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REVIEW ARTICLE

INTRACELLULAR PATHOGENESIS OF *FRANCISELLA TULARENSIS*

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Summary

Intracellular pathogen *F. tularensis* is a causative agent of tularemia disease and belongs to the most hazardeous pathogen worldwide, categorized by the Center for Disease Control and Prevention, USA (CDC) as a category A agent. However, no safe and licensed vaccine for prevention a *F. tularensis* infection is available for vaccination. Tularemia is manifested by several forms depending on a route of infection and virulence of a *F. tularensis* strain. Essential to a development of the disease is the ability to infect, survive and proliferate inside the mononuclear phagocytes, such as macrophages or dendritic cells. Therefore, this review will discuss aspects of *F. tularensis* intracellular fate within host macrophages, modulate host signaling pathways to benefit *Francisella* infection and finally, summarize bacterial determinats involved in the process of phagosomal escape and intracellular replication.

Key words: Francisella tularensis; tularemia; intracellular trafficking; phagosome; intracellular replication; macrophage; Francisella Pathogenicity Island; virulence

ABBREVIATIONS

AIM, absence in melanoma
Aph, autophagosome
BMMs, bone marrow macrophages
CDC, Center for Disease Control and Prevention
EE, early endosome
EEA-1, early endosomal antigen 1
ERK, extracellular signal-regulated kinase
FCP, Francisella-containing phagosome
FPI, Francisella pathogenicity island
hMDMs, human monocyte-derived macrophages
IFN, interferon
Igl, intracellular growth locus
IL, interleukin
iNOS, inducible nitric oxide synthase
LAMP-1, lysosomal-associated membrane protein 1
LE, late endosome
LPS, lipopolysaccharide
LVS, live vaccine strain
MAP, mitogen-activated protein
Mgl, macrophage growth locus

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MyD, myeloid differentiation
NADPH, nicotinamide adenine dinucleotide phosphate
NK, natural killer
NLRs, nucleotide-binding oligomerization domain-like receptors
Pdp, pathogenicity determinant protein
PEC, peritoneal exudate cell
PI3K, phosphoinositide 3-kinase
PRRs, pattern recognition receptors
Syk, spleen tyrosine kinase
TLRs, toll-like receptors
TNF, tumor necrosis factor
vATPase, vacuolar ATPase

INTRODUCTION

Francisella tularensis (*F. tularensis*) is characterized as a Gram-negative, facultative intracellular pathogen that causes tularemia disease in both humans and animals. *F. tularensis* is easily aerosolized, disseminated and if untreated it results in illness with high mortality rate. Strikingly, as few as 10 bacteria of virulent strain can cause disease in humans. Due to all these reasons, *F. tularensis* has been classified by the Center for Disease Control and Prevention, USA (CDC) as a category A agent [1-3].

F. tularensis belongs to the class of γ-proteobacteria of the order *Thiotrichales*. The family *Francisellaceae* consists of only one genus, *Francisella*, which is further divided into 3 subspecies: *Francisella tularensis*, *Francisella novicida* and *Francisella philomiragia*. Additionaly, there are three subspecies of *F. tularensis*: subsp. *holarctica*, subsp. *tularensis* and subsp. *mediasiatica*, which differ in the virulence in humans [4]. Generally, *F. tularensis* is found primarily in the Northern part of the Hemisphere. Clinically important is the highly virulent subspecies *tularensis*, which is found mainly in North America. This subspecies has a very low infection dose in humans, since less than 10 bacteria can cause severe disease. Subspecies *tularensis* is also known as a type A and is further divided into two clades (AI and AII) [5]. *F tularensis* subsp. *holarctica*, also known as a type B, is located throughout the Northern Hemisphere and has milder clinical manifestations in humans compared to type A. *F. tularensis* subsp. *mediasiatica* was isolated in the central part of Asia and it is of the same virulence as a subsp. *holarctica*. *F. tularensis* is a zoonotic bacterium, transmitted via arthropod vectors, most frequently by tick, flies or mosquitos. Common hosts of *F. tularensis* are wild rabbits, hares, ground squirells, rodents, birds or fish. However, it has been described that 190 species of mammals, 88 invertebrates, 23 birds and 3 amphibians are susceptible to the infection [6]. The exact mechanism of tularemia transmission from a vector to a host is not known. Additionaly, the bacterium has not been detected in a salivary gland of a primary human biting tick. Therefore, it has been supposed that the bacterium is transsmited from a tick mechanicaly from the feces directly to a skin wound [7].

