life styles and the infection of nutritionally variable and distinct host niches (Alteri and Mobley, 2012; Rohmer et al., 2011). Diversity and redundancy typically precludes rational therapeutic intervention targeting pathogen metabolism broadly (Becker et al., 2006; Steeb et al., 2013). We hypothesized a highly conserved metabolic enzyme known as the serine hydroxymethyltransferase (GlyA) may be an exception to this. Homologs to this protein exist in the vast majority of bacterial pathogens and persist even in the highly reduced genomes of *Rickettsia prowazekii*, *Borrelia burgdorferi*, and *Mycoplasma genitalium* (Andersson et al., 1998; de Crecy-Lagard et al., 2007; Fraser et al., 1995). To evaluate the role of the serine hydroxymethyltransferase in pathogenesis, we investigated the *glyA* homolog of *F. tularensis*. In terms of host niches, *F. tularensis* is promiscuous and exposure to this agent results in a disseminating infection involving multiple cell-types, organs, and even an extracellular bacteremic phase (Forestal et al., 2007; Hall et al., 2008; Horzempa et al., 2010a). Thus, *F. tularensis* was a reasonable choice to determine if *glyA* is important for virulence at multiple host sites, a critical feature in the search for therapeutic targets against diverse pathogens. Our findings suggest that, despite its attractive conservation among pathogens, the serine hydroxymethyltransferase is not essential for the virulence of *F. tularensis*. It is, however, required for serine prototrophy and contributes to pathogenesis in vivo.

The *glyA* homolog of *F. tularensis* was successfully deleted in both LVS and Schu S4, and resulted in a serine auxotrophic strain (Fig. 6 and Fig. 11). This work confirmed our earlier findings with *gcvT* and suggests that *F. tularensis* relies on the GCS and GlyA for serine prototrophy. This pathway facilitates the degradation of glycine to obtain 5'10-mTHF by the

GCS and the subsequent utilization of this 5'10-mTHF to synthesize serine from glycine by SHMT (Ravnikar and Somerville, 1987). This appears to phenotypically distinguish *Francisella* as the loss of *glyA* does not typically result in strict serine auxotrophy (Bogard et al., 2012; Pizer, 1965). Furthermore, the successful deletion and characterization of *glyA* in *F. tularensis* demonstrates that this enzyme is not essential for bacterial viability. This is in contrast to a previous study in *F. tularensis* subsp. *novicida* which labeled *glyA* as a candidate essential gene (Gallagher et al., 2007). This finding may have been a false-positive in this screen, perhaps due to the slow growth phenotype of some *glyA* deficient strains (Fig. 6-8). Alternatively, it remains possible that *glyA* is essential in the *novicida* but not the *holarctica* (LVS) or *tularensis* (Schu S4) subspecies.

While loss of *glyA* was not lethal to *F. tularensis*, two phenotypically distinct variants were identified in LVS Δ*glyA* (Fig. 7). These variants were initially distinguished by their appearance on chocolate agar (Fig. 7). Importantly, these variants were both serine auxotrophs and the primary metabolic distinction between these strains appeared to be related to glycine (Fig. 8). Indeed, the large variant appeared to not require exogenous glycine for growth, while the small variant exhibited very poor growth without supplementation (Fig. 8). Importantly, GlyA is typically a major source of glycine biosynthesis in other bacteria and deficiency in this enzyme often results in glycine auxotrophy (Bogard et al., 2012; Pizer, 1965). Furthermore, previous work on *glyA* mutants in *E. coli* identified spontaneous pseudo-revertants that restored glycine prototrophy (Fraser and Newman, 1975). Based on this study and our results with *F. tularensis*, pseudo-reversion may be a common confounding variable in studies on *glyA*. None theless, these data suggest LVS Δ*glyA* LV is a pseudo-revertant that retains serine auxotrophy while overcoming an exogenous glycine requirement.

Metabolic pathways can be circumvented through a variety of mechanisms, often involving conditional enzymatic redundancy (Patrick et al., 2007). Thus, one plausible mechanism behind the LVS Δ*glyA* LV phenotype involves *glyA*-independent glycine biosynthesis. The upregulation of an alternative glycine biosynthetic pathway in the large variant may facilitate glycine independence while preserving serine auxotrophy. To our knowledge, the only other source of glycine biosynthesis in *F. tularensis* is a pathway involving the threonine dehydrogenase (TDH) (Newman et al., 1976). Thus, we hypothesized that upregulation of TDH may account for the suppression of phenotypes in the LVS Δ*glyA* LV strain. In support of this, similar pseudo-revertants in *E. coli* were found to upregulate this pathway and had up to 60-fold higher levels of TDH activity (Newman et al., 1976) In contrast to our expectations and the previous *E. coli* results, the TDH activity of the *F. tularensis* pseudo-revertant strain was relatively similar to both wild-type and the isolated small variant (Fig. 10). This was true in all conditions tested and suggested that increased TDH activity is not the mechanism of suppression in *F. tularensis*. It remains plausible, however, that the TDH is still involved with this process. For example, it has been proposed that mutations that increase the intracellular pool of threonine may help facilitate the TDH reaction during replication (Fraser and Newman, 1975). This would not alter TDH activity in a bacterial lysate but would provide additional substrate for TDH activity during growth. Ultimately, the mechanistic explanation for the pseudo-reversion of LVS Δ*glyA* LV remains unknown.

