FipB, an Essential Virulence Factor of *Francisella tularensis* subsp. *tularensis*, Has Dual Roles in Disulfide Bond Formation

Aiping Qin,^a^ Yan Zhang,^a^ Melinda E. Clark,^a^ Meaghan M. Rabideau,^a^ Luis R. Millan Barea,^a^ Barbara J. Mann^a^,^b^,^a^b

Department of Medicine, Division of Infectious Diseases and International Health,^a^ and Department of Microbiology, Immunology, and Cancer Biology,^b^ University of Virginia, Charlottesville, Virginia, USA

FipB, an essential virulence factor of *Francisella tularensis*, is a lipoprotein with two conserved domains that have similarity to disulfide bond formation A (DsbA) proteins and the amino-terminal dimerization domain of macrophage infectivity potentiator (Mip) proteins, which are proteins with peptidyl-prolyl cis/trans isomerase activity. This combination of conserved domains is unusual, so we further characterized the enzymatic activity and the importance of the Mip domain and lipid modification in virulence. Unlike typical DsbA proteins, which are oxidases, FipB exhibited both oxidase and isomerase activities. FipA, which also shares similarity with Mip proteins, potentiated the isomerase activity of FipB in an in vitro assay and within the bacteria, as measured by increased copper sensitivity. To determine the importance of the Mip domain and lipid modification of FipB, mutants producing FipB proteins that lacked either the Mip domain or the critical cysteine necessary for lipid modification were constructed. Both strains replicated within host cells and retained virulence in mice, though there was some attenuation. FipB formed surface-exposed dimers that were sensitive to dithiothreitol (DTT), dependent on the Mip domain and on at least one cysteine in the active site of the DsbA-like domain. However, these dimers were not essential for virulence, because the Mip deletion mutant, which failed to form dimers, was still able to replicate intracellularly and retained virulence in mice. Thus, the Mip domains of FipB and FipA impart additional isomerase functionality to FipB, but only the DsbA-like domain and oxidase activity are essential for its critical virulence functions.

*Francisella tularensis* is the causative agent of tularemia, a vector-borne disease that can also be contracted by inhalation of aerosolized material (1). There are several subspecies of *F. tularensis* that vary in virulence. *Francisella tularensis* subsp. *tularensis* is the most virulent subspecies and the only subspecies classified by the Centers for Disease Control and Prevention as a tier 1 select agent (http://www.selectagents.gov/select%20agents%20and%20toxins%20list.html), which is the designation given to those biological agents that have the greatest potential to be misused as biological weapons.

*F. tularensis* is an intracellular pathogen that escapes the phagosome and replicates in the cytoplasm. FipB (*Francisella* infectivity potentiator B; encoded by the FFT1103 locus) is an essential virulence factor of *F. tularensis* subsp. *tularensis* that is required for phagosomal escape and intracellular replication (2). *F. tularensis* fipB mutants are also completely avirulent in mice; mice survive and show no symptoms after challenge with as many as $10^{10}$ CFU (2). The FipB protein consists of a combination of two conserved domains (http://www.ncbi.nlm.nih.gov/cdd/), the amino-terminal dimerization domain (FKBP_N) found in macrophage infectivity potentiator (Mip) proteins (3) and a disulfide bond formation A (DsbA)-like domain (4, 5). So far, this combination of domains has been found only in *Francisella* and in the related species *Fangia hongkongensis* (6). Mip proteins are typically lipoproteins that form homodimers via their alpha-helical amino-terminal FKBP_N domain and also contain a peptidylprolyl cis/trans isomerase (FKBP_C) domain (7, 8). The *fipB* gene is transcribed in an operon with *fipA*, which encodes a short polypeptide of 96 amino acids that also has similarity to FKBP_N, though it has only ~37% identity to the FKBP_N-related region of FipB (2, 9).

