

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

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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 FTT1103 locus) is an essential virulence factor of *F. tularensis* subsp. *tularensis* that is required for phagosomal escape and intracellular replication (2). *F. tularensis* subsp. *tularensis* *fipB* mutants are also completely avirulent in mice; mice survive and show no symptoms after challenge with as many as 10¹⁰ 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 cysteine-supplemented Mueller-Hinton agar (MHA/c) plates or in Trypticase soy broth supplemented with cysteine (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 and 3 mg/ml L-glutamine and differentiated into macrophage-like cells in 24-well plates containing 100 ng/ml phorbol 12-myristate 13-acetate (PMA) over 48 h. The cells were then washed with phosphate-buffered saline (PBS) and recovered in RPMI without PMA for 24 h before infection. Bacteria were added to cells in tissue culture wells in 24-well plates, centrifuged at $1,000 \times g$ for 8 min, and incubated at 37°C, 5% CO₂. After 1 h, the cells were washed, and then the medium was replaced with fresh medium containing 50 µg/ml gentamicin. At the indicated time points, the cells were washed and then lysed with 0.1% sodium deoxycholate in PBS for 5 min or in water for 10 min. The number of bacteria was determined by plating serial dilutions on MHA/c plates. These experiments were repeated at least three times. The Student two-tailed *t* 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 bacteria were inoculated into the nares. The actual challenge dose was confirmed by parallel viable plate counting. The mice were monitored twice daily. Mice showing signs of irreversible mortality were humanely euthanized, and death was considered to occur within 24 h.

Subcellular localization of FipB. Whole-cell lysates were prepared from an overnight bacterial culture by mixing equal amounts of an overnight culture and 2× SDS-PAGE loading buffer and then boiling for 5 min. Membrane fractions and culture supernatants were prepared from a 25-ml overnight culture. Bacteria were concentrated by centrifugation, and culture supernatants were filtered through a 0.22-µm syringe filter, followed by trichloroacetic acid (TCA) precipitation and resuspension in 1× SDS-PAGE sample buffer. Soluble and insoluble membrane fractions were prepared by suspending bacterial pellets in 1 volume of lysis buffer (0.75 M sucrose, 10 mM Tris, 20 mM EDTA), adding 300 µg/ml of lysozyme, and then incubating on ice for 2 h. After this incubation, 4 volumes of cold water was slowly added, with occasional rocking. The samples were incubated at room temperature (RT) for 30 min, and then protease inhibitor cocktail was added. The samples were subjected to 5 cycles of freezing and thawing in liquid nitrogen and at RT. Samples were then pelleted in a microcentrifuge at $12,000 \times g$ for 30 min to remove unbroken bacteria. The supernatants were passed through a 0.22-µm syringe filter and then spun at $165,000 \times g$ for 2 h at 4°C in an ultracentrifuge. Supernatants containing soluble proteins were TCA precipitated and then suspended in 1× SDS-PAGE buffer. Insoluble pellets containing outer and inner membrane proteins were washed with 10 mM HEPES and then suspended in 1× SDS-PAGE loading buffer. Samples were applied to SDS-PAGE for protein separation, and specific proteins were visualized by Western blotting. Antibodies included rabbit anti-FipB (2), rabbit anti-

ti-FupA (14), anti-IgIB1 (BEI Resources), and anti-GroEL (Sigma-Aldrich).

Proteinase K treatment. Bacteria grown on MHA/c plates were suspended in PBS containing 5 mM Mg²⁺ to an optical density at 595 nm (OD₅₉₅) 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 OD₅₉₅. 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). pACYC vectors containing the *fipA*, *fipB*, or *fipAB* gene were introduced into JCB571 by electroporation.

PDI detector assay. To test for isomerase activity, PCR products corresponding to the *fipA*, *fipB*, or *fipAB* gene were inserted into the pACYC184 plasmid in the tetracycline resistance gene and then introduced into RGP665 (a generous gift from J. C. Bardwell) by electroporation. RGP665 is a $\Delta dsbC$ *E. coli* mutant that carries a protein disulfide isomerase (PDI) detector plasmid, which contains an engineered β -lactamase with two nonconsecutive cysteine substitutions (S81C and T108C) (15). Incorrect disulfide bond formation leads to increased sensitivity to ampicillin. In the presence of a protein disulfide isomerase, the β -lactamase can be refolded into a functional conformation and therefore confers increased ampicillin resistance on the host strain. Accordingly, bacterial cultures were grown in LB medium to an OD₅₉₅ of 1 with appropriate antibiotics added. The bacteria were then diluted 1:100 and grown at 37°C in a shaker for 3 h with or without 2.5 mg/ml ampicillin. The cells were then serially diluted and plated on LB plates with appropriate antibiotics for plasmid maintenance, but without ampicillin.

