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Components of the type six secretion system are substrates of *Francisella tularensis* Schu S4 DsbA-like FipB protein

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ABSTRACT

FipB, an essential virulence factor in the highly virulent Schu S4 strain of *F. tularensis* subsp. *tularensis*, shares sequence similarity with Disulfide Bond formation (Dsb) proteins, which can have oxidoreductase, isomerase, or chaperone activity. To further explore FipB's role in virulence potential substrates were identified by co-purification and 2D gel electrophoresis, followed by protein sequencing using mass spectrometry. A total of 119 potential substrates were identified. Proteins with predicted enzymatic activity were prevalent, and there were 19 proteins that had been previously identified as impacting virulence. Among the potential substrates were IglC, IglB, and PdpB, three components of the *Francisella* Type Six Secretion System (T6SS), which is also essential for virulence. T6SS are widespread in Gram-negative pathogens, but have not been reported to be dependent on Dsb-like proteins for assembly or function. The presented results suggest that FipB affects IglB and IglC substrates differently. In a *fipB mutant* there were differences in free sulfhydryl accessibility of IglC, but not IglB, when compared to wild-type bacteria. However, for both proteins FipB appears to act as a chaperone that facilitates proper folding and conformation. Understanding the role FipB plays the assembly and structure in this T6SS may reveal critical aspects of assembly that are common and novel among this widely distributed class of secretion systems.

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Introduction

Francisella tularensis subsp. *tularensis* is a tier 1 select agent, which is a biological agent determined by the US. Dept. of Health & Human Services to be a potentially severe threat to human or animal health. Concerns over documented and potential use of *F. tularensis* as a biological weapon, and reports of the development of weaponized strains that are resistant to antibiotics and vaccines have led to increased interest in defining mechanisms of virulence as a means of identifying new targets for therapy and immune protection.[1,2]

There are several subspecies of *F. tularensis* that vary in virulence. *Francisella tularensis* subsp. *tularensis* causes the most severe disease, while *F. tularensis* subsp *holarctica* strains cause a milder disease. Commonly used lower virulence model strains include the Live Vaccine Strain (LVS), an attenuated *F. holarctica* strain, and *Francisella novicida*, a closely related species that has low virulence in humans, but is virulent in mice. Although they vary significantly in virulence, all of the subspecies are quite similar at the genomic level (>98 %).[3] There are few recognizable virulence factors encoded in the *Francisella* genome, but several have been identified that are required for virulence and phagosomal escape.[4-8] Included in this group of virulence factors are FipB, as well as components of the *Francisella* Type Six Secretion System (T6SS).

FipB is an essential virulence factor that is required for phagosomal escape, and intracellular survival.[9] *FipB* mutants are also completely avirulent in mice.[9] FipB protein has similarity to DsbA proteins, which are periplasmic oxidoreductases that catalyze disulfide bond formation in

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nascent proteins.[10,11] *E. coli*, as well as many other Gram-negative bacteria, also contain a separate but related protein DsbC, which is an isomerase that refolds incorrectly folded proteins.[12] However, a separate DsbC protein has not been identified in *Francisella* strains. We, and others have found that FipB has both oxidoreductase and isomerase activities.[13,14] Recombinant FipB has also been demonstrated to have chaperone activity using an *in vitro* assay.[15]

