

Characterization of the attenuated *Francisella tularensis* strain FSCo43

- with special focus on the gene *pdpC*

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Life is change

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Abstract

Francisella tularensis is a highly infective, intracellular bacterium. It is capable of infecting a wide range of mammals and causes the disease tularemia in humans. As a result of its high infectivity there have been a lot of efforts made to create a generally available vaccine against this pathogen.

One potential vaccine candidate is the FSCo43 strain, a spontaneous mutant that has acquired mutations making it attenuated for replication both in *vitro* and in the experimental mouse model. However, it was noted that it afforded protection against challenge with a highly virulent *F. tularensis* strain. The aim of this thesis has been to delineate the mechanisms of its attenuation to better understand *F. tularensis* pathogenesis and to obtain a better knowledge about the prerequisites of protective immunity against this potent pathogen.

Microarray and whole-genome sequencing revealed four mutations in the attenuated FSCo43 strain that were not present in the virulent SCHU S4 isolate. One of these mutations has been described earlier as it results in a fusion protein also found in other attenuated strains. Among the other differences, two mutations were identical nonsense mutations in a duplicated gene region known as the *Francisella* pathogenicity island (FPI). The affected gene, *pdpC*, is coding for PdpC (pathogenicity determinant protein C). We found that these mutations resulted in a truncated form of PdpC, and also that the downstream gene was severely downregulated due to these mutations. Further, our studies revealed that the intracellular phenotype of the FSCo43 strain differed from other tested strains in that a small portion of the intracellular bacteria were able to escape the phagosome and multiply within the host, while the majority of intracellular bacteria stayed confined to the phagosome.

We wanted to study the specific function of *pdpC* and therefore deleted both copies of it in the virulent SCHU S4 strain as well as the Live Vaccine Strain, an empirically attenuated strain often used as a model for the virulent strains of *F. tularensis*. The resulting mutants showed an attenuated phenotype; no intracellular growth in murine cells, and no virulence in mice. When studying the intracellular localization of the LVS $\Delta pdpC$ mutant, we found that it was uniformly located adjacent to phagosomal membrane-like structures but that the membrane was markedly disrupted. Further, this mutant induced an MOI-dependent cytotoxicity, measured by LDH release, and also the release of IL-1 β , an inflammatory cytokine not induced by phagosomally contained mutants. Studies on markers for host cell death revealed that the LVS $\Delta pdpC$ mutant induced mitochondrial instability, phosphatidylserine (PS) presentation, and TUNEL-specific DNA fragmentation in infected cells, rather similar to the wildtype strain, despite its lack of replication.

This study reveals that the *pdpC* gene is an important gene required for *F. tularensis* virulence. We also show that non-replicating intracellular bacteria can induce host cell death, hypothesizing that release of bacterial components in the host cell cytosol is required for this induction.

The FSCo43 mutant showed a unique phenotype where a small subset of bacteria was able to escape the phagosome and replicate in the host cell. This was also seen in the *pdpC* deletion mutant of SCHU S4, but not with the LVS Δ *pdpC*. However, regardless of genetic background, the Δ *pdpC* mutant had an effect on phagosomal escape; either by affecting the phagosomal membranes in a unique way or by allowing phagosomal escape of a small proportion of the bacteria.

Enkel sammanfattning på svenska

Francisella tularensis är den bakterie som orsakar harpest, också kallad tularemi. Sjukdomen förekommer hos däggdjur och smittspridning till människor tros främst ske via myggor och fästingar men även vid kontakt med infekterade djur då man antingen andas in bakterien eller genom att bakterien via ett sår eller en rispa kan ta sig in i kroppen. Symptomen hos människor beror på smittvägen; om infektion spridits via bett eller en rispa resulterar det i ett svårläkt sår och uppsvullnade lymfknotor samt mer ospecifika influensaliknande symptom som värk och feber.

Det finns flera underarter av *F. tularensis* varav två har visats kunna smitta människor med normalt immunförsvar: subsp. *holarctica* (Typ B) och subsp. *tularensis* (Typ A). Den förra (Typ B) har främst hittats på norra halvklotet, och i Sverige rapporteras det in ca 300 fall per år. Den senare (Typ A) har bara isolerats i Nordamerika, är väldigt sjukdomsframkallande, och resulterar i hög dödlighet om obehandlad. Båda typerna är väldigt smittsamma och det krävs endast ett tiotal bakterier för att orsaka sjukdom. På grund av sin smittsamhet har *F. tularensis* länge setts som ett potentiellt biologiskt stridsmedel och har därför ingått i många skyddsprogram där man försökt skapa ett effektivt vaccin. Tularemi behandlas relativt effektivt med antibiotika, det svåra är att identifiera sjukdomen eftersom många symptom är väldigt ospecifika.

Idag finns det ett vaccin som ges till personer som är särskilt utsatta för smitta, främst laborativ personal som arbetar med *F. tularensis*. Vaccinet kallas för LVS, Live Vaccine Strain, och är ett attenuerat (försvagat), levande vaccin. Vid vaccination med denna stam utsätts kroppen för en försvagad infektion och har då tid att generera ett skyddande immunsvår. Detta vaccin fungerar till viss del mot båda underarterna men är ineffektivt vid aerosol smitta (smitta genom inandning), som ger den mer allvarliga formen av tularemi, och bör inte heller ges till personer med nedsatt immunförsvar. Stora satsningar har gjorts för att skapa ett bättre vaccin som både ska vara effektivt men samtidigt säkert att använda för hela befolkningen.

F. tularensis är en intracellulär bakterie vilket betyder att den använder sin värdorganismers celler för att föröka sig. Eftersom värdorganismen har ett väl utvecklat immunförsvar för att skydda sig mot just denna typ av infektion måste bakterien ha utvecklat en rad egenskaper för att kringgå detta skydd. Idag finns mycket kunskap om många intracellulära bakterier, men eftersom de är väldigt olika skiljer sig deras strategier åt. Det har publicerats relativt många vetenskapliga artiklar om virulensstrategin hos *F. tularensis* men mycket är fortfarande oklart, eller passar ännu inte in i den stora bilden.

För några år sedan identifierade forskare ett gammalt patientisolat av en Typ A stam, som var försvagad och dessutom gav ett bättre skydd än det gamla

vaccinet. För att kunna licensiera en bakteriestam som vaccin krävs att man vet exakt varför bakterien är attenuerad.

Målet med min avhandling är att identifiera vilka gener som gjorde denna stam, FSCo43, försvagad, samt försöka utreda hur dessa gener passar in i virulensstrategin hos *F. tularensis*, för att därigenom bidra med information som i slutändan kan användas vid skapandet av ett effektivt vaccin.

En av de gener som var muterade i FSCo43 var *pdpC* och genom att fullständigt mutera bort genen i LVS har vi studerat dess funktion. Vi har lyckats visa att denna LVS $\Delta pdpC$ mutant saknar förmåga att föröka sig i celler, och att mutanten inte är virulent i möss. Däremot interagerar den med värdcellen på ett liknande sätt som LVS, vilket betyder att den härmar en LVS infektion men utan att kunna sprida sig mellan cellerna, och borde ge ett bra immunsvaret vid vaccinering.

Våra studier i den mer virulenta SCHU S4 stammen visar däremot att deletion av endast *pdpC* inte ger något skydd mot luftburen smitta. FSCo43 däremot ger ett bättre skydd, troligtvis för att den har två ytterligare mutationer, men kanske också för att den fortfarande uttrycker en liten del av *PdpC*. En sådan kombination av mutationer är också intressant ur aspekten att det anses säkrare att skapa ett levande vaccin med fler än en mutation för att minska risken att det muterar tillbaka vid kontakt med andra bakterier.

Slutligen, min avhandling ökar kunskapen om funktionen hos genen *pdpC* samt fördjupar våra kunskaper om vad som händer i cellen vid en *F. tularensis* infektion. Studierna på FSCo43 har visat att det är högst sannolikt att det går att skapa ett bra levande vaccin med en högvirulent Typ A stam som bakgrund.

List of papers

This thesis is based on the following papers, which are referred to by their Roman numerals (I-IV).

Paper I

Andreas Sjödin, Kerstin Svensson, **Marie Lindgren**, Mats Forsman, and Pär Larsson. Whole-genome sequencing reveals distinct mutational patterns in closely related laboratory and naturally propagated *Francisella tularensis* strains. 2010. PLoS ONE 5(7) e11556. doi:10.1371/journal.pone.0011556

Paper II

Marie Lindgren, Linda Tancred, Igor Golovliov, Wayne Conlan, Susan M. Twine, and Anders Sjöstedt. Characterization of the spontaneous mutant FSCo43 of *Francisella tularensis* SCHU S4. *Manuscript*.

Paper III

Marie Lindgren, Jeanette E. Bröms, Lena Meyer, Igor Golovliov, and Anders Sjöstedt. The *Francisella tularensis* LVS $\Delta pdpC$ mutant exhibits a unique phenotype during intracellular infection. 2013. BMC Microbiology 13(20). doi:10.1186/1471-2180-13-20

Paper IV

Marie Lindgren, Kjell Eneslätt, Jeanette E. Bröms, and Anders Sjöstedt. Importance of PdpC, IglC, IglI, and IglG for modulation of a host cell death pathway induced by *Francisella tularensis* LVS. *Manuscript*.

Abbreviations

BSL	Bio-safety level
DNA	Deoxyribonucleic acid
FPI	<i>Francisella</i> pathogenicity island
FSC	<i>Francisella</i> strain collection
GFP	Green fluorescent protein
LDH	Lactate dehydrogenase
LVS	Live vaccine strain
LAMP-1	Lysosomal-associated membrane protein 1
MOI	Multiplicity of infection
ORF	Open reading frame
PAI	Pathogenicity island
PAMPs	Pathogen-associated molecular patterns
PS	Phosphatidylserine
PCR	Polymerase chain reaction
PI	Propidium iodide
ROS	Reactive oxygen species
RT-qPCR	Reverse transcriptase quantitative PCR
RNA	Ribonucleic acid
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
TLR	Toll-like receptor
TEM	Transmission electron microscopy

1 Introduction/Background

1.1 Aim

- To determine the mechanisms of attenuation of the spontaneous *F. tularensis* strain FSC043
- To define the function of the PdpC for the pathogenesis of *F. tularensis*

1.2 History and taxonomy

The Gram-negative bacterium *Francisella tularensis* was first identified as a pathogen in 1911 since it was the causative agent of a plague-like outbreak among rodents (Sjöstedt, 2007). It was given the name *Bacterium tularensis* after the Tulare County in California, where it was originally found, and it was later renamed *Bacterium tularensis* by Edward Francis, who was the first researcher to describe the bacterium and grow it *in vitro*. Over the years, this pathogen has been known by several designations, including *Bacterium tularensis* and *Pasteurella tularensis*. Its current name was given to honor Francis, who performed numerous investigations of tularemia and *F. tularensis*.

Throughout the years, *F. tularensis* has been grouped together with many different species of bacteria but was eventually grouped in a family, *Francisellaceae*, containing only one genus but several species, including *tularensis*. The species *F. tularensis* comprises several subspecies, two of which are of clinical relevance: subspecies *holarctica*, and subspecies *tularensis* (Table 1). The *holarctica* subspecies, Type B, can be found in most of the Northern hemisphere whereas the *tularensis* subspecies, Type A, is essentially located to North America (Johansson et al., 2004). The *holarctica* subspecies has been known to cause a less severe form of tularemia, which is the name of the disease caused by *F. tularensis*.