Detection of free sulfhydryls. Overnight cultures were pelleted by centrifugation and then suspended in 10% TCA at 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 dithiothreitol (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 Na₂HPO₄, 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 Na₂HPO₄, 500 mM NaCl

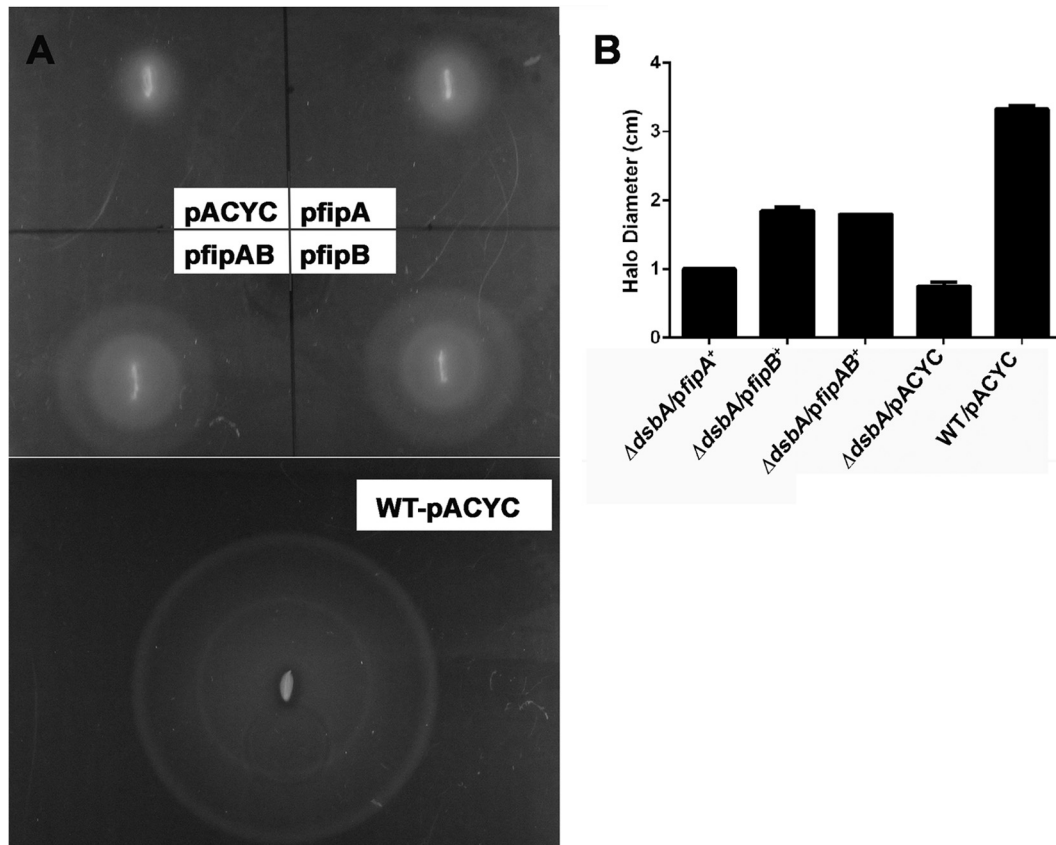


FIG 1 *fipB* complements *E. coli dsbA*. (A) $\Delta dsbA$ strains expressing *fipA*, *fipB*, or *fipAB* or carrying an empty vector, pACYC187, or wild-type (WT) bacteria with pACYC187 were stabbed into soft agar plates and grown for 18 h at RT. (B) Comparison of halo diameters of complemented strains, as well as wild-type *E. coli* carrying the empty vector. Strains expressing *fipB* or *fipAB* restored motility, while complementation with *fipA* was similar to the vector-only control. The error bars indicate standard deviations.

