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Research Article

RESEARCH ARTICLE

Cooperation of both, the FKBP_N-like and the DSBA-like, domains is necessary for the correct function of FTS_1067 protein involved in *Francisella tularensis* virulence and pathogenesis

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One sentence summary: Our study corroborates the finding emphasizing importance FTS_1067 isomerase activity in *Francisella tularensis* virulence and supports the cooperation of domains in establishing of the full function of this protein.

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ABSTRACT

Francisella tularensis the etiological agent of tularaemia is one of the most infectious human pathogen known. Our knowledge about its key virulence factors has increased recently but it still remains a lot to explore. One of the described essential virulence factors is membrane lipoprotein FTS_1067 (nomenclature of *F. tularensis* subsp. *holarctica* strain FSC200) with homology to the protein family of disulphide oxidoreductases DsbA. Lipoprotein consists of two different domains: the C-terminal DsbA_Com1-like domain (DSBA-like) and the N-terminal FKBP-type peptidyl-prolyl *cis/trans* isomerases (FKBP_N-like). To uncover the biological role of these domains, we created bacterial strain with deletion of the DSBA-like domain. This defect in gene coding for lipoprotein FTS_1067 led to high *in vivo* attenuation associated with the ability to induce host protective immunity. Analyses performed with the truncated recombinant protein showed that the absence of DSBA-like domain revealed the loss of thiol/disulphide oxidoreductase activity and, additionally, confirmed the role of the FKBP_N-like domain in the FTS_1067 oligomerization and chaperone-like function. Finally, we verified that only full-length form of FTS_1067 recombinant protein possesses the isomerase activity. Based on our results, we proposed that for the correct FTS_1067 protein function both domains are needed.

Keywords: oligomerization; virulence factor; copper ions; protein disulphide isomerase; chaperone

INTRODUCTION

Francisella tularensis (F. tularensis) is a Gram-negative facultative intracellular bacterium and causative agent of the zoonotic disease tularaemia. Due to its extreme virulence, low infectious dose, ease of aerosol dissemination, and the capacity to cause severe illness and death, *F. tularensis* has been classified as a category A biological agent of bioterrorism (Dennis *et al.* 2001). The availability of genomic information and the appropriate molecular tools has facilitated various studies where several putative virulence factors have been identified. The conserved lipoprotein FTS_1067 (in FSC200 genome, FTT_1103 in SchuS4 genome) has been proved as one of the most powerful factors of *F. tularensis* virulence and bacterial pathogenesis (Thakran

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et al. 2008; Qin *et al.* 2009; Straskova *et al.* 2009). Bioinformatic analysis of FTS_1067 proposed the presence of a signal peptide on position 1–21 and a lipid group on Cys22 (Straskova *et al.* 2009) (Damborsky and Sebestova, pers. comm.). This lipidation is probably linked to its ability to activate TLR2-mediated signalling (Thakran *et al.* 2008). Further post-translational modification associated with this protein is O-glycosylation (Balonova *et al.* 2010). There are two functional domains, the amino terminal domain to FKBP-type peptidyl-prolyl isomerase which shares homology with the terminal dimerization domain of macrophage infectivity potentiator (Mip) proteins and the carboxy terminal domain DsbA_Com1_like. The exact role of these domains, especially the FKBP_N-like domain, in the biological function of the protein FTS_1067 was not fully clarified yet, but there is a large amount of knowledge about the proteins with structural homology. Peptidyl-prolyl isomerases are important for *cis/trans* isomerization of peptide bonds in oligopeptides, probably also in extracytoplasmic proteins (Missiakas, Betton and Raina 1996). FTS_1067 N-terminal domain shares a homology with FkpA of *Escherichia coli* (Riboldi-Tunnicliffe *et al.* 2001), which also has a chaperone activity (Arié, Sassoon and Betton 2001), and with *Legionella pneumophila* Mip (Saul *et al.* 2004). Both of these proteins exist in dimeric state. The C-terminal domain has been identified to be homologous to a protein family of the thiol/disulphide oxidoreductase family DsbA_Com1_like. The C-terminal domain contains the conserved catalytic active site CXXC, which is typical for DsbA proteins and protein disulphide isomerases (PDI), as well. Members of these protein families are responsible for introduction or correction of disulphide bonds into synthetized proteins and are important for folding of extracytoplasmic proteins including known virulence factors (Yu and Kroll 1999). In this study, we aimed to elucidate the function of both FTS_1067 domains using either mutants or recombinant proteins with selectively deleted DSBA-like or FKBP_N-like domains.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions

The *F. tularensis* and *E. coli* strains and plasmids used in this study are listed in Table 1. *Francisella tularensis* FSC200 and derivative strains were routinely cultured at 37°C on McLeod agar enriched for bovine haemoglobin (BD Diagnostics) and IsoVitaleX (BD Diagnostics) or in chemically defined Chamberlain's medium (Chamberlain 1965) with shaking at 200 rpm. For stress tolerance tests, the following conditions were used: 2% additional NaCl, medium adjusted to pH 4, growth at 42°C, presence of 0.03% hydrogen peroxide, $CuCl_2$ in a final concentration of 20 μM and iron-depleted Chamberlain's medium. *Escherichia coli*

Table 1. Bacterial strains, plasmids and primers used in this study.

Strain	Description	Source
Francisella tularensis		
FSC200	WT *F. tularensis* subsp. *holarctica*, clinical isolate	*Francisella* Strain Collection (FSC) of the Swedish Defense Research Agency, Umeå, Sweden
FSC200/ΔFTS_1067	FSC200 Δ*fts_1067*	Straskova *et al.* (2009)
cFSC200	FSC200/ΔFTS_1067 + pKK289-FTS_1067	Schmidt *et al.* (2013)
FSC200/ΔDSBA-like	FSC200/ΔFTS_1067 + pKK289-FTS_1067ΔDSBA	This study
FSC200/ΔFKBP_N-like	FSC200/ΔFTS_1067 + pKK289-FTS_1067ΔFKBP_N	Schmidt *et al.* (2013)
Escherichia coli		
TOP10	F$^-$ *mcrA* Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lacZ*ΔM15 Δ*lacX74 recA1 ara*D139 Δ(*ara-leu*)7697 *galU galK rpsL* (Str^R) *end*A1 *nup*G	Invitrogen
rFTS_1067	BL21 (DE3) + pET28b-FtdsbA	Schmidt *et al.* (2013)
rFTS_1067/ΔDSBA-like	TOP10 + pBAD/FTS_1067ΔDSBA Myc-His	This study
rFTS1067/ΔFKBP_N-like	BL21 (DE3) + pET28b-FtdsbA ΔFKBP_N	Schmidt *et al.* (2013)
Plasmid	**Description**	**Source**
pCR4-TOPO	Cloning vector, *lac* promoter, Km^R, Amp^R	Invitrogen
pKK289KmGFP	*gfp* gene under the control of the LVS GroES promoter, Km^R	Bönquist *et al.* (2008)
pKK289-FTS_1067ΔDSBA	pKK289Km + FTS_1067ΔDSBA, Km^R	This study
pBAD/*Myc*-His	Expression vector, *araBAD* promoter, C-terminal polyhistidine tag, C-terminal *c-myc* epitope, Amp^R	Invitrogen
pBAD/FTS_1067ΔDSBA *Myc*-His	pBAD/*Myc*-His + FTS_1067ΔDSBA, Amp^R	This study
Primer	**Sequence**	
A	5′-TAA ATT TCA TAT GAC TAA GAA AAA ACT T-3′	
B	5′- AGA GCT CTT ATA GAT CAG AGT TAT AGA T -3′	
C	5′- TAC CAT GGG CAC TAA GAA AAA ACT T -3′	
D	5′- TCT CGA GTA GAT CAG AGT TAT AGA T -3′	

strains were cultured in Luria–Bertani broth or on Luria–Bertani agar plates. When required, antibiotics were used at the following concentrations: kanamycin 20 μg mL^{-1} (*F. tularensis*) or 50 μg mL^{-1} (*E. coli*); ampicillin 50 μg mL^{-1} (*E. coli*).

