The oxidative stress response of *Francisella tularensis*

Marie Honn

Department of Clinical Microbiology
Laboratory for Molecular Infection Medicine Sweden (MIMS)
Umeå University
Umeå 2016

Responsible publisher under swedish law: the Dean of the Medical Faculty

ISBN: 978-91-7601-415-8
ISSN: 0346-6612; 1781
Front cover by Marie Honn
Elektronisk version tillgänglig på http://umu.diva-portal.org/
Tryck/Printed by: Print & Media
Umeå, Sweden 2016

To boldly go where no one has gone before

- Star Trek

Table of Contents

Abstract

Francisella tularensis is capable of infecting numerous cell types, including professional phagocytes. Upon phagocytosis, *F. tularensis* resides within the phagosome before escaping into the cytosol to replicate. Phagocytes constitute a hostile environment rich in ROS, which are employed as a means of killing pathogens. ROS interact with and disrupt the function of vital molecules such as DNA, proteins and bacterial structures. Iron potentiates the danger of ROS through the Fenton reaction where ferrous iron reduces H_2O_2 causing the formation of highly reactive hydroxyl radicals and anions. Low levels of ROS are formed during normal aerobic metabolism and pathogens thus have a need for defense mechanisms to handle the ever present levels of ROS but even more so to combat the onslaught of ROS experienced within a host.

This thesis was focused on the investigation of the iron status and oxidative stress response of *F. tularensis*; thereby identifying key players controlling the bacterial iron content, its adaptation to oxygen-rich environments and defense against ROS.

We identified subspecies-specific differences in iron content, where *F. tularensis* subsp. *tularensis* was found to contain significantly less iron than strains of subsp. *holarctica*. The reduced iron content resulted in an increased tolerance to H_2O_2, despite simultaneously causing a decrease in the activity of catalase - the iron-dependent enzyme responsible for degrading H_2O_2 in *F. tularensis*. This strongly suggests that the restricted iron uptake and storage by subsp. *tularensis* strains is beneficial by rendering the bacteria less susceptible to H_2O_2, thereby evading the toxic effects of the iron-driven Fenton reaction. This evasion is likely to be an important part of the higher virulence displayed by subsp. *tularensis* as compared to subsp. *holarctica*.

We further identified that the global regulator, MglA, is important for the adaptation of LVS to oxygen-rich environments. Deletion of *mglA* from LVS resulted in a mutant, Δ*mglA,* with impaired defense to oxidative stress, as manifested by an inability to grow to wild-type levels under aerobic conditions, an accumulation of proteins with oxidative damage, a suppressed expression of iron-uptake related genes, an increased catalase activity, and an increased tolerance to H_2O_2. This phenotype was reversed in a microaerobic environment. We therefore conclude that MglA is an important factor for the defense of LVS to oxidative damage under aerobic conditions and speculate that MglA is of greatest importance in oxygen-rich foci.

We also studied the role of OxyR in LVS by creating a Δ*oxyR* mutant as well as a double mutant, Δ*oxyR*/Δ*katG*. The *in vitro* response of these mutants, as well as of Δ*katG*, to defined ROS was assessed using H_2O_2, the O_2^- generating agent paraquat, and the $ONOO^-$ generator SIN-1. Δ*oxyR* was more susceptible to all ROS than LVS as was Δ*katG*, with the exception of O_2^- Strikingly, Δ*oxyR*/Δ*katG* was significantly more susceptible to all ROS tested compared to either single deletion mutant. LVS, Δ*oxyR* and Δ*katG* replicated efficiently in bone marrow-derived macrophages whereas Δ*oxyR*/Δ*katG* showed no replication. In mice, the Δ*oxyR* mutant displayed impaired replication in liver, but intact replication vs. LVS in spleen. Collectively, our results demonstrate an important role of OxyR in the oxidative stress response and virulence of *F. tularensis*, and further reveal overlapping roles of OxyR and catalase in the defense against ROS. The results thus shed new light on the complexity of ROS defense in *F. tularensis*.

Sammanfattning på svenska

Francisella tularensis (*F. tularensis*) är den bakterie som orsakar sjukdomen tularemi, på svenska kallad harpest. *F. tularensis* är en mångsidig bakterie som kan infektera såväl människor som mer än 200 andra däggdjursarter, men även insekter och amöbor. Det finns flera underarter – subspecies - varav två, subsp. *tularensis* och subsp. *holarctica* är betydelsefulla för infektioner av människor. Bakterien smittar på flera sätt: via sjuka djur, insektsbett, förorenat vatten eller mat och inandning av t.ex. förorenat damm eller droppar av kontaminerad vätska. Sjukdomsbilden inkluderar influensaliknande symptom som hög feber, muskel- och huvudvärk, men även andra symptom som varierar beroende på smittoväg. Hudsmitta, ger svårläkta sår och svullnad av närliggande lymfkörtlar; intag av kontaminerad mat eller dryck ger symptom i mun, svalg eller mage och smitta via inandning ger lunginflammation, vilket är den allvarligaste formen av tularemi.

F. tularensis kan infektera många cell-typer, inklusive makrofager - immunceller som i normala fall tar upp och oskadliggör invaderande mikroorganismer. Även *F. tularensis* tas upp av makrofager men undkommer avdödning och utnyttjar istället den skyddade intracellulära miljön för att föröka sig. Vid upptag – fagocyctos - av mikroorganismer bildar makrofagerna en membranomsluten vakuol – fagosom – runt mikroorganismen i fråga. Genom modifieringar av fagosomen utsätts mikroorganismen för en mängd ämnen ämnade att oskadliggöra den. Utmärkande för *F. tularensis* är dock att den bryter sig ut ur fagosomen och på så sätt kommer ut i cellens cytosol, där den sedan förökar sig massivt.

En av de strategier makrofager utnyttjar för avdödning av mikroorganismer är reaktiva syreföreningar, så kallade ROS, för att orsaka oxidativ stress. ROS förekommer i stora mängder i fagosomen där de reagerar med bakteriella strukturer, proteiner och molekyler som skadas och detta medför att viktiga funktioner i mikroorganismerna slås ut. Ytterligare ett skydd mot infektion är nutritionell immunitet, som innebär att värdorganismen försvårar för invaderande mikroorganismer att få tillgång till näringsämnen. Av vikt för denna avhandling är att detta försvar leder till en minimering av mängden järn tillgängligt för mikroorganismer. Järn ingår i en uppsjö av proteiner som i sin tur ingår i en mängd viktiga cellulära processer och är därför essentiell för nästan alla organismer. Effektiva mekanismer för järnupptag är därför av stor vikt, men måste regleras noggrant för att undvika överskott av järn. Överskottet riskerar annars att bidra till oxidativ stress genom att reagera med väteperoxid (H_2O_2) i Fenton reaktionen, där oerhört reaktiva hydroxyl radikaler ($HO^{\cdot}$) bildas. För att överleva och föröka sig i en värdorganism behöver *F. tularensis* följdaktligen både ett kraftfullt

försvar mot oxidativ stress och en effektiv hantering av järn – både vad gäller upptag och regleringen av upptaget. Denna avhandling har fokus på mekanismer som är involverade i denna järn-balans, samt på mekanismer involverade i försvar mot oxidativ stress i *F. tularensis*.

Bakterier har utvecklat komplexa system för att möta värdorganismens attacker i form av ROS och nutritionell immunitet. En viktig del i regleringen av dessa mekansimer är transkriptionsfaktorer. Transkriptionsfaktorer är proteiner som reglerar överförandet – transkriptionen - av informationen i DNA till RNA, som sedan fungerar som ritningar vid byggandet av proteiner. Transkriptions-faktorer kontrollerar vilka gener som uttrycks och under vilka förhållanden. De är därför av oerhörd vikt både för att upprätthålla grundläggande biologiska funktioner, och för att möjliggöra anpassningar till olika förhållanden.

Vi har identifierat subspecies specifika skillnader i järnbalansen hos *F. tularensis*, där subsp. *holarctica* innehåller väsentligt mer järn än subsp. *tularensis*. Vi visar även vikten av detta järninnehåll i relation till känsligheten för H_2O_2-inducerad oxidativ stress, där ett högre järninnehåll ger en ökad känslighet. Utöver skillnaderna mellan subspecies visar vi att den icke-virulenta subsp. *holarctica* stammen LVS innehåller avsevärt mycket mer järn än virulenta stammar av denna subspecies, vilket även påverkar H_2O_2 känsligheten. Vi visar även att en bidragande orsak till det förändrade järn-innehållet i LVS är att den bär en fusion mellan två gener, *fupA* och *fupB*, som i andra stammar är separata.

Vi har även studerat transkriptions-faktorer och funnit att både MglA och OxyR fyller viktiga funktioner i *F. tularensis* respons till oxidativ stress. Genom att studera mutanter där vi tagit bort – deleterat – vissa gener, och undersöka defekterna de uppvisar, har vi kunnat visa att MglA är avgörande för *F. tularensis* förmåga att anpassa sig till syre-rika miljöer och skydda sig mot den oxidativa stress dessa miljöer innebär. Avsaknaden av MglA leder därför till oxidativ skada på proteiner och sämre tillväxt-förmåga hos bakterien. Även transkriptionsfaktorn OxyR är viktig vid oxidativ stress och vi visar att den är involverad i försvaret mot flera ROS; att dess funktion till viss del överlappar med den skyddande effekten av enzymet katalas, samt att den är nödvändig för full virulens i möss. Genom att studera en mutant där vi deleterat både *oxyR* och genen för katalas påvisar vi även överlappande effekter av OxyR och katalas för bakteriens skydd mot ROS.

Sammanfattningsvis visar våra resultat nödvändigheten av robusta försvars- och kontroll-mekanismer i dessa sammanhang och att sådana mekanismer utgör ett komplext nätverk hos *F. tularensis*. Avhandlingen bidrar således med viktig ny kunskap inom ämnesområdet.

List of Papers

Papers included in the thesis

I

Lindgren H, **Honn M,** Salomonsson E, Kuoppa K, Forsberg Å, Sjöstedt A. Iron content differs between *Francisella tularensis* subspecies *tularensis* and subspecies *holarctica* strains and correlates to their susceptibility to H_2O_2-induced killing. Infect Immun2010;79:1218–24.

II

Honn M, Lindgren H, Sjöstedt A. The role of MglA for adaptation to oxidative stress of *Francisella tularensis* LVS. BMC Microbiology. 2012;12:14.

III

Honn M, Lindgren H, Sjöstedt A. OxyR - an important regulator of the oxidative stress response in *Francisella tularensis* LVS. (*Manuscript*)

Papers not included in the thesis

IV

Lindgren H, **Honn M**, Golovlev I, Kadzhaev K, Conlan W, Sjöstedt A. The 58-kilodalton major virulence factor of *Francisella tularensis* is required for efficient utilization of iron. Infect Immun. 2009;77:4429–36

Abbreviations

CAS	Chrome Azurol Sulfonate
CDM	Chamberlain's Defined Media
C-CDM	Chelated-CDM
CFU	Colony Forming Unit
DFO plates	Deferoxamine C-CDM-GC agar plates
DNA	Deoxyribonucleic acid
FeoB	Ferrous iron transporter B
FPI	*Francisella* Pathogenicity Island
Fsl	*Francisella* siderophore locus
Fur	Ferric uptake regulator
LVS	Live Vaccine Strain
MC plates	McLeod agar plates
MglA	Macrophage growth locus A
mRNA	messenger RNA
NOS	Nitric Oxide Synthase
OD	Optical Density
PCR	Polymerase Chain Reaction
qRT-PCR	Quantitative Real-Time PCR
RNA	Ribonucleic acid
RNAP	RNA Polymerase
ROS	Reactive Oxygen Species
SIN-1	3-morpholinosydnonimine hydrochloride
SOD	Superoxide Dismutase
VC	Viable count
wt	wild-type

1 Introduction

1.1 *Francisella tularensis* – an overview

Francisella tularensis is a Gram-negative, facultative intracellular coccobacillus and is the causative agent of the potentially lethal disease tularemia [1]. The disease was first described in the early 20th century as a plague-like disease of rodents caused by a bacterium capable of infecting numerous mammals as well as arthropods [2]. Already early on it was suspected the bacterium may be capable of infecting humans, a suspicion which was confirmed only a few years after the disease was first described [3]. The name of the disease, tularemia, and the initial name of the causative bacterium, *Bacterium tularense*, were both proposed by Edward Francis, in recognition of Tulare County, where the bacterium first was observed and the disease was endemic in the rodent population. The pioneering work of Edward Francis was later honored by giving the bacterium its current name: *Francisella tularensis* [4].

1.1.1 Transmission, prevalence and disease

As was recognized already by the early pioneers, *Francisella* is flexible in its choice of hosts, as demonstrated by the fact that it has been reported in at least 250 different species ranging from mammals to arthropods and even protozoa [1]. Tularemia is a zoonotic disease, meaning it can transmit from animals to humans; and just as its broad range of hosts, *Francisella* is very versatile when it comes to the route of infection.

Transmission can occur through contamination of skin lesions when handling infected animals, through bites of infected arthropod vectors (e.g. mosquitoes or ticks), through ingestion of contaminated food or water, or through inhalation of aerosols or contaminated material [1]. The influence of arthropod vectors such as ticks and mosquitoes is substantial, with ticks being important vectors in the United States and continental Europe, while mosquitoes are the predominant mode of transmission in Sweden; mosquito bites having been identified as the single largest risk factor for contracting tularemia in Sweden [1]

The mode of transmission and the subspecies of *F. tularensis* causing infection strongly influence the clinical manifestations of the disease. There are two clinically important subspecies of *F. tularensis*: subsp. *tularensis* and subsp. *holarctica*. Subsp. *tularensis* is geographically confined to North America while *holarctica* is found all across the Northern Hemisphere.

Subsp. *tularensis* causes an aggressive disease with high mortality if left untreated, whereas subsp. *holarctica* infections are only rarely lethal [1]. Despite this difference in virulence between subsp. *tularensis* and *holarctica,* the two subsp. are very similar on a genetic level. The genetic differences are limited to 9 genes unique to subsp. *tularensis*, and a further 20 genes being active only in this same subspecies. Individual deletion of five of the uniquely encoded genes has been shown not to affect the virulence of the prototypic *subsp. tularensis* strain SCHU S4 in mice [5]. This implies that the difference in virulence is likely to be caused not by the absence or presence of a single gene, but rather by the combined effect of differences in the regulation of several genes.

The most common mode of transmission of tularemia is through bites from infected arthropod vectors. The resulting form of tularemia – ulceroglandular tularemia - is characterized by the formation of ulcers at the infection site and a swelling of the local draining lymph nodes and is rarely lethal although it may take months to recover fully. Oculoglandular tularemia is the result of a direct inoculation of the eye, oropharyngeal and gastrointestinal tularemia result from ingestion of contaminated food or water, respectively, and the most severe form of the disease – respiratory tularemia – is transmitted through inhalation of aerosols or contaminated material. Such airborne infection, requiring no more than 10, or indeed fewer, organisms to establish infection, causes respiratory tularemia and is by far the most lethal mode of transmission with mortality rates reaching above 30% for untreated subsp. *tularensis* infections. Typhoidal tularemia is a rare form of tularemia with septicemia without ulcers or enlarged lymph nodes. Ulceroglandular tularemia is rarely lethal, even when it is the result of a subsp. *tularensis* infection [1, 6].