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 $\Delta dsbC$ *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 Stedium 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, $\Delta dsbC$ 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- $\mu\text{g}/\text{ml}$ 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 CuCl_2 and incubated at 37°C with 200 rpm shaking. Growth of the bacteria was monitored by measuring the culture OD_{595} 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 dsbA* 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

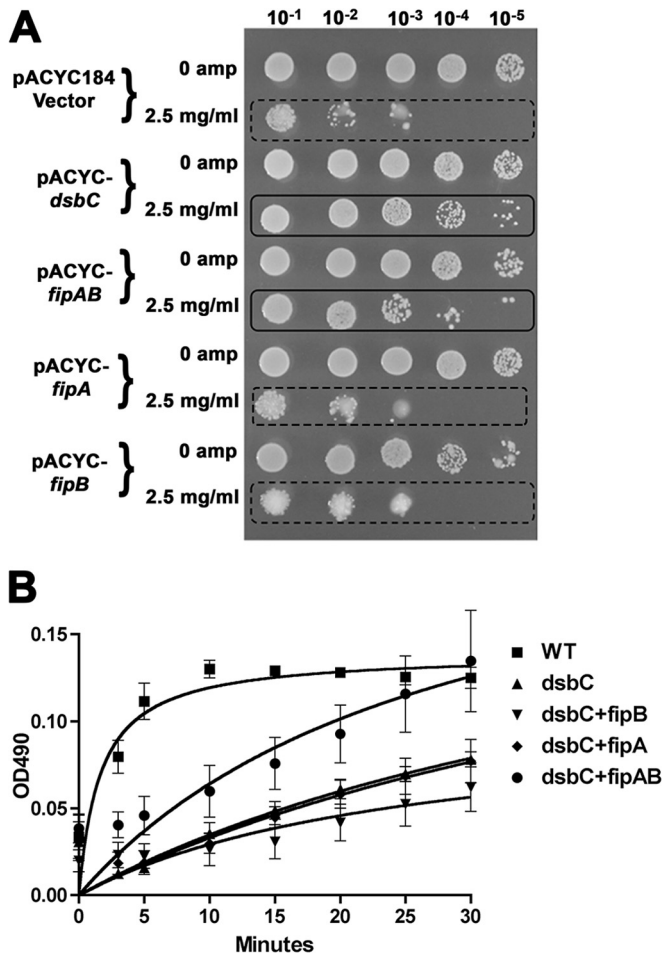


FIG 2 FipB demonstrates isomerase activity in *E. coli* that is dependent on *fipA*. (A) Cultures of a *dsbC* strain carrying the PDI detector plasmid and the indicated *fip* genes were grown in the presence of ampicillin (amp); the next day, they were serially diluted and spotted on LB plates without ampicillin. The solid boxes highlight cultures that were considered positive for isomerase activity, and the dashed boxes indicate cultures that were considered negative. (B) Recombinant FipB with or without FipA was incubated with supernatant from the *dsbC* strain used in panel A, which contains the PDI detector plasmid and produces the engineered, misfolded β -lactamase. β -Lactamase activity was measured upon the addition of nitrocefin. Cleavage of the substrate was measured every minute at a wavelength of 490 nm for 30 min at 30°C. Reaction mixtures were tested in triplicate. A supernatant from the wild-type PDI detector strain was used as a positive control. Using 2-way analysis of variance (ANOVA), FipAB was statistically significantly different from FipB, starting at 10 min; FipA and FipB were not statistically significantly different. The error bars indicate standard deviations.

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

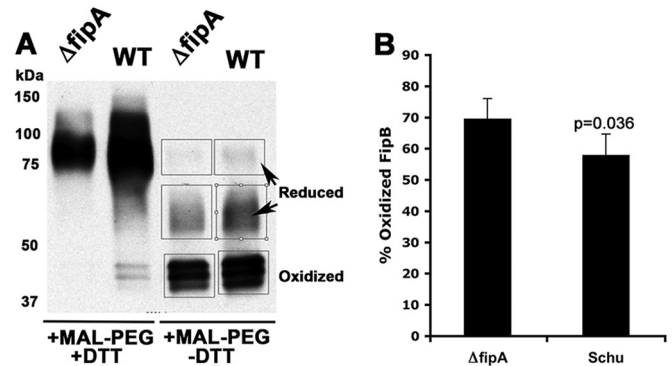


FIG 3 *fipA* influences the oxidation state of FipB. Overnight cultures of each strain were TCA precipitated and then treated with DTT and MAL-PEG, as indicated. (A) Western blot of labeled cultures with anti-FipB antibody. The boxed bands were subjected to densitometry scans. (B) Percentages of total FipB (the three boxed bands in panel A) that were present in the oxidized state as determined by densitometry scans. The data are combined from three separate labeling experiments. The *P* value was determined using Student's *t* test. The error bars indicate standard deviations.