Complementation *in trans*

A partial *trans*-complemented strain with deletion of the DSBA-like domain was constructed by using *in-frame* deletion mutant FSC200/ΔFTS_1067, previously referred as FSC200/ΔDsbA (Straskova *et al.* 2009). A DNA sequence coding for *fkbpn-like* domain and surroundings was amplified by PCR with primers A and B described in Table 1. Restriction sites for *Nde*I and *Sac*I were introduced into these primers. The PCR product was cloned into the pCR4-TOPO vector (Invitrogen) and sequenced (Generi Biotech, Czech Republic). The *fkbp_n-like* fragment was then ligated into the shuttle vector pKK289KmGFP (a kind gift from Åke Forsberg, Umeå University, Sweden) under the control of LVS GroES promoter (Bönquist *et al.* 2008) using *Nde*I and *Sac*I restriction sites. The final construct pKK289-FTS_1067ΔDSBA was introduced into *F. tularensis* subsp. *holarctica* FSC200/ΔFTS_1067 by electroporation (Rodriguez *et al.* 2008). The newly constructed mutant strain was denoted as FSC200/ΔDSBA-like. It is deletion mutant (complete gene deletion) with N-terminal *fkbp_n-like* domain *trans*-complemented. The ability of the partial *trans*-complemented strain to produce truncated protein was confirmed by LC-MS/MS and immunodetection.

Evaluation of protein expression level in cell lysates

The wild-type (WT) strain FSC200, the FSC200/ΔDSBA-like mutant strain and the cFSC200 (full-length gene complementation *in trans*) (Schmidt *et al.* 2013) were grown overnight at 37°C with shaking at 200 rpm in the Chamberlain's medium containing appropriate antibiotic. The overnight cultures were diluted to a final optical density at 600 nm of 0.1 in complete Chamberlain's medium, and then grew it at 37°C with shaking at 200 rpm until its OD_{600} reached to 0.8. Then harvested the culture by centrifugation (7300 rpm, 15 min, 4°C), discarded the supernatant, washed the pellet with cold 50 mM Tris buffer (pH 7.5), spun down (7300 rpm, 15 min, 4°C) and resuspended the pellet into 50 mM Tris buffer (pH7.5) with protease inhibitor cocktail Complete EDTA-free (Roche) and Benzonase Nuclease (Sigma-Aldrich). The cell lysis of the WT and mutant strains was performed by using a French press (three passages at 16 000 psi). The concentration of proteins was quantified by using a BCA protein assay kit (Sigma-Aldrich). The SDS-PAGE was used to separate the proteins and then the separated proteins were transferred from gel to PVDF membrane by using the semi-dry Western blotting procedure. For the visualization of transferred proteins, we used polyclonal rabbit antibody raised against FTT_1103 protein (1:1000) (Apronex, Czech Republic) as the primary antibody and the polyclonal swine anti-rabbit immunoglobulins/HRP (1:1000) (DAKO) as the secondary antibody.

Cloning, overproduction and purification of recombinant protein rFTS_1067/ΔDSBA-like

The *fkbp_n-like* domain coding region was amplified by PCR with primers C and D containing the restriction sites for *Nco*I and *Xho*I (Table 1). *Francisella tularensis* subsp. *holarctica* strain FSC200 chromosomal DNA was used as the template. The *fkbpn-like* fragment was ligated into the pBAD/*Myc*-His vector (Invitrogen) which has a polyhistidine tag on the C-terminus. The rFTS_1067/ΔDSBA-like construct was verified by sequencing then transformed it into *E. coli* One Shot TOP10 Competent Cells (Invitrogen). For recombinant protein purification, the cells grew in LB medium at 37°C until culture OD_{600} reached to 0.6, and then L-arabinose was added to a final concentration of 0.008% to induce recombinant protein expression. After 24-h cultivation at 25°C, the bacteria were harvested by centrifugation (8000 × g, 15 min, 4°C). The pellet was washed with cold phosphate-buffered saline (PBS), centrifuged (8000 × g, 10 min, 4°C) and resuspended into PBS containing 350 mM NaCl and protease inhibitors cocktail Complete EDTA-free (Roche). The bacterial lysate was prepared by using French press by three passages at 16 000 psi. The lipoprotein-enriched fraction was isolated by using Triton X-114 (Thakran *et al.* 2008). The final lipoprotein-enriched fraction was resuspended in binding buffer (100 mM HEPES, pH 7.5, 10 mM imidazole, 500 mM NaCl) and diluted to final concentration of detergent under 2%. The diluted sample was mixed with His-Link Purification Resin (Promega) and incubated for 4 h at 4°C. The resin was loaded onto the column and washed three times with wash buffer (100 mM HEPES, pH 7.5, 125 mM imidazole, 500 mM NaCl). Finally, the recombinant protein was eluted from the resin with high imidazole concentration elution buffer (100 mM HEPES, pH 7.5, 500 mM imidazole). The elution fraction was dialyzed against 5 mM HEPES, pH 7.5 at 4°C, and then the Amicon Ultra Centrifugal Filters (Merck-Millipore) were used to get concentrated dialyzed protein. The purity of the recombinant protein was evaluated by using SDS_PAGE and Coomassie R-250 Blue Staining. The full-length and the rFTS_1067/ΔFKBP_N recombinant proteins were purified as described previously (Schmidt *et al.* 2013).

Isolation and infection of macrophages

Mouse bone marrow cells were isolated from dissected femur of 6–10 weeks old female BALB/c mice (Velaz, Czech Republic). The ends of the bones were cut off, and the bone marrow cells were flushed out by DMEM (Dulbecco's Modified Eagle Medium + GlutaMAX-I (Invitrogen)). The cells were cultured in DMEM supplemented with 10% foetal bovine serum (FBS, Gibco), 10% L929-conditioned medium (source of macrophage-colony stimulating factor), and 50 U mL^{-1} penicillin and 50 μg mL^{-1} of streptomycin cocktail (Sigma-Aldrich) and incubated at 37°C in 5% CO_2 for 7 days to differentiate to bone marrow-derived macrophages. For the cell infection experiments, the cells were seeded to a 24-well tissue culture plate at a density 5 × 10^5 cells per well and the WT or the mutant strains were used at a multiplicity of infection (MOI) of 50. Tissue culture plates were centrifuged at 400 × g for 5 min to start the infection and then incubated for 30 min at 37°C with 5% CO_2. Extracellular bacteria were then killed by gentamicin treatment (5 μg mL^{-1}) for 30 min. Finally, the cells were washed three additional times with PBS before the complete cultivation medium without antibiotic was added. At selected time points (1, 6, 12, 24 and 48 h) after infection, the cells were lysed by 0.1% sodium deoxycholate and the number of viable intracellular bacteria was determined by viable counts (CFU). The lysates were serially diluted in PBS and plated on McLeod agar plates. Plates were incubated for 3 days at 37°C in the presence of 5% CO_2.

Infection of mice and protection assays

Groups of five female BALB/c mice 6–10 weeks old were inoculated subcutaneously (s.c.) with 200 μL of bacterial suspension with infection doses of 10^2, 10^3, 10^4, 10^5 and 10^6 CFU/mouse for

the mutant strains FSC200/ΔDSBA-like and FSC200/ΔFKBP_N-like and 10^2 CFU/mouse for the WT strain. The control group of mice was inoculated with sterile saline only. Mice were observed every day for 21 days to monitor symptoms of infection or mortality. The actual doses administered for each strain were determined after 3 days cultivation of the serially diluted bacteria plated on McLeod agar plates at 37°C and 5% CO_2.

For the protection studies, mice vaccinated with the mutant strains in previous experiment were challenged subcutaneously 21 days post-infection with 10^2 CFU/mouse of the WT strain and monitored for at least next 21 days.

Organ dissemination of the mutant strains

To examine the bacterial burden in target organs, groups of female BALB/c mice 6–10 weeks old were infected with 10^5 CFU/mouse of the FSC200/ΔDSBA-like or the FSC200/ΔFKBP_N-like mutant strains or with 10^2 CFU/mouse of the WT strain. After 1, 3, 5, 7, 14, 21 and 28 days post-infection, groups of three mice were sacrificed, and the numbers of bacteria in livers, spleens and lungs were determined by plating of homogenized samples on McLeod agar plates supplemented with kanamycin 20 μg mL^{-1} (in case of the complemented strains) and penicillin 100 U mL^{-1}. After 3 days of incubation at 37°C in 5% CO_2, the bacterial colonies were quantified and the number of CFU per organ was calculated.