Table 1. Comparison of Type A and B subspecies of *F. tularensis*.

Subspecies	Type	Prevalence	Severity of illness (humans)	Infectivity
<i>tularensis</i>	A	North America	Severe	High
<i>holarctica</i>	B	Northern hemisphere	Mild	High

1.3 Vaccine development

Due to its high infectivity, as few as 10 bacteria are enough to cause disease, *F. tularensis* has been placed on the Category A list by the CDC (Centers for

Disease Control and Prevention). The Category A includes *Yersinia pestis* (plague) and *Bacillus anthracis* (anthrax), as well as three other high priority organisms. All these organisms are considered to pose a major public threat if ever used in bioterrorism. A lot of research has been made on these six agents, and after September 11, 2001, a lot of resources were focused on speeding up the process of making vaccines and treatments against the Category A agents. The main focus of the tularemia field is to create an effective vaccine against infection by the Type A subspecies, including infection caused by the inhalation route.

F. tularensis has historically been used in several warfare agent programs (Dennis et al., 2001), and already in the middle of the 20th century, the Live Vaccine Strain (LVS) was approved for use as a vaccine (Wayne Conlan and Oyston, 2007). This is an attenuated strain of the Type B subspecies and it has been used to vaccinate high-risk personnel but has never been licensed as a public vaccine. The LVS is the most commonly studied *F. tularensis* strain and is used as a model for the virulent Type A and Type B strains. A problem with LVS is that it does not give satisfactory protection against inhalational tularemia and it is also presumed to be too virulent to be safely given to immunocompromised persons. Vaccination using LVS is accomplished by adding a drop of live bacteria on the skin followed by scarification to mix the bacteria with the blood. Numerous studies of the immune responses of former tularemia patients and vaccinees illustrate the good protective properties obtained either by a natural infection or vaccination, and that the acquired immunity is extremely long-lived, for at least 25 years (Eneslätt et al., 2011).

When *F. tularensis* enters the host, the host is rarely aware of the infection. The bacteria are able to enter the host's cells without being recognized as a threat, and are able to proliferate for several days, before the immune response is activated (Elkins et al., 2003). The host, of course, has a number of different strategies to detect intruders. Due to long and successful evolution, however, *F. tularensis* are able to evade most of these initial defense mechanisms. During infection, there are some signal pathways that are activated, indicating that the infection is not completely silent to the host. This probably means that the most likely strategy to obtain a good vaccine against tularemia requires the use of live bacteria and not a subcomponent vaccine. The cell-mediated immune response that seems essential for eradication of the infection does not seem to be completely triggered without the use of live bacteria. Although several attempts of using killed bacteria or bacterial lysates as vaccines against *F. tularensis* has been reported, none of these methods confer sufficient protection to be accepted as a vaccination strategy.

1.4 Hosts and infection routes

Tularemia is generally described as a disease with flu-like symptoms: fever, nausea, joint pain, fatigue, and swollen lymph nodes close to the site of infection (Sjöstedt, 2007). The route of infection does seem to play a crucial role for manifestations of symptoms and their severity. The disease can be contracted via various routes and normally the tick/mosquito transmission is the most common. There are reports of infection by ingestion of contaminated food or water, through lesions on the skin, and the most severe one, infection by inhalation. This last route of infection may result in lethal progress of the disease, at least when caused by the Type A subspecies. Treatment is usually a course of antibiotics but one danger of the disease has been that the symptoms are unspecific and that routine testing for *F. tularensis* is rarely done. As previously mentioned, the main route of transmission of infection is believed to be by arthropod vectors and the current model considers Type A subspecies to be more often tick-borne while the Type B subspecies is adapted for transfer via ticks or mosquitoes, although this differs from region to region (Mörner et al., 1988; Telford and Goethert, 2011). *F. tularensis* is able to infect a large number of mammals, including rabbits and other lagomorphs, which is why the disease is sometimes referred to as rabbit fever; there are several reports describing how the disease has wiped out entire rabbit populations. In humans, the estimated incubation time can be as short as 2-5 days (Svensson et al., 2009).

As has been mentioned, *F. tularensis* is able to infect a multitude of hosts and the virulence of the bacterium differs among different hosts. The LVS, which is attenuated in humans, is still highly virulent in laboratory mice, which are commonly used as an animal model. Other mammalian models include rats and monkeys. The highly virulent SCHU S4 strain, which was isolated from an ulcer in the early 1900's, is of course even more virulent in these small animal models. To study the infection route via arthropods, the well-known *Drosophila melanogaster* model has been used. Other eukaryotic models include the nematode *Caenorhabditis elegans* and amoeba. There are also fish model systems established for the study of the fish pathogens in the *Francisella* family.

1.5 Intracellular infection model

The main aim of a pathogen is to multiply. In order to multiply, the pathogen requires nutrients and a safe environment. The intracellular pathogens have evolved strategies to use the living cell as a safe-house for proliferation and source of nutrients. But there is a long way to go from entering the body to being able to live and thrive. Well inside its multicellular host, the pathogen must find a host cell to hide inside to prevent discovery by the host's intricate immune system, a defense system that has co-evolved with pathogens over the ages.

1.5.1 Uptake and replication

The immune system consists of a large number of cells specialized to either attack foreign matter, or warn and engage other cells in the attack. Among the first types of immune cells to encounter pathogens are the leukocytes (white blood cells), such as the phagocytic neutrophils and macrophages. These cells are recruited to the site of infection and specialize in engulfing potentially harmful matter, such as bacteria. Many intracellular bacteria have adapted to live inside these phagocytic cells, and allow themselves to be phagocytosed but are then able to evade the cell's attempts to destroy them.

Phagocytes are able to separate foreign and non-foreign particles by the aid of receptors on the cell's surface. The most common receptors used to recognize microbes are the Toll-like receptors (TLR) (Akira and Takeda, 2004; Beutler, 2009). These receptors bind to pathogen-specific molecules such as LPS, a surface molecule found on Gram-negative bacteria. This target binding initiates an inflammatory cascade, among other things leading to secretion of cytokines.

F. tularensis is taken up by phagocytic cells by a method similar to regular phagocytosis (Clemens et al., 2012), although it is unclear if the bacteria induces the uptake, or passively allow themselves to be engulfed. What is known is that this bacterium's LPS is only weakly inflammatory (Sandström et al., 1992; Telepnev et al., 2003), but that something expressed by the bacteria is recognized by the TLR2 receptors (Katz et al., 2006).

During the process of phagocytosis, the bacteria are internalized in a membrane compartment known as the phagosome (Figure 1). This structure will later be fused with the lysosome resulting in acidification and exposure to highly antibacterial enzymes, thereby leading to killing of the internalized bacteria. *F. tularensis* is able to prevent this acidification (Clemens et al., 2004), and within at most a few hours, the bacteria can be found in the cell cytoplasm, where they can replicate to high numbers. By preventing the host cell from signaling to other cells that it has become infected, the bacteria are hidden from the hostile surroundings and can focus on nutrient acquisition and growth.

The initial phase of a *F. tularensis* infection is silent with regard to response from the immune system (e.g., cytokine response). The first signs of cytokine response in mouse cells are seen after 24 hours. This gives the bacteria a lot of time to spread and since it usually is via means of inflammation that the host knows it is sick, this gives the bacteria a head start and probably results in a prolonged time of illness, or in smaller mammals, sepsis and death.

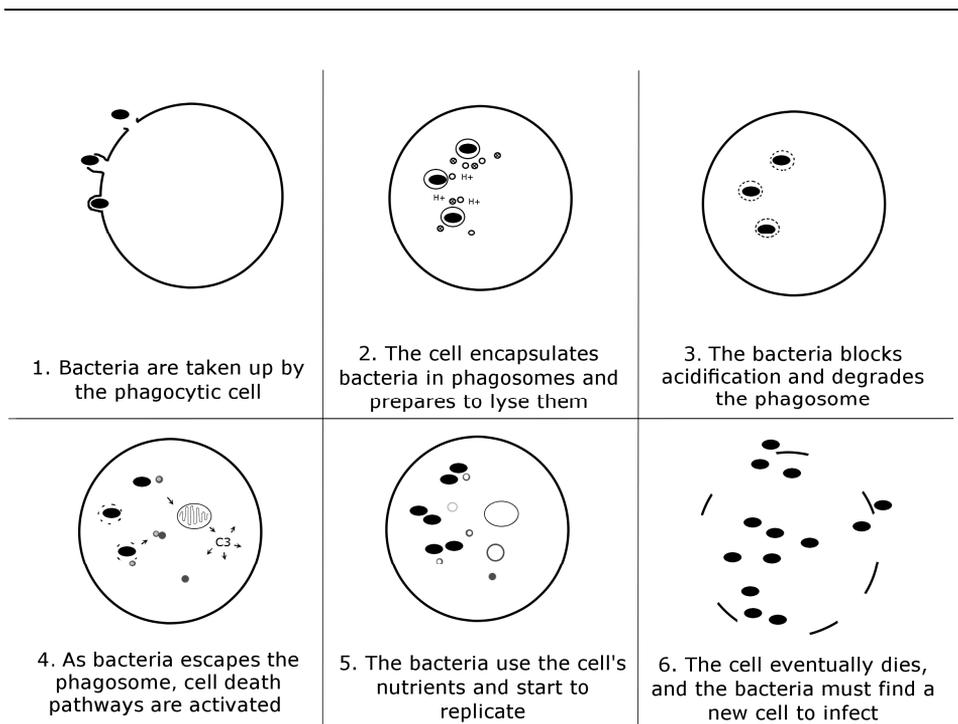


Figure 1. Intracellular bacteria infection model. Note that step 4 and 5 occur simultaneously.

1.6 Programmed cell death

During the time when the bacteria are internalized, escape, and multiply, several other warning systems have been activated in the infected cell. One of them is programmed cell death, a tightly controlled self-destruction mechanism that can be induced to get rid of cells that either poses a direct threat to other cells, or are redundant and take up valuable nutrients for other cells (Ashida et al., 2011; Lamkanfi and Dixit, 2010). There are many different kinds of cell death but in this chapter we will focus on only two of them: apoptosis and pyroptosis.

1.6.1 Apoptosis

The number of cells in a multicellular organism has to be tightly regulated, through division and through deletion, in order to keep tissues and organs healthy, and at a constant size. Apoptosis is one of the most studied of these eradication mechanisms. This mechanism allows single cells to be removed from the cell population and occurs regularly in a multicellular organism. Failure to induce cell death often leads to autoimmune or inflammatory diseases. A physically injured cell will undergo necrosis by bursting, spilling its contents, and causing inflammation in the surrounding tissue. In contrast, the

apoptotic cell will break down from inside, shrink, and, by displaying certain markers on its surface, will be rapidly phagocytosed by other cells (Fink and Cookson, 2005; Wickman et al., 2012). These phagocytosing cells can then utilize the dead cell's resources for themselves at the same time as preventing the inflammatory reaction that would have followed if the cell contents uncontrollably leaked out into the surroundings.

As apoptosis is often used by the host cell to protect the host against the infection, intracellular bacteria such as *Mycobacterium* have developed strategies to prevent apoptosis in order to protect their safe environment (Menaker and Jones, 2003), while others like *Yersinia* are able to induce apoptosis in order to prevent phagocytosis (Bergsbaken and Cookson, 2009). This may also be a strategy to aid cell to cell spread, with minimal immune signaling.