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 thio-reactive 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 $\Delta fipA$ 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 only one MAL-PEG molecule. This was not too surprising, because based on the structure of *E. coli* DsbA, the second cysteine is buried in the molecule and not as accessible (22). As predicted, there was significantly more oxidized FipB in the $\Delta fipA$ strain than in the wild type (70%; *P* = 0.038) (Fig. 2B), supporting our premise that FipA helps to regulate 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

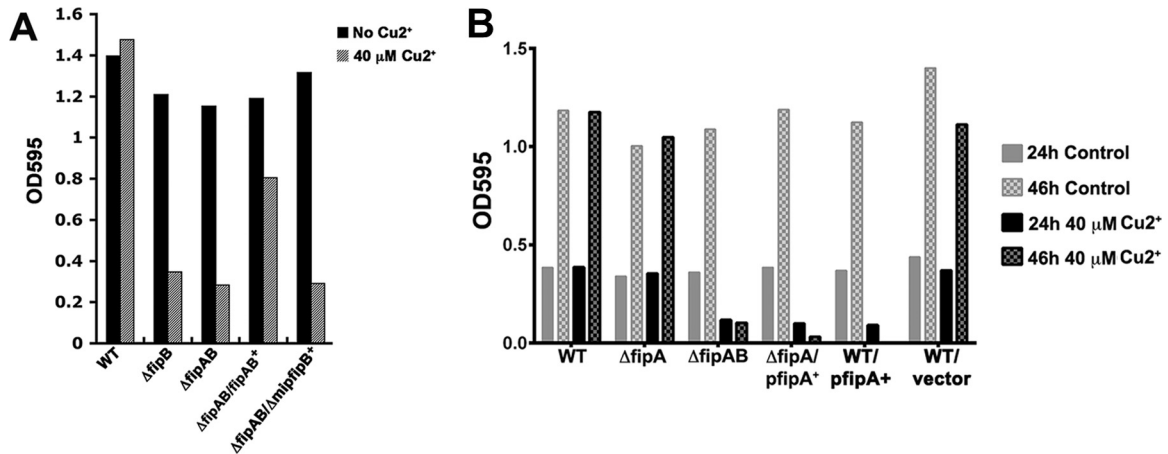


FIG 4 (A) A Schu S4 *fipB* mutant is more sensitive than the wild type to CuCl_2 . Bacteria were grown at 37°C for 21 to 24 h with shaking in TSB/c supplemented with $40\ \mu\text{M}$ CuCl_2 . The $\Delta fipAB$ mutant was complemented in *cis* (indicated with a plus) with wild-type *fipAB* or Δmip *fipB*. (B) A Schu S4 *fipA* mutant is more sensitive to copper than the wild type. The indicated strains were grown at 37°C with shaking in TSB/c supplemented or not with $40\ \mu\text{M}$ CuCl_2 at the indicated time points. The WT and the $\Delta fipA$ mutants were complemented with *fipA*⁺ in *trans*, indicated by “p.”

wild-type Schu S4 was unimpaired in $40\ \mu\text{M}$ 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 $\Delta fipA$ 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 $\Delta aa27-117$, where FipB::AS indicates FipB with the insertion of two amino acids, alanine and serine, and $\Delta aa27-117$ indicates the deletion of amino acids 27 to 117) were constructed (Fig. 5) by integrating the engineered genes into the *blaB* locus in a $\Delta fipAB$ 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 $\Delta aa27-117$) 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 5% 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 $\Delta aa27-117$). 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