1.1.2 Biowarfare- and bioterrorism-agent – nefarious uses for F. tularensis

Due to the low infectious dose and potentially high mortality rate, *F. tularensis* has long been a biowarfare agent. It was used for this nefarious purpose by Japan already during the 1930's and during the cold war stockpiled by both the US and Soviet Union. Moreover, the US military also developed weapons for the aerosol dissemination of the bacterium. After the 2001 terrorist attacks in the United States there was an increased concern about biosecurity, including the possibility that *F. tularensis* may be used as a bioterror agent. The possible bioterror-threat caused the American Center for Disease Control and prevention (CDC) to classify *F. tularensis* as a Tier 1 Select Agent – a classification reserved for the group of agents and toxins deemed most likely of being deliberately misused with devastating effects

with regard to the number of casualties/public health and safety, or effects to society by way of damaging economy, infrastructure or public confidence.

To date there is no licensed vaccine against tularemia but the Live Vaccine Strain – LVS, an attenuated subsp. *holarctica* strain, is used to vaccinate laboratory personnel. It is also extensively used for research as it is attenuated in humans but retains its virulence in mice. Concerns about efficacy and safety, especially for immunocompromised people, along with an unclear mechanism of protection, have prevented LVS from becoming licensed for use as a vaccine for the general population [7]. Substantial research efforts have therefore been made to better understand *F. tularensis* with the ultimately goal being the development of a new vaccine against this agent.

1.1.3 Intracellular niche – phagosomal escape

F. tularensis is capable of infecting numerous cell types and belongs to a group of pathogens with the ability to infect professional phagocytes like macrophages [8], one of the most important cell-types responsible for eliminating invading pathogens [9]. Pathogens seeking refuge in these professional 'killer cells' have evolved different strategies of survival within this environment designed for killing pathogens. Phagocytes will normally engulf – phagocytose – pathogens forming a compartment known as a phagosome around them. This phagosome becomes acidified and eventually fuses with a lysosome to form a very hostile compartment known as a phago-lysosome, where the pathogens are subsequently destroyed [10]. Intracellular pathogens have evolved different strategies for evading this deadly pathway in macrophages. Some, like *Mycobacterium tuberculosis*, modify the phagosome to prevent phagosomal-lysosomal fusion and create a favorable niche in which to replicate [10]. Others like *Listeria monocytogenes*, *Burkholderia pseudomallei* and indeed *F. tularensis,* escape the phagosome prior to phagosomal-lysosomal fusion and go on to replicate within the cell cytosol [4, 11–13]. The mechanisms of this escape differ between pathogens; *L. monocytogenes* secretes listerolysin O, a pore-forming toxin critical for escape through degradation of the phagosome [11], *B. pseudomallei* relies on BopA through an as of yet unknown mechanism [12].

F. tularensis relies on the genes of the *Francisella Pathogenicity Island* – FPI for escape from the phagosome, by mechanisms as of yet unknown. Escape from the phagosome occurs within, at most, a few hours. *F. tularensis* is released into the cytosol along with bacterial components and DNA. Once in the cytosol *F. tularensis* proceeds to multiply massively. When

sufficient levels of bacterial component are present, assembly of the AIM2 inflammasome is triggered. The actions of the AIM2 inflammasome eventually lead to host cell death through pyroptosis, thus releasing *F. tularensis* into the extracellular environment and enabling spread to additional cells [13]. An overview of the intracellular lifestyle of *F. tularensis* is depicted in (Fig. 1)

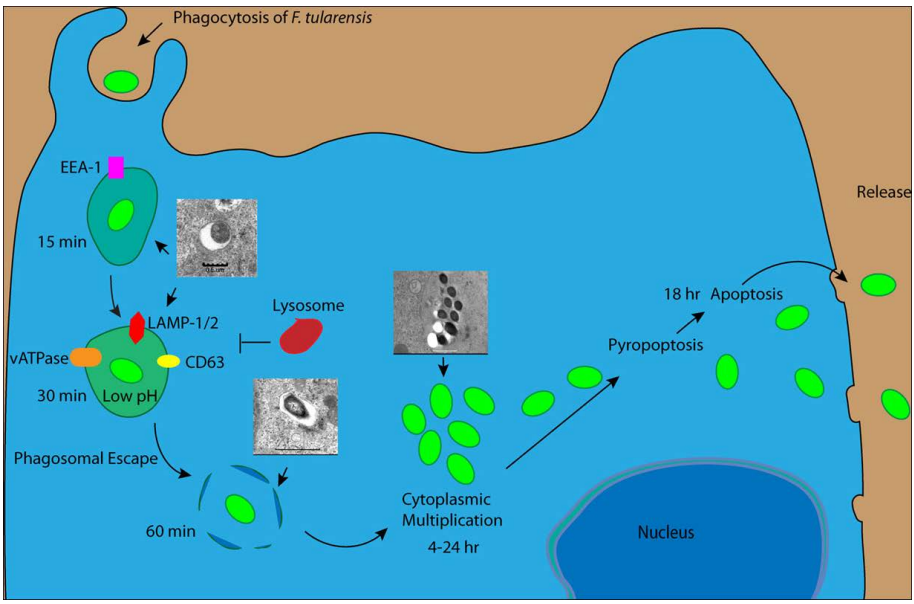

Figure 1: An overview of the intracellular lifestyle of *F. tularensis* showing phagocytosis, phagosome maturation with important phagosomal markers, phagosomal escape, bacterial multiplication and release from the host cell. Figure from Asare *et al.* [13]

1.2 The necessity and danger of iron and oxygen

For the first billion years of life on Earth, iron, the fourth most abundant element in the Earth's crust was much more readily available than it is now, simply because oxygen was absent. During this time of freely available iron, it and another abundant element, sulfur, were incorporated into many early enzymes in the form of iron-sulfur (Fe-S) clusters. The ability of iron to change between the ferrous state (Fe^{2+}) and the ferric (Fe^{3+}) state gave enzymes the ability to transfer electrons. This and other chemical properties of iron made it a versatile element which was incorporated into many proteins. Iron thus became an integral part of many cellular functions and metabolic pathways, ranging from DNA synthesis to glycolysis. Further on in Earth's history, oxygen became abundant in the Earth's atmosphere through

the action of photosynthetic organisms. This introduction of molecular oxygen changed the availability of iron dramatically by switching the predominant form of iron from the relatively soluble ferrous state to the extremely insoluble ferric state. What had once been a readily available nutrient, upon which life had by now become almost universally dependent, was now a scarce commodity. The frequent use of different forms of Fe-S clusters now became a burden, and a threat. The threat was posed by the same culprit as before – oxygen [14, 15].

The chemical properties of molecular oxygen (0_2) predispose it to reduction to water with the formation of reactive intermediates, so called Reactive Oxygen Species (ROS). These intermediates are prone to react with other molecules and in doing so they disrupt their functions [16]. The intermediates, which will be described in more detail in section 1.2.2.2, are indiscriminant and subsequently disrupt the function of a wide array of vital cellular components, such as DNA, lipids, and proteins. Moreover, ROS in combination with iron can become an extremely toxic cocktail, potentially devastating for aerobic life, due to a chemical reaction known as the Fenton reaction. In this reaction ferrous iron reduces hydrogen peroxide (H_2O_2) – one of the reactive intermediates of oxygen reduction – and causes the formation of the hydroxyl radical ($HO^{\cdot}$), a ROS of an extraordinarily reactive, and therefore dangerous, nature [14] (Fig. 2). The effect of the emergence of oxygen in the Earth's atmosphere was thus two-fold – it reduced the bioavailability of iron and rendered it a potentially lethal element [14, 15]. In this thesis, I have focused on the challenge this poses to bacteria with a special focus on the bacterium *F. tularensis* with regard to iron homeostasis (section 1.2.1), and oxidative defense systems (section 1.2.2).

1.2.1 Iron acquisition – battling nutritional immunity

Virtually all forms of life on Earth have an absolute requirement for iron [14], *F. tularensis* being no exception [17]. In the host-pathogen setting, iron-uptake is a veritable war, with both sides trying to gain access to and to control this precious, but dangerous commodity. Like most organisms on earth, *F. tularensis* requires iron for survival and growth, a fact complicated by the host's nutritional immunity - the efforts of the host to keep nutrients, in this case iron, unavailable to pathogens. This sequestration of iron is achieved by binding it tightly to molecules such as transferrin and lactoferrin [10, 18, 19]. As other pathogens, *F. tularensis* has evolved strategies to circumvent the iron-restriction posed by the host.

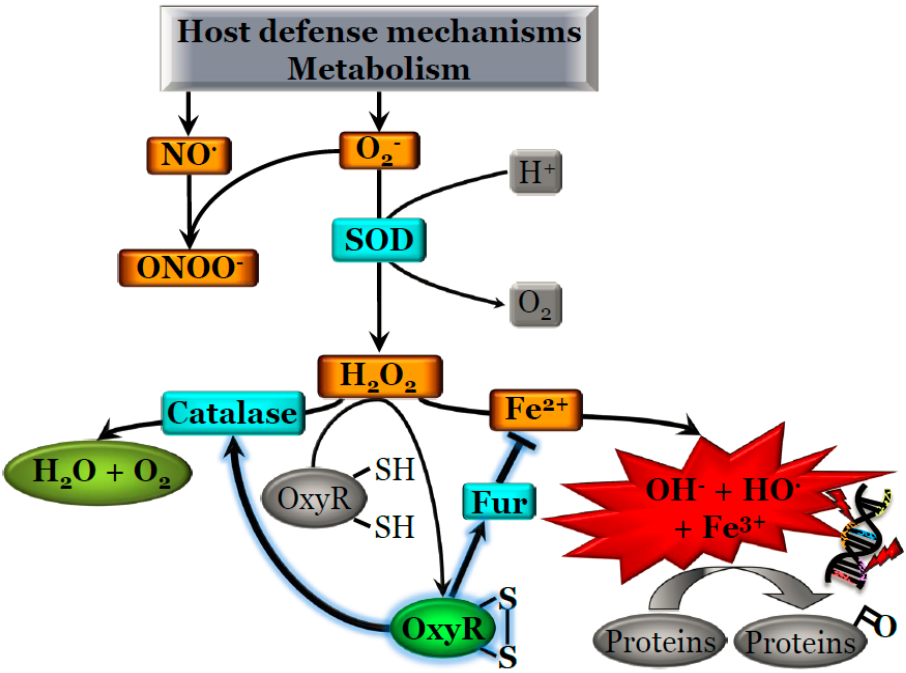

Figure 2: A schematic overview of the ROS central to this thesis, their relation to each other, and the actions of important enzymes. Also depicted are: the Fenton reaction, the H_2O_2-mediated activation of OxyR and the actions of OxyR on H_2O_2 degradation and on the Fenton reaction.

Bacteria typically use several strategies for securing sufficient uptake of this hard to access element. Genomically, this redundancy is seen as the presence of genes encoding several different iron uptake systems. These systems rely heavily on the piracy of iron from of iron-containing molecules, e.g. transferrin and lactoferrin, either by binding to receptors at the bacterial surface, or by the use of siderophores - iron scavengers with affinities for iron that supersede even those of the host iron-sequestration proteins – thus allowing the pathogens to steal iron from the very proteins meant to keep them safely out of reach. Bacterial strategies also involve the lysis of erythrocytes to access the iron-containing hemoglobin and heme therein, others rely on uptake of Fe^{2+} through the *feo* system either under low-oxygen pressure, when Fe^{2+} is more readily available, or through the secretion of ferric reductases that convert Fe^{3+} to Fe^{2+} [10, 14, 18–20]. Utilizing heme as a source of iron has great potential for pathogens since 80% of the host's iron-pool is found in heme, whereas not even 1% is bound to transferrin [10].

F. tularensis differs from the norm by harboring unusually few genes homologous to iron uptake systems of other bacteria. The known iron-uptake related genes of *F. tularensis* are the genes of the *Francisella siderophore locus* (*fslA-F)* [21, 22], the *ferrous iron transporter B (feoB)* [23] and the *Fe utilization protein A* (*fupA*) [24]. Iron uptake in *F. tularensis* becomes even more enigmatic when considering that it has the ability to grow with heme as the sole iron source but does not encode any heme-uptake systems known from other Gram-negative bacteria [25]. All other systems described so far rely on a specific outer membrane receptor, usually powered by the TonB complex, a periplasmic transport protein and an ABC transport system for the final transport across the inner membrane [26]. *F. tularensis* does, however, not encode any homologues of TonB or the other constituents of the known heme-internalization machineries but instead relies on the presence of the systems it uses for acquisition of other forms of iron, namely the siderophore- and Feo-systems and FupA [25].

Below follows an overview of the known iron-uptake systems of *F. tularensis*.

1.2.1.1 FupA vs FupA/B - Genomic subspecies differences

Despite originating from a virulent subsp. *holarctica* strain, LVS has become attenuated through repeated passage on media [7]. LVS differs from other *holarctica* strains by a number of genomic deletions. One such deletion affects the *fupA* gene, which in SCHU S4 exists as a separate gene adjacent to the *fupB* gene, but due to a partial deletion of both genes exists as a *fupA/B* gene-fusion in LVS (Fig. 3). FupA and the FupA/B fusion protein are both located to the outer membrane [27, 28]. We have previously shown that FupA serves a function in the iron-uptake in SCHU S4, by both siderophore-dependent and independent mechanisms and find it likely that it serves this function in all strains with an intact *fupA* gene [24]. The siderophore-independent mechanism of FupA relates both to uptake of ferrous iron [29] and to iron homeostasis [24]. Like FupA, the FupA/B fusion protein is involved in uptake of both ferrous iron [28, 29] and siderophore-bound ferric iron [30]. Deletion of *fupA/B* in LVS or deletion of *fupA* in SCHU S4 results in lower intracellular replication as well as a strong attenuation in mice [24, 30, 31]. In paper I, we investigated the impact the gene-fusion of *fupA* and *fupB* has on iron-uptake and content and tolerance to oxidative stress in LVS.

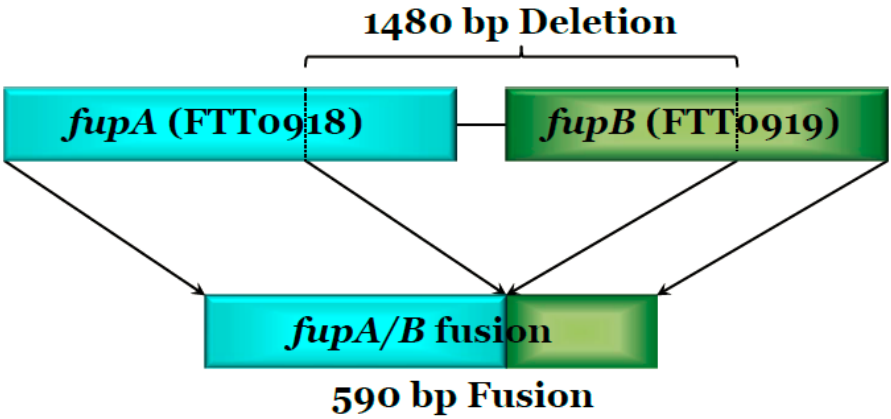

Figure 3: A schematic view of the separate *fupA* and *fupB* genes of virulent *F. tularensis* strains and the *fupA/B* gene fusion in LVS.