As far as we are aware, this is the first report that attempts to distinguish and evaluate a Δ*glyA* pseudo-revertant for in vitro virulence phenotypes. This is a particularly relevant endeavor, as the ability to circumvent a highly conserved gene is not only surprising but also hampers its viability as a drug target. Our findings strongly suggest that the pseudo-revertant

phenotype is relevant during intramacrophage replication as significant differences were found between the large and small variant of Δ*glyA* in all conditions (Fig. 9). The large variant was capable of significantly higher levels of intracellular replication in both primary mouse macrophages and human macrophages in standard culture conditions (Fig. 9A and 9C). This finding was in line with our previous results in broth culture suggesting an enhanced fitness of the large variant (Fig. 8). Interestingly, this pattern did not hold true when the two variants were cultured in nutritionally limiting media (Fig. 9B). In these conditions, the small variant grew significantly better than the large variant, which failed to increase in CFU after invasion (Fig. 9B). This result was somewhat puzzling and suggests that the underlying mechanism of pseudo-reversion is not universally beneficial in all nutritional environments. Further, these results suggest that the pseudo-reversion phenotype is conditionally detrimental and can impede the intracellular replicative capacity of LVS Δ*glyA*. One plausible mechanism may be that the pseudo-revertant aberrantly and uncontrollably degrades essential nutrients. For example, mutations that facilitate the degradation of threonine into glycine may partially repress *glyA* phenotypes but would consequently demand higher threonine concentrations. This increased threonine demand is likely met in standard DMEM culture conditions, where the cells are abundantly supplied with high levels of nutrients. This level of threonine, however, may not be accessible during intramacrophage replication in nutritionally minimal (MEM) culture conditions. Since *F. tularensis* appears to be a threonine auxotroph and threonine is required for protein synthesis, the aberrant degradation of threonine in MEM may be disastrous.

The virulent *F. tularensis* subsp. *tularensis* Schu S4 is metabolically distinct from the attenuated *F. tularensis* subsp. *holarctica* LVS. Studies in broth culture revealed that Schu S4, unlike LVS, was capable of robust growth in serine-free media even without the supplementation

of glycine (Fig. 6A and 11A). This result suggests that Schu S4 is less reliant on exogenous and serine-derived glycine and may have an increased capacity for threonine-derived glycine production. Interestingly, the Schu S4 Δ*glyA* strain was phenotypically identical to the LVS Δ*glyA* LV strain in CDM (Fig. 8 and 11). Indeed, both of these *glyA* deficient strains were serine auxotrophs but had no requirement for exogenous glycine supplementation (Fig. 8 and 11). Two possibilities exist to explain this finding. The first is that our Schu S4 Δ*glyA* isolate is already a pseudo-revertant, while the second is that metabolic differences between LVS and Schu S4 preclude the necessity for reversion. Critically, Schu S4 Δ*glyA* was capable of intramacrophage replication comparable to wild-type in both DMEM and MEM (Fig. 12.) This feature distinguishes this strain from both LVS variants, which have growth defects in either DMEM or MEM (Fig. 9A and B). This strongly suggests that the Schu S4 Δ*glyA* is not simply a pseudo-revertant and is phenotypically distinct as a result of metabolic differences between LVS and Schu S4. Several metabolic differences between these subspecies are known, including differences in iron metabolism and alanine biosynthesis (Miller et al., 2013) (Lindgren et al., 2011). Despite these known differences, no clear explanation is available to account for the reduced glycine requirement of the Schu S4 strain in CDM. It remains plausible that the regulation and expression level of glycine biosynthetic genes differs in Schu S4 and favors threonine degradation. In addition to the bacterial side of metabolism, these subspecies may differ in their manipulation of host metabolism. Indeed, Schu S4 utilizes the recently discovered host mechanism known as ATG5-independent autophagy to acquire nutrients while LVS appears to rely on ATG5-dependent processes during intracellular replication (Steele et al., 2013). Although further studies are needed, these subspecies clearly differ in their strategies of nutrient acquisition and thus may encounter different metabolic environments during intramacrophage

replication. These differences may contribute to the distinct intracellular growth phenotypes of LVS and Schu S4 *glyA*-deficient strains observed in this study.

The primary goals of this study were to assess the role of *glyA* in the metabolism of *F. tularensis* and to determine if this conserved enzyme is fundamental for pathogenesis throughout the host. While loss of *glyA* resulted in in vitro attenuation of intracellular growth in LVS, *glyA* appeared largely dispensable for intracellular replication of Schu S4 (Fig. 8 and Fig. 12). Recent studies have challenged the predicative value of in vitro intramacrophage replication and several strains have been found to be attenuated in the absence of intramacrophage growth defects (Alkhuder et al., 2010; Russo et al., 2011). Further, a strain that appeared entirely incapable of intramacrophage replication was only modestly attenuated in vivo (Horzempa et al., 2010a). Despite this possibility, *glyA* only modestly contributed to the in vivo virulence of *F. tularensis* (Fig. 13). Of note, *glyA* contributed in a site-specific manner to bacterial burden and provided the largest fitness advantage in the blood (Fig. 14). Since *Francisella* is primarily extracellular in the blood, this may be due to the lower levels of amino acids in plasma in comparison to the cytosol (Bergstrom et al., 1974; Canepa et al., 2002; Forestal et al., 2007). Interestingly, *glyA*-mediated metabolism also appeared to contribute to fitness moderately in the liver and spleen and slightly in the lung (Fig. 14). This suggests that while serine biosynthesis and other *glyA* reactions do not contribute to in vitro intracellular growth, they may contribute to replication in vivo. Alternatively, the reduced burden at this time may simply be a result of the greater than three log reduction in bacteremia. Overall, serine auxotrophy as a result of loss of *glyA* may significantly impair bacteremia but ultimately appears largely inconsequential to the lethality of *F. tularensis*.

While potentially attractive therapeutic targets, the requirement of many metabolic pathways for pathogenesis at nutritionally diverse host niches remains unknown. To our knowledge, this study represents the first time the bacterial serine hydroxymethyltransferase (*glyA*) has been characterized systemically during infection. Utilizing *F. tularensis* as a model, the role of *glyA* at various host niches was observed. This metabolic enzyme contributed to pathogen fitness minutely in the lung, moderately in the liver and spleen, and greatly in the blood. As the *glyA* of *F. tularensis* was found to be essential for serine prototrophy, these findings may fail to translate to pathogens with redundant serine biosynthetic pathways. It may remain feasible, however, to target redundant mechanisms of serine biosynthesis with the goal of inducing serine auxotrophy. Despite the conserved nature of *glyA* and its contribution to bacteremia, this enzyme proved to be ultimately dispensable for the lethality of *F. tularensis*. Thus, while the targeting of *glyA* and serine biosynthesis may cripple pathogens that facilitate lethality through bacteremia, this provides little reprieve to infections at other sites. This study contributes to the growing understanding of host-pathogen metabolic interactions and underscores the importance of examining the contribution of bacterial metabolism at multiple host niches.