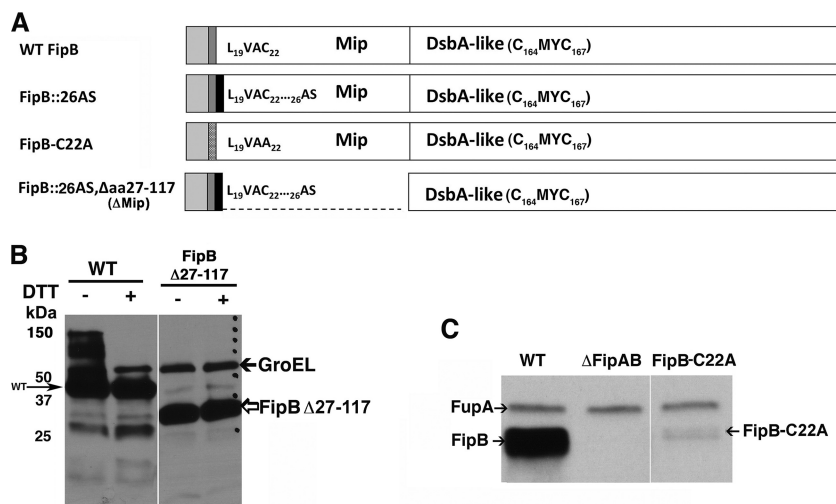


FIG 5 Construction and expression of *fipB* deletion and point mutants. (A) Diagram showing the domain structure of each mutant. Each construct contains the signal peptide (light-gray box), followed by the conserved lipobox motif (LVAC). In the FipB-C22A construct, the conserved cysteine (C) has been 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 *fipA* and the native promoter, was integrated into a $\Delta fipAB$ mutant of Schu S4 by homologous recombination at the *blaB* locus. (B) Western blot of a nonreducing SDS-PAGE showing the expression of the Mip deletion construct (FipB $\Delta 27-117$). Whole-cell lysates were separated by SDS-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.

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 also formed these complexes in enhanced portions, but an AMYA mutant did not. Since there is only one available Cys in the CMYA and AMYC mutants, these results also indicate that these ~ 125 -kDa complexes are dimers, though the apparent molecular mass is more than twice the monomer size of ~ 47 kDa. The size of this complex is likely due to aberrant migration of the protein, because purified recombinant FipB also forms higher-molecular-mass complexes on nonreducing SDS-PAGE that migrate at a size that is larger than twice the monomer size (data not shown). The FipA protein appears to play a role in dimer formation, because although the deletion of *fipA* did not affect dimer formation, over-

expression of *fipA* prevented the formation of these complexes (Fig. 8C). However, overexpression of *fipA* by episomal complementation of a $\Delta fipA$ mutant restored the intracellular-growth defect of the $\Delta fipA$ mutant, suggesting that this dimer form of FipB is not critical for intracellular growth (Fig. 9).

Several members of the Mip family have been reported to be surface exposed (7, 8). We observed, using several different 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 oxidoreductase 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