1.2.1.2 The ferrous iron transporter – FeoB

FeoB is a commonly used bacterial transporter of ferrous iron. Iron transport through FeoB seems to be of particular importance in low oxygen conditions, when ferrous iron dominates over ferric iron. The presence of a FeoB has been shown to be important for colonization of the gut and virulence of several pathogens, including, but not restricted to; *E. coli, Helicobacter pylori, Shigella flexneri* and *Legionella pneumophila* [32]. FeoB is also important for virulence of *F. tularensis*, since a deletion of *feoB* results in lower bacterial burdens in organs of infected mice [23] and a partial deletion of the gene has also been seen to reduce virulence in mice [33]. The FeoB-system is typically encoded in an operon containing *feoA, feoB* and *feoC*, but some systems lack *feoA*, *feoC* or even both. FeoB forms a channel in the inner membrane of Gram-negative bacteria and transports iron from the periplasm into the cytoplasm [32]. In *F. tularensis*, transport of Fe^{2+} across the outer membrane is mediated by FupA in virulent strains and FupA/B in LVS (see section 1.2.1.1) [28, 29]. Through its action as a transporter of ferrous iron from the periplasm to the cytosol FeoB is also required for iron-uptake from heme, a feature unique for *F. tularensis* [25].

1.2.1.3 Siderophores – ferric iron scavengers

Siderophores are low molecular weight iron chelators with both high specificity and affinity to the poorly soluble ferric iron, which predominates in oxygenated environments. Usage of siderophores is a common strategy employed to access ferric iron and siderophores are produced and secreted

by many bacteria as well as yeasts, fungi, and even plants, upon iron starvation [14, 34]. As mentioned previously, siderophores are an important part of the armamentarium of bacteria seeking to survive within a host since they bind iron with an even greater affinity than the iron-sequestration proteins of the host. Many pathogens therefore utilize siderophores as weapons in the war of iron, and over 500 different siderophores have been identified [35]. This strategy gives pathogens the ability to plunder iron from host sources, an opportunity used by e.g. *Pseudomonas aeruginosa* and *Staphylococcus aureus*, which use siderophores to sequester iron from transferrin, and in the case of *P. aeruginosa*, also from lactoferrin [36]. The utilization of siderophores therefore provides a means by which pathogens may circumvent the iron-restriction imposed by the host, and thereby overcome the host's nutritional immunity.

The *Francisella* siderophore system components are encoded by the *Francisella siderophore locus* (*fsl*), which is an operon consisting of six genes – *fslA-F*. FslA is responsible for siderophore synthesis, FslB, FslC and FslD are predicted to play roles in synthesis or uptake of the siderophore, and FslE serves as a siderophore receptor for virulent strains [22, 30, 37]. The *fslE* gene and its paralog *fupA*, are both unique to *Francisella*, and are predicted to form β-barrels in the outer membrane of the virulent SCHU S4 strain [29]. As discussed in 1.2.1.1, both the *fupA* gene of SCHU S4 and the *fupA/B* fusion gene of LVS play roles in uptake of siderophore-bound iron [24, 30]. In Gram-negative bacteria, siderophore systems classically involve a TonB-dependent outer-membrane receptor, a periplasmic binding-protein and an ABC-transporter [20]. *Francisella* does, however, not encode a TonB homologue and the exact mechanism by which it takes up siderophore-bound iron has yet to be clarified [22, 30].

The *fslA, B, C, D* and *E* genes are up-regulated in intracellular *F. tularensis*, indicating a siderophore-mediated iron-uptake in this environment [38]. This iron uptake mechanism is however, not crucial in these settings as is evident by the capacity for intra-macrophage growth, as well as full virulence in mice, seen when deleting *fslA* in LVS [21, 33] or either *fslA* or *fslE* in SCHU S4 [24, 29]. This stands in contrast to the situation in the intracellular pathogens *Bacillus anthracis* [39] and *Mycobacterium tuberculosis* [40, 41], which require siderophores for normal growth in macrophages and virulence in animal models. Iron is nevertheless important in these settings for *F. tularensis*, as is revealed when deleting genes of the *fsl* operon in concert with other iron-uptake related genes. Deletion of both *fslE* and *fupA* in SCHU S4 resulted in a reduced capacity for intra-macrophage growth and a marked attenuation in mice [29]. Further, the deletion of both *fslA* and *feoB* in LVS also resulted in a strain attenuated for growth in macrophages and

virulence in mice [33]. The results for both SCHU S4 and LVS show the flexibility of *F. tularensis,* in terms of the form of iron it uses, as the presence of either siderophore or ferrous iron uptake systems, was enough for intra-macrophage replication and virulence in mice. The loss of both systems was, however, not tolerated.

Collectively these results clearly show the importance of iron uptake for the success of *F. tularensis* in both macrophages and hosts, and also show the overlap between systems, allowing one system to step in when another fails.

1.2.1.4 The Ferric up-take regulator – Fur

Regulation of iron-uptake related genes is of utmost importance in order to avoid an overload of iron that could drive the Fenton reaction and cause oxidative damage. Genes involved in iron-uptake are commonly regulated by the *ferric uptake regulator* (Fur). It functions as a homodimer and acts as a positive repressor, meaning that it can only repress gene expression if it has access to its co-repressor, in this case ferrous iron. When bound to iron, the Fur dimer will bind DNA at specific sequences known as Fur-boxes, which are located up-stream of the controlled genes. Binding of the Fur-Fe^{2+} complex blocks the promotor of the controlled gene, making it inaccessible to RNA-polymerase (RNAP), which is therefore unable to bind and initiate transcription. When iron levels are low, Fe^{2+} releases from Fur, which then dissociates from the Fur-box, making the promoter accessible for RNAP, which then initiates transcription. Gene-expression of iron uptake related genes is thereby prevented when iron levels are high and permitted when the levels are low [14, 42, 43]. Both the *fur* gene and a Fur box are found up-stream of the *fsl* operon of *F. tularensis*. In *E. coli*, expression of the *fur* gene is influenced by the internal redox state. Under H_2O_2 induced oxidative stress, the transcription factor OxyR is activated and in turn induces the transcription of *fur* (Fig. 2) [14].

1.2.2 The deadly chemistry of oxygen – and the antioxidant systems of bacteria

Oxidative stress is the imbalance caused by the presence of more reactive oxygen species (ROS) than the defense systems – antioxidants – can disarm. The excess ROS react with vital macromolecules such as proteins, lipids and DNA, thereby disrupting their functions. ROS are produced both during normal aerobic metabolism and by hosts as a defense mechanism during infection. Host phagocytes increase their production of ROS, a process known as an 'oxidative burst', as a means of killing phagocytosed pathogens. There is therefore a constant need for defense mechanisms to combat the

ever-present ROS produced during normal metabolism but an even greater need for pathogens intending to survive the onslaught of ROS experienced within a host [44]. These systems include, but are not restricted to, the superoxide dismutases (SODs), glutathione peroxidases, alkyl-hydroperoxide reductases and catalases. This neutralization of host ROS also functions to restrict macrophage signaling and cytokine production to minimize the response to the presence of the pathogen [45].

1.2.2.1 Respiratory burst - chemical warfare at the cellular level

Phagocytes, such as the macrophages favored by *F. tularensis*, constitute a hostile environment rich in ROS, which are produced in a process known as a 'respiratory burst', as a means of killing phagocytosed pathogens. This respiratory burst is mediated by the NADPH oxidase, or phox, as it is also known. This membrane-bound protein catalyses the reduction of vast amounts of O_2 to O_2^- upon phagocyte activation. The O_2^- is dismutated to H_2O_2, which in turn may oxidize Cl^- to the bactericidal compound hypochlorite. The membrane spanning properties of phox allows the directed production of O_2^- into membrane-bound cellular compartments, like the phagosome [46]. The respiratory burst is a vital component of the defense against invading microorganisms, as is underlined by the hyper-susceptibility to infection seen in individuals with a defective phox enzyme, a condition called chronic granulomatous disease (CGD) [47]. Phox is activated by binding of e.g. bacteria, fungi and soluble inflammatory mediators to receptors at the cell surface [48]. *F. tularensis* has the ability to inhibit the function of phox [49] but nevertheless, mice lacking phox ($p47^{phox-/-}$) are extremely susceptible to infection with LVS [50]

1.2.2.2 Reactive Oxygen Species – actions and detoxification

Reactive oxygen species all have in common that they all, as the name implies, contain oxygen and are highly reactive. The reactiveness of ROS is caused by the chemical structure of these compounds, most of which are radicals. Radicals contain unpaired electrons which cause them to readily react with other molecules, as if they were to find a partner for their lonely electron. ROS therefore rapidly react with molecules in their surroundings, including vital macromolecules such as proteins, lipids and DNA, thereby disrupting their functions [16]. Briefly presented below are the properties of ROS most important to this thesis and how the bacteria can neutralize them.

Superoxide (O_2^-) and superoxide dismutases (SODs)

The superoxide radical O_2^- has the ability to act both as an oxidant and as a reductant but the reducing environment of the cellular cytosol makes it act as an oxidant. Being charged, O_2^- cannot readily pass over membranes, but can nevertheless traverse through cellular ion channels. One of the primary targets of O_2^- is the iron-sulfur cluster (4Fe4S) of a class of enzymes involved in e.g. the citric acid cycle of aerobic metabolism. Once oxidized, these iron-sulfur clusters decompose and release free iron, which can in turn drive the Fenton reaction [16, 51]. O_2^- can also react with nitric oxide (NO) to yield the powerful oxidant peroxynitrite ($ONOO^-$). However, in the presence of SODs, which catalyzes the dismutation of O_2^-, the anion is readily converted to H_2O_2 and oxygen. It should be emphasized that the concentration of, and distances between the reactants and detoxifying enzymes as well as the surrounding conditions e.g pH greatly influence the chemistry that takes place [52].

SODs are categorized based on their metal cofactor into MnSOD (SodA), FeSOD (SodB) and CuZnSOD (SodC), of which *F. tularensis* encodes the latter two. SodB (FeSOD) is important for oxidative stress defense as it binds iron, thereby limiting the amount of Fe^{2+} available for the Fenton reaction, and, dismutating O_2^- thus preventing it from reacting with DNA, lipids and proteins as well as preventing O_2^- from reacting with NO to produce $ONOO^-$ (Fig. 2). The importance of keeping the levels of O_2^- low are emphasized by the essential role of SodB for LVS, making it impossible to create a deletion mutant. A mutant with reduced SodB levels (*sodB*$_{Ft}$) was, however constructed and displayed increased susceptibility to O_2^- and H_2O_2 as well as reduced virulence in mice [53]. SodC (CuZnSOD), on the other hand, is non-essential for *F. tularensis* survival but its O_2^- detoxifying activity nonetheless serves an important function for intra-macrophage survival and full virulence in mice. SodB and SodC have also been suggested to have cooperative roles, rather than redundant functions, as has been reported in other bacterial species. This is supported by the phenotype of a double mutant with reduced expression of *sodB* and a deletion of *sodC*. This mutant displayed an increased sensitivity to ROS, decreased survival in activated macrophages and attenuation in mice, as compared to the single *sodB*$_{Ft}$ or Δ*sodC* mutants [53, 54].

Hydrogen peroxide (H_2O_2) and catalase

H_2O_2 is a naturally occurring compound, formed not only through the dismutation of O_2^- as shown above, but also e.g. through reactions between sulfur and oxygen under certain conditions. Apart from being formed within organisms, H_2O_2 is also a threat from outside as many organisms, bacteria as well as plants and animals, actively secrete it as a weapon. H_2O_2 is small,

relatively stable and uncharged, and can therefore pass through biological membranes. It is only a weak oxidant, but in combination with ferrous iron (Fe^{2+}), it drives the Fenton reaction giving rise to $HO^{\cdot}$, which is as stated, an extremely potent oxidant. $HO^{\cdot}$ will react with, and thereby disrupt the function of, most organic compounds, including vital cellular macromolecules, as soon as it encounters them [14, 55]. H_2O_2toxicity arises, in part from its reaction with DNA-associated iron, which gives rise to DNA damage through the Fenton reaction; but predominantly from the inactivation of iron-containing enzymes. Such enzymes are important in several metabolic pathways and their inactivation leads to disruption of growth [55]. Catalase has an important role in preventing the devastating Fenton reaction to occur since this enzyme converts H_2O_2 to oxygen and water (Fig. 2) [42, 44].

The *Francisella* catalase is encoded by the *katG* gene and is essential for tolerance to H_2O_2 and is also important for protection against $ONOO^-$ [56]. *In vitro* studies comparing the subsp. *tularensis* strain SCHU S4 to the subsp. *holarctica* strains FSC200 and LVS have shown SCHU S4 to possess a significantly higher resistance to oxidative stress mediated by H_2O_2 than the subsp. *holarctica* strains. Seemingly paradoxically, the SCHU S4 strain is less dependent on catalase for virulence than the *holarctica* strains, despite its higher tolerance for H_2O_2, indicating the presence of additional or alternative approaches for avoiding radical formation through the Fenton reaction [57]. In paper I we investigated the basis for this apparent paradox and demonstrated that the basis lay in a subspecies difference in iron homeostasis.

Peroxynitrite and AhpC

$ONOO^-$ is a highly reactive and bactericidal ROS. Its protonated form, peroxynitrous acid, has the ability to pass through lipid bilayers, thus allowing it both to pass from the extracellular space into cells and out from one cell to another [52, 58]. The high reactivity and toxicity of $ONOO^-$ is caused by its ready conversion to the radicals $HO^{\cdot}$, $NO_2^{\cdot}$ and $CO_3^{\cdot}$. $ONOO^-$ is mainly generated through the reaction of NO with O_2^- [58, 59], which in turn are generated through the actions of the nitric oxide synthase NOS and phox (see section 1.2.2.1) , respectively. There are three forms of NOS: endothelial (eNOS), neuronal (nNOS) and inducible (iNOS) [60]. Production of NO through iNOS is induced as a response to macrophage activation by interferon-gamma (IFN-γ) and is important for macrophage killing of several intracellular pathogens, including *Mycobacterium tuberculosis* and *Salmonella typhimurium* [61]. $ONOO^-$ has a vital role in the IFN-γ induced killing of LVS by murine macrophages [62].