Apoptosis is also known as caspase-mediated cell death. The mechanism, or pathway, for apoptosis is similar in all animal cells and rely on the activity of certain proteases, called caspases. There are effector caspases and initiator caspases, and most of them are present in the cell in a pro-caspase form and must be cleaved in order to become activated. The activated caspases can then proceed to cleave and activate other caspases that in turn will cleave proteins and fragment the DNA, all which is part of the process of cell death (Figure 2). The initial activation of caspases is triggered either by an extracellular event (extrinsic pathway), or by an internal mechanism (intrinsic pathway) (Lamkanfi and Dixit, 2010).

The extrinsic pathway is mainly activated by the binding of ligands, such as FasL and TNF- α , to specific receptors on the cell surface, leading to activation of effector caspases 3 and 7, and subsequent DNA fragmentation and apoptotic bodies, membrane-bound vesicles containing the degraded cell (Slee et al., 1999). These vesicles are taken up by other cells and the nutrients inside are recycled. Cells can also, during stress, induce apoptosis from within. This is referred to as the intrinsic pathway and involves the mitochondria. In this pathway, mitochondria-specific ROS accumulation is followed by depolarization of the mitochondrial membrane, allowing release of the electron carrier molecule cytochrome *c* into the cell cytosol (Gross et al., 1999; Slee et al., 1999). Cytochrome *c* will bind to the adaptor protein Apaf-1, and the apoptosome is formed. This structure will, in turn, activate caspase-9 that will start to activate other caspases such as the effector caspase-3 leading to the activation of caspase-8, and finally DNA fragmentation and the formation of apoptotic bodies

Due to the potential destructive force of apoptosis, it is tightly regulated. A protein family known as the Bcl-2 family is involved in the regulation of the activation of caspases, for example by preventing cytochrome *c* release, but also by stimulating the release of this protein (Gross et al., 1999). p53 is another

regulator of apoptosis. It is involved in DNA damage control and induces apoptosis when DNA damage occurs (Taylor et al., 2008). There are an additional number of different inhibitors and promoters of apoptosis in every cell, but the complete pro- and anti-apoptotic pathways are far from being fully elucidated.

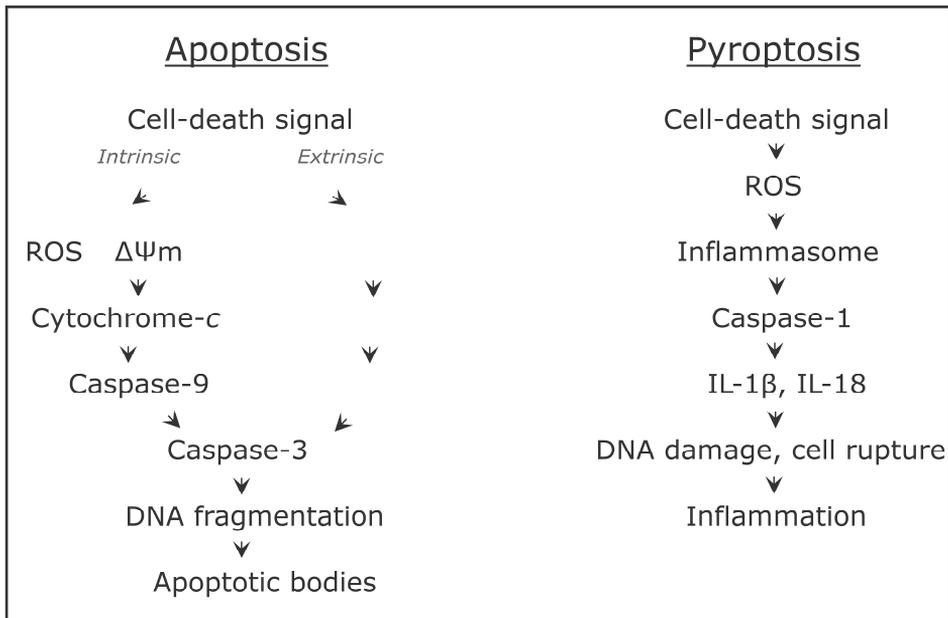


Figure 2. Cell death pathways.

1.6.2 Pyroptosis and the inflammasome

The term pyro, meaning “fire”, refers to the inflammatory outcome of pyroptosis when the cell contents leak out into the environment. This form of cell death was previously grouped together with necrosis, due to the end result. Pyroptosis is a recently characterized form of cell death induced by intracellular bacteria such as *Salmonella* (Brennan and Cookson, 2000) as well as *Francisella* (Henry and Monack, 2007). Pyroptosis is also known as caspase-1 dependent cell death, and only occurs in caspase-1 expressing cells such as monocytes. By definition, caspase-1 is activated during pyroptosis, but not during apoptosis, making the expression of this cytokine one way to distinguish these two types of cell death, but several other markers have been identified (Table 2). Caspase-1 is activated upon assembly of the protein complex known as the inflammasome, and will in turn activate the inflammatory cytokines IL-1 β and IL-18 (Jones et al., 2011, 2010). The secretion of these two pro-inflammatory cytokines results in the inflammatory reaction seen at the completion of pyroptosis (Figure 2).

During *Francisella* infection, as the phagosomal membrane is disrupted, bacterial compounds and DNA leak out into the host cell's cytoplasm. When the concentration of these compounds is high enough – indicating a certain number of intracellular bacteria – the protein complex known as the inflammasome is assembled. There are different kinds of inflammasomes and the AIM2 inflammasome is the one activated during infections with *Francisella* (Jones et al., 2010). The AIM2 protein, which is present in the cell cytosol, binds to *Francisella* DNA, and then to the ASC protein, forming the inflammasome. The inflammasome will cleave pro-caspase-1 to caspase-1. Caspase-1 will cleave pro-IL-1 β , and the mature form together with IL-18 is secreted from the cell, causing an inflammatory reaction (Henry and Monack, 2007). *Francisella*-induced inflammasome formation leads to host cell death via the pyroptotic pathway, and the release of DNA also activates the type I interferon response, including IFN- β (Henry and Monack, 2007). It has been suggested that the amount of DNA released from the phagosomes is too little to activate the inflammasome complex, and that the production of IFN- β is required to trigger a feedback loop leading to more killing of *Francisella* with subsequent DNA release followed by activation of the AIM2 inflammasome.

Table 2. Cell death comparison.

	Apoptosis	Pyroptosis
Apoptosis-specific DNA fragments	+	-
Caspase-1 activation	-	+
Caspase-3 activation	+	-
Caspase-9 activation	+	-
Cell swelling	-	+
Cytochrome <i>c</i> release	+	-
Inflammatory	-	+
Maintained membrane integrity	+	-
Maintained mitochondrial integrity	-	+

The induction of apoptosis and pyroptosis can both be triggered by infection. The big difference between these two pathways is that apoptosis leads to a non-inflammatory cell death whereas pyroptosis leads to inflammation in the surrounding tissues. Still, pyroptosis and apoptosis are to some extents overlapping. Our knowledge about these cell death mechanisms is far from complete and it is unclear if several cell death pathways can be active in the same cell at the same time.

1.7 The *Francisella* pathogenicity island

Many *Francisella* isolates have been sequenced, and most of them have been *F. tularensis* strains. This facilitates mutagenesis and gene manipulation that can be used to knock out a specific gene to see what it was required for. When *F. tularensis* sequences have been compared, one large cluster of genes has been receiving a lot of attention; the *Francisella* pathogenicity island (FPI). A pathogenicity island (PAI) is defined as a cluster of genes involved in the virulence (infection strategy) of an organism. It is also often considered that a true PAI should have been acquired by horizontal gene transfer, *i.e.* transferred from one species to another, rather than acquired due to mutations in the organism's own genome. Due to this latter part, the GC and AT contents of a PAI often differs from that in the actual genome of the organism. The debate of whether or not the FPI is a true pathogenicity island has not been resolved, but since it has GC content different from the remaining genome of *F. tularensis* and is surrounded by transposon regions, this indicates that this region has migrated from another species in the past. It is also clear that large parts of the FPI are conserved among the *Francisella* species and subspecies. The FPI region is duplicated in both Type A and Type B subspecies of *F. tularensis*, in contrast to the *F. novicida* species that only has one copy of the pathogenicity island. Some of the gene products of the FPI are secreted and it has been suggested that the FPI is encoding for a secretion system similar to the Type VI secretion system found in many other bacteria (Bröms et al., 2010; Nano and Schmerk, 2007).

2 Results

2.1 FSCo43

The initial purpose of my project was to identify the cause of attenuation in Type A strain FSCo43. The progenitor of this strain, the SCHU strain, was originally isolated from a patient in 1941 demonstrating that it was virulent at the time of isolation; the FSCo43 strain was derived from the variant SCHU S4 that was first isolated in 1951 (Eigelsbach et al., 1951). Over time this isolate was passaged on plates and acquired mutations, before it was finally stock frozen and entered into the *Francisella* Strain Collection as number 43. It was not until later, when the collection was screened for attenuated strains, that the deviating phenotype of the strain was discovered. Twine et al. reported in 2005 that this strain was attenuated in mice, macrophages, and also that it conferred rather prominent protection when mice were immunized with it. It also resulted in less inflammation in the mouse skin, compared to the LVS vaccine, when it was administered intradermally. As the FSCo43 is a Type A *F. tularensis* strain, these findings indicated that it was possible to construct a live vaccine from the virulent subspecies. A Type A vaccine strain would have an antigenic composition more similar to virulent type A strains than LVS has, resulting in a strain more efficient as a vaccine against Type A *F. tularensis*.

2.1.1 Gene expression

In the published article, Twine et al. discussed the reason for attenuation in this strain (Twine et al., 2005). They found that the FSCo43 strain had a fusion gene consisting of the N-terminus of *fupA* (ORF *FTT0918*), and the C-terminus of *fupB* (ORF *FTT0919*), identical to the fusion found in the attenuated subsp. *holarctica* strain LVS (Figure 3). For a list of the genes used in this thesis see Table 3.

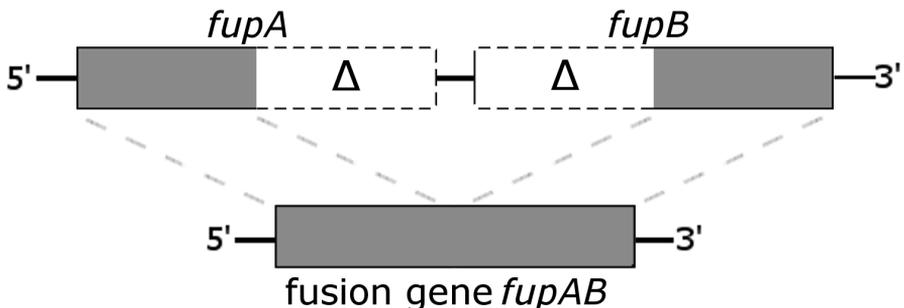


Figure 3. Schematic view of the fusion gene in FSCo43 and LVS.

The presence of this fusion gene was not enough to explain all the differences, however, so the protein expression was compared between FSCo43 and the parental, virulent SCHU S4 strain. Eight proteins were found to be differentially expressed in the attenuated FSCo43 strain and the first part of my project was to see whether this differential expression also was seen on a genetic level. By isolating mRNA, transcribing it to cDNA, and running quantitative PCR amplifying these eight genes, I was able to show that there was only one difference on a genetic level; the expression of the FPI-localized gene *pdpE* was decreased in FSCo43.

Due to the interesting location of *pdpE*, the expression of all the other genes of the pathogenicity island was tested; the largest, and most prominent, difference was that the gene right next to *pdpE*, *pdpC* (pathogenicity determinant protein C), was not expressed at all with the primer pair I used. Since the wildtype subsp. *tularensis* has two copies of the pathogenicity island, we thought that maybe FSCo43 had somehow lost one of them, explaining the downregulation seen in these two genes. This hypothesis was disproven as PCR on genomic DNA revealed identical DNA fragments of both FPIs in FSCo43 as well as SCHU S4. At the time, there were only a few genes on the island that had been studied in more detail, but by using bioinformatics, it was hypothesized that the FPI consisted of two large operons, and that it was at the end of the largest operon that *pdpC* and *pdpE* were located (Nano and Schmerk, 2007). What was interesting was that upstream of *pdpC*, there was a small non-coding region large enough to hold a separate promoter for *pdpC* and 1355. A brief inspection of the genomic area in question indicated that there could very well be a promoter there; nucleotide sequences resembling the -10 and -35 elements were present in roughly the correct locations. However, as the mRNA transcript of prokaryotic operons can include an unknown number of genes, we could test this by running PCR on cDNA obtained from the laboratory LVS strain, on which the FPI is very similar to that of SCHU S4. One primer targeted the end of ORF 1353 and the other in the beginning of 1354, and if an amplicon was obtained the transcript spanned over both genes, and they most likely had the same promoter. The only promoter we identified using this method was the one already proposed to be located upstream of the FPI gene *FTT1344* (Nano and Schmerk, 2007). So it seemed that the putative promoter mutation was not the explanation for the downregulation of the two genes.

2.1.2 Sequencing FSCo43

The next step was to sequence the region. First, the two FPI copies were separated using PCR after which the genomic region in question was divided into eight parts, cloned into a vector and sequenced. The results were obvious; in the entire region only one difference was consistent among the different

clones sequenced: the deletion of one adenine (A) in a sequence of 8, approximately 630 nucleotides into the *pdpC* gene (Figure 4). This deletion was found in both copies and was confirmed when the entire FSCo43 genome was sequenced a few years later, as described in PAPER I.

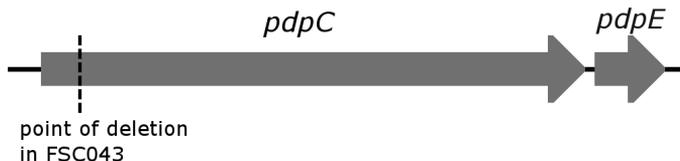


Figure 4. Point of deletion in the *pdpC* gene of *F. tularensis* strain FSCo43.

This deletion of one A resulted in a stop codon only a few bases downstream the deletion, leading to a truncated protein. Since we had already seen that the expression of *pdpC* was downregulated, we hypothesized that the deletion was the cause of reduced expression. The primers we used for qPCR targeted the middle of the gene, downstream the deletion, but when we used primers targeting the area upstream of the mutation, it was clear that this region of the gene was expressed at similar levels as the wildtype, and thus was unaffected by the deletion.

Table 3. Gene names and annotations used in this thesis.

ORF	Gene name	Comment
<i>FTLo439</i>	<i>FTLo439</i> ^a	Fusion gene between <i>fupA</i> and <i>fupB</i> , containing deletion of the 3' region of <i>fupA</i> and 5' region of <i>fupB</i> . Present in LVS and FSCo43
<i>FTTo615</i>		Gene with nonsense mutation in FSCo43
<i>FTTo918</i>	<i>fupA</i>	Also known as 12A. N-terminus present in FTLo439 fusion protein
<i>FTTo919</i>	<i>fupB</i>	Also known as 12B. C-terminus present in FTLo439 fusion protein
<i>FTT1354</i>	<i>pdpC</i>	FPI gene, main focus of this thesis. Contains a nonsense mutation in FSCo43
<i>FTT1355</i>	<i>pdpE</i>	FPI gene directly downstream <i>pdpC</i> . Downregulated in FSCo43
	<i>iglA</i>	FPI gene, mutant is attenuated and used as a negative control
	<i>iglC</i>	FPI gene, mutant is attenuated and used as a negative control
	<i>iglG</i>	FPI gene, LVS Δ <i>iglG</i> shows intermediate attenuation in cells
	<i>iglI</i>	FPI gene, LVS Δ <i>iglI</i> shows intermediate attenuation in J774 cells

^a This annotation (SCHU S4 Δ *FTLo439*) has been used in this thesis to describe a mutant containing the same fusion between *fupA* and *fupB* that is found in LVS and FSCo43.

There are a number of regulatory mechanisms of both transcription and translation in prokaryotes. Among the described mechanisms there are a few that can explain the reduced expression we see in genes *pdpC* and *pdpE*. It is generally believed that the termination of transcription in prokaryotes is either Rho-dependent or Rho-independent (Boudvillain et al., 2010). In the former, the Rho protein reduces the binding between mRNA and DNA template so that the mRNA molecule is released, in the latter, the mRNA molecule itself forms a hairpin and this formation of a hairpin secondary structure is enough to break the binding between mRNA and template, but it is also possible that the mutation renders the transcript unstable, making it hard for the RNA polymerase to bind. Another theory is about polarity, which states that the RNA polymerase becomes detached after initiation of a premature stop codon (nonsense mutation) (Gowrishankar and Harinarayanan, 2004). This could explain why there seem to be some but not full expression of the *pdpE* gene. However, it does not explain why the distal part of *pdpC* seems to be completely untranscribed, while *pdpE* has a reduced - but measurable, expression.

Aside from the already known fusion of genes *fupA* and *fupB*, and the single mutation in both copies of the FPI gene *pdpC*, there was only one more genomic difference in the attenuated FSCo43, compared to the virulent wildtype SCHU S4; the deletion of two nucleotides in ORF *FTT0615*, a gene coding for a protein of unknown function but with homology to metal ion transporters. The mutation in *FTT0615* was also a nonsense mutation, resulting in a truncated protein. A truncated protein often loses its function, and in the case of PdpC, only 1/5 of the protein was expressed. In *FTT0615* about half the protein was theoretically still expressed in FSCo43. Due to time limitations, we decided to omit the *FTT0615* from further studies, as nothing in the literature, or in its localization on the genome, indicated that it contributed to virulence.

2.1.3 Complementation

Although there are three genetic events separating the FSCo43 and the SCHU S4 strains, we decided to focus on only two of them for complementation; the fusion protein FTLo439 and the deletion in both copies of the FPI gene *pdpC*.

When studying mutations, it is very useful to include complemented strains in the experiments. When making complemented strains you start with the mutant strain and genetically modify it to express the gene that is mutated. In most cases this means reintroducing the gene that is faulty in the mutant, but in the case of FSCo43 there are several mutations and in order to know which one of them is the cause of a certain phenotype, you can create several complemented strains, complementing one gene at a time. A fully complemented strain should have the same phenotype as the wildtype strain, in this case, SCHU S4.

As already mentioned, we decided to ignore the *FTT0615* mutation and instead focus on the other mutations. After failing to create a fully complemented strain in *trans* (on a plasmid, in this case), we successfully replaced the fusion gene with the two original genes *fupA/fupB* in *cis* (on the chromosome, in this case). Thereafter we were able to restore expression of genes *pdpC* and *FTT1355* (*pdpE*) in *trans*, but in order to restore the correct expression levels, it would be best to have all complementations in *cis*. This would also enable further genetic modifications such as introducing the GFP-expressing gene.

2.1.4 Intracellular phenotype

F. tularensis has developed a highly successful strategy to survive intracellularly, it escapes the phagosome and replicates in the cytosol. Mutants that are unable to escape the phagosome also seem incapable of replicating. When studying the intracellular localization of FSCo43, and SCHU Δ *pdpC*, we realized that a small proportion of bacteria were able to escape the phagosome and replicate, while the large majority of bacteria were trapped inside a phagosomal membrane. More detailed studies on these two populations in FSCo43, using electron microscopy, confirmed that the first group seemed to emulate the wildtype phenotype with degradation of the phagosomal membrane and multiplication in the cytoplasm. This is very similar to what Long et al. saw with their intron *pdpC* mutant in SCHU S4 (Long et al., 2012).

2.1.5 Animal studies

Vaccination with the FSCo43 strain protects mice against intradermal wildtype infection. A SCHU S4 strain with the *FTL0439* fusion gene also worked well as a vaccine strain, even against intranasal challenge, while vaccination with FSCo43 only protected half of the mice from an intranasal challenge. The problem with the *FTL0439* mutant was that it was highly virulent; only ten bacteria could be used of this mutant compared to ten million of the FSCo43 strain.

2.2 PdpC

2.2.1 General information

The estimated open reading frame (ORF) of *pdpC* is (in LVS and SCHU S4) 3987 nucleotides, and the estimated protein size is 156 kDa. This is a fairly large protein in a bacterium and the sheer size makes it hard to work with on a molecular level. When screening the protein using various bioinformatic tools not much was found. PdpC contains putative transmembrane regions, which may be expected for a protein this size. Reviewing the published data on this gene and protein not much can be found, to date. It got its name “pathogenicity

determinant protein” from its location in the well-studied FPI (Figure 5), and is required for intracellular growth in SCHU S4 (Long et al., 2012), but not in *F. novicida* (Read et al., 2008; Åhlund et al., 2010). The *F. novicida* strains harbor only one copy of the FPI, and although most of the region is identical to *F. tularensis*, *pdpC* is not. It is slightly smaller in *F. novicida*, and with more than 70 amino acids difference, the protein function may be different in the two species. In contrast, PdpC is conserved among the Type A and B strains of *F. tularensis*, indicating that their function may be conserved, which is why we decided to start working on the BSL2 strain LVS. Using whole-cell lysate and centrifugation, we were able to determine the localization of PdpC to be in the inner membrane, with a small fraction in the cytoplasm.

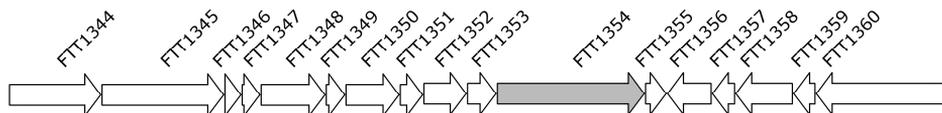


Figure 5. The *Francisella* pathogenicity island. The *pdpC* gene (FTT1354) is in grey.

2.2.2 SCHU S4 Δ pdpC

The attenuation seen in FSCO43 can be traced back to only four genomic events. One of these events is the fusion of the *fupA* and *fupB* genes, and when recreating this mutation in a SCHU S4 background, the resulting mutant SCHU S4 Δ FTLO439 was much more virulent than the FSCO43 strain. Therefore, we concluded that the fusion mutation was not enough to explain the FSCO43 phenotype. As already mentioned, we have focused instead on the FPI gene *pdpC*, which contains a nonsense mutation in both copies. The first thing we did was to create a deletion mutant of *pdpC*, *i.e.* we removed the two copies of the gene, leaving only a few nucleotides, and making sure not to disrupt the reading frame to ensure unaltered expression of the downstream gene *pdpE*. Since the downregulation of the *pdpE* gene in FSCO43 could be due to its *pdpC* mutation, we tested the expression of all FPI genes in the SCHU S4 Δ pdpC mutant. There were no differences in expression of the other FPI genes between the Δ pdpC mutant and the wildtype SCHU S4 strain.

As seen in Paper II, the SCHU S4 Δ pdpC mutant was completely avirulent in both cells and mice, and was unable to cause cytotoxicity in the infected cell. However, it had a similar phenotype to that of FSCO43, with a small subset of bacteria replicating, as evident by LAMP-1 colocalization experiment. Despite this, there was no intracellular net growth of the mutant, and it was non-protective against an intranasal challenge, in contrast to the FSCO43 strain, which afforded significant, but not complete, protection. Further, the expression of genes in the FPI was indistinguishable, in the SCHU S4 Δ pdpC mutant, compared to the wildtype strain. All in all, this indicated that the attenuation

seen in FSC043 was the combined result of several mutations. Our hypothesis is that the fusion gene *FTL0439* together with expression of the much truncated form of PdpC could explain the FSC043 phenotype, but this needs to be verified experimentally.

2.3 LVS Δ pdpC

All work with the virulent *F. tularensis* strains requires a BSL3 environment, and due to the many safety regulations and considerations, the types of assays that can be performed in BSL3 are limited. To get around this, but still work with the *pdpC* gene, we created a similar deletion mutant in the attenuated model strain LVS, which can be handled under regular BSL2 restrictions and therefore is less tedious and complicated to work with.

2.3.1 Expression patterns

The two FPI copies in subspecies *tularensis* and *holarctica* are essentially identical, which is why we expected the expression patterns of the FPI genes in SCHU S4 Δ pdpC and LVS Δ pdpC to be similar. This turned out to be false, as the expression of a number of FPI genes in the LVS Δ pdpC mutant was significantly lower compared to the expression in wildtype LVS (Paper III). In addition, a number of FPI proteins were downregulated in the LVS mutant, indicating that the function of PdpC is not the same in LVS and SCHU S4.

2.3.2 Intracellular growth

Most of the previous publications involving *pdpC* deal with *F. novicida* (U112), which is a non-virulent *Francisella* species. The published data on the *novicida* Δ pdpC mutant reveals it to be as virulent as the wildtype in *Drosophila* attenuation studies (Åhlund et al., 2010), and it has never been detected to be required for virulence in any screen. I have also confirmed that the transposon Δ pdpC mutant in U112 grows as well as wild type in J774 cells, in contrast to the SCHU S4 Δ pdpC, which was avirulent. As LVS is more closely related to SCHU S4 than to *novicida*, we expected the LVS Δ pdpC strain to behave similar to the SCHU S4 mutant.

As with the SCHU S4 mutant, we infected the mouse macrophage-like cell line J774 A.1 with LVS and LVS Δ pdpC. LVS is harmless to humans with a normal immune system, but is highly virulent in mice and various cell types. We found that the uptake of the mutant was similar to that of LVS, but that the mutant strain did not replicate over a period of 72 hours, in contrast to the wildtype, which replicated at least 2 logs over a period of 24 hours, whereafter the number of cells declined due to the infection. The cells were visually inspected during the experiment and it was clear that both the LVS- and Δ pdpC- mutant

infected cells differed morphologically from uninfected cells, or cells infected with the attenuated $\Delta iglC$ mutant. This difference in morphology indicated that the $\Delta pdpC$ mutant had some kind of negative impact on the host cells. We collected supernatants to evaluate cell death by measuring the amount of LDH, a cytoplasmic enzyme that can only be found in the supernatant due to cell leakage. The amount of LDH was high in cells infected with the LVS strain, and low in uninfected cells, or cell infected with the $\Delta iglC$ mutant. There was a lack of consistency when it came to LDH levels in $\Delta pdpC$ mutant infected cells; in some experiments there was no difference to uninfected cells, while in other the levels of LDH were similar to those seen in LVS infected cells. This led us to suspect that the different outcomes were linked to the multiplicity of infection (MOI), *i.e.* the number of bacteria used to infect each cell. To test this hypothesis we used different MOI and collected supernatant samples. The results showed that the higher MOI we used for the $\Delta pdpC$ mutant, the higher the LDH release was. Regardless of MOI there was never any replication recorded for the $\Delta pdpC$ mutant. It should also be mentioned that the MOI had only limited impact on an LVS or $\Delta iglC$ mutant infection.

2.3.3 Intracellular localization

It was clear that the $\Delta pdpC$ mutant had an effect on the host cell, but that this effect was not linked to replication. The next step was to study the intracellular localization of the mutant, *i.e.* if it was able to escape the phagosome.

Bacteria engulfed by a macrophage will be internalized in membrane structures known as phagosomes. The phagosomal membrane contains the late endosomal marker LAMP-1 (Lysosomal-associated membrane protein 1). By using a fluorescent antibody against this protein the membrane can be visualized, and by infecting cells with GFP-expressing bacteria we can get an estimation of the proportion of bacteria localized within phagosomes.

The LAMP-1 antibody binds to the phagosomal membrane as well as the cell membrane, making it easy to discern individual cells and individual phagosomes. The $\Delta iglC$ mutant is known to be clearly localized to the phagosomes, while the phagosomes of LVS infected cells are disrupted within a few hours, which we could confirm. The $\Delta pdpC$ mutant was also clearly localized to intact phagosomes, although less prominent than those containing the $\Delta iglC$ mutant. We decided to examine the cells by transmission electron microscopy (TEM) and found that although the phagosomal membrane around the $\Delta pdpC$ mutants seemed intact using immunofluorescence, there was clear fragmentation of the membrane when viewed using TEM. So, the $\Delta pdpC$ mutant did not seem to fully escape from the phagosomal membrane, but was able to disrupt it and therefore colocalize with molecules present in the cell cytoplasm.

2.3.4 General cytokine expression

Cytokines are molecules used for various kinds of cell signaling and allow cells to communicate with each other. Cytokines play an important role in the immune system and are used as markers for infection or apoptosis. We compared the cytokine expression of cells infected with LVS to that of cells infected with the $\Delta pdpC$ mutant. Uninfected cells and cells infected with the attenuated $\Delta iglC$ mutant were used as controls. It was evident that the cytokine expression, of both genes and proteins in cells infected with the $\Delta pdpC$ mutant was much more similar, but not identical, to that of cells infected with LVS. The expression in uninfected cells and cells infected with the $\Delta iglC$ mutant was completely different from the other infections.

2.3.5 IL-1 β

One molecule that can be detected in the cell supernatant is the cytokine IL-1 β . The secretion of this cytokine has been linked to the assembly of the protein complex known as the inflammasome (Fernandes-Alnemri et al., 2009; Jones et al., 2010). In an LVS-infected cell the amount of IL-1 β increases dramatically within a few hours after infection, while the amount in uninfected cells, or cell infected with the $\Delta iglC$ mutant, is below limit of detection even 24 hours after infection. The IL-1 β levels in $\Delta pdpC$ mutant-infected cells were similar to those of LVS at 5 hours, but did not increase as much as for LVS up to 24 hours after infection. It has previously been postulated that there needs to be replication in order for the inflammasome to be assembled, but as the $\Delta pdpC$ mutant infection induced IL-1 β secretion there seems to be enough with only the leakage of DNA and/or other bacterial molecules, out into the cell cytoplasm in high enough amounts, to activate the inflammasome.

2.3.6 TNF- α

On the cell surface there are a number of receptors for various PAMPs (pathogen-associated molecular patterns) which enable the cell to bind to pathogen specific molecules and in turn activate the immune system. One type is represented by the TNF (Tumor Necrosis Factor) receptors. They are major activators for intrinsic apoptosis. Most cells have TNF receptors 1 and 2 and if TNF binds to receptor 1 the apoptotic pathway is activated (Parameswaran and Patial, 2010; Silke, 2011). Binding of the receptor can also stimulate inflammatory response from the cell.

The TNF molecules are secreted by infected cells in order to activate apoptosis either in the surrounding cells or in the secreting cell, in response to an infection. LVS has been shown to inhibit *Escherichia coli* LPS-induced secretion of TNF- α and we have seen that the same is true for the LVS $\Delta pdpC$ mutant. In

contrast, there is no inhibition of TNF- α secretion by the attenuated \DeltaiglC mutant. *E. coli* LPS is used to induce TNF- α secretion, since *F. tularensis* LPS is non-inflammatory, and does not induce the secretion of this molecule (Sandström et al., 1992; Telepnev et al., 2003).

2.3.7 MitoSOX

Reactive oxygen species are by-products of the respiratory chain, and are mainly accumulated in the mitochondrion. This organelle is also the central starting point for many cell death processes. There have been many publications linking the accumulation of mitochondria-specific ROS to cell death (Alfadda and Sallam, 2012), both through the apoptotic mechanism (Li et al., 2003) and via inflammasome activation (Zhou et al., 2011). To see whether or not infection with *F. tularensis* increased ROS accumulation we used MitoSOX to label mitochondria-specific superoxides.

We could see that after 12 hours, a large portion of the cells infected with LVS was MitoSOX positive, *i.e.*, they had started to accumulate measurable levels of mitochondria-located ROS. In contrast, uninfected cells, and cells infected with the \DeltaiglC mutant were not MitoSOX positive at this time. About 10% of the cells infected with the $\Delta pdpC$ mutant were MitoSOX positive at 12 hours, but this number increased to 40% after 24 hours, a value similar to that of LVS-infected cells. The proportion of uninfected cells or cells infected with the attenuated \DeltaiglC mutant stayed below 10% even after 24 hours.

2.3.8 MitoScreen

Apoptosis is induced in different ways, but they are generally grouped into two groups; the extrinsic and intrinsic pathways. In the extrinsic pathway, apoptosis is induced by outside stimuli such as the binding to TNF receptors or Fas ligand. The intrinsic pathway is activated by intracellular stimuli and initially causes the mitochondrial membrane to become permeable. We have used the JC-1 dye to study apoptosis using flow cytometry. This dye passes through the cell membrane and into the mitochondria, where it accumulates and form red-fluorescing dimers. If the mitochondrial membrane loses its integrity the dye will leak out in the form of monomers, which fluoresces in green. An apoptotic cell will thus contain the green form of the dye, and a non-apoptotic cell will be red (See Paper IV).

We used this dye to label cells infected with *Francisella* and saw that most of the LVS-infected cells were apoptotic (green) 24 hours post infection, but only half of the cells infected with the $\Delta pdpC$ mutant were apoptotic at this time. At 48 hours the proportion of red and green cells were similar (apoptotic) in cells

infected with LVS and the $\Delta pdpC$ mutant, and completely different from that of uninfected cells or cells infected with the $\Delta iglC$ mutant.

2.3.9 Phosphatidylserine (PS)

Phosphatidylserine (PS) is expressed on the cell surface as an “eat me” signal of apoptotic cells, showing phagocytes that the expressing cell is targeted for phagocytosis. By using fluorescent annexin A5 (annexin V), a molecule that binds to PS, we were able to count the number of apoptotic cells in a cell population. By simultaneous staining with propidium iodide (PI), a dye that can only enter the cell through a ruptured membrane, we were able to discriminate between healthy cells, intact but apoptotic cells, and cells that were apoptotic and had a ruptured membrane.

After 24 hours, most of the cells infected with LVS or the $\Delta pdpC$ mutant, were either early apoptotic (annexin V positive, PI negative) or late apoptotic (annexin V and PI positive). Most of the cells in the population of uninfected cells or cells infected with the $\Delta iglC$ mutant were healthy (annexin V and PI negative). Similar results were obtained after 48 hours with the exception that at this time point, most of the LVS-infected cells were dead, whereas $\Delta pdpC$ -infected cells were almost exclusively (88%) apoptotic.

2.3.10 Caspase-9

There are a number of caspases (cysteine-aspartic proteases) involved in apoptosis. They are generally divided into two groups with respect to their function, effector caspases and initiator caspases. Caspase-9 is one of the initiator caspases and its function is to activate effector caspases. As all caspases are present in an inactive form in the cell, they must be activated to be functional, usually through cleavage. This activation is tightly controlled due to the devastating effects the caspases have on the cell. The protein becomes smaller as it is cleaved, something that has been taken advantage of when it comes to detect active and inactive caspases. We used Western blot to detect active caspase-9 in cells infected with *F. tularensis*, and could see that cells infected with LVS contained large amounts of cleaved caspase-9 after 24 hours, something that was not seen with the $\Delta pdpC$ mutant.

2.3.11 Caspase-3

Caspase-3 is one of the effector caspases; in the intrinsic pathway it is activated by caspase-9 and is a relatively late marker of apoptosis. It is required for the DNA fragmentation that is among the final steps of apoptosis (Lamkanfi and Dixit, 2010). We labeled cells infected with various *F. tularensis* strains with an antibody specific for activated caspase-3, and found that after 24 hours, cells

infected with LVS or the $\Delta pdpC$ mutant had a high proportion of caspase-3-positive cells, in contrast to uninfected, or $\Delta iglC$ mutant-infected cells. Since caspase-9 is considered to be required for caspase-3 activation, but active caspase-9 was not detected in $\Delta pdpC$ infected cells, it is possible that the caspase-3 activation is due to some other mechanism.

2.3.12 TUNEL

In the final stages of apoptotic cell death, the cell's DNA is fragmented by an apoptosis-specific enzyme. These fragments can be labeled using TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) (Gavrieli et al., 1992). In this method, a specific enzyme incorporates labeled UTPs at the 3' end of the DNA fragments, allowing for estimation of the number of cells undergoing this type of cell death. Using this method, we could see that DNA fragmentation had already started at 24 hours post infection in LVS-infected cells. In the cells infected with the $\Delta pdpC$ mutant, there seemed to be a delay as hardly any cells were TUNEL-positive at 24 hours but a large proportion was at 48 hours. In comparison, only a few of the uninfected cells and cells infected with the $\Delta iglC$ mutant became TUNEL-positive, and the number did not seem to increase even up until 72 hours post infection.

It must be noted that this is not a true quantitative assay; one limitation is that, as the final stages of apoptosis are reached, the cells rupture. Many of these ruptured cells may have been TUNEL-positive but as we only measure the number of cells that at the time are TUNEL-positive, the results may be slightly misleading since a lot of previously TUNEL-positive cells are missed, as is likely for LVS-infected cells at 48 hours post infection.

2.3.13 Complementation of LVS $\Delta pdpC$

In order to verify that the phenotype seen in the LVS $\Delta pdpC$ mutant was due to the absence of *pdpC* and not an effect of the cloning procedure, we complemented the mutant with the wildtype *pdpC* gene, and were able to restore much of the wildtype phenotype. We could therefore conclude that the differences seen between LVS and the $\Delta pdpC$ mutant was due to the absence of the *pdpC* gene and the encoded protein.

3 Discussion

3.1 FSCo43

The intracellular life cycle of *F. tularensis* is dependent on escape from the phagosome, as well as the ability to multiply within the cytosol of cells. The spontaneous mutant FSCo43 demonstrates limited intracellular growth, and less cytotoxicity compared to SCHU S4. A small subset of the FSCo43 population seems to be able to escape the phagosome, essentially as the wildtype SCHU S4, and is able to replicate intracellularly. A similar finding, with a small proportion of replicating bacteria, was also seen in a SCHU S4 $\Delta pdpC$ mutant, and has also been reported for a SCHU S4 *pdpC* intron mutant (Long et al., 2012). These three mutants differ significantly on the genomic level; whereas the deletion mutant contain only a few nucleotides of the *pdpC* gene, the intron mutant harbors the duplicated gene in its entirety although disrupted by an intron at nucleotide 608. FSCo43 also contains both *pdpC* copies but with a deletion of one nucleotide about 630 base pairs from the start codon, leading to a nonsense mutation, and truncated protein. Thus, the FSCo43 mutant and the SCHU S4 $\Delta pdpC$ intron mutant are likely to express similar portions of PdpC. However, the authors of the $\Delta pdpC$ intron manuscript show by RT-PCR that the initial part of the gene was not expressed (Long et al., 2012). In contrast, we have been able to show, using qPCR, that the initial part of *pdpC* is expressed in FSCo43, and also confirmed the expression of the truncated protein via Western blot, something that was not tested with the intron mutant. The intron inserted so far (630 nt) into the gene should theoretically not have an effect on upstream nucleotides; the initiation of transcription should start as usual at the start codon, making it hard to explain why there is no evidence of transcription of the beginning of the gene in the intron mutant. It would be expected that the intron *pdpC* mutant and the FSCo43 strain should have a similar expression of the *pdpC* gene and protein. However, as the intracellular phenotype is very similar between all three *pdpC* mutants, FSCo43, *pdpC* intron mutant, and the *pdpC* deletion mutant, one can expect that the small part of *pdpC* that is still expressed, at least in FSCo43, is not responsible for this particular phenotype.

Another interesting feature of the FSCo43 strain is that the gene downstream *pdpC*, *pdpE*, is significantly downregulated. There is no effect on the expression of *pdpE* in our SCHU S4 $\Delta pdpC$ deletion mutant, but a similar downregulation was seen by Long et al., in the *pdpC* intron mutant. For FSCo43, this downregulation could be the effect of the nonsense mutation, which in some cases induces detachment of the RNA polymerase, with reduced expression of downstream genes as a result. Of course, for the intron mutant the downregulation seen could be a case of polar effect, where unwanted disruption

of transcription arises from a mutation, which is relatively common when disrupting genes rather than removing them.

Apart from the mutation in the *pdpC* gene, there are two more genetic differences that separate FSCo43 from SCHU S4: the nonsense mutation in *FTT0615*, and the fusion of ORFs *fupA* and *fupB*. There is little known about the *FTT0615* gene and its homologues, but in a study in *F. novicida*, the *FTT0615* homologue was identified as one of the genes negatively selected in the spleen of mice (Weiss et al., 2007), but not identified in any other whole-genome screens. Thus, little evidence suggests that the *FTT0615* gene is important for *F. tularensis* virulence, and as the other mutations seemed more likely to contribute to the FSCo43 attenuation, we ignored the *FTT0615* gene in most of our studies of the FSCo43 strain. We did, however, measure the expression of the *FTT0615* gene in SCHU S4 using qPCR, and found that it was expressed at a very low level in agar-grown bacteria, making it difficult to compare to the expression of the gene in FSCo43.

The gene known as FTLO439 in LVS is in FSCo43 the result of a fusion between the *fupA* and *fupB* genes. The encoded protein of *fupA* was shown to be involved in iron uptake, and thus given the name “Ferric uptake protein A” (Lindgren et al., 2009), while the downstream gene became known as *fupB*. The former has been shown to be required for virulence whereas the latter was not required (Twine et al., 2005). Due to its similarities to the *fsIE* gene, the *fupA* (*FTT0918*) has been extensively studied in both SCHU S4, and in LVS, as the fusion gene *FTLO439* (FTLO439). Reintroduction of the *fupA* gene in LVS has been shown to increase its virulence, and when complementing with both *fupA* and another defective gene, *pilA*, LVS virulence was restored to wildtype levels (Salomonsson et al., 2009), indicating that the attenuation seen in both LVS and FSCo43 are largely dependent on the lack of an intact *fupA*. However, a *fupA* deletion mutant in SCHU S4 did not have the same phenotype as the FSCo43 strain, indicating that another of the mutations also contributed to the FSCo43 virulence, and as described earlier, this mutation appear likely to be the nonsense mutation in the FPI-located gene *pdpC*. It could also be that the partial expression of *fupA* resulting from the presence of the *FTLO439* gene resulted in the FSCo43 attenuation. However, we were unable to separate the intracellular phenotype of the $\Delta fupA$ and $\Delta FTLO439$ mutants as measured by intracellular growth, phagosomal escape, and cytotoxicity (LDH-release). However, the mouse vaccination studies showed a clear difference between the mutants; the *FTLO439* mutant could only be used at a very low dose (10 bacteria) but gave good immunity against wildtype challenge both when using the intradermal and intranasal route. The *fupA* mutant afforded good protection against intradermal challenge, but intermediate protection against intranasal challenge, but could be used at a higher dose (10^3 bacteria). In comparison, both the $\Delta pdpC$ mutant, and FSCo43 could be administered at a very high dose (10^7

bacteria) but did only fully protect mice against intradermal challenge, although intranasal challenge with FSCo43 resulted in an intermediate protection similar to that seen with the *fupA* mutant. If the FSCo43 phenotype is exclusively a result of the *FTLO439* mutation, these two strains would show similar phenotypes. However, since 10 million bacteria of FSCo43 does not cause illness in mice, whereas no more than 10 bacteria of the *FTLO439* mutant can be given without causing aggressive disease, the phenotype of FSCo43 must depend on another mutation as well. In contrast to the aforementioned strains, a SCHU S4 mutant lacking the *pdpC* gene affords no protection against an intranasal wildtype-challenge in mice.

As already mentioned, the difference between the $\Delta FTLO439$ mutant and FSCo43, seen both in cells and animals, clearly shows that the partial deletions of the *fupA* and *fupB* genes, in the FSCo43 strain, are not enough to explain its phenotype. It does, however, seem likely that the combined effects of the highly attenuating $\Delta pdpC$ mutation and the *FTLO439* mutation, that gives intermediate attenuation but good protection, would result in a phenotype similar to that seen in FSCo43. It could also be possible that the small part of PdpC that is still expressed contributes to the FSCo43 phenotype. Generally, non-replicating mutants do not confer an efficacious good immune response, but FSCo43 seems to be an exception; although not conferring potent protection, this mutant has a significantly higher protective ability compared to the $\Delta pdpC$ SCHU S4 mutant, despite its limited capability to replicate in cells.

It is well-known that survival from a severe infection often leads to efficient immunity against the disease, but taken into account that a vaccine strain should be safe to use in immunocompromised persons, both the $\Delta fupA$ and $\Delta FTLO439$ mutants are poor vaccine candidates due to their relatively high virulence. Since FSCo43 can be administered at such high numbers without causing lethal infection, it means that it is safer to use in humans. Unfortunately, this strain does not give enough protection against an intranasal challenge to be a good enough vaccine candidate. One can speculate that perhaps restoration of *FTT0615* or *pdpC* would result in a strain with slightly more intracellular growth and thus more virulent and better at raising an immune response, resulting in better protection even against an intranasal challenge.

3.2 LVS $\Delta pdpC$

Due to the impact of the *pdpC* mutation on the FSCo43 phenotype, and since little had been published about this gene, we created a *pdpC* deletion mutant in the live vaccine strain. The resulting LVS $\Delta pdpC$ strain did not replicate intracellularly and was avirulent in mice, although the mutant was capable of spreading to the liver and spleen. Using LAMP-1 staining, we determined that

the mutant bacteria were colocalized with phagosomal membranes; however, when inspecting the cells closer, using electron microscopy, we discovered that these membranes were fragmented. In contrast, LVS bacteria were free in the cytoplasm, and the *ΔiglC* mutant was surrounded by intact membranes.

This clearly illustrates the benefits on using several methods to study one event; if we had been satisfied with the results from the LAMP-1 experiment, we would never have discovered the fragmented phagosomal membranes. This would have led to difficulties explaining the *ΔpdpC* mutant's ability to affect the host cell similar to LVS, something that we now can explain by the ability of the bacteria to interact with the host cell's cytoplasm through the disrupted phagosomal membrane. The fragmented membranes were not clearly observed using immunofluorescence and this can be explained by the strong fluorescence emitted by the LAMP-1 antibody. The light tends to spread diffusely, making it hard to see if the membranes surrounding the bacteria are intact or not. Visualization of the fragments would also require high resolution of the fluorescence microscope.

It was clear that the fragmented membranes enabled the *ΔpdpC* mutant to interact with the host cell's cytoplasm, as we could see by its capability of inducing IL-1 β secretion, an event that is generally believed to be linked to the formation of the protein complex known as the inflammasome. The inflammasome is assembled upon contact with bacterial DNA, or other effectors. It has previously been suggested that bacterial replication was necessary for the inflammasome assembly and subsequent secretion of inflammatory cytokine IL-1 β . Apart from IL-1 β , infection with this mutant also inhibits TNF- α secretion from cells treated with TNF- α -inducing *E. coli* LPS, and MOI-dependent cytotoxicity, as measured by the cytosolic protein LDH. The impact of the number of bacteria per cell, or multiplicity of infection (MOI), indicates that the more bacteria that are present, the more can be taken up by the phagocytic cell. This is often up for discussion, as some suggest that the maximum number of bacteria that can be phagocytosed by any one cell is quite low, while others claim that the macrophage, which is capable of engulfing very large particles, is also able to ingest large quantities of bacteria. The MOI dependency in this case, seems to be irrelevant for replication, as the LVS replicated to equal numbers regardless of which MOI was used. The MOI dependency on LDH release after infection with the *pdpC* mutant could be that the number of cells with activated cell death pathways, of which the inflammasome is part, may be increased, resulting in more cell death. It could also indicate that as more bacteria are taken up into phagosomes, the faster the inflammasome is formed, and cell death pathways may be activated.

Although many FPI mutants show similar phenotypes, with no intracellular replication, complete attenuation in the mouse model, and no phagosomal

escape, there are a few exceptions. Among these are the mutants of *iglG* and *iglI*, both of which have an intermediate phenotype regarding phagosomal escape and intracellular replication.

Several members of the FPI have been shown to be essential for virulence in both the cell and mouse model. Interestingly, several of the FPI genes and proteins are downregulated in a $\Delta pdpC$ LVS mutant, indicating that the $\Delta pdpC$ gene or protein, has an impact, direct or indirect, on the expression or regulation of these genes. As many of these genes are suggested to be involved in the phagosomal escape, could this reduced expression of these genes account for the phagosomal phenotype seen in the $\Delta pdpC$ mutant?

The FPI is present in its entirety in *F. novicida* and *F. tularensis* species. The main difference between the species is that in the *F. tularensis* strains, there are two identical copies of the FPI, whereas *F. novicida* only harbors one copy. Most of the genes and intergenic regions in the FPI are almost identical in both species. However, the large *pdpC* gene is not. There are about 70 amino acids that differ between the species, indicating that this gene may have different functions in the various species.

The $\Delta pdpC$ mutant does not seem to replicate in the mouse model, although it is capable of spreading to the liver and spleen. Based on the mutant's ability to elicit cytopathogenicity, one could postulate that it would have protective abilities if used to immunize mice. This has not yet been tested using the LVS mutant, and the SCHU S4 mutant only gave protection against an ID challenge. The particular phenotype seen by the $\Delta pdpC$ mutant in LVS, with the fragmented phagosomal membrane, has not been confirmed nor excluded for the SCHU S4 $\Delta pdpC$, or FSC043 strain. This fragmentation of the phagosomal membrane resembles a phenotype observed in *Mycobacterium*, where the perforation of the phagosomal membrane was performed by the ESX-1 secretion system. It was also demonstrated that the ability of the bacterium to come in contact with the cytosolic content was a prerequisite for virulence (Manzanillo et al., 2012).

3.3 Cell death pathways

A cell can respond to an infection in different ways, but the objective - from the cell's point of view - is to limit the infection and prevent further spread of the pathogen. This can be achieved by activation of cell death pathways, including apoptosis and pyroptosis (Fink and Cookson, 2005). It has been shown that *F. tularensis* is able to inhibit the apoptotic pathway in neutrophils, which is beneficial for the bacteria as it protects its safe environment and nutrient source, especially since neutrophils are short-lived cells that usually undergo apoptosis after a few days. In contrast, our results indicate that, in the long-

lived macrophages, infection with LVS activates several cell death mechanisms such as IL-1 β secretion, superoxide accumulation, decrease in mitochondrial membrane potential, activation of caspases 3 and 9, phosphatidyl (PS) expression, and DNA fragmentation.

It has been shown for *F. novicida* that assembly of the inflammasome, or rather activation of caspase-1, is essential for IL-1 β release (Mariathasan et al., 2005). However, a recently published article show that upon LVS infection, IL-1 β is released even when cells are treated with a caspase-1 inhibitor, suggesting that this may not be the case for *F. tularensis* (Bröms et al., 2011). The assembly of the inflammasome, in *Francisella*-infected cells, has been suggested to require physical contact between the bacterial DNA and AIM2 and that strains with unstable outer membranes are hypercytotoxic as they induce a very high IL-1 β release, presumably due to large amounts of DNA released (Peng et al., 2011).

Since LVS induces less IL-1 β release in comparison to *F. novicida* strain U112, and J774 cells secretes much lower levels of this cytokine than purified mouse cells such as BMMs (bone marrow derived macrophages) and PECs (peritoneal exudate cells), we used the latter two cell types for these experiments. Since almost all other cell infection experiments were performed exclusively on J774 cells, one can argue that the IL-1 β release seen in BMMs and PECs infected with either LVS or the $\Delta pdpC$ mutant may not correspond to what would be the results in J774 cells. Regardless of which field of science you look at, the conclusions drawn from one experiment are often generalized in order to apply to the issue addressed. As an example of this we have cell types. There are vast numbers of cell lines and cell types available. What you choose depends on your hypothesis, and issues such as established methods, cost, availability, and so on. The focus of this thesis has been an intracellular bacterium, and it has therefore been essential to select cell types that this pathogen is able to infect. As it is a model for the real infection, the cells used should resemble what the bacteria would encounter in real life. *F. tularensis* is a pathogen adapted to mammals and it would make little sense for us to use, for example, a fish cell line and try to draw conclusions about the infection in mice. The use of cell lines in comparison to isolating cells from tissue is also a question that needs to be addressed, cell lines are often viable over an extended time period, and results are more easily repeated than when using isolated cells. A cell line can be kept viable for several months and give the same results the last week as on the first, whereas isolated BMMs, for example, will drastically drop in viability within a few weeks, and due to this, an experiment can rarely be repeated using the same isolation of cells.

In order to test the replicating ability of $\Delta pdpC$, we infected not only J774 cells, but also BMMs, PECs, and HMMs (human monocyte-derived macrophages). In none of these cell types did we observe any replication of either $\Delta pdpC$ or $\Delta iglC$,

while both LVS and the complemented $\Delta pdpC$ strain replicated to expected numbers. The cytotoxicity, as recorded by LDH release, was high for LVS and the complemented $\Delta pdpC$ mutant, and low for $\Delta iglC$. Infection with the $\Delta pdpC$ mutant in BMMs resulted in an elevated LDH release, in some of the experiments, probably due to a different MOI used than expected. This indicates that the phenotype seen in $\Delta pdpC$ -infected J774 cells is of relevance for other cell types. Since we utilize the mouse model, with a mouse cell line, and mouse cells, we suggest that the J774 is a good and reliable model to study the function of bacterial proteins in the intracellular environment. The wildtype infection behaves similarly in other cell types tested and the negative control ($\Delta iglC$) is completely attenuated, including lack of escape from the phagosomal compartment. Of course, the results obtained in J774 cells can not be automatically extrapolated to all other cell types; however, it is likely that the phenotype observed in J774 murine macrophage-like cells is very similar to the phenotype in other macrophages. All experiments, except for the IL-1 β release, have utilized J774 cells.

It is clear that several cell death mechanisms are activated in cells infected with LVS or $\Delta pdpC$, but since J774 cells infected with LVS do not induce IL-1 β secretion (Telepnev et al., 2003), we cannot conclude that the non-inflammatory and inflammatory pathways are active at the same time. What we can conclude is that both LVS and the $\Delta pdpC$ mutant are capable of inducing both inflammatory and non-inflammatory cell death. It is hard to say if the host or the bacteria will benefit most from an inflammatory or non-inflammatory cell death; an inflammatory outcome will lead to recruitment of more immune cells to the site of infection, but at the same time provide new targets for the bacteria to infect. A non-inflammatory event will protect surrounding tissues from damage, but allow the bacteria to invade new cells without the host being able to mount a protective immune response.

TNF- α is a potent regulator of cell signaling. It can induce apoptosis by binding to specific receptors in the cell membrane, or it can inhibit apoptosis by inducing release of NF- $\kappa\beta$, a molecule involved in regulation of inflammatory genes. It is also activated by oxidative stress (Samuhasaneeto et al., 2009). TNF- α can be released by the cell in response to recognition of pathogen-specific molecules such as LPS. Both LVS and $\Delta pdpC$ inhibited the release of TNF- α upon stimulation of cells using *E. coli* LPS, indicating that the extrinsic pathway will not be activated by TNF- α release in a cell population infected with either of these two strains.

It has been previously demonstrated that LVS induces apoptosis in J774, as evident from PS-expression, DNA fragmentation and nucleosome formation, and that this induction was MOI-dependent (Lai et al., 2001). This MOI-dependency means that the number of bacteria used to infect a cell population

has a direct effect on the outcome of the experiment, which is not generally the case. For example, MOI has a very small, if any, effect on the intracellular net growth of LVS; using a MOI of 100 or 500 will result in the same relative replication, compared to the uptake. The uptake of intracellular *Francisella*, on the other hand, has been reported to be very ineffective. A MOI of 200 means that there are 200 bacteria per cell, and with effective uptake, the majority of cells would contain at least 100 bacteria. This is not the case with *F. tularensis*; generally only a few bacteria are taken up by each cell, regardless of the MOI. As a result of this, some research groups working with *Francisella* have started to use the term “effective MOI”, indicating the number of bacteria estimated to be *taken up* by the cells, rather than the number available for uptake. Other groups are using various opsonizing agents in order to increase uptake.

Apart from the MOI-dependency, Lai et al. 2001 showed that in order to induce cell death, the bacteria have to be alive and inside the cell. However, they also concluded that the bacteria need to be replicating to activate cell death, something that appears not to be required, based on our data from the non-replicating, but still cell death-inducing, $\Delta pdpC$ mutant. However, taking into account the results seen in the phagosomally located $\Delta iglC$ mutant, it appears that bacteria need to be in contact with the cell cytoplasm for induction to occur. The LVS deletion mutants of $\Delta iglG$ and $\Delta iglI$ further contribute to this hypothesis. These two mutants have an intermediate intracellular phenotype; slightly delayed phagosomal escape, delayed growth, reduced cytotoxicity, and diminished induction of IL-1 β secretion, and no inhibition of TNF- α secretion, which is in contrast to $\Delta pdpC$, that has a TNF- α phenotype identical to LVS (Table 4). Both the $\Delta iglG$ and $\Delta iglI$ mutants were included in the cell death study, but none of them was able to induce cell death as potently as LVS, or the $\Delta pdpC$ mutant. In fact, the $\Delta iglI$ mutant induced almost no cell death markers, similar to $\Delta iglC$. This further illustrates that replication does not automatically lead to induction of cell death.

It has already been reported that there are vast differences in the intracellular phenotype between the model strains U112 (*F. novicida*), LVS (attenuated Type B), and SCHU S4 (virulent Type A). For example, when compared side-by-side, the U112 induces a significantly higher IL-1 β release than LVS in BMMs (Bröms et al., 2011). As mentioned earlier, LVS infection in neutrophils prevents apoptosis (Schwartz et al., 2012), whereas infection of mice with SCHU S4 resulted in more extensive DNA-fragmentation than did LVS (Wickstrum et al., 2009). The reason for this variation may first of all be that the evolutionary adaptations of these three strains are different. For U112, an environmentally adapted strain, it could be that the induction of the inflammatory cell death is the method-of-choice, while in the mammalian-adapted LVS, and SCHU S4

strains, induction of the non-inflammatory pathways provides better possibilities for survival and further replication.

Table 4. Summary of intracellular phenotypes of *F. tularensis* strains in J774 cells, unless otherwise noted.

	LVS	\DeltaiglC	$\Delta pdpC$	\DeltaiglG	\DeltaiglI
Intracellular growth	+++	-	-	+++	++
Cytotoxicity	+++	-	+	+	+
IL-1 β release (PEC)	+++	-	++	++	+
TNF- α release	-	+++	-	+++	+++
ROS-accumulation	+++	-	++	+	-
Depolarization Ψ_m	+++	-	+++	+	-
PS-expression	+++	-	++	+	-
Caspase-9 activation	+++	-	-	+	-
Caspase-3 activation	+++	+	+++	++	+
DNA fragmentation	+++	-	++	+	+

We have reported about a mutant unable to replicate intracellularly, but still able to induce secretion of IL-1 β in BMMs, and PECs, and activation of cell death markers in J774 cells. Most of the events reported are delayed during infection with the $\Delta pdpC$ mutant, perhaps due to the mutant's lack of replication, leading to a lower amount of PAMPs for the eukaryotic cell to respond to and thus a delayed induction of cell death pathways. What clearly separated the LVS and $\Delta pdpC$ infection was the caspase-9 activation. This was detected in LVS within 24 hours, whereas no caspase-9 was detected for 48 hours in mutant-infected cells. One could argue that Western Blot is a less sensitive method than cell counting using flow cytometry, as the other methods are based upon, but nevertheless, the amount of cell death markers observed in the other assays was almost always as strong in $\Delta pdpC$ mutant-infected cells, as in LVS-infected cells, if accounting for the delay. Caspase-9 activation is believed to be a prerequisite for caspase-3 activation in the intrinsic pathway, and if caspase-9 is not activated there will be no activation of caspase-3. However, caspase-3, with subsequent DNA fragmentation, can be activated also by the extrinsic pathway. It is therefore possible, that although caspase-9 is never activated in $\Delta pdpC$ -infected cells, possibly due to lack of replication, caspase-3 may be activated by another pathway (Figure 6). The only two events measured, that were obviously slower in $\Delta pdpC$ -infected cells in comparison to LVS-infected, was the early onset of ROS accumulation, and DNA-

fragmentation. All other markers, save caspase-9, were found to be activated to the same levels in both LVS- and $\Delta pdpC$ -mutant infected cells.

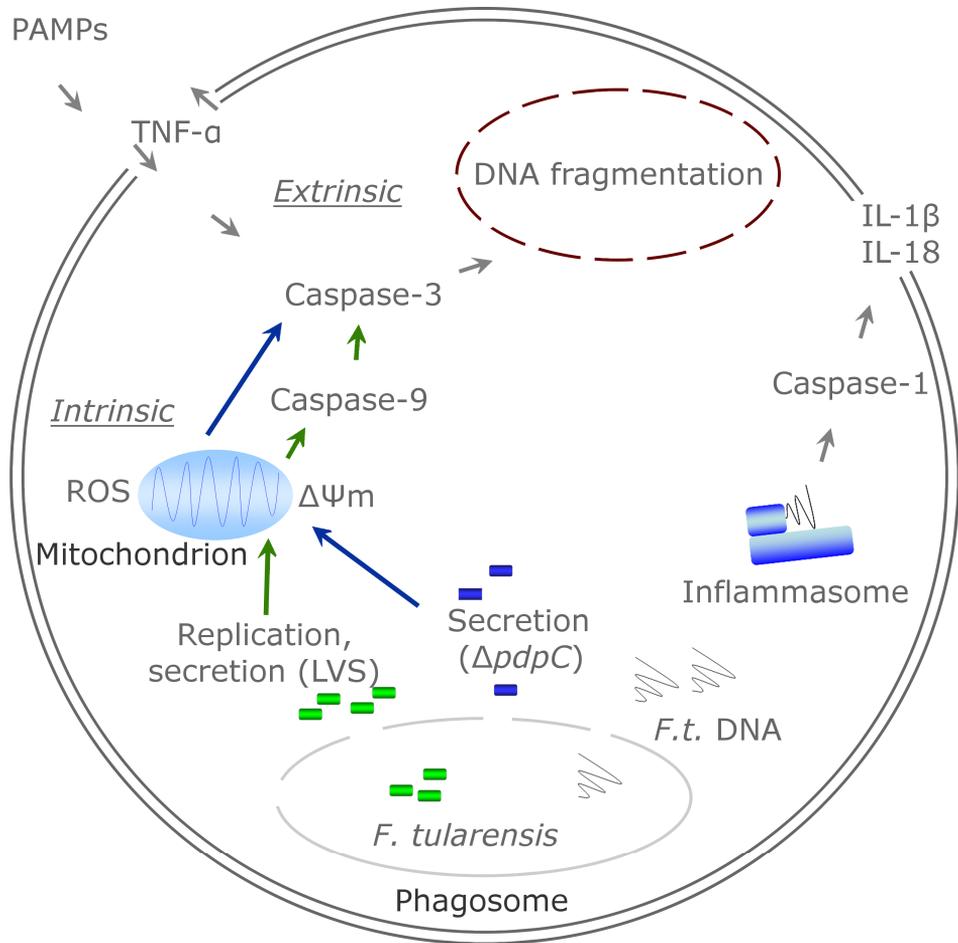


Figure 6. Model of induction of cell death pathways by *F. tularensis*. The bacteria are able to induce both inflammatory and non-inflammatory cell death in the infected cell. The LVS $\Delta pdpC$ mutant (blue arrows) does not induce activation of caspase-9 but potentially activates all other markers measured.

There are a number of intracellular pathogens capable of affecting the cell death pathways in macrophages. For some of them, the macrophage is not the main host and it is therefore beneficial for those bacteria to quickly induce a non-inflammatory host cell death, silently escape, and invade other types of cells instead. For a pathogen adapted to intracellular replication in macrophages, the induction of cell death can facilitate spread, and the benefit of activating an inflammatory cell death can be that more target cells are drawn to the location of the bacteria. Recent findings show that *F. tularensis* inhibits apoptosis in

neutrophils, which are very short-lived cells. The prevention of cell death by *F. tularensis* in these cells may be due to the fact that as a slow-replicating bacterium, *F. tularensis* requires its host to survive for a longer period of time than is natural for them. In contrast, the long-lived macrophage, provides a long-lasting source of nutrients and protection, and does not naturally undergo apoptosis within a few days, allowing the bacteria to replicate in peace.

With this thesis we have contributed with a lot of information regarding the host-pathogen interaction during intracellular infection. We have shown that replication is not necessary to induce a cytosolic immune response, but confirmed that physical interaction between the bacteria and the host cell's cytoplasm seems to be essential for a number of cellular responses, such as inflammasome activation, and induction of cell death pathways. Further, we have results indicating that complete protection against a pathogen may not require a fully replicating strain, and that it is possible to create a vaccine from a Type A strain.

Conclusions

The main aim of this project was to identify the reasons for attenuation in the Type A *F. tularensis* strain FSCo43. This has been accomplished on a genomic level; using various methods of sequencing we were able to show that only four genomic events distinguished the FSCo43 mutant and the fully virulent SCHU S4 strain. By creating various mutants simulating these genomic events we have been able to separate the impact of the fusion gene *FTL0439* and the FPI gene *pdpC*. We have also characterized the FSCo43 strain with respect to intracellular localization, genomic and proteomic patterns, as well as vaccine properties in mice. What seems to stand out is the mutation of *pdpC*; it gives the mutant a dual phenotype in cells, where a small part of the population is able to multiply intracellularly. This was also seen in the complete deletion mutant.

Apart from studying the FSCo43 we created a *pdpC* deletion mutant in LVS and have characterized it thoroughly, both with respect to basic properties such as gene expression and infectivity in mice, but also using studies of its intracellular life cycle that revealed that the mutant, although unable to escape and replicate, is able to affect the host cell from within a partially degraded phagosome, similar to that of wildtype LVS. It did not display the same phenotype as the SCHU S4 mutant, with a subset of escaping, and replicating bacteria, but it further contributes to the hypothesis that PdpC is, directly, or indirectly, involved in phagosomal escape.

The fragmented phagosome, around LVS Δ *pdpC* bacteria, allowed the pathogen to interact with the host cell's cytoplasm, leading to activation of a number of cell death markers such as ROS accumulation, mitochondrial membrane instability, presentation of phosphatidylserine on the cell surface, caspase-3 activation, and DNA fragmentation. The mutant was also able to induce IL-1 β secretion from the infected cell, a process known to require assembly of the inflammasome, indicating that replication is not required for inflammasome assembly.

In short, we have characterized the gene and protein PdpC in various assays, and apart from these more focused studies, this thesis contributes to the overall knowledge about bacteria-host cell interaction.

Glossary

Apoptosis	A process of programmed cell death, usually non-inflammatory
Caspase	Cysteine-aspartic proteases, proteins that are essential for various types of cell functions, such as apoptosis
Complementation	Regarding mutants, to restore the mutation in order to create a non-mutant
Cytokine	Proteins used for cell-signaling, essential for the immune system
Cytoplasm	The internal compartment of a cell
Flow cytometry	A laser-based method used to label and count cells
IL-1 β	Interleukin 1 β , a pro-inflammatory cytokine released by macrophages
Inflammasome	A protein complex activated in response to infection, activates caspase-1
MOI	Multiplicity of infection, an estimate of the number of bacteria used to infect one cell, <i>e.g.</i> MOI 200 = 200 bacteria per cell.
Operon	A part of the genome that is under the control of a single promoter
ORF	Open reading frame, section of the genome containing the expressed part of a gene
Phagocyte	An immune cell with the function to engulf foreign matter
Phagosome	A structure formed around particles engulfed by phagocyte
PI	Propidium iodide, a compound used to label dead cells, membrane impermeable
promoter	The binding site for RNA polymerase and the initial start of transcription
Pyroptosis	Caspase-1-mediated cell death, inflammatory
Stop codon	A sequence of nucleotides that signals the end of translation
TLR	Toll-like receptor, a receptor on the surface of eukaryotic cells, recognizes various pathogen molecules
TNF- α	Tumor necrosis factor α , a cytokine involved in the immune system, can induce apoptosis
Wildtype	The original, non-mutated strain from which mutants are derived

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