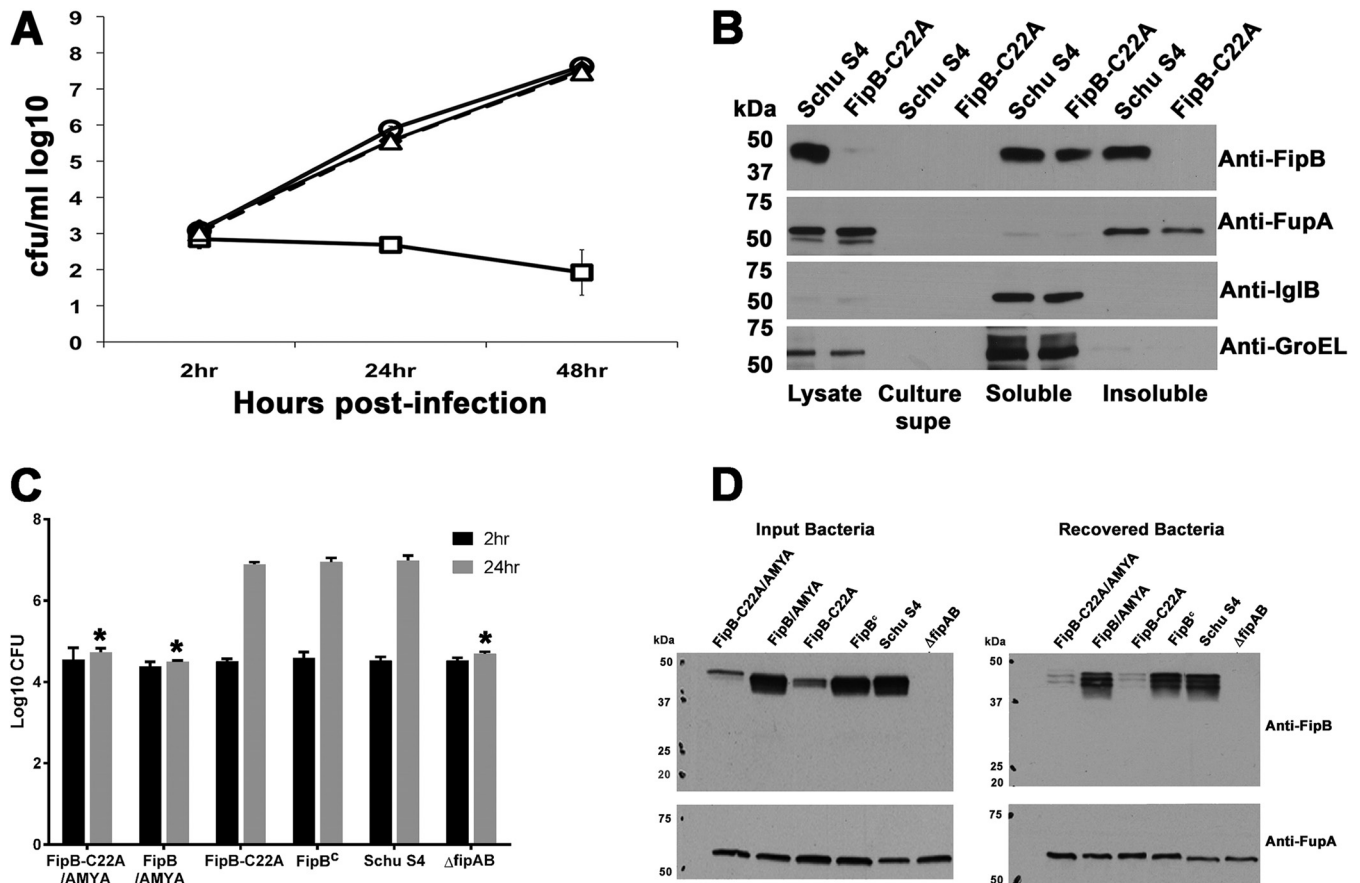


FIG 6 The lipobox mutant (FipB-C22A) is competent for intracellular replication. (A) A549 cells were infected at a multiplicity of infection (MOI) of 100:1 and then lysed at the indicated times postinfection. The lysates were diluted and plated to enumerate intracellular bacteria. The data are representative of at least three independent experiments, each using an average of three independent wells. The strains used were Schu S4 (◆) and mutants expressing $\Delta fipAB$ (□), FipB-C22A (△), or FipB::26AS (○). The error bars indicate standard deviations. (B) The lipobox mutant, FipB-C22A, does not associate with the membrane fraction. Bacteria were lysed and fractionated as described in Materials and Methods. Western blots of subcellular fractions of wild-type Schu S4 and strain BJM1100, which produces the lipobox mutant FipB-C22A, were prepared as described in Materials and Methods and incubated with the indicated antibodies. FupA is an outer membrane protein involved in ferrous iron transport, IgIB is part of the *F. tularensis* subsp. *tularensis* type VI secretion system, and GroEL is a chaperone protein. FipB-C22A was faintly visible in total lysates and the soluble fraction, which was 25 \times concentrated. supe, supernatant. (C) PMA-differentiated THP-1 cells were infected with the indicated strains at an MOI of 50:1 and then lysed at 2 and 24 h postinfection. Negative controls included strains expressing FipB and FipB-C22A with mutated active sites (AMYA) and the $\Delta fipAB$ mutant. All mutated *fipB* genes were integrated into the *bla* site in the $\Delta fipAB$ mutant. The FipB^c strain has the wild-type *fipB* gene integrated into the *bla* site of a $\Delta fipAB$ mutant. The data are representative of at least three independent experiments, each using an average of three independent wells. The strains indicated by asterisks were significantly different from wild-type Schu S4 ($P < 0.0001$). (D) Western blots depicting FipB in input bacteria and bacteria recovered from host cells at 24 h from the experiment depicted in panel C. Anti-FupA antibodies were used as a loading control.

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* subsp. *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 coimmunoprecipitation, pull-downs, or bacterial two-hybrid interaction (28). We were able to detect FipA only when it was over-

TABLE 1 Lipobox and Mip deletion mutants retain virulence

| Strain (genotype) | Intranasal infection dose(s) ^a (avg CFU/mouse) | Days to death postinfection ^b | No. of survivors/total no. of mice (%) |
|---|---|--|--|
| Schu S4 | 12 and 24 | 5, 5, 5, 4, 4, 5 | 0/6 (0) |
| BJM1103 (<i>fipB</i> AMYA) | 24 and 45 | No deaths | 12/12 (100) |
| BJM1076 (<i>fipB</i> CMYC) | 10 and 100 | 6, 6, 7, 6, 6, 6 | 0/6 (0) |
| BJM1100 (<i>fipB</i> -C22A) | 31 | 5, 5, 5, 5 | 2/6 |
| | 71 | 5, 5 | 4/6 |
| | 210 | 5, 5, 5 | 0/3 |
| | 21 | 6, 6, 8 | 0/3 |
| | 4 | 6, 9, 10 | 0/3 |
| | All tested doses | | 6/21 (29) |
| BJM1099 (<i>fipB</i> ::26AS) | 47 and 65 | 6, 6, 6, 6 ^c | 8/12 (67) |
| BJM1111 (<i>fipB</i> ::26AS Δaa27–117) | 37 | 5, 5, 5, 5, 5, 5 | 0/6 |
| | 79 | 5, 5, 5, 5 | 2/6 |
| | All tested doses | | 2/12 (17) |

^a Two values represent two independent trials with the indicated doses.

^b 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.

^c 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-

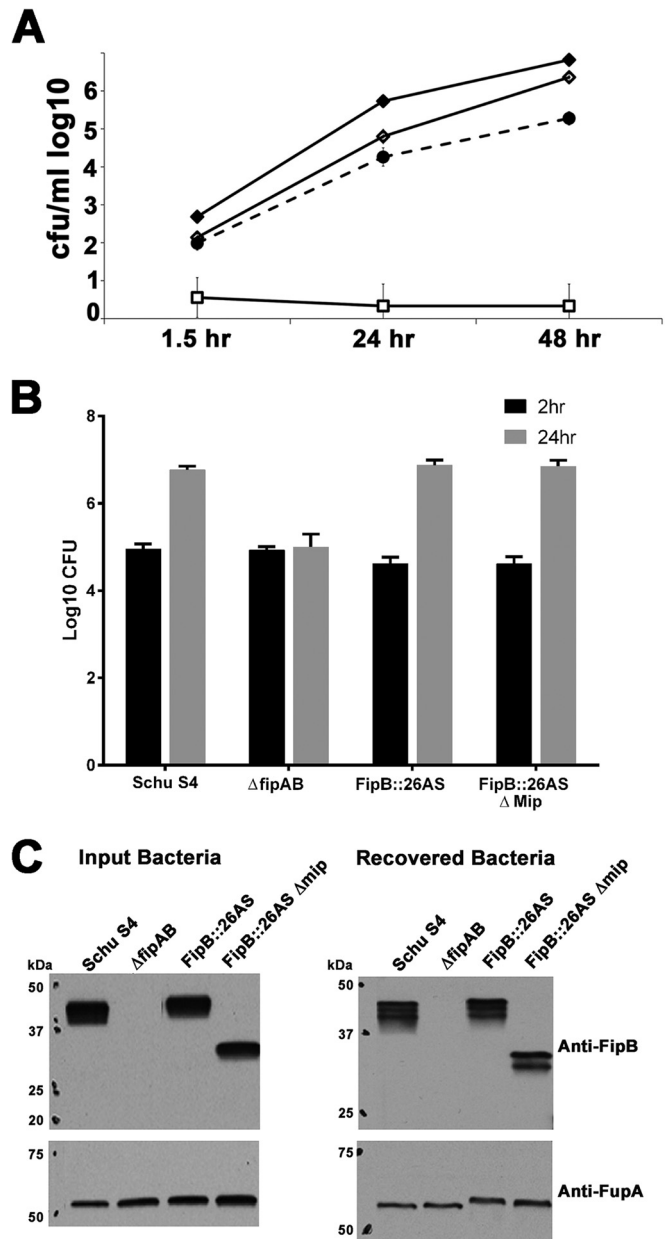


FIG 7 The Mip domain deletion mutant is competent for intracellular replication. (A) A549 cells were infected with the indicated strains and then lysed at the indicated times postinfection. The lysates were diluted and plated to enumerate intracellular bacteria. The data represent an average of results from three independent wells and are representative of at least three independent experiments. The strains and MOIs used were as follows: Schu S4 (◆), 130:1; Δ*fipAB* mutant (□), 133:1; FipB::AS Δaa27–117 mutant (Δ*mip*) (●), 197:1; mutant with Δ*fipAB* in *cis* complemented with *fipAB*::AS26 (◇), 232:1. The MOIs were verified by plate counts. The error bars indicate standard deviations. (B) Differentiated THP-1 cells were infected with the indicated strains and then lysed at 2 or 24 h postinfection. The MOIs used were as follows: Schu S4, 39:1; Δ*fipAB* mutant, 112:1; FipB::AS Δaa27–117 mutant (Δ*mip*), 104:1; mutant with Δ*fipAB* in *cis* complemented with *fipAB*::AS26, 69:1. The data represent an average of results from three independent wells and are representative of at least three independent experiments. (C) Western blots depicting FipB in input bacteria and bacteria recovered from host cells at 24 h from the experiment depicted in panel B. Anti-FupA antibodies were used as a loading control.