T6SSs are quite widespread among Gram-negative bacteria.[16] Some are key for host-pathogen interactions, but others function more in bacteria-bacteria encounters, and may be important for colonization of an environmental niche. The *Francisella* T6SS is clearly an outlier T6SS sharing only five out of 13 core conserved T6SS proteins leading to some speculation as to whether the *Francisella* T6SS functions as a secretion system.[17] However, demonstration of a contractile sheath by cryo-electron microscopy [18] and secretion of effector proteins into the host cytoplasm have largely confirmed that the genomes of *Francisella* species encode a functional T6SS.[19-21] A model of the *Francisella* T6SS structure, which resembles other T6SSs and is supported by the atomic structure,[18] proposes that two proteins, IglA and IglB, form heterodimers and polymerize to assemble the contractile sheath. A third protein, IglC, forms hexamers and assembles as a nanotube inside the IglA/IglB sheath.[22] This model for IglC is based on the structure of Hemolysin coregulated protein, (Hcp), a conserved component of T6SSs.[22] It has been suggested that IglC serves as the functional equivalent of Hcp, though they share little sequence similarity. IglC, like Hcp, is also secreted.[19,20] Mutations in *iglA*, *iglB* and *iglC* are severely attenuating, and mutant bacteria fail to escape into the cytoplasm.[18,22,23]

DsbA activity is important for the function of many virulence factors in other bacteria including the assembly and function of Type Three Secretion Systems.[24-26] We therefore reasoned that the avirulence of a Δ*fipB* mutant was due to improper folding of key *Francisella* virulence factors. To better understand the function and role of FipB in virulence we sought to identify its substrates. Among the identified substrates were three components of the T6SS, IglB, IglC, and PdpB. Although this makes sense phenotypically, because both Δ*fipB* and mutants in the T6SS are highly attenuated and fail to escape the phagosome, in other organisms T6SS have not been reported to require DsbA for function, and the crystalized structure of IglC does not suggest intramolecular disulfide bonds.[27] Our data suggest that FipB is required for proper protein folding of the T6SS components. The dependence of the T6SS on FipB may be an adaptation to the loss of some of the conserved elements found in other T6SSs.

Results

Identification of FipB substrates

Functional and mutational analyses of the FipB protein and gene support a role for the FipB protein in disulfide bond formation, including an essential dependence on the two cysteines found in the conserved active site of DsbA proteins, CXXC (Cysteine- any amino acid- any amino acid-Cysteine).[14,28] We hypothesize that the avirulence of the Δ*fipB* mutant is due to the inability to produce functional critical virulence factors. To identify factors that were dependent on FipB, we sought to co-purify FipB with its substrates. In *E. coli* it was reported to be difficult to co-purify DsbA with its substrates. Kadokura *et al.* speculated that the interaction of *E. coli* DsbA with its substrates was rapid and transient, making co-purification difficult.[29] They performed a mutational analysis to identify mutations that would slow down the oxidation reaction or trap substrates. Mutation of the conserved *cis*-proline residue P151, which is located in the substrate-binding pocket of *E. coli* DsbA,[30] enhanced the interaction of *E. coli* DsbA with its substrates and facilitated their identification.[29] Mutation of the second cysteine in conserved active site of DsbA proteins, CXXC to alanine [29] also resulted in substrate trapping.[31]

FipB has a *cis*-proline residue located nearly the same distance (just one 1 amino acid farther) from the conserved enzymatic CXXC site as in *E. coli* DsbA. The structure of FipB has not been solved, but we reasoned that mutations in these corresponding residues, C167A and P286T would similarly trap FipB substrates. His-tagged versions of wild-type, C167A (CXXA), and P286T FipB proteins were introduced *in trans* via a plasmid encoding these proteins into a Δ*fipB* mutant of Schu S4, a virulent *F. tularensis* subsp. *tularensis* strain. His-tagged FipB was purified using a metal binding column, and then the purified material was separated on SDS-PAGE using non-denaturing or denaturing sample buffer, transferred to a nylon membrane, and incubated with anti-FipB antibody (Fig. 1). High molecular sized complexes containing FipB were observed under non-denaturing conditions for wild-type, CXXA and P286T versions of FipB, although there were fewer complexes detected with FipB-P286T. Note that FipB alone migrated as a triplet between the 37 and 50 kDa markers. This is a typical pattern of FipB migration on Western blots; these isoforms are likely due to differences in glycosylation.[32]

Two methods were used to identify the proteins that co-purified with FipB, 2D gel electrophoresis followed by mass spectrometry sequencing of individual spots (Fig. S1), and mass spectrometry sequencing of purified His-tagged FipB complexes without further separation.