Growth kinetics and stress survival assays

The FSC200 and the mutant strains were grown overnight at 37°C with shaking at 200 rpm in Chamberlain's medium and appropriate antibiotic. The cultures were diluted to a final optical density at 600 nm of 0.1 in complete Chamberlain's medium containing additional 2% NaCl (osmotic stress) or with pH 4.0 (acid stress) or in iron-depleted Chamberlain's medium. For testing sensitivity to the $CuCl_2$, the overnight cultures were diluted to a final optical density at 600 nm of 0.1 in complete Chamberlain's medium supplemented with $CuCl_2$ to a final concentration of 20 μM (higher concentration of the $CuCl_2$ impaired the growth of the WT strain). The cultures were grown for 24 h at 37°C with shaking. For heat resistance tests, the overnight cultures were diluted in the same way in complete Chamberlain's medium and diluted cultures were grown for 24 h at 42°C with shaking. The growth kinetics was assessed by measuring optical density at 600 nm using multimode microplate reader FLUOstar Optima (BMG Labtech). The growth curves obtained from FLUOstar Optima were similar as curves from traditional cultivation in cultivation flasks (data not shown). Every strain was measured in pentaplicate, and experiments were independently repeated at least three times. Data represent the mean ± standard deviation from all experiments. For oxidative stress survival assay, 0.03% hydrogen peroxide was used. The overnight cultures were diluted to a final optical density at 600 nm of 0.1 in complete Chamberlain's medium and were grown at 37°C with shaking until they reached stationary phase. The cultures were thereafter adjusted with hydrogen peroxide to a final concentration of 0.03%. The aliquots of cultures were withdrawn 10, 20, 30 and 40 min after addition of hydrogen peroxide, serially diluted and plated on McLeod agar enriched for bovine haemoglobin (BD Diagnostics) and IsoVitaleX (BD Diagnostics). The number of viable bacteria was enumerated after 72 h of incubation at 37°C. Each experiment was independently repeated at least three times, and data represent the mean ± standard deviation from all experiments.

Assay for disulphide oxidoreductase activity

The ability of the recombinant proteins to catalyse the reduction of human insulin (Sigma-Aldrich) in the presence of dithiothreitol (DTT) was determined by following the rate of precipitation of insulin B chain from the solution (Holmgren 1979). The reaction mixture contained 150 μM insulin solution in 100 mM potassium phosphate buffer, pH 7.0, 2 mM EDTA and 5 μM thioredoxin (Sigma-Aldrich) or 5 μM recombinant proteins for reaction catalysis. The reaction was started by adding DTT to a final concentration of 0.33 mM, and the measurement of turbidity of the reaction mixture was performed at 650 nm every 30 s for 1 h on NanoPhotometer (Implen). The uncatalysed insulin reduction by DTT was measured as a negative control reaction without addition of any recombinant protein or thioredoxin.

Chaperone-like activity assay

The measurement of chaperone-like function of the recombinant proteins was based on monitoring the activity of thermally inactivated citrate synthase (CS, Sigma-Aldrich) in the presence or absence of recombinant protein (Buchner, Grallert and Jakob 1998). For this purpose, we used the Citrate Synthase Assay Kit (Sigma-Aldrich). Porcine heart CS was dialyzed and concentrated into 40 mM HEPES-KOH buffer, pH 7.5. The protein concentration of CS was determined using Pierce BCA Protein Assay Kit (Thermo Scientific), and thereafter CS was diluted into 40 mM HEPES-KOH buffer, pH 7.5 to final concentration of 30 μM. Thermal inactivation was achieved by incubation of CS at 43°C in 40 mM HEPES-KOH buffer, pH 7.5, for 16 min. The chaperone activity of recombinant proteins was tested in final 4-fold molar excess concentration of CS (monomer). Bovine serum albumin (Sigma-Aldrich) in equimolar and 10-fold molar excess concentration of CS (monomer) was used as a control for non-specific CS protection (data not shown). All measurements were performed on NanoPhotometer (Implen), where change of turbidity was measured every 10 s for 2 min using 412 nm. All assays were performed at least three times.

Assay for isomerase-like activity

First *in vitro* assay was inspired by Hiniker, Collet and Bardwell (2005). All variants of FTS_1067 recombinant proteins (20 μM) were reduced for 10 min on ice with 10 mM DTT. After reduction, we used PD MiniTrap G-25 (GE Healthcare) to remove DTT by buffer exchange into 25 mM Tris, pH 7.5 or 25 mM HEPES, pH 7.5 (for the rFTS_1067/ΔDSBA-like). Next, we oxidized 50 μM bovine pancreatic RNase A (Serva), which was diluted into 300 mM NaCl, 50 mM sodium phosphate, pH 6.0, with 50 μM $CuCl_2$ and 2 mM H_2O_2 during the incubation at 25°C for 30 min. $CuCl_2$ and H_2O_2 were removed and buffer was exchanged by gel filtration (PD MiniTrap G-25) to 300 mM NaCl, and 50 mM sodium phosphate, pH 7.5. To test the ability of reduced recombinant proteins to refold the incorrect disulphide bond and restore the activity of $CuCl_2$/H_2O_2-oxidized RNase A, aliquots of 20 μM reduced recombinant proteins and 20 μM oxidized RNase A were mixed together and incubated for 2 h at 25°C. After incubation, the cCMP was added to a final concentration of 4 mM and its hydrolysis was followed at 296 nm for 300 s on NanoPhotometer (Implen). The cCMP hydrolysis of native RNase A was set to 100% activity and the activity of RNase A in all other samples was expressed as a percentage of native RNase A activity. Activity of the oxidized RNase A coincubated with reduced PDI (Sigma-Aldrich) and

activity of $CuCl_2/H_2O_2$-oxidized RNase A were used as positive and negative controls, respectively.

In the second *in vitro* assay, we used thermally unfolded RNase A in the assay testing the PDI activity of the recombinant proteins (Fu and Zhu 2011). We mixed 20 μM native RNase A (Serva) with 20 μM reduced variants of FTS_1067 recombinant proteins in 10 mM sodium phosphate buffer, pH 7.5. These mixtures were thermally treated for 30 min at 65°C and afterwards cooled down for 30 min at 25°C. Enzyme activity of RNase A was determined by monitoring of cCMP hydrolysis. Reaction buffer contained mixture of proteins in 50 mM Tris-HCl buffer, pH 8.0 with 4.5 mM cCMP and reaction was initiated by adding of EDTA into a final concentration of 0.4 mM. Enzymatic activity was measured at 412 nm every 30 s for 20 min. All data represent the mean ± standard deviation from three independent experiments.

Blue native polyacrylamide electrophoresis (BN-PAGE)

For determination of the oligomeric state, we used BN-PAGE. We prepared mini-gels with linear 4.5–16% gradient and running buffers according to protocols described earlier (Schägger and von Jagow 1991; Jänsch *et al.* 1996). The sample containing recombinant protein was treated with 2% (v/v) dodecyl-β-D-maltoside (Sigma-Aldrich) in 50 mM HEPES buffer. Into the 20 μL of sample, 1 μL of 5% w/v Coomassie Brilliant Blue G-250 (Serva) in 750 mM 6-aminohexanoic acid was added. For designation of the molecular weight, NativeMark Unstained Protein Standard (Invitrogen) was used. The BN-PAGE was performed on ice. For the first 30 min of the run, the voltage was set at 100 V, and then was increased to 200 V until the run ended. Halfway the run the blue cathode running buffer was changed to colourless to clear the background for immunoblotting. Finally, the gel was washed with 0.1% sodium dodecyl sulphate (SDS) solution for 15 min, and then with transfer buffer (25 mM Tris-base, 192 mM glycine and 20% methanol). The proteins were transferred from gel to PVDF membrane by using the semi-dry Western blotting procedure. For visualization of transferred proteins, we used anti-His horseradish peroxidise-conjugated antibody (1:2000) from Sigma-Aldrich and anti-*myc* horseradish peroxidase-conjugated antibody (1:5000) from Invitrogen and chemiluminiscent detection with the Chemiluminiscence Blotting Substrate (POD) (Roche).

Statistical analysis

The statistical significance of the results was analysed using an one-way ANOVA followed by Bonferroni's multiple comparisons test performed using GraphPad Prism version 6.00 for Windows, GraphPad Software, La Jolla, CA, USA.

RESULTS

Detection of the truncated protein in cell lysate

As we expressed the *fkbp_n-like* domain from non-native promoter GroES on plasmid, we first controlled its expression via LC-MS/MS analysis. Next, we evaluated the expression level using immunodetection of protein in the whole cell lysate and compared it with the expression of the FTS_1067 protein in the WT strain and *trans*-complemented strain cFSC200 (Schmidt *et al.* 2013). The expression of the truncated protein was slightly weaker than the expression of FTS_1067 protein in the WT strain, but it was very similar to that of the protein FTS_1067 produced by *trans*-complemented strain cFSC200 (Fig. S1-A, Supporting Information). Immunodetection of unrelated protein IglC was done as an internal control of expression (Fig. S1-B, Supporting Information).

In vivo and *in vitro* characterization of the FSC200/ΔDSBA-like mutant

In the previously published studies (Qin *et al.* 2009; Straskova *et al.* 2009), the deletion mutant strain for full-length *dsbA* gene in type A and type B *F. tularensis* strains was shown to be attenuated in *in vitro* and *in vivo* models of infection. Here, we used the complete gene deletion mutant FSC200/ΔFTS_1067 with N-terminal *fkbp_n-like* domain *trans*-complemented (FSC200/ΔDSBA-like) to determine the importance of DSBA-like domain for ability of *F. tularensis* to cause disease in a murine model of infection. Groups of BALB/c mice were infected subcutaneously with the WT FSC200 strain, the FSC200/ΔDSBA-like, the FSC200/ΔFKBP_N-like mutant (Schmidt *et al.* 2013) or the cFSC200 complemented mutant strain (not shown). The course of infection was followed for 21 days.

All mice infected with parental strain as well as with the cFSC200 strain died by day 6 post-infection. In contrast, all mice infected by the mutant strains survived at all doses. For better clarity, we showed only data for the lowest and the highest doses of the mutant strains (Fig. S2, Supporting Information). These results demonstrate that virulence is attenuated in both mutant strains and thus both the DSBA-like domain together with the FKBP_N-like domain contribute to *F. tularensis* virulence.

To investigate the protective effect of the mutants, the survivors were subsequently infected with 10^2 CFU/mouse of the WT strain and monitored for the mortality through a period of 21 days. We observed dose-dependent protection against the WT strain where the full protection was achieved after immunization with the doses of 10^5 and 10^6 CFU/mouse (Fig. 1). From the group of mice immunized with the FSC200/ΔDSBA-like mutant at a dose of 10^2 CFU/mouse, we obtained only one survivor. Three mice survived the FSC200 challenge after vaccination with a dose of 10^3 CFU/mouse, and finally from the group immunized with a dose of 10^4 only one mouse succumbed the FSC200 infection. The very similar data were obtained in the case of the FSC200/ΔFKBP_N-like mutant strain.

To investigate the fate of bacteria inside the host tissues, we infected mice with 10^5 CFU/mouse of the mutant strains or 10^2 CFU/mouse of the WT strain and followed the kinetics of infection by assessing the numbers of viable bacteria in the spleens, livers and lungs. At selected time points, post-infection groups of three mice were sacrificed and the numbers of viable bacteria were determined by plating diluted tissue homogenates (Fig. 2). The FSC200/ΔDSBA-like mutant was initially detected in all organs and the maximum of replication was reached the third day after infection (almost 10^2 CFU in lungs, over 10^5 CFU in spleens and livers) but then the numbers of bacteria declined. The FSC200/ΔDSBA-like mutant was eliminated from lungs at 7th day, from livers at 14th day and from spleens at 21st day. There were not any macroscopic markers of infection on the monitored organs and none of the mouse exhibited any symptoms of illness. These results correspond to those of the FSC200/ΔFTS_1067 deletion mutant (Straskova *et al.* submitted) and indicate that the FSC200/ΔDSBA-like mutant is able to infect mice and to persist for some time in the organs but it cannot replicate as efficiently as the WT to cause disease. The FSC200/ΔFKBP_N-like mutant bacteria showed the similar elimination from lungs and livers. But in case of spleens, the bacteria

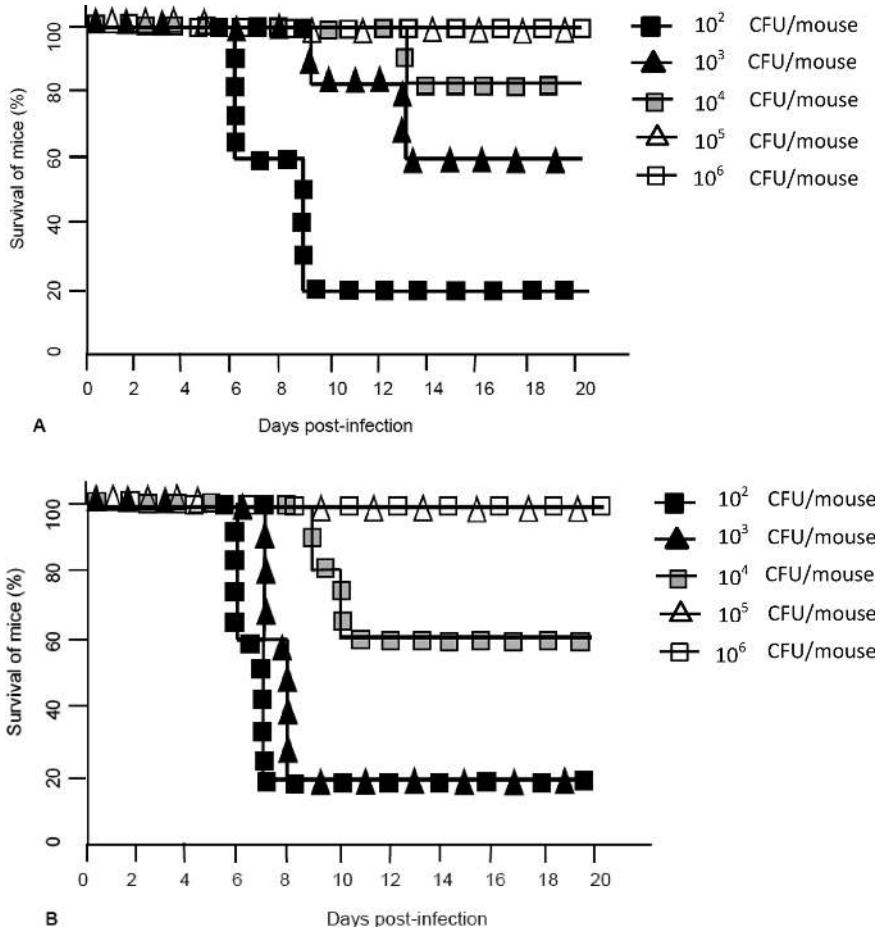

Figure 1. Protection against *F. tularensis* FSC200 infection. (**A**) Groups of five BALB/c mice were infected by the FSC200/ΔDSBA-like mutant strain at doses of 10^2 (black squares), 10^3 (black triangles), 10^4 (grey squares), 10^5 (white triangles) and 10^6 (white squares) CFU/mouse and after 21 days reinfected by the WT strain FSC200 (s.c., 3×10^2 CFU/mouse). (**B**) Groups of five BALB/c mice were infected by the FSC200/ΔFKBP_N-like mutant strain at doses of 10^2 (black squares), 10^3 (black triangles), 10^4 (grey squares), 10^5 (white triangles) and 10^6 (white squares) CFU/mouse and after 21 days reinfected by the WT strain FSC200 (s.c., 3×10^2 CFU/mouse).

persisted during the whole observed time interval and some of the mice exhibited clinical sings of illness.

To examine the ability of the FSC200/ΔDSBA-like mutant to enter and replicate within the macrophages, we infected cultures of primary bone marrow-derived macrophages. The results showed that there are no differences between the FSC200/ΔDSBA-like mutant and the WT strain in their ability to invade into macrophages. But there is a noticeable defect in the intracellular multiplication at 12 h post-infection. At this time point, the WT strain reached more than 2×10^7 CFU ml^{-1} while the mutant strain only 8×10^5 CFU ml^{-1}. And this trend continued at 24 and 48 h post-infection. The number of viable bacteria of the WT strain further increased to 4×10^8 CFU ml^{-1} at 24 h and to 7×10^8 CFU ml^{-1} at 48 h. In contrast, the number of viable bacteria of the mutant strain reached 1×10^7 CFU ml^{-1} at 24 h and 4×10^7 CFU ml^{-1} at 48 h post-infection (Fig. 3). To exclude the possibility of general growth defect, the mutant strain was tested for its growth in Chamberlain's medium and the growth kinetics did not differ from that of the WT strain (data not shown). These data suggested that the DSBA-like domain might be important for the intracellular survival and replication within the infectious model of murine macrophages.

Next, we repeated this experiment and except above-mentioned strains we also used the following mutant strains: the FSC200/ΔFTS_1067, the cFSC200 (complemented *in trans*) and the FSC200/ΔFKBP_N-like strain. Our results illustrate that the multiplication of both strains with partial domain deletion is decreased compared to the WT strain but, on the other side, it is significantly higher than that of the deletion mutant strain FSC200/ΔFTS_1067. The statistically significant differences were observed in the time intervals 24 and 48 h post-infection comparing the mutants with deleted domains to the full-length deletion mutant strain (Fig. 3).

To determine if the DSBA-like domain is involved in stress tolerance, we monitored the growth and the survival of the WT strain and the mutant strain under various conditions. The most significant differences between these two strains we observed in the oxidative stress survival assay performed with H_2O_2. The FSC200/ΔDSBA-like mutant strain was much more sensitive to the oxidative stress than the WT strain. The evident growth defect was observed in all tested time intervals with the statistical significance at the time intervals 30 and 40 min. To find out if this phenotype is connected only with the deletion of the DSBA domain or not, we performed this experiment with the FSC200/ΔFKBP_N-like and the FSC200/ΔFTS_1067 strains, as well. The *trans*-complemented strain cFSC200, which showed the same H_2O_2 resistance as the WT strain, was used as a control documenting that the *trans*-complementation is not responsible for this changed phenotype (Fig. 4A). As results clearly showed the increased sensitivity of all deletion mutants to the

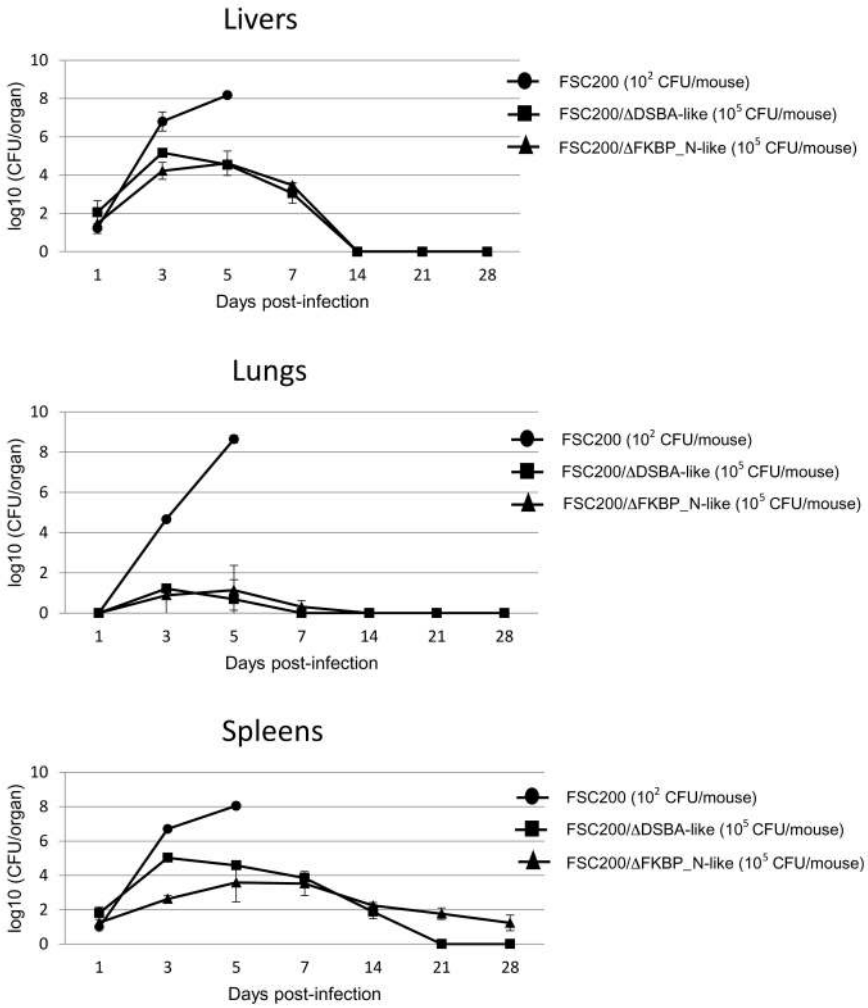

Figure 2. Organ dissemination of the FSC200, the FSC200/ΔDSBA-like and the FSC200/ΔFKBP_N-like. Group of mice were infected with the WT strain FSC200 (10^2 CFU/mouse, dots) and the mutant strains FSC200/ΔDSBA-like (10^5 CFU/mouse, squares) and the FSC200/ΔFKBP_N-like (10^5 CFU/mouse, triangles). Groups of three mice were sacrificed 1, 3, 5, 7, 14, 21 and 28 days after the infection, and numbers of the viable bacteria were determined by plating diluted tissue homogenates. The error bars represent the standard deviations of the three independent values of CFU.

oxidative stress, we further follow up the influence of copper ions, which are another oxidative agent. This experiment confirmed that all deletion mutants are much more sensitive to the oxidative stress than the WT strain (Fig. 4B). The statistical significance was analysed at the 24 h time point. All deletion mutant strains reached after 24 h cultivation significantly lower OD than the WT strain. Our results just document that both the DSBA-like and the FKBP_N-like domains contribute to the process of bacterial adaptation to the oxidative stress stimuli.

Functional assays with the recombinant proteins

The recombinant protein rFTS_1067/ΔDSBA-like does not contain the CXXC motif associated with thioredoxin and disulphide oxidoreductases; hence, it should not exhibit this type of enzyme activity. The lack of this activity was really confirmed using the assay based on reduction of human insulin (Fig. S3, Supporting Information). It was also shown that the rFTS_1067/ΔFKBP_N-like did not exhibit this activity (Fig. S3, Supporting Information) (Schmidt *et al.* 2013). Only the full-length recombinant protein was active, suggesting that both domains participate in this process.

Then we focused on the examination of possible role of the FKBP_N-like in disulphide isomerase activity. In previous tests, all deletion mutants showed higher sensitization to the oxidative environment which suggests the role of the FTS_1067 in isomerization of incorrect disulphides. To verify the existence of functional isomerase activity and to determine which protein domains are necessary for this function, we tested for the isomerase activity the rFTS_1067/ΔDSBA-like, the rFTS_1067/ΔFKBP_N-like and the rFTS_1067. For this purpose, we employed two different enzyme assays. First, we studied the ability of the reduced variants of recombinant proteins to refold the incorrect disulphide bonds in $CuCl_2/H_2O_2$-oxidized RNase A and thus restore its enzyme activity. The $CuCl_2/H_2O_2$-oxidized RNase A exhibited only 20% cCMP hydrolysis comparing to the native RNase A. Coincubation of oxidized RNase A with the rFTS_1067 led to the restoration of its activity to almost 50%. A similar increase in activity was observed when the oxidized RNase A was coincubated with the reduced PDI (Sigma-Aldrich) (62%). However, the presence of neither the rFTS_1067/ΔDSBA-like nor the rFTS_1067/ΔFKBP_N-like did not help to restore the RNase A activity (Fig. 5A).

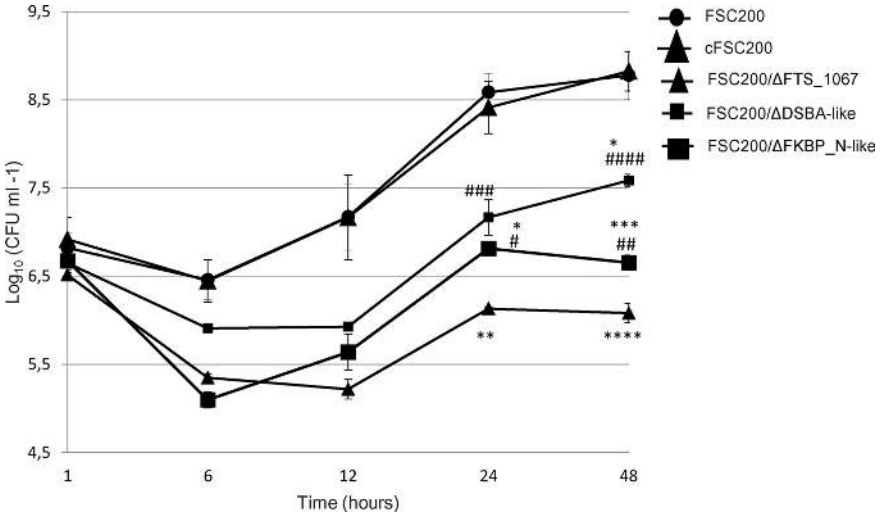

Figure 3. Intracellular growth of the FSC200 and the mutant strains. The cell line of primary murine bone marrow-derived macrophages was infected at a MOI 50:1 with the FSC200 (dots), the cFSC200 (big triangles), the FSC200/ΔFTS_1067 (small triangles), the FSC200/ΔDSBA-like (small squares) and the FSC200/ΔFKBP_N-like (big squares). The numbers of viable intracellular bacteria were determined by viable counts (CFU). The data represent mean values ± standard deviations of the three independent experiments, each performed in triplicate. The statistical significance was analysed using a one-way ANOVA followed by the Bonferroni's multiple comparisons test; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$ (comparing the FSC200/ΔFTS_1067, the FSC200/ΔDSBA-like, the FSC200/ΔFKBP_N-like with the FSC200); #, $P < 0.05$; ##, $P < 0.01$; ###, $P < 0.001$; ####, $P<0.0001$ (comparing the FSC200/ΔDSBA-like, the FSC200/ΔFKBP_N-like with the FSC200/ΔFTS_1067).

Second enzyme assay took advantage of the fact that the RNase A thermally unfolded at 65°C under non-reductive conditions preserves its native disulphide bonds intact. Therefore, during cooling the unfolded RNase A refolds and adopts back its enzyme activity. However, coincubation of the RNase A with the PDI during thermal unfolding disrupts its native disulphide bonds resulting in the loss of RNase A function (Fu and Zhu 2011). Similarly, as in a previous experiment the level of cCMP hydrolysis of thermally treated and cooled RNAse A was set to 100% activity (Fig. 5B, column a) and the activity of RNase A in all other samples was expressed as a percentage of thermally treated and cooled RNase A activity. In accordance with previous assay, only the presence of the rFTS_1067 during the thermal inactivation of RNase A led to the partial loss of its activity (51%). The coincubation of enzyme with the rFTS_1067/ΔDSBA-like or the rFTS_1067/ΔFKBP_N-like had only neglected effect on RNase A activity (Fig. 5B). The obtained results are in agreement with our hypothesis that the protein FTS_1067 also has the PDI activity and they also show that both the DSBA-like and the FKBP_N-like domains are necessary for the correct isomerase activity.

Previously (Schmidt *et al.* 2013), it was shown that the FTS_1067 protein has an *in vitro* chaperone activity, but the activity of the FKBP_N-like domain itself was not determined. So, here we monitored *in vitro* chaperone activity of the FKBP_N-like domain by measuring the enzyme activity of thermally inactivated CS. CS is inactivated by incubation at 43°C which leads to aggregates formation. The degree of its inactivation can be monitored by measuring the remaining activity of enzyme (Buchner, Grallert and Jakob 1998). First, we determined the activity of native CS and this activity was set to 100%. Then, the native CS was incubated with or without the rFTS_1067/ΔDSBA-like at 43°C for 16 min and in the selected intervals aliquots were withdrawn and the remaining activity of thermally inactivated or chaperone protected CS was measured. It is visible from Fig. 6 that the rFTS_1067/ΔDSBA-like protein is able to slow down the thermal aggregation of CS but the aggregation is not completely suppressed. Next, we monitored the activity of thermally inactivated CS in the presence or absence of the rFTS_1067/ΔFKBP_N-like or the rFTS_1067.Our results confirmed that also these variants of the FTS_1067 protein have this activity, but contrary to Schmidt *et al.* (2013), the rFTS_1067/ΔFKBP_N-like domain exhibits only very weak chaperone activity with statistical significance at time interval of 2 min. On the other hand, the rFTS_1067 and the rFTS_1067/ΔDSBA-like have comparable chaperone activities with statistical significance in all tested time intervals. These results show that the rFTS_1067/ΔDSBA-like exhibits similar *in vitro* chaperone activity as the WT form of recombinant protein and thus the FKBP_N-like domain is responsible for the chaperone activity of FTS_1067 protein.

Finally, we decided to investigate a potential role of the FKBP_N-like peptidyl-prolyl isomerase domain in oligomerization using BN-PAGE followed by immunoblotting. On SDS-PAGE, the rFTS_1067/ΔDSBA-like migrates as a protein with Mw less than 20 kDa protein, on BN-PAGE as a protein with molecular weight less than 66 kDa. For the estimation of its molecular mass, we used its relative mobility in gel (Pallová *et al.* 2007). By this enumeration, the Mw for the rFTS_1067/ΔDSBA-like oligomer was calculated as 56 kDa. Based on our results, we can conclude that recombinant protein appeared in native and non-reducing environment just in oligomeric state, probably as a trimer (Fig. 7).

DISCUSSION

Francisella tularensis is a highly infectious bacterium belonging to a category A agent of bioterrorism (Dennis *et al.* 2001). Successful development of the efficient vaccines and therapeutics against *F. tularensis* must be based on the comprehensive knowledge of the molecular mechanisms of its virulence. The conserved lipoprotein FTS_1067 with the homology to a protein of family disulphide oxidoreductases is one of the key virulence factors of *F. tularensis* (Qin *et al.* 2009; Straskova *et al.* 2009). In the present study, we focused on the characterization of its biological function with special focus on the role of FKBP_N domain employing selective deletion mutants.

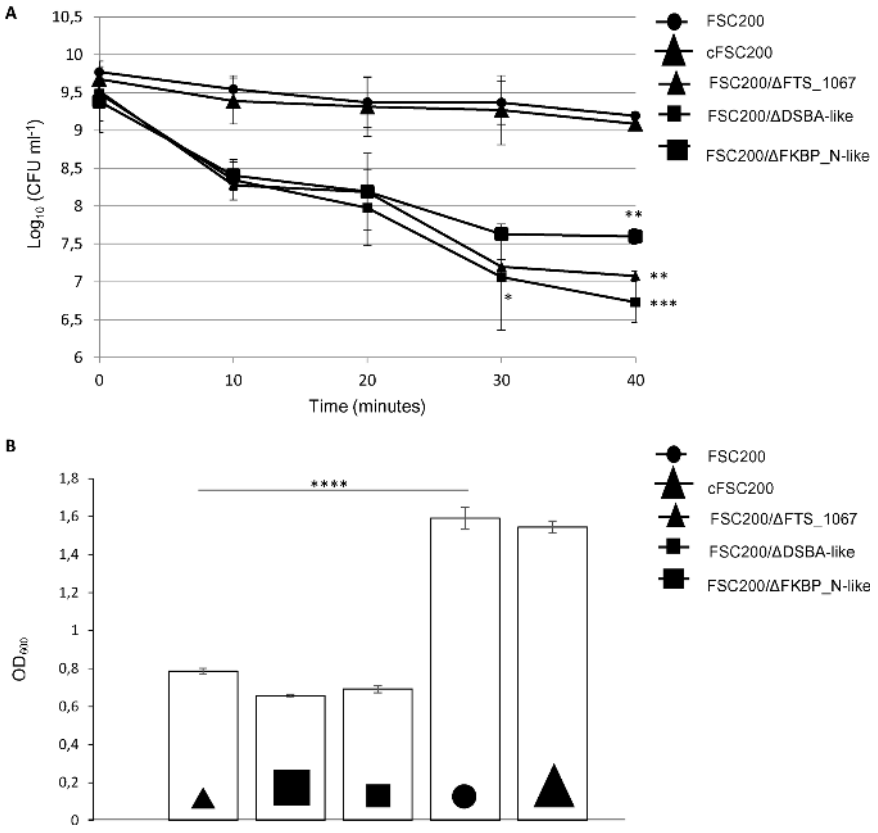

Figure 4. Oxidative stress survival and growth assay. (**A**) Survival of the bacterial strains after addition of 0.03% hydrogen peroxide. The aliquots of the bacterial cultures were diluted and plated on McLeod agar enriched for bovine haemoglobin and IsoVitaleX. The numbers of viable bacteria were enumerated after 72 h of incubation at 37°C. (**B**) Growth of the bacterial strains in complete Chamberlain's medium supplemented with $CuCl_2$ to a final concentration of 20 μM for 24 h in 37°C. Each experiment was independently repeated at least three times, and the data represent the mean ± standard deviations from all experiments. The FSC200 (dots), the cFSC200 (big triangles), the FSC200/ΔFTS_1067 (small triangles), the FSC200/ΔDSBA-like (small squares) and the FSC200/ΔFKBP_N-like (big squares). The statistical significance was analysed using a one-way ANOVA followed by the Bonferroni's multiple comparisons test; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$ (comparing the FSC200/ΔFTS_1067, the FSC200/ΔDSBA-like and the FSC200/ΔFKBP_N-like with the FSC200).

Here, we demonstrate the importance of the C-terminal DSBA-like domain for virulence in *in vitro* and *in vivo* murine models of tularaemia infection. We prepared the FSC200/ΔDSBA-like mutant strain expressing only the FKBP_N-like domain by *trans*-complementation of the full-length deletion mutant strain FSC200/ΔFTS_1067. Using immunoblotting, we found out that the cellular level of the FKBP_N-like domain did not reach the level of the full-length FTS_1067 protein in WT strain; nevertheless, it was the same as the level of the full-length FTS_1067 protein in *trans*-complemented strain cFSC200. Since the cFSC200 strain exerts *in vitro* and *in vivo* the same virulent phenotype as the WT strain (Schmidt *et al.* 2013, this study), we are convinced that our data on the FSC200/ΔDSBA-like mutant strain just do not reflect the effect of the weaker expression of the truncated protein. The absence of the DSBA-like domain led to the diminished proliferation of the mutant strain within the murine macrophages and *in vivo* attenuation in murine model of BALB/c mice. These results are consistent with recently published observations (Qin *et al.* 2009, 2014; Straskova *et al.* 2009). However, unlike the full-length deletion mutant strain FSC200/ΔFTS_1067 (Straskova *et al.* 2009), the DSBA-like mutant was unable to induce full protection against the original virulent strain challenge and likewise in case of the FKBP_N-like mutant (Schmidt *et al.* 2013) only dose-dependent protection was observed. We have shown that the bacterial dissemination in target organs following s.c. infection with the DSBA-like mutant strain is similar to that of the FSC200/ΔFTS_1067 (Straskova *et al.* submitted). The highest bacterial loads were found in spleens followed by livers and lungs. Importantly, the DSBA-like mutant strain bacteria were eliminated from all analysed organs earlier than those of the FSC200/ΔFTS_1067 strain showing the less ability of the DSBA-like mutant strain to resist to the host immune response. In case of the FSC200/ΔFKBP_N-like mutant strain, the bacterial burden in the target organs was comparable to that of the FSC200/ΔDSBA-like with the exception of the spleens, where the mutant bacteria persisted during whole tested time interval.

Using the stress tolerance assays, we demonstrated the role of C-terminal DSBA-like and surprisingly also N-terminal FKBP_N domain in the adaptation to oxidative stress. Basically, the full-length deletion mutant strain FSC200/ΔFTS_1067, as well as the FKBP_N-like and the DSBA-like mutant strains were all defective in replication under the presence of either hydrogen peroxide or copper chloride in medium. During the oxidative stress, cysteine residues become susceptible to oxidation and they are prone to generate the unwanted disulphide bonds which can lead to protein misfolding (Hiniker, Collet and Bardwell 2005). As it has been already mentioned above, *fts_1067* gene encodes protein with CXXC motif where the second X codes for tyrosine. Location of the polar amino acid in this position is much more typical for the disulphide isomerases than for the oxidoreductases (Fabianek, Hennecke and

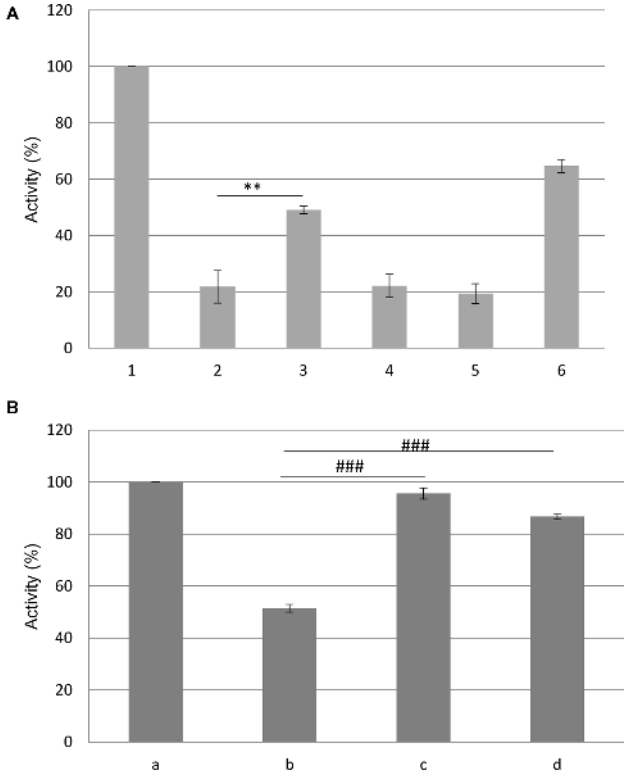

Figure 5. Assay for the isomerase-like activity. (**A**) The measurement of the ability of reduced recombinant proteins to refold incorrect disulphide bonds and restore the activity of copper/hydrogen peroxide-oxidized RNase A. The activity of native RNase A was set to 100% activity (1), oxidized RNase A (2), oxidized RNase A with reduced rFTS_1067 (3), oxidized RNase A with reduced rFTS_1067/ΔDSBA-like (4), oxidized RNase A with reduced rFTS_1067/ΔFKBP_N-like (5) and oxidized RNase A with reduced PDI (6). (**B**) The measurement of the activity of RNase A after thermal inactivation and subsequent cooling. The activity of thermally treated RNase A without any recombinant protein was set to 100% (a), the activity of thermally treated RNase A after coincubation with reduced rFTS_1067 (b), reduced rFTS_1067/ΔDSBA-like (c), reduced rFTS_1067/ΔFKBP_N-like (d). The statistical significance was analysed using a one-way ANOVA followed by Bonferroni's multiple comparisons test; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (comparing the activity of oxidized RNase A with the activity of oxidized RNase A coincubated with reduced rFTS_1067); #, $P < 0.05$; ##, $P < 0.01$; ###, $P < 0.001$ (comparing the activity of thermally treated RNase A coincubated with reduced rFTS_1067 with the activity of thermally treated RNase A coincubated with reduced rFTS_1067/ΔDSBA-like or reduced rFTS_1067/ΔFKBP_N-like).

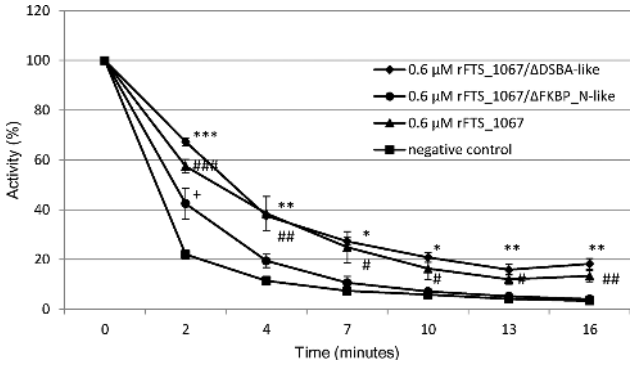

Figure 6. Monitoring the activity of thermally inactivated CS. The activity of native CS was determined and this activity was set to 100%. Then the CS was incubated together with 0.6 μM rFTS_1067/ΔDSBA-like (black rhombuses) or with 0.6 μM rFTS_1067/ΔFKBP_N-like (black dots) or with 0.6 μM rFTS_1067 (black triangles) in 43°C for 16 min and at the selected intervals the aliquots were withdrawn and the remaining activity of chaperone protected CS was measured. The activities of thermally inactivated CS without any recombinant proteins (black squares) were measured as a negative control. The statistical significance was analysed using a one-way ANOVA followed by Bonferroni's multiple comparisons test; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (comparing the activity of thermally inactivated CS with the activity of CS coincubated with 0.6 μM rFTS_1067/ΔDSBA-like); +, $P < 0.05$; ++, $P < 0.01$; +++, $P < 0.001$ (comparing the activity of thermally inactivated CS with the activity of CS coincubated with 0.6 μM rFTS_1067/ΔFKBP_N-like); #, $P < 0.05$; ##, $P < 0.01$; ###, $P < 0.001$ (comparing the activity of thermally inactivated CS with the activity of CS coincubated with 0.6 μM rFTS_1067).

Thöny-Meyer 2000; Anwar, Sem and Rhen 2013). So, our results showing the role of both FTS_1067 domains in the adaptation to oxidative stress might suggest that the disulphide isomerase activity is dominant for the protein encoded by the *fts_1067* gene. In *E. coli*, the lack of gene coding for the periplasmic disulphide isomerase DsbC resulted in copper chloride sensitization, while deletion of the *dsbA* gene did not (Hiniker, Collet and Bardwell 2005). Recently, Achard *et al.* (2012) described the role of copper ions in mouse macrophage responses to *Salmonella* and they came to conclusion that the copper ions contribute effectively to the host defence against the pathogen. This might indicate the role of the FTS_1067 protein in struggle mechanism of *Francisella* against the host immune defence machinery. Using two different enzyme assays, we proved that both the DSBA-like and the FKBP_N-like domains are necessary for functional isomerase activity and the loss of each of them is reflected by strong attenuation (Schmidt *et al.* 2013, this study). Therefore, we can conclude that the lack of the FTS_1067 isomerase activity resulting in the defect in recovery of oxidative stress-induced protein misfolding diminishes *Francisella* virulence. Additionally, as a result of the loss of isomerase activity the inflicted proteins can accumulate in periplasmic space (Straskova *et al.* 2009). From the studies performed in *E. coli* system, it results that the efficiency of the PDI as a catalyst of disulphide bond formation in the course of oxidative folding is markedly improved when the peptidyl-prolyl *cis/trans* isomerase is present simultaneously (Schönbrunner and Schmid 1992). Thus, we hypothesize that the FKBP_N-like domain might be supportive to the DSBA-like domain and thus sustain its catalytic activity.

In the course of the manuscript preparation, Qin *et al.* (2014) published their results also showing the role of FTT_1103 (SchuS4 homologue of FTS_1067, FipB) in protein disulphide isomerization and the importance of the FKBP_N-like domain in this phenomenon. Nevertheless, their study did not prove the role of the FKBP_N-like domain and, more generally, isomerase activity in *F. tularensis* virulence. Additionally, they observed that both isomerase and oxidase activities of the FTT_1103 are dependent on its reduced state that is maintained by direct interaction of FipB with *F. tularensis* Mip orthologue FipA protein (FTT_1102) (Qin *et al.* 2014). Our data also support the importance of the FTS_1067 reduction to act at least as an isomerase; nevertheless, the role of the FTS_1068 (FTT_1102 homologue in FSC200 strain) in the FTS_1067 isomerase activity is questionable. We had previously generated the FSC200/ΔFTS_1068 strain and this mutant strain retained its virulence in mice and its ability to proliferate in BMMs was not affected (data not shown). Hence, at least in the FSC200 strain the activity of the FTS_1067 is not dependent on the presence of the FTS_1068. Ren *et al.* in their very recent paper dealing with identification of the FTL_1096 (*F. tularensis* subsp. *holarctica* LVS homologue of the FTS_1067) substrates also found that this protein alone possessed both oxidoreductase and isomerase activities. They proved that the isomerase activity is tightly connected with the virulence and it is

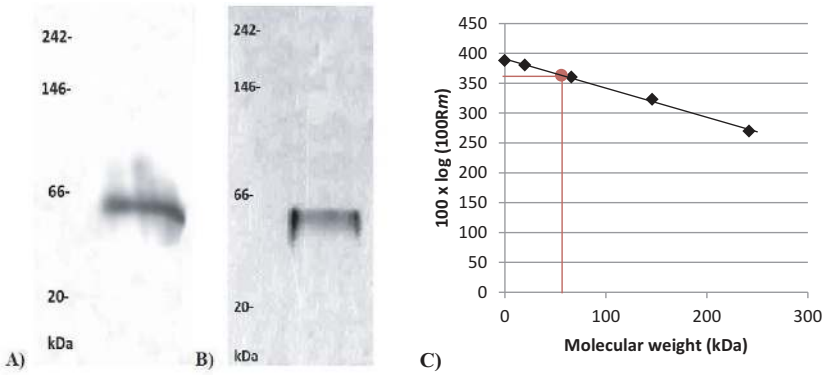

Figure 7. Determination of the oligomeric state of the rFTS_1067/ΔDSBA-like using BN-PAGE. Purified recombinant protein was loaded onto BN_PAGE with linear gradient 4.5–16%. The protein was transferred onto the PVDF membrane using semi-dry Western blotting and detected using anti-His antibody (**A**) and anti-*myc* antibody (**B**). Enumeration of the molecular mass of the recombinant protein. Rhombuses represent points of the trend line, dot is measured relative motility of the recombinant protein.

regulated by the *cis*-Pro motif exhibiting single amino acid polymorphism between *F. tularensis* subsp. *tularensis* (type A) and *F. tularensis* subsp. *holarctica* (type B) strains. However, in this publication the exact role of particular protein domains was not investigated (Ren, Champion and Huntley 2014).

It was published that bacterial Mip proteins occur as homodimers interacting via their FKBP_N-like domains (Horstmann *et al.* 2006). Our findings support the role of this domain in the FTS_1067 oligomerization. In fact, the BN-PAGE analysis demonstrated that the FKBP_N-like domain exists probably in trimeric state. This stands in contrast to the findings published by Schmidt *et al.* (2013) and Qin *et al.* (2014), in which the existence of dimers is predicted although the molecular mass of their proteins is slightly larger. They suppose the association of DsbA monomers via CXXC motif and the formation of disulphide bridges. Considered that there is no cysteine in the rFTS_1067/ΔDSBA-like and the protein appeared only in one oligomeric form (no monomer); it must be another mechanism for the FTS_1067 oligomerization. Moreover, recent paper dealing with the electrochemical characterization of the FTT_1103 recombinant protein proved the creation of oligomeric, probably trimeric structures of the FTT_1103 and there was also shown that the oligomerization process is not dependent on the existence of disulphide bridges (Večerková *et al.* 2014). The precise course of FTS_1067 oligomerization needs to be further clarified.

As was mentioned above, the FKBP_N-like domain shares a homology with FkpA of *E. coli* (Riboldi-Tunnicliffe *et al.* 2001), which has a chaperone activity (Arié, Sassoon and Betton 2001). Molecular chaperones are able to influence the thermal aggregation of CS and suppress it effectively (Buchner, Grallert and Jakob 1998). The chaperone activity for the rFTS_1067 and the rFTS_1067/ΔFKBP_N-like was confirmed previously (Schmidt *et al.* 2013). In the mentioned paper, it was shown that the rFTS_1067 protein has an *in vitro* chaperone activity and even that the deletion of the FKBP_N domain resulted in the increase of the activity. In spite of that the activity of the FKBP_N-like domain itself was not determined, it was concluded that only the DSBA-like domain and not the FKBP_N-like domain is responsible for the chaperone activity. In contrast, our results obtained in this study demonstrate the ability of the rFTS_1067/ΔDSBA-like to slow down the thermal aggregation of CS. So we proved *in vitro* chaperone activity of the rFTS_1067/ΔDSBA-like and showed that the FKBP_N-like domain is responsible for this activity, and thus might be necessary for the proper folding and protecting of substrate proteins of the FTS_1067.

In conclusion, our data support the role of the FKBP_N-like domain as well as the DSBA-like domain in the PDI and oxidoreductase activities and also we confirm the role of the FKBP_N-like domain in chaperone activity of the FTS_1067 protein. In contrast to previous studies, we uncovered oligomerization function of the FKBP_N-like domain that is not dependent on oxidation of cysteine residues. The current study also corroborates the finding emphasizing importance the FTS_1067 isomerase activity in *F. tularensis* virulence and taken together supports the necessity of the cooperation of both domains in establishing of the full function of this bifunctional protein.

SUPPLEMENTARY DATA

Supplementary data is available at FEMSPD online.

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Conflict of interest. None declared.

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