AhpC – alkyl hydroperoxide reductase - belongs to the peroxiredoxin family of antioxidant enzymes, and is ubiquitously found in nature, having been identified in organisms ranging from bacteria, to yeasts, mammals and plants. Peroxiredoxins degrade peroxides and in some cases also $ONOO^-$, a capacity identified in certain bacterial, as well as yeast and mammalian peroxiredoxins. [63]. AhpC has been shown to decompose $ONOO^-$ in e.g. *Salmonella typhimurium, Mycobacterium tuberculosis* and *Helicobacter pylori* [64]. AhpC has also recently been shown to be important for the detoxification of O_2^- and $ONOO^-$ in SCHU S4, and to be important for full virulence of this strain in mice [56].

1.3 Transcription factors and defense against ROS.

Transcriptional regulation is of great importance to bacteria as it allows them to adapt their gene-expression to suit their needs and current surroundings, e.g. activating or repressing expression of genes important during nutrient deficiency, or other forms of stress such as cold, heat, osmotic or oxidative stress. Some transcriptional interactions, important for basic cellular functions such as amino acid synthesis, iron transport and the SOS response, are conserved between bacterial species, but the majority of factors are more variable, mostly depending on the environmental niche occupied by different organisms, a plasticity important for evolutionary adaptation to new challenges. Organisms with similar life-styles thus often utilize similar regulatory networks for the regulation of orthologous genes, while organisms with different habitats display different networks [65, 66].

Attempting to shed light on the relatively unexplored regulation of oxidative stress defense mechanisms in *F. tularensis,* this thesis has explored the functions of two transcription factors, namely MglA and OxyR. These transcription factors, which will be discussed in more detail below, are involved in oxidative stress defenses in *F. novicida*, in the case of MglA [67], and in several different bacterial species in the case of OxyR [68–70].

1.3.1 MglA

The global transcription factor MglA (Macrophage growth locus A) plays a complex role in gene-regulation in *F. tularensis*. It forms a heterodimer with SspA, associates to the RNA-polymerase (RNAP) and PigR, and binds DNA at the promoter-sequences of virtually all *F. tularensis* genes, despite only regulating the transcription of a subset of them [71–73]. The specificity of the regulation is mediated by a 7 base pair DNA sequence called PRE (PigR response element), which is found in the promoter regions of regulated genes [71] (Fig. 4).

In *Francisella novicida*, the regulatory actions of the three transcription factors in the MglA-SspA-PigR complex are necessary for the regulation of a multitude of genes, including the genes of the *Francisella* pathogenicity island (FPI), as well as approximately 100 genes outside the FPI [74]. The *F. novicida* MglA has been shown to induce several proteins involved in oxidative stress response, including glutathione synthetase, peroxiredoxins, glutaredoxin and thioredoxin, but also to repress catalase. The importance of MglA for oxidative stress response in *F. novicida* was also evident in a *mglA* deletion mutant in *F. novicida*, which had reduced amounts of oxidative stress related proteins in general, but an increased amount of catalase, and was shown to be more sensitive to O_2^-, and more resistant to H_2O_2, than the wt [67].

MglA-SspA-PigR function as a complex also in *F. tularensis* [71, 73, 75] where its regulatory actions are required for phagosomal escape and replication in macrophages and are therefore necessary for virulence [72, 76]. The majority of the available data concerning gene expression affected by MglA stems from work performed in *F. novicida*, and the gene expression data presented in paper II therefore provides a valuable addition by providing data from *F. tularensis*. The work presented in this paper also provides further insight into the importance of MglA by showing its necessity for adaptation to oxygen-rich environments [77].

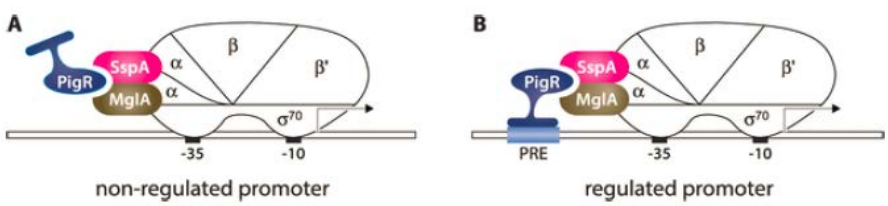

Figure 4: Model of gene-regulation by the MglA-SspA-PigR complex, and the conformation of PigR at A) a non-regulated promoter and B) a regulated promoter with PigR binding the PigR response element (PRE). Figure from Ramsey *et al.* [71].

1.3.2 OxyR

OxyR is a well documented and widely spread regulator of oxidative stress response, which is found in e.g. *E. coli*, *Salmonella enterica* [68] *Haemophilus influenzae* [69] and *Moraxella catarrhalis* [70]. In *E. coli,* OxyR regulates the expression of a large group of genes, including *katG*, encoding catalase, and *ahpCF*, encoding alkyl hydroperoxide reductase. The

E. coli OxyR is involved in the protection against several stresses; including H_2O_2, heat, and lipid peroxidation-mediated damage. The OxyR of *H. influenzae* is important for bacterial fitness and for protection against peroxides. It regulates the expression of several genes in response to ROS, including *hktE*, encoding catalase and *pgdX*, encoding a fused peroxiredoxin/glutaredoxin [68]. In *M. catarrhalis*, OxyR regulates the expression of *katA*, encoding catalase, as well as *ahpC* and *ahpF*, which encode alkylhydroperoxide reductase, and is important for tolerance to H_2O_2. *F. tularensis* harbors an OxyR homologue, which shares 36 % protein sequence identity to the *E. coli* OxyR, but the function of which has not yet been described.

OxyR functions as a homotetramer in which each subunit contains two critical cysteines. Depending on their redox status, these cysteines will, or will not, form disulfide bridges, causing a conformational change in the tetramer complex (Fig. 2). This change in conformation changes the affinity for binding motifs upstream of regulated genes, the expression of which will thus depend on the redox state of OxyR and ultimately of the redox state of the organism [69, 78]. In *E. coli*, OxyR plays a major role in the defense against the oxidative and nitrosative stress since it is activated by both H_2O_2 and nitrosylating agents [79] and regulates the expression of genes involved in the protection against both types of stress [80, 81]. The effects of H_2O_2 are combated on two fronts; by OxyR driving both the expression of catalase, which degrades H_2O_2 to oxygen and water, and the expression of the Ferric uptake regulator (Fur). Fur, in turn, down-regulates the expression of genes involved in iron uptake, thus limiting the amount of available iron with which H_2O_2 can combine in the Fenton reaction (Fig. 2) [43, 82].
In paper III, we show that OxyR plays an important role in oxidative stress defense in *F. tularensis*, and we also shed light on the interplay between OxyR and catalase in ROS tolerance.

2 Aims of the thesis

This thesis was focused on the investigation of the iron status and oxidative stress response of *F. tularensis*, thereby identifying key components for regulation of the *F. tularensis* iron content, adaptation to oxygen rich environments, and defense against ROS.

3 Methodological considerations

This section is intended to give a better understanding of the Results and Discussion later in this thesis by introducing and discussing the methods chosen for the work presented, the reasoning behind them as well as their pros and cons.

3.1 Mutagenesis

The work presented in this thesis has been performed using various strains and mutants of *F. tularensis* subs. *holarctica* and *tularensis*. Mutagenesis has been performed by in-frame deletion as described by Golovliov *et al.* [83] and discussed below.

3.1.1 In-frame deletion

When constructing deletion mutants, we have chosen to create them in-frame, thereby deleting the entire gene of interest from the genome of the strain and not leaving any foreign DNA in the mutant, in order to avoid the polar effects that otherwise may arise from insertion-based mutagenesis. In-frame deletion mutagenesis requires the creation of a suicide vector containing regions homologous to the genetic sequence up- and down-stream of the gene to be deleted (flanking regions). These sequences are typically 1000-1500 bp long and are amplified by PCR to have sticky ends where they would otherwise connect to the gene to be deleted. The sticky ends are joined in a second PCR, creating the final deletion construct - a sequence homologous to the regions preceding and following the gene to be deleted, but lacking the sequence of the actual gene. The construct is thereafter enzymatically digested and ligated into a suicide vector, which is unable to replicate in *F. tularensis*. The vector is transformed into *Escherichia coli* S-17-λpir, which is used to transfer the vector to *F. tularensis* through conjugal mating. Since the vector lacks an origin of replication functional in *F. tularensis* it can only be maintained in *F. tularensis* if it integrates into the genome by recombination of the deletion construct and the homologous regions of the *F. tularensis* genome. The resulting transconjugants are selected by incubation on media containing an antibiotic for which the vector confers resistance, and polymyxin for counter-selection of the donor *E. coli*. This ensures that survival is only possible for *F. tularensis*, and only if it harbors the vector. To force a second recombination event, where the suicide vector recombines out of the *F. tularensis* genome, sucrose is added to the growth media. The suicide vector contains the *Bacillus subtilis* gene *sacB*, which encodes an enzyme that converts sucrose

to a high-molecular-weight compound lethal to Gram-negative bacteria. The only clones left will thus be *F. tularensis* deleted for the gene of interest and no longer harboring the suicide vector [83, 84].

3.1.2 Complementation

Complementation is the process where a gene is introduced to a strain from which is absent, either as a consequence of natural processes where the gene has been lost/truncated/inactivated in a strain, or caused by deliberate deletion. Complementation is performed to ensure that re-introduction of the gene restores the phenotype of the strain or deletion mutant to that of strains with the intact, functional gene, thereby confirming the phenotype as being caused by the deletion. Complementation can be performed in *cis* or in *trans*, the difference being how and where the gene is re-introduced. *Cis*-complementation denotes a re-introduction of the gene into the chromosome of the organism, whereas complementation *in trans* relies on re-introduction on a plasmid – a separate genomic structure, which does not integrate into the chromosome of the organism. Both methods require the amplification of the gene of interest by PCR and the cloning into a vector. For complementation *in trans*, the vector is then introduced into the deletion mutant by cryotransformation and the complementation is complete. Complementation in *cis* is considerably more complex and time-consuming, requiring transformation of the vector into an *E. coli* strain, chromosomal integration, and excision of the plasmid using the same arduous procedure described in section 3.1.1.

In Paper I we constructed a *cis*-complemented strain designated FSC697 – an LVS strain complemented with the *FTT0918* gene (*fupA*) which is normally present as a fusion of *FTT0918* and *FTT0919* (*fupB*) in LVS but is found as a separate gene (as is *fupB*) in other *F. tularensis* strains of both subsp. *holarctica* and *tularensis* origin.

The complementation of LVS with the full-length *fupA* gene, was assumed to lead to an increased virulence as Salomonsson *et al.* showed this to be the case when complementing another LVS strain with this gene [85]. Complementing a pathogen with properties not found in the original strain, so called gain-of-function experiments, is a form of "dual-use" research – research can be used in the aid of science and society but also for other, nefarious purposes. Such research therefore always raises important ethical issues concerning the safety from a bio-security standpoint. Will the strain or procedure be a potential source of bio-terrorism? Is the need for the strain or procedure really so great that it outweighs the risk of aiding those who may use it for acts of terrorism?

The risk of dual-use of the complementation made in paper I was however deemed non-existent, since the strain used was the attenuated LVS strain. Even if complementation with full-length *fupA* was to restore its lost virulence, the introduction of this single gene would not be expected to cause virulence to reach that of the naturally occurring virulent strains of subsp. *tularensis*. The complementation therefore did not produce a strain expected to be more virulent than the naturally occurring strains. Any potential bio-terrorists wishing to "improve" the virulence or other properties of *F. tularensis,* and in possession of the knowledge and equipment to do so, would presumably base their efforts on a highly virulent strain of subsp. *F. tularensis*, not on a less virulent strain of subsp. *holarctica*, and most certainly not on the attenuated LVS strain. Neither was the procedure in any way novel, but is long since established and published and therefore openly available to all who wish to read how to perform it. The production of the complemented LVS strain was therefore not deemed a risk for dual-use.

In Paper II we constructed a *trans*-complemented strain designated FUU301 - a strain where the *mglA* gene had been deleted and was re-introduced – complemented- by introduction of a plasmid carrying the *mglA* gene.

3.2 Growth media and environmental conditions

In the three papers presented, we have used different growth media and growth conditions in order to investigate different aspects of iron metabolism and tolerance to oxidative stress. We have used media with and without iron, both in liquid and solid form, as well as media to indicate release of siderophores. We have further grown bacteria in both atmospheric and sub-atmospheric oxygen concentrations.

3.2.1 Preparation of media

When depleting the bacterial iron pool in order to study siderophore secretion or other effects of iron starvation, it is imperative that the growth media used contain as low levels of iron as possible. Achieving a completely iron-free medium is likely to be impossible since small amounts iron can be found virtually everywhere, but iron levels low enough to be considered to be functionally 'iron-free', in the sense that they retain mere trace amounts of iron - too low to support bacterial growth- is achievable.

Chamberlain's defined medium (CDM) is a synthetic medium widely used for cultivation of *F. tularensis* and was routinely used in papers I-III for growth in liquid media. However, several assays in papers I-III required use of iron-free media and to this end we set out to remove iron from CDM.

When preparing iron-free CDM, it was not sufficient to exclude the $FeSO_4$ normally added to CDM since too much iron was present in other components of this complex medium. The preparation of iron-free CDM instead required active removal of iron, which was accomplished through the addition of Chelex-100, which efficiently chelates all divalent cations, to CDM prepared without iron. The medium was chelated twice for 24 h, each time at 4°C with rotation, to ensure that as much iron as possible was chelated. Chelex-100 was removed by filtration and desired cations, in this case: $ZnCl_2$, $CuSO_4$, $MnCl_2$ and $CaCl_2$, were added back to the medium. $FeSO_4$ was added to the medium when specific concentrations of iron were desired. The chelated medium, designated C-CDM for Chelated-CDM, was stored in plastic bottles to avoid the contamination of iron leaking from glass bottles during storage.

McLeod agar plates (MC plates) are composed of 3.6% (wt/vol) GC agar base, 1% (wt/vol) hemoglobin and 1% (vol/vol) IsoVitaleX and were routinely used for growth on solid medium. In the instances where iron free plates were required, these were prepared by combining C-CDM and 4% GC-agar in a 1:1 ratio, alternatively, the iron chelator deferoxamine (25 mg/ml) was added to C-CDM prior to the addition of GC-agar (DFO plates). For detection of siderophore secretion, plates containing chrome azurol sulfonate (CAS-plates) were prepared by adding CAS-Fe(III)-hexadecyltrimethylammonium solution to the GC-agar prior to addition of C-CDM.

The differences in preparation and definition of iron-free media are a result of methods developing over time with the insights of the problem of residual iron.

3.2.2 Aerobic vs microaerobic growth

F. tularensis was routinely grown at 37°C in 5% CO_2 when on solid media and in 37°C with rotation in normal air, when grown in liquid media. In paper II, we asked if the poor growth of Δ*mglA* could be alleviated by growth in a less oxidizing environment. Accordingly, we incubated the bacteria in an incubator in which the oxygen concentration could be reduced by the addition of N_2 as well as the CO_2 (microaerobic conditions).

When working with a specialized environment, like the microaerobic environment described above, there is always a risk of disturbing that environment when e.g. sampling liquid cultures. The opening of a microaerobic incubator invariably leads to an influx of O_2 and the incubator was therefore opened as little as possible, and was preferentially only used

for one experiment at a time, as to not expose the bacteria within to unwanted levels of O_2 when opening the incubator to sample other experiments. A completely microareobic handling of the bacteria was, however, not achievable since preparation, measurements of optical density (OD), sampling, sample preparation and assays had to be performed in the atmospheric conditions of the normal lab-space. All such breaches of microaerobic incubation were therefore performed as swiftly as possible. These considerations were of particular importance when preparing protein- or RNA-samples for down-stream assays, such as measurement of catalase activity, detection of oxidized proteins and gene expression studies, where it was important that the bacteria did not have time to react in any substantial way to the higher oxygen pressure outside of the incubator. The extent to which the bacteria did have time to react is uncertain but considering our modus operandi, we consider the effects to have been kept to a minimum.

3.3 Iron content and iron scavenging

As described in section 1.2, the intracellular presence of Fe^{2+} is hazardous due to the risk of production of radicals through the Fenton reaction. In paper I and II, we measured the total iron content of different *F. tularensis* strains. In paper II, we also measured the iron content of the culture media in which the strains had been grown (paper II) and investigated if growth in a microaerobic environment had an effect on the internal iron pool by virtue of being a less oxidizing environment with a lower risk of intracellular iron causing the production of radicals through the Fenton reaction. Measurements were performed by use of the ferrozine assay – a colorimetric assay based on the absorbance of light at 562 nm by ferrozine when it is bound to Fe^{2+}. This is a cheap, rapid and reliable method for measuring total iron content of samples [86]. Sample preparation comprises bacterial lysis, release of protein-bound iron (by use of $KMnO_4$) and reduction of Fe^{3+} to Fe^{2+} (by use of ascorbic acid), which is subsequently bound to the ferrozine-containing detection reagent. The reduction of Fe^{3+} allows binding of ferrozine to the total amount of iron present in the sample, not only the ferrous iron. For detection of iron in culture medium, the bacteria were removed by centrifugation and the resulting bacteria-free culture media was mixed with the ferrozine containing detection reagent.

In paper II, we compared the siderophore-secreting abilities of LVS and the Δ*mglA* strain by use of the CAS-plate assay (see section 3.2.1). This is a simple, straight-forward, colorimetric method for detecting secretion of siderophores, which has successfully been used in several previous studies to measure siderophore secretion of *F. tularensis* [21, 24, 87]. Chrome azurol sulphonate (CAS) is a dye which is blue when bound to Fe^{3+} and orange

when not. The removal or iron requires a compound with high affinity for iron, such as a siderophore. The addition of iron-bound CAS to plates therefore results in blue plates which serve as indicators of siderophore secretion, where secretion of siderophores is seen as the formation of orange halos around bacterial colonies. The CAS-plate assay thus provides a simple and reliable assay for the detection of siderophore secretion.

3.4 Stress susceptibility and detection

The studies performed in papers I-III include a set of stress susceptibility assays in which the bacteria are subjected to various compounds causing oxidative stress.

H_2O_2 susceptibility was assessed by incubating bacteria in the absence or presence of H_2O_2 in the culture medium. In paper I-II, the bacteria were thereafter serially diluted and plated to give the read-out in the form of viable counts (VC), which require the enumeration of the colony forming units (CFU) on each plate. This procedure, which has been the standard approach in our lab, is however time–consuming, laborious and produces vast amounts of waste. When optimizing H_2O_2 concentrations to use with the Δ*oxyR*/Δ*katG* strain we therefore tried assessing H_2O_2 susceptibility in a growth assay instead. We found that this approach, which is considerably less time-consuming, laborious and waste-producing, gave a clear read-out which corresponded well to comparisons made with V.C. at different time points (data not shown). Moreover, this approach enabled us to follow the effects of susceptibility over time in a simple, efficient way. In paper III, we therefore opted to alter our approach and to measure H_2O_2 by this new, OD-based method.

In paper I, the catalase assay and H_2O_2 susceptibility assay both use bacteria grown on solid media, either iron-replete MC -plates or iron-depleted DFO-plates. This was in line with the desire to compare effects of high/low bacterial iron content on catalase activity and H_2O_2 susceptibility in different strains of *F. tularensis*.

In paper II, the questions asked, and therefore the approach, was different. Here, we did not focus on the influence of the internal iron pool, but instead on the influence of oxygen pressure. We aimed at determining if the poor growth of Δ*mglA* was a result of a reduced capacity for adaptation to growth in aerobic environments. The decision was therefore made to subject the strain to several rounds of incubation (overnight on MC -plates, overnight in CDM and sub-culturing in CDM) to increase this stress prior to running the catalase and H_2O_2 susceptibility assays. Cultures were grown with rotation to

ensure maximal gas-exchange in the culture media. This regime was established to allow maximal differentiation between the aerobic and microaerobic environments in terms of oxygen pressure, thereby exacerbating the possible stress-induced effects in Δ*mglA*.

In paper III, the focus was to determine the effects of the deletion of the *oxyR* gene in LVS, and where time allowed, the effects of deleting *katG* as well as *oxyR* and *katG* in concert. Strains were grown overnight in CDM prior to being analyzed for catalase and H_2O_2 susceptibility assays. For H_2O_2 susceptibility, the strains were, as discussed above, grown in CDM in the presence/absence of H_2O_2 and the read-out was OD. The OD-values at early log-phase and stationary phase are presented in paper III as they represent two distinct phases of the life of a bacterial culture and enable the presentation of results in a way aiding visual clarity of the results and significant differences between strains and treatments. When preparing samples for the catalase assay one set of samples was pre-incubated with 0.03 mM H_2O_2 in order to stress the strains into increasing their H_2O_2 degrading capacity, thus forcing an increase in catalase activity.

The catalase assay is based on the measurement of absorption of light of 240 nm wavelength (A_{240} nm), which is the wavelength at which H_2O_2 has its peak absorption. Immediately before measurement of A_{240} nm commences H_2O_2 is added to the samples and A_{240} nm is thereafter measured over time. The rate of degradation depends on the H_2O_2 degrading abilities of the samples and thus on the activity of catalase. The assay can be performed using purified protein samples, as in paper I, or whole bacteria sampled from bacterial cultures, as in papers II and III. Each method has its pros and cons; where the protein method provides specificity by excluding the plethora of non-protein components of a whole-culture sample, thereby eliminating the possibility of parts of the H_2O_2 added to the assay being degraded by reacting with e.g. nucleic acids or lipids, it also requires several additional experimental steps when preparing the protein, which are naturally not needed for the whole-culture approach. With each additional step, the risk of contamination, degradation, change in activity and/or loss of protein increases and there is therefore a risk of altering the catalase activity during the preparation of the samples. Provided all samples within the group to be compared are treated the same way, any alteration should however, be the same in all samples, therefore not affecting the catalase activity of the samples relative to each other.

In papers I and II, the catalase activity is expressed in units, where one unit is defined as the amount of catalase needed to decompose 1 μmol of H_2O_2 per minute at 25°C, whilst the activity in paper III is expressed as the reduction

in absorption per OD_{600} ($\Delta A_{240}/OD_{600}$) over a 10 min measurement. The method used for calculating catalase activity and the way it is expressed differs between the papers and reflects the changes in execution of the assay (purified protein vs whole bacteria) as well as changes in the way considered the optimal way of presenting the data, but does in no way alter the internal relationship between the strains within the experiments.

In paper III, we investigated the susceptibility of strains not only to H_2O_2 but also to additional sources of oxidative stress, namely O_2^- and $ONOO^-$. Susceptibility to O_2^- was assessed by exposing bacteria to the O_2^- generator paraquat dichloride hydrate, which generates O_2^- through the reaction with components of the respiratory chain in bacteria, causing the reduction of O_2 to O_2^- [88]. Susceptibility to paraquat-mediated stress was assessed by way of a disc diffusion assay where plates (here MC plates) were coated with a layer of bacteria (here approx. 3 x 10^5 CFU/plate) and allowed to dry. A sterile filter-disc was thereafter placed in the center of each plate to which the substance of choice was applied (here 10 µl of paraquat in H_2O). Plates were thereafter incubated at 37°C for 4 days during which time the bacteria multiplied and paraquat diffused from the filter-disc out onto the plate. The presence of paraquat will hinder bacterial replication, causing the formation of a clear zone – an inhibition zone - around the disc. The diffusion of paraquat from a point in the center of the plates, rather than the coating of the entire plate, creates a gradient of paraquat with the concentration inversely correlated to the distance to the disc. The size of the zone will thus vary with the susceptibility of the strain to paraquat.

The disc diffusion assay was chosen since it requires only a small volume of dissolved paraquat per plate (10 µl), unlike the comparatively large culture-volumes (2.5 ml) needed for OD-based assay used for determining H_2O_2 susceptibility. The small volume required in the disc diffusion assay allows for screening of susceptibility using several different concentrations of this expensive compound, which would not have been reasonable with the OD-based assay.

In paper III, we studied the susceptibility to $ONOO^-$ - induced stress in addition to the H_2O_2 and O_2^- - induced stress already discussed. $ONOO^-$ is a highly reactive and bactericidal ROS formed through the reaction between nitric oxide (NO) and O_2^-. Experimentally SIN-1 (3-morpholinosydnonimine hydrochloride) can be used to mimic a continuous exposure to $ONOO^-$. SIN-1 slowly decomposes thus releasing both NO and O_2^- that under physiological conditions combine to generate 10 µM $ONOO^-$/min/mM SIN-1 [58, 59]. $ONOO^-$ provided in this manner can enter from the extracellular environment since it can pass through lipid bilayers [52, 58].

Strains were incubated in PBS with or without the addition of SIN-1 for a total of 4 h, with equal amounts of SIN-1 added at the start of the experiment and again after 2 h to ensure stable levels of $ONOO^-$ [62]. Unlike the H_2O_2 susceptibility testing performed in this paper (paper III), the $ONOO^-$ susceptibility testing was not OD-based, but VC-based. The decision to retain this read-out, despite the draw-backs discussed previously, was made due to the substantial amount of SIN-1 which would have been required for volumes large enough for OD-measurements, and the expensive nature of this compound. We attempted to use the disc diffusion assay used for O_2^- susceptibility testing also for $ONOO^-$ testing, since this assay requires even smaller volumes of $ONOO^-$ than does the VC based assay, but were unable to obtain any inhibition zones even when increasing the concentration in excess of 100 fold compared to the concentration used in the liquid assay in paper III (data not shown). We speculate that this lack of inhibition is due to the rapid formation of $ONOO^-$ by SIN-1, meaning that SIN-1 simply does not have time to diffuse out from the disc before it is forms $ONOO^-$ which reacts with the immediate surroundings.

In paper II, we wished to compare the amount of oxidized proteins in the LVS, Δ*mglA* and FUU301 strains to assess the possibility that the poor growth of the Δ*mglA* strain was linked to oxidative stress, which may have resulted in oxidative damage to proteins (see section 1.2). We furthermore wished to determine the effect cultivation of the strains in a low oxygen (microaerobic) environment would have compared to cultivation in an environment with atmospheric (aerobic) levels of oxygen. To this end, we used the OxyBlot Protein Oxidation Detection Kit, which detects carbonyl groups introduced at lysine, arginine, proline and threonine residues by metal catalyzed oxidation [89]. During the assay, these carbonyl groups are derivatized into a form detectable by standard immunostaining procedures. As the method was used in order to compare the total amount of damage to proteins, rather than to identify specific proteins with damage, we analyzed the samples by dot-blot, rather than Western blot. This allowed for the blotting of serial dilutions of several samples on the same membrane, thus allowing inter-sample comparison of the signal of each consecutive dilution.

3.5 Gene expression studies

Gene expression studies were performed by using quantitative real-time PCR (qRT-PCR), which offers the possibility to compare gene-expression levels by analyzing cDNA (complementary DNA) synthesized from the mRNA (messenger RNA) of a biological sample. Since mRNA levels reveal the transcriptional activity of their corresponding genes qRT-PCR is a useful tool for detecting both differences in gene-expression between strains, and

differences caused by the influence of e.g. different environments or stresses. Differences in mRNA level between samples are detected as a difference in the number of cycles of amplification needed before the sample can be detected by the instrument. The method may not offer the extensive screening possibilities of microarrays but is well suited for analysis of sub-sets of genes and is an available and well established method well in our lab and thus suited our needs. This method was therefore used for all gene-expression studies performed throughout the papers in this thesis.

3.6 Cell and mouse models

The cells used in Paper III –bone marrow-derived macrophages (BMDMs) were chosen as they are primary murine macrophages. As they are primary cells BMDMs must be isolated from fresh mouse bone marrow and cannot be propagated for generation after generation as is the possible with cell-lines.

To determine the replicative ability of Δ*oxyR* within the macrophages, cells were infected for 1.0 h with LVS or Δ*oxyR* at a MOI of 10. Incubation with gentamicin is commonly used as a way of removing bacteria remaining in the media after the infection-stage but was not done in these experiments since BMDMs take up gentamicin since it affects the viability of intracellular *F. tularensis* [90, 91]. Remaining extracellular bacteria were instead removed by rinsing the monolayers with fresh media. After 0 and 24 h incubation the macrophages were lysed, serially diluted and plated for determination of viable bacteria.

In order to determine the virulence of the Δ*oxyR* deletion mutant, female C57BL/6 mice were infected subcutaneously with LVS or Δ*oxyR* at a dose of 1 x 10^4 cfu/mouse. The mice were monitored for signs of illness and were euthanized by CO_2 asphyxiation after three days. Moribund mice would have been euthanized upon discovery, but this was never needed. The number of viable bacteria in spleens and livers were determined by homogenizing the organs in PBS and plating dilutions on MC plates. Infections were performed on groups of six mice per strain a total of three times. One mouse infected with Δ*oxyR* was excluded from the analysis due to presenting atypical characteristics both before and after infection. All animal experiments were approved by the Local Ethical Committee on Laboratory Animals, Umeå, Sweden (no. A 1-09, A 99-11 and A 67-14).

4 Results and Discussion

4.1 Paper I:

Iron content differs between *Francisella tularensis* subspecies *tularensis* and subspecies *holarctica* strains and correlates to their susceptibility to H2O2-induced killing.

As discussed previously (see section 1.2.1), iron is an essential nutrient to virtually all known organisms, including *F. tularensis*, and hosts therefore utilize iron-sequestering proteins to restrict iron availability as a means to hamper the growth of pathogens. *F. tularensis* therefore requires efficient iron-uptake to thrive in a host, but must at the same time regulate this uptake as to avoid oxidative stress (see section 1.2.2).

In paper I, we followed up on both our previous work regarding the iron-metabolism related to *fupA* [24] and the work related to the apparent differences in strategies for handling oxidative stress between LVS and virulent strains [57]. In these previous publications, we showed that *in vitro*, the subsp. *tularensis* strain SCHU S4 demonstrated a significantly higher resistance to oxidative stress mediated by H_2O_2 than the subsp. *holarctica* strains FSC200 and LVS, of which the former, a clinical isolate, was the more resistant one. Despite its higher tolerance for H_2O_2, the SCHU S4 strain was less dependent on catalase for its virulence than the subsp. *holarctica* strains, indicating the use of additional or alternative strategies for avoiding the toxic effects of the Fenton reaction [57]. The role of *fupA* in iron metabolism and the genomic differences regarding *fupA* and *fupA/B* (Fig. 3, section 1.2.1.1) indicated a high probability for a differential regulation of iron up-take between virulent strains and LVS. In light of the essential nature of iron for *F. tularensis* and the efforts made by hosts to restrict access of iron to invading pathogens, such dissimilarity may well be linked to the difference in virulence between the strains.

Here, we investigated how traits related to iron metabolism differ between *F. tularensis* subsp. *tularensis* and subsp. *holarctica*, and how these differences, through their link to oxidative stress, influenced the resistance to H_2O_2-mediated killing; we further discussed the implication this had on the great difference in virulence between these subspecies. The main body of experiments was performed using one subsp. *tularensis* strain, SCHU S4 and three subsp. *holarctica* strains: LVS, the virulent strain FSC200 and the FSC697 strain - an LVS strain complemented with *fupA*. Such a complementation of LVS with *fupA* has been shown to increase the virulence

of another LVS strain to the level of fully virulent subsp. *holarctica* strains [85].

4.1.1 Impact of the iron pool on H_2O_2 susceptibility

The total iron content of the strains was determined using the ferrozine assay and was found to be significantly larger in all subsp. *holarctica* strains than in SCHU S4, with LVS approximately containing a staggering 7 times the amount of iron of found in SCHU S4 and was followed by FSC200, which contained 6 times as much iron as SCHU S4 but still significantly less iron than LVS ($P < 0.001$). Complementation of LVS with *fupA* (FSC697) led to a decreased iron pool compared to LVS and FSC200 ($P < 0.001$) but the strain still contained approximately 4 times the amount of iron found in SCHU S4 ($P < 0.001$). The difference in iron-content between the subspecies was further confirmed by examining the iron-content of 9 additional strains of subsp. *holarctica* and 11 strains of subsp. *tularensis*, all of which presented the same pattern of elevated iron content of subsp. *holarctica* strains compared to subsp. *tularensis* strains. The average iron content between the subspecies differed by approximately 3-4 times.

As discussed previously, iron is both a necessity, through its incorporation in e.g. many enzymes, and a liability through its capacity to generate radicals by combining with H_2O_2 in the Fenton reaction. We therefore wished to determine if the difference of the iron pool of LVS, FSC200, FSC697 and SCHU S4 impacted their tolerance to H_2O_2. The influence of the iron pool was clearly seen as FSC697, which had lower iron content than LVS, also survived better when exposed to 1 mM H_2O_2 ($P < 0.001$). Underscoring the role of iron for the H_2O_2 susceptibility, both LVS and FSC697 became less susceptible to H_2O_2 when depleted of their iron pool prior to the H_2O_2 challenge ($P < 0.001$). In fact, the Fe-depleted LVS and FSC697 were equally susceptible to H_2O_2, which was in agreement with the below-detection limit levels of iron in both strains. SCHU S4 and FSC200 strains were unaffected by the 1 mM H_2O_2 concentration and were therefore additionally tested with 6 mM H_2O_2. SCHU S4 was found to be less susceptible to killing than FSC200 ($P < 0.05$), which was in accordance with the smaller iron pool of the former strain. In line with the results of the other subsp. *holarctica* strains, iron-depleted FSC200 became less susceptible to H_2O_2 when it contained less iron. In contrast to the *holarctica* strains, the H_2O_2 susceptibility of SCHU S4 increased dramatically when iron-depleted, causing a reduction in survival from nearly 20 % to around 2 %.

4.1.2 Gene expression

The difference in iron content was reflected by the gene-expression of the strains, since the *holarctica* strains (LVS, FSC200 and FSC697) showed significantly higher expression of the siderophore synthesis genes of the *fsl* operon, as well as the *ftn* gene, which encodes the iron-storage protein ferritin, than the *tularensis* strain SCHU S4. No other differences in expression were detected, neither in the iron-uptake related *feoA, feoB, fur* and *fupA* genes, nor in the oxidative stress defense related *oxyR, katG, gpx, ahpC1, sodB, sodC, trxA1, trxB, gshA, gshB, grxA, grxB, msrA1* and *msrA2* genes.

4.1.3 Catalase activity

The difference in H_2O_2 susceptibility was not reflected in the catalase activity of the strains. Our results showed that SCHU S4, FSC200 and LVS all had similar catalase activities under iron-replete conditions, whereas FSC697 had a lower activity, significantly different from both SCHU S4 and LVS. In line with the iron-dependency of the catalase enzyme, the activity decreased in all strains upon iron-depletion. In view of this and the decreased susceptibility of the *holarctica* strains to H_2O_2, it appears that a low intracellular iron pool is beneficial with regard to H_2O_2 susceptibility even if the catalase activity is reduced. On the other hand, the reduced catalase activity in iron-starved SCHU S4 may contribute to its increased susceptibility to H_2O_2. However, the catalase activity was still pronounced and therefore it is likely that some other protective mechanism is also hampered in the strain upon iron-depletion.

4.1.4 Conclusions

In conclusion, the results in paper I show that *F. tularensis* subsp. *tularensis* and subsp. *holarctica* have fundamental differences in iron-homeostasis that influence not only internal iron levels, but also susceptibility to oxidative stress mediated by H_2O_2. The subspecies are therefore differentially equipped for handling the iron deficiency and oxidative stress encountered in a host setting. The disparity in iron homeostasis may thus ultimately be central to the difference in virulence between subsp. *tularensis* and *holarctica*. Further, it was clear that the *fupA/B* fusion of LVS contributed to the additional increase in the internal iron pool seen for this strain in comparison to other subsp. *holarctica* strains.

4.2 Paper II:

The role of MglA for adaptation to oxidative stress of *Francisella tularensis* LVS

As discussed in section 1.3, transcriptional regulation is of great importance to bacteria and the global regulator MglA of *F. novicida* (discussed in 1.3.1) is important for the regulation of a large number of genes, and influences the amounts of proteins important for oxidative stress response [67]. We therefore sought to determine if MglA is involved in response to oxidative stress in *F. tularensis* also, by studying the effects of a deletion of *mglA* in *F. tularensis* LVS.

4.2.1 Phenotype of ΔmglA in an aerobic miliue

In paper II, we investigated the basis for the reduced growth observed when *mglA* (Δ*mglA*) was deleted from the LVS genome. Apart from the poor growth already mentioned, the effects of deleting *mglA* were wide-ranging. The oxy-blot method revealed that ΔmglA contained significantly more oxidized proteins than did LVS. By real time PCR it was demonstrated that ΔmglA expressed genes of the fsl operon at a significantly reduced level compared to LVS. The catalase activity of ΔmglA was elevated relative to LVS ($P < 0.01$), as determined by the catalase activity assay, and as a likely consequence of this, the mutant was also found to be less susceptible to H_2O_2 induced killing than LVS ($P < 0.01$).

In strains with intact regulation of siderophore synthesis and secretion, iron depletion will lead to secretion of siderophores. By use of real time PCR analysis of the *fsl* operon, and the CAS assay for detection of siderophore secretion by iron starved LVS and Δ*mglA*, it was demonstrated that there is no inherent defect in the ability of Δ*mglA* to regulate the siderophore operon and to secrete siderophores. Thus, the effect of deleting *mglA* has other consequences, causing this strain to restrict expression of iron-uptake related genes and up-regulating the catalase activity. Down-regulation of iron-uptake related genes, as well as increasing catalase activity are well-known strategies for coping with oxidative stress as they prevent the formation of $HO^{\cdot}$ through the Fenton reaction (see sections 1.2 and 1.2.2.2 respectively) [14, 42, 44]. In view of this, the phenotype of Δ*mglA* indicates that this strain experiences a higher level of oxidative stress than LVS, and that this is the basis for its poor growth compared to LVS.

4.2.2 Phenotype of ΔmglA in a microaerobic milieu

The conclusion that Δ*mglA* experiences a higher level of oxidative stress than LVS was further supported by the results of the assays performed on bacteria grown in microaerobic conditions. The sub-atmospheric oxygen concentration provided an environment less prone to oxidizing events, such as the Fenton reaction, and was found to be beneficial for Δ*mglA*, rendering a normalization of many of the previously aberrant properties of this strain. Indeed, the strain not only grew as well as LVS, it also contained lower amounts of oxidized proteins, again comparable to LVS, and expressed the *fsl* operon at the same level as LVS. The previously abnormally high catalase activity and associated resistance to H_2O_2 was also reversed in this permissive environment.

The microaerobic environment also affected LVS, causing a reduced growth rate, increased iron-sequestration and an increase in H_2O_2 susceptibility, consistent with our findings in paper I, connecting high iron levels to increased H_2O_2 susceptibility. Similar to LVS Δ*mglA* was more susceptible to H_2O_2 under the aerobic condition despite iron levels comparable to those in the aerobic environment. Given the dissociation between the internal iron pool and catalase activity, the basis for the increased susceptibility to H_2O_2 seems likely to differ between the strains; with the increased susceptibility of LVS being caused by the increased iron pool, which in combination with H_2O_2 cause oxidative damage through the Fenton reaction, while it is reasonable to assume that the susceptibility of Δ*mglA* instead was a result of the decrease in the ability to detoxify H_2O_2 due to the lower catalase activity. The increased susceptibility of the strains to H_2O_2 was thus mediated through an increase in the toxic effects of the Fenton reaction in both cases, but by different mechanism, one through an increase of the reaction itself and the other through a decreased preventive ability.

4.2.3 Conclusions

This is the first report demonstrating the function of MglA in oxidative stress defense in *F. tularensis*. Based on the results presented in paper II, we draw the conclusion that while MglA is of less importance in oxygen-restricted environments, it is absolutely crucial to the successful adaptation of LVS to oxygen-rich environments.

4.3 Paper III:

OxyR - an important regulator of the oxidative stress response in *Francisella tularensis* LVS

As discussed in section 1.3, transcriptional regulation is of great importance to bacteria for their ability to adapt to different stimuli. Several transcription factors are conserved between bacterial species, including factors involved in transcriptional regulation of stress responses such as the oxidative stress response. In paper III, we have investigated one such transcription factor – OxyR. As discussed in section 1.3.2, OxyR is a widely spread regulator of oxidative stress response of which *F. tularensis* harbors a homologue. In this paper, we aimed to investigate whether the *F. tularensis* OxyR homologue served a function in oxidative stress response, as well as the mechanisms underlying such function. To this en an in frame deletion mutant of *oxyR* was generated in LVS (Δ*oxyR*). Gene expression studies revealed lower expression of several antioxidant genes in the Δ*oxyR* strain than in LVS, indicating a positive influence of OxyR on expression of these genes. We therefore hypothesized that OxyR plays a part in the oxidative stress defense of LVS and also that the effects of deleting *oxyR* could be augmented by further crippling the defense system by deleting genes encoding ROS-degrading enzymes.

4.3.1 H_2O_2 stress and catalase activity

One of the down-regulated genes in Δ*oxyR* relative to LVS was *katG*, which is important for H_2O_2 tolerance [57]. We therefore deleted *katG* in the Δ*oxyR* background, generating a Δ*oxyR*/Δ*katG* double deletion mutant, and also included Δ*katG* in the study. The tolerance of Δ*oxyR* and Δ*oxyR*/Δ*katG* to H_2O_2 was assessed and compared to that of LVS and Δ*katG* by exposing the strains to H_2O_2 during growth in the liquid broth medium CDM. These experiments revealed a hierarchy with regard to H_2O_2 susceptibility, LVS being the least susceptible followed by Δ*oxyR*, which in turn was less susceptible than Δ*katG*. In accordance with expectations, the Δ*oxyR*/Δ*katG* double mutant was hyper-susceptible to H_2O_2, and was indeed much more affected by H_2O_2 than Δ*katG*, displaying not only the same phenotype as Δ*katG* when grown with 0.2 mM H_2O_2, i.e., no growth, but also failed to grow with 0.02 mM H_2O_2, a concentration at which Δ*katG* was able to reach wild type (wt) growth after 22 h.

We further investigated the response to H_2O_2 by studying the H_2O_2 degrading capacity of the strains using the catalase activity assay. Strains were incubated with or without H_2O_2 for 4 h and whole culture samples were

used in the catalase assay. LVS and Δ*oxyR* displayed equal catalase activities in the non-stimulated setting, thereby demonstrating that the basal catalase activity is not dependent upon OxyR. Pre-stimulation of LVS with H_2O_2 resulted in an increase of catalase activity compared to the non-stimulated setting ($P < 0.05$) but not for Δ*oxyR*. This resulted in the strains having significantly different levels of catalase activity in the pre-stimulated setting ($P < 0.05$). As expected the activity of Δ*oxyR*/Δ*katG* and Δ*katG* was significantly lower than in both LVS and in Δ*oxyR*.

In summary, the results demonstrated that both OxyR and catalase influenced the survival of LVS when challenged with H_2O_2. The results show that catalase was able to confer a degree of protection against H_2O_2 in the absence of OxyR, albeit to a reduced extent. The results further show the converse to be true; in the absence of KatG, OxyR has the capacity to confer a certain degree of protection against H_2O_2.

4.3.2 O_2^- stress

The gene-expression study of Δ*oxyR* further revealed a reduced expression relative to LVS of *sodB* and *sodC*, the genes encoding the SODs of *F. tularensis*. As described in 1.2.2.2, both SodB and SodC play important roles in the defense against oxidative stress by dismutating O_2^- in *F. tularensis* and are important for *F. tularensis* intra-macrophage survival, as well as for virulence in mice. SodB is also important for limiting the Fenton reaction by means of being an iron-containing FeSOD and thus binding iron, preventing it from partaking in the Fenton reaction [53, 54]. In agreement with the reduced expression of *sodC* and *sodB* in Δ*oxyR* and with the impact of SODs on O_2^- tolerance, Δ*oxyR* displayed a heightened susceptibility to this ROS. The Δ*katG* strain, on the other hand, tolerated O_2^- to the same extent as LVS, whereas Δ*oxyR*/Δ*katG*, as for H_2O_2, was more susceptible to H_2O_2 than the other strains. The results thereby demonstrate that catalase was dispensable for O_2^- tolerance but only in the presence of OxyR.

Based on the previously published results for the SOD mutants, and our findings in paper III, we believe it is likely that the decreased expression of *sodB* and *sodC* in Δ*oxyR* contributed to the increase in susceptibility to O_2^- and H_2O_2 observed for this strain through an increase in O_2^- mediated damage, as well as an increase in Fenton-mediated toxicity. We further hypothesize that the increase in Fenton-mediated toxicity caused by the reduction in *sod* expression, in combination with the lack of catalase, may account for the increase in O_2^- sensitivity observed for the Δ*oxyR*/Δ*katG* even though a lack of catalase alone did not cause such an increase in susceptibility.

4.3.3 ONOO⁻ stress

The most striking result of the gene expression study was the large reduction in expression of *ahpC2* (hereafter referred to as *ahpC*) in the Δ*oxyR* strain compared to LVS. As discussed in section 1.2.2.2, AhpC is known to be involved in protection against $ONOO^-$ in several bacterial species. Furthermore, the *ahpC* gene of *F. tularensis* is divergently encoded from *oxyR*. Such divergent transcription, i.e transcription of genes occurring in the opposite genomic direction, is a feature common for genes under the transcriptional regulation of OxyR [92, 93], indicating that this may be the case for the *F. tularensis ahpC* also. In subsp. *tularensis* strain SCHU S4, AhpC has recently been found to confer tolerance to O_2^- and $ONOO^-$ [56]. In agreement with this, and the reduced expression of *ahpC*, Δ*oxyR* was found to be highly susceptible to $ONOO^-$ generated by SIN-1 *in vitro*. Δ*katG* was as susceptible to $ONOO^-$ as Δ*oxyR*. As for H_2O_2 stress, Δ*oxyR*/Δ*katG* was hyper-susceptible to $ONOO^-$, manifested as a 100-fold reduction in survival compared to either single mutant, when exposed to $ONOO^-$. Collectively the results demonstrated the importance of both OxyR and catalase for the defense against $ONOO^-$.

The importance of AhpC to LVS is further emphasized by the apparent absolute requirement of *ahpC* for this strain. Despite repeated efforts, deletion of *ahpC* was not possible neither in Δ*oxyR*, nor in LVS, which is in stark contrast to the SCHU S4 strain where deletion of *ahpC* did not pose a problem [56]. It should, however, be noted that creation of a Δ*oxyR*/Δ*katG* mutant was not possible in the SCHU S4 strain [5, 56]. This, both indicates an overlap in the protective mechanisms conferred by these genes, and points to the crucial nature of ROS protection for the survival of the strain. The difference in importance of AhpC between the subsp. *tularensis* SCHU S4 strain and the subsp. *holarctica* LVS strain indicates a disparity in ROS defense between these strains and may contribute to the difference in characteristics, such as virulence between them.

4.3.4 BMDM and mice

Despite the diminished tolerance to several important ROS, Δ*oxyR* was able to replicate to the same extent as LVS in BMDMs, despite these being professional phagocytes and therefore constitute a hostile environment rich in ROS. The same was also true for Δ*katG*, which replicated to a similar extent as LVS and Δ*oxyR*, despite an increased ROS susceptibility. In contrast, Δ*oxyR*/Δ*katG* failed to replicate at all in BMDMs. The results thus clearly showed that although dispensable on their own, *oxyR* and *katG* could not be lost in concert without seriously affecting the ability for intracellular

replication. This suggests that OxyR and catalase are able to provide compensatory effects and, to a certain degree, “cover” for each other.

Despite showing no replication defect *in vitro,* Δ*oxyR* was unable to replicate to the same extent as LVS in the livers of mice. Infection of mice with Δ*oxyR* caused fewer symptoms of illness, and resulted in significantly fewer bacteria being recovered from the livers, than from mice infected with LVS. These results therefore demonstrate that the importance of efficient ROS defense is of greater importance *in vivo* than *in vitro* and thereby highlight the importance of *in vivo* testing when studying these systems.

4.3.5 Conclusions

To conclude, the results from paper III demonstrate the importance of a robust oxidative stress defense system for the success of *F. tularensis*, *in vitro* as well as *in vivo*. The results further provide new insights in the roles of both OxyR and catalase in LVS, demonstrating the interconnection between them and shedding new light on the complexity of ROS defense.

5 Summary and Conclusions

This thesis highlights the importance of iron-homeostasis, transcriptional regulation and the management of ROS for the tolerance of *F. tularensis* to oxidative stress and oxygen-rich environments. The work presented demonstrates the impact of the bacterial iron pool as well as of the transcription factors MglA and OxyR in this context.

We successfully identified fundamental differences in iron-homeostasis between *F. tularensis* subsp. *tularensis* and subsp. *holarctica*, and demonstrated how these affected the ability of the subspecies to withstand oxidative stress in the form of H_2O_2. We further determined that the *fupA/B* fusion of the LVS strain further altered the iron homeostasis of this strain compared to other subsp. *holarctica* strains, as well as its susceptibility to H_2O_2.

The importance of transcriptional regulation for the adaptation of *F. tularensis* to oxidative stress is accentuated as we demonstrated the crucial function of both MglA and OxyR in these processes. Specifically, MglA was found to be pivotal for adaptation of LVS to oxygen-rich environments, and OxyR for tolerance to several ROS. Moreover, the results showed that OxyR and catalase had compensatory effects with regard to ROS detoxification.

Collectively, this thesis provides new insights into the web of interconnecting oxidative stress defense systems of *F. tularensis*.

- ➔ *F. tularensis* subsp. *tularensis* contains less iron than subsp. *holarctica*.
- ➔ LVS contains more iron than virulent subsp. *holarctica* strains
- ➔ A larger iron pool is associated with a higher susceptibility to H_2O_2.
- ➔ The *fupA/B* gene-fusion of LVS contributes to the additional increase in the internal iron pool seen for this strain in comparison to other subsp. *holarctica* strains.
- ➔ MglA is crucial for adaptation to oxygen-rich environments.
- ➔ OxyR is not necessary for basal catalase activity, but is required for increased activity upon H_2O_2 stimulation.
- ➔ The protective functions of OxyR and catalase against O_2^-, H_2O_2 and $ONOO^-$ are partially overlapping

Acknowledgements

> If we knew what it was we were doing, it would not be called research, would it?
>
> - *Albert Einstein*

This thesis represents the fulfillment of a childhood dream – the dream to do research - to explore the unknown, learn what no one has known before and understand how things work.

A thesis requires a lot of hard work and dedication, but also, and possibly most importantly – people. A work of this scope is not possible without the advice, help and support of a lot of people. I would like to take this opportunity to thank all those who made this possible.

Anders – thank you for taking me on all those years ago and giving me the chance to do research in your group, and for allowing me to attend courses and conferences in different parts of the world.

Helena – co-supervisor, mentor and friend. I cannot tell you how much I value all you have taught me about *Francisella*, lab-work in general and so many things beyond that. Thank you for all your support and all our discussions about work, politics, kids and life in general.

Marie L – my partner in crime, my comrade in arms, my fellow "exil-Jämte" ☺. Thank you for all the laughter, help and support you have given, both in and out of work, and for all our great discussions about work and life beyond work. I'm glad we still see each other!

Lena M – I'm so glad you joined our group! I have enjoyed our talks, both of work and life, immensely. Thank you for all your help and support, both in and out of the lab. We have made a great team - you, me and Marie L – the three musketeers ☺. I miss our talks and lunches at Taj Mahal!

I would also like to thank all the people at **Clinical Bacteriology and the "fika-corner"**, both past and present, for the interesting discussions, fun parties and nice trips. ***Jeanette*** – The master of lab-tricks! Thank you for your help, for sharing the little tricks that help when things get stuck, and for chats about life outside work! ***Malin*** – for your calm and your friendship, ***Mattias*** – for sharing and listening, and for your wonderful sense of humor! ***Igor*** – for your calm, expertise and help, ***Kjell*** – for being positive and helpful, ***Lena L*** – for lab-related tips, ***Maggan*** – for chats and administrative expertise, ***Alicia*** – for radiating positive energy, ***Olena*** – for lifting our spirits, ***Kun*** – for calm and humor, ***Gurram*** – for your help and positive attitude, ***Nasibeh*** – for bringing color to our lives, ***Daniel*** – for your sense of humor, ***Anna*** – for interesting discussions, ***Athar and***

Eram – for new perspectives, ***the members of group CU*** – ***Hanna, Ava, Anna TS, Marios, Pedro, Madhu, Sujan –*** for chats, laughter and parties, ***Gunborg and Lena N-H*** – for help with administrative things, ***Åsa and rest of the staff at TBK*** – for providing endless amounts of media and plates. And of course, thanks to all others who have worked in the lab during my years here, and to anyone I might have forgotten!

Life is so much more than work, and I would therefore like to thank some great friends whom have been with me throughout this journey – our "Umeå family": ***Annasara and Andreas*** – for always caring, and being there when we need you! And for moving to Sävar, so we ended up there too ☺, ***Reine and Sofia*** - for all the talks, encouragement and super nice photographs! Too bad you moved so far away! ***Annika and Calle*** – for talks, crafts-evenings and laughter; and to the whole gang: thanks for all our dinners, board game nights, exploration trips and all the laughter! Also to my great friends from home ***Linnéa and Jonas***, and ***Jenny and Niklas*** – for the laughs we share whenever we meet.

A special thank you goes to the first teacher who introduced me to the fields of biology and chemistry – ***Knut*** – easily the most inspirational teacher anyone ever had! Thanks for making science interesting and fun!

Last, but certainly not least, I would like to thank my family. My loving parents ***Margareta and Tim*** – for always supporting me and believing I could do this: ***Mamma*** – for your love, support and encouragement, for all our talks about life, and for always listening and helping out! ***Papa*** – for your love, all the practical things you have taught me, and all our discussions of science, culture, crafts and more. My wonderful brothers ***Dan and Erik*** – for the laughs (and fights) we had growing up: ***Dan*** – for being there when I need you and for giving me place to stay when I first moved to Umeå, ***Erik*** – for all the things we did together growing up and for the talks and laughs we still share when we meet.

To my wonderful husband ***John*** – you are my rock, my safe harbor in the storm and the person who understands me the best. Thank you for taking care of the kids and the house while I've been cooped up writing this thesis! Thank you for all the time we have had so far, all the laughter and adventure, I look forward to the rest of our lives and I love you with all my heart!

To the two most precious people in my live – my children – ***Samuel and Ian*** – you give me perspective, allow me to play again, to show you the wonders of this world and to re-discover them with you. You make my life complete and I am forever grateful to have you. I love you to the moon and back, to the stars and beyond and to a galaxy far, far away!

References

1. Oyston PCF, Sjöstedt A, Titball RW: **Tularaemia: bioterrorism defence renews interest in *Francisella tularensis*.** *Nat Rev Microbiol* 2004, **2**:967–78.

2. McCoy GW, Chapin CW: **Further observations on a Plague-Like Disease of rodents with a preliminary note on the Causative Agent, *Bacterium tularense*.***. *J Infect Dis* 1912:61–72.

3. Chapin CW: **Review of our knowledge of *Bacterium tularense*.** *Am J Public Health (N Y)* 1921, **11**:529–32.

4. Sjöstedt A: **Tularemia: history, epidemiology, pathogen physiology, and clinical manifestations.** *Ann N Y Acad Sci* 2007, **1105**:1–29.

5. Kadzhaev K, Zingmark C, Golovliov I, Bolanowski M, Shen H, Conlan W, Sjöstedt A: **Identification of genes contributing to the virulence of *Francisella tularensis* SCHU S4 in a mouse intradermal infection model**. *PLoS One* 2009, **4**.

6. Ellis J, Oyston PCF, Green M, Titball RW: **Tularemia**. *Society* 2002, **15**:631–646.

7. Wayne Conlan J, Oyston PCF: **Vaccines against *Francisella tularensis*.** *Ann N Y Acad Sci* 2007, **1105**:325–50.

8. Sjöstedt A: **Intracellular survival mechanisms of *Francisella tularensis*, a stealth pathogen.** *Microbes Infect* 2006, **8**:561–7.

9. Price J V, Vance RE: **The Macrophage Paradox**. *Immunity* 2014, **41**:685–693.

10. Schaible UE, Kaufmann SHE: **Iron and microbial infection**. *Nat Rev Microbiol* 2004, **2**:946–953.

11. Flannagan RS, Cosío G, Grinstein S: **Antimicrobial mechanisms of phagocytes and bacterial evasion strategies**. *Nat Rev Microbiol* 2009, **7**:355–366.

12. Allwood EM, Devenish RJ, Prescott M, Adler B, Boyce JD: **Strategies for intracellular survival of *Burkholderia pseudomallei***. *Front Microbiol* 2011, **2**(AUG):1–19.

13. Asare R, Abu Kwaik Y: **Exploitation of host cell biology and**

evasion of immunity by *Francisella tularensis*. *Front Microbiol* 2011, **1**(JAN):1–14.

14. Andrews SC, Robinson AK, Rodrí-guez-Quiñones F: **Bacterial iron homeostasis**. *FEMS Microbiol Rev* 2003, **27**:215–237.

15. Imlay JA: **Iron-sulphur clusters and the problem with oxygen.** *Mol Microbiol* 2006, **59**:1073–82.

16. Fridovich I: **Oxygen: how do we stand it?.** *Med Princ Pract* 2013, **22**:131–7.

17. Fortier AH, Leiby DA, Narayanan RB, Asafoadjei E, Crawford RM, Nacy CA, Meltzer MS: **Growth of *Francisella tularensis* LVS in macrophages: the acidic intracellular compartment provides essential iron required for growth.** *Infect Immun* 1995, **63**:1478–1483.

18. Skaar EP: **The battle for iron between bacterial pathogens and their vertebrate hosts**. *PLoS Pathog* 2010, **6**:1–2.

19. Barber MF, Elde NC: **Buried Treasure: Evolutionary Perspectives on Microbial Iron Piracy**. *Trends Genet* 2015, **31**:627–636.

20. Faraldo-Gómez JD, Sansom MSP: **Acquisition of siderophores in Gram-negative bacteria**. *Nat Rev Mol Cell Biol* 2003, **4**:105–116.

21. Deng K, Blick RJ, Liu W, Hansen EJ: **Identification of *Francisella tularensis* genes affected by iron limitation**. *Infect Immun* 2006, **74**:4224–4236.

22. Sullivan JT, Jeffery EF, Shannon JD, Ramakrishnan G, Al SET, Acteriol JB: **Characterization of the Siderophore of *Francisella tularensis* and Role of *fslA* in Siderophore Production**. *J Bacteriol* 2006, **188**:3785–3795.

23. Thomas-Charles CA, Zheng H, Palmer LE, Mena P, Thanassi DG, Furie MB: **FeoB-mediated uptake of iron by *Francisella tularensis***. *Infect Immun* 2013, **81**:2828–2837.

24. Lindgren H, Honn M, Golovlev I, Kadzhaev K, Conlan W, Sjöstedt A: **The 58-kilodalton major virulence factor of *Francisella tularensis* is required for efficient utilization of iron.** *Infect Immun* 2009, **77**:4429–36.

25. Lindgren H, Lindgren L, Golovliov I, Sjöstedt A: **Mechanisms of Heme Utilization by *Francisella tularensis*.** *PLoS One* 2015, **10**:e0119143.

26. Tong Y, Guo M: **Bacterial heme-transport proteins and their heme-coordination modes**. *Arch Biochem Biophys* 2009, **481**:1–15.

27. Huntley JF, Conley PG, Hagman KE, Norgard M V: **Characterization of *Francisella tularensis* outer membrane proteins.** *J Bacteriol* 2007, **189**:561–74.

28. Ramakrishnan G, Sen B: **The FupA/B protein uniquely facilitates transport of ferrous iron and siderophore-associated ferric iron across the outer membrane of *Francisella tularensis* live vaccine strain**. *Microbiol (United Kingdom)* 2014, **160**(PART 2):446–457.

29. Ramakrishnan G, Sen B, Johnson R: **Paralogous outer membrane proteins mediate uptake of different forms of iron and synergistically govern virulence in *Francisella tularensis tularensis***. *J Biol Chem* 2012, **287**:25191–25202.

30. Sen B, Meeker A, Ramakrishnan G: **The *fslE* Homolog, FTL_0439 (fupA/B), Mediates Siderophore-Dependent Iron Uptake in *Francisella tularensis* LVS**. *Infect Immun* 2010, **78**:4276–4285.

31. Twine S, Byström M, Chen W, Forsman M, Golovliov I, Johansson A, Kelly J, Lindgren H, Svensson K, Zingmark C, Conlan W, Sjöstedt A: **A mutant of *Francisella tularensis* strain SCHU S4 lacking the ability to express a 58-kilodalton protein is attenuated for virulence and is an effective live vaccine.** *Infect Immun* 2005, **73**:8345–52.

32. Cartron ML, Maddocks S, Gillingham P, Craven CJ, Andrews SC: **Feo – Transport of Ferrous Iron into Bacteria**. *BioMetals* 2006, **19**:143–157.

33. Pérez NM, Ramakrishnan G: **The Reduced Genome of the *Francisella tularensis* Live Vaccine Strain (LVS) Encodes Two Iron Acquisition Systems Essential for Optimal Growth and Virulence.** *PLoS One* 2014, **9**:e93558.

34. Chu BC, Garcia-Herrero A, Johanson TH, Krewulak KD, Lau CK, Peacock RS, Slavinskaya Z, Vogel HJ: **Siderophore uptake in bacteria and the battle for iron with the host; a bird's eye view.** *Biometals* 2010, **23**:601–11.

35. Ratledge C, Dover LG: **Iron metabolism in pathogenic bacteria.** *Annu Rev Microbiol* 2000, **54**:881–941.

36. Saha R, Saha N, Donofrio RS, Bestervelt LL: **Microbial siderophores: A mini review**. *J Basic Microbiol* 2013, **53**:303–317.

37. Ramakrishnan G, Meeker A, Dragulev B: ***fslE* is necessary for**

siderophore-mediated iron acquisition in *Francisella tularensis* Schu S4. *J Bacteriol* 2008, **190**:5353–5361.

38. Wehrly TD, Chong A, Virtaneva K, Sturdevant DE, Child R, Edwards JA, Brouwer D, Nair V, Fischer ER, Wicke L, Curda AJ, Kupko JJ, Martens C, Crane DD, Bosio CM, Porcella SF, Celli J: **Intracellular biology and virulence determinants of *Francisella tularensis* revealed by transcriptional profiling inside macrophages**. *Cell Microbiol* 2009, **11**:1128–1150.

39. Cendrowski S, MacArthur W, Hanna P: ***Bacillus anthracis* requires siderophore biosynthesis for growth in macrophages and mouse virulence**. *Mol Microbiol* 2004, **51**:407–417.

40. De Voss JJ, Rutter K, Schroeder BG, Su H, Zhu Y, Barry CE: **The salicylate-derived mycobactin siderophores of *Mycobacterium tuberculosis* are essential for growth in macrophages.** *Proc Natl Acad Sci U S A* 2000, **97**:1252–1257.

41. Reddy PV, Puri RV, Chauhan P, Kar R, Rohilla A, Khera A, Tyagi AK: **Disruption of Mycobactin Biosynthesis Leads to Attenuation of *Mycobacterium tuberculosis* for Growth and Virulence**. *J Infect Dis* 2013, **208**:1255–1265.

42. Troxell B, Hassan HM: **Transcriptional regulation by Ferric Uptake Regulator (Fur) in pathogenic bacteria.** *Front Cell Infect Microbiol* 2013, **3**:59.

43. Hantke K: **Iron and metal regulation in bacteria**. *Curr Opin Microbiol* 2001, **4**:172–177.

44. Betteridge DJ: **What is oxidative stress?** *Metabolism* 2000, **49**(2 Suppl 1):3–8.

45. Melillo AA, Bakshi CS, Melendez JA: ***Francisella tularensis* antioxidants harness reactive oxygen species to restrict macrophage signaling and cytokine production**. *J Biol Chem* 2010, **285**:27553–27560.

46. Dupré-Crochet S, Erard M, Nüβe O: **ROS production in phagocytes: why, when, and where?** *J Leukoc Biol* 2013, **94**:657–70.

47. Panday A, Sahoo MK, Osorio D, Batra S: **NADPH oxidases: an overview from structure to innate immunity-associated pathologies**. *Cell Mol Immunol* 2015, **12**:5–23.

48. Nunes P, Demaurex N, Dinauer MC: **Regulation of the NADPH Oxidase and Associated Ion Fluxes During Phagocytosis**. *Traffic*

2013, **14**:1118–1131.

49. McCaffrey RL, Schwartz JT, Lindemann SR, Moreland JG, Buchan BW, Jones BD, Allen L-AH: **Multiple mechanisms of NADPH oxidase inhibition by type A and type B *Francisella tularensis*.** *J Leukoc Biol* 2010, **88**:791–805.

50. Lindgren H, Stenmark S, Chen W, Tärnvik A, Sjöstedt A: **Distinct roles of reactive nitrogen and oxygen species to control infection with the facultative intracellular bacterium *Francisella tularensis*.** *Infect Immun* 2004, **72**:7172–82.

51. Liochev SI, Fridovich I: **Superoxide and iron: partners in crime.** *IUBMB Life* 1999, **48**:157–61.

52. Szabó C, Ischiropoulos H, Radi R: **Peroxynitrite: biochemistry, pathophysiology and development of therapeutics.** *Nat Rev Drug Discov* 2007, **6**:662–80.

53. Bakshi CS, Malik M, Regan K, Melendez JA, Metzger DW, Pavlov VM, Sellati TJ: **Superoxide dismutase B gene (*sodB*)-deficient mutants of *Francisella tularensis* demonstrate hypersensitivity to oxidative stress and attenuated virulence.** *J Bacteriol* 2006, **188**:6443–8.

54. Melillo AA, Mahawar M, Sellati TJ, Malik M, Metzger DW, Melendez JA, Bakshi CS: **Identification of *Francisella tularensis* live vaccine strain CuZn superoxide dismutase as critical for resistance to extracellularly generated reactive oxygen species.** *J Bacteriol* 2009, **191**:6447–56.

55. Imlay JA: **Transcription Factors That Defend Bacteria Against Reactive Oxygen Species**. *Annu Rev Microbiol* 2014, **69**:150612172656007.

56. Binesse J, Lindgren H, Lindgren L, Conlan W, Sjöstedt A: **Roles of reactive oxygen species-degrading enzymes of *Francisella tularensis* SCHU S4.** *Infect Immun* 2015, **83**:IAI.02488–14–.

57. Lindgren H, Shen H, Zingmark C, Golovliov I, Conlan W, Sjöstedt A: **Resistance of *Francisella tularensis* strains against reactive nitrogen and oxygen species with special reference to the role of KatG.** *Infect Immun* 2007, **75**:1303–9.

58. Murphy MP, Packer MA, Scarlett JL, Martin SW: **Peroxynitrite: a biologically significant oxidant.** *Gen Pharmacol* 1998, **31**:179–86.

59. Hogg N, Darley-Usmar VM, Wilson MT, Moncada S: **Production of**

hydroxyl radicals from the simultaneous generation of superoxide and nitric oxide. *Biochem J* 1992, **281 (Pt 2**:419–24.

60. Bogdan C: **Nitric oxide synthase in innate and adaptive immunity: an update**. *Trends Immunol* 2015, **36**:161–178.

61. Maggio S, Takeda K, Stark F, Meierovics AI, Yabe I, Cowley SC: **Control of *Francisella tularensis* Intracellular Growth by Pulmonary Epithelial Cells**. *PLoS One* 2015, **10**:e0138565.

62. Lindgren H, Stenman L, Tärnvik A, Sjöstedt A: **The contribution of reactive nitrogen and oxygen species to the killing of *Francisella tularensis* LVS by murine macrophages.** *Microbes Infect* 2005, **7**:467–75.

63. Sue GR, Ho ZC, Kim K: **Peroxiredoxins: A historical overview and speculative preview of novel mechanisms and emerging concepts in cell signaling**. *Free Radic Biol Med* 2005, **38**:1543–1552.

64. Bryk R, Griffin P, Nathan C: **Peroxynitrite reductase activity of bacterial peroxiredoxins.** *Nature* 2000, **407**:211–5.

65. Lozada-Chavez I: **Bacterial regulatory networks are extremely flexible in evolution**. *Nucleic Acids Res* 2006, **34**:3434–3445.

66. Madan Babu M, Balaji S, Aravind L: **General trends in the evolution of prokaryotic transcriptional regulatory networks.** *Genome Dyn* 2007, **3**:66–80.

67. Guina T, Radulovic D, Bahrami AJ, Bolton DL, Rohmer L, Jones-Isaac KA, Chen J, Gallagher LA, Gallis B, Ryu S, Taylor GK, Brittnacher MJ, Manoil C, Goodlett DR: **MglA regulates *Francisella tularensis* subsp. *novicida* (*Francisella novicida*) response to starvation and oxidative stress.** *J Bacteriol* 2007, **189**:6580–6586.

68. Chiang SM, Schellhorn HE: **Regulators of oxidative stress response genes in *Escherichia coli* and their functional conservation in bacteria**. *Arch Biochem Biophys* 2012, **525**:161–169.

69. Whitby PW, Morton DJ, VanWagoner TM, Seale TW, Cole BK, Mussa HJ, McGhee PA, Bauer CYS, Springer JM, Stull TL: ***Haemophilus influenzae* OxyR: Characterization of Its Regulation, Regulon and Role in Fitness**. *PLoS One* 2012, **7**:e50588.

70. Hoopman TC, Liu W, Joslin SN, Pybus C, Brautigam CA, Hansen EJ: **Identification of gene products involved in the oxidative stress response of *Moraxella catarrhalis*.** *Infect Immun* 2011, **79**:745–55.

71. Ramsey KM, Osborne ML, Vvedenskaya IO, Su C, Nickels BE, Dove SL: **Ubiquitous Promoter-Localization of Essential Virulence Regulators in *Francisella tularensis***. *PLOS Pathog* 2015, **11**:e1004793.

72. Charity JC, Blalock LT, Costante-Hamm MM, Kasper DL, Dove SL: **Small molecule control of virulence gene expression in *Francisella tularensis*.** *PLoS Pathog* 2009, **5**:e1000641.

73. Rohlfing AE, Dove SL: **Coordinate control of virulence gene expression in *Francisella tularensis* involves direct interaction between key regulators.** *J Bacteriol* 2014, **196**:3516–26.

74. Brotcke A, Weiss DS, Kim CC, Chain P, Malfatti S, Garcia E, Monack DM: **Identification of MglA-regulated genes reveals novel virulence factors in *Francisella tularensis***. *Infect Immun* 2006, **74**:6642–6655.

75. Charity JC, Costante-Hamm MM, Balon EL, Boyd DH, Rubin EJ, Dove SL: **Twin RNA Polymerase–Associated Proteins Control Virulence Gene Expression in *Francisella tularensis***. *PLoS Pathog* 2007, **3**:e84.

76. Bönquist L, Lindgren H, Golovliov I, Guina T, Sjöstedt A: **MglA and Igl proteins contribute to the modulation of *Francisella tularensis* live vaccine strain-containing phagosomes in murine macrophages.** *Infect Immun* 2008, **76**:3502–10.

77. Honn M, Lindgren H, Sjöstedt A: **The role of MglA for adaptation to oxidative stress of *Francisella tularensis* LVS**. *BMC Microbiol* 2012:14.

78. Green J, Rolfe MD, Smith LJ: **Transcriptional regulation of bacterial virulence gene expression by molecular oxygen and nitric oxide.** *Virulence* 2014, **5**:794–809.

79. Hausladen A, Privalle CT, Keng T, DeAngelo J, Stamler JS: **Nitrosative stress: activation of the transcription factor OxyR.** *Cell* 1996, **86**:719–729.

80. Marshall HE, Merchant K, Stamler JS: **Nitrosation and oxidation in the regulation of gene expression.** *FASEB J* 2000, **14**:1889–1900.

81. McLean S, Bowman L a H, Sanguinetti G, Read RC, Poole RK: **Peroxynitrite toxicity in *Escherichia coli* K12 elicits expression of oxidative stress responses and protein nitration and nitrosylation**. *J Biol Chem* 2010, **285**:20724–20731.

82. Zheng M, Doan B, Schneider TD, Storz G: **OxyR and SoxRS regulation of fur.** *J Bacteriol* 1999, **181**:4639–43.

83. Golovliov I, Sjöstedt A, Mokrievich A, Pavlov V: **A method for allelic replacement in *Francisella tularensis***. *FEMS Microbiol Lett* 2003, **222**:273–280.

84. Pelicic V, Reyrat JM, Gicquel B: **Expression of the *Bacillus subtilis sacB* gene confers sucrose sensitivity on mycobacteria.** *J Bacteriol* 1996, **178**:1197–9.

85. Salomonsson E, Kuoppa K, Forslund A-L, Zingmark C, Golovliov I, Sjöstedt A, Noppa L, Forsberg A: **Reintroduction of two deleted virulence loci restores full virulence to the live vaccine strain of *Francisella tularensis*.** *Infect Immun* 2009, **77**:3424–31.

86. Riemer J, Hoepken HH, Czerwinska H, Robinson SR, Dringen R: **Colorimetric ferrozine-based assay for the quantitation of iron in cultured cells.** *Anal Biochem* 2004, **331**:370–5.

87. Kiss K, Liu W, Huntley JF, Norgard M V, Hansen EJ: **Characterization of *fig* operon mutants of *Francisella novicida* U112**. *FEMS Microbiol Lett* 2008, **18**:1199–1216.

88. Hassan M, Fridovich I: **Paraquat and *Escherichia coli***. 1979, **254**:10846–10852.

89. Stadtman ER: **Oxidation of Free Amino Acids and Amino Acid Residues in Proteins By Radiolysis and By Metal-Catalyzed reactions**. 1993:797–821.

90. Lindgren H, Sjöstedt A: **Gallium potentiates the antibacterial effect of gentamicin against *Francisella tularensis***. *Antimicrob Agents Chemother* 2015, **60**:AAC.01240–15.

91. Sutera V, Caspar Y, Boisset S, Maurin M: **A new dye uptake assay to test the activity of antibiotics against intracellular *Francisella tularensis***. *Front Cell Infect Microbiol* 2014, **4**(March):1–7.

92. Hahn J-S, Oh S-Y, Roe J-H: **Role of OxyR as a peroxide-sensing positive regulator in *Streptomyces coelicolor* A3(2).** *J Bacteriol* 2002, **184**:5214–22.

93. Maddocks S, Oyston PCF: **Structure and function of the LysR-type transcriptional regulator (LTTR) family proteins**. *Microbiology* 2008, **154**:3609–3623.