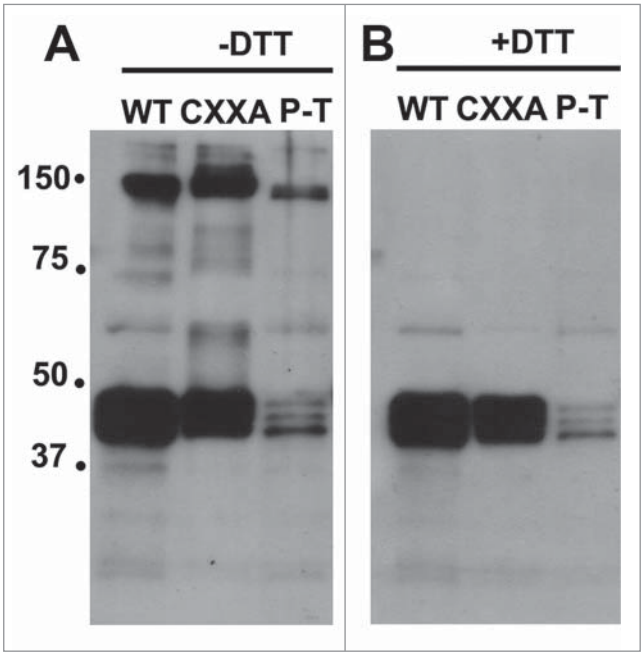

Figure 1. FipB migrated as high molecular weight complexes that were sensitive to reduction. FipB protein was purified from strains expressing His-tagged wild-type FipB, FipB-CXXA, or FipB P286T, using a metal affinity column. FipB was eluted from the column and run on separate SDS-PAGEs using sample buffer without (−DTT, Panel A) or with DTT (+ DTT, Panel B), and then transferred to a nylon membrane for Western blotting. FipB complexed with co-purified substrates were visualized with anti-FipB antibody.

The sequencing of seven individual spots from the 2D gel identified 52 proteins (Table S1). Samples for direct sequencing of co-purified material included His-tagged FipB-P286T, and FipB-CXXA as well as a His-tagged FipB-AXXA as a negative control. Overall 107 proteins were identified by co-purification with FipB-CXXA or FipB-P286T in this analysis. Only 29 proteins were identified from the FipB-P286T sample. Three proteins were unique to FipB-P286T, but these proteins were only identified by one or two peptides. We failed to detect any protein that co-purified with the His-tagged FipB-AXXA mutant, supporting a requirement for a disulfide bond interaction between FipB and its substrates. In total 119 proteins were identified by total sequencing and 2D gel analyses (Table S1). Forty out of the 52 proteins (77%) detected by 2D gel separation were also identified by sequencing the total co-purified material.

A summary of predicted subcellular location and Clusters of Orthologous Genes (COG) categories classification of all 119 proteins detected by 2D gel or total sequencing of His-tagged purified FipB-CXXA and FipB-P286T is shown in Figure S2. The subcellular location of many of the proteins (45%, 53/119) could not be predicted. Twenty-nine percent (35/119) were predicted to be cytoplasmic, and 26% (31/119) in the outer membrane, secreted, or found in multiple locations. A diverse set of 20 COG categories was represented. The top six categories, ranging from 16 to 9%, were: i) Unknown, ii) Energy production and conversion, iii) Translation, ribosome structure, and biogenesis, iv) Cell envelope biogenesis and outer membrane, v) Amino acid transport and metabolism, and vi) Posttranslational modification, protein turnover, chaperones. Eighty-two percent of the proteins had two or more cysteines. Eleven proteins had one cysteine and 11 had no cysteines, which was unexpected. Since we failed to detect any co-purifying proteins with the AXXA mutant of FipB, we anticipated that there would have been at least one cysteine in the protein (see Table S1). It is possible that these proteins interact with FipB indirectly though interaction with a substrate or through a non-specific interaction with a substrate. Five out of the 22 had a predicted cytoplasmic location, and another three had significant similarity to translation machinery, supporting the later possibility. Ten were identified by only one or two peptides; however, others were identified by greater than 15 peptides.