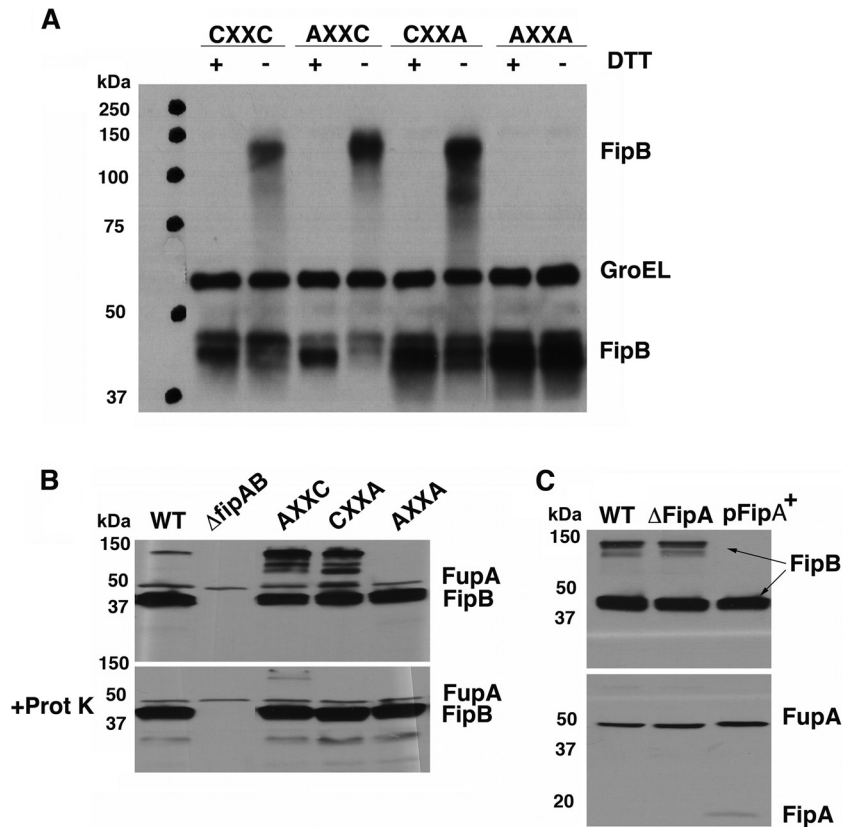


FIG 8 FipB forms a dimer via cysteines in the enzymatic active site that is sensitive to proteinase K treatment and inhibited by overexpression of *fipA*. (A) Immunoblots of strains complemented in *cis* with *fipB* genes with mutations in the active-site cysteines. Bacteria were lysed in the presence or absence of DTT. The blots were incubated with anti-FipB and anti-GroEL antibodies. (B) Bacterial pellets were resuspended in PBS (top) or 10 μ g/ml of proteinase K (bottom) and incubated for 30 min at 37°C. After incubation, samples were boiled in 2 \times nonreducing sample buffer, separated by SDS-PAGE, transferred to a nylon membrane, and then incubated with both anti-FipB and anti-FupA antibodies. (C) Immunoblotting was first performed with anti-FipB antibody, and immunoblots were then stripped and incubated with both anti-FupA and anti-FipA antibodies.

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). How-

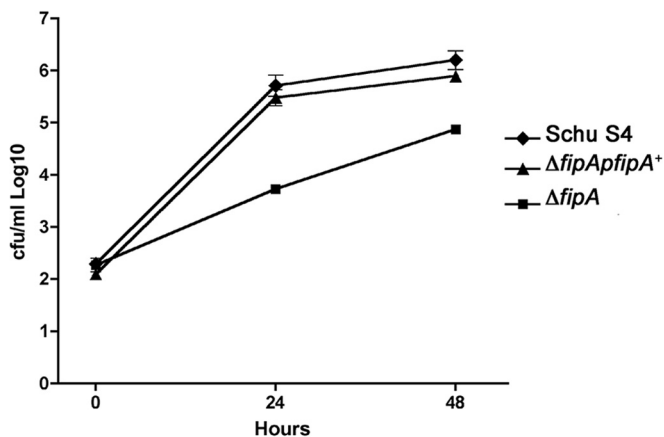


FIG 9 Complementation of the $\Delta fipA$ mutant restores the intracellular-growth defect. A549 cells were infected at an MOI of 100:1, as described in Materials and Methods, and then lysed at the indicated times postinfection to determine CFU/ml. The error bars indicate standard deviations.

ever, 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, *in vivo*, 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 *amnK*, *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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