To develop the disease, *F. tularensis* enters and replicates itself in host cells, initially within macrophages. Entry of *F. tularensis* into macrophages is arranged by looping phagocytosis. Once inside the macrophages, *Francisella* is harboured within the *Francisella*-containing phagosome (FCP), which gradually acquires maturation markers of early and late endosomes. To overcome the macrophage defence strategies, *F. tularensis* rapidly escapes from late endosomes into the cytosol. The specific factor/s responsible for the process of phagosomal escape is not described but a *Francisella* pathogenicity island (FPI), potentially encoding type VI secretion system, seems to play a key role in the process of phagosome biogenesis and subsequent escape into the cytosol. In this review, we will focus on the individual aspects of *Francisella* intracellular lifestyle, manipulation of host cell signaling by *F. tularensis*, together with a summary of bacterial factors that contribute to a successful replication within a host cell cytosol.

TULAREMIA

Tularemia is an acute, febrile disease with flu-like symptoms. Depending on the route of infection and an infecting biovar, several different forms of tularemia are recognized. Generally, an infection begins by entering of a pathogen through mucous membranes or skin (e.g. by direct contact with infected insects or by manipulation with infected animals or water) or by inhalation of aerosolized bacteria [8, 9]. The most common form of tularemia is a ulceroglandular form, representing approximately 90% of all tularemia cases [10]. In this form, a host is infected via skin or a mucous membrane by a direct contact with an infected material or as a result of a vector-borne transmission. Following inoculation, a microbe targets a host sensitive organ, such as

lymph nodes, lung, spleen, liver and kidney, where it replicates. [10]. At a place of entry, an ulcer begins to form and this is followed by swelling of regional lymph nodes. An incubation period is between 3 to 6 days post infection, but can last up to 21 days [11, 12]. After an incubation period, the disease is manifested with rapid onset of fever and flu-like symptoms. An ulceroglandular form has a mortality rate of about 5%. The most serious form of tularemia is a respiratory form with a mortality level of up to 30% - 60%, if untreated [12]. This form develops upon inhalation of aerosolized microbes or as a secondary developed pneumonia due to organ-bacterial dissemination from another site of the infection. The infection results in a systemic disease with variable symptoms depending on the inhaled subtype. Typically, a respiratory form of the disease is manifested by a sore throat and swelling of the lymph nodes in lungs followed by onset of fever, chills, headache and flu-like symptoms [11]. An infection by direct contamination of an eye with *F. tularensis* can result in an oculoglandular form of tularemia, which represents 1 - 4% of all tularemia cases. A patient generally suffers from conjuctivitis of an eye, purulent secretion and swelling of eyelids [11]. Other minor forms of tularemia are an oropharyngeal and a gastrointestinal form (ingestion of contaminated water or food) and a typhoidal form [13]. In general, tularemia is treated with antibiotics where streptomycin is recommended as the first choice of treatement with tetracyclines as potential alternatives. Among others, ciprofloxacin, gentamicin, doxycyclin and chloramphenicol are used [9, 14, 15]. Ampicillin and penicillin derivates are ineffective since *F. tularensis* encodes β-lactamases in the genome that hydrolyzes penicillin [16].

INTRACELLULAR LIFESTYLE OF *F. TULARENSIS*

F. tularensis is able to survive and multiply inside a variety of host cell types. In mammals, *F. tularensis* infects different phagocytic (macrophages, dendritic cells or B cells) and non-phagocytic cells (hepatocytes, lung epithelial cells)[8, 17]. Macrophages are believed to be the first target upon *Francisella* entry into a host.

Entry of F. tularensis into host cells

Uptake of *F. tularensis* into host cells occurs by a unique process termed looping phagocytosis [18]. This type of uptake involves formation of asymmetric pseudopod loops and it is dependent upon presence of a complement factor C3 and a complement receptor CR3 [18]. Moreover, *F. tularensis* may also use a scavenger receptor or a Fcγ receptor for entry [19]. In the absence of serum opsonization a bacteria enters via a mannose receptor or a nucleolin [20-22]. Recently, an interest of cholesterol-rich lipid domains, so called lipid rafts, on an incoming process of *F. tularensis* has been reported. It has been shown that a depletion of plasma membrane cholesterol inhibits *Francisella* entry into a host cell. It is hypothesized that an interaction of *Francisella* with lipid rafts may act as a signaling platform, e.g. linking a process of *F. tularensis* at a cell membrane to a cytoskeleton and intracellular signaling pathways [23].

F. tularensis within host cell phagosome

In general, phagocytosis is a process when microbial or inert particles are internalized by a phagocytic cell into a newly formed organelle originating from a plasma membrane, called phagosome [24-26]. Immediatelly after formation, phagosomes go through several maturation steps that start with an early endosomal stage, followed by a late endosomal stage when late endosomes acquire proton vacuolar ATPase pump (vATPase) and become acidified. Acidification triggers fusion of a late endosome-like phagosome with degradative lysosomes. The newly-formed phagolysosome behaves as a degradative compartment for invading microbes. Overall, this series of transient interactions is very rapid and is complete within minutes of phagosome biogenesis [24-26]. Therefore, successful intracellular pathogens evolved strategies of how to circumvent this inconvenient process. These strategies include (i) a modification of phagosome maturation at a distinct stage of an endosomal-lysosomal pathway (such as an infection caused by *Legionella*), (ii) an acclimatization to unpleasant acidic conditions within a phagolysosme (such as *Coxiella*) or (iii) an escape from a phagosome into a cell cytosol (such as in the case of *Listeria* or *Shigella*)[27, 28].

Following an uptake, *F. tularensis* enters an endocytic pathway and is found in a membrane bound vacuole named a *Francisella*-containing phagosome (FCP). The FCP acquires a marker of early endosomes, such as an early endosomal antigen 1 (EEA-1) and Rab5 GTPase within 5 minutes post infection. Shortly afterwards, the FCP interacts with a late endosome under a control of Rab 7 GTPase and is characterized by late endosomal markers, a

lysosomal-associated membrane protein 1 and 2 (LAMP-1, LAMP-2) within 15-30 min after the uptake [29-34]. However, before the fusion of the FCP with degradative lysosomes, *F. tularensis* escapes an endocytic pathway and then a cytosol serves as a replicative niche [35] (Figure 1). Consequently, the FCP does not merge with a lysosomal hydrolase cathepsin D or with a marker of an acidic compartments lysotracker [29]. However, the kinetics of a phagosomal escape together with an acquisition of endosomal markers is quite intricate depending on a type of a *Francisella* strain used, an infection model used and an experimental setup used by various laboratories.