To narrow the list of putative substrates to those with greater confidence we applied a more stringent filter. Proteins that were identified in at least two out of the three samples, CXXA, P286T samples, or 2-D gel, or by four or more peptides in at least one sample are listed in Table 1. We also eliminated any gene that was likely essential. Essentiality was judged based on the failure to isolate a transposon mutant in that gene in a saturated comprehensive library of *F. novicida* (Francisella.org) [33] and bioinformatics. Thirteen proteins that fell into this category were annotated to have roles in such functions as in translation, transcription and various cytosolic metabolic functions. These proteins are highlighted in Table S1. While this filter is not foolproof,

Table 1. List of putative FipB substrates

Annotation	Locus	#Cys	Predicted Subcellular Location	Previously identified Virulence Phenotype
Unknown function	FTT0014	2	Unknown	
Unknown function	FTT0066	11	Outer Membrane	Identified as DsbA substrate in LVS [38]
Unknown function Conserved membrane	FTT0101	3	Unknown	No phenotype in LVS, [38] defect lung colonization in LVS [42]
Acid phosphatase/ *acpA*	FTT0221	4	Extracellular	Intracellular, defective intracellular growth and in mice in in FN, [43] mild defects in SCHU S4 [44]
Cytochrome BD-I terminal oxidase subunit I	FTT0279	3	Inner Membrane	
Cytochrome BO terminal oxidase subunit I	FTT0282	3	Inner Membrane	
Pyrrolidone-carboxylate peptidase	FTT0296	3	Periplasmic	Identified as DsbA substrate in LVS, [38] attenuated in FN, [45] increased expression in SCHU S4-infected macs [46]
Chaperone Hsp90	FTT0356	2	Cytoplasm	Attenuated in FN, [45] increased expression in SCHU S4-infected macs [46]
DipA	FTT0369	4	Multi/Extracellular/ Lipoprotein	Identified as DsbA substrate in LVS, [38] defective intracellular growth and in mice in SCHU S4 [47]
Glutamate dehydrogenase (NADP+)	FTT0380	4	Cytoplasm	
Periplasmic L-asparaginase II precursor/ *ansB*	FTT0464	2	Periplasmic	
Unknown function/ membrane	FTT0505	0	Inner/Outer Membrane	
FopA	FTT0583	2	Outer Membrane	Identified as DsbA substrate in LVS, [38] defective intracellular growth and in mice in SCHU S4 [47]
DNA/RNA endonuclease family	FTT0610	3	Extracellular	No defect LVS, [38] increased expression in SCHU S4-infected macs [46]
Beta-lactamase	FTT0611	2	Periplasmic	Identified as DsbA substrate in LVS, [38] increased expression in SCHU S4-infected macs [46]
KatG Catalase	FTT0721	0	Outer membrane	Defect lung colonization in LVS, [42] role in resistance to reactive oxygen species [48]
OmpA-related protein	FTT0831	4	Unknown	Attenuated in LVS and SCHU S4 [49]
OmpA- related protein	FTT0842	7	Outer Membrane	Growth defect in U937 macrophages and S2 insect cells [50]
Unknown function	FTT0989	1	Unknown	Attenuated in FN, [51] increased expression, but no intracellular defect in SCHU S4 [46]
LPS transport/LptA	FTT1025	4	Unknown	Identified as DsbA substrate in LVS, [38] No TN in FN library [33]
D-alanyl-D-alanine carboxypeptidase/ *dacD*	FTT1029	3	Inner Membrane	Identified as DsbA substrate in LVS, [38] increased expression in SCHU S4-infected macs ,[46] growth defect in S2 insect cells [50]
Serine-type D-Ala-D-Ala carboxypeptidase/ DacB	FTT1039	2	Unknown	Pleiotropic nutrient and stress phenotypes in *F. novicida* [52]
FKBP-type peptidyl-prolyl cis-trans isomerase (Mip)	FTT1043	4	Outer membrane /Lipoprotein	Identified as DsbA substrate in LVS [38]
Choloylglycine hydrolase family	FTT1109	1	Unknown/Lipoprotein	Growth defect in S2 insect cells [50]
Unknown function	FTT1242	2	Outer Membrane	Decreased expression in SCHU S4-infected macs, [46] repressed by PmrA in FN, [53] growth defect in S2 insect cells [50]
Peptidoglycan hydrolase	FTT1246	5	Unknown	
Multidrug resistance efflux pump membrane fusion subunit	FTT1257	0	Unknown	Attenuated in LVS [54]
Chaperone protein DNAK	FTT1269	3	Periplasm	
PdpB1/IcmF	FTT1345/ 1700	5	IM	Identified as DsbA substrate in LVS, [38] attenuated in FN [20, 21, 55]
Intracellular growth locus, subunit C	FTT1357/ 1712	4	Multi/Extracellular	Attenuated in LVS [42] and FN, [20, 45] increased expression in SCHU S4-infected macs [46] and attenuated in SCHU S4 [56]
Intracellular growth locus, subunit B	FTT1358/ 1713	6	Multiple	Attenuated in SCHU S4 [57]
Galactose-proton symporter, MFS	FTT1473	10	Inner Membrane	
Tfp pilus assembly protein PilW	FTT1496	4	Unknown	
Omp26	FTT1542	0	Unknown	