4.0 SUMMARY AND DISCUSSION

In order to grow successfully and replicate, living organisms must acquire nutrients from their surroundings. For bacterial pathogens, this environment is often one of a multitude of host niches. Pathogens must therefore possess the metabolic pathways required to exploit and thrive within their respective host site. As anatomical locations are nutritional distinct, it stands to reason that pathogens that colonize different host niches will require different metabolic pathways (Rohmer et al., 2011). Despite this complexity and the obvious importance of metabolism, the host-pathogen interaction over nutrients lacks sufficient clarity. Further, little is known in regards to which metabolic pathways contribute to pathogen fitness in these variable host environments. Increased knowledge of bacterial metabolism during infection may provide useful insights for rational therapeutic interventions (Zhang and Rubin, 2013). For instance, a specific niche, such as the lung, may enforce a particular metabolic requirement on the pathogen and therapeutics targeting this avenue would not only be effective but may also spare other sites with beneficial commensals, such as the gut. In contrast, certain metabolic pathways would be expected to be beneficial or essential in multiple environments, providing targets for systemic infections. Overall, the metabolic pathways that contribute to pathogen fitness require further investigation, specifically in regards to the nutritional diversity of host sites.

Francisella tularensis is an intracellular pathogen and the causative agent of a systemic debilitating disease known as tularemia (Francis, 1925). Utilizing this bacterium as a model,

the contribution of a metabolic pathway can be assessed during intracellular infection of multiple cell-types, of various organs (lung, liver, spleen), and during an extracellular bacteremic phase (Forestal et al., 2007; Hall et al., 2008; Horzempa et al., 2010a). Thus, evaluation of the role of metabolism in pathogen fitness using *F. tularensis* provides a distinct advantage over other less promiscuous pathogens with narrow host niche specificity. Taking advantage of this, I examined the contribution of one-carbon metabolism to the pathogenesis of *F. tularensis*. One-carbon metabolism involves the generation of 5'10-methylenetetrahydrafolate (5'10-mTHF) and its utilization for DNA synthesis, protein translation, and the production of various amino acids (Dev and Harvey, 1982; Kikuchi et al., 2008). 5'10-mTHF is the product of two metabolic pathways within one-carbon metabolism, the glycine cleavage system (GCS) and the serine hydroxymethyltransferase (SHMT) (Dev and Harvey, 1982; Kikuchi et al., 2008; Pizer, 1965). Previously, the GCS of *F. tularensis* was found to be transcriptionally upregulated during intramacrophage replication, to be induced at the protein level in bacteria harvested from mouse spleen, and has been identified in an in vivo negative selection screen (Twine et al., 2006; Wehrly et al., 2009; Weiss et al., 2007). The SHMT enzyme of *F. tularensis* was listed as a candidate essential gene but also has been loosely linked to intramacrophage replication and in vivo virulence by transposon mutagenesis in the *novicida* subspecies (Tempel et al., 2006; Weiss et al., 2007). These indirect screens suggested one-carbon metabolism may contribute to *Francisella* virulence. As such, direct investigation of the role of one-carbon metabolism in *F. tularensis* pathogenesis was warranted to establish the pathogenic value of this pathway throughout the host.

One potential source of 5'10-mTHF, the one-carbon donor that is at the core of one-carbon metabolism, is through GCS-mediated glycine degradation (Dev and Harvey, 1982;

Kikuchi et al., 2008). In chapter 2, the contribution of the GCS to *Francisella* metabolism, in vitro virulence, and in vivo pathogenesis was investigated. Mutagenesis of the glycine cleavage system protein T (*gcvT*), an essential member of this pathway, was performed in LVS, an attenuated strain, and Schu S4, a fully virulent strain. Through the utilization of these mutants, it was revealed that the GCS was required for serine prototrophy in both LVS and Schu S4 (Fig. 1). Further, it was demonstrated that, despite its effects on metabolism, the GCS of *F. tularensis* had no role in cellular invasion, intracellular replication, complement resistance, or immune stimulation when evaluated in standard tissue culture medium (Fig. 2 and Appendix A and B). In contrast to the unimportance of the GCS in our initial in vitro virulence assays, the GCS was found to contribute to pathogenesis in a murine model of pneumonic tularemia (Fig. 3). Strains deficient in *gcvT* resulted in significantly lower bacterial burden in the spleens, liver, and blood of infected animals (Fig. 4). In order to reconcile the in vivo and in vitro results, I evaluated the role of the GCS during in vitro intracellular replication in a minimal culture medium (Fig. 5). In this environment, the GCS significantly contributed to the intracellular replication of *F. tularensis* in a serine-dependent fashion (Fig. 5). Overall, the work performed in chapter 2 characterized a previously unappreciated contribution of the GCS to the pathogenesis of *F. tularensis*. To our knowledge, this is the first time the GCS has been found to contribute to bacterial pathogenesis during mammalian infection. Finally, this chapter also raises an important cautionary note regarding nutritionally replete tissue culture medium employed in most standard in vitro assays.

In addition to the GCS-mediated glycine degradation, 5'10-mTHF can also be generated through SHMT-mediated serine degradation (Dev and Harvey, 1982; Pizer, 1965). In chapter 3, the role of the SHMT in *F. tularensis* metabolism and virulence was assessed. Successful

mutagenesis of the SHMT (*glyA*) homolog in LVS and Schu S4 revealed that this gene was clearly not required for viability. The *glyA* gene was, however, required for serine prototrophy in both LVS and Schu S4 (Fig. 6, 8, and 11). Furthermore, loss of *glyA* in LVS appeared to result in an increased glycine requirement that was frequently suppressed by pseudo-reversion (Fig. 6-8). The *glyA*-deficient variants in LVS had distinct phenotypes in regards to intracellular replication as pseudo-reversion proved to be beneficial in nutritionally rich media, while detrimental in nutritionally minimal media (Fig. 9). The mechanism of pseudo-reversion remains unknown, but does not appear to directly involve manipulation of threonine dehydrogenase activity (Fig. 10). In contrast to LVS, deletion of *glyA* in Schu S4 did not result in variant formation and failed to alter the bacterium's glycine requirement or level of intracellular replication in either nutritionally rich or nutritionally minimal culture media (Fig. 11 and 12). The contribution of *glyA* to the pathogenesis of virulent *F. tularensis* was found to be quite modest in a mouse model of pneumonic tularemia (Fig. 13). While ultimately only contributing modestly to mortality, an intact *glyA* was associated with increased bacterial burden at all host sites (Fig. 13). The magnitude of this phenotype varied per site, however, with a minor effect on lung burden, a moderate effect on spleen and liver burden, and a large effect on bacteremia (Fig. 13). The findings of chapter 3 thus clarify the essentiality of *glyA* while providing insight into the contribution of this gene to *F. tularensis*. To our knowledge, this study represents the first time *glyA* has been characterized at various host sites during a systemic bacterial infection. The capacity of this bacterium to maintain close to wild-type virulence upon the loss of *glyA* also highlights an unexpected metabolic robustness of *F. tularensis*.

One of the most basic findings of this work is that disruption of either the GCS or the SHMT leads to serine auxotrophy in *F. tularensis* (Fig. 1 and 6). This result suggests that *F.*

tularensis exclusively utilizes a system known as the threonine utilization (Tut) cycle to facilitate serine production (Ravnikar and Somerville, 1987). This pathway begins with the degradation of threonine to 2-amino-3-oxobutyrate by the threonine dehydrogenase (TDH), which is subsequently converted to glycine by a 2-amino-3-oxobutyrate CoA ligase (Ravnikar and Somerville, 1987). The glycine derived by this process is utilized in two down-stream reactions. First, a portion of this glycine pool is degraded into 5'10-mTHF, carbon dioxide, and ammonia by the GCS (Ravnikar and Somerville, 1987). Secondly, remaining glycine is combined with 5'10-mTHF to generate serine, a reaction facilitated by SHMT (Ravnikar and Somerville, 1987). This cycle was initially described as an alternative serine biosynthetic pathway in *E. coli* and could only be assessed in the absence of the major serine biosynthetic pathway, *serABC* (Ravnikar and Somerville, 1987; Umbarger et al., 1963). This system likely explains the ability of *F. tularensis* to grow, albeit somewhat poorly, in CDM lacking both serine and glycine, as both of these can be derived from threonine (~17 mM in standard CDM) with the Tut cycle (Fig. 1) (Chamberlain, 1965). To our knowledge, this is the first time the Tut cycle has been implicated as the primary and exclusive serine biosynthetic pathway in a bacterial pathogen. Interestingly, these results defied our initial expectations. Members of the *F. tularensis* subsp. *holarctica*, such as LVS, have intact homologs to *serA* and *serC* but harbor a frameshift mutation in the *serB* homolog (Champion et al., 2009). In contrast, members of the *F. tularensis* subsp. *tularensis*, such as Schu S4, are annotated to possess intact homologs of *serABC* (Champion et al., 2009; Larsson et al., 2005). Despite this difference, both strains required the GCS and SHMT to maintain serine prototrophy, indicating that these proteins were, at least in these assay conditions, failing to contribute to serine biosynthesis (Fig. 1 and 6). Future studies should be devoted to understanding the contribution of the *serA* and *serC* homolog in *F. tularensis*. While

the data presented herein suggest that these proteins do not produce serine, it is plausible that they contribute to other metabolic reactions. In fact, *serA* and *serC* have been found to contribute to certain mechanisms of pyridoxine (vitamin B6) synthesis (Lam and Winkler, 1990). Pyridoxine biosynthesis has been found to contribute to the virulence of bacterial pathogens and ultimately this may provide a rational explanation as to the presence of these proteins in *F. tularensis* (Grubman et al., 2010). Only the highly virulent subspecies, *tularensis*, possesses an intact *serB* homolog (Champion et al., 2009). Intriguingly, a bacterial homolog of *serB* has recently been identified as directly suppressing host cell immune signaling, independent of any role in metabolism (Takeuchi et al., 2013). Given the abundant mechanisms of immune modulation in *Francisella*, it is tempting to postulate that the homolog of *serB* in the *tularensis* subspecies may function in a similar fashion. This gene thus warrants future investigation and should be evaluated for a possible non-metabolic role in *F. tularensis* subsp. *tularensis*. In conclusion, the Tut cycle is the sole serine biosynthetic pathway in *F. tularensis*.

While investigating one-carbon metabolism, several metabolic differences between LVS and Schu S4 were noted: 1) Schu S4 grew substantially better than LVS in CDM lacking serine and glycine (Fig. 1, 6, and 11); 2) LVS exhibited a diauxic shift in CDM, which was alleviated with supplemental glycine, while Schu S4 exhibited no such shift (Fig. 1); 3) loss of the GCS resulted in a growth defect in CDM in both strains but could be completely restored by 25 mM serine in only Schu S4 (Fig. 1); 4) loss of SHMT in LVS resulted in poor growth on chocolate agar and in CDM broth, which was frequently suppressed by a pseudo-revertant, while no such defects were present in Schu S4 (Fig. 7, 8, and 11). Interestingly, no clear genetic difference exists between these strains that would explain these phenotypes and the definitive mechanism remains unknown. Hypothetically, however, all of these phenotypes could be explained by an

increase in glycine acquisition or a decrease in glycine requirement. Thus, future studies should compare these strains by examining the contribution of the TDH, which supplies glycine through threonine degradation (Fraser and Newman, 1975). To our knowledge, this enzyme represents the only other source of glycine besides SHMT in *F. tularensis* and may thus be required to facilitate differences between LVS and Schu S4. Ultimately, even if the TDH is required for these differences, it may prove challenging to rationally identify the exact mechanism behind this. Alterations in intracellular amino acid content, amino acid transport, or feed-back inhibition could all alter TDH functionality without affecting the level of TDH transcripts or protein. Therefore, it may be more suitable to investigate these differences through a genome-wide assay, such as a microarray, comparing LVS and Schu S4 in CDM broth. Finally, since the pseudo-revertant of LVS appears to possess a SchuS4-like glycine requirement, genome sequencing of this variant may provide valuable insight into this phenomenon.

One-carbon metabolism provided a pathogenic fitness advantage in *F. tularensis* which varied substantially between host niches. In a mouse model of pneumonic tularemia, one-carbon metabolic reactions were required for wild-type kinetics of mortality as the loss of either the GCS or the SHMT delayed mortality (Fig. 3 and 13). Taking advantage of the systemic nature of this infection, distinct host sites including the lung, liver, spleen, and blood of infected animals were evaluated. Enumeration of bacterial burden at various host sites indicated that loss of either 5'10-mTHF producing branch (GCS or SHMT) led to a negligible effect in the lung, a moderate decrease in the spleen and liver, and a pronounced reduction in the blood (Fig. 4 and 14). It is likely that the large reduction in bacterial burden in the blood is the result of *F. tularensis* being primarily extracellular in this compartment (Forestal et al., 2007). Indeed, the nutritional content of the blood is significantly below that of the cytosol, a fact that is likely to

put great stress on a metabolic mutant (Bergstrom et al., 1974; Canepa et al., 2002). While this ultimately may rationalize the drastic reduction in the blood, it fails to explain the difference between organs. It is intriguing that the lung represents an environment where the loss of GCS or SHMT in *F. tularensis* has little consequence during infection (Fig. 3 and 14). It is plausible that host proteases which are known to be activated in the lung during *Francisella* infection, provide a nutritional source to a bacterium capable of degrading host peptides (Alkhuder et al., 2009; Malik et al., 2007). Although it is somewhat unlikely, it is also plausible that the infected host cells of the lung possess a distinct nutritional content that accommodates GCS or SHMT deficient *F. tularensis* strains. Overall, a definitive mechanism for this site specific contribution remains to be identified but is likely linked to the general availability of nutrients at each site.

Since one-carbon metabolic reactions may be contributing to the synthesis of glycine, serine, purines, thymidine, pantothenate, and formylmethionyl-tRNA in *F. tularensis,* identifying the attenuating metabolic defects in GCS or SHMT deficient strains may prove complex and challenging (Guillon et al., 1992; O'Donovan and Neuhard, 1970; Ravnikar and Somerville, 1987; Stauffer and Brenchley, 1978; Webb et al., 2004; Weissbach and Brot, 1991; Zhang et al., 2008). However, evaluation of the available information on *F. tularensis* metabolism provides some indication of the relative importance of these metabolites. For instance, a defect in pantothenate production is not likely as a contributing factor since a Schu S4 pantothenate auxotroph is fully virulent during murine infection (Miller et al., 2013). *F. tularensis* Schu S4 strains that are purine auxotrophs are entirely avirulent in mouse models and so it remains plausible that a reduction in purine biosynthesis could be associated with the phenotypes of GCS or SHMT deficient strains (Pechous et al., 2008). In regards to amino acid biosynthesis, our studies indicate that one-carbon metabolic reactions are essential to serine prototrophy in *F.*

tularensis (Fig. 1, 6 and 8). To our knowledge, no direct assessment of thymidine or formylmethionyl-tRNA biosynthesis has been performed in *Francisella*. Future studies could address the contribution of these compounds to *F. tularensis* virulence by mutagenesis. For instance, genetic disruption of thymidylate synthase and methionyl-tRNA formyltransferase would be expected to result in thymidine auxotrophy and loss of formylation of methionyl-tRNA respectively (Guillon et al., 1992; O'Donovan and Neuhard, 1970). Thus, while it is clear that loss of the GCS or the SHMT leads to serine auxotrophy, the effect on other metabolites remains unknown. Determining the contribution of these down-stream metabolites will help clarify the role of one-carbon metabolism in *F. tularensis* pathogenesis.

While the two branches of 5'10-mTHF production are both required for the full virulence of *F. tularensis*, the GCS provides greater pathogenic fitness to *F. tularensis* than SHMT. Upon comparison of the mutant strains (Schu S4 Δ*gcvT* v.s. Schu S4 Δ*glyA*), the loss of the GCS was found to result in a greater delay in mortality in our murine model (Fig. 3 and 13). Further, while these mutant strains were indistinguishable in the lung and spleen, Schu S4 Δ*gcvT* infected animals had at least a half log lower bacterial burden in the liver and blood (Fig. 4 and 14). This finding suggests that hepatic burden and bacteremia may be useful predictors of lethality from *F. tularensis* infection in this mouse model. Interestingly, this inequality suggests that *F. tularensis* preferentially utilizes GCS-mediated glycine degradation as a source of 5'10-mTHF in the liver and blood of the host instead of SHMT-mediated serine degradation. In the blood, this possibility is supported by the availability of these amino acids, as the levels of glycine are higher than those of serine in plasma (Sumiyoshi et al., 2004). Thus, in the nutritionally limited environment of the blood, a higher relative availability of glycine over serine may favor GCS activity. Furthermore, it is plausible that *F. tularensis* obtains glycine from the host indirectly.

For example, *F. tularensis* has been found to uptake and degrade the host molecule, glutathione (Alkhuder et al., 2009; Ireland et al., 2011). While these studies focused on the acquisition of cysteine from glutathione degradation, it is important to recognize that degradation of glutathione also provides glycine to the bacterium (Alkhuder et al., 2009; Ireland et al., 2011). Importantly, glutathione is present in the plasma and is also abundant specifically in the liver (Michelet et al., 1995; Wu et al., 2004). Thus, it would not be surprising that 5'10-mTHF production at these sites favors degradation of the more abundant amino acid, glycine. It is also possible that the increased attenuation of Schu S4 Δ*gcvT* is associated with serine utilization. While the Schu S4 Δ*gcvT* and Schu S4 Δ*glyA* strains are both serine auxotrophs, they may have different serine requirements (Fig. 1, 6, and 8). For example, Schu S4 Δ*gcvT* must acquire sufficient exogenous serine for both protein synthesis and 5'10-mTHF production through SHMT-mediated serine degradation. In contrast, Schu S4 Δ*glyA* only requires exogenous serine to synthesize proteins and must rely on GCS-mediated glycine degradation to supply 5'10-mTHF. The dual-use of serine for protein and 5'10-mTHF synthesis upon GCS deficiency would be expected to further hobble a serine auxotroph in serine-limiting environments, such as the host's blood. This hypothesis is further supported by the observation that Schu S4 Δ*gcvT* is strongly attenuated for intracellular replication when the assay is performed in serine-limiting culture medium, while Schu S4 Δ*glyA* exhibits no such defect (Fig. 5 and 12). Regardless of the exact mechanism behind this inequality, it is clear that one-carbon metabolic products provide a fitness advantage in vivo and their presence is required for wild-type levels of bacterial burden.

Future studies should examine the feasibility of creating a *F. tularensis* strain deficient in both the GCS and SHMT. If obtainable, this double mutant would provide a useful tool to confirm several hypotheses. For one, this strain would allow confirmation that the GCS and the

SHMT pathways are the only sources of 5'10-mTHF in *F. tularensis*. If this is the case, this strain should be a serine auxotroph, a thymidine auxotroph, a pantothenate auxotroph, lack de novo purine biosynthesis, and lack formylation of methionyl-tRNA (Guillon et al., 1992; O'Donovan and Neuhard, 1970; Ravnikar and Somerville, 1987; Stauffer and Brenchley, 1978; Webb et al., 2004; Weissbach and Brot, 1991; Zhang et al., 2008). Further, since *Francisella* strains deficient in de novo purine biosynthesis are completely attenuated, a Schu S4 strain lacking both these pathways would be expected to also be completely attenuated (Pechous et al., 2008). This warrants particular interest since neither GCS-deficient nor SHMT-deficient Schu S4 strains are attenuated for lethality (Fig. 3 and 13). Ultimately, any discrepancies in these results may indicate unique features in *F. tularensis* metabolism or possible novel nutritional host-pathogen interactions. Of interest, initial attempts to generate a double deletion of these pathways in LVS using homologous recombination have been unsuccessful. Since this strain is expected to be auxotrophic for several nutrients, it is plausible that the double deletion is not obtainable on standard chocolate agar. Future attempts should thus focus on generation of this strain on supplemented chocolate agar containing abundant purines, thymidine, serine, glycine, and pantothenate. In support of this approach, a mutant deficient in both of these pathways has been recently reported in *E. coli* and was maintained with thymidine supplementation (Waller et al., 2010).

The knowledge gained from studying one-carbon metabolism in *F. tularensis* may be of medical benefit. While inhibition of either the GCS or SHMT did not ablate lethality following murine infection, it resulted in an over three log reduction in bacteremia (Fig. 3,4,13 and 14). Interestingly, human lethality from tularemia is substantially slower than mouse lethality and uncontrolled bacteremia has been implicated as the primary cause of death in human disease

(Foshay, 1937). Mutation of either the GCS or SHMT may thus be useful in the generation of a safe rationally attenuated vaccine strain. Additionally, pharmacological disruption of one-carbon metabolism in *F. tularensis* may provide a directed therapy against bacteremia and lethality in humans. Therapeutics that target host one-carbon metabolism and related folate metabolism are currently being developed as cancer treatments and these therapies should be evaluated for efficacy against bacterial metabolism (Locasale, 2013). Furthermore, the results of this work strongly highlight the nutritional stress on bacteria that are extracellular in the host's blood. To our knowledge, our work on one-carbon metabolism is the first to suggest that serine auxotrophy is associated with a dramatic limitation of bacteremia. Since many bacteria possess not only one-carbon metabolism but also a glycolytic serine biosynthetic pathway *serABC*, a combinatorial approach will likely be required to achieve serine auxotrophy. Thus, future studies should initially evaluate whether or not other pathogens are similarly attenuated by serine auxotrophy in bacteremia models. Since bacteremia caused by antibiotic resistant strains is a major public health concern and novel antibiotics are desperately needed, this weakness should be thoroughly examined for possible exploitation (Wisplinghoff et al., 2004).

The strengths of this study include the use of two *F. tularensis* subspecies, the generation and utilization of deletion mutants with accompanying genetic complementation, and the robustness of the in vivo phenotypes. Limitations include the lack of biochemical and enzymatic assays for the GCS and SHMT and the use of a mouse model of pneumonic tularemia. While mice are the most common animal model for tularemia, one must acknowledge that they are far more susceptible to *F. tularensis* infection than humans (Stundick et al., 2013). In light of these strengths and weaknesses, the contribution of one-carbon metabolism to the pathogenesis of *F. tularensis* was assessed. The GCS and SHMT of *Francisella* were functionally required for

serine biosynthesis in our studies. This establishes the first time, to our knowledge, that one-carbon metabolism has been linked to serine prototrophy in a bacterial pathogen. Further, this body of work identifies the first example of the GCS providing a pathogenic fitness advantage to a bacterium in a mammalian infection model. SHMT was found to provide only a modest advantage to *F. tularensis* during infection, a contribution weaker than that of the GCS. As far as we are aware, the SHMT has previously always been the predominant source of bacterial 5'10-mTHF. Ultimately, the GCS and SHMT represent metabolic pathways that enhance the fitness of *F. tularensis* during infection. Critically, the utilization of an *F. tularensis* infection model has revealed a site-specific role for one-carbon metabolism. Indeed, the GCS and SHMT played a major role in bacteremia, contributed moderately to splenic and hepatic bacterial burden, and appeared largely inconsequential in the lungs. These findings highlight the value of investigating the metabolic interaction between host and pathogen at nutritionally distinct host niches. Future studies must be designed to continue to strengthen our understanding of bacterial metabolism during mammalian infection with the hopes of exploiting this critical interaction.

APPENDIX A

CYTOKINE RELEASE FOLLOWING EXPOSURE TO *F. TULARENSIS* STRAINS

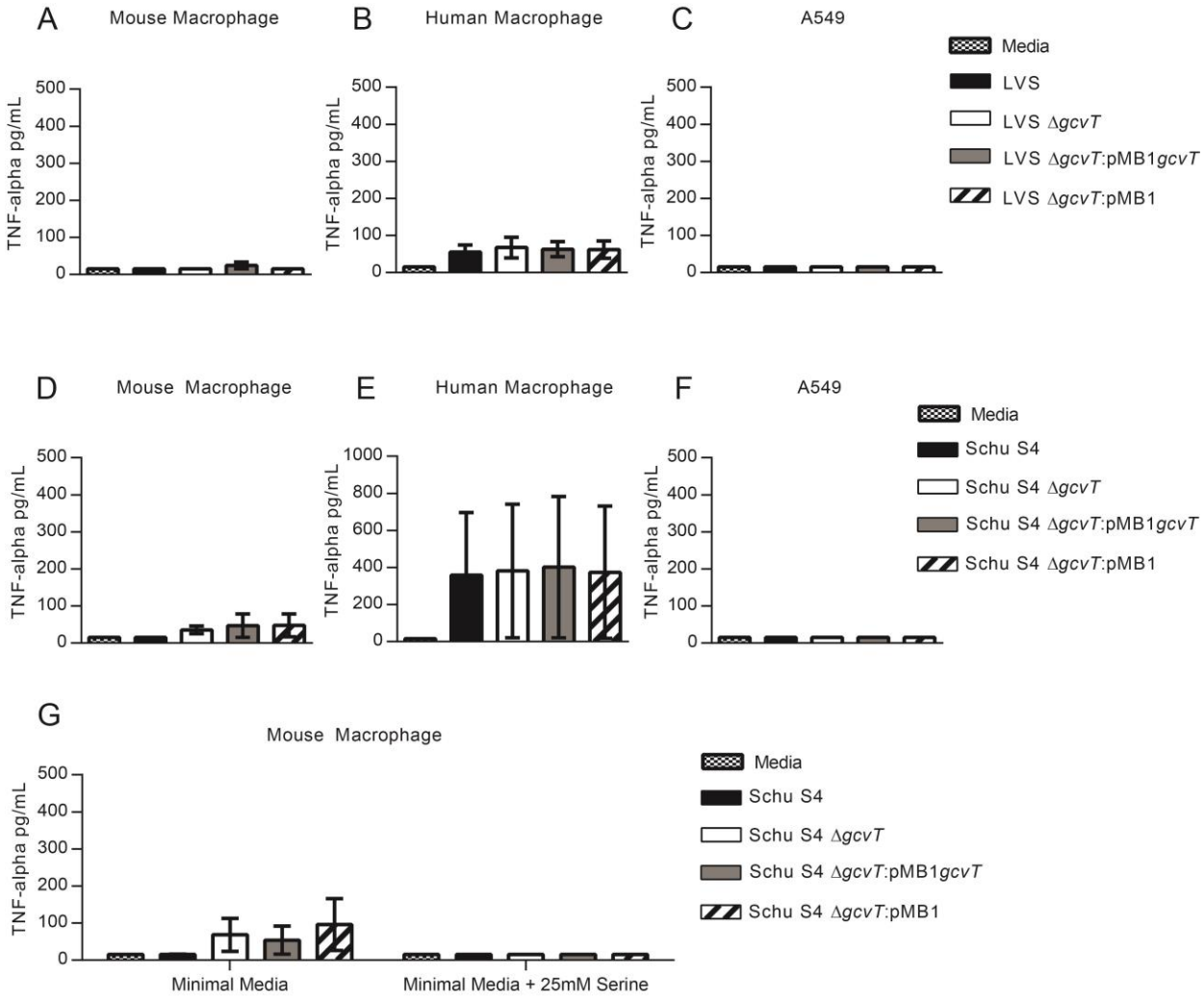

Figure 17. The glycine cleavage system of *F. tularensis* is not required to limit TNF-alpha release in vitro.

Overnight TSB-C broth cultures of LVS (A-C) and Schu S4 strains (D-F) were pelleted, resuspended in PBS, and added to mouse macrophages (A,D,G), human macrophages (B,E), or A549 cells (C,F) at a MOI of 10. Bacteria were incubated with cells for approximately 24 hours in either 20% FBS, 25 mM HEPES, 2 mM glutaMAX, 1 mM sodium pyruvate, and 1 X MEM non-essential amino acids (A,D), 1% AB human serum, DMEM, 2 mM glutaMAX, and 25 mM HEPES (B,E), Ham's F-12 (Kaighn's modification) with 10% FBS and 25 mM HEPES (C,F) or Minimal Essential Media (MEM, Gibco) with 10% FBS, 2 mM glutaMAX, and 25 mM HEPES (G). After this incubation, supernatants were harvested and an ELISA was performed to determine the concentration of TNF-alpha. The Human TNF-alpha ELISA was performed using DuoSets (R&D Systems) while the murine TNF-alpha ELISA employed a matched antibody pair (eBiosciences). The limit of detection of these assays was approximately 15 pg/mL. Values below this concentration were set at the limit of detection. Data are expressed as the mean ± SEM of at least two independent experiments. No significant difference ($p < 0.05$) was detected between strains in any cell type or culture condition by one-way ANOVA followed by a Bonferroni multiple comparison correction.

APPENDIX B

COMPLEMENT RESISTANCE OF *F. TULARENSIS* STRAINS

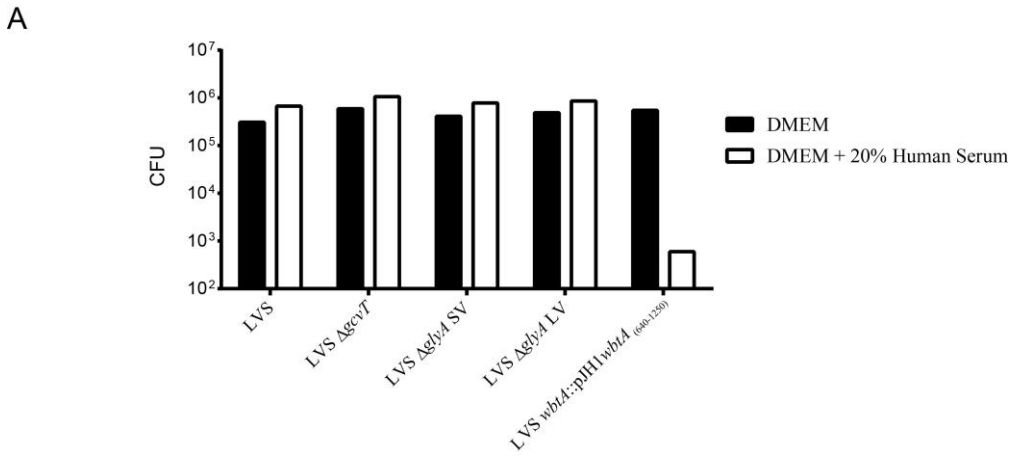

Figure 18. The glycine cleavage system and the serine hydroxymethyltransferase do not contribute to complement resistant in *F. tularensis*.

Overnight TSB-C broth cultures of LVS strains (A) were pelleted, resuspended in DMEM, and diluted to approximately 1 x 10^6 bacteria/mL in either DMEM or DMEM with 20% AB human serum (Complement-Replete Gem Cell; Gemini Bio-Products). Bacteria were then incubated at 37 °C with shaking (250 RPM) for approximately one hour. After this period, cultures were serially diluted and plated as drips on chocolate agar. CFU were enumerated by visual inspection following colony formation. LVS *wbtA*::pJH1*wbtA*$_{(640\text{-}1250)}$ was generated by Dr. Joseph Horzempa and contains a disruption of *wbtA*. This disruption was created through the genomic integration of a suicide vector, pJH1*wbtA*$_{(640\text{-}1250)}$, which contains internal homology to the *wbtA* gene of LVS (nucleotide 640 to 1250). As disruption of *wbtA* results in loss of the O-antigen capsule, the primary mechanism of complement resistance in *F. tularensis*, this strain serves as a positive control. These data are from a single experiment.

APPENDIX C

GENETIC CONFIRMATION OF MUTAGENESIS

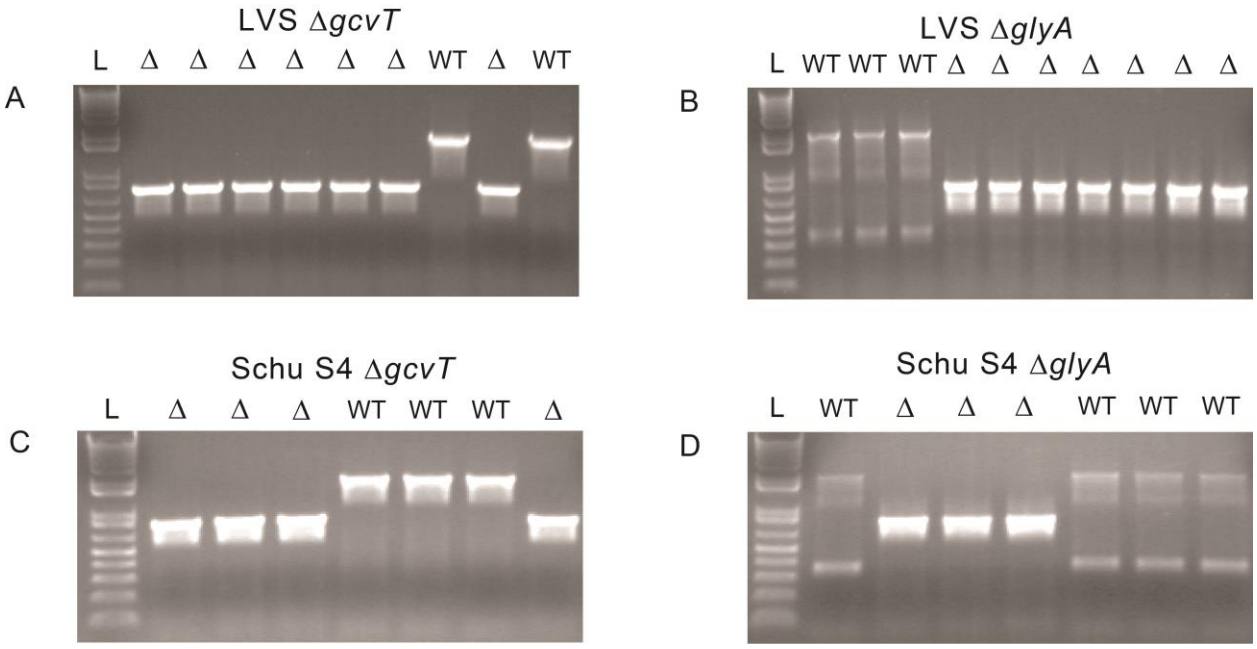

Figure 19. PCR analysis of candidate deletion mutants.

Screening of candidate deletion mutants in LVS (A-B) and Schu S4 (C-D) was performed on isolated colonies following resolution of merodiploids as previously described (Horzempa et al., 2010b). Primers (see Materials and Methods in Chapter 2 (*gcvT*) and Chapter 3 (*glyA*)) were designed flanking *gcvT* (523 bp upstream, 108 bp downstream) and *glyA* (539 bp upstream, 118 bp downstream). For *gcvT* (A, C), a wild-type locus is expected to amplify a 1,708 bp product and a mutant locus is expected to amplify a 719 bp product. For *glyA* (B, D), a wild-type locus is expected to amplify a 1,911 bp product and a mutant locus is expected to amplify a 741 bp product. In some

instances, *glyA* primers yielded a non-specific band of approximately 350 bp in addition to the expected wild-type product size. Of note, this banding pattern was also observed with wild-type LVS genomic DNA (data not shown). Determination of resolution pattern is indicated above each well (WT = wild-type, Δ = deletion mutant, L = 1 Kb Plus DNA Ladder (Invitrogen)). Deletion mutants utilized in these studies were selected from those strains with a (Δ) PCR designation (indicated above each well).

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