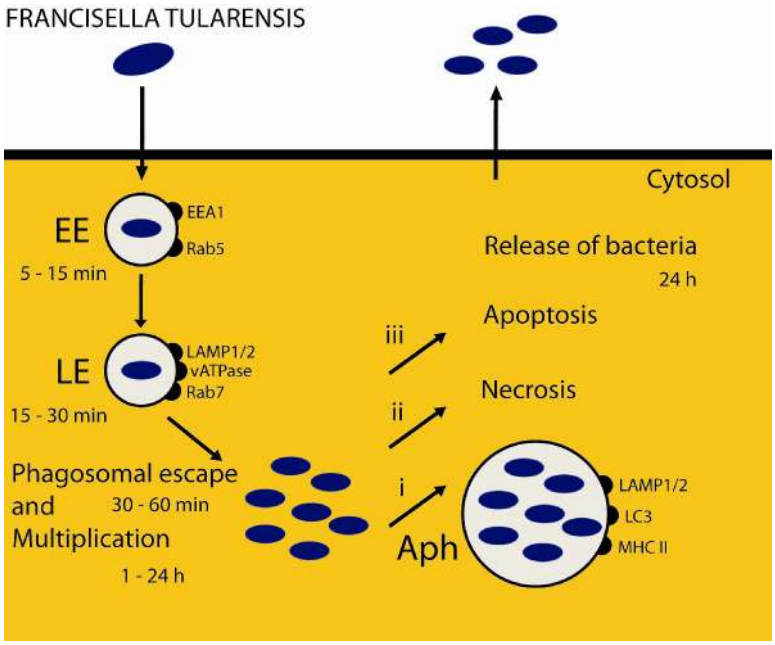

Figure 1. Model of a *F. tularensis* trafficking within murine macrophages
Upon entry, the FCP matures into an early endosome (EE) that is characterized by the EEA 1 antigen and Rab5. The maturation progress into the late endosome (LE) is characterized by the late endosomal markers Rab7, LAMP 1/2 together with an acquistion of the proton vATPase pump. The acidified LE provides alarming conditions to F. *tularensis* to rapidly escape the LE stadium into the cell cytosol. Subsequently, *F. tularensis* rapidly multiplicates within the cell cytosol between 1h and 24 hrs post the infection. Following the replication F. tularensis (i) re-enters the endosomal-lysosomal pathway through the autophagosme-like vacuole (Aph) or (ii) induces the necrosis or (iii) apoptosis of the host cell. Finally, *F. tularensis* leaves the damaged host cell and continues in the infection of neighbouring cells.

F. tularensis phagosomal escape and multiplication in cell cytosol

It has been reported that *F. tularensis* begins to escape from a phagosome within 15 - 30 min after an infection in both human and murine macrophages [36, 37]. An important signal for bacteria to escape into a cytosol is phagosomal acidification. Studies using a specific inhibitor of a proton vATPase pump, Bafilomycin A1, resulted in a delay of a *Francisella* escape into a cytosol [36]. This suggests that there is an unknown factor involved in a disruption and an escape of *Francisella* from phagosmes which is activated by an acidic pH in early stages after an infection. A mechanism by which *F. tularensis* escapes into a cell cytosol is not well understood but a possible role of a type VI secretion machinery has been recently discovered [38]. Additionally, several genes located in FPI together with their transcription regulators have been shown to be also required for a bacterial escape into a cytosol (Table1). However, an inhibition of acidification did not inhibit intracellular induction of FPI proteins, which demonstrates that this response is not dependent on a phagosomal pH [37].

Once in a cytosol, *F. tularensis* multiplicates to high numbers resulting in an induction of apoptosis

Table 1. Factors involved in a process of a *Francisella* phagosomal escape and a cytosolic multiplication

Role or function	Protein/gene	Strain/host cells	Reference(s)
Phagosomal escape	IglA*	LVS/J774	[58]
	IglB*	LVS/J774	[58]
	IglC*	*F. novicida*/U937 and hMDMs	[34]
		LVS/J774	[29]
	IglD*	LVS/J774	[29]
	IglH*	*F. tul.* subsp. *holarctica* FSC200/BMMs	[60]
	IglI	LVS/J774	[61]
		F. novicida U112/BMMs	[38]
	IglG	LVS/J774	[61]
	VgrG*	*F. novicida* U112/BMMS	[38]
	PdpA*	*F. novicida* U112/J774/BMMs	[62]
	MglA*	*F. novicida* U112/J774	[66]
		F. novicida U112/U937 and hMDMs	[34]
		LVS/J774A.1	[29]
	MigR*	LVS/BMMs	[68]
	FevR*	LVS/BMMs	[68]
	CarA*	LVS/BMMs	[76]
	DsbA*	SchuS4/J774	[81]
		F. tul. subsp. *holarctica* FSC200/BMMs	[60]
	FTT1676*	SchuS4/BMMs	[65]
	FTT0383*	SchuS4/BMMs	[65]
	AcpA	*F. novicida* U112/THP-1/BMMs	[88]
Intracellular growth	IglA	*F. novicida* U112/J774	[57]
		LVS/J774	[58]
	IglB	LVS/J774	[58]
	IglC	LVS/J774	[59]
		F. novicida U112/U937 and hMDMs	[34]
	IglD	LVS/J774; *F. novicida* U112/hMDMs	[29]
	IglH	*F. tul.* subsp. *holarctica* FSC200/BMMs	[60]
	IglI	*F. novicida* U112/J774	[38]
	PdpA	*F. novicida* U112/BMMs	[56]
	PdpB	*F. novicida* U112/BMMs	[63]
	VgrG	*F. novicida* U112/J774	[38]
	MglA	*F. novicida* U112/J774	[66]
		F. novicida U112/U937 and hMDMs	[34]
		LVS/J774	[29]
	MglB	*F. novicida* U112/J774	[66]
	MigR	LVS/BMMs	[68]
	FevR	*F. novicida* U112/BMMs; LVS/BMMs	[68, 70]
	PmrA	*F. novicida* U112/THP-1/J774	[67]
	PurA,PurF	*F. novicida* U112/J774	[75]
	PurMCD	LVS/J774/PEC	[73, 74]
	CarA, CarB, PyrB	LVS/BMMs	[76]
	DsbB	SchuS4/HepG2/J774	[83]
	DsbA	SchuS4/HepG2	[72]
		SchuS4/J774	[81]
		LVS/J774	[82]
	FTT0383	SchuS4/BMMs	[65]
	FTT0369c	SchuS4/BMMs	[65]
	FTT1676	SchuS4/BMMs	[65]
	AcpA	*F. novicida* U112/THP-1/BMMs	[88]

*****Proteins involved in both procesess of a phagosomal escape and an intracellular replication.

of infected cells [39]. This cycle is terminated by a disruption of a cell membrane and a subsequent infection of neighbouring cells (Figure 1). Interestingly, at later stages of an intracellular infection, some species of *Francisella*, instead of inducing immediate apoptosis, re-enter an endocytic pathway and are located in a LAMP-1 positive vacuole, termed a *Francisella*-containing autophagic vacuole (Figure 1) [31, 40]. This phenomenon has been observed only in murine macrophages and does not occur in human macrophages [41]. Additionally, it has been shown that in human monocytes several autophagy-related genes are down-regulated after an infection with *F. tularensis*, suggesting that *Francisella* suppresses a host autophagy response at a gene expression level [42, 43].

MODULATION OF HOST CELL SIGNALLING PATHWAYS BY *F. TULARENSIS*

Similarly to other intracellular bacteria, *F. tularensis* is able to subvert host defensive strategies [44]. Intracellular signals that are involved in the process of *F. tularensis* phagocytosis are mediated via a tyrosine kinase (Syk). Activation of Syk results in activation of a mitogen-activated protein kinase (MAP), originally called an extracellular signal-regulated kinase (ERK), through a protein kinase C. This cascade results in actin polymerization and induction of phagocytosis [45-47]. It has been shown that another signaling pathway, a phosphoinositide 3-kinase PI3K/Akt pathway, is not affected during *Francisella* internalization into murine macrophages [46]. After internalization into a host cell, *F. tularensis* alters bactericidal processes. It prevents induction of a respiratory burst including a disruption of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase assembly [48]. Another step in evasion of an immune system is an interference of *F. tularensis* with interferon-gamma (IFN-γ) signaling. IFN-γ is a major mediator of macrophage activation and also triggers an inducible nitric oxide synthase (iNOS) production. Therefore, a supression of an IFN-γ release by *F. tularensis* is an essential strategy of how to circumvent a macrophage defence system [49].

Host cells recognize conserved bacterial molecules expressed only by pathogens with a variety of receptors, so called pattern recognition receptors (PRRs). There are two groups of PRRs depending on a cell localization: membrane-bound Toll-like receptors (TLRs) and cytosolicly localized nucleotide-binding oligomerization domain-like receptors (NLRs). Activation of a proinflammatory response in macrophages occurs via TLR2, since *F. tularensis* provides a biologicaly inactive lipopolysaccharide (LPS) unable to bind to TLR4. Signaling through TLR2 results in an activation of a myeloid differentiation factor MyD88. MyD88 activates PI3K and thereafter Akt. Active Akt promotes a downstream localized NF-κB resulting in a cytokine production and a host cell survival. However, *Francisella* activates an inositol phosphatase SHIP resulting in an inhibiton of a PI3K/Akt pathway and a production of pro-inflammatory cytokines [50]. Inside a host cell cytosol, *Francisella* activates a multiprotein complex called an absence in melanoma 2 (AIM2) inflammasome [51, 52]. As an infection sensor, an AIM2 inflammasome activates caspase-1 which triggers a production of proinflammatory cytokines IL-1β and IL-18 [53, 54]. Activation of an inflammasome is criticaly dependent on a type-I interferon signaling [55].

BACTERIAL FACTORS AFFECTING AN INTRACELLULAR FATE OF *F. TULARENSIS*

Microorganisms are commonly equipped with various virulence factors whose production is crucial for a microbe invasion, a replication inside host cells and for an evasion of cell immune responses. Despite a high virulence, *Francisella* does not encode any exotoxin, and pathogenicity probably relies on its ability to replicate inside host cells causing a tissue damage and an organ failure. More than 250 gene products have been identified up to now to be important for a multiplication within mammalian cells. These genes can be grouped into the following functional categories: *Francisella* Pathogenicity Island, transcriptional regulators, metabolism, chaperones and genes with various functions.

Francisella Pathogenicity Island

The *Francisella* pathogenicity island (FPI) is a ~ 30 kb region identified within a genome of *F. tularensis* [56]. An importance of a FPI region is implied by its duplication in all *F. tularensis* subspecies but not in *F. novicida* species [56]. Two transcriptional units have been verified as being required for a virulence. The first one is an intracellular growth locus *iglABCD* and the second

one is a pathogenicity determinant protein unit *pdpABCD* [56]. A key role of FPI genes has been documented in their ability to modulate a phagosome biogenesis and an intracellular growth.

Deletion mutants for each gene within an *iglABCD* operon have been prepared and characterized for an *in vitro* virulence. Potential interacting partners IglA and IglB have been shown to be required for a phagosmal escape and an intramacrophage growth of *F. novicida* or an LVS strain within murine J774 macrophages [57, 58]. In contrast, *iglD* was identified to be important for a replication of *F. novicida* and also for an LVS strain within a cytosol [33]. However, there is a controversy about a phagosomal escape of an *iglD* mutant, since in the case of an IglD deletion in a *F. novicida* strain the mutant behaved in the same way as a parental strain when examining an intracellular trafficking [33]. On the contrary, the mutant constructed in the LVS strain showed an impaired phagosomal escape into a host cell cytosol [29]. This controversy pointed out the differences between *Francisella* strains used, host models and examination methods of trafficking events. An *iglC* deletion mutant constructed in a *F. tularensis* LVS strain is unable to replicate inside a cell cytosol of J774 cells and also it is unable to escape from phagosomes [59]. Recently, participation of additional FPI proteins in an intramacrophage growth and a phagosomal escape has been desribed for proteins IglH, IglG and IglI, where only an IglH protein is involved in both a phagosomal escape and an intracellular replication [60, 61].

A non-polar deletion mutant constructed for the first cistron of the second largest FPI operon, *pdpA*, is also unable to replicate within a murine J774 cell line but it is capable of a small amount of proliferation within BMMs [62]. Nevertheless, a *pdpA* mutant associates within late endosomes indicating an impaired phagosomal escape into a cell cytosol [62]. Another mutant from a pdp operon, pdpB, is also unable to replicate inside a cytosol of primary murine BMMs, however, a phagosomal escape was not tested [63]. In contrast, a *pdpD* mutant replicates within BMMs with the same trend as a parental strain [64].

Interestingly, an expression profile of FPI genes showed the maximum of expression at the end of a cytosolic replication suggesting a role for FPI genes during late stages of an infection [65]. It is suggested that FPI encodes a type VI-like secretion apparatus responsible for a modulation of a phagosome biogenesis and for facilitating an escape of bacteria into a cell cytosol [38]. Recently, an evidence for a secretion of VgrG and IglI proteins into a cell cytosol was provided [38]. It is generally assumed that all proteins encoded by FPI genes actively participate on countering a host defense. However, their exact role in pathogenesis of the disease is still unclear.

Transcriptional regulators

Transcriptional regulators are important factors enabling bacteria to survive in diverse environmental niches. Several regulatory genes have been identified in *F. tularensis* up to day including MglA, SspA, PmrA, FevR, MigR and Hfq. It has been shown that deletion mutants for each of transcription regulators are attenuated for a virulence in *Francisella* [66-70]. These data suggest that a contribution of transcription regulators to a *F. tularensis* intracellular survival is likely a result of a deregulation of effector proteins, rather than a direct participation of a particular gene in a virulence process.

The first described transcription regulator in *Francisella*, a macrophage growth locus MglA, regulates 102 genes in total [63, 66]. The majority of these regulated genes are involved in a *Francisella* metabolism, an environmental information processing or are located within FPI [63]. It has been shown that a deletion of MglA, as well as a gene of the next operon MglB, resulted in a defective intracellular replication within host cells [66]. In addition, a deletion of MglA resulted in an inability of *F. tularensis* to disrupt a phagosome and to escape into a cell cytosol [29, 34]. Recently, PmrA, another regulatory protein physically interacting with MglA, has been described [67]. PmrA is a DNA-binding protein involved in a two-component system required for a bacterial sensing. A transcription factor PmrA is phosphorylated by KdpD, which is a membrane-bound sensor kinase sensing environmental signals [71]. PmrA regulates 65 genes, majority of them are located around PmrA or within FPI [67]. A deletion of *pmrA* in a *F. novicida* strain resulted in an impaired intracellular growth in both murine macrophages (J774.A1) and human macrophages (THP-1)[67]. The other transcription regulator, termed a *Francisella* effector of a virulence regulation FevR (also called PigR), is required for regulating the same set of genes as MglA and in turn is positively regulated by a MglA/SspA regulon [70]. Importantly, it has been shown that FevR contributes to a replication in

macrophages, since a *fevR* mutant was unable to replicate inside murine BMMs [70]. A macrophage intracellular growth regulator (MigR) is another regulator of an *iglABCD* operone encoded within FPI [68]. MigR is also crucial for a *Francisella* growth in macrophages and contributes to a blockade of a neutrophil NADPH oxidase activity [68]. The only transcription regulator acting mostly as a repressor on a *Francisella* gene expression is a bacterial RNA-binding protein Hfq. Hfq is described to regulate, directly or indirectly, an expression of several genes in *Francisella*, including 10 genes inside FPI [69]. However, it has been shown that a deletion mutant constructed for a Hfq protein in both LVS and a clinical virulent isolate of a *F. tularensis* subsp. *holarctica* FSC200 strain does not play a major role in an intracellular multiplication inside murine macrophages. Nevertheless, it is important for an *Francisella* virulence *in vivo* [69].

Virulence factors with an important role in *Francisella* metabolism

To be able to survive in various life conditions *F. tularensis* has to adjust its metabolism to nutrients and physical conditions of surroundings. Recently, due to the development of genome-wide genetic screens, several virulence factors related to metabolic and nutritional functions have been identified. For example an aromatic amino acids biosynthetic pathway has been identified to be crucial for a *Francisella* virulence. Inactivation of genes within this pathway, such as *aroA* or *aroB*, led to an attenuated phenotype [72]. Furthermore, a characterization of auxotrophic mutants at pyrimidine (*carA*, *carB* and *pyrB*) and purine biosynthesis pathways (*purF*, *purA* or purine biosynthetic locus *purMCD*) showed a contribution of these genes to an *in vitro* growth of a *F. tularensis* LVS strain or a *F. novicida* strain [73-76]. In addition, it is generaly known that a cytosol of a host cell serves as a source of nutritions for invading bacteria. The only direct proof of a *Francisella* nutrient utilization from a host cell cytosol is existing for a tripeptide glutathione (γ-glu-gly-cys) which is obvious due to a dependency of *Francisella* on a high concentration of cystein [77].

Chaperones

Chaperones are generally expressed in bacteria to provide a protection against stressful conditions during an intracellular multiplication of bacteria in a host. *F. tularensis* disposes of six genes encoding proteins with a chaperone characterization. Those are partner proteins of DsbA and DsbB which act together in a process of a disulfide bond formation in newly synthetised proteins [78], a heat shock protein ClpB [79], a peptidase Lon (ATP dependent protease), CphB (Cyanophycinase) and a heat shock protein HtpG [80].

It has been shown that a deletion mutant for a DsbA protein, encoded by a FTT1103 gene, is essential for a phagosomal escape and an intracellular replication of a *F. tularensis* subs. *holarctica* strain as well as for a highly virulent *F. tularensis* subs. *tularensis* strain SchuS4 in a murine J774 cell line and primary murine BMMs [60, 81, 82]. Moreover, transposon mutant constructed in a *F. tularensis* subsp. *tularensis* Schu S4 strain for a DsbB protein is defective in an intracellular growth in HepG2 cells as well as in J774 macrophages [83]. Next chaperone is a heat-shock protein ClpB. ClpB is also involved in a process of a protein folding and it is expressed under heat shock conditions. It has been shown that a ClpB protein contributes to an intracellular growth of *F. tularensis* LVS and *F. novicida* in murine but not in human macrophages [79, 84]. Chaperones Lon, CphB and HtpG have been identified to be implicated in an intracellular growth of *Francisella* using *in vitro* genetic screens [80, 84, 85]. Moreover, a transposon mutant for HtpG has been found to be important also for an *in vivo* virulence [86] . Generally, chaperones play an important role in a *Francisella* virulence due to their pleiotropic function, such as an adaptation to different environmental conditions during an infection (heat shock proteins) or a protein quality control (disulfide oxidoreductase activity).

Other bacterial factors involved in an intracellular survival

A transcriptional profiling analysis of virulence determinants involved in an intracellular growth inside a murine macrophage infected with a SchuS4 strain has revealed several important loci required for a proliferation [65]. Deletion mutants constructed for three of them, specifically FTT0383, FTT0369c and FTT1676, have been identified to be required for an intracellular replication of a SchuS4 strain within BMMs. Moreover, mutants for FTT0383 and FTT1676 genes showed a delayed escape from a late phagosome indicating an involvement of these proteins in an optimal phagosomal escape and a further cytosolic replication of a highly virulent strain inside murine macrophages [65].

Contribution of acid phosphatases to an intracellular lifestyle and an intraphagosomal escape of *Francisella* is well documented. *Francisella* disposes of at least four acid phosphatases: AcpA, AcpB, AcpC and Hap, where AcpA contributes greatly to a total phosphatase enzyme activity and therefore has been characterized in great detail [87-91]. Recently, it has been shown that AcpA is secreted *in vitro* into a culture supernatant. Strikingly, it has been shown that AcpA is secreted and transclocated across a phagosomal membrane into a cytosol during an infection with a *F. novicida* as well as with a SchuS4 strain [92].

CONCLUSIONS

Understanding a process of a *F. tularensis* intracellular fate within mammalian cells is a crucial step in revealing a pathogenesis of this highly virulent microorganism. Therefore, during the last few years, researchers have focused on determining an intracellular cycle using a variety of *F. tularensis* strains (*F. novicida*, subsp. *holarctica* LVS strain or subsp. *tularensis* SchuS4 strain) and host cell models (macrophage cell lines, murine or human primary macrophages). Despite slight discrepances, mainly in a time-setting of particular maturation steps, it has been shown that FCP is arrested at a late endosomal stage, prior to a fusion with degradative lysosomes. Thereafter, *F. tularensis* rapidly escapes into a cell cytosol where extensively replicates. Several proteins have been detected to be participating in a process of a phagosomal biogenesis and mutants generally constructed for these proteins were also impaired in a cytosolic intracellular multiplication. Neverthelless, a molecular mechanism together with a significant factor/s responsible for a degradation of a phagosomal membrane and an escape of bacteria into a cytosol remains unexplored. Moreover, following a *Francisella* infection, a host cell starts up its defense mechanisms to limit a replication of bacteria which means that *Francisella* has to respond by several different strategies to counteract a disadvantageous host defense mechanism.

Future research of a *Francisella* intracellular fate should be targeted on a description of a molecular mechanism covering a process of a disruption of a phagosomal membrane and a characterization of molecular functions of identified bacterial and host factors in order to understand a *Francisella* pathogensis at a molecular level.

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