(*continued*)

Table 1. (*Continued*)

Annotation	Locus	#Cys	Predicted Subcellular Location	Previously identified Virulence Phenotype
HlyD family secretion protein/*emrA*	FTT1654/ FTN_0029	4	Unknown	Identified as DsbA substrate in LVS, [38] decreased expression in SCHU S4-infected macs [46]
Unknown function	FTT1676	3	Unknown	Attenuated in SCHU S4-infected mice [46]
Heat Shock Protein ClpB	FTT1769	2	Cytoplasm	Attenuated in LVS [58]

and may eliminate valid substrates or retain some nonphysiological interactions, it allowed for selection of higher confidence FipB-substrate interactions. Using these criteria, 37 putative substrates were identified. With one exception all had two or more cysteines, and only two were predicted to have a cytoplasmic location. Proteins with predicted enzymatic activity were prevalent (14/37). Of particular note were 19 putative FipB substrates that have been previously identified as impacting virulence in various screens for attenuated mutants in mice, or for defects in intracellular growth in various cells types. Among the known virulence factors identified were DipA, FopA, KatG, EmrA1, and three components of the T6SS.

FipB is required for the proper folding of T6SS components

Intramolecular disulfide bond interactions among T6SS components have not been explored, though the solved crystal structure of IglC does not suggest any intramolecular disulfide bonds.[27] IglB, which has six cysteines, interacts with IglA to form the contractile sheath through the interdigitation of strands from both proteins.[18] PdpB, which has five cysteines, is proposed to be an inner membrane component.[34] IglC has four cysteines, and is predicted to comprise the core of the sheath and is also secreted into the host cytoplasm.[19,20,34]

If these T6SS components were FipB substrates, then one would expect that the oxidation state of their cysteines would be altered in a FipB mutant. To examine the oxidation state of the cysteines of IglB and IglC, TCA-precipitated cell lysates were labeled with the sulfhydryl-reactive reagent AMS. AMS irreversibly reacts with free sulfhydryls, and adds 500 Da to the molecular weight of the protein. PdpB was not analyzed by this method because even if all 5 cysteines were labeled, this would only add 2.5 kDa to a 127 kDa protein, which would be difficult to detect with this gel system.

IglB was labeled with AMS, with or without treatment with the reducing agent TCEP, as evidenced by the slight decrease in band migration in AMS treated samples in both in wild-type and Δ*fipB* strains, indicating that IglB has free sulfhydryl groups that are not influenced by FipB (Fig. 2A). However, in samples that were not treated with AMS and TCEP there was less IglB detected in both strains (Fig. 2A and 2B). IglB polymerizes with IglA to form the T6SS sheath, so one might expect that as part of the polymerized sheath IglB would not be able to enter the gel.[18] Since the amount of IglB was less under nonreducing conditions this could suggest that the formation of the sheath is sensitive to reducing agents. Another related possibility is that epitope recognized by the IglB-specific monoclonal antibody is more accessible when the protein is labeled with AMS or reduced. We were unable to detect IglB with this IglB monoclonal in any wild-type lysate on SDS-PAGE when nonreducing loading buffer was used, but we could detect some IglB in Δ*fipB* lysates (data not shown). Of note was that more IglB was detected in all Δ*fipB* lysates compared to wild-type, lending support to the idea that FipB influences the conformation of IglB (Fig. 2A and 2B).

Since IglC has free cysteines then one might expect to see up to four additional bands corresponding to AMS-labeled IglC. There was an increase in the amount of IglC with 3 or 4 AMS- labeled cysteines in the Δ*fipB* mutant compared to wild-type bacteria (Fig. 2A), indicating that there were more free accessible cysteines in IglC in the Δ*fipB* mutant. However, we also observed an unexpected result; when lysates from wild-type bacteria were first reduced with TCEP before labeling with AMS, a treatment that should result in the labeling of all cysteine residues, there was no AMS labeling (Fig. 2A). This result was also seen when DTT was used as the reducing agent (data not shown). This result suggests that IglC forms a tightly folded, reduction-resistant protein that is dependent on FipB, and that reduction with TCEP also helps to protect free cysteines from labeling. We also observed that, similar to IglB, there was overall more IglC protein detected in most of Δ*fipB* mutant lysates, (Fig. 2A and 2C). The exception was the lysate that had not been treated with AMS or TCEP. As mentioned above a possible explanation for this result is that the epitope recognized by the anti-IglC monoclonal antibody is more exposed in the Δ*fipB* mutant. Take all together these data support the conclusion that in the absence of FipB IglB and IglC do not assume their native conformations.

To further explore the effect of FipB on IglC His-tagged recombinant IglC and FipB were purified and