In Gram-negative bacteria, DsbA is a periplasmic protein that catalyzes disulfide bond formation by donating its disulfide bond to a nascent protein. DsbB, an inner membrane protein, reoxidizes DsbA so that it can fold another nascent protein (10). In a separate pathway, DsbC, a periplasmic disulfide bond isomerase that is a dimer with reduced cysteines in its active state, refolds proteins that have been misfolded by DsbA (11). DsbC is restored to its reduced state by an inner membrane protein, DsbD. FipB is an outer-membrane-anchored lipoprotein that, although not unique, is not typical of most DsbA-like proteins (12). In a number of other pathogens, DsbA is key for the folding, and thus the assembly and function, of a variety of critical virulence factors (12). Therefore, we hypothesized that FipB was a variant DsbA protein that acts as an oxidoreductase and is required for the folding of critical virulence factors. However, in this paper, we demonstrate that FipB has both oxidase and isomerase functions. We provide evidence that FipA and the Mip domain potentiate the isomerase activity of FipB. FipB represents an unusual model for a bacterial protein disulfide isomerase, because the active form does not appear to be a homodimer. Isomerase activity was not essential for FipB’s role in virulence, but we suggest that isomerase activity is important for repairing misfolded proteins that result from oxidative stress induced by redox-active metals, such as copper.

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**MATERIALS AND METHODS**

**Bacterial strains and media.** All bacterial strains and plasmids used in this study are listed in Table S1 in the supplemental material. *Francisella* strains were grown on cytochrome-supplemented Mueller-Hinton agar (MHA/c) plates or in Trypticase soy broth supplemented with cytochrome (TSB/c). Studies involving Schu S4 and derivatives of that strain were carried out in an approved biosafety level 3 laboratory. *Escherichia coli* strains were grown in Luria-Bertani (LB) broth or on LB agar plates with kanamycin (50 µg/ml), ampicillin (100 µg/ml), tetracycline (10 µg/ml), or chloramphenicol (35 µg/ml) as required.

**Construction of mutant strains.** Site-directed mutation, plasmid construction, and in cis complementation by homologous integration into the blaB locus were performed as described previously (9). Disruption of *blaB*, which encodes beta-lactamase resistance, is otherwise phenotypically neutral and provides a convenient screening mechanism (13).

**Intracellular-replication assay.** Human lung epithelial A549 cells were propagated in high-glucose Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum. Human monocyte THP-1 cells were maintained in RPMI containing 10% fetal bovine serum. Human monocyte THP-1 cells were infected with *F. novicida* or *F. tularensis*, which encodes beta-lactamase resistance, is otherwise phenotypically neutral and provides a convenient screening mechanism (13). After incubation at RT for 18 h, the motility from the inoculation site was measured. An *E. coli* (JCB571) *dsbA* mutant (kindly provided by J. C. Bardwell) was used as the parent strain (5).

**Proteinase K treatment.** Bacteria grown on MHA/c plates were suspended in PBS containing 5 mM MgCl$_2$ to an optical density at 595 nm (OD$_{595}$) of 1 and incubated with 10 µg/ml proteinase K at 37°C for 30 min. Proteinase K digestion was quenched by the addition of 2 mM EGTA. The bacterial pellets were washed with protease inhibitor cocktail (Sigma-Aldrich), resuspended in 1× SDS-PAGE buffer, and then boiled for 5 min before loading onto SDS-PAGE for Western blot detection.

**Motility assay.** Overnight cultures of each strain were adjusted to the same OD$_{595}$. Bacteria were stabbed into a soft agar (0.2%) plate with LB medium containing 35 µg/ml chloramphenicol to maintain plasmid selection. After incubation at RT for 18 h, the motility from the inoculation site was measured. An *E. coli* (JCB571) *dsbA* mutant (kindly provided by J. C. Bardwell) was used as the parent strain (5). The actual challenge dose was repeated at least three times. The Student two-tailed test was used for statistical analysis.

**Mouse model of tularemia.** All mouse studies were approved by the University of Virginia Animal Care and Use Committee. For intranasal inoculation, 9- to 12-week-old C57BL/6 mice (Jackson Laboratory) were anesthetized with a ketamine-HCl–xylazine mixture. Twenty microliters of *F. novicida* or *F. tularensis* in PBS was inoculated into the nares. The actual challenge dose was repeated at least three times. The Student two-tailed test was used for statistical analysis.

**Detection of free sulphydryls.** Overnight cultures were pelleted by centrifugation and then suspended in 10% TCA for 4°C overnight. The precipitates were pelleted and washed in ice-cold acetone, dissolved in buffer (1 M Tris, pH 8.0, 0.1% SDS, 1 mM EDTA) containing 20 mM methoxypolyethylene glycol-maleimide (MAL-PEG) (Sigma-Aldrich), and incubated at RT for 30 min in the dark. After incubation, TCA was added to the samples at a 10% final concentration, and incubated on ice for 30 min. Protein pellets were washed twice with ice-cold acetone. The MAL-PEG-labeled samples were suspended in SDS-PAGE buffer containing diethiothreitol (DTT) for SDS-PAGE and Western blot analysis. Reduced controls were prepared by first incubating samples in buffer (1 M Tris, pH 8.0, 0.1% SDS, 1 mM EDTA) that contained 10 mM DTT at RT for 30 min, followed by TCA (10%) precipitation and acetone washing before MAL-PEG treatment. Untreated negative controls were also prepared by the same procedures but without DTT or MAL-PEG in the buffer. Western blot films were scanned, and bands were quantitatively analyzed using ImageQuant software. FipB bands on Western blots were visualized with polyclonal anti-FipB (2). Rabbit anti-GroEL serum (Sigma-Aldrich) or guinea pig anti-FupA (a gift from Girija Ramakrishnan) (14) served as the loading control.

**FipB and FipA protein purification.** The fipB gene, minus the signal peptide and lipobox, was synthesized with an *E. coli* codon bias and ligated into the pHis-parallel 1 vector with a 6×His tag at the amino terminus (16). The fipA gene was amplified by PCR using Schu S4 DNA as the template and then inserted into plasmid pET-20b (Novagen) in frame with the vector-provided 6×His tag. Each clone was verified by DNA sequencing. Proteins were purified from lysates of induced cultures using Talon beads (Clontech). The beads were washed with wash buffer (50 mM Na$_2$HPO$_4$, 500 mM NaCl, containing 0.5 ml of 10-mg/ml stock aprotinin and 0.5 ml of 10-mg/ml stock leupeptin, pH 8.0), followed by a wash with 10 ml of the wash buffer, which contained 10 mM imidazole. The proteins were eluted with 500 mM imidazole in a 50 mM Na$_2$HPO$_4$, 500 mM NaCl buffer.
buffer at pH 6.8 and then dialyzed with PBS. Purification was analyzed by SDS-PAGE, followed by silver stain (Bio-Rad) to assess purity. Proteins were aliquoted and stored at −20°C. The protein concentration was assessed using the BCA protein kit (Pierce) with bovine serum albumin (BSA) as the standard.

**Assay for protein disulfide isomerase activity.** Isomerization activity was measured using the TEM1 β-lactamase assay described by Kpadeh et al. (17). Briefly, spent culture supernatants were collected from wild-type and dsbC E. coli strains containing the engineered TEM1 β-lactamase and concentrated 24-fold on a Vivaspin 2 spin column with a molecular weight cutoff of 10,000 (Sartorius Stedim Biotech). Samples were examined by SDS-PAGE, followed by staining with Gelcode Blue (Thermo Scientific) to determine the relative concentration of β-lactamase in each fraction. For the enzyme reaction, ΔdsbC supernatants were added to 200 μM FipA and/or FipB. The tubes were incubated for 15 min at 37°C. After incubation, samples were transferred to a 96-well plate, and β-lactamase activity was measured upon the addition of 10 μl nitrocefin (500-μg/ml stock solution). Cleavage of the substrate was measured every minute at a wavelength of 490 nm for 30 min at 30°C on a Synergy H4 Hybrid reader (Biotek). Reaction mixtures were tested in triplicate.

**Copper sensitivity assay.** Overnight cultures of *F. tularensis* or *Francisella novicida* were inoculated into 2 ml of TSB/c with or without CuCl2 and incubated at 37°C with 200 rpm shaking. Growth of the bacteria was monitored by measuring the culture OD595 at 24 h and 48 h.

**RESULTS**

FipB has both oxidoreductase and isomerase activities in *E. coli*. We, and others have found that purified recombinant FipB has oxidoreductase activity in vitro using the insulin precipitation assay (18). To demonstrate that FipB is capable of oxidoreductase activity in vivo, we complemented an *E. coli* dsbA mutant with FipB and showed that it could restore motility, which is a DsbA-dependent activity in *E. coli* (Fig. 1). In this experiment, the *fipB* gene was introduced on the low-copy-number plasmid pACYC184 into *E. coli* with or without the *fipA* gene. Motility was restored, though not to wild-type levels (Fig. 1). The presence of the *fipA* gene in addition to *fipB* did not influence the degree of motility. The growth of the strain expressing *fipA* alone had slight enhancement of growth compared to the vector control. We tested the growth of each of these strains in culture and found that the *fipA* strain grew slightly better than the vector control (see Fig. S1 in the supplemental material). Why this strain has a growth advantage is unclear.

Homodimerization of DsbC, the *E. coli* protein disulfide isomerase, is a requirement for isomerase activity (19). Because FipB has a Mip-like dimerization domain, we hypothesized that FipB might have isomerase activity. To determine whether FipB has any isomerase activity, we used an *E. coli*-based PDI detector system developed by Ren and Bardwell (15). In this system, a plasmid carrying a *bla* gene that has been engineered to encode two extra cysteines is introduced into a *dsbC* mutant. In the absence of *dsbC*, a higher portion of the β-lactamase that is produced is misfolded, and thus, these strains have increased sensitivity to...
ampicillin. The introduction of a functional dsbC gene increases resistance to ampicillin. Using the PDI detector strain, we were able to demonstrate that FipB has isomerase activity in E. coli, but only when FipA was present (Fig. 2A). These results suggest that FipA influences the enzymatic activity of FipB.

An in vitro assay of isomerase activity was used to test for isomerase activity of recombinant FipB and the influence of the addition of FipA (17). Only FipB plus FipA demonstrated isomerase activity over background levels (Fig. 2B), further supporting isomerase activity of FipB and a FipA requirement.

FipA influences the oxidation state of FipB. As demonstrated in an E. coli system, DsbC is a homodimer with two CXXC motifs that are in a reduced state (11). When DsbC encounters a misfolded protein, it first reduces the protein and then refolds it into its proper conformation. DsbC is returned to a reduced state by interaction with the inner membrane protein DsbD, which transfers reducing equivalents from the cytoplasm. The F. tularensis species lacks a recognizable DsbD or orthologs of other proteins that play similar roles (20). Since FipA was required for FipB isomerase activity in E. coli, we predicted that FipA would influence the oxidation state of FipB. We used MAL-PEG, a thioreactive compound that forms a covalent bond with free sulfhydryls and shifts the molecular mass of the protein by 5 kDa, to detect the oxidation states of FipB in the presence or absence of FipA. Cultures of wild-type and ΔfipA strains were treated with MAL-PEG in the presence or absence of DTT (Fig. 3). The percentage of FipB labeled by MAL-PEG for each strain was calculated by densitometry scans of the Western blots. In the samples treated with DTT and MAL-PEG, the migration of FipB was similarly increased to an apparent size of 75 to 100 kDa. Since FipB has two cysteines, one would have predicted only an increase of 10 kDa, but it appears that MAL-PEG significantly alters the migration of this protein on SDS-PAGE. Aberrant migration on SDS-PAGE after MAL-PEG modification has been observed by others (21). The addition of MAL-PEG without DTT produced three bands representing oxidized FipB, reduced FipB with one MAL-PEG molecule, and reduced FipB with two MAL-PEG molecules. These results indicated that in wild-type Schu S4, on average, 58% of FipB is in an oxidized form. Most of the reduced FipB was modified by copper oxidation (23). Therefore, to demonstrate that FipA helps to regulate the oxidation state of FipB, we needed to show that FipA influences the oxidation state of FipB.

FipB has isomerase activity in Francisella, as measured by copper sensitivity. In E. coli, DsbC is required to refold proteins misfolded by copper oxidation (23). Therefore, to demonstrate that FipB has isomerase activity in Francisella, the copper sensitivities of fipB mutants of Schu S4 were examined. The growth of
wild-type Schu S4 was unimpaired in 40 μM copper (Fig. 4). However, when fipB or fipAB was deleted, these strains were significantly more copper sensitive than wild-type bacteria (Fig. 4), supporting FipB’s role as an isomerase in Francisella. The fipA mutant was also more sensitive than the wild type to copper, which is consistent with our in vitro and E. coli data that implied a role for FipA in potentiating isomerase activity (Fig. 4). Both wild-type and ΔfipA strains that overexpressed fipA were also more sensitive to copper, suggesting a dominant-negative effect of FipA. Deletion of the Mip domain led to increased copper sensitivity, indicating that the domain is also critical for isomerase activity. F. novicida strain U112 and a fipB mutant had similar copper sensitivity profiles (see Fig. S2 in the supplemental material).

The amino-terminal Mip domain and the lipobox motif are not essential for virulence. As a potential DsbA-like protein, FipB has two atypical features: a lipobox motif, which specifies the addition of amino-terminal fatty acyl chains, and an FKBP_N domain characteristic of Mip proteins. To determine the importance of these domains in the essential virulence function of FipB, mutants that produced FipB with an altered lipobox (FipB-C22A) or deleted Mip domain (FipB::AS Δaa27–117), where FipB::AS indicates FipB with the insertion of two amino acids, alanine and serine, and Δaa27–117 indicates the deletion of amino acids 27 to 117) were constructed (Fig. 5) by integrating the engineered genes into the blab locus in a ΔfipA mutant of the Schu S4 strain of F. tularensis subsp. tularensis, along with wild-type fipA and the native promoter. To construct the Mip domain deletion mutant, two additional amino acids, alanine and serine, were introduced after the lipobox sequence. The addition of these two amino acids to an otherwise wild-type FipB did not negatively affect intracellular growth (Fig. 6A and B) but did, surprisingly, impact in vivo virulence (Table 1) (see Discussion). Expression of these genes was verified by Western blotting (Fig. 5B and C). Under nonreducing conditions, FipB formed higher-molecular-mass complexes. In Schu S4, FipB migrates as three isoforms, due at least in part to glycosylation differences (24). The lipobox mutant was poorly expressed, and protein was visible only after an extended exposure time (Fig. 5C and 6D).

The intracellular-growth phenotypes of the lipobox mutant (FipB-C22A) and the Mip deletion mutant (FipB::AS Δaa27–117) in the human lung epithelial cell line A549 and in the human macrophage-like cell line THP-1 were determined using a gentamicin protection assay (Fig. 6 and 7). The lipobox mutant (FipB-C22A) replicated very similarly to wild-type bacteria in both cell lines, despite very low levels of detectable protein, indicating that the amino-terminal cysteine residue, and thus the fatty acyl modification, were not required for intracellular replication. The Mip deletion mutant was also found to be competent for intracellular replication (Fig. 7), though replication was reduced compared to wild-type levels. To rule out the possibility of contamination with wild-type bacteria restoring growth, the FipB proteins from input bacteria and from bacteria recovered from cells infected for 24 h were compared using Western blots (Fig. 6D and 7C). Using the FupA protein to normalize the values, the amounts of FipB-C22A protein in the input and recovered bacteria were 16% and 3% of the wild-type level, respectively. The FipB detected in cells infected with the Mip deletion mutant was the same size as the FipB with Mip deleted in the input bacteria. These Western blots indicated that contaminating bacteria were not responsible for the observed wild-type or near-wild-type levels of intracellular bacterial growth in the lipobox and Mip deletion mutant strains.

Both the lipobox and the Mip deletion mutants retained virulence in mice (Table 1). Although 6 out of 21 mice (29%) survived infection with the lipobox mutant (FipB-C22A), this level of survival was not statistically different from that of mice infected with the wild type or the in cis wild-type complement, where 100% of the mice died (Fisher’s exact test). Only 2 out of 12 mice (17%) survived infection with the Mip deletion mutant (fipB::26AS Δaa27–117). However, infection with the strain that produced a FipB protein with two extra amino acids, AS, was not as virulent as infection with wild-type Schu S4 (67% survived). This result was surprising, because the FipB::AS strain did not exhibit any intracellular growth defects (Fig. 7). The fipB gene in this strain was resequenced to confirm that the AS insertion was still present. Western blots of bacteria recovered from the organs of infected mice ruled out the potential for contamination with wild-type bacteria in mice infected with FipB-C22A and FipB::AS ΔMip strains (see Fig. S3 in the supplemental material). All of the mice...
infected with a mutant that expressed a fipB gene with a mutation at the active site (AMYA) survived infection. A fipAB deletion mutant, as well as other CMYC mutants, have been previously shown to be avirulent at greater than 10^6 CFU (2, 9).

Cell fractionation demonstrated that the FipB-C22A protein was found in the soluble fraction, indicating that association of FipB with the membrane fraction was not essential for intracellular replication and virulence (Fig. 6B).

**FipB forms a surface-exposed dimer that is sensitive to reduction.** In other bacteria, Mip proteins are homodimers mediated by the amino-terminal domain (FKBP_N) (3). Therefore, we predicted that FipB would also dimerize via this domain. The higher-molecular-mass complexes that were observed on Western blots with wild-type protein were not detectable when the Mip domain was deleted (Fig. 5B). However, these complexes were also sensitive to reducing conditions. FipB has three cysteine residues, one in the lipobox, which is acylated, and two in the active site, which in DsbA proteins is characterized by a CXXC motif; in FipB, this motif is CMYC. Mutations in the CMYC motif were constructed with alanine residues substituted for each cysteine. Wild-type FipB formed a higher-molecular-mass complex under nonreducing conditions (Fig. 8A). CMYA, and AMYC mutants of FipB were not surface exposed. We tested the surface exposure of FipB using techniques (immunofluorescence, biotin labeling, and immunoprecipitation [data not shown]), that at least some portion of FipB was surface exposed. We tested the surface exposure of FipB using proteinase K digestion and found that only the higher-molecular-mass complexes of FipB were sensitive to digestion (Fig. 8B). Whether surface-exposed FipB has any function is unclear, but since FipB dimers were not detected in the Mip deletion mutant or the fipA overexpression strain and these strains were still able to replicate intracellularly, it appears that this dimer complex and surface exposure do not play essential roles in FipB-mediated virulence functions.

**DISCUSSION**

In this paper, we have demonstrated that the DsbA-related protein FipB is a bifunctional protein that acts as both an oxido-reductase and a protein disulfide isomerase. In many bacteria, these functions are carried out by separate pathways (10). The DsbA/DsbB pathway forms disulfide bonds in nascent proteins, and the DsbC/DsbD pathway refolds misfolded proteins by first reducing incorrect disulfide bonds and then reforming the correct bonds. For DsbC to function as an isomerase, it needs to be in a reduced state. Dimerization of DsbC protects it from being oxidized by DsbB. The isomerase activity was somewhat unexpected, because FipB does not appear to form a functional dimer. Using protease sensitivity, we were able to detect only surface-exposed dimers of FipB that formed through the cysteines in the active site. The

**FIG 5** Construction and expression of fipB deletion and point mutants. (A) Diagram showing the domain structure of each mutant. Each construct contains the signal peptide (light-gray box), followed by the conserved lipobox motif (LVAC). In the FipB-C22A construct, the conserved cysteine (C) has been changed to an alanine (A). The black boxes indicate the insertion of 2 amino acids, alanine and serine (AS), after amino acid 26 as a result of the insertion of an NheI restriction site that was used to create the constructs. The endpoints of the deletions are indicated in the construct name; the numbering includes the amino-terminal signal peptide. Each mutant gene, along with wild-type fipB and the native promoter, was integrated into a ΔfipAB mutant of Schu S4 by homologous recombination at the b1b locus. (B) Western blot of a nonreducing SD-PAGE showing the expression of the Mip deletion construct (FipB Δ27–117). Whole-cell lysates were separated by SD-PAGE and then transferred to a nylon membrane. The membranes were incubated with both anti-FipB and anti-GroEL antibodies (loading control). Note that only the wild-type protein forms higher-molecular-mass complexes. (C) Western blot demonstrating the expression of FipB-C22A in a whole-cell lysate. Membranes were incubated with both anti-FipB and anti-FupA (used as a loading control) antibodies.
periplasmic exposed forms of FipB appear to be monomers, though we cannot rule out the possibility that transient dimers form. In *E. coli*, DsbC is returned to its reduced state via an inner membrane protein, DsbD, that provides reducing equivalents it receives from cytoplasmic thioredoxin (25). Bioinformatic searches of *F. tularensis* have not revealed any DsbD ortholog. There are other related families of proteins that can also provide reducing equivalents, such as CcdA and ScsB, but there are no orthologs of these protein families either (20). Interestingly, there is a strain of a *Francisella* sp., Tx077308, which contains a DsbD ortholog (F7308_1227) that is lacking in other sequenced strains and species, suggesting that this gene may have been a later addition to the strain’s genome. Strain Tx077308 belongs to a clade that is quite distant from the *F. tularensis* subspecies (26). However, the strain encodes both FipA and FipB proteins that are 79% and 73% identical, respectively, to the Schu S4 FipA and FipB proteins. Regardless, *Francisella* must have some other mechanism for maintaining FipB in a reduced state. We found that the short peptide FipA, which is cotranscribed with *fipB*, influenced the proportion of FipB in the oxidized state. When *fipA* was deleted, there was an increased amount of oxidized FipB. In *E. coli*, one function of the dimerization domain of DsbC is to prevent interaction with and oxidation by DsbB; when the dimerization domain of DsbC is mutated, DsbC can complement a *dsbA* mutant (27). These results suggest that *F. tularensis* subspp. *tularensis* FipA and FipB directly interact and prevent oxidation by DsbB. Our model is that FipA helps to maintain FipB in a reduced state through interaction with the Mip domain of FipB. The *in vitro* isomerase assay suggests that there is a direct FipA-FipB interaction (Fig. 2B), but we have been unable to demonstrate this by communoprecipitation, pulldowns, or bacterial two-hybrid interaction (28). We were able to detect FipA only when it was over-

![FIG 6](image_url)
TABLE 1 Lipobox and Mip deletion mutants retain virulence

<table>
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<tr>
<th>Strain (genotype)</th>
<th>Intrapulmonary infection dose(s) (avg CFU/mouse)</th>
<th>Days to death postinfection</th>
<th>No. of survivors/total no. of mice (%)</th>
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<tr>
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<td>24 and 45</td>
<td>No deaths</td>
<td>12/12 (100)</td>
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<td>79</td>
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<td></td>
<td>All tested doses</td>
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<td>2/12 (17)</td>
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*Two values represent two independent trials with the indicated doses.

The number of days after challenge that mice showed the first signs of irreversible mortality and were euthanized. Survivors were all monitored for >21 days.

Two deaths occurred in each trial.

expressed (Fig. 7B), so the protein is either present at levels below our limits of detection or unstable, which may limit our ability to detect a direct interaction. The dominant-negative effects of fipA overexpression on copper sensitivity suggest that FipA may need to dissociate for isomerase activity and interaction with substrates to occur. Overexpression of fipA, however, did not affect intracellular growth (Fig. 9). One potential explanation for this paradox is that in the reducing environment of the host cytoplasm, the dominant-negative effects of FipA have less impact or that only a low level of isomerase activity is required for late-stage bacterial growth. Thus far, Francisella is one of only a few bacteria that lack a recognizable member of the DsbD superfamily, which provides the reducing equivalents to periplasmic protein disulfide isomerase. The chimeric nature of FipB and its potential interaction with FipA may be an adaptation to the absence of DsbD. Regardless, F. tularensis is an unusual paradigm for disulfide bond formation and isomerase activity. FipB is not the only bifunctional DsbA-related protein. The DsbA2 protein of Legionella pneumophila also has both oxidase and protein disulfide isomerase activities (17). DsbA2 has an amino-terminal domain that functions in dimer formation, but the domain is not related to the FKBP_N domain of Mip proteins. A bifunctional DsbA has also been engineered in E. coli by fusing the FKBP_N domain to DsbA (19). This chimeric protein formed dimers, had both oxidase and isomerase activities, and conferred modest copper resistance.

In Francisella tularensis subsp. holarctica, using the thermal aggregation of citrate synthase assay, Schmidt et al. have shown that FipB, which they refer to as DsbA, has chaperone activity in vitro (29). This result suggests a third function for FipB. However, not all of their data were consistent with ours. They found that deletion of the Mip domain in FipB rendered the strain avirulent and that a recombinant protein lacking the Mip domain was not able to reduce insulin. In contrast, our FipB strain with Mip deleted exhibited only partial attenuation in intracellular growth. The deletion of these genes in both subspecies results in a highly attenuated or avirulent phenotype (2, 29), but there are sequence differ-
ences between the *F. tularensis* subsp. *tularensis* FipB protein and the *F. tularensis* subsp. *holarctica* ortholog, DsbA (30), including a Thr-to-Ala change in a tripeptide that has been found to be critical for the resolution of substrate binding in *E. coli* DsbA (31). However, the most likely explanation is the difference in the extent of the deletion; our mutant lacked amino acids 27 to 117, while Schmidt et al. deleted amino acids 46 to 143. A BLAST search with known DsbA and Mip proteins indicates that DsbA similarity begins around amino acid 124 and that Mip similarity ends around amino acid 127. These authors have shown that their Mip deletion protein lacks oxidoreductase activity, so we suggest that the extent of their deletion disrupted the functions of both domains. When constructing our Mip deletion mutant, 2 amino acids (AS) were inserted as the result of the insertion of a restriction site. We tested the phenotype of a strain that expressed full-length FipB that, except for the insertion of AS, was otherwise wild type. Intracellular replication of this strain was similar to that of wild-type strains (Fig. 7); however, the strain exhibited a higher level of attenuation than the Mip deletion strain (Table 1). This result indicated that the insertion of AS was not neutral, but the deletion of the Mip domain somewhat mitigated this effect. It also indicated that in *Francisella*, the intracellular-replication phenotype might not always indicate the level of *in vivo* virulence. The lack of correlation between intracellular growth and *in vivo* virulence has been observed for several other *Francisella* gene mutants, in particular *annK*, *pdpD*, *iglG*, and *iglI* (32, 33).

In a number of bacterial pathogens, DsbA proteins have been shown to be required for the function of critical virulence factors,
including the assembly of type III secretion machinery, flagella, pili, and toxins (12). We speculate that FipB is required for the folding or function of key virulence factors; however, specific substrates that reflect FipB’s essential role in virulence have yet to be characterized. In the live vaccine strain (LVS), several proteins that increased in abundance in a fipB (dsbA) mutant were identified using a proteomic-labeling technique, but their link to virulence has not been established (18). In E. coli, where DsbA and DsbC have been the best studied, a clear phenotype for DsbA is evident, including loss of motility and decreased virulence (34–36), and multiple substrates have been identified (5, 31). Initially, although DsbC was characterized as having protein disulfide isomerase activity, few substrates could be identified, and dsbC mutants did not have a clear phenotype (37). However, Hiniker et al. have shown that DsbC is required for copper resistance (23). They found that copper catalyzed the formation of incorrect disulfide bonds that then could be repaired to the correct state by DsbC and proposed that the primary role of DsbC is to refold misfolded proteins formed during oxidative stress. Consistent with our isomerase assay results, we found that FipB, the Mip domain of FipB, and FipA were all required for copper resistance. These results, along with our in vitro data, support the role of FipB as a protein disulfide isomerase in F. tularensis subsp. tularensis and suggest that the isomerase activity of FipB may be more critical during oxidative stress and perhaps more critical for survival in the vector or environmental reservoirs. Combining its oxidase and isomerase activities in one protein, and possibly eliminating the need for DsbD, may contribute to its success as a low-dose pathogen that can survive in many hosts.

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