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Characterization of the Role of Transcriptional Regulator of AraC/XylS Family in Tularemia Pathogenesis

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ABC	ATP-Binding Cassette
ADP	Adenosine Diphosphate
$AgNO_3$	Silver Nitrate
ATP	Adenosine Triphosphate
ATPase	Adenosine Triphosphate Hydrolase
BAL	Broncho-Alveolar Lavage
BSL2	Bio-Safety containment Level-2
CDC	Centers for Disease Control
CDM	Chamberlain's Defined Medium
CFU	Colony Forming Units
CHP	Cumene Hydroperoxide
ChIP	Chromatin Immunoprecipitation
CLC	Capsule-Like Complex
CT	Cycle Threshold
DMEM	Dulbecco's Modified Eagle's Medium
EMSA	Electrophoretic Mobility Shift Assay
FBS	Fetal Bovine Serum
FCP	Francisella Containing Phagosome
FPI	Francisella Pathogenicity Island
HDPs	Host Defense Peptides
HTH	Helix-Turn-Helix
H_2O_2	hydrogen peroxide
IFN-γ	Interferon-gamma
IM	Inner Membrane

IP	Intraperitoneal
KDO	2-Keto-3-Deoxy-Octulsonic acid
LPS	Lipopolysaccharide
LVS	Live Vaccine Strain
MATE	Multidrug and Toxic Compounds Extrusion
MDMs	Monocyte-Derived Macrophages
Meme	Multiple Expectation maximization for Motif Elicitation
MEP	Multidrug Efflux Pump
MFS	Major Facilitator Superfamily
MHB	Mueller-Hinton broth
MOI	Multiplicity Of Infection
MPL	Monophosphoryl Lipid A
NADPH	Nicotinamide-Adenine Dinucleotide Phosphate
NMR	Nuclear Magnetic Resonance
NO	Nitric Oxide
OM	Outer Membranes
PAMPs	Pathogen Associated Molecular Patterns
PBMCs	Peripheral Blood Mononuclear Cell
PBS	Phosphate-Buffered Saline
PMN	Polymorphonuclear leukocytes
RNAP	RNA Polymerase
RNA-Seq	RNA Sequencing
RND	Resistance Nodulation Division
ROS	Reactive Oxygen Species
SMR	Small Multidrug Resistance

TBH	Tert-Butyl Hydroquinone
Th1	T helper 1
Th17	T helper 17
TCA cycle	Tricarboxylic Acid cycle
TCS	Two Component System
TGF-β	Transforming Growth Factor-beta
TLR4	Toll-Like Receptor 4
TNF-α	Tumor Necrosis Factor-alpha
VNTR	Variable-Number Tandem Repeat

ABSTRACT

The Tier 1 Select Agent, *Francisella tularensis* causes an acute and fatal disease known as tularemia. Many studies have devoted enormous efforts to understand how *F. tularensis* avoids host defense mechanisms, replicates within an extremely secure immune system, and eventually causes the deadly disease tularemia. The extremely high virulence of *Francisella* depends on its ability to manipulate gene expression according to the surrounding environment. This process requires the involvement of unique transcriptional regulators. *Francisella* possesses very few transcriptional regulators, and a majority of them characterized to-date have been shown to regulate genes involved in virulence and cellular functions. The role of a transcriptional regulator of *F. tularensis* belonging to the AraC/XylS in gene regulation and virulence remains uncharacterized to-date. This study characterized the role of AraC in gene regulation, intramacrophage survival, and virulence of *F. tularensis*.

In specific aim 1, we generated a deletion mutant (Δ*araC*) of *FTL_0689* gene encoding AraC of *F. tularensis* Live Vaccine Strain (LVS), and its transcomplemented strain (Δ*araC*+p*araC*). Characterization of the Δ*araC* mutant demonstrated that AraC does not regulate genes involved in arabinose utilization. Genomic organization of the *araC* gene suggested that it may have a role in the regulation of a unique multidrug efflux pump (MEP) located downstream of it. Our results revealed that the phenotype of the Δ*araC* mutant mirrors that of the *emrA1* and the *silC* mutants, the components of the MEP. However, these phenotypic similarities are not due to the direct regulation of MEP genes by AraC. Further characterization revealed that AraC is involved in providing resistance against oxidative stress.

In specific aim 2, we investigated the role of AraC as a global transcriptional regulator in *F. tularensis* LVS strain. We studied gene expression profiles of the wild type *F. tularensis* LVS and the Δ*araC* mutant under normal and oxidative stress conditions. The results revealed that AraC serves as a transcriptional regulator only when the bacteria are exposed to oxidative stress conditions. AraC is involved in the regulation of virulence genes encoded on Francisella Pathogenicity Island, stress response genes, energy production, genes encoding enzymes in the tricarboxylic acid (TCA) cycle, metabolism and regulatory proteins indicating its role as a global regulator. The differential expression of these genes also impaired the ability of the Δ*araC* mutant to survive in macrophages and attenuated its virulence in mice.

Collectively, this study identified and characterized a novel transcription factor, AraC, required for adaptation of *Francisella* to oxidative stress conditions encountered outside or within a host.

1. INTRODUCTION

1.1. *Francisella tularensis*

F. tularensis is a Gram-negative bacterium that is extremely infectious and as few as ten bacteria can cause a deadly zoonotic disease [1, 2]. In 1912, *F. tularensis* was first discovered in Tulare County, California as the causative agent of a disease in ground squirrels that resembled plague [3]. After that, Edward Frances greatly devoted his efforts to studying this bacterium and discovered that it causes a disease with different clinical manifestations in human and named that disease "tularemia" [4]. *Francisella* genus was then named after him as a reward, and the species *tularensis* was derived from the Tulare County [5] *Francisella* descends from the gamma-subclass of the phylum proteobacteria as classified according to small subunit RNA sequencing and is the only genus within the *Francisellaceae* family [6]. The *Francisella* genus branches out into two species: *tularensis* and *philomiragia*, which were categorized based on their fatty acid and genetic material identity [6, 7]. *F. tularensis* has been subdivided into four major subspecies: *F. tularensis* subsp. *tularensis* (Type A or also referred to as Schu S4) and *F. tularensis* subsp. *holarctica* (Type B), which are virulent for humans while the other two subsp., *F. tularensis* subsp. *novicida* (referred to in this study as *F. novicida*) and *F. mediasiatica* are known to be avirulent [8]. One more subspecies of *F. tularensis* isolated in Japan has been identified and is an *F. tularensis* subsp. *holarctica* variant [9] (**Figure 1**). Furthermore, molecular studies including Multiple-Locus Variable-number Tandem Repeat (VNTR) Analysis subdivided Type A strains into two genetically unrelated clades known as type A1 and type A2 [10, 11], Type B strain into: B1, B2, B3, B4, and B5 [11], and currently, *F. tularensis* subsp. *mediasiatica* was also subdivided into three clades: M1, M2, and M3 [12].

The geographic distribution of *F. tularensis* differs between Type A and Type B strains [13]. While Type B is less common in North America and prevalent throughout Europe and Asia, Type A is confined to North America and was also isolated from Central Europe [11, 13]. In addition, the avirulent strains *F. novicida* and *mediasiatica* were isolated in the Great Salt Lake, Utah from Ogden Bay salt water in 1950 [3] and Central Asia, respectively [14].

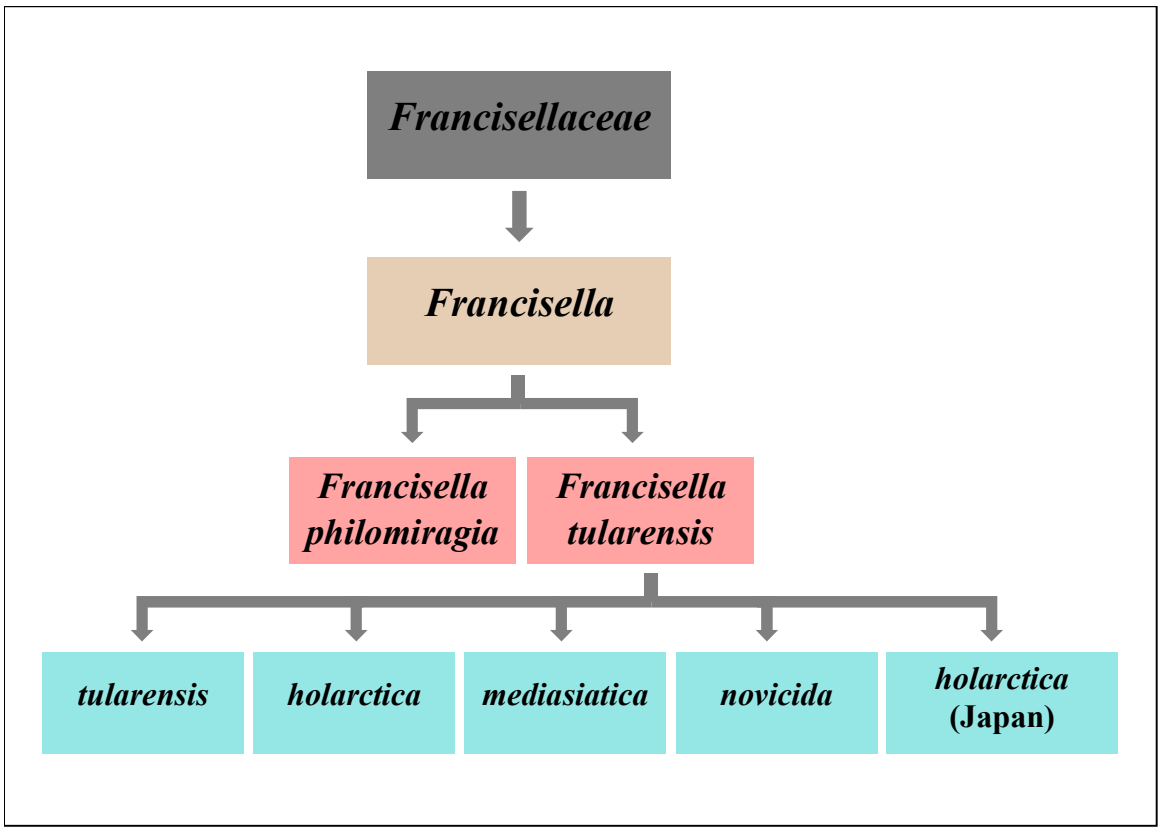


Figure 1. Classification of *Francisella*.

1.1.1. Disease transmission

Type A and Type B strains of *F. tularensis* are highly contagious. They can be acquired through insect bites, direct contact with diseased animals, ingestion of contaminated food or water, or by inhaling bioaerosols [3]. A majority of *F. tularensis* infection around the world is caused by Type B strains [15, 16]. The high mortality rate in North America as a result of *F. tularensis* infection is related to Type A strains, whereas Type B strains are less severe for the disease across the Northern Hemisphere. This indicates that the natural distribution of Type A and Type B in addition to the severity of the disease is distinct between the two subspecies of *F. tularensis*. However, there is no record of *F. tularensis* subsp. *mediasiatica* infection in humans [3]. In contrast, *F. novicida* can infrequently cause illness in immunocompromised humans but is highly lethal for rodents [17]. In addition, preliminary investigations indicate that the bacterial morphology of this subspecies resembles the pathogenic *F. tularensis* strain as they share about 78% to 97% of the genetic material [7]. Nevertheless, it is less virulent, metabolizes sucrose, and does not show any immunologic reaction when administered into *F. tularensis* immunized rabbits [18]. This suggests that *F. novicida* is an excellent tool for *Francisella* and tularemia research [17].

1.1.2. Clinical signs and symptoms

The onset of tularemia is very fast causing flu-like symptoms such as high temperature, sore throat, headache, chills, and general bodily weakness or discomfort [19]. The severity of the disease depends on various factors including the route of administration, bacterial dosage, and infecting *Francisella* subspecies [20]. Moreover, different portals of entry can show the different clinical manifestation of tularemia in humans [21, 22]. For instance, direct contact

with infected animals or transmission through insect bites can ulcerate the target area and result in glandular and ulceroglandular tularemia, which is the most frequent form of the disease. This will lead to systemic infection due to bacterial drainage to the nearest lymph node [23]. There is also oculoglandular and oropharyngeal tularemia that are caused by eye infection or ingestion of contaminated materials, respectively. The most acute form of the disease in humans is pneumonic tularemia which is induced by bacterial inhalation but fortunately, this occurs infrequently under natural conditions [23, 24].

1.1.3. Bioweapon potential

The concern of using *F. tularensis* as a potential bioterrorism weapon has emphasized the importance of classifying it as a Tier 1 Select Agent by the U. S. CDC. The reason is that *F. tularensis* has low infectious dosage, ease of transmission, and the ability to cause high rates of morbidity and mortality [24]. Presently, vaccines against tularemia are not available [25]. In spite of the development of LVS from a Type B strain in the former Soviet Union, it is not approved for human immunization in the United States because it is not safe or effective against respiratory tularemia caused by Type A strains of *F. tularensis* [25, 26]. The Russian vaccine strain was used to derive the LVS by several passages on peptone cysteine agar [25]. Although LVS is not used to vaccinate humans, it is being used in research studies for several reasons. One is that the LVS is safe to handle in biosafety level 2 laboratories. Secondly, it has both high virulence in mice and the ability to replicate identically to the virulent strains. In addition to that, the virulence genes in Type A and Type B strains are found to be conserved in the LVS, which favored using LVS to a large extent in the majority of *F. tularensis* studies [27, 28].

1.2. Intracellular life cycle of *F. tularensis*

F. tularensis is a non-obligatory intracellular bacterium which can infect different cells including phagocytes such as monocyte-derived macrophages (MDMs) or polymorphonuclear leukocytes (PMN) and non-phagocytes such as respiratory epithelium and fibroblasts [28-30]. While macrophages are one of the primary and crucial cells that interact with *F. tularensis* [23] and are the focus of most studies, the other cell types that bacteria prefer for infection are due to unknown reasons [28-30]. The life cycle of *F. tularensis*, upon its entry into macrophages, is very distinctive since it prevents phagosome maturation and escapes into the cytosol for replication [24, 30] (**Figure 2**).

The innate immune response initially comes into play and activates defense mechanisms including complement system, antibodies, and host defense peptides (HDPs) once *F. tularensis* infection is established [23]. If the bacteria fail to resist any of these mechanisms, they will be defeated, and the infection will be cleared [31]. However, this is not the case with *F. tularensis*. It utilizes several structural adjustments to overcome the innate immune response and avoid any bacteriolysis that allows immune recognition due to the induction of the proinflammatory cytokines and, then, activates a robust adaptive immunity [23] (**Figure 3**).

Phagocytes engulf *F. tularensis* rapidly, within about an hour of infection [29], by receptor-mediated phagocytosis and enclose it in a phagosomal compartment containing antimicrobial compounds required for bacterial degradation [23]. These antimicrobials generated by the phagocytic cells are as a result of a multi-component assembly of a membrane-attached enzymatic complex, known as nicotinamide-adenine dinucleotide phosphate (NADPH) oxidase responsible for turning molecular oxygen into negatively charged and highly toxic superoxide radicals [32]. Thus, at the time of phagocytosis, NADPH

oxidase subunits aggregate in the phagosomal vacuole to assemble and produce reactive oxygen species (ROS) [23], which, however, *F. tularensis* can resist extremely efficiently [33].

Accordingly, *Francisella* fights these microbicidal mechanisms by some unique mechanisms and by the escape into the cytosol [23]. *F. tularensis* phagocytosis not only supports bypassing extracellular immune defense factors [23] but also avoids the stimulation of pro-inflammatory cytokines [29]. In addition, *F. tularensis* suppresses immune recognition by stimulating transforming growth factor β (TGF-β), prostaglandin E2, or production of other anti-inflammatory cytokines via undiscovered mechanisms [29]. When *Francisella* is in the cytosol, it undergoes rapid replication in spite of the presence of various immune defense components such as inflammasome [23].

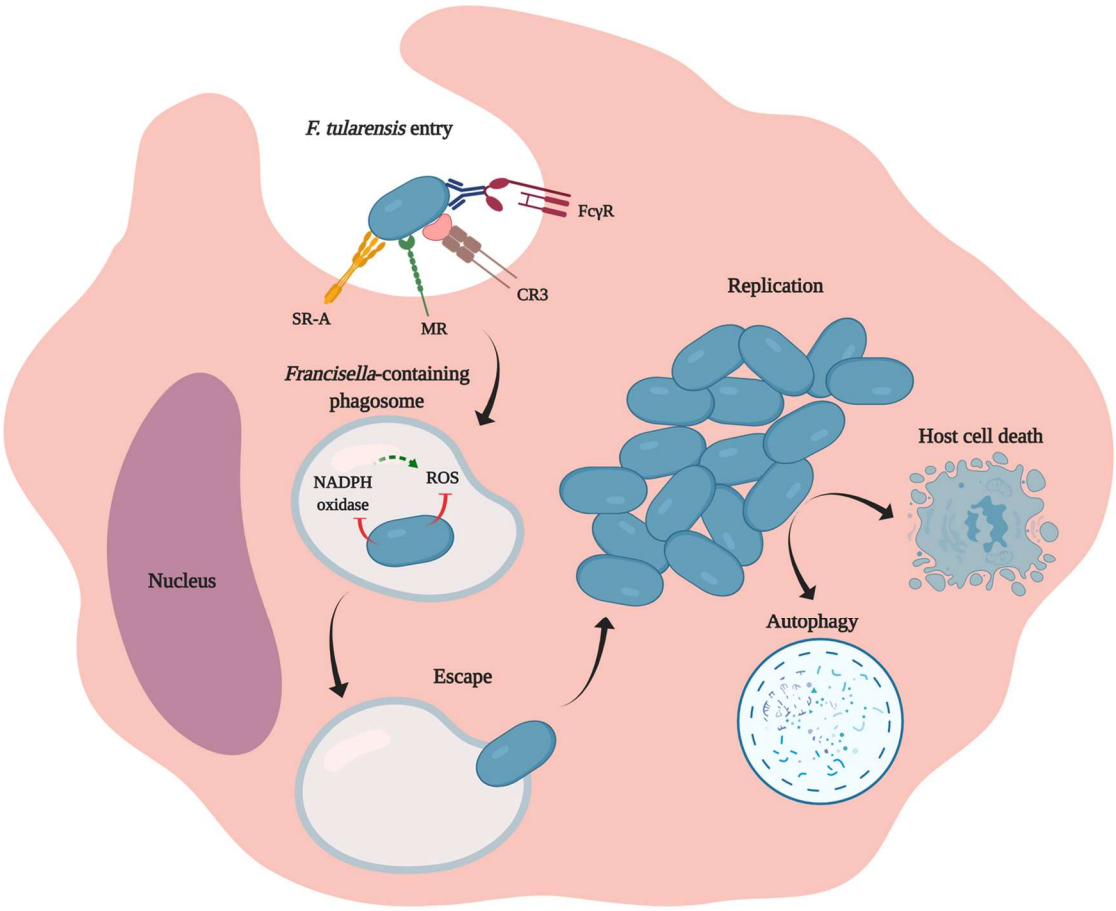


Figure 2. Intracellular life cycle of *F. tularensis*. *Francisella* is internalized by various-receptors mediated phagocytosis into a phagosome known as *Francisella* Containing Phagosome (FCP). *Francisella* must evade antimicrobial molecules such as NADPH oxidase that produce ROS and cause respiratory burst. *Francisella* then disrupts the phagosome and escapes into the cytosol to undergo several rounds of multiplications resulting in host cell death. This will be followed by bacterial release to infect new cells. Modified from Jones *et al*., 2012 [23].

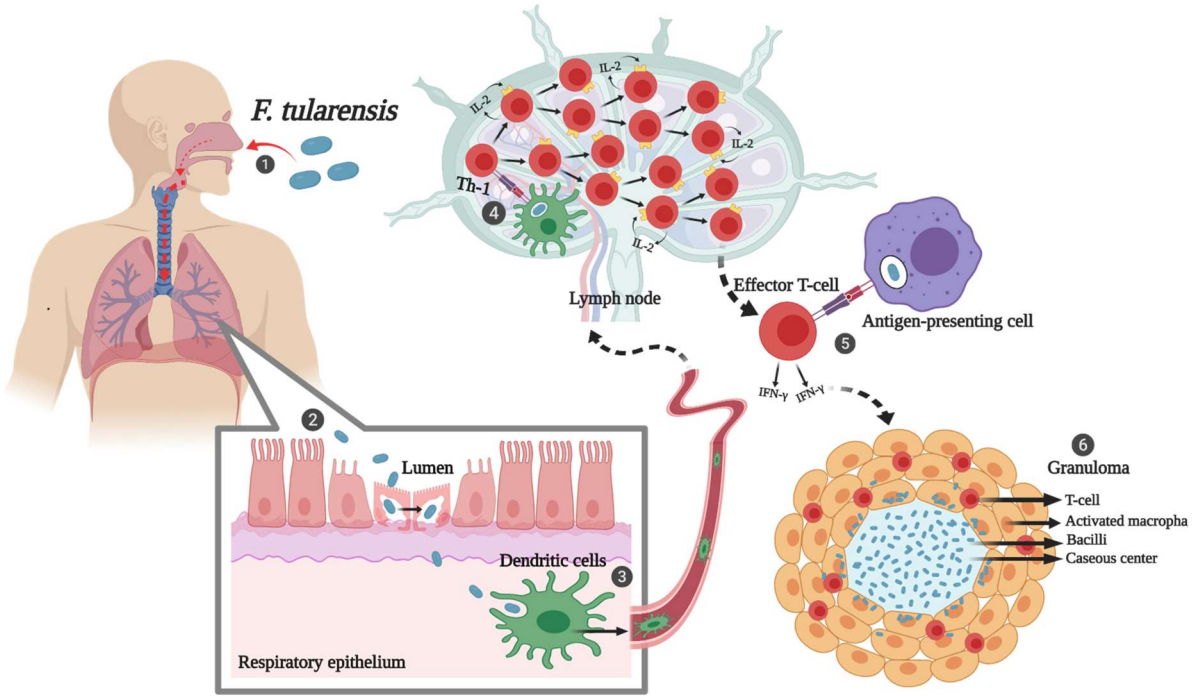


Figure 3. Stimulation of extreme immune response upon failure to defeat *F. tularensis* infection. Inhalation of bioaerosoles containing *F. tularensis* results in bacterial entry into the lamina propria lining of the lung where antigen presenting cells ingest bacteria and transfer them into the regional lymph nodes. The bacteria will then be presented to T-cells that will proliferate and produce Interferon-gamma (IFN-γ) and activate macrophages. If the immune system fails to clear the bacteria, cytokine storm will be produced and bacterial infection will end in granuloma formation. Modifed from Murray P. R., 2009 [34].

1.3. Immunity to *Francisella*

After *Francisella* infection was established, anti-*Francisella* antibodies and other immunogenic molecules are produced in the host against the bacterial antigen, lipopolysaccharides (LPS) [35]. Vaccination with LVS also induced the production of potent serum antibodies: IgM, IgA, and IgG within two weeks and was found to specifically target *F. tularensis* LPS [36]. The multispecific IgM antibodies mostly induce the early protection against *F. tularensis* LVS infection through the intradermal and intranasal routes [37]. Furthermore, the mucosal IgA was found to be involved in the protection against *F. tularensis* LVS as it was isolated from the infected host sera and bronchoalveolar lavage (BAL) [38, 39]. Another indication of the IgA involvement is that vaccination through the intradermal route did not protect against LVS intranasal challenge whereas using the intranasal route for vaccination protected against other routes of administration [35, 40, 41]. Unfortunately, human vaccination with LVS did not produce enough anti-*Francisella* antibodies that protect against the virulent *Francisella* strain. However, vaccination with killed *Francisella* successfully induced the production of anti-*Francisella* antibodies but stimulated weak or no cellular immunity [36, 42].

Interestingly, using LVS, Schu S4, or monophosphoryl lipid A (MPL) component of LPS to perform nanoparticles incorporating lysate provides immunity against the LVS challenge in mice [37]. Not only does the nanoparticles incorporating lysate produce serum antibodies, but it also stimulates and expands the production of T-cell dependent cytokines [35]. *Francisella*, as an intracellular pathogen, avoids detection by antibodies unless translocated to the extracellular environment where antibodies take place and thus, support the induction of T-cells response [35]. Consequently, T-cells proliferate and stimulate the

production of T helper 1 (Th1)-type cytokines particularly the INF-γ [36]. INF-γ is an important cytokine for T-cells priming to LVS as injecting mice intraperitoneally (IP) with anti-INF-γ antibodies results in a very high morbidity rate unless the mice were challenged with the lowest dose of LVS [43]. Furthermore, *in vitro* stimulation of immunity using T-cells isolated from mice with INF-γ deficiency does not successfully prevent LVS intramacrophage replication [43]. Proliferated T-cells also induce the production of tumor necrosis factor-alpha (TNF-α), which activates responding memory T-cells [44].

The establishment of robust memory T-cells is an essential factor in the protection against intracellular organisms [45]. After T-cells recognize the antigen presented on the surface of antigen presenting cells, they proliferate and differentiate into memory T-cells. The peripheral blood mononuclear cell (PBMCs) post-LVS vaccination contained memory $CD4^+$ and $CD8^+$ T-cells that induce the production of cytokines IL-17 and IL-22, which are produced by T helper 17 (Th17) cells, a subset of memory T-cells [46]. The level of pulmonary IL-17 is very crucial for successful vaccination against the virulent strain Schu S4 [47]. Depleting IL-17 with neutralizing antibodies at the time of the secondary challenging has been observed to increase the bacterial burden in mice lungs [47]. Furthermore, $CD4^+$ and $CD8^+$ T-cell responders of PBMCs of tularemia patients or after vaccination with LVS exhibited a phenotype of effector memory T-cells that have the ability to induce cell lysis, target variety of tissues, and persist in the host sera for up to 25 years after the first establishment of pneumonic tularemia [36, 45]. Moreover, B-cells were found to contribute to the secondary infection to *F. tularensis* as they function similarly to antigen presenting cells as well as cytokine producing cells [48].

1.4. Virulence factors

F. tularensis is a firm, long-living organism that has the ability to tolerate low temperatures and to exist for several weeks in different conditions including wet soil, dried grass, and even on decomposed dead animals [24]. *Francisella* is also a non-spore-forming bacteria enveloped by a thin layer of lipopolysaccharide [24]. At present, the virulence factors of *F. tularensis* and the mechanisms that it uses to subvert host's immune response are not clear [2]. Studies have shown that although LVS is attenuated for virulence in humans, it still causes extreme illness in animals and results fatal disease in rodents similar to human infection [26]. LVS can certainly adjust to intra-phagosomal life [49, 50] in mice macrophages and subsequently cause structural damages to them [2]. Managing virulence mechanisms in *Francisella* is not controlled by a specific protein or system. It is a concurrent activity of various systems that help *Francisella* to abolish immune defenses, enter host cells, subvert phagosome antimicrobials by modifying phagolysosome maturation, escape to the cytosol and replicate with host recognition avoidance, manipulate cell survival/death, and spread to neighboring cells for new infection [19]. Some of the currently studied virulence factors are located within *Francisella*'s envelope which are comprised of the capsule, LPS, outer and inner membranes (OM / IM), periplasm, and bacterial secretion systems [19].

1.4.1. *Francisella* capsule

The production of the capsule by *F. tularensis* is completely sustained in most of the biovars [51]. It mostly consists of lipids, around 51%, and the remaining is amino acids and carbohydrates (35 % and 21% respectively) [52]. The density of the capsule is about 0.02 – 0.04 μm and it is translucent according to Hood *et al*., who revealed that it has a virulent

function in *F. tularensis* [52]. Further studies demonstrated that the capsular materials composed of polysaccharides that were similar to one of the LPS component (O-antigen) [51] solely, but no other parts of the LPS, such as 2-keto-3-deoxyoctulsonicacid (KDO) and lipid A, were found by the mass spectrometry and nuclear magnetic resonance (NMR) analysis [51, 53]. Additionally, the sugar composition of the capsule was found to be distinct among *F. tularensis* strains such as Schu S4, whose capsule contains two sugars: mannose and rhamnose; whereas LVS capsule has three: mannose, glucose, and galactose [53]. Some studies have also shown that different strains produce various capsular-like molecules either due to the bacterial growth conditions or the surrounding environment, which could be significant to the virulence pathway [19]. In addition, LVS capsule production was higher when the bacteria were grown on Chamberlain's medium and resulted in a more aggressive bacterial infection in mice upon intravenous inoculation [54].

In contrast to virulent *F. tularensis* strains, Schu S4, and LVS, *F. novicida* neither produces capsule nor its O-antigen [55, 56]. However, studies have found that *F. novicida* posses a locus that is highly identical to that responsible for capsule-like complex (CLC) glycosylation of *F. tularensis* [55]. This CLC is an extracellular component that is produced by *F. tularensis* during certain growth conditions [53]. This may imply that CLC is a potential virulence factor of *F. novicida* [55].

Capsule formation in *F. tularensis* substantially contributes to *F. tularensis* evasion of any intracellular immune responses and in general, any *in vivo* or *in vitro* virulence mechanisms [19]. It can protect *Francisella* from being opsonized either by complement or antibodies; however, it is not yet understood if it is essential for serum resistance or contributes to this mechanism in association with other factors [19]. There were many attempts to design

the *F. tularensis* vaccine that targets capsule because of their surface localization [19]. Nevertheless, conflicting results have been observed when Schu S4 or the LVS capsular proteins are used for intraperitoneal immunization, as Schu S4 did not show any protection in guinea pigs or in mice [52]. On the contrary, LVS capsular proteins protect mice only against a homologous challenge; however, it did not protect when challenged with Schu S4 intraperitoneally [51].

1.4.2. Lipopolysaccharide

Another virulence factor that *F. tularensis* employs for immune evasion is LPS. It consists of lipid A, the O-antigen, and the middle domain which is made of oligosaccharide [57]. *F. tularensis* lipid A differs from the *Escherichia coli* (*E. coli*) lipid A in that it is tetraacylated and has 16-18 extent of carbon [58, 59]; while the latter is hexaacylated with 12-14 stretch of carbons [19]. Lipid A can trigger the immune system as most Gram-negative bacteria can modify it for virulence mechanisms [60]. The polysaccharide repeats of the O-antigen; however, are conserved among the virulent strain Schu S4 and the LVS; but differ from those of *F. novicida* as detected by mass spectrometry and NMR analysis [61-64] (**Figure 4**).

Studies have demonstrated that LPS-targeted vaccines show protection only against homologous challenge with the same species [65]. For instance, using *F. novicida lpxF* strain for immunization protected mice only from wild type *F. novicida* challenge, but not from challenge with Schu S4 or subsp. *holarctica* strains [65]. Interestingly, 60-95% of *Francisella* lipid A, unlike that of other Gram-negative bacteria, was found to be missing the core or the O-antigen domains, and these are known as free lipid A [66-68]. In addition, *Francisella* can

control the length of the acyl chains according to the surrounding temperature, and this is an essential virulence mechanism since infecting mice subcutaneously with *F. novicida lpxD1* mutant (LPXD1 is an enzyme that adds carbons to acyl chains) was not virulent [69]. These LPS alterations are significant for *Francisella* to lower the chances to be recognized by one of the host LPS detectors, such as LPS-binding proteins, Lymphocyte antigen 96 (MD-2), or Toll-Like Receptor 4 (TLR4) [70-72].

Normally, *Francisella* LPS is insufficient to stimulate B-cells. However, at an extreme high dose, such as 75 μg, *F. novicida* LPS can promote murine white blood cells to proliferate [19]. *E. coli* LPS, on the other hand, enhances B-cell activation via LPS recognition proteins [19]. Although *Francisella* alters LPS and avoids innate immune system detection, the classical pathway of the complement system is induced by some strains such as LVS [73]. The C3b subunit of the classical pathway opsonizes *F. tularensis* and facilitates its internalization into host cells. However, the bacteria have to avert the genesis of the membrane attack complex by the complement components [19]. Studies revealed that Schu S4, LVS, and *F. novicida* are resistant to complement activation because this process depends on the O-antigen subunit of LPS [74]. Thus, the studies above suggest that *F. tularensis* controls the phagocytosis process due to the presence of the O-antigen subunit in any of the capsule or LPS [19]. Furthermore, TLR4 was found to be deactivated to a great extent because of LPS alteration and subsequently, it resulted both in evading the immune system and enhancing the virulence mechanism in general [19]. Taken together, *Francisella* depends on the O-antigen, either in LPS or capsule together with the plasmin, to evade the host's first line of defense, the complement system. This, in turn, avoids a robust immune response that would normally be induced via pro-

inflammatory release when bacteria are detected and ultimately killed by pathogen-associated molecular patterns (PAMPs) [23].

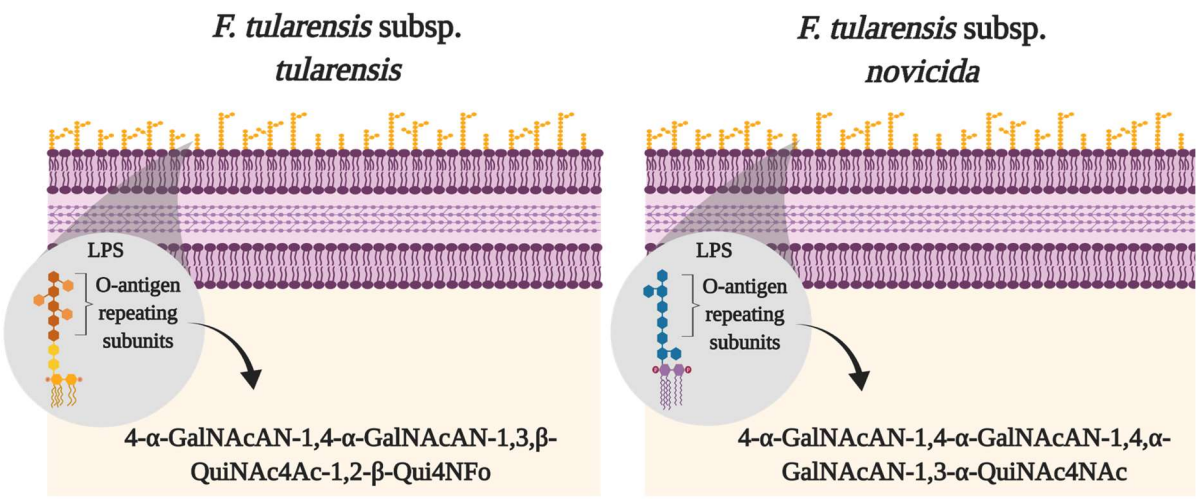


Figure 4. O-antigen of *F. tularensis* versus *F. novicida*: The O-antigen repeating molecule of *F. tularensis* subsp. *tularensis* is distinct from that of the *F. novicida.*

1.4.3. Type IV pili

Most of the Gram-negative organisms possess Type IV pili which act as an essential virulence factor as it helps in the attachment to the host cell, gives the bacteria a special movement technique known as twitching motility, helps bacterial assembly and DNA acquisition [75, 76]. In *F. tularensis*, studies have demonstrated that growth conditions can modify the shape of Type IV pili; for instance, culturing LVS on agar produces horn-like Type IV pili while growing LVS on broth produces elongated cylindrical fiber-like Type IV pili [77]. The Type IV pili are observed on *F. tularensis* Schu S4 and *F. novicida* [78, 79]. Some studies, on the other hand, reported that no pili are detected neither on subsp. *holarctica* strain nor *F. novicida*, suggesting that the presence of *pili* depends either on *Francisella* strain or on growth conditions among different laboratories [80, 81].

The pilus is composed of five or six pilin subunits; from PilE1 to PilE6 [77, 79]. Genomic sequencing of *F. tularensis* indicated that Schu S4 and *F. novicida* have the six subunits while Type B strain has many mutations or deletions in *pilE1, pilE2, or pilE3* [79]. LVS, on the other hand, has *pilE1* deleted and *pilE2* and *pilE3* containing premature stop codons [77]. In particular, PilE1 was found to be playing an important role in *Francisella* virulence. During infection, LVS forms several glycosylated multimers of PilE1 on its surface. It is this glycosylation of Type IV pili that makes it infectious [80]. Previous reports on mice subcutaneously infected with either Schu S4 or Type B strain was also found to have the virulence factor PilE1 and was determined to be responsible for the entire virulence mechanisms of these strains [80, 82]. Furthermore, Salomonsson *et al*., reported that trans-complementation of *pilE1,* along with or without other genes in LVS, makes the bacteria highly virulent [83]. Studies also noted that the proteins which are important for Type IV pili assembly

in *Francisella* play an important role in the virulence [19]. Thus, like other Gram-negative bacteria, *Francisella* expresses Type IV pili that enhance bacterial virulence and intracellular survival.

1.4.4. *Francisella* secretion systems

Most Gram-negative bacteria have Type I secretion system that facilitates toxins and drugs transport and thereby assists in the virulence mechanisms [84-86]. Three ortholog genes of the outer membrane, *tolC*, of *E. coli* and other bacteria were detected in *F. tularensis* by the genomic and bioinformatic analysis. These genes are *tolC*, *ftlC*, and *silC* with the locus tags *FTL_1865*, *FTL_1107*, and *FTL_0686*, respectively [87], and are found to be essential for LVS resistance to antimicrobial drugs, dyes, and detergents [87, 88]. The structure of Type I secretion system is characterized by the association between three subunits: the OM TolC, an IM ATPase, and a transmembrane protein, forming a three-component system [89]. In a study using Schu S4 *tolC* mutants, it was found that they were partially attenuated following intradermal mice infection as mice survived two more days as compared with those which received wild type bacteria [90]. LVS *tolC* mutants were unable to survive and therefore could not spread within the host cells, leading to conditions which were permissive for the pro-inflammatory cytokines cascade, which in combination, led to macrophage death. This suggests that TolC might be contributing to other unknown virulence pathways [91, 92]. Another protein on *F. tularensis* OM, SilC, identical to silver cation efflux proteins, has some sequence similarity with TolC and FtlC [93]. A study conducted from our lab has characterized the role of SilC in *F.tualrensis* LVS and demonstrated that this OM protein is involved in the resistance to antibiotics, oxidants, and silver. In addition, SilC is also crucial for *F. tularensis*

virulence *in vivo* and *in vitro* [87]. Another study on OM orthologs of Schu S4 showed that TolC was required for *in vivo* virulence when bacteria were administered intranasally and intradermally. However, FtlC was required for virulence using the intradermal route of administration, indicating that *F. tularensis* virulence is dependent on the route of entry to the host [94].

Gram-negative bacteria also have Type III and Type IV secretion systems that are essential for the bacterial virulence mechanism and are characterized by infusing effector proteins through the plasma or internal membranes [95-97]. *F. tularensis*, especially Schu S4, lacks any of Type III or Type IV secretion system orthologs or functional proteins [98]. Although studies have identified some of the virulent genes that share homology with Type III and Type IV secretion systems in *F. tularensis* subsp. *mediasiatica*, these genes are determined to be non-functional in *Francisella* species. To date, no intact or functional Type III or Type IV secretion systems have been detected in *Francisella* except Type IV pili biogenesis system that has some subunits shared with Type III secretion system while the majority of other subunits are homologous to components of Type II secretion system [88, 99, 100].

Additionally, studies on *F. novicida* indicated that Francisella pathogenicity island (FPI) encodes Type VI secretion system [101, 102], which is described by infusing the effector proteins into host cells or other bacterial cells in a similar manner to the T4 bacteriophage injecting mechanism [103, 104]. Two proteins, IglA and IglB, compose the outer sheath of Type VI secretion system and are found to be arranged together when the bacteria are under stress, such as during phagocytosis, an alkaline environment (5% KCl), and oxygen availability [105], suggesting that Type VI secretion system might be important for *Francisella* virulence. Other important subunits, such as DotU and VgrG, also promote phagocytosis resistance and

virulence during intradermal or intranasal mice infection [101, 106]. Moreover, LVS uses Type VI secretion system to secrete IglC, IglE, IglF, IglI, IglJ, PdpA, PdpE, and VgrG effector proteins into host macrophages, while IglC, IglE, PdpA, and PdpE proteins are secreted by *F. novicida*, suggesting that every species has its specific Type VI secretion effector proteins [107]. Type VI secretion system effector proteins are not fully characterized despite the involvement of this secretion system in the virulence of *F. novicida* in a mouse model [7].

1.5. The Multidrug efflux pump (MEP) system

F. tularensis have evolved other mechanisms to avoid or counter the host immune system [23]. These mechanisms depend on the nature of the host defenses that the bacteria encounter. For example, when *F. tularensis* confront host defense peptides, it can repulse the interaction between them and avoid their antimicrobial effect by managing its surface charge as these peptides are usually positively charged [23]. However, some antimicrobial peptides have no surface charge or can bear the same charge as the bacteria, which makes it difficult for *F. tularensis* to resist them by modifying its surface charge. Thus, *F. tularensis* uses another strategy that employs the MEP system to help diffusing antimicrobial drugs out of the bacterium [23].

F. tularensis subverts host defensins and other microbicidal components by encoding one or more of the MEPs similar to other bacteria including *Neisseria*, [108], *Pseudomonas* [109], and *Salmonella* [110]. One of the most commonly studied MEP systems in *F. tularensis* is known as the AcrAB/TolC efflux pump [23]. The MEP is composed of IM AcrAB [111] and OM TolC components [88, 91]. The importance of this system, specifically TolC subunit, is due to its activity toward antibiotics such as ß-lactam, tetracycline, aminoglycoside, and

quinolone and also toward detergents including bile salts and dyes effective against Gram-negative bacteria [88, 91, 111]. *In vivo* studies have shown that disrupting the MEP subunits leads to attenuation of virulence in mice [88, 91, 111].

There are two categories of the MEP: the ATP-binding cassette (also known as ABC) transporters and the secondary transporters [112]. The fuel for ABC transporter depends on Adenosine triphosphate (ATP) while the secondary transporters depend on either sodium or protons electron gradients [112]. Furthermore, the secondary transporters are further subcategorized into four super-families according to their compositional similarities [84]. These four subcategories are: major facilitator superfamily (MFS), resistance nodulation division (RND) family, multidrug and toxic compounds extrusion (MATE) family, and small multidrug resistance (SMR) family [84] (**Figure 5**). While the RND superfamily is specific for most Gram-negative organisms, the other efflux pumps can be found in any of Gram-positive or negative organisms [113].

Studies have found that 31 MFS transporters are encoded by *F. tularensis* genome [112] and the virulent strain Schu S4 has 15 active ABC transporters that are detected *in silico* [114]. In a study of LVS multidrug transporter, AcrAB RND, the AcrB subunit was categorized as an important virulence factor in mice along with its essential role in antibiotics and antimicrobial peptides avoidance [111]. However, another study using Schu S4 subspecies demonstrated that not only AcrB is required for drug resistance, AcrA also plays an important role in the efflux of dyes and detergents but not for virulence in mice [115].

Interestingly, an additional role of the multidrug efflux pump is the involvement in resisting the oxidative stress mechanism [116-118]. Ma *et al*., (2014) reported that EmrA1 of *F. tularensis* LVS contributes to virulence in mice as it influences the production of the

antioxidant enzymes, SodB and KatG, that are required for resisting oxidative stress and thus, intramacrophage survival. [112]. *acrB* and *tolC* mutants, however, secreted SodB or KatG enzymes as detected in the culture filtrate and also did not show any susceptibility towards oxidants, yet, only sensitive to detergents and dyes [88, 111, 115].

The AcrAB/TolC efflux pump is regulated by the AcrR transcriptional regulator (a repressor) [113], which is a member of the TrtR family transcriptional regulators present in most bacteria that contain helix-turn-helix (HTH) domain specific for DNA binding [119]. This DNA binding motif is substantially conserved in most of TrtR family members [119]. In addition, proteins encoded by members of TrtR family regulate several genes that are required for resisting multidrugs and osmotic shock, catabolic enzymes, antibiotics biosynthesis, and virulence of most bacteria [119]. Hence, TrtR family members are essential for bacterial adjustment to various surrounding conditions [119]. Furthermore, AcrAB efflux pump was found to be activated by members of the AraC/XylS family; MarA, SoxS, and Rob [120-122]. However, they are not required for regulating the efflux in response to bacteria stress [123-128]. Studies suggested that during stress, the expression of *acrAB* is regulated by a unique *mar-sox* pathway that is independent of AcrR [119].

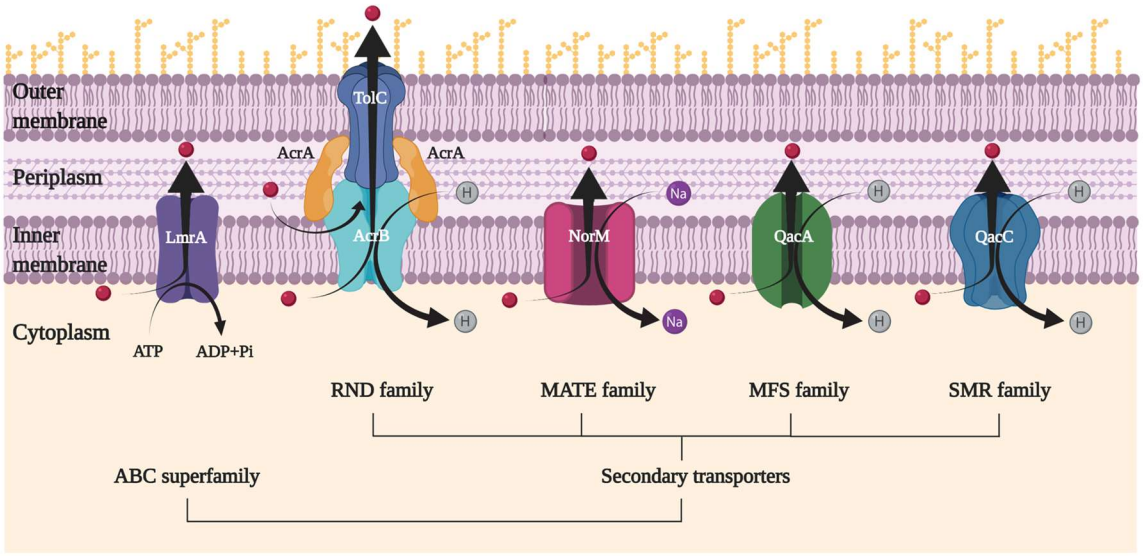


Figure 5. Superfamilies of bacterial efflux pumps and their substrates. Modified from Piddock, 2016 [129].

1.5.1. *F. tularensis* transcriptional regulators

Francisella possesses very few transcription regulators. A majority of these has been shown to regulate genes involved in virulence [130]. In general, bacteria produce transcription factors that bind to either specific or nonspecific DNA domains. The determination of whether the transcription factor is a repressor or activator depends on where the binding domain is located and on its sequence contents [130]. Transcriptional regulators are affected by several environmental factors such as high or low temperature, iron availability, oxidative pressure, and other antimicrobial compounds within macrophages. However, the molecular pathway behind the regulation needs further studies [130].

Three transcriptional regulators regulate FPI: MglA (macrophage growth locus protein A), SspA (stringent starvation protein A), and PigR (pathogenicity island gene regulator) [131, 132]. MglA and SspA are members of SspA family, and they function as a heterodimer that attaches to RNA polymerase (RNAP) [131, 133, 134] to regulate expression of virulent genes [135]. The SspA-MglA complex was also found to be involved in the bacterial response to stringent amino acid starvation leading to alarmone release, which is a guanosine-pentaphosphate ((p)ppGpp) [136]. PigR, or FevR (*Francisella* effector of virulence regulation) [130] in *F. novicida,* is a DNA binding protein that is recruited to the SspA-MglA-RNAP complex [132, 137]. It associates with the SspA and MglA to upregulate expression of other virulence genes that are not in the FPI [135]. In addition, about 100 genes that might be virulent were found to be regulated by SspA-MglA-PigR [136, 138]. *fevR/pigR* are positively regulated by MigR (macrophage intracellular growth regulator or also known as CaiC) to manipulate FPI gene expression. As most transcriptional regulators have DNA binding domain [139], MigR lacks this domain but still regulates the transcription of *fevR/ pigR* indirectly by

interacting with either of two genes: RelA or SpoT (stringent response regulators), which leads to the production of alarmone and thus controls *fevR* expression for virulence mechanism [130].

Studies have shown that response to the stringent starvation is not only exclusive to the binding of PigR to the complex, another transcriptional regulator, known as PmrA, was also found to be involved. PmrA is a DNA binding protein too that is homologous to that in *Salmonella* spp. where it functions as a response regulator of the two-component system (TCS) [140]. It is phosphorylated by the histidine kinase KdpD at the PhoP phosphorylation site (Asp51) [141] to form a member of the orphaned TCS in *F. tularensis* [130]. Another member of the TCS in *F. tularensis* is QseC that contributes to the virulence mechanism and regulation [140, 142]. Therefore, *F. tularensis* activates the TCS (PmrA and KdpD) leading to the binding of PmrA to promoters and then to the MglA-SspA-RNAP complex or freeing RNAP to regulate virulence gene transcription [143].

Besides the above-mentioned transcriptional regulators, iron limitation causes stress on *F. tularensis* that requires the presence of specialized ferrous transcriptional regulator to manipulate iron gene expression. Fur is a ferric uptake regulator that binds to ferrous iron, when available, to downregulate iron genes [130]. These genes are upregulated by Fur in case of the iron-limiting environment [130]. They are located downstream of *fur* in *F. tularensis* and are known as *F. tularensis* siderophore locus (*fsl*) or *F. tularensis* iron genes (*fig*) [144, 145].

In addition to iron availability, temperature fluctuation during *Francisella* infection is another environmental stress. Before entering warm-blooded hosts (37°C), *Francisella* can survive in a 25°C environmental temperature and subsequently requires to adapt by modifying

the Lipid A composition of its LPS [146]. Studies demonstrate that *F. tularensis* has only two sigma factors (the housekeeping σ^{70} and the stress-related σ^{32}), unlike *E. coli* and *Bacillus subtilis* that have six to eight sigma factors [130]. σ^{32} in *F. tularensis* is known as RpoH and is responsible for inducing the heat shock proteins [147-149]. Interestingly, several heat shock and virulence proteins of *F. tularensis* LVS are induced when exposed to higher temperatures [130]. Nevertheless, only 13 of the upregulated genes have a promoter binding site for σ^{32}, and none of these genes is located in the FPI [149]. This indicates that there is an indirect path of σ^{32} to regulate virulence genes [130].

Another crucial transcriptional regulator in *F. tularensis* that is involved in the oxidative stress resistance is known as OxyR, which is a member of LysR transcriptional regulator family [150]. Like most of transcriptional regulators, members of this family has an HTH motif in the N-terminal and a regulatory domain in the C-terminal [150]. In several bacteria, the regulation of oxidative stress is manipulated by many regulators including OxyR, SoxR, PerR, and RNA polymerase of stationary phase (RpoS) [151]. Other regulators that act on resisting oxidative stresses, especially to reduce the effect of RNS and iron-sulfur development, include NorR and IscR [152]. *E. coli* overcomes hydrogen peroxide (H_2O_2) toxicity, heat shock, oxidative stress, and death by phagocytosis by regulating essential genes using the transcriptional regulator OxyR [153]. This requires oxidation of Cys199 and Cys208, conserved loci, as a result of H_2O_2 stimuli [154]. SoxR, on the other hand, is involved in resistance of superoxide radicals, nitric oxide (NO), or H_2O_2 at higher concentrations [155]. *F. tularensis*, as revealed from sequencing, has OxyR and RpoS homologs only when it lacks SoxR/SoxS regulators [98]. OxyR in *F. tularensis* plays a central role in regulating the genes that are essential for ROS and oxidative stress resistance as well as bacterial survival and the

virulence mechanisms *in vivo* [156]. OxyR binds directly to the promoter regions of antioxidant enzyme genes alkyl hydroperoxidase (*ahpC*) and catalase (*katG*) to regulate their expression [156].

Additionally, by analyzing the genome of *F. tularensis,* we have identified a novel transcriptional regulator that is homologous to AraC regulators present in other Gram-negative bacteria. The AraC regulator belongs to the commonly known positive transcriptional regulators, the AraC/XylS family. They are characterized by the presence of high sequence similarity of amino acids that are expanding for a length of 100-residues to form two HTH DNA binding motif. This motif is unique and conserved among AraC/XylS family members. The first detected and biochemically studied AraC transcriptional regulator is the one that controls L-arabinose operon in *E. coli*. It functions as an activator of the *araBAD* operon as well as others, *araFGH* and *araE* [139], to manipulate L-arabinose utilization and catabolism [157] **(Figure 6)**.

AraC regulator has also been identified in *Erwinia chrysanthemum*, *Citrobacter freundii*, and *Salmonella* and functions similarly to that of the *E. coli.* Members of the AraC/XylS family are involved in the regulation of several mechanisms in bacteria. Some of them control the metabolic functions of specific elements including carbon, amino acids, alcohol, and herbicides. Others are responsible for regulating the secretion of virulence proteins such as siderophores, urease, and those required for attachment to epithelial cells, composing cellular capsule, or invasion. Some members of the AraC/XylS family also play a central role in regulating bacterial response to alkylating agents, oxidative stress, antibiotics, and other stress environments [139]. Nonetheless, the role of AraC regulator in *F. tularensis* is not known yet.

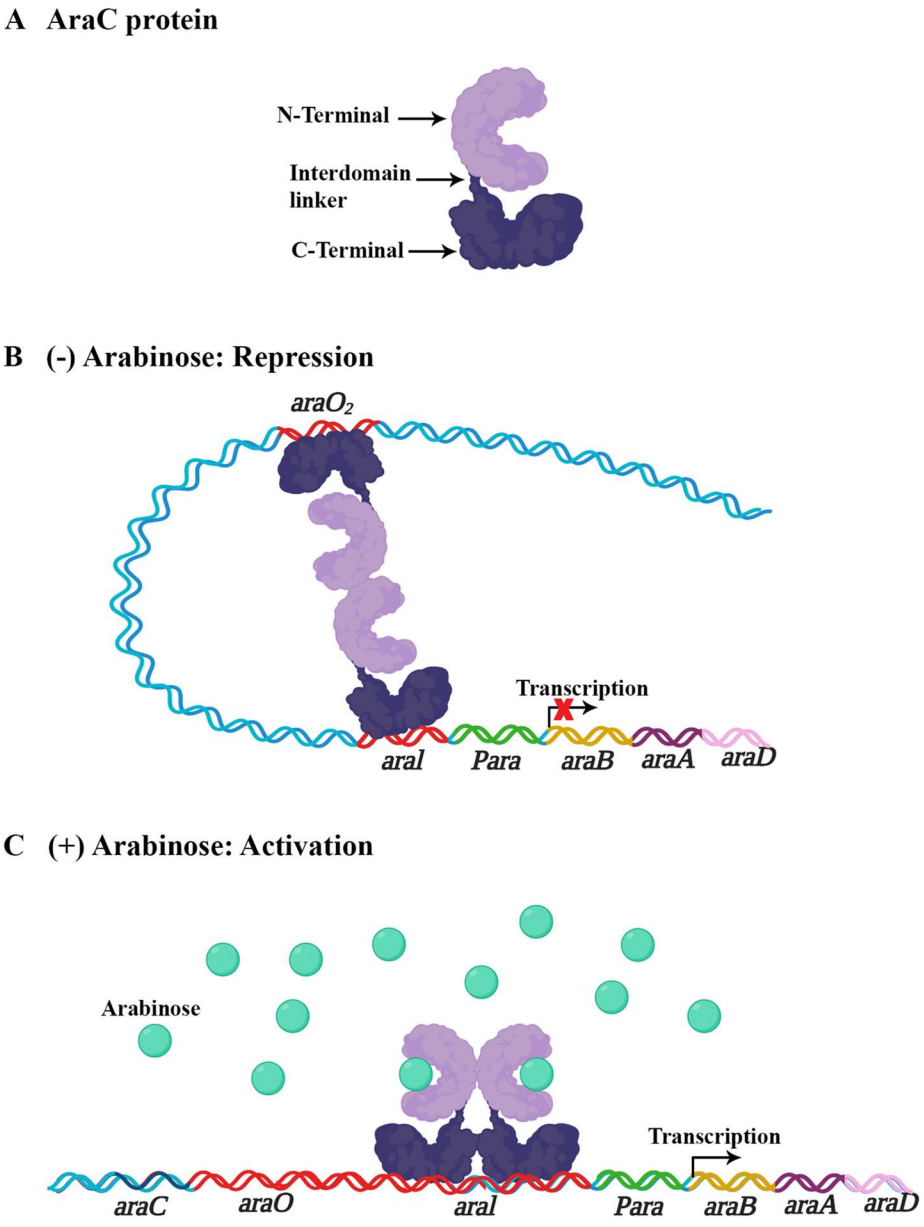


Figure 6. Schematic diagram of transcriptional regulation of L-arabinose operon by AraC in *E. coli*. (a) AraC monomer structure. (b) When arabinose is absent, two AraC monomers bind to I_1 and O_2 causing DNA to bend and form a loop that halks transcription of *ara*BAD by RNA polymerase. (c) When arabinose is available, allosteric changes will occur in AraC dimer resulting in the release of the O_2 site and binding of the dimer to I_1 and I_2 .This will facilitate RNA polymerase transcription of *ara*BAD. Modified from Yang *et al*., 2011 [158].

2. HYPOTHESIS AND SPECIFIC AIMS

Several studies have devoted enormous efforts to understand how *F. tularensis* avoids host defense mechanisms, replicates within an extremely secure immune system, and eventually causes the deadly disease tularemia. The extremely high virulence of *Francisella* depends on its ability to manipulate gene expression according to the surrounding environment. This process requires the involvement of unique transcriptional regulators. *Francisella* possesses very few transcriptional regulators, and a majority of them characterized to date has been shown to regulate genes involved in virulence and cellular functions. The role of a transcriptional regulator of *F. tularensis* belonging to the AraC/XylS in gene regulation and virulence remains uncharacterized to date.

The goal of this study was to characterize the role of AraC as a transcriptional regulator in *F. tularensis*. Previous studies focused on understanding the role of AraC transcriptional regulator in *E. coli* reported that *araC* encodes a protein that is required for regulating arabinose utilization genes [130-134]. Other studies, on the other hand, found that members of the AraC family of transcriptional regulators are involved in regulating virulence-associated genes. [110]. This study aimed at characterizing the novel role of AraC as a transcriptional regulator in *F. tularensis.* In several Gram-negative bacteria, *araC* is transcribed divergently from and controls *araBAD* operon. While in *F. tularensis,* it is transcribed divergently from an operon encoding *EmrA* MEP. Thus, the overall hypothesis set forth was: "*AraC may have a unique role in transcriptional regulation of multidrug efflux pump genes in addition to the genes involved in general metabolism as well as the stress response*." The following specific aims were proposed to address this hypothesis:

Specific Aim 1: Phenotypic characterization of the *araC* gene deletion mutant (Δ*araC*) of *F. tularensis* LVS. AraC transcriptional regulator is known in most Gram-negative bacteria for its role in regulating the *araBAD* operon. Because the role of *araC* in *F. tularensis* has not been defined, in this study several potential functions of *araC* were to be examined to determine the exact role of this gene. A clean gene deletion mutant (Δ*araC*) and the transcomplemented strain (Δ*araC*+*paraC*) of *F. tularensis* LVS were generated and characterized for the role of AraC in **1)** sugar utilization and metabolism; and **2)** resistance to oxidative stress and antibiotics due to its proximity to the genes encoding Emr MEP.

Specific Aim 2: Investigation of the role of AraC as a global transcriptional regulator in *F. tularensis* LVS. The role of AraC as a transcriptional regulator in *F. tularensis* was determined by **1)** deep RNA sequencing analysis to obtain a signature expression profile of genes in Δ*araC* mutant in presence or absence of oxidative stress; **2)** confirmation of differentially expressed genes by quantitative real-time PCR; and **3)** establishing the role of AraC in transcriptional regulation of differentially expressed genes in intramacrophage survival and virulence in mice.

The results from these studies were expected to provide comprehensive information regarding the genes that were under the control of AraC in absence or presence of oxidative stress. It was also our expectation that these studies would uncover the role of AraC in transcriptional regulatory mechanisms of *F. tularensis*.

3. MATERIALS AND METHODS

3.1. Bacterial strains

The bacterial strains used in this study are listed in **Table 1**. The wild type *F. tularensis* LVS used in this study was obtained from BEI Resources, Manassas, VA. The deletion mutant of *araC* (*FTL_0685*) gene (Δ*araC*) of *F. tularensis* LVS and its transcomplemented strain (Δ*araC*+p*araC*) were generated in Dr. Meenakshi Malik's laboratory at Albany College of Pharmacy and Health Sciences (ACHPS), Albany, NY. A transposon insertion mutant in *emrA1* gene of *F. tularensis* LVS available in our laboratory and used in previously published works was also used in this study. The *E. coli* DH-5α and S17 strains were obtained from Invitrogen.

The *Francisella* strains were cultured on Mueller-Hinton (MH) chocolate agar plates (BD Life Sciences) and incubated for 48 hours at 37ºC and 5% CO_2. Individual colonies were selected and inoculated at MH broth (MHB) enhanced with Isovitalex (BD Biosciences, San Jose, CA), Ferric Pyrophosphate, Glucose, Anhydrous Calcium Chloride, and Hydrous Magnesium Chloride. The bacterial cultures were incubated at 37ºC with constant shaking until the bacterial growth reached the mid-log-phase. Bacterial aliquots were snap-frozen in liquid nitrogen and stored at -80ºC. The *emrA1* mutant was grown on MH Chocolate agar plates, or MHB supplemented with Kanamycin (10µg/ml). For the transcomplemented strain (Δ*araC*+p*araC*), Hygromycin (100µg/ml) was added to the media instead of Kanamycin. All work was performed under containment of Biosafety level 2 (BSL2).

Table 1. List of bacterial strains, plasmids, and primers used in this study

Strains, plasmids, and primers	Description	Source or reference
Strains		
F. tularensis LVS	Wild type strain	ATCC
F. tularensis Δ*araC* mutant	Deletion mutant of *araC* gene of *F. tularensis* LVS	This study (Dr. Malik's Lab.)
F. tularensis emrA1 mutant	LVS FTL_0687::Tn5 Kan^r	[112]
F. tularensis araC transcomplemented strain (Δ*araC*+p*araC*)	*F. tularensis* LVS, Δ*araC*, pMM09 (pMP822+*araC*), $Hygro^r$	This study (Dr. Malik's Lab.)
E. coli	F–Φ80lacZΔM15Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 (rK–, mK+) *pho*A *sup*E44 λ– *thi*-1 *gyr*A96 *rel*A1	Invitrogen
Plasmids		
pMP822	*E. coli-Francisella* shuttle vector, $Hygro^r$	[159]
pJC84	*E. coli-Francisella* suicide vector, Kan^r	[160]
pMM03	pJC84 + fused flanking fragment of *araC* gene, Kan^r	This study
pMM014	pMP822 + *araC*, $Hygro^r$	This study
Primers for *araC* gene deletion		
MP135	5'-CAAGGATCCAAATAGATTGTGTTAGCATTGCAC-3'	
MP137	5'-TTTATCTGTACTCTTTCACTAGAGCATACTTTGTCCTTTTTTCACCA-3'	
MP139	5'-TGGTGAAAAAAGGACAAAGTATGCTCTAGTGAAAGAGTACAGATAAA-3'	
MP140	5'-TGATGTCGACGCAATACTCAAGTGGAACAACTGG-3'	
Primers for confirmation of *araC* gene deletion		
MP037	5'-CCGGATCCATGAAATTTGAATTACCAAAAC-3'	

	MP 038	5’-CGCTGCAGCTAATCAGCGAATTGCTCAGAAAC-3’
	MP192	5’-ATGGGTGTTGCCATCAAATAGG-3’
	MP193	5’-TGTTGTCGCCAACGTGAAA-3’
Primers for transcomplementation		
	MP372	5’-CAAGGTTCCATGATACGGGAAGAGATCACATAT-3’
	MP373	5’-TGATCTCGAGTCAGTTCTTATAAATATTTTTTATC-3’
Primers for qRT-PCR		
SilC	MP015	5’-AAGCCAAGTTAGTGCTGCATATTT-3’
	MP016	5’-AAGCCAAGTTAGTGCTGCATATTT-3’
emrA1	MP017	5'-GTGCATCTTGTAAAGAGCCAGCATC-3'
	MP018	5'-CAGCCAAACTAAGCGCACAGTCAT-3'
emrB	MP019	5’-TCCTAATCCCTGAATAGCCGTTGT-3’
	MP020	5’-AGGTGTTGCCGCTATTATTGGTGC-3’
groES	MP280	5’-ACCCATGATATCTTCTTCTCTC-3’
	MP281	5’-AGAGTATTAGTTCGTCGTGCAG-3’
groEL	MP282	5’-TCTTCAAAGCCTTTGCCTTC-3’
	MP283	5’-TGTCACTGCAGGTATGAATCC-3’
ClpB	MP284	5’-TTTCATAATCCTGACCTTGCA-3’
	MP285	5’-GGCGGCTATCTAACTGAACAT-3’
FTL_1957	MP286	5’-AGATATAACAGAAGATGAAGCTGC-3’
	MP287	5’-TAGGGATGTTCAAGCTTAGTAC-3’
dnaJ	MP564	5’- GCCAAGGTACTATTCGTAGACAAC -3’
	MP565	5’- ATTAGAGCCAGAATCACCCTCA -3’
dnaK	MP562	5'-TAGTGGGTGGACAAACTCGTATG-3'
	MP563	5'-TCTCAATACCTAGAGAAAGCGGTG-3'

3.2. Construction of Δ*araC* gene deletion mutant of *F. tularensis* LVS

The plasmids and primer sequences used for generation and confirmation of the deletion mutant of *F. tularensis araC* gene and the transcomplemented strain are listed in **Table 1**. pJC84 suicide vector that allows for SacB-dependent allele replacement was used as reported earlier [160] for the generation of *in-frame* gene deletion of *araC* gene (*FTL_0689*) in *F. tularensis* LVS. For deletion of *araC* gene, a 5' fragment composed of a 1152bp sequence from upstream to the start codon of *FTL_0689* to the start codon was amplified by PCR using MP135 and MP137 primers. In addition, a 3' fragment of a 2081bp from downstream of *FTL_0689* to the stop codon was also amplified by PCR using MP139 and MP140 primers. By overlapping extension PCR using Pfx enzyme with primers MP135 and MP140, both fragments were merged to generate a larger fragment (2233bp). Since MP135 contained a *BamH1* site and MP140 with a *SalI* site, the merged fragment was cloned into the *BamH1* and *SalI* sites of pJC84 to generate pMM03. After that, PCR confirmation is performed. For electroporation, *F. tularensis* LVS were cultured in MH-chocolate agar plate for 48 hours at 37ºC with 5% CO_2. A starting OD_{600} of 0.05 *F. tularensis* LVS colonies was prepared in a 30 ml MHB and incubated at 37ºC with shaking. Once the suspension reached an OD_{600} of approximately 0.5, the bacteria were centrifuged at 4ºC for 5 minutes at 4000 g. Then, the supernatant was discarded; the pellet was resuspended in the same volume of 0.5M sucrose solution for washing and then resuspended in 100µl of 0.5M sucrose solution. After that, 1µg of pMM03 was added to the bacterial suspension and incubated on ice for 10 minutes. Electroporation was conducted using an Eppendorf Eporator at 2,500 V, 200Ω, 25µF. Immediately after electroporation, 1ml of pre-warmed MHB was added and bacteria were incubated for another 2-3 hours at 37ºC with agitation. Then, the electroporated bacteria were

centrifuged at 4°C for 5 minutes at 4000 g and resuspended in 100µl of 0.2M KCL. The bacteria were selected for antibiotic resistance on MH-chocolate agar plates containing 25µg/ml kanamycin. Following that, sucrose counterselection was performed by growing 0.5 OD_{600} of independent kanamycin resistant clones on MHB at 37°C with shaking and then supplemented with sucrose solution to make a final concentration of 5% and incubated for additional 2 hours.

After incubation, the bacteria were serially diluted and plated on MH-chocolate agar supplemented with 8% sucrose and incubated at 37°C with 5% CO_2 for 48 to 72 hours. To verify loss of Kanamycin resistance marker, sucrose resistant clones were streaked into MH-chocolate agar plates containing 25µg/ml Kanamycin. The sucrose resistant and Kanamycin sensitive clones were screened for the loss of *araC* gene using PCR. For *araC* gene deletion confirmation, a duplex colony PCR was performed using the primer sets: *araC* gene specific (MP192/MP193) and internal control-*sodB* gene (MP037/MP038) **(Figure 7).** To confirm the *in-frame FTL_0689* deletion, we conducted genomic DNA sequencing for PCR positive clones.

For the transcomplemented strain (Δ*araC*+p*araC*), the sequence of *F. tularensis araC* gene was amplified using the primers MP372 and MP373 with the enzyme Pfx. BamHI and XhoI restriction enzymes were used to digest the amplified sequence and then cloned into *E.coli-Francisella* shuttle vector pMP822 [159] to generate pMM014. This plasmid was then confirmed using PCR and DNA sequencing and was electroporated into Δ*araC* mutant at 2,500 V, 200Ω, 25µF. To confirm the transcomplementation of *araC* gene, the bacteria were selected in MH-chocolate agar plates containing 200µg/ml hygromycin. The hygromycin-resistant colonies were screened later with PCR.

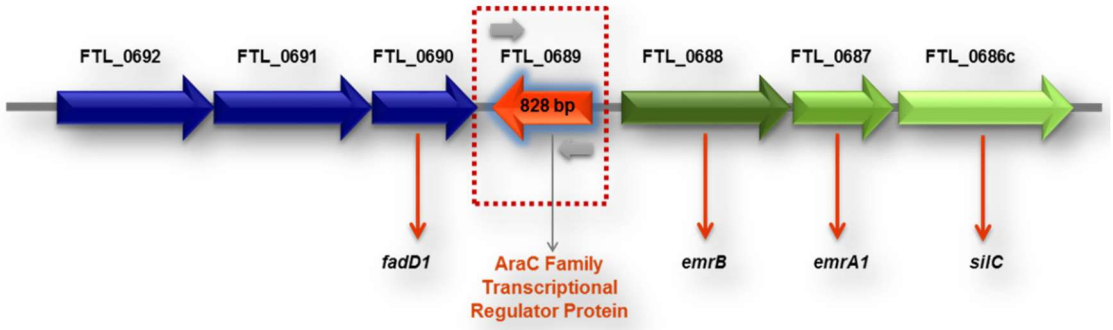


Figure 7. The genomic organization of the *araC* gene (*FTL_0689*) of *F. tularensis* LVS. The gene presents upstream of the three MEP genes *emrB, emrA1,* and *silC*. The grey arrows represent the location of the primers used to screen *araC* gene deletion.

3.3. Determination of the role of AraC in sugar utilization

The AraC of *F. tularensis* LVS was evaluated for its role in arabinose sugar utilization. Two different experiments were approached by growing the bacteria on MH chocolate agar plates with or without the sugars and by generating growth curves in the presence or absence of sugars.

3.3.1. Growth in arabinose versus glucose containing chocolate agar

Mueller Hinton (MH)-chocolate agar plates containing glucose or arabinose sugars were prepared in our lab. Wild type *F. tularensis* LVS or Δ*araC* mutant were grown overnight in MHB at 37ºC with shaking. Bacteria were washed twice using 1×Phosphate-Buffered Saline (PBS) to remove any excess growth media. *E. coli* DH-5α or *E. coli* S17 used as positive controls. Bacteria were streaked on MH chocolate agar plates supplemented with 1% glucose or 1% arabinose and incubated 37ºC with 5% CO_2 for 48 hours. The bacterial growth pattern of Δ*araC* mutant was compared to that of the controls.

3.3.2. Bacterial growth curve using Chamberlain's Defined medium (CDM)

A chemically defined medium known as Chamberlain's medium was prepared as described previously [161]. The sugar concentration in the CDM was 0.4% and the media were prepared at three different conditions: 1) +glucose/-arabinose, 2) -glucose/+arabinose, and 3) -glucose/-arabinose. *F. tularensis* LVS or the Δ*araC* mutant were cultured at 37ºC with 5% CO_2 for 48 hours and washed twice with 1× PBS before inoculated into CDM. Bacteria at 0.2 OD_{600}, which corresponded to 1×10^9 CFU/ml, were inoculated into the CDM and incubated at 37ºC with shaking. OD_{600} was recorded every 4 hours, and growth curves were generated using

GraphPad Prism 6 software.

3.4. Disc diffusion assays

Disc diffusion assay, also known as Kirby-Bauer assay for antibiotic sensitivity testing, is a technique using sterile paper discs containing antibiotic or any other compound to determine bacterial sensitivity to that specific antibiotic/compound. *F. tularensis* LVS or the Δ*araC* mutant was grown on MH-chocolate agar plate and incubated for 48 hours at 37ºC and 5% CO_2. The bacterial suspension was prepared using sterile 1× PBS and adjusted to 2.0 OD_{600}. A thick bacterial layer was spread on MH chocolate agar using sterile cotton swabs (Puritan Medical Products Company, Guilford, Maine). Antimicrobial susceptibility test discs including: Polymyxin B (300IU/IE/UI), Nalidixic acid (30µg), Chloramphenicol (30µg), Streptomycin (10µg), Erythromycin (15µg), Gentamycin (10µg), Novobiocin (30 µg), or Tetracycline (30µg) (BD BBL™ Sensi-Disc™) were placed over the bacterial lawn and incubated at 37 ºC and 5% CO_2 for 48 hours or until bacterial lawn was thick enough to observe antibiotic sensitivity indicated by a zone of clearance around the disc. Using a ruler, the bacterial sensitivity was determined by measuring the zone of inhibition around antibiotic discs (in mm). If the bacterial susceptibility to a certain antibiotic is more, a larger zone of inhibition is formed around that antibiotic disc.

3.5. Determination of the sensitivity of *F. tularensis* Δ*araC* mutant to oxidants

The sensitivity of the Δ*araC* mutant to various oxidants was determined using bacterial killing assay and by generating bacterial growth curves as described above. The bacterial growth curve used for bacterial sensitivity was generated using MH-broth, instead of CDM.

3.5.1. Bacterial killing assay

The bacterial killing assay is a direct method to determine the sensitivity of the bacteria to certain oxidative compounds. The procedure was performed according to the previously published protocols from our lab [112, 162, 163]. In brief, peroxides [1mM H_2O_2, tert-butyl hydroquinone (TBH) and cumene hydroperoxide (CHP)] or superoxide-generating compounds (20 mg/ml menadione, 50 mg/ml pyrogallol, 50 mg/ml paraquat) were serially diluted in a 96 well-plate using MHB or 70% ethanol in case of CHP and paraquat. Wild type *F. tularensis* LVS or the Δ*araC* mutant was adjusted to 0.2 OD_{600}, and an equal amount of bacteria were added to each well containing the serially diluted compounds. The 96 well-plate was incubated for 1 and 3 hours at 37°C and 5% CO_2 and then the bacteria were spotted on MH-chocolate agar plates using a multichannel pipette. In another approach, bacterial suspensions were exposed to H_2O_2, pyrogallol, or paraquat for 1 hour with shaking at 180 rpm and 37°C. Bacteria were serially diluted (10-fold) and plated on MH chocolate agar for 48 hours at 37°C and 5% CO_2. Colony forming units (CFUs) of bacteria were counted and plotted as Log_{10} CFU/ml.

3.5.2. Cell culture assay

Murine Raw 264.7 macrophage cells ($1x10^6$ cells/well) were seeded in a 24-well tissue culture plate. Dulbecco's Modified Eagle's medium (DMEM) (Corning, without L-glutamine and Sodium Pyruvate) was used in this experiment and was supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS), 2% L-glutamine, and 1% Sodium Pyruvate. The cells were infected with *F. tularensis* LVS or the Δ*araC* mutant at a multiplicity of infection (MOI) of 1, 10, 100, or 500. Cells were centrifuged for 10 minutes at 1000 rpm to synchronize bacterial infection and incubated at 37°C and 5% CO_2 for 4 and 24 hours. Two hours post-

infection, medium from the infected cells was replaced with DMEM containing gentamycin (250µg/ml) to kill extracellular bacteria and was replaced with DMEM without any antibiotics after one hour of incubation. The cells were lysed with 0.1% sodium deoxycholate at 4 and 24 hrs post-infection and then (10µl) of 10-fold serial dilution of the lysed cells was spotted on MH chocolate agar plates for enumeration of bacterial CFUs. Plates were incubated at 37ºC and 5% CO_2 for 48 hours and figure was generated using GraphPad Prism 6 software **(Figure 8).**

3.6. Mouse survival studies

The role of AraC in virulence was investigated by infecting wild type C57BL/6 mice with *F. tularensis* LVS or the Δ*araC* mutant intranasally after anesthetizing them through intraperitoneal injection of ketamine and xylazine. Mice were inoculated with four different doses ($1x10^2$, $1x10^3$, $1x10^4$, or $1x10^5$ CFUs) in a volume of 20µl (10µl/nare) of *F. tularensis* LVS or the Δ*araC* mutant. Morbidity and mortality of the infected mice were observed for 15 days. Body weight and survival were recorded daily. Survival was expressed as the Kaplan-Meier survival curve, and the data were analyzed statistically by the Log-rank test **(Figure 9).**

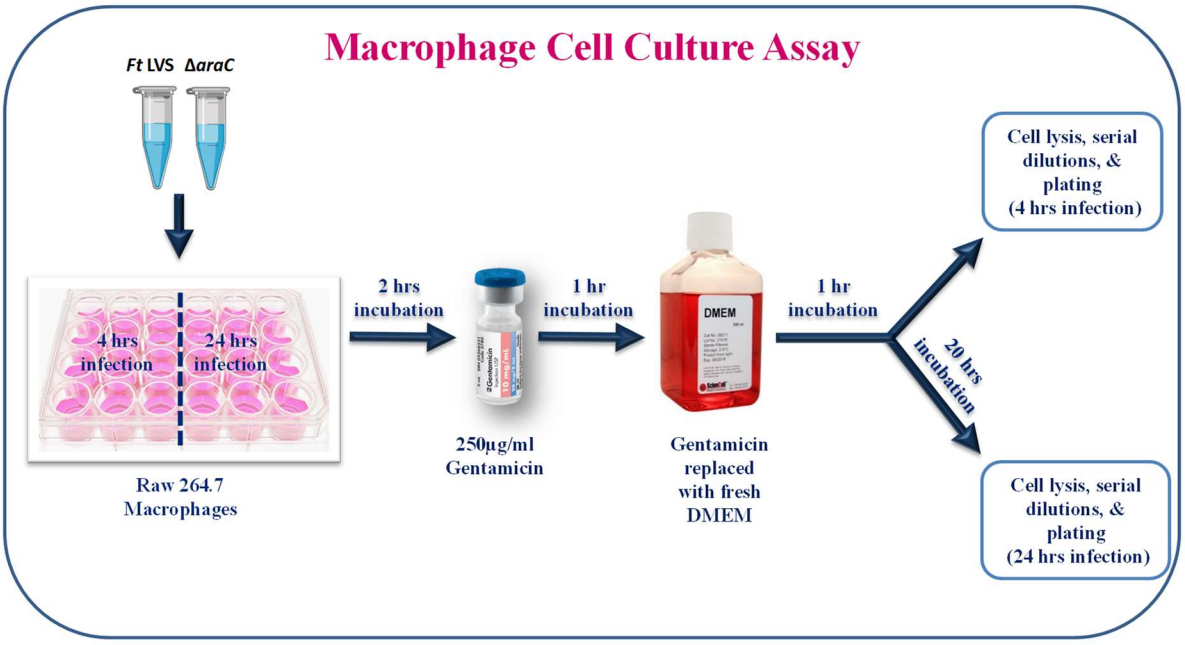


Figure 8. Design of macrophage cell culture assay.

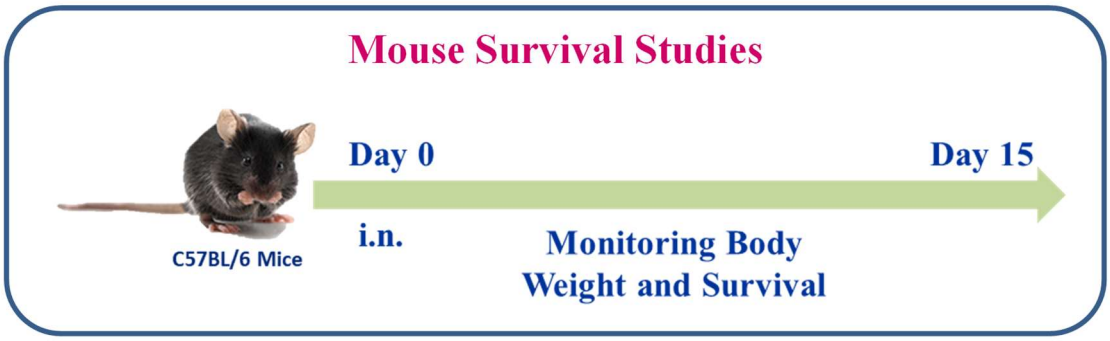


Figure 9. Mouse survival studies. C57BL/6 mice were infected with varying doses of *F. tularensis* LVS and the Δ*araC* mutant intranasally (n=5 mice/bacterial strain). Morbidity and mortality of the infected mice were monitored and recorded daily for 15 days.

3.7. RNA extraction

To determine the role of AraC as a global transcriptional regulator, we profiled whole transcriptome of the Δ*araC* mutant in response to oxidative stress. This technique provides an quantitative measurement of RNA expression level [164] during oxidative stress. The superoxide-generating compound, menadione, was used for treatment. Menadione (20 mg/ml) was dissolved in 70% ethanol and filtered using 0.2m syringe filter (MILLEX-GP, Millipore Express PES membranes, Millipore Ireland Ltd, Cork, IRL). *F. tularensis* LVS or the Δ*araC* mutant were suspended in MHB (0.2 OD_{600}), and menadione was added for a final concentration of 1.25mM. For untreated control, MHB was added alternatively, and bacterial tubes were incubated for 1 hour at 37ºC with 5% CO_2. Bacteria were then centrifuged at 10000 rpm for 10 minutes at room temperature, and pellets were resuspended in 990 µl lysis buffer (from Purelink RNA mini kit/Invitrogen) supplemented with 10µl 2-mercaptoethanol following the manufacturer's protocol. The bacteria were then lysed by vortexing, and 1 ml TRIzol reagent (Invitrogen™) was added to the lysate and mixed well. The lysate was incubated at 25ºC for 4 minutes. Cold chloroform was added to the lysate and mixed well. The mixture was incubated for 2-3 minutes and centrifuged at 12000 rcf for 15 minutes at 4ºC until an aqueous colorless solution containing the RNA is separated. After transferring the clear solution into another tube, isopropanol was added, mixed, and incubated for 10 minutes at room temperature. After that, the Spin cartridges with the collection tubes supplemented with the kit were used to purify RNA. RNA samples were treated with DNase using Invitrogen™ (TURBO DNA-free Kit). Pure RNA samples were stored at -80ºC.

3.8. RNA sequencing

Purified RNA samples were sent to the Genomics Core Laboratory at the New York Medical College for sequencing. Differentially expressed genes were evaluated based on $\log_2$(fold change) value. The differential expression of *F. tularensis* LVS and Δ*araC* mutant genes was analyzed based on $\log_2$(fold change) of genes in the Δ*araC* mutant as compared to those in wild type *F. tularensis* LVS in the untreated, and the menadione treated samples. For the downregulated genes, the expression of the *araC* gene which has been deleted in the Δ*araC* mutant was taken as 100%. Any gene that represented less than 50% of the $\log_2$(fold change) of the *araC* gene was considered as downregulated. However, to further narrow the number of genes identified as under the control of AraC, we considered the *p*-values and any value less than 0.05 was considered significant. All the data analyzed were cumulative of two independent experiments **(Figure 10)**.

3.9. Quantitative real-time polymerase chain reaction (qRT-PCR)

qRT-PCR was used to confirm differentially expressed genes from the RNA sequencing experiments. Gene-specific primers used for qRT-PCR are listed in **Table 1**. All qRT-PCR experiments were performed using 96-well plates (MicroAmp Optical 96-Ell Reaction Plate, Applied Biosystems, Inc., Foster City, CA, USA). Samples denaturation, reverse transcription, and amplification were conducted using a 7500 Fast Real-Time PCR System (Applied Biosystems, Inc., Foster City, CA, USA). The C_T (cycle threshold) values of both *F. tularensis* LVS and the Δ*araC* mutant were provided by the qRT-PCR instrumentation. These C_T values are readily imported into Excel sheets to calculate ΔC_T of each of the wild type and Δ*araC* mutant for every tested gene. For example, if we assumed that the wild type

F. tularensis is A and the Δ*araC* mutant is B, then the calculations are: $\Delta C_T A = C_{T\ (target)} A - C_{T\ (control)} A$ and $\Delta C_T B = C_{T\ (target)} B - C_{T\ (control)} B$. Next, the $\Delta\Delta C_T$ was calculated for every target gene using the formula: $\Delta\Delta C_T = \Delta C_T B - \Delta C_T A$. After that, expression normalization was calculated as $2^{-(\Delta\Delta CT)}$. This indicated the fold change in gene expression of the Δ*araC* mutant as compared to the *F. tularensis*.

3.10. Statistical analysis

All results were analyzed using GraphPad Prism 6 software and were represented as Mean ± Standard Deviation (SD). Comparisons among experimental groups were made using one-way ANOVA. Survival data were plotted as the Kaplan-Meier survival curves, and significance was statistically calculated by the Log-rank test. The statistically significant data are considered as p-value < 0.05.

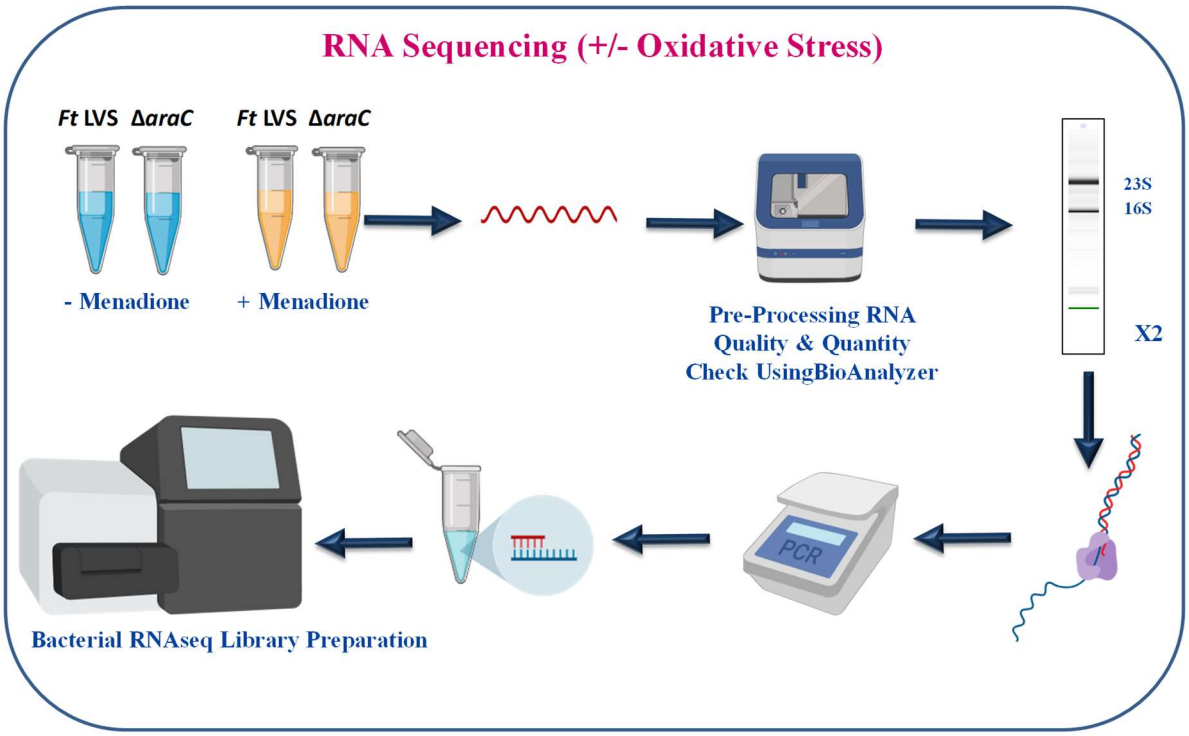


Figure 10. RNA sequencing protocol.

4. RESULTS

The primary goal of this study was to investigate the role of AraC as a transcriptional regulator of *F. tularensis,* and to understand its role in tularemia pathogenesis. In literature, AraC has been reported to be a member of the AraC/Xyls transcriptional regulator family [139, 165-167]. AraC has been shown to regulate L-arabinose metabolism in *E. coli* and serves both as an activator as well as a repressor [139, 168]. The role of AraC in *F. tularensis* LVS is not characterized to date. In this study, we elucidated the role of AraC in gene regulation of *F. tularensis*.

Our overall hypothesis was "*AraC may have a unique role in transcriptional regulation of multidrug efflux pump genes in addition to the genes involved in general metabolism as well as the stress response.*" We developed two specific aims to address our hypothesis.

Specific Aim 1: Phenotypic characterization of the *araC* gene deletion mutant (Δ*araC*) of *F. tularensis* LVS.

4.1. The genomic organization of the *araC* gene of *F. tularensis* LVS

The genomic organization of *araC* gene is shown in **Figure 11A**. The *araC* gene of *F. tularensis* is transcribed divergently from the downstream operon encoding for components EmrB, EmrA1, and SilC of MFS type MEP **(Figure 11B)**. All the other *Francisella* strains including the virulent *F. tularensis* Schu S4 and *F. novicida* exhibit an identical genomic organization to that observed in *F. tularensis* LVS. A similar genomic organization is also present in *Burkholderia pseudomallei* (*B. pseudomallei*). In *E. coli, Yersinia pestis,* and *Salmonella typhi,* this gene is annotated as a transcriptional regulator rather than *araC* and is transcribed in a similar direction as the upstream genes. Thus, the genomic organization of

araC gene in *Francisella* is unique. This also implies that AraC may have a unique role in tularemia pathogenesis.

Sequence comparison of AraC transcriptional regulator within *Francisella* subspecies revealed high similarity, ranging from 97% to 100%. However, AraC showed less homology when aligned with the transcriptional regulator present in a similar genomic location in other pathogenic bacterial species. **Table 2** shows the identity percentage of some Gram-negative pathogenic bacteria, including *B. pseudomallei* (51.35 %), *S. typhi* (47.62 %), *Y. pestis* (42.42 %), and *E. coli* (33.33 %), with the AraC amino acid sequence of *F. tularensis* LVS. We determined that the AraC amino acid sequence of *F. tularensis* is highly conserved in almost all *Francisella* subspecies. Multiple sequence alignment in **Figure 12** using Clustal W demonstrated that AraC protein sequences of all the pathogens used in this alignment exhibited a conserved C-terminal domain (highlighted grey), with a consensus motif GxxxxxxFxxxxxxxxxxxP (G/F/P). This consensus sequence starts at amino acid 244 and ends at amino acid 263 of AraC (FTL_0689).

Moreover, to demonstrate the protein binding motif logo sequence, we used one of the most commonly utilized software, Meme (Multiple Em for Motif Elicitation), a visually rich and informative logo representation of sequence alignment. The alignment identified the C-terminal and N-terminal conserved sequences of AraC protein of *F. tularensis* LVS compared to *E. coli* AraC and *Y. pestis* VirF. **Figure 13A** demonstrates the conserved DNA binding motif in the C-terminal domain, which is similar to the binding domain that we revealed using Clustal W. In addition, we identified two protein binding motifs located in the N-terminal. **Figure 13B** represents the signature motif of HPxM, and **Figure 13C** is the second binding motif WxH. These N-terminal domains are unique, present in AraC of a few pathogens

including all *Francisella* strains. Collectively, bioinformatic analysis of AraC suggests that AraC transcriptional regulator of *Francisella* is unique and may be responsible for a specialized role in the virulence of *Francisella*.

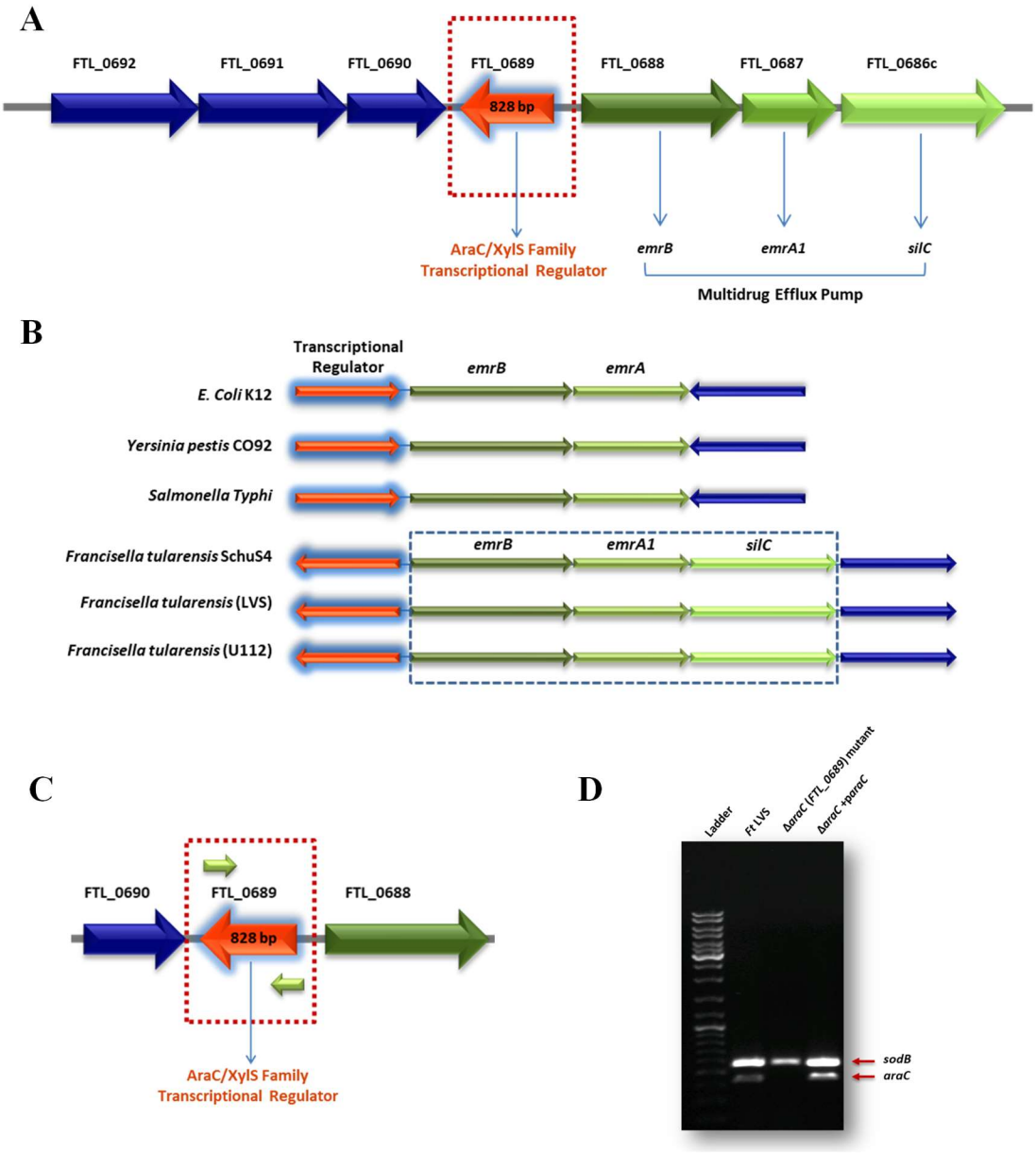


Figure 11. Generation of the Δ*araC* deletion mutant. (A) Genomic organization of the *F. tularensis* LVS multidrug efflux pump (MEP) genes showing the presence of the *araC* transcriptional regulator upstream of this set of genes and is annotated as *FTL_0689*. **(B)** schematic diagram showing *araC* transcriptional regulator of *F. tularensis* (red box) is transcribed divergently from the MEP components (blue dashed box), unlike other organisms. **(C)** Deletion of *araC* and primer locations for the screening of *araC* mutant indicated by grey arrows. **(D)** Confirmation of *araC* deletion by the PCR. The wild type *F. tularensis* LVS and *sodB* gene primers were used as positive and internal controls, respectively.

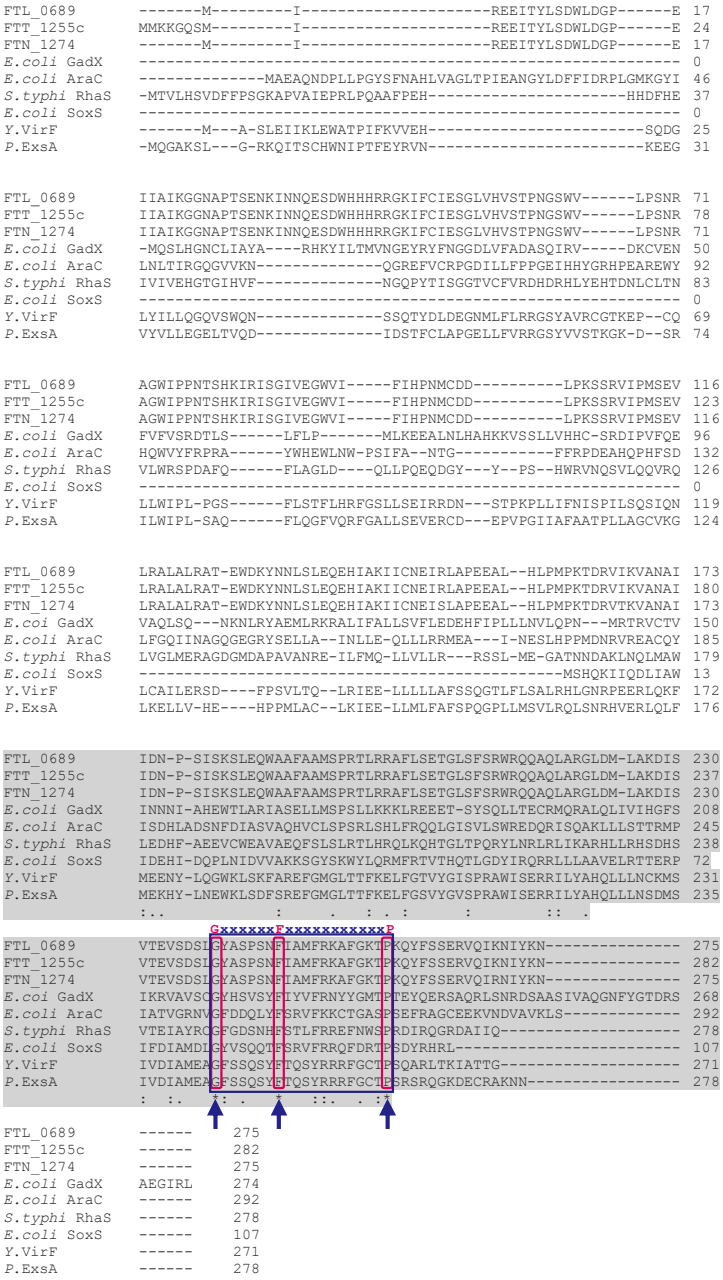


Figure 12. Protein sequence alignment of the AraC of *F. tularensis* and XylS/AraC family proteins from other pathogens. Multiple alignments were generated using Clustal W software. The C-terminal region (highlighted in grey) shows a conserved DNA binding motif GxxxxxxFxxxxxxxxxxxP (conserved residues are represented by red boxes and arrows). The numbers in the right indicate amino acids location.

Table 2: *F. tularensis* LVS AraC sequence alignment with other bacterial strains

Pathogen	Locus tag	Product	Identity	Accession
F. tularensis subsp. *tularensis* Schu S4	FTT_1255c	AraC family transcriptional regulator	99.65%	YP_170213
F. novicida U112	FTN_1274	Transcriptional regulator, AraC family	98.91%	ABK90155
E. coli O25b:H4	WLH_02767	Transcriptional regulator GadX	32.63%	ANK04028
E. coli K-12	U069_c0078	AraC family putative transcriptional regulator protein	33.33%	AKK15826
E. coli	N/A	DNA-binding transcriptional regulator SoxS	27.27%	AAA24640
S. enterica subsp. *enterica* serovar Typhi	CJP42_0176	HTH-type transcriptional activator RhaS	26%	AXR57446
Y. enterocolitica	RLY99535	Virulence regulon transcription al activator VirF	28%	RLY99535
P. aeruginosa PAO1	PA1713	transcriptional regulator ExsA	20.46%	AE004598_1

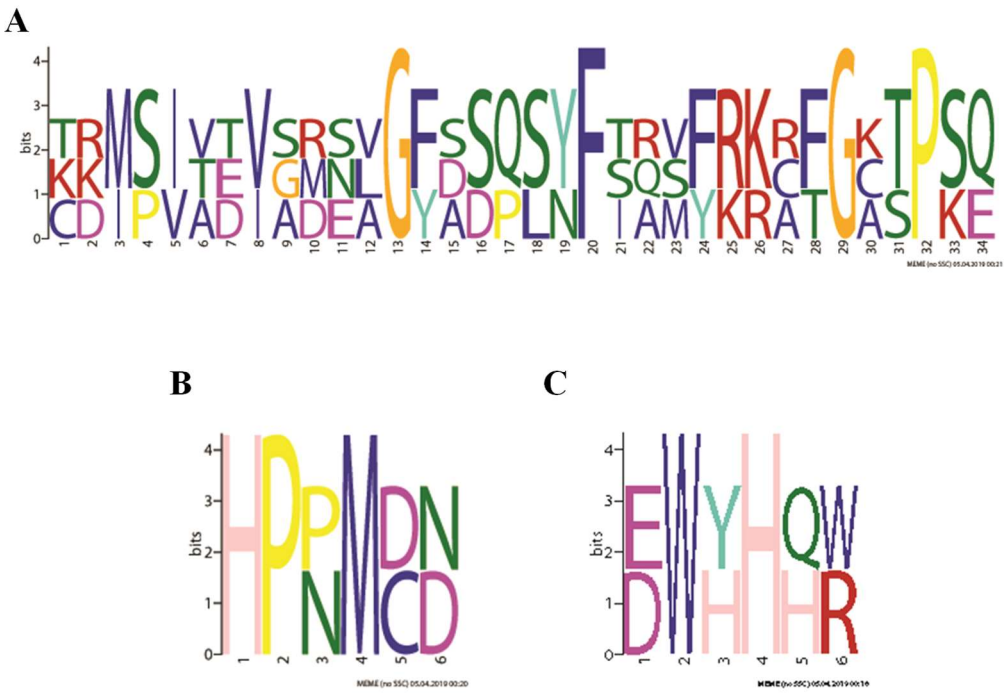


Figure 13. AraC binding motifs are conserved. (A) AraC C-terminal region showing the DNA binding domain (GxxxxxxFxxxxxxxxGxxP). **(B and C)** Protein binding motifs in the N-terminal region of AraC.

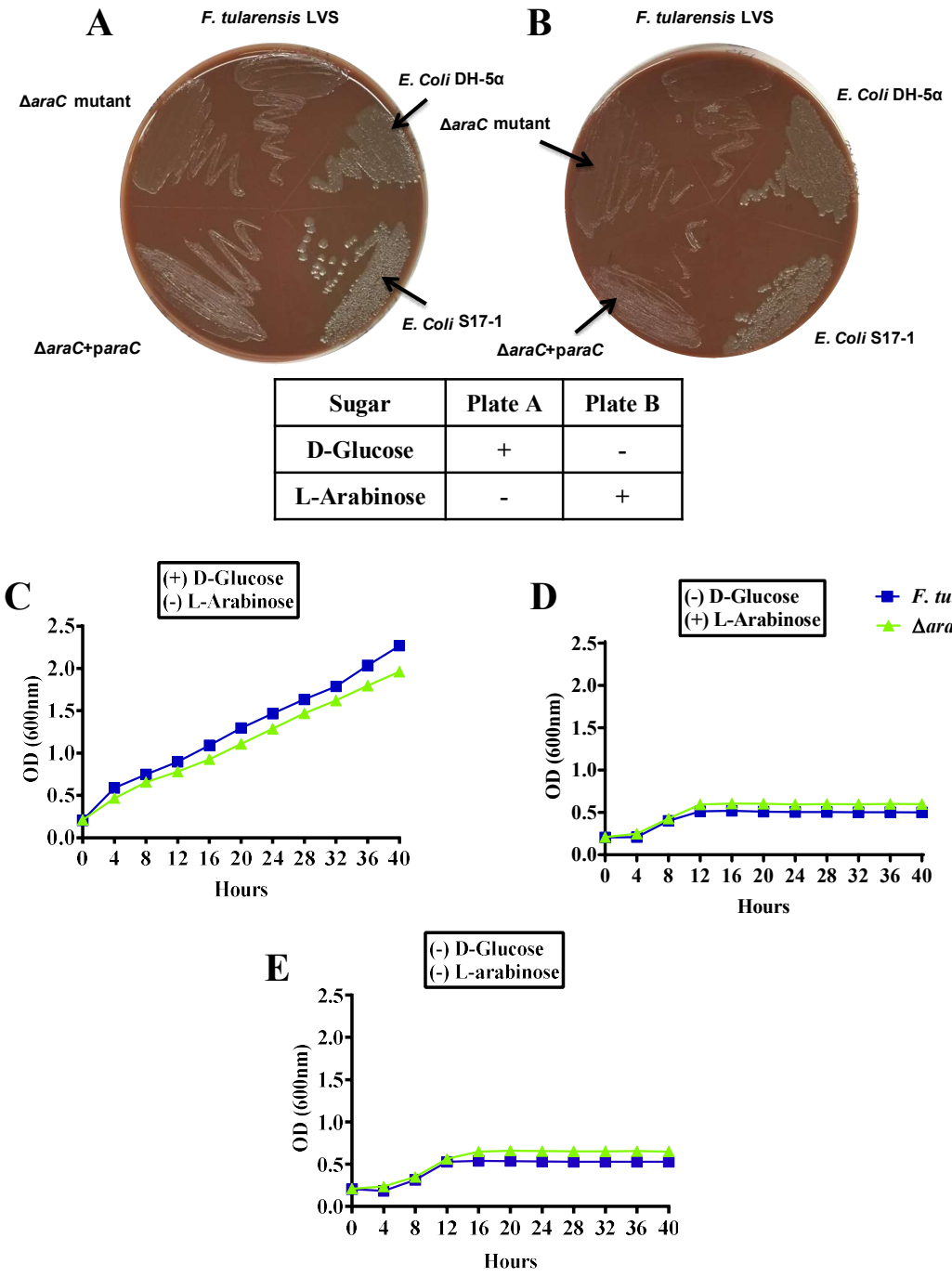
4.2. Generation of *araC* gene deletion mutant and the transcomplemented strain

The *araC* in-frame gene deletion mutant (Δ*araC*) of *F. tularensis* LVS was generated by an allelic replacement method and confirmed by PCR. Primers internal to the *araC* gene amplified a fragment of 500 bp in the wild type *F. tularensis* LVS, while the absence of this amplicon indicated deletion of *araC* gene **(Figure 11C and 11D)**. The full-length *araC* gene was amplified in the transcomplemented strain indicating the restoration of the *araC* gene in the Δ*araC* mutant. Primers specific to the *sodB* gene were used as internal control and a PCR fragment corresponding to the *sodB* gene was amplified in all the three strains tested, indicating that the absence of *araC* gene-specific fragment in the Δ*araC* mutant is not due to the lack of input DNA. Furthermore, the in-frame gene deletion in the Δ*araC* mutant was additionally confirmed by DNA sequencing (data not shown).

4.3. Determining the involvement of AraC in L- arabinose utilization and metabolism

To determine if AraC transcriptional regulator is responsible for L-arabinose metabolism, we tested the ability of the Δ*araC* mutant to grow in the presence of L-arabinose containing media. We prepared MH-chocolate agar plates containing 10g of either the D-glucose or L-arabinose. The plates were then streaked with *F. tularensis* LVS, the Δ*araC* mutant, or the transcomplemented strain. Two strains of *E. coli*, DH-5α and S17.1, were used to serve as positive controls as both these strains can grow in the presence of arabinose. Our results demonstrated that the Δ*araC* mutant, the wild type *F. tularensis* LVS, and the transcomplemented strain as well as *E. coli* DH-5α, and S17 strains were able to grow sufficiently in the presence of D-glucose **(Figure 14A)**. However, all three *F. tularensis* strains showed traces of visible growth in the presence of L-arabinose, as compared to the *E. coli*

strains **(Figure 14B)**. These results demonstrate that all *F. tularensis* LVS strains fail to grow in the presence of L-arabinose indicating that *Francisella* cannot utilize L-arabinose as a carbohydrate source and that D-glucose is the preferred sugar source for *F. tularensis* LVS. A trace amount of growth for the wild type *F. tularensis*, the Δ*araC* mutant and the transcomplemented strains were observed when grown on MH-chocolate agar plates containing L-arabinose as the sole sugar source. This indicated that either the *Francisella* strains poorly utilize L-arabinose or that components of the MH-agar media were supporting this scant growth. To address this notion, we generated bacterial growth curves using CDM with three different sugar compositions: one with neither glucose nor arabinose, one with D-glucose only, and the other with arabinose as the sole carbohydrate source. Both the Δ*araC* mutant and the wild type *F. tularensis* LVS grew very well in glucose-containing CDM (**Figure 14C**). Both strains grew at a similar rate and reached an approximate OD_{600} of 2.4 after 40 hours of incubation. However, when the CDM was supplemented with L-arabinose only **(Figure 14D)** or with no sugar **(Figure 14E)**, both the wild type and the mutant strains reached an OD_{600} of 0.5 after a short period of growth and then entered into a stationary phase, indicating their growth was largely affected by the lack of appropriate sugars in the medium. Collectively, these results demonstrate that D-glucose is essential for the growth of *F. tularensis* and it cannot utilize L-arabinose. Furthermore, these results also demonstrate that AraC of *F. tularensis* LVS is likely not involved in the regulation of L-arabinose metabolism.



Sugar	Plate A	Plate B
D-Glucose	+	-
L-Arabinose	-	+

Figure 14. Growth of *F. tularensis* LVS strains in the presence of different carbohydrate substrates. The wild type *F. tularensis* LVS, the Δ*araC* mutant, or the transcomplemented strains were grown in MH-chocolate agar containing glucose **(A)**, or arabinose **(B)**. *E. coli* DH-5α and *E. coli* S17.1 were used as positive controls. Growth curves of the wild type *F. tularensis* LVS and Δ*araC* mutant in CDM supplemented with glucose **(C)**, arabinose **(D)**, or in the absence of sugar **(E)**. (n = 3)

4.4. Investigating the role of AraC in the regulation of EmrA1 MEP components

In the genomic organization (**Figure 11A),** *araC* of *F. tularensis* is divergently transcribed from the genes encoding major facilitator superfamily type multidrug efflux pump genes: *emrB*, *emrA1*, and *silC*. Such a unique organization indicated that AraC might be involved in the regulation of these downstream genes. Thus, we first tested the expression levels of *emrB*, *emrA1*, and *silC* genes in the Δ*araC* mutant and compared them with those in the wild type *F. tularensis* LVS by qRT-PCR. The results shown in **Figure 15** indicated that expression of all these three genes was significantly downregulated in the Δ*araC* mutant in stark contrast to the wild type *F. tularensis* LVS, suggesting that AraC may have a role in the regulation of the components of the Emr multidrug efflux pump.

4.4.1. The sensitivity of Δ*araC* mutant to oxidants

We next investigated to confirm if the phenotype of the Δ*araC* mutant is similar to those described for the *emrA1* and Δ*silC* mutants. EmrA1 is a membrane fusion protein and SilC is an outer membrane protein of major facilitator superfamily type MEP in *F. tularensis* LVS [87, 112]. Loss of both *emrA1* and *silC* results enhanced sensitivity of these mutants towards oxidative stress. Therefore, we investigated if AraC contributes to the resistance of *F. tularensis* LVS towards oxidants via regulating MEP components *emrA1* and *silC.* We investigated the sensitivity of Δ*araC* mutant to peroxides and superoxide (O_2^-) generating compounds.

4.4.1.1. The Δ*araC* mutant of *F. tularensis* LVS is sensitive to hydrogen peroxide

To determine the sensitivity of Δ*araC* mutant to peroxides, we used organic peroxides and H_2O_2 in a spot assay. The *emrA1* mutant was used for a comparison. We performed bacterial killing assay by exposing wild type *F. tularensis* LVS, the Δ*araC* mutant, the transcomplemented strain Δ*araC*+p*araC*, and the *emrA1* mutant to varying concentrations of H_2O_2, incubated for one and three hours, and plated on MH-chocolate agar to determine the bacterial viability. The results showed an enhanced killing of the Δ*araC* mutant when exposed to increasing concentrations of H_2O_2 for one and three hours as compared to the wild type *F. tularensis* LVS. The transcomplementation restored the wild type phenotype and showed similar sensitivity after one and three hours of incubation. The *emrA1* mutant showed an enhanced killing as compared to the wild type *F. tularensis* LVS after one hour of exposure to H_2O_2 **(Figure 16A)**. These results indicate that Δ*araC* mutant, similar to the *emrA1* mutant, is sensitive to the increasing concentration of H_2O_2.

We next performed the bacterial killing assay to quantitate the bacterial numbers after exposure to H_2O_2. We exposed wild type *F. tularensis* LVS, the Δ*araC* mutant, the transcomplemented strain, and the *emrA1* mutant (adjusted to an OD_{600} of 0.2) to 8 mM of H_2O_2, a concentration that had been demonstrated bacterial killing in spot assays. Ten-fold dilutions of the bacterial strains after one hour of exposure to H_2O_2 were then plated on MH-chocolate agar plates. The results showed that about 40% of the Δ*araC* and the *emrA1* mutants were killed, which was significantly higher than the percentage of bacterial killing (28%) observed for the wild type *F. tularensis* LVS after one hour of exposure. The transcomplemented strain showed a significant increase in killing as compared to the wild type *F. tularensis* LVS and failed to restore the wild type phenotype possibly due to the loss of the

transcomplementation plasmid in the absence of antibiotic selection pressure **(Figure 16B)**. Furthermore, the bacterial counts revealed an approximately 12-fold decrease in the viability of Δ*araC* and the *emrA1* mutants as compared to the wild type *F. tularensis* LVS **(Figure 16C)**. Collectively, these results demonstrate that loss of *araC*, similar to the *emrA1* mutant, is associated with enhanced sensitivity of the Δ*araC* mutant to H_2O_2.

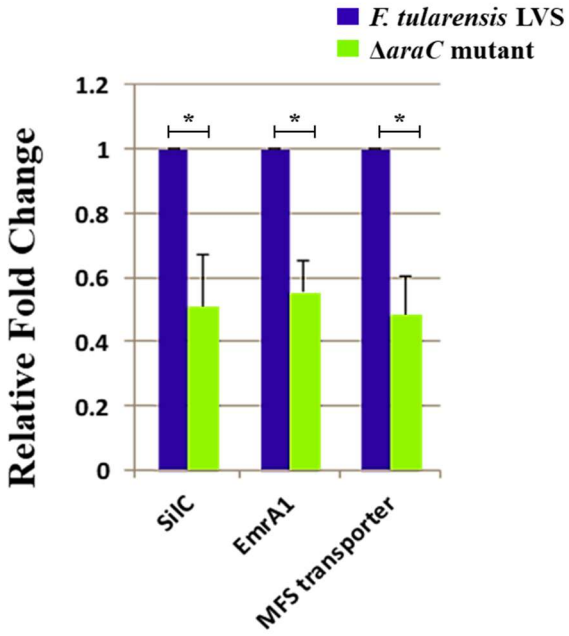


Figure 15. qRT-PCR analysis of *silC*, *emrA1*, and *emrB* genes. The data shown are representative of three independent experiments. Data are analyzed using Two-tailed student T-test. *p<0.05.

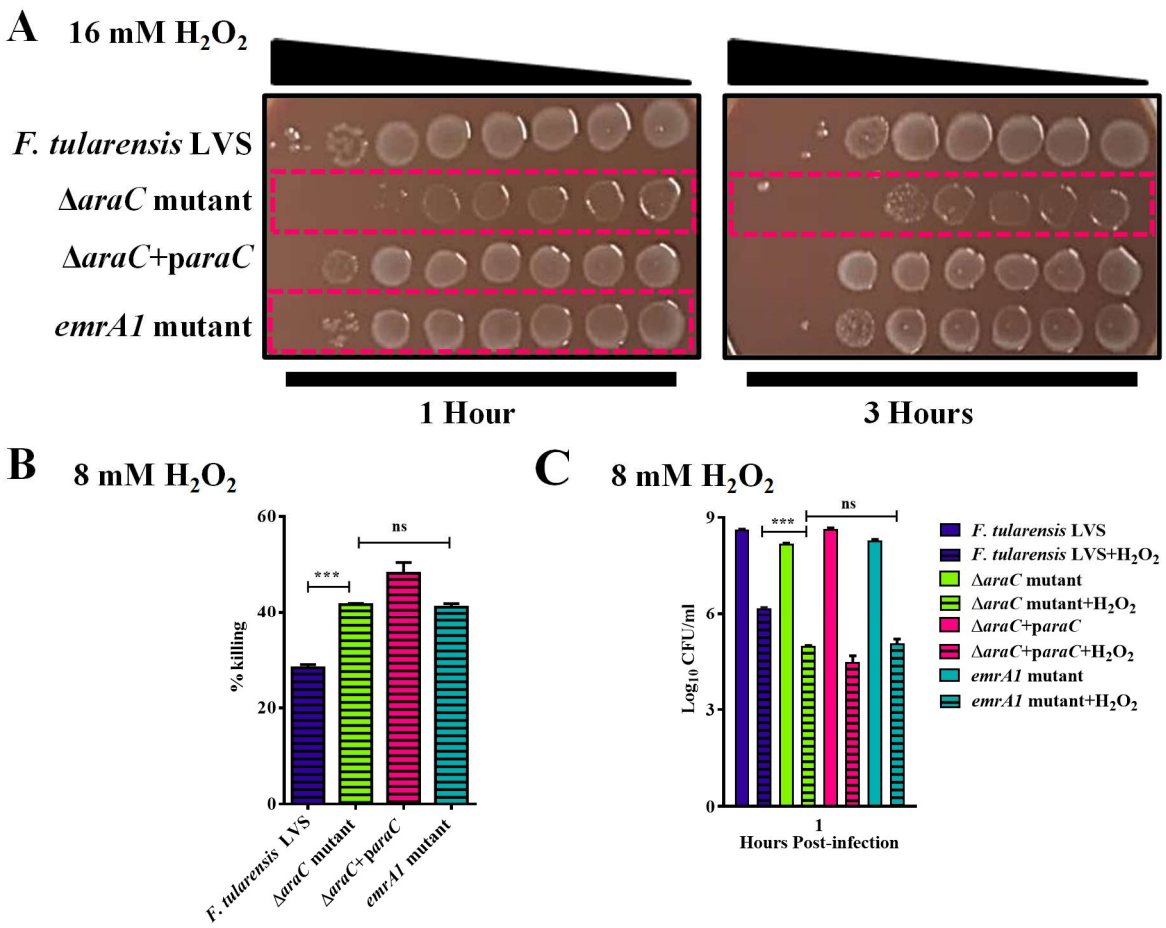


Figure 16. The sensitivity of the Δ*araC* mutant to H_2O_2. (A) Wild type *F. tularensis* LVS, the Δ*araC* mutant, Δ*araC*+p*araC*, or the *emrA1* mutant were exposed to the indicated concentration of H_2O_2 for one hour and three hours in a spot assay. Experiments are representative of two independent experiments. **(B and C)** Bacterial killing assay using 8 mM H_2O_2. The data were analyzed using one-way ANOVA. The data from the bacterial killing assay experiments are cumulative of three independent experiments. ***p<0.001.

4.4.1.2. The Δ*araC* mutant is sensitive to organic peroxides

The sensitivity of the Δ*araC* mutant was also determined against organic peroxide, TBH. Bacteria were exposed to varying concentrations of TBH, and the bacterial viability was determined by spot assay. Bacterial viability was similarly reduced in both the wild type *F. tularensis* LVS and the Δ*araC* mutant after 1 hour of exposure to TBH. However, after three hours of incubation, there was an enhanced killing of the Δ*araC* mutant as compared to the wild type *F. tularensis* LVS, indicating that prolonged exposure of the Δ*araC* mutant to TBH results enhanced sensitivity to TBH (**Figure 17A**).

Next, we investigated sensitivity of the Δ*araC* mutant to CHP. The Δ*araC* mutant exhibited enhanced sensitivity to CHP after one hour and three hours of exposure to CHP as compared to the wild type *F. tularensis* LVS (**Figure 17B**). We further confirmed these results by performing disc diffusion assay. Significantly larger zones of inhibition (11.25±0.25 mm) were observed for the Δ*araC* mutant as compared to those observed for the wild type *F. tularensis* LVS (9.25±0.25 mm) and the transcomplemented strain (10.25±0.25 mm) when exposed to a 0.1M concentration of CHP. The difference was lost when a higher concentration of 0.5M of CHP was used (**Figure 17C**). Taken together, these results demonstrate that the Δ*araC* mutant is highly sensitive to organic peroxides TBH and CHP. This phenotype has also been demonstrated for both the *emrA1* and Δ*silC* mutants of *F. tularensis* LVS as well as Schu S4 [87, 112].

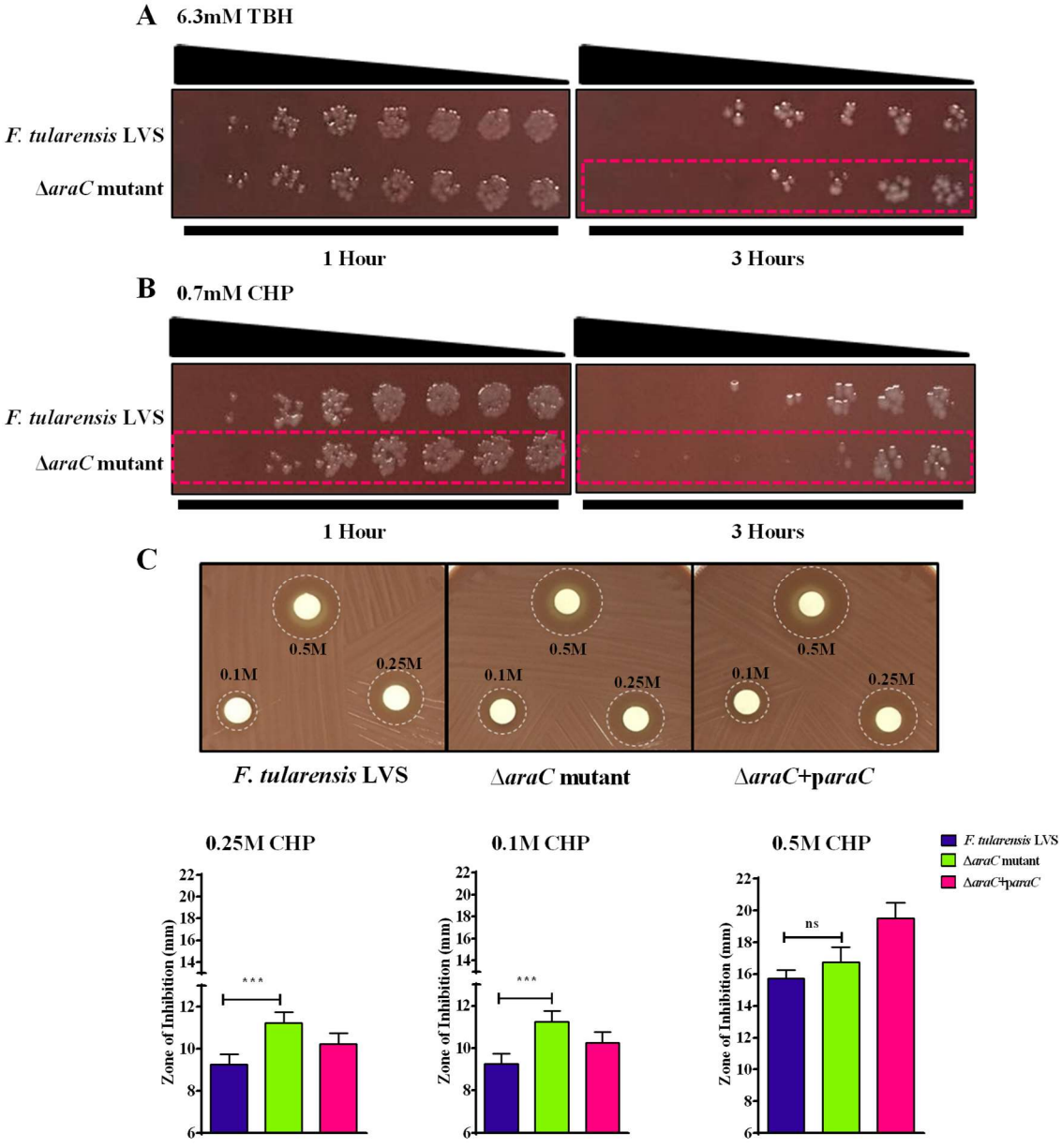


Figure 17. Sensitivity of Δ*araC* mutant to organic peroxides. Spot assay of *F. tularensis* LVS and the Δ*araC* mutant at varying concentrations of TBH starting from 6.3 mM **(A)** and CHP starting from 0.7 mM CHP **(B)**. Disc diffusion assay of the wild type *F. tularensis* LVS, the Δ*araC* mutant, and the transcomplemented strain using the indicated concentrations of CHP **(C)**. Zones of inhibition from bacteria treated with 0.25 M, 0.1 M, or 0.5 M CHP are shown**.** The data shown in C are cumulative of two independent experiments and are analyzed using one-way ANOVA. *** $p<0.001$.

4.4.1.3. The Δ*araC* mutant of *F. tularensis* LVS is sensitive to superoxide-generating compounds

Both the *emrA1* and Δ*silC* mutants are also sensitive to superoxide-generating compounds. Since we observed that the Δ*araC* mutant had higher sensitivity towards peroxides, similar to the *emrA1* and *silC* mutants, we also tested sensitivity of the Δ*araC* mutant to superoxide-generating compounds pyrogallol, paraquat, and menadione.

Enhanced killing of the Δ*araC* mutant was observed when exposed to increasing concentrations of pyrogallol, at one and three hours post-exposure, as compared to the wild type *F. tularensis* LVS. The trans-complementation did not restore the wild type phenotype. The *emrA1* mutant as reported earlier showed enhanced killing similar to the Δ*araC* mutant after one and three hours of exposure to pyrogallol (**Figure 18A**).

Next, we confirmed our observations by performing disc diffusion assay by exposing the wild type *F. tularensis* LVS, Δ*araC* mutant, and the transcomplemented strain to sterile discs impregnated with 50mg, 25mg, or 12.5mg of pyrogallol. The plates were incubated, and zones of inhibition were measured in mm **(Figure 18B)**. The Δ*araC* mutant exhibited significantly larger zone of inhibition (19.00±0.41) as compared to that of the wild type *F. tularensis* LVS (17.25±0.48).

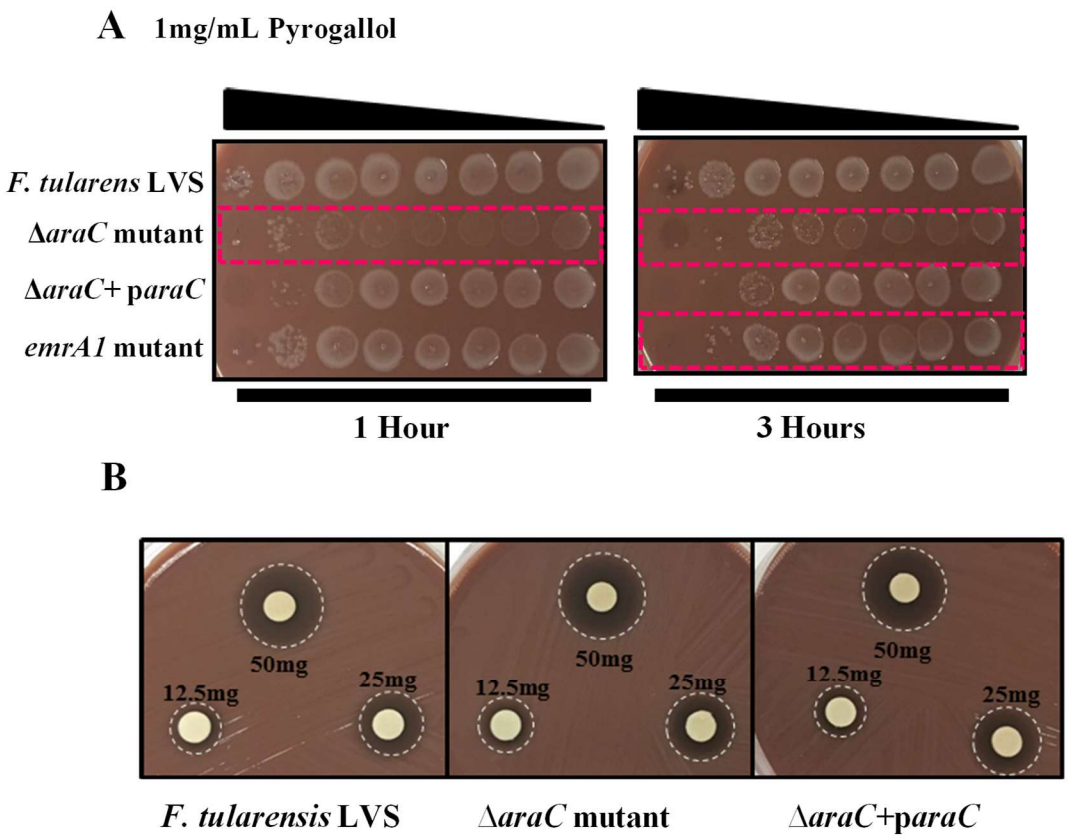


Figure 18. Sensitivity of Δ*araC* mutant to pyrogallol. (A) Spot assay of *F. tularensis* LVS, the Δ*araC* mutant, Δ*araC*+p*araC*, and the *emrA1* mutant. Bacteria were exposed to a concentration gradient of pyrogallol for one and three hours and plated on MH-chocolate agar plates. **(B)** Disc diffusion assay using indicated concentrations of pyrogallol and zones of inhibition for 50 mg/mL concentration are shown. The data shown are cumulative of two independent experiments and are analyzed using one-way ANOVA. *p<0.05.

We also tested sensitivity of the Δ*araC* mutant to superoxide-generating compound paraquat which generates superoxide radicals intracellularly. However, we did not observe any enhanced susceptibility of the Δ*araC* mutant to paraquat when tested by spot assay, bacterial killing assay or the disc diffusion assay. Similar results were also observed for the *emrA1* mutant (**Figure 19**). Results similar to those observed for paraquat were obtained when the sensitivity of the Δ*araC* mutant was tested against another superoxide-generating compound menadione (**Figure 20**). These results demonstrate that the Δ*araC* mutant exhibits enhanced sensitivity only towards pyrogallol as compared to the wild type *F. tularensis* LVS but not towards paraquat or menadione. Moreover, the patterns of the sensitivity of the Δ*araC* and the *emrA1* mutant to pyrogallol and paraquat are identical. These results indicate that the sensitivity of the Δ*araC* mutant to superoxide-generating compounds varies depending on the location and mechanism of generation of superoxide radicals. Taken together, these results demonstrate that the Δ*araC* mutant exhibits an identical oxidant sensitive phenotype similar to that observed for the *emrA1* mutant.

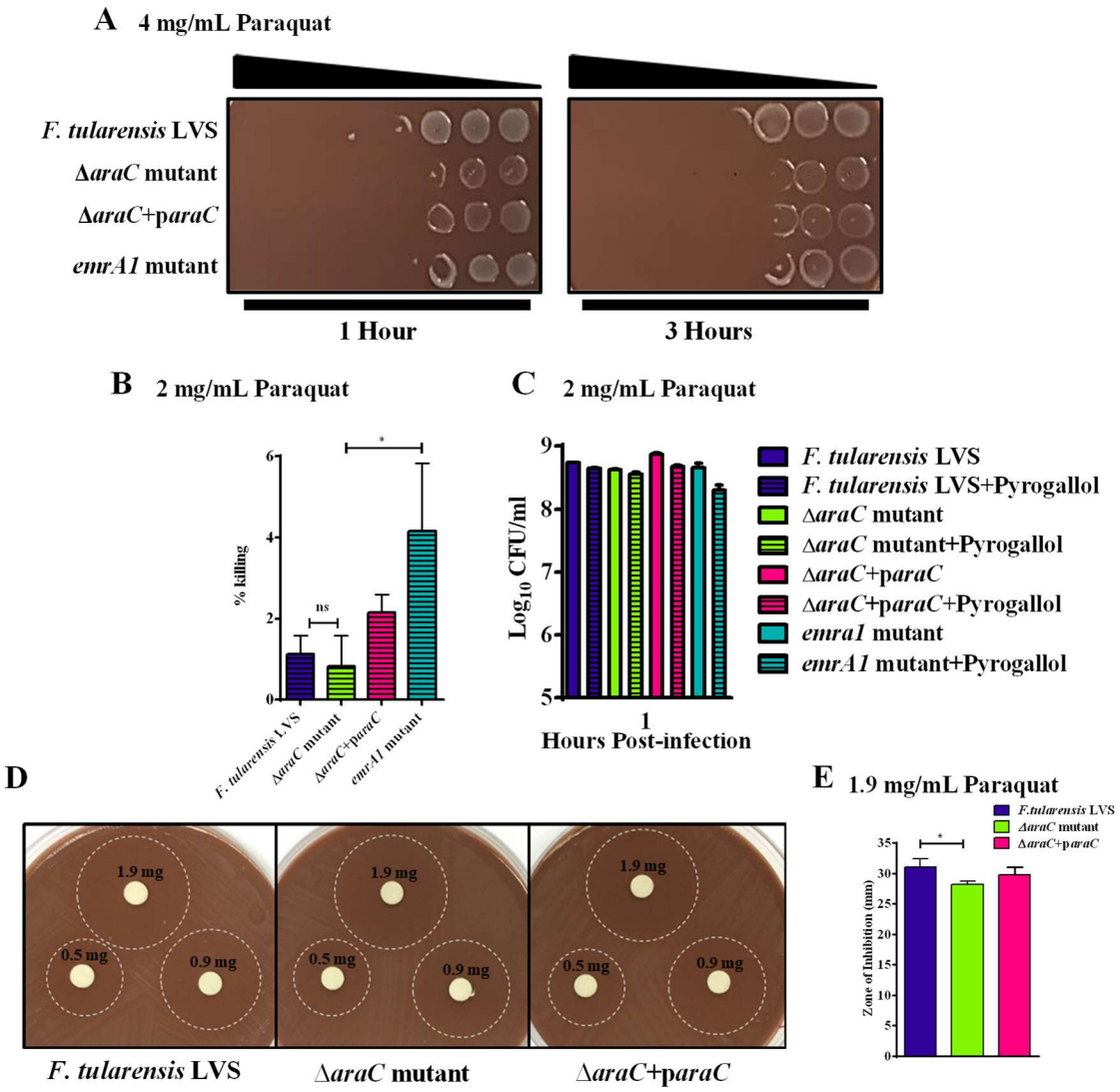


Figure 19. Sensitivity of Δ*araC* mutant to paraquat. (A) Spot assay of *F. tularensis* LVS, Δ*araC* mutant, trans-complement, and *emrA1* mutant. **(B)** percentage of bacterial killing and **(C)** CFUs after exposing to 0.5 mM paraquat, expressed as Log_{10} CFU/mL. **(D)** Disc diffusion assay using the indicated concentrations of paraquat. **(E)** Zones of inhibition measurement when bacteria were treated with 0.9 mg of paraquat, expressed in mm. The data shown are cumulative of three independent experiments. The data are analyzed using one-way ANOVA. *p<0.05.

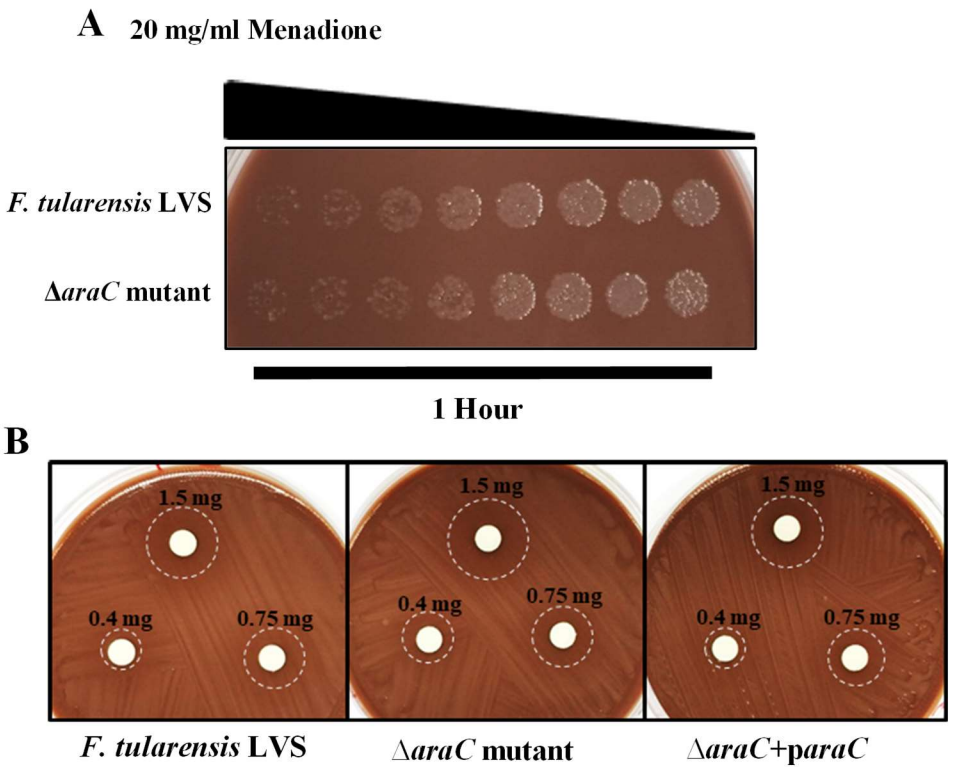


Figure 20. Sensitivity of Δ*araC* mutant to menadione. (A) spot assay of Δ*araC* mutant as compared to *F. tularensis* LVS. **(B)** Disc diffusion assay using the indicated concentrations of menadione. **(C)** Measurement of the zones of inhibition for bacteria treated with 1 µg menadione, expressed in mm. The data shown are cumulative of three independent experiments. The data are analyzed using one-way ANOVA.

4.4.2. AraC is not involved in resistance to silver compounds

In addition to providing resistance to oxidants, EmrA1 and SilC also contribute towards resistance to silver compounds [87]. We tested sensitivity of the Δ*araC* mutant to varying concentrations of silver nitrate in a spot assay to determine its contribution to resisting silver compounds. The results indicated no enhanced sensitivity of the Δ*araC* mutant to increasing concentrations of silver nitrate as compared to the wild type *F. tularensis* LVS. However, the *emrA1* mutant was found to be highly sensitive to silver nitrate following one and three hours of exposure (**Figure 21**). These results suggest that AraC is not involved in resistance to silver compounds.

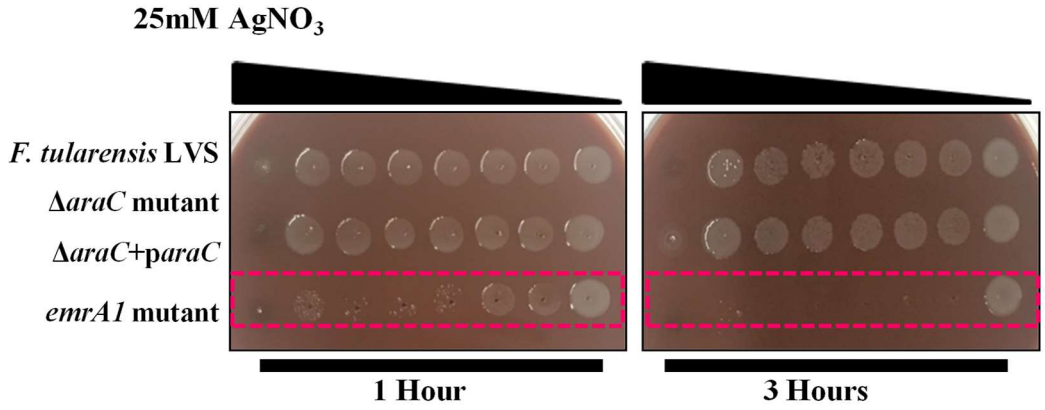


Figure 21. Sensitivity of Δ*araC* mutant to Silver nitrate ($AgNO_3$). Spot assay of *F. tularensis* LVS, Δ*araC* mutant, and *emrA1* mutant. 25 mM of silver serially diluted in a 96-well plate and incubated for one and three hours and plated using a replica plating method. The results are representative of three independent experiments conducted.

4.4.3. AraC is not involved in rendering resistance towards antibiotics

The MEP components EmrA1 and SilC of the Emr multidrug efflux pump are required for efflux of antibiotics and antibiotic resistance [87, 112]. We further investigate if AraC, similar to EmrA1 and SilC, is involved in antibiotic resistance. We tested sensitivity of the Δ*araC* mutant to Streptomycin (10 μg), Gentamicin (10 μg), Novobiocin (30 μg), Erythromycin (15 μg), Tetracyclin (30 μg), Nalidixic acid (30 μg), Polymyxin B (300 units), and Chloramphenicol (30 μg). As shown in **Figure 22,** the Δ*araC* mutant was found to be equally sensitive to all the antibiotics tested as the wild type *F. tularensis* LVS. This result suggests that AraC does not contribute to antibiotic resistance.

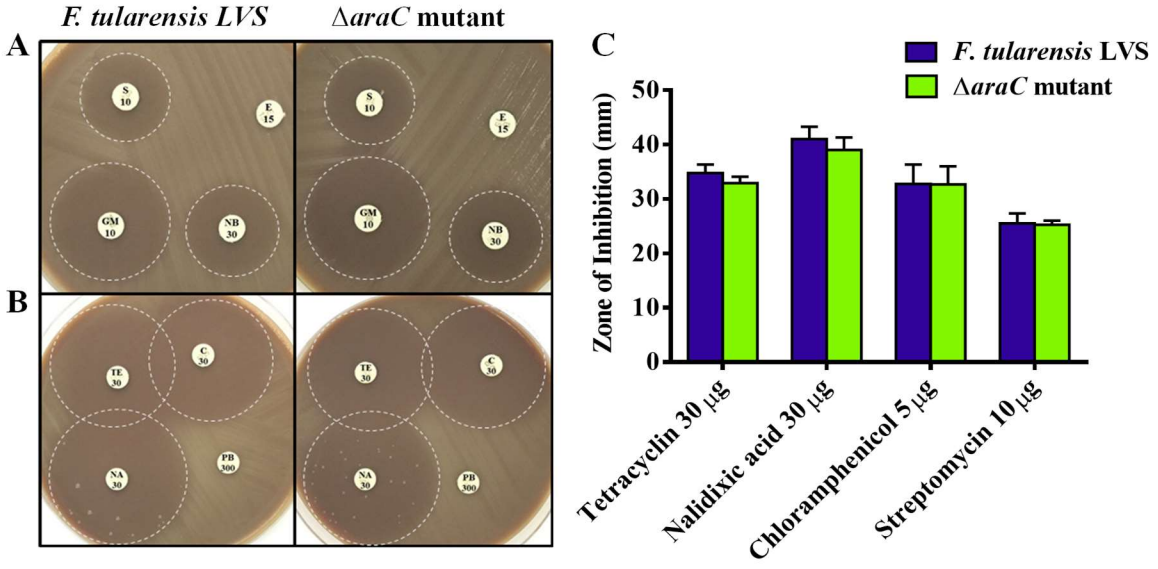


Figure 22. The Δ*araC* mutant does not exhibit enhanced sensitivity towards antibiotics. Disc diffusion assay of the Δ*araC* mutant and *F. tularensis* LVS using Streptomycin, Gentamicin, Novobiocin, and Erythromycin in **(A)**, Tetracyclin, Nalidixic acid, Polymyxin B, and Chloramphenicol in **(B)**. Measurement of zones of inhibition of the indicated antibiotics and their concentrations **(C)**. The results are cumulative of three independent experiments. The data are represented as Mean±SEM.

Specific Aim 2: Investigation of the role of AraC as a global transcriptional regulator in *F. tularensis* LVS

Our preceding results demonstrated the involvement of AraC in oxidative stress resistance. In Specific Aim 2, we investigated the genes that are being controlled by AraC transcriptional regulator. Activation of AraC/XlyS transcriptional regulators requires the presence of a substrate such as arabinose that activates them. Studies conducted in Specific Aim 1 failed to identify the specific substrate of AraC of *F. tularensis*. However, the Δ*araC* mutant did demonstrate sensitivity towards oxidative stress. We hypothesized that "*AraC of Francisella gets activated in the presence of oxidative stress and regulates the expression of genes required for survival under the oxidative stress conditions*." To address this hypothesis, we investigated the transcriptome of the Δ*araC* mutant when exposed to oxidative stress and compared to that of *F. tularensis* LVS.

We observed that the Δ*araC* mutant was sensitive to several superoxide-generating compounds and peroxides. However, Δ*araC* mutant did not exhibit any enhanced sensitivity towards superoxide-generating compound menadione **(Figure 20)**. We chose menadione to induce the oxidative stress as this oxidant would not kill either the wild type or the mutant bacteria but would induce an oxidative stress response. Wild type *F. tularensis* LVS and the Δ*araC* mutant bacteria were either left untreated or treated with menadione (1.25 mM menadione) and incubated for 1 hour at 37ºC. RNA was isolated and RNA sequencing was performed.

Differential gene expression of *F. tularensis* LVS and the Δ*araC* mutant was analyzed based on Log_2(fold change) of the gene expression levels comparing the Δ*araC* mutant to the wild type *F. tularensis* LVS and comparing menadione treated samples to the untreated. For

the downregulated genes, the expression of the *araC* gene which has been deleted in the Δ*araC* mutant was taken as 100%. Any genes that represented less than 50% of the Log_2(fold change) of the *araC* gene was considered as downregulated. However, to further narrow the number of genes identified as under the control of AraC, we considered the *p*-values and any value less than 0.05 was considered significant. All the data analyzed were cumulative of two independent experiments.

4.5. Determination of differentially expressed genes in the Δ*araC* mutant not exposed to oxidative stress

At first, we identified differentially expressed genes in the Δ*araC* mutant in comparison to the wild type *F. tularensis* LVS when bacteria were not exposed to oxidative stress. The Log_2(fold change) of the *araC* gene in the Δ*araC* mutant as compared to the wild type *F. tularensis* LVS was observed to be -0.67. Thus, 50% of this value (=-0.3) was used as a cut-off to determine the downregulated genes in the Δ*araC* mutant. The analysis showed that only the *araC* gene (*FTL_0689*) was significantly downregulated in the Δ*araC* mutant not exposed to menadione. These results demonstrate that AraC is not required for gene regulation of *F. tularensis* under normal growth conditions.

4.6. Determination of differentially expressed genes in the Δ*araC* mutant exposed to oxidative stress

Next, we identified the differentially expressed genes in the Δ*araC* mutant as compared to the wild type LVS when the bacteria were exposed to oxidative stress. The lowest expression with a log_2(fold change) of -1.08 was observed for the Δ*araC* mutant. Based on this, we

selected a value of -0.5 or +0.5 Log_2(fold change) as a cut-off to determine the differentially expressed genes in the Δ*araC* mutant.

4.6.1. Exposure of Δ*araC* mutant to oxidative stress results in downregulation of genes encoded on Francisella Pathogenicity Island (FPI)

The RNA Sequencing results identified five downregulated genes encoded as an operon within the FPI. The expression of these genes remained unaltered when the Δ*araC* mutant was not exposed to oxidative stress (**Figure 23).** These genes encode factors of *F.* tularensis essential for intracellular survival and virulence [169]. The *pdpC* gene encodes a pathogenicity determinant protein that does not contribute to intracellular survival but is required for virulence in mice. On the other hand, *pdpE* is neither required for virulence nor intracellular survival. The *iglJ* gene product is required for intracellular growth, cytopathogenicity and virulence in mice, whereas *iglI* plays an important role in the phagosomal escape, cytopathogenicity, and virulence in mice [170]. The *dotU* gene product is an essential structural component of the Type VI secretion system (T6SS) of *F. tularensis* and is absolutely required for the functioning of T6S. Overall, these results indicate that AraC regulates expression of virulence-associated genes under oxidative stress conditions.

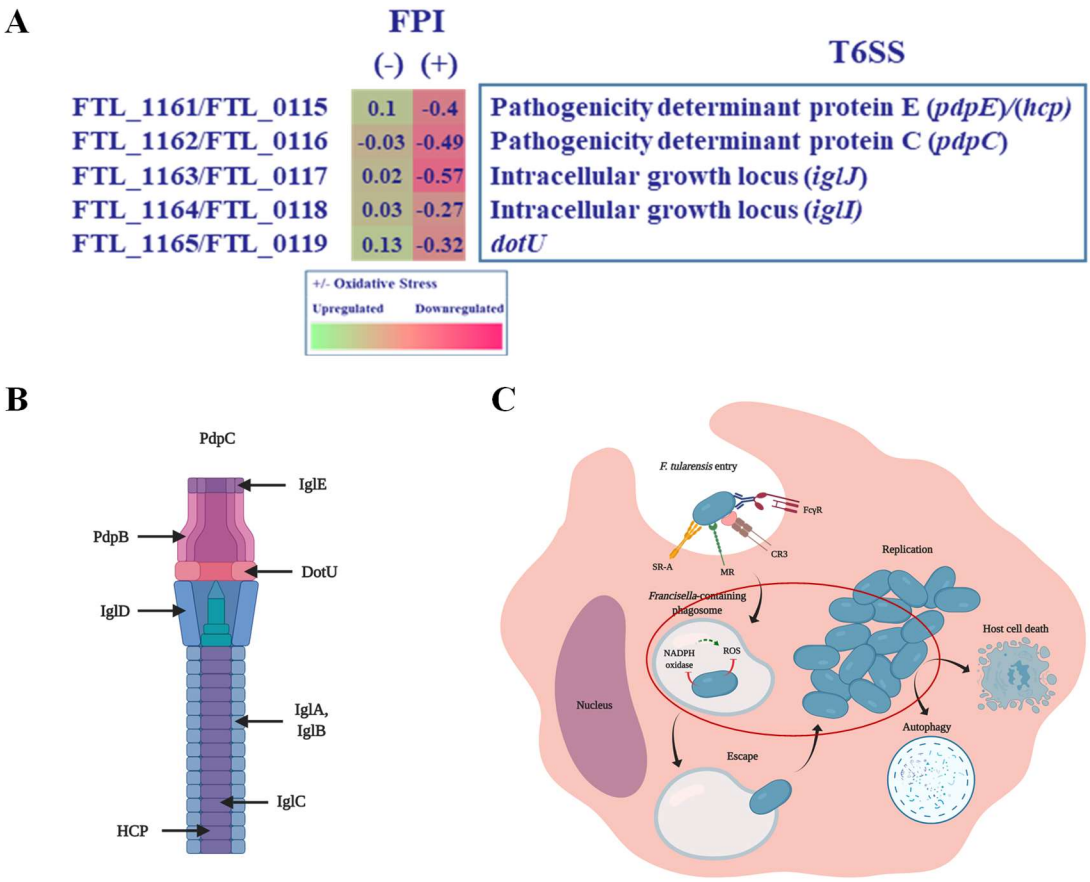


Figure 23. AraC regulates the expression of FPI genes when exposed to oxidative stress. **A.** Expression profile of FPI genes in the Δ*araC* mutant upon exposure to menadione. The operon is indicated by the box. Data shown are from two independent experiments and expressed as Log_2(fold change). **B.** The structure of T6SS. Modified from Clemens. D. L., Lee. B., Horwitz. M. A., 2018 [171] **C.** T6SS is required for the intracellular replication phase of *F. tularensis* indicated by red circle. Modified from Jones *et al*., 2012 [23].

4.6.2. The Δ*araC* mutant exhibits a dose-dependent attenuation of intramacrophage growth

During its intracellular residence, *F. tularensis* is exposed to oxidative stress. We next verified if the downregulated FPI genes in the Δ*araC* mutant when exposed to oxidative stress is associated with its impaired survival in macrophages. We used Raw macrophage cell line as host. We infected murine raw macrophages with the wild type *F. tularensis* LVS, the Δ*araC* mutant, and the transcomplemented strain at 1, 10, 100, and 500 MOIs**.** The cells were lysed at 4 and 24 hours post-infection to determine the numbers of bacteria that invaded cells and replicated intracellularly. The results revealed that all the three *Francisella* strains invaded the macrophages with equal efficacy irrespective of the MOI used and identical bacterial numbers were recovered from all the three strains tested at 4 hours post-infection. At 24 hours, the differences were more prominent. At an MOI of 1, significantly less Δ*araC* mutant bacteria (4.1±0.0 Log_{10} CFU/ml) were recovered from the infected macrophages as compared to the wild type strain (6.3±0.0 Log_{10} CFU/ml). Transcomplementation of the Δ*araC* mutant partially restored the wild type phenotype (**Figure 24A**). At an MOI of 10 infection dose, there were fewer bacteria recovered from the macrophages infected with the Δ*araC* mutant (6.8±0.0 Log_{10} CFU/ml) as compared to the wild type *F. tularensis* LVS (7.1±0.0 Log_{10} CFU/ml) **(Figure 24B)**. Similar results were observed when the infection dose was increased to 100 MOI with macrophages infected with the Δ*araC* mutant harbored significantly fewer bacteria (8.3±0.0 Log_{10} CFU/ml) than the wild type (8.5±0.0 Log_{10} CFU/ml) at 24 hours post-infection **(Figure 24C).** At a saturating infection dose of 500 MOI, the difference in numbers of bacteria recovered from the Δ*araC* mutant infected cells and the wild type *F. tularensis* infected macrophages was lost and equal numbers of bacteria were recovered from both groups of

infected macrophages (**Figure 24D).** Altogether, results from the macrophage assays demonstrate that Δ*araC* mutant is attenuated for intramacrophage survival. The attenuation of intramacrophage growth of Δ*araC* mutant is dose-dependent. These results also corroborate findings of the gene expression studies that AraC-dependent gene expression of the FPI genes is required for intramacrophage survival of *F. tularensis*.

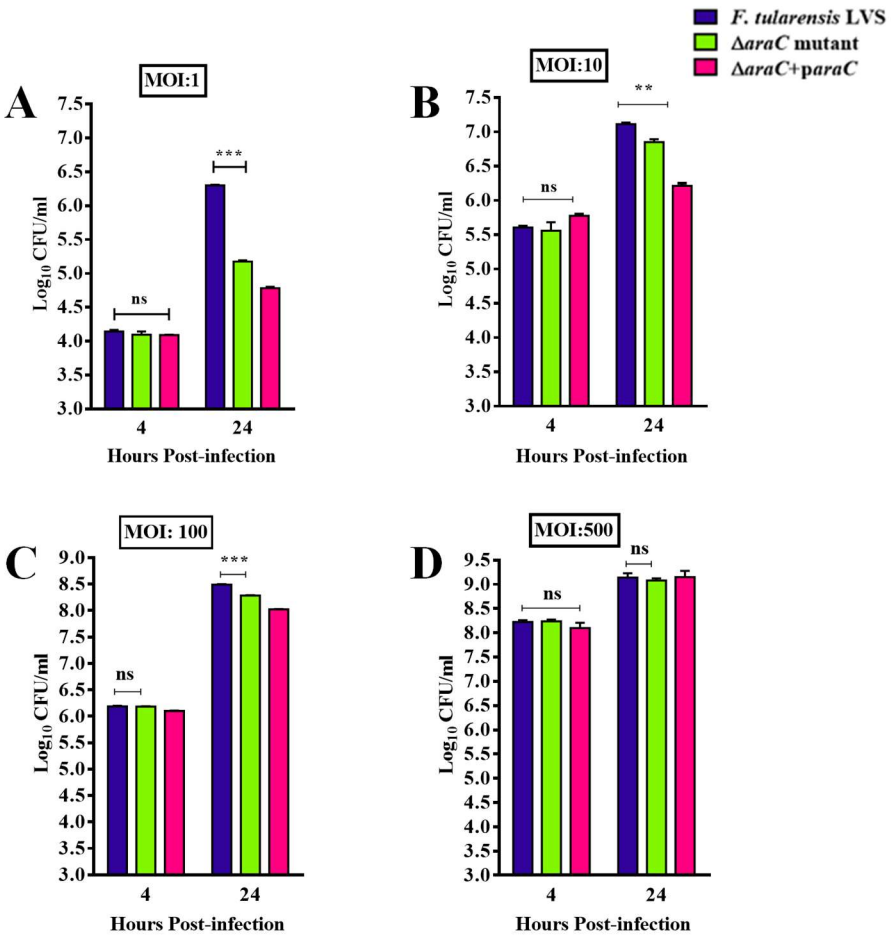


Figure 24. The Δ*araC* mutant is attenuated for intramacrophage survival. Dose dependent attenuation of intramcarophage growth of Δ*araC* mutant at an MOI: 1 **(A)**, 10 **(B)**, 100 **(C)**, and 500 **(D).** The data shown are cumulative of three independent experiments and are analysed using one way ANOVA. *** $p<0.001$; ** $P<0.01$.

4.6.3. The Δ*araC* mutant exhibits attenuation of virulence in mice in a dose-dependent manner

To further establish that AraC-dependent regulation of FPI genes observed under oxidative stress is not only required for intramacrophage growth and survival, but also for virulence in mice, we performed *in vivo* experiments in a mouse model of tularemia. We infected C57BL/6 mice intranasally with incremental doses ($1x10^2$, $1x10^3$, $1x10^4$, or $1x10^5$ CFUs) of *F. tularensis* LVS or the Δ*araC* mutant and monitored their survival and body weights daily for 15 days **(Figure 25)**. It was observed that the Δ*araC* mutant was attenuated for virulence as compared to the wild type *F. tularensis* LVS. However, a dose-dependent increase in virulence of the Δ*araC* mutant was observed. All of the mice infected with $1x10^2$ and $1x10^3$ CFUs of the Δ*araC* mutant survived the infection whereas 20% mice infected with $1x10^2$ CFUs and 40% of those infected with $1x10^3$ of the wild type *F. tularensis* LVS succumbed to infection **(Figures 25A and 25B, left panels)**. All mice infected with $1x10^4$ and $1x10^5$ of *F. tularensis* LVS also succumbed to the infection by day 10. However, 20% of mice infected with similar doses of the Δ*araC* mutant survived the infection. All infected mice lost body weight during the first 3-6 days and then gradually regained their original body weight indicating that all mice received the infection. Collectively, these results demonstrate that AraC by regulating the FPI genes contributes to intramacrophage growth and virulence in mice in a dose-dependent manner.

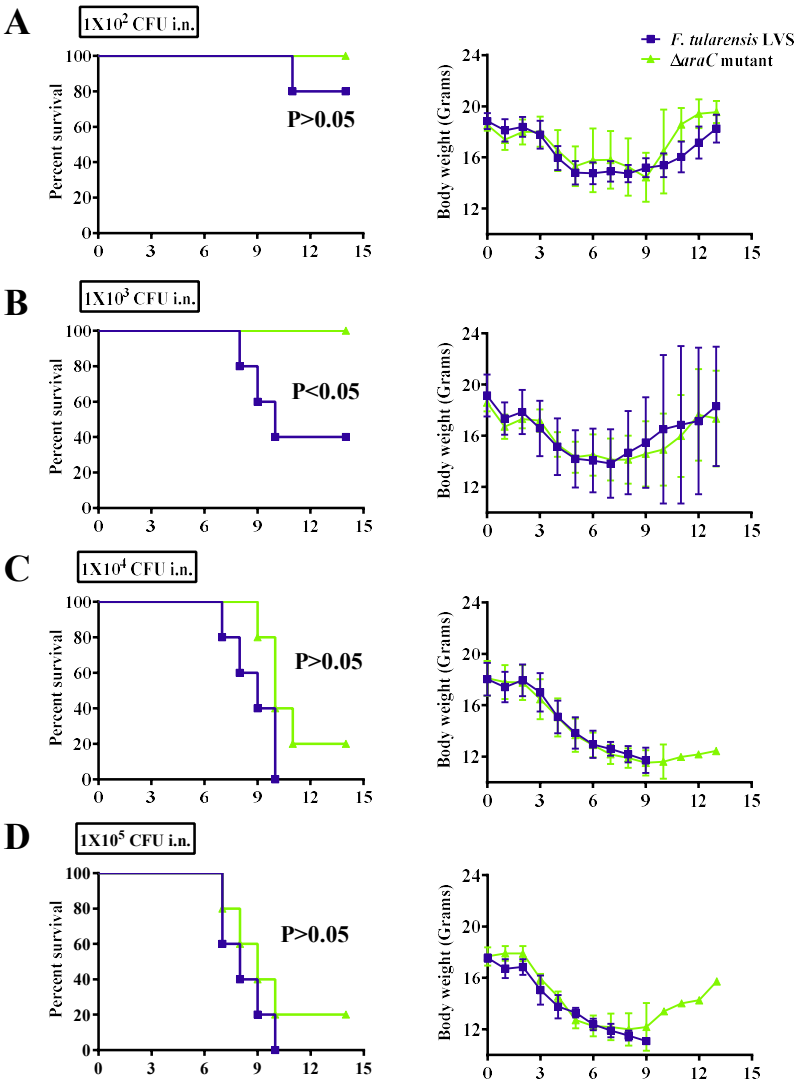


Figure 25. The Δ*araC* mutant exhibits a dose-dependent attenuation of virulence in mice: C57BL/6 mice inoculated with $1x10^2$ **(A)**, $1x10^3$ **(B)**, $1x10^4$ **(C)**, or $1x10^5$ **(D)** CFU of *F. tularensis* LVS or the Δ*araC* mutant intranasally. Survival and body weights of infected mice (n=5/group) were recorded for 15 days. The survival curves are plotted as Kaplan-Meier survival curve (left panels). Body weights represented as Mean ±S.D. are shown in the right panels.

4.6.4. Exposure of Δ*araC* mutant to oxidative stress results in differential expression of stress response genes

RNA Sequencing analysis also unveiled differential expression of another three genes, part of a stress response operon, in the Δ*araC* mutant. Expression of these genes was significantly affected upon treatment with menadione. These three downregulated genes encode AroA, RnhA, and a hypothetical protein. *rnhA* gene (-0.48 Log_2-fold change) encodes Ribonuclease H, which protects bacteria from killing by oxidative stress [172]. *rnhA* is a multi-functional protein which is also actively involved in bacterial growth as simultaneous deletion of *rnhA*, *rnhB*, and *rnhC* leads to lethality in *Bacillus subtilis* [173]. The findings indicate that AraC could be involved in regulation of *F. tularensis* ribonuclease H abundance under oxidative stress **(Figure 26A)**.

Interestingly, another gene, *rpoH*, which encodes a sigma factor that shares homology with the σ^{32} heat-shock family, was also downregulated in the Δ*araC* mutant irrespective of its exposure to oxidative stress [149]. RpoH modulates expression of several genes including stress response genes *groEL/S*, *clpB*, *FTL_1957*, *dnaK*, *dnaJ*, and *hsp40* [174]. To investigate whether the absence of Δ*araC* could change the expression profile of these heat shock proteins while exposed to oxidative stress, expression of *groES, clpB, FTL_1957 dnaK, dnaJ,* and *hsp40* was determined by qRT-PCR. It was observed that expression of *gorEL, dnaK, dnaJ* and *hsp40* genes that are primarily involved in heat-shock response was significantly upregulated in the Δ*araC* mutant. Collectively, these results indicate that AraC serves to positively regulate the expression of *rpoH* which in-turn negatively regulates the expression of heat-shock proteins **(Figure 26B)**.

Further, to verify the participation of AraC in regulating heat-shock response, we conducted thermal resistance experiment in which bacteria were exposed to a high temperature (48ºC), higher than the homeostatic temperature (37ºC). The transcomplemented strain was utilized as a positive control because *araC* in the transcomplemented vector is under the control of GroEL promotor, activated at high temperature. **Figure 26C** shows that the viabilities of all these three *Francisella* strains were not affected when exposed to a temperature of 37ºC for 1 hour. The findings indicate that at the physiological temperature, AraC exerts no benefit or hindrance to *F. tularensis* growth.

Next, we induced high-temperature stress by growing the bacteria at 48ºC (**Figure 26D)**. At the start point, the CFU/ml for these three *Francisella* strains were set approximately equal. After 1 hour of incubation, these three strains exhibited a significant decrease in the viable bacterial count. The numbers of the Δ*araC* mutant bacteria (7.8±0.0 Log_{10}CFU/ml) were significantly higher than the wild type *F. tularensis* LVS (7.5±0.0 Log_{10}CFU/mL). The transcomplemented strain survived better than the former two *Francisella* strains tested, with significantly higher numbers of bacteria (8.3±0.0 Log_{10}CFU/mL) survived at the higher temperature. Overall, the results indicate that AraC of *F. tularensis* LVS regulates the expression of heat-shock proteins negatively and facilitates its survival under extreme stress conditions.

In addition to the aforementioned, *aroA* gene (-0.55 Log_2-fold change) was also downregulated in Δ*araC* mutant in response to oxidative stress. *aroA* encodes 3-phosphoshikimate 1-carboxy-acyltransferase, which is an enzyme required in the shikimate pathway responsible for the bio-synthesis of enolpyruvyl shikimate-3-phosphate, inorganic phosphate, folate, and aromatic amino acids [175] (**Figure 26E)**. This pathway is important

for bacterial metabolism and homeostasis [175]. The finding that downregulation of the *aroA* RNA abundance in Δ*araC* mutant after treatment with menadione implied that AraC is involved in modulation of *F. tularensis* metabolism.

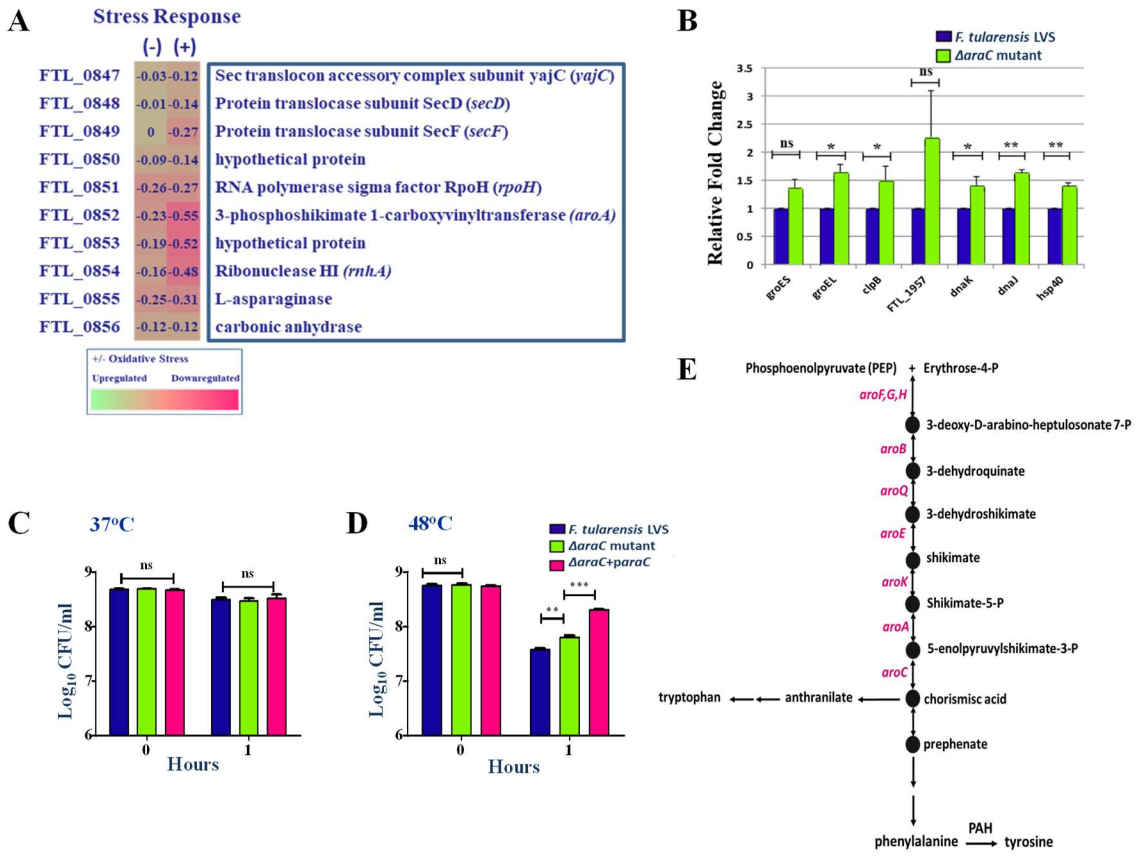


Figure 26. AraC regulates stress response genes of *F. tularensis* LVS. (A) Expression profile of genes involved in heat and stress response in the Δ*araC* mutant as compared to the wild type *F. tularensis* LVS untreated or treated with menadione. Data are cumulative of 2 independent experiments **(B)** qRT-PCR analysis of indicated heat-shock proteins in the Δ*araC* mutant and the wild type *F. tularensis* LVS treated with menadione. The data shown are cumulative of three independent experiments. Data were analyzed using Two-tailed student T-test. **(C)** CFU of the Δ*araC* mutant, the wild type *F. tularensis* LVS, and the transcomplemented strain on exposure to 37°C versus 48°C for I hour **(D). (E)** The shikimate pathway required for aromatic amino acid synthesis showing the involvement of *aroA* gene in the sixth step. Modified from Lutke-Eversloh, T. & Stephanopoulos. G., 2005 [176]. The results are cumulative of three independent experiments. The data were analyzed using one way ANOVA. *** $p<0.001$, ** $p<0.01$, * $p<0.05$, ns= non-significant.

4.6.5. Genes responsible for energy production during oxidative stress are differentially regulated in the Δ*araC* mutant

In addition to FPI and heat-shock proteins, RNA sequencing identified a set of genes responsible for the production of ATPases that were downregulated in the Δ*araC* mutant in the presence of oxidative stress (**Figure 27A)**. These genes transcribed on a single operon are required for catalysis, synthesis, and hydrolysis of ATP [177]. They are also part of trans-membrane proton (H^+) translocator in prokaryotes [178]. ATP synthases are composed of two molecules: F_0 is attached to the periplasmic membrane and F_1 extends to the cytoplasmic space and forms the catalytic region of the enzyme [177] **(Figure 27B)**. RNA Sequencing analysis revealed downregulation of ATP synthase subunits, except A subunit of F_0 was upregulated in the Δ*araC* mutant post-exposure to menadione. Three of the genes affected to the greatest extent were those encoding ATP synthase subunits of F_1 molecule: γ, β, and AtpC (ε). Altogether, these results demonstrate that AraC of *F. tularensis* differentially regulate the expression of genes involved in energy production under oxidative stress.

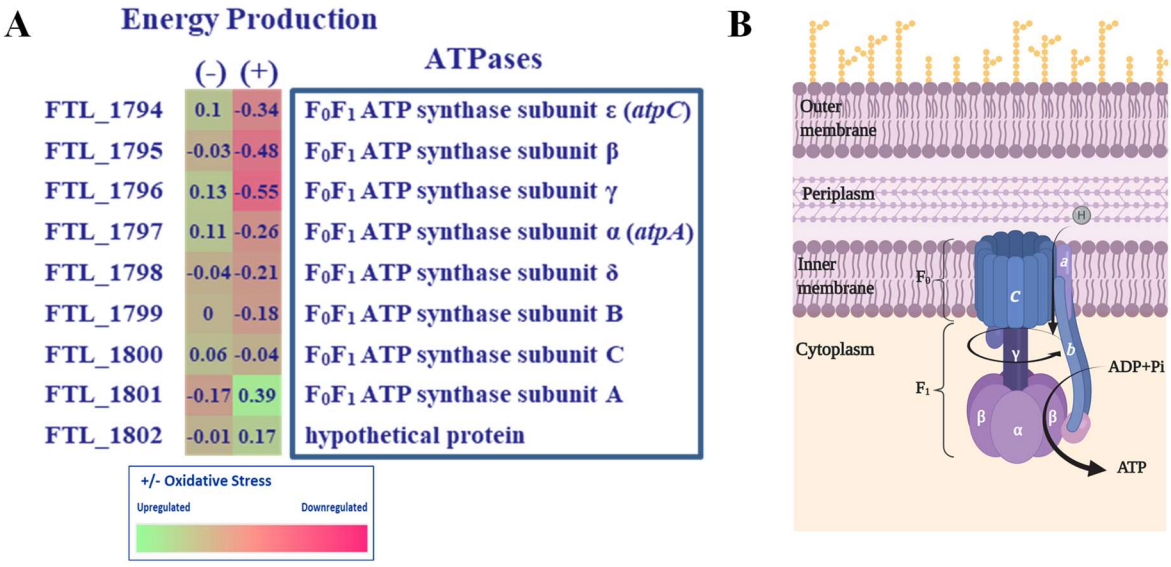


Figure 27. AraC differentially regulates the expression of genes involved in energy production during oxidative stress. (A) Gene expression profile shows differentially expressed ATP synthase genes in the Δ*araC* mutant in the normal versus oxidative stress conditions. The data are derived from two independent experiments. **(B)** Illustration of the ATPase structure showing the main two molecules F0 and F1 and their subunits. Modified from Sobti, M., *et al*., 2016 [179].

4.6.6. AraC of *F. tularensis* positively regulates key components of TCA cycle during oxidative stress

Exposure of the Δ*araC* mutant to menadione downregulated expression of genes involved in metabolism encoded on four different operons. One of these genes encodes the enzyme pyruvate dehydrogenase subunits: E1, E2, and E3, required for the biosynthesis of Acetyl-CoA, an enzyme that initiates the TCA cycle [180]. Among the three subunits, E3 or dihydrolipoyl dehydrogenase encoded by *lpd* was downregulated in the Δ*araC* mutant exposed to oxidative stress as compared to the wild type *F. tularensis* LVS. The other two subunits, E1 pyruvate dehydrogenase (AceE) and E2 digydrolipoyl transacetylase (AceF), were also downregulated in the Δ*araC* mutant. The results demonstrate that AraC activates the TCA cycle in *F. tularensis* under oxidative stress **(Figure 28)**.

The expression of genes encoded on another operon was downregulated in the Δ*araC* mutant. This operon comprised of the genes required for the biosynthesis of pantothenate (vitamin B_5), which is the central component of coenzyme-A [181]. The RNA Sequencing data analysis revealed that *coaX* gene encoding type III pantothenate kinase was downregulated in the Δ*araC* mutant as compared to the wild type when subjected to oxidative stress. The analysis also exhibited downregulation of aspartate-1-decarboxylase *panD* gene, required for the formation of β-alanine from L-aspartate [182], the *panC* gene encoding the pantothenate synthase that employs β-alanine and L-pantoate for the production of pantothenate [183]. The expression levels of *panB* (ketopantoate hydroxymethyltransferase) and *panG* (ketopantoate reductase) were also downregulated in menadione treated Δ*araC* mutant as compared to the wild type *F. tularensis* LVS. Enzymes, PanB and PanG, catalyze the conversion of 2-oxoisovalerate to 2-dehydropantoate and pantoate, respectively [182]. Altogether, these results

indicate that, under oxidative stress, AraC of *F. tularensis* positively activates the pantothenate biosynthesis pathway that is required for coenzyme-A formation to initiate the TCA cycle [181, 184].

Furthermore, exposing Δ*araC* mutant to oxidative stress altered the expression of genes encoded on an additional metabolic operon. This operon harbors three genes; *pgk*, *pyk*, and *fba*. The *pgk* gene encodes phosphoglycerate kinase, an enzyme that catalyzes the formation of ATP from ADP [185]. The *pyk* gene, encoding for pyruvate kinase [186], which is involved in the production of ATP and pyruvate, crucial for several metabolic pathways including the TCA cycle [186]. Moreover, the *fba* gene was downregulated to a greater extent in the Δ*araC* mutant post-exposure to menadione. This gene encodes fructose-bisphosphate aldolase class II (FBA), which is required for the formation of fructose 1,6-bisphosphate (FBP) through the gluconeogenesis and glycolysis pathways in *Francisella*. [187]. FBA is required for bacterial virulence and is essential for intracellular survival, indicating the significance of these metabolic pathways in bacterial pathogenesis [187]. Thus, downregulation of this operon suggests that AraC of *F. tularensis* positively regulates glucose metabolic pathways in addition to the TCA cycle during oxidative stress to sustain intracellular survival of *F. tularensis*.

In addition to the genes above, RNA expression levels of a set of metabolic genes were changed in the Δ*araC* mutant when treated with menadione. This operon encodes key enzymes that are implicated in the TCA cycle. The two most downregulated genes were *sucA* and *sucB* encoding oxoglutarate dehydrogenase subunits E1 and E2, respectively. This enzyme catalyzes the transformation of 2-oxoglutarate into succinyl-CoA in the TCA cycle [188] and reduction in the RNA expression level of these genes indicate that oxoglutarate dehydrogenase activity of *F. tularensis* is deactivated in the Δ*araC* mutant during oxidative stress. Furthermore, the

genes, *tpiA*, *glmM,* and *apt*, encoding triosephosphate isomerase, phosphoglucosamine mutase and adenine phosphoribosyltransferase required for gluconeogenesis pathway, were all downregulated in the Δ*araC* mutant during oxidative stress. The remaining genes within the operon were impacted to a lower extent in the Δ*araC* mutant, upon treatment with menadione, including *secG* that codes for the preprotein translocase subunit SecG and genes that code for the succinate dehydrogenase enzyme contributing to the TCA cycle and oxidative phosphorylation pathway [189, 190]. Succinate dehydrogenase is composed of 4 subunits: SdhA, SdhB, SdhC, and SdhD, and their expression remained unaltered in the Δ*araC* mutant as compared to the wild type **(Figure 28)**.

Collectively, the RNA sequencing data analysis indicate that inducing oxidative stress in the Δ*araC* mutant diminishes the expression of various genes encoding key elements of the metabolic pathways; TCA, and electron transport channels. Thus, AraC as a transcriptional regulator plays a significant regulatory role in the metabolism of *F. tularensis* required for survival and pathogenesis during oxidative stress.

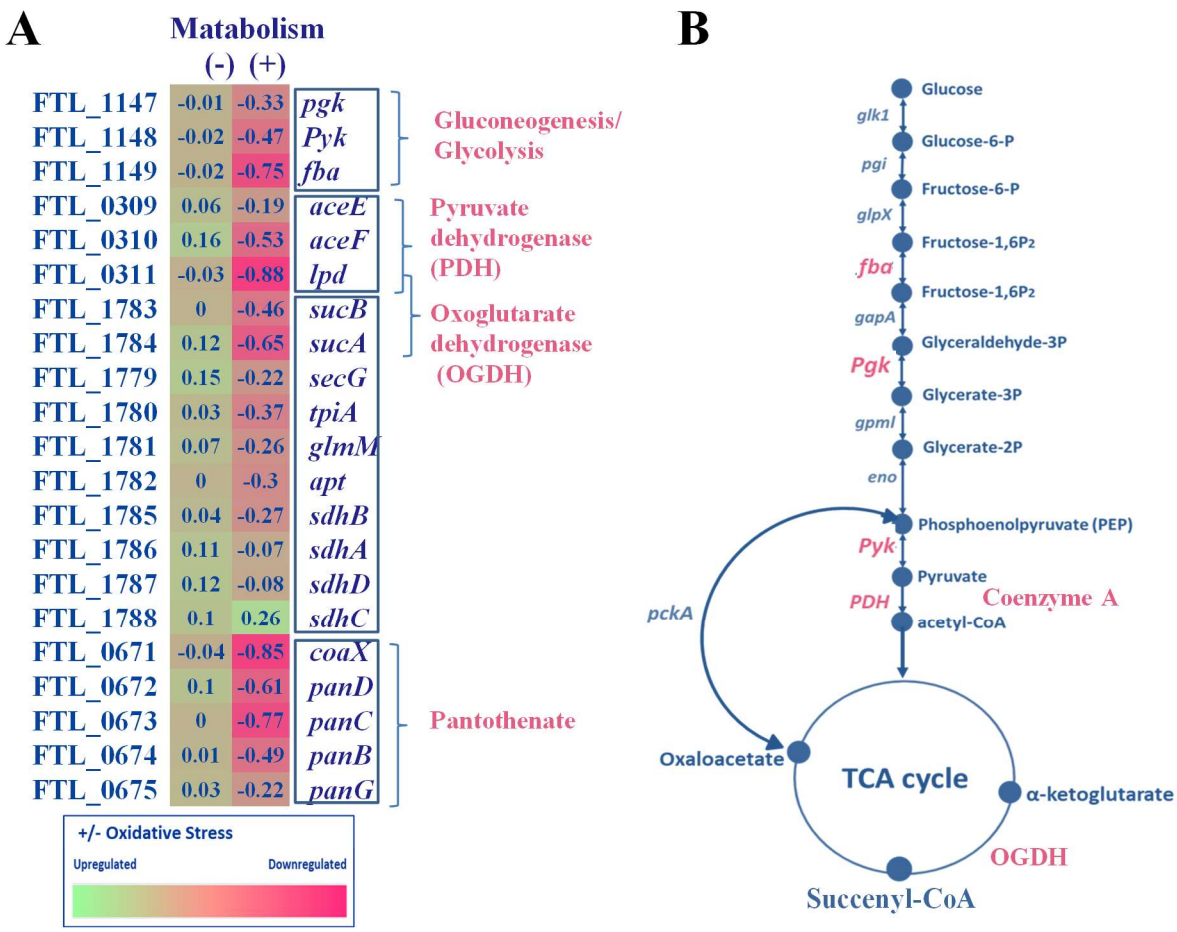


Figure 28. AraC is involved in the regulation of genes encoding key metabolic enzymes. **(A)** Gene expression profile of the Δ*araC* mutant as compared to the wild type *F. tularensis* LVS of genes encoded on four operons (operons labeled by blue boxes). Genes prodcut composing TCA cycle key enzymes are labled by blue parenthesis to the right. The Log_2(fold change) values are indicated. The data represent the average of gene expression from two independent experiments. **(B)** Pathway of glucose metabloism ending in TCA cycle. Enzymes downregulated by Δ*araC* mutant during oxidative stress are highlited in red. Modified from Seidl, K., *et al*., 2009 [191].

4.6.7. AraC-dependent transcription of regulatory proteins

We identified an additional set of genes encoded on operons that were differentially expressed in the Δ*araC* mutant as compared to the wild type *F. tularensis* LVS after induction of oxidative stress (**Figure 29A**). One of the operons is composed of genes encoding: *trkA*, amino acid antiporter, and a hypothetical protein that was downregulated to a great extent when the Δ*araC* mutant was exposed to the oxidative stress. The *trkA* gene encodes a potassium uptake protein. Moreover, the expression level of two genes: *rpoB* and *rpoC*, on another operon was significantly downregulated in the menadione treated Δ*araC* mutant. These genes encode two of the RNA polymerase core proteins: the *β* (RpoB) and *β'* (RpoC) subunits [149] (**Figure 29B**), indicating that AraC is also involved in regulating *F. tularensis* transcriptional process during oxidative stress. Another gene (*FTL_1051*) that encodes NAD(P)/FAD-dependent oxidoreductase was upregulated in the Δ*araC* mutant as compared to the wild type *F. tularensis* LVS. This enzyme is known to catalyze the redox reactions of nicotinamide adenine dinucleotide NAD(P)H or NAD(P)+ [192]. However, no studies have done to determine the role of this enzyme in *F. tularensis* during oxidative stress. Altogether, these results indicate that AraC of *F. tularensis* displays a regulatory effect on the structural components of RNA polymerase and gene products involved in the redox reactions during oxidative stress.

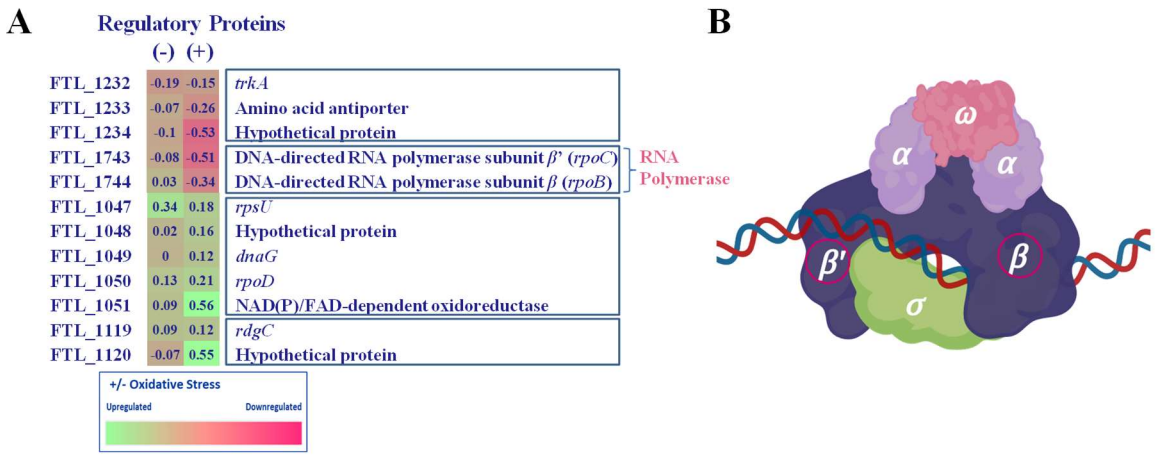


Figure 29. AraC-dependent transcription of regulatory proteins. (A) Gene expression profile of regulatory genes within four operons of the Δ*araC* mutant. (operons labeled by blue boxes to the right). The Log_2(fold change) values are indicated. Data presented are cumulative of two independent experiments. **(B)** RNA polymerase Holoenzyme structure modifed from Johnson Chavarria, E.M., 2016 [193]. The structure is composed of five molecules and sigma factor to initiate RNA synthesis. Red circles indicate core subunits (β and β') encoded by *rpoB* and *rpoC,* respectively, and are downregulated by Δ*araC* mutant in response to oxidative stress.

4.6.8. Transcriptional units differentially regulated by AraC of *F. tularensis*

Apart from regulating the RNA expression levels of genes encoded on operons, AraC of *F. tularensis* exhibited a significant regulatory effect on a few transcriptional units during oxidative stress. **Figure 30** represents the differentially expressed genes in the Δ*araC* mutant as compared to the wild type *F. tularensis* LVS. The *araC* gene which is transcribed as a single transcription unit remained downregulated to the greatest extent irrespective of the treatment with menadione in the Δ*araC* mutant. The *pyrH* gene that encodes uridylate kinase enzyme to catalyze the synthesis of pyrimidine was also downregulated [194], suggesting that AraC is involved in nucleotide damage repair in response to oxidative stress. Another gene in the Δ*araC* mutant that was largely affected by menadione treatment was *FTL_0426* coding for type I DNA topoisomerase. This enzyme is required for DNA supercoil repair [195], indicating that AraC is also involved in the regulation of DNA repair mechanisms in *F. tularensis* during oxidative stress. Furthermore, two genes *FTL_0024* and *FTL_0031* were significantly upregulated in Δ*araC* mutant compared to the wild type. These genes encode serine/threonine transporter and acid phosphatase, respectively, indicating that AraC negatively regulates genes required for transport of amino acids (serine and threonine) during the conditions of oxidative stress. Additionally, since acid phosphatase is essential for glucose and G6P phosphorylation and dephosphorylation mechanisms in bacteria [196], AraC of *F. tularensis* is possibly involved in the negative regulation of G6P to suppress the glucose metabolism and, thus, deactivate the TCA cycle.

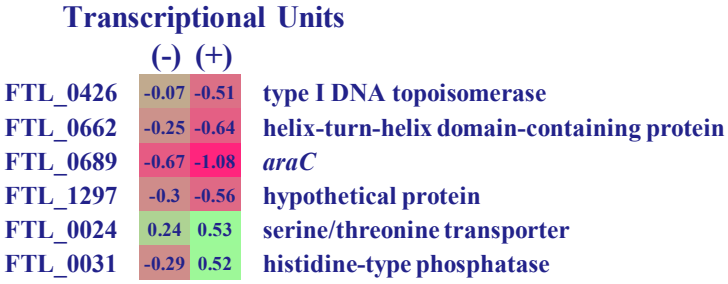


Figure 30. Transcriptional units differentially regulated by AraC of *F. tularensis*. Gene expression profile displaying the genes transcribed as single transcription units that are regulated by AraC. The Log_2(fold change) values are indicated. The data represent the average expression from two independent experiments.

5. DISCUSSION

As a Tier 1 Select Agent, *F. tularensis* has a high infectivity rate and rapid onset of the disease known as tularemia [19]. Many studies have devoted enormous efforts to understand how *F. tularensis* avoids host defense mechanisms, replicates within an extremely secure immune system, and eventually causes the deadly disease tularemia. Understanding virulence mechanisms of *F. tularensis* is a fundamental approach that will certainly assist in decoding the pathogenesis and navigating to the proper development of tularemia vaccine and therapeutics. Furthermore, the ability of *F. tularensis* to survive the host bactericidal mechanisms and replicate intracellularly is attributed to its unique regulation of virulence genes. Gene regulation of *F. tularensis* virulence is complicated and is adaptable to the accurate detection of environmental stimuli and host response to infection [137, 197]. Also, gene regulation employs regulatory molecules and transcriptional regulators that dictate the differential gene expression [197]. Transcriptional regulators are categorized based on their structure, function, sequence conservation of DNA binding motifs, and amino acids alignment [119, 197].

The AraC/XylS family is one of the largest families of bacterial transcriptional regulators [139, 197]. Members of the AraC/XylS family can alter gene expression, mostly, positively [119, 139, 197]. In rare cases, gene expression can be either negatively altered by members of the family such as the CelD of *Y. pestis* [198], or both positively and negatively by the AraC of *E. coli* and YbtA of *Y. pestis* [139, 199]. Transcriptional regulators are additionally classified into two categories based on the regulation of their own transcription [139]. The first category has signal receptors within the same protein that respond to the availability of substrates to activate or repress genes expression [139]. Examples of this type of transcriptional regulators

are AraC, XylS, RhaR, and UreR [139, 157, 200-202]. The second category requires the activity of another regulator (activator or repressor) to control gene expression in a two-component regulatory system manner such as MarA, SoxS, and TcpN [120, 139, 203, 204]. The AraC/XylS family proteins operate three unique features, which are regulation of virulence factors, stress response, and metabolism of carbons [139].

In the present study, we have characterized the role of a transcriptional regulator; AraC, a member of the AraC/Xyls family, in intramacrophage survival and virulence of *F. tularensis*. To date, no study has been conducted characterizing the regulatory role of AraC in *Francisella*. Thus, the findings from this study are novel. Sequence alignment with previously characterized AraC/XylS family proteins confirmed the presence of the unique HTH DNA binding domain in the C-terminal residues of AraC. This signature DNA binding motif is conserved in all members of the AraC/XylS family [139, 205]. The *FTL_0689* gene encodes AraC of *F. tularensis* LVS.

In this study, we considered several aspects for the investigation of the role of AraC transcriptional regulator in tularemia pathogenesis. First, AraC is an extensively studied transcriptional regulator in *E. coli* that is involved in the metabolism of L-arabinose [139, 197, 206]. Since L-arabinose metabolism has never been studied in *F. tularensis*, we considered investigating this phenotype. Second, the *araC* gene of *F.tularensis* is located upstream of the operon encoding Emr type MEP and transcribed in a divergent direction. Given the fact that most AraC/XylS family regulators are transcribed divergently from their target gene or operon [205], and since we have previously reported the involvement of MEP components, the *emrA1* and *silC,* in *F. tularensis* virulence [87, 112], we hypothesized that *araC* of *F. tularensis* LVS regulates the expression of the MEP components. Third, members of the AraC/XylS family

such as AraC of *E. coli* and YbtA of *Y. pestis* had been reported to exert their regulation in *cis* or in *trans* [139, 199], proposing a possibility for AraC to regulate *F. tularensis* genes in *trans.* Given the rationales above, we hypothesized that AraC of *F. tularensis* is a unique transcriptional regulator that plays an important role in tularemia pathogenesis. An AraC gene deletion mutant was generated in this study and characterized phenotypically with regards to L-arabinose utilization, sensitivity to oxidants, heavy metal ions, and antibiotics. Further, we investigated the global gene regulation of AraC in *F. tularensis* LVS in response to oxidative stress.

Results from sugar utilization experiments unveiled that AraC transcriptional regulator is not involved in L-arabinose utilization as *F. tularensis* LVS is deficient for growth in the presence of L-arabinose. It also highlighted that the availability of D-glucose is indispensable for the growth of *F. tularensis* LVS and is the sugar source of choice. These findings demonstrate that unlike the AraC transcriptional regulator of *E. coli* involved primarily in L-arabinose metabolism, the AraC of *F. tularensis* LVS has a distinct function other than the carbon metabolism.

The genomic organization of the Emr type multidrug efflux pump (MEP) of *F. tularensis* is unique, composing of three adjacent genes *emrB*, *emrA1*, and *silC* that constitute an operon [87, 112]. The transcription of the *araC* gene in the opposite direction of *emrB*, *emrA1*, and *silC* genes suggested a possible involvement of AraC in the regulation of these MEP genes. A similar genomic structure of MEP is present in *Pseudomonas aeruginosa* (*P. aeruginosa*) with MexR transcriptional regulator transcribed divergently from the *mexAB-oprM* multidrug efflux system [118]. In response to oxidative stress, MexR transcriptional regulator detaches from the promotor and functions as an activator to initiate the MEP genes expression [118]. Moreover,

Campylobacter jejuni (*C. jejuni*) possesses a *cmeGH* multidrug efflux pump that is controlled by Fur repressor transcribed divergently upstream of the operon [116]. Therefore, as a transcriptional regulator, *araC* was hypothesized to control the expression of the components of the MEP. This notion was investigated by using *emrA1* mutant as a comparison tool throughout this study and supported by results from qRT-PCR analysis that MEP components are downregulated in the Δ*araC* mutant in response to oxidative stress, suggesting a possible role for AraC in MEP gene regulation.

Oxidant sensitivity testing was performed using peroxides and superoxide-generating compounds. Results from peroxides sensitivity testing demonstrated that similarly to the *emrA1* mutant, the Δ*araC* mutant is sensitive to increasing concentrations of H_2O_2. Additionally, the Δ*araC* mutant exhibited high sensitivity to organic peroxides as compared to the wild type *F. tularensis* LVS. A similar phenotype has also been reported for the *emrA1* and Δ*silC* mutants of *F. tularensis* LVS [87, 112]. On the other hand, results from the sensitivity testing of superoxide-generating compounds revealed an enhanced sensitivity of the Δ*araC* mutant towards pyrogallol but not against paraquat or menadione. Studies on *emrA1* and Δ*silC* mutants have demonstrated a high sensitivity to these superoxide-generating compounds [87, 112].

The selective sensitivity of Δ*araC* mutant to one superoxide-generating compound but not to others can be attributed to the mechanism of action of each compound. Paraquat is a redox cycling compound that disrupts the electron transport channel in the bacterial cell membrane by inhibiting the reduction of NADP to NADPH during the redox reactions resulting in the production of highly microbicidal reactive oxygen species (ROS) such as superoxide anion, singlet oxygen, hydroxyl, and peroxyl radicals [207, 208]. Menadione is a

redox-cycling compound that induces oxidative stress in bacteria by acting first as aminoglycoside to disrupt the cell wall and plasma membrane [209] and then transporting into the bacterial cell where it converts molecular oxygen into superoxide radicals and H_2O_2 as a result of reduction by redox enzymes [210]. Both the compounds act by disrupting the bacterial plasma membrane to induce the production of ROS. However, several factors can induce the resistance of Δ*araC* mutant to both paraquat and menadione. One of them is the presence of the outer membrane that inhibits the permeability of these superoxide-generating compounds into the cytoplasmic membrane of the bacteria [211]. In addition, *F. tularensis* produces the antioxidant enzymes, SodB and SodC, that maintain the redox homeostasis and hinder the production of ROS [30]. Since Δ*araC* mutant did not exhibit enhanced sensitivity toward paraquat and menadione, we perceived that the production of the antioxidant enzymes is not disrupted by the *araC* gene deletion. Secretion of SodB and KatG enzymes is impaired in the *emrA1* mutant [87, 112]. Thus, AraC due to its impact on the expression of the MEP genes may lower the secretion of these antioxidant enzymes but may not completely abolish it and therefore mirror the phenotype of the *emrA1* mutant.

Furthermore, pyrogallol, a polyphenolic compound with an antibacterial effect reported on a wide range of Gram-negative as well as Gram-positive bacteria [212-215]. Pyrogallol is a type of catechins that kills bacteria by interaction with the cytoplasmic membrane, resulting in disruption of membrane permeability [215, 216]. In a study conducted on epigallocatechin gallate mechanism of action on *Bacillus subtilis* demonstrated that this compound attaches to and disrupts the outer membrane proteins, including transport systems of the bacteria, and then generates H_2O_2 and cerium [216] that consequently leads to ROS production. Another study also demonstrated that epigallocatechin gallate attaches to the porin protein in the outer

membrane of *E. coli* to diminish its activity [217]. The sensitivity of Δ*araC* mutant to pyrogallol as compared to the wild type suggests that Δ*araC* mutant may have encountered membrane protein damage. We speculate that AraC may be important for maintaining membrane integrity of *F. tularensis* through the regulation of genes involved in this function. Since the *emrA1* and Δ*silC* mutants exhibited extreme sensitivity to pyrogallol [87, 112], and as we indicated previously that members of the AraC XylS family function in a two-component regulatory manner [139], we proposed that AraC may participate in MEP components regulation.

The results from this study demonstrated that AraC of *F. tularensis* is not involved in resistance to heavy metals. Similar to the wild type *F. tularensis* LVS, Δ*araC* mutant was not sensitive to treatment with silver nitrate. Resistance to silver compounds had been proposed to be inherited among different *Francisella* species [87]. Silver nanoparticles used as an antibacterial cure for various fish infections are not effective in treating cases of *Francisella noatunensis* [218]. In contrary to Δ*araC* mutant, studies on heavy metals sensitivity unveiled that *emrA1* and Δ*silC* mutants are extensively sensitive to silver compounds [87, 112]. This indicated that MEP components of *F. tularensis* are required for heavy metal resistance but AraC does not contribute to this resistnace.

Furthermore, the results from this study revealed that AraC of *F. tularensis* is not involved in antibiotic resistance. Antibiotic susceptibility testing against various classes of antibiotics unveiled that Δ*araC* mutant is identical to the wild type *F. tularensis* LVS in antibiotic sensitivity. In contrary, EmrA1 and SilC together were reported to be responsible for the effusion of some antibiotic subsets including streptomycin, nalidixic acid, and chloramphenicol [87]. Additionally, transcriptional regulators of other bacterial species

including AarP (*Providencia stuartii*), MarA and Rob (*E. coli*), and RamA (*Klebsiella pneumoniae*) are involved in regulation in regards to antibiotics, organic compounds, and heavy metals stressors [139, 219, 220]. Altogether, we concluded that AraC transcriptional regulator of *F. tularensis* does not directly contribute to the regulation of MEP genes.

Phenotypic characterization of Δ*araC* mutant suggested that AraC transcriptional regulator of *F. tularensis* may be involved in the regulation of the oxidative stress response. Therefore, investigation of the global regulatory role of AraC of *F. tularensis* was performed to obtain novel understanding about its role in response to oxidants. The results from whole transcriptome data analysis in the absence of oxidant stressor revealed that despite the loss of *araC* gene in the mutant, the transcription profiles of both the Δ*araC* mutant and the wild type *F. tularensis* LVS were identical. These results demonstrated that AraC transcriptional regulator of *F. tularensis* does not exert its regulatory role when bacteria are growing in rich homeostatic growth conditions. These results also suggested that AraC gets activated only when the bacteria are exposed to certain stress conditions. Since the Δ*araC* mutant exhibited sensitivity towards oxidants, our primary hypothesis was that AraC plays a regulatory role under the conditions of oxidative stress. SoxS, an AraC/XylS family transcriptional regulator of *E. coli* is induced by oxidative compounds including paraquat and menadione to activate the transcription of the SoxRS regulon [120, 139, 204].

Our results demonstrate that activation of the AraC transcriptional regulator of *F. tularensis* in response to oxidative stress induced by menadione resulted in the differential expression of a multitude of genes some of which are transcriptional units and/or co-transcribed as operons. These genes are involved in key pathways that contribute to tularemia pathogenesis. Whole transcriptome analysis of bacteria treated with menadione confirmed the

notion that AraC transcriptional regulator requires to be induced by a stressor to employ its regulation. The results from this study unveiled the involvement of AraC in the positive regulation of some FPI genes in response to oxidative stress. Components of the FPI were determined to constitute a Type VI secretion system of *F. tularensis* [221]. We demonstrated that Δ*araC* mutant treated with menadione exhibited a significant downregulation of a subset of FPI genes. Thus far, several studies have published the fundamental role of FPI genes in intramacrophage replication and/or *Francisella* virulence [102, 222-225]. In *F. novicida*, *iglJ*, *iglI*, and *iglG* were found to be crucial for Type VI secretion system assembly [226]. We observed that *iglJ* and *iglI* genes were significantly downregulated in Δ*araC* mutant following menadione treatment, indicating that type VI secretion system assembly may be impaired in the mutant. The FPI genes *iglJ*, *iglI*, or *iglG* however, have been reported to be indispensable for intramacrophage replication and, specifically, the phagosomal escape [226].

Our results demonstrated the downregulation of *dotU* gene in the Δ*araC* mutant. DotU is one of the Type VI secretion system core proteins [170]. In *Legionella pneumophila,* IcmF-DotU homologs are required for Type IV secretion system stability but not for its functionality [227]. However, DotU is crucial for type VI secretion system functionality in *F. tularensis* [170]. DotU protein is implicated in the secretion of other FPI proteins: Hcp and VgrG of *Vibrio cholerae* and *P. aeruginosa* [101]. In *F. tularensis*, DotU is an essential virulence factor required for intracellular growth and virulence in mice [170]. Furthermore, the results exhibited a significant reduction in *pdpC* gene expression of Δ*araC* mutant in response to the oxidant. While PdpC in conjugation with PdPD were found to be partially involved in intramacrophage growth of *F. novicida* and Schu S4, they are extensively required for the complete virulence *in vivo* [228, 229].

The results also revealed the downregulation of *pdpE* and *Hcp* genes in Δ*araC* mutant after exposure to menadione. It was reported that PdpE structure and full functionality is affected by *pdpC* gene integrity [228]. A defect in *pdpC* gene results in the transcription of a disrupted PdpE protein. Similarly, the downregulation of *pdpE* gene in Δ*araC* mutant can be caused either by exposure to menadione or due to the downregulation of the *pdpC* gene. Another study also published the possibility of the presence of a sec secretion signals in PdpE due to its ability to translocate into the outer membrane of *Francisella* [170]. In an attempt to define the role of PdpE in *F. tularensis*, it was demonstrated that PdpE protein sequence exhibited poor homology with the Hcp protein. However, both proteins were found to perform distinct functions [170]. While *pdpE* is dispensable for type VI secretion system assembly, phagosomal escape, or virulence in mice in both of the *F. novicida* [226] and *F. tularensis* LVS [230], its role is yet to be determined. The Hcp protein, on the other hand, is one of type VI secretion system major structural proteins [231]. It forms a tube-like channel in *F. tualrensis* that is in conjunction with VipA and VipB sheathing to facilitate the translocation of a principle virulence protein, VgrG, effector domain into its target location [170]. The downregulated gene expression profile of the FPI genes in the Δ*araC* mutant, also corroborates with attanuated intramacrophage survival and virulence of *F. tularensis* in mice in a dose-dependent manner.

Besides the FPI encoded genes, the whole transcriptome analysis of the Δ*araC* mutant under oxidative stress also revealed the involvement of AraC of *F. tularensis* LVS in the regulation of two genes, *aroA,* and *rnhA*, that are a part of heat stress related operon. The role of AroA in *Francisella* is not determined yet. However, in *Salmonella*, AroA is involved in the shikimate pathway that catalyzes the synthesis of aromatic amino acids from glucose [232]. Attenuation of the *aroA* gene, commonly used to study metabolic pathways, results in

accumulation of pyruvate within the bacteria and consequently cause osmotic pressure [233]. Therefore, AroA is essential to maintain bacterial homeostasis through the shikimate pathway. In addition, it was reported that *aroA* gene deletion of *Salmonella* resulted in aromatic amino acids auxotrophy and attenuation of virulence [232]. In agreement with the role of AroA in *Salmonella*, oxidative stress resulted in the downregulation of *aroA* gene in the Δ*araC* mutant that can further explain the attenuation of intramacrophage growth and virulence in mice.

AraC of *F. tularensis* positively regulated the *rnhA* gene that encodes ribonuclease H. In many Gram-negative bacteria such as *Yersinia*, *Salmonella*, *Helicobacter pylori* (*H. pylori*), and *Shigella flexneri*, ribonucleases are known to function as virulent factors [234]. A study reported that RnhA contributes to *Mycobacterium smegmatis* (*M. smegmatis*) defense against the destruction by oxidative stress [172]. In the same study, it was reported that deletion of *rnhA* gene has no impact on the bacterial viability [172]. Interestingly, deletion of both *rnhA* and *rnhB* genes in *M. smegmatis* enhanced bacterial sensitivity to H_2O_2 [172], which explains the sensitivity of the Δ*araC* mutant to H_2O_2 observed in this study. Other studies also demonstrated that *rnhA* in conjugation with *rnhB* and *rnhC* genes are required for *B. subtilis* viability [173]. Therefore, we propose that AraC by regulating the epression of *rnhA* gene protects *F. tularensis* against oxidative stress.

In addition, we have observed that the expression level of *rpoH* gene was not altered in Δ*araC* mutant in response to oxidative stress and remained downregulated. However, results from qRT-PCR unveiled the significant upregulation of heat shock proteins (GroEL, DnaK, DnaJ, and Hsp 40) in the Δ*araC* mutant after menadione treatment. In *E. coli*, GroES and GroEL proteins were discovered to be induced in response to various stressors including heat shock, oxidative stress, nutrient deprivation, and SOS [235]. Additionally, DnaK protein was

also detected when exposing bacteria to peroxides, UV radiation, nutrient deprivation, and some antimicrobial agents [235]. Similarly, a study on *F. tularensis* reported that RpoH is required for the positive regulation of heat shock proteins when bacteria are under different stress conditions including high temperature or oxidative stress [149]. Therefore, although heat shock proteins are mostly induced in response to high-temperature stress, other stressors also promote their induction. We confirmed this notion as well as the role of AraC in regulating heat shock genes by conducting a thermal resistance experiment. The result demonstrated that in the physiologic temperature, the growth of Δ*araC* mutant is similar to the wild type strain. However, when growing the bacteria in extremely high temperature, the Δ*araC* mutant exhibited significantly enhanced viability as compared to the wild type *F. tularensis* LVS. The viability of the transcomplemented strain was significantly higher than both the Δ*araC* mutant and the wild type *F. tularensis* LVS the due to the presence of the GroEL promotor in the transcomplementation vector that governs the expression of *araC*. These findings support our qRT-PCR results and demonstrate that AraC is required for the regulation of heat shock proteins in *F. tularensis* in response to heat and oxidative stresses.

The results from whole transcriptome analysis further highlighted the involvement of AraC of *F. tularensis* LVS in the positive regulation of F_1F_0 ATP synthases in response to oxidative stress. Treatment with menadione induced the significant downregulation of the ATPase subunits in Δ*araC* mutant as compared to the wild type *F. tularensis* LVS. ATPases are known for their role in the generation of the electrochemical gradient to facilitate the penetration of protons, ions, and metabolic molecules through cell membrane [236, 237]. ATPases are required by pathogens to tolerate acidic environments. In *Streptococcus mutans*, ATPase gene is upregulated when the bacteria is under acidic pH stress [238]. Additionally,

ATPases are crucial for energy conveyance in several pathogens. ATPases catalyze the conversion of ADP and inorganic phosphate into ATP [239]. Few studies have also reported the response of the ATPases during oxidative stress. In *E. coli*, YchF protein has an ATPase activity that is sensitive to H_2O_2 exposure. It was reported that OxyR transcriptional regulator causes the downregulation of *ychF* in response to oxidative stress [240]. The downregulation of ATPases in the Δ*araC* mutant in response to oxidative stress indicates that AraC stimulates ATPases to support oxidant resistance. The results allow us to speculate that oxidative stress inhibits energy production in the Δ*araC* mutant, and consequently impeds other vital mechanisms.

The results from this study further demonstrated the engagement of AraC in the regulation of the TCA cycle in *F. tularensis* during oxidative stress. Several studies have linked the TCA cycle with the virulence of *F. tularensis* during stress conditions [187, 188, 241, 242]. We observed the downregulated gene expression of key TCA cycle enzymes in the Δ*araC* mutant after menadione treatment. The operon encoding pyruvate dehydrogenase enzyme was significantly downregulated. The pyruvate dehydrogenase is an enzyme that catalyzes the synthesis of the first enzyme, Acetyl-CoA, of the TCA cycle [180]. It has three subunits: E1: pyruvate dehydrogenase (AceE), E2: dihydrolipoamide transacetylase (AceF), and E3: dihydrolipoamide dehydrogenase (Lpd) [188]. We also observed downregulation of another enzyme, oxoglutarate dehydrogenase, encoded on another operon. The oxoglutarate dehydrogenase promotes the conversion of α-ketoglutarate (or 2-oxoglutarate) into succinyl-CoA in the TCA cycle. Oxoglutarate dehydrogenase is also composed of three subunits: E1: 2-oxoglutarate dehydrogenase (SucA), E2: dihydrolipoamide succinyltransferase (SucB), and E3: lipoamide dehydrogenase (Lpd) [188]. In *F. novicida*, a study had shown the requirement

of the E1(AceE) subunit of the pyruvate dehydrogenase enzyme in intramacrophage growth [243]. In addition, another study have demonstrated the contribution of E3 (Lpd) subunit to *F. tularensis* LVS virulence [148]. Other than its major role in metabolism, the E3 (Lpd) subunit is a component of *M. tuberculosis* defense mechanism against oxidative stress [244]. Furthermore, double deletion of *sucA* and *suB* genes in *S.* Typhimurium attenuated the bacteria for virulence in mice [245]. In accordance with the results from studies explained above, exposing AraC deficient bacteria to the oxidant repressed the expression of all three subunits both pyruvate dehydrogenase and oxoglutarate dehydrogenase. Moreover, we have observed that Δ*araC* mutant is attenuated for intramacrophage growth and virulence in mice. Therefore, it appears that regulation of the TCA cycle of *F. tularensis* LVS by AraC is also associated with intramacrophage survival and virulence especially when the bacteria are experiencing an oxidative stress environment.

The results from whole transcriptome analysis denoted that AraC regulates the pantothenate metabolism in *F. tularensis*. The pantothenate, also known as vitamin B_5, is an essential metabolic molecule that is a central component of the CoA of the TCA cycle [182]. In *E.coli* and *S.* Typhimurium, the *de novo* synthesis of pantothenate molecule relies on their ketopantoate reductase genes [246]. In contrast, *F. tularensis* virulent Schu S4 strain lacks these genes, but still synthesizes the pantothenate molecule [182]. *F. tularensis* LVS, on the other hand, harbors the ketopantoate reductase genes and loss of any of these genes renders the bacteria auxotrophic for pantothenate [182]. The ketopantoate reductase genes in *F. tularensis* are composed of a group of five genes (*coaX*, *panD*, *panC*, *panB,* and *panG*) that promote the enzymatic reaction of pantothenate synthesis. *F. novicida* requires the *coaX* gene for the successful dissemination into mice organs [247]. Moreover, a striking increase in the

expression level of *F. tularensis* ketopantoate reductase genes post-invasion of bone marrow-derived macrophages has been reported [160]. Similarly, lack of both PanC and PanD in attenuates the virulence of *M. tuberculosis* in mice [248]. These studies established the significance of pantothenate for *F. tularensis in vivo* and *in vitro* intracellular life cycles. Our analysis indicated that all of the five ketopantoate reductase genes were significantly downregulated after exposing the Δ*araC* mutant to menadione. Therefore, we infer from these observations that during oxidative stress, *F. tularensis* employs the AraC transcriptional regulator to synthesize the pantothenate that consequently contributes to the formation of CoA enzyme. The acetylation of CoA enzyme then initiates the TCA cycle.

Our observations further indicate that AraC is also involved in the positive regulation of glucose metabolism in *F. tularensis* LVS. Three genes *pgk*, *pyk*, and *fba* were significantly downregulated in the Δ*araC* mutant after menadione treatment. PGK (phosphoglycerate kinase) is an essential enzyme for the breakdown of glucose into pyruvate and subsequently, the release of high energy [185]. Correlation between phosphoglycerate kinase and bacterial virulence has not been completely established. However, it has been reported that PGK is required for *Corynebacterium glutamicum* (*C. glutamicum*) growth as bacteria with the *pgk* gene deletion is deficient for glucose metabolism [249]. Furthermore, deletion of the pyruvate kinase encoding gene (*pyk*) in *C. glutamicum* resulted in a growth defect even in the culture media supplemented with glucose [250]. In contrast, in this study, it was observed that both the *pgk* and *pyk* genes were downregulated in the Δ*araC* mutant but the growth rate of the Δ*araC* mutant remained similar to the wild type *F. tularensis* LVS when grown in CDM supplemented with D-glucose **(Figure 14C)**. In *Enterococcus faecalis,* PYK activation is promoted by fructose 1,6-bisphosphate (FBP) [186], which is then metabolized by the enzyme

fructose-bisphosphate aldolase class II (FBA). The FBA is indispensable for the viability during acute and chronic stages of several bacterial pathogens including *M. tuberculosis* [251]. Several efforts to disrupting the *fba* gene in *E. coli*, *B. subtilis*, and *P. aeruginosa* have failed [187]. Moreover, in addition to the bacterial pathogens, *Toxoplasma gondii*, an intracellular parasite, requires the *fba* gene for survival, multiplication, and pathogenesis [252]. A study has shown that the FBA plays an important role in *Francisella* cytosolic replication only if gluconeogenesis substrate is available but is not required required for the bacterial escape from the phagosome [187]. Additionally, deletion of *fba* gene in *Francisella* induces the expression of the antioxidant enzyme KatG [187]. Since OxyR regulates the expression of *katG* [156], it has been proposed that *fba* serves as a secondary regulator for *katG* [187]. In the present study, the expression of *katG* gene was not affected by *araC* deletion. Therefore, we suggest that AraC positively regulates the gluconeogenesis and glycolysis pathways in *F. tularensis* to enhance the cytosolic replication. Further, as the end product of glucose metabolism is pyruvate, which is a key molecule in initiating the TCA cycle, AraC may contribute to the regulation of the TCA cycle indirectly through the carbon metabolism.

We have also noted the involvement of AraC in the positive regulation of *tpiA* gene encoding triosephosphate isomerase, an enzyme required for glucose metabolism and TCA cycle. Bacterial glycolytic enzymes are different than those of the eukaryotes in their involvement in pathogenesis [253, 254]. In *Staphylococcus aureus*, *Lactobacillus plantarum*, and *Paracoccidioides brasiliensis,* TPI is essential for attachment to host cells in addition to its function in glycolysis [156]. In *M. tuberculosis*, *tpi* is required for intracellular growth [255]. Deletion of *tpi* in *M. tuberculosis* attenuates the bacterial virulence *in vivo* [255]. Conversely, TPI in other bacteria, including *E. coli* and *S.* Typhimurium, is involved in, but

not required for pathogenesis [256]. Consistent with these observations, it appears that AraC-mediated regulation of TPI may contribute to the virulence of *F. tularensis*.

The RNA-seq data analysis also demonstrated the downregulation of an additional gene (*glmM*) in menadione treated Δ*araC* mutant that is involved in glucose metabolism. The *glmM* gene encodes for phosphoglucosamine mutase enzyme that catalyzes the conversion of glucosamine-6-phosphate into glucosamine-1-phosphate in the cytosol [257]. The end product of this reaction is essential for the synthesis of bacterial peptidoglycan [258]. In addition, GlmM enzyme is essential for virulence of Gram-positive bacteria. However, several reports demonstrated that the GlmM enzyme is required for the survival of most Gram-negative bacteria including *E. coli*, *S.* Typhimurium, *P. aeruginosa*, and *H. pylori* [259-261]. Similarly, the downregulation of *glmM* in the Δ*araC* mutant during oxidative stress further explains the attenuation of virulence of the Δ*araC* mutant. Furthermore, the defect in glucose metabolism due to the downregulation of GlmM can also cause a subsequent shortening of the TCA cycle and reduce bacterial fitness in response to oxidative stress. Energy production impeded by the glucose metabolism defect thus may cause the Δ*araC* mutant to become less viable intracellularly than the wild type *F. tularensis* LVS. Although not investigated in this study, the downregulation the *glmM* may also result in a defect in the Δ*araC* mutant peptidoglycan layer and thus may affect the bacterial integrity and permeability and increase susceptibility to oxidants.

The results from the whole transcriptome analysis of the Δ*araC* mutant also revealed the downregulation of adenine phosphoribosyltransferase encoding gene (*apt*) after treatment with menadione. The adenine phosphoribosyltransferase catalyzes the conversion of free adenine into AMP [262], which can further be utilized for the interconversion into ADP and/or ATP.

AMP also contributes to the synthesis of the acetyl-CoA, the central enzyme in the TCA cycle [263]. Furthermore, AMP is a key constituent of the RNA [263]. Additional genes that were downregulated within the same metabolic operon included *secG*, encoding the SecG translocon subunit, as well as Succinate dehydrogenase encoding genes (*sdhA*, *sdhB*, *sdhC*, *sdhD*). Collectively, these results indicate that AraC may play an essential role in *F. tularensis* pathogenesis by positively regulating the AMP synthesis thereby promoting the TCA cycle.

The results from this study further informed that AraC of *F. tularensis* also controls the expression of antiporters and regulatory proteins. One of these genes encodes an amino acid antiporter. This antiporter is an arginine (ArgP) transporter. Attenuation of ArgP results in a delayed phagosomal escape and defect in cytosolic replication [264]. A study has established that this arginine transporter in *F. novicida and F. tularensis* LVS support metabolic pathways during intracellular invasion [264, 265], highlighting the significance of arginine for *Francisella* pathogenesis. Furthermore, it was also determined that deficiency in arginine acquisition caused a defect in the production of ribosomal proteins [264]. Suppression of the synthesis of ribosomal proteins is commonly observed in organisms exposed to various stress conditions [264]. Therefore, arginine plays a crucial role in promoting the phagosomal escape and enhancing intracellular replication. In agreement with the literature, exposing Δ*araC* mutant to oxidative stress resulted in the downregulation of *argP*, thus, may limit the arginine acquisition and defective protein synthesis ultimately resulting in attenuation of intramacrophage growth.

The results from this study additionally revealed that AraC positively regulates the expression of other regulatory genes, *rpoB,* and *rpoC*. These genes encode two key components, the *β* (RpoB) and *β'* (RpoC) that constitutes the catalytic core of RNA polymerase

[266]. RpoB and RpoC were both downregulated in the Δ*araC* mutant after menadione treatment, indicating inhibition of protein synthesis, thus reducing the viability intracellularly than the wild type bacteria. The downregulation of the *rpoB* and *rpoC* in conjunction with downregulation of *argP* further indicate the suppression of protein synthesis in the Δ*araC* mutant. Furthermore, the NAD(P)/FAD-dependent oxidoreductase encoding gene was upregulated in the Δ*araC* mutant in response to oxidative stress. This enzyme catalyzes the redox reactions NAD(P)H or NAD(P)+. The induction of NAD(P)H or NAD(P)+ indicates a defect in the electron transport chain homeostasis, which subsequently reduces the bacterial viability.

In addition to the role of AraC in regulating operons, the results from whole transcriptome analysis revealed the involvement of AraC in regulating genes transcribed as single transcriptional units. Data analysis demonstrated that type I DNA topoisomerase was downregulated in the Δ*araC* mutant when treated by menadione. Inhibition of topoisomerase I had been exploited as a target for therapeutics in several organisms including *E. coli*, *Y. pestis*, and *Streptococcus pneumoniae* [267, 268], indicating its importance in pathogenesis. *F. tularensis* requires the activity of topoisomerase I to repair DNA damages and supercoils exerted by exposing to the oxidative agents. Our results demonstrate that AraC negatively regulates Topoisomerase I when *F. tularensis* is exposed to oxidative stress.

Furthermore, the data analysis indicated that AraC of *F. tularensis* also negatively regulates *pyrH* gene that encodes the uridylate (UMP) kinase enzyme. *E. coli* utilizes the UMP kinase to establish *de novo* pyrimidine nucleotides synthesis [269], providing evidence of the importance of the *pyrH* gene. Similarly, *F. tularensis* employ the UMP kinase to compensate for nucleotides damage as a result of oxidative stress.

Lastly, the results from this study revealed the involvement of AraC in the negative regulation of two genes of *F. tularensis*. One of these genes encodes serine/threonine transporter. These results indicate that along with arginine transporter, additional amino acid transporters such as serine/threonine transporter are also under the control of AraC. *Legionella pneumophila* harbors MFS threonine transporter, which contributes to the intracellular bacterial replication [270]. Both *Francisella* and *Legionella* mostly rely on amino acids as the main carbon [271]. It was observed that exposing Δ*araC* mutant to oxidative stress significantly upregulated serine/threonine transporter gene indicating that in response to oxidative stress, AraC of *F. tularensis* restricts the amino acid uptake and relies on arginine to conserve energy. Moreover, the induction of the serine/threonine transporter in Δ*araC* mutant also indicates that mutant is hunting for every nutritional source to maintain its viability.

The other gene that is negatively regulated by AraC in regards to oxidative stress is the one encoding acid phosphatase. Acid phosphatase is an enzyme that catalyzes inorganic phosphates mobility [272]. It is also engaged in the eukaryotic and prokaryotic phosphor-relay system [272]. Acid phosphatase is required by *Francisella* strains for intramacrophage growth [273, 274]. However, the virulent strains (type A and B) strains do not employ acid phosphatases to overcome intracellular oxidative burst [274]. *F. tularensis* virulent strains rely on *fevR* to overcome NADPH oxidase [274]. There are at least five acid phosphatases identified in *Francisella* [272], which are encoded by distinct genes than the one induced in our analysis. The gene encoding acid phosphatase was significantly upregulated in Δ*araC* mutant after treatment with menadione, indicating that this gene may not involved in virulence of the wild type.

Collectively, the results obtained from the present study demonstrate that AraC transcriptional regulator exerts a global regulatory role on several key pathways of *F. tularensis* LVS. We determined that AraC regulates FPI genes and contributes to the *in vivo* and *in vitro* virulence of the *F. tularensis* LVS strain. We also unraveled the regulatory role of the AraC in heat shock proteins in response to oxidants and heat stress. Additionally, AraC plays an important role in regulating *F. tularensis* LVS metabolic pathways, specifically the TCA cycle, by targeting key enzymes that are indispensable for the cycle. We further observed the engagement of the AraC in the regulation of DNA repair pathways as well as controlling protein synthesis of *F. tularensis* during oxidative stress. Altogether, we conclude that AraC belonging to the AraC/XylS family of transcription regulator plays an essential role in oxidative stress response of *F. tularensis* LVS.

Significance of the study and future directions

This study provides a wide range of knowledge about *F. tularensis* LVS response to oxidative stress from the point view of gene regulation. It provides a powerful tool for tularemia therapeutic applications. Up until now, efforts have been focused on developing effective tularemia treatment with little success. The present study characterized a global transcription regulator that controls several pathways in *F. tularensis* LVS and thus, can be utilized as an effective target to develop effective tularemia therapeutics.

In the future, we aim to determine the exact mechanism of AraC-dependent regulation of these virulence and metabolic pathways by identifying binding domains in the promoter regions of the regulated genes/operons by chromatin immunoprecipitation (ChIP) sequencing and electrophoretic mobility shift (EMSA) assays.

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