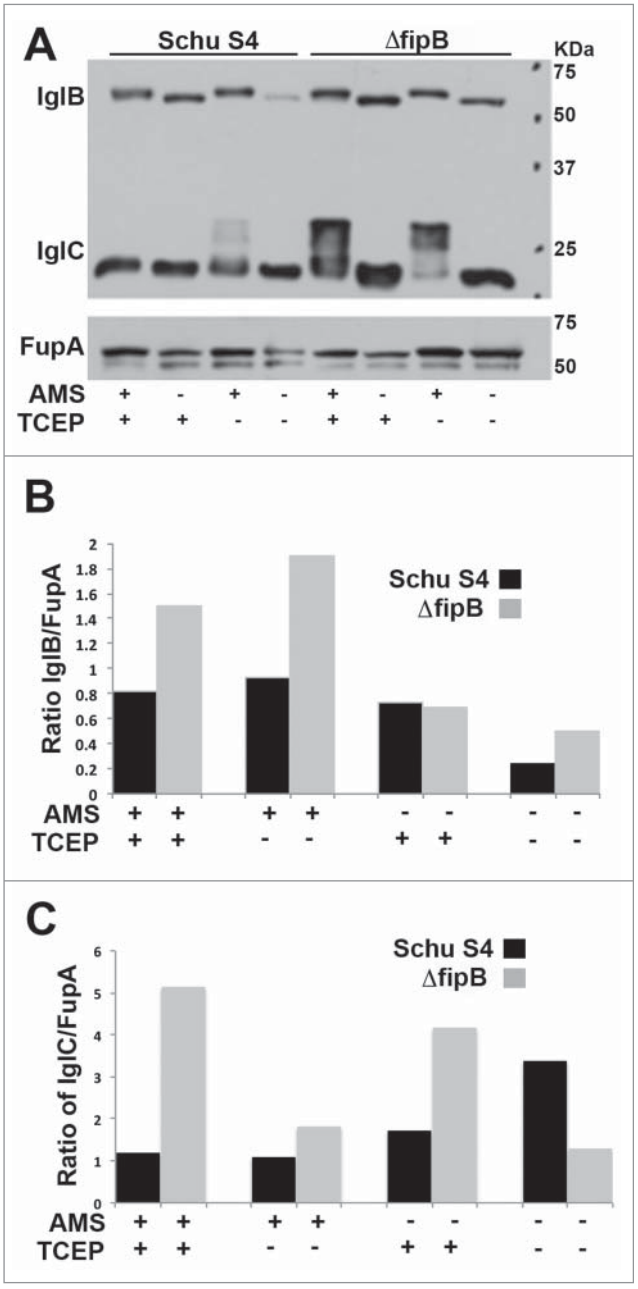

Figure 2. Accessibility of free sulfhydryls in IglB and IglC in wild-type and Δ*fipB* mutant bacteria. Panel A) Total bacterial lysates were labeled with AMS, a reagent that reacts with free sulfhydryls and adds 500 Da. Some samples were first treated with TCEP, to reduce existing disulfide bonds. Samples were separated on 4–15% SDS gel before transfer to PVDF membranes for immunoblots. Proteins were visualized with anti-IglC and IglB monoclonal antibodies. The same blot was stripped and then rehybridized with anti-FupA antibody. Blots are representative of at least three blots. Panels B& C) Blots were scanned by densitometry, and the amount of IglB (Panel B) or IglC (Panel C) was compared to the loading control FupA.

used in an *in vitro* assay to examine the ability of FipB to affect the oxidation or reduction of IglC's cysteines (Fig. 3A). Recombinant His-IglC alone formed multiple higher molecular weight forms that were not influenced by the presence of up to 50 μM DTT. However, when FipB was added the number of higher molecular weight bands decreased and there was increased AMS labeling. Although this in an *in vitro* assay, it demonstrated the ability of FipB to directly reduce IglC intermolecular disulfide bonds.

We looked at another FipB substrate, FopA, and detected a higher molecular weight band corresponding to FopA only in Δ*fipB* mutant lysates (Fig. 3B), suggesting that prevention of incorrect disulfide bonds can occur *in vivo*.

Growth in KCl is deleterious to Schu S4 strains

If IglC, IglB or PdpB were FipB substrates, then one would predict that the T6SS would not be functional in a Δ*fipB* mutant. Testing whether the T6SS is functional in a Δ*fipB* mutant is challenging because in *Francisella* the secretion of effectors had only been detected in the cytoplasm of host cells.[19-21] Since the Δ*fipB* mutant does not grow intracellularly this cannot be directly assessed. However, Clemens *et al.* found that secretion of T6SS effectors could be induced in *F. novicida* by adding 5% KCl to the growth media.[18] When we tried this protocol with Schu S4 we found that the addition of 5% KCl to the growth media drastically inhibited growth. Schu S4 and the Δ*fipB* mutant did grow when 2.5% KCl was to the added to the growth media, though cultures failed to reach a similar OD_{595} in stationary phase as bacteria grown without added KCl (Fig. S3). Attempts were made to determine if T6SS effectors were secreted under this condition. Culture supernatants were examined for presence of secreted effector IglC in the presence of added KCl (Fig. 4). Non-secreted T6SS component IglB, FopA, an outer membrane protein, and LPS were used as controls for cell lysis or decreased membrane integrity. IglC was detected in KCl-treated culture supernatants from both wild-type and Δ*fipB* strains. However, we also detected IglB, and FopA in the same supernatants, indicating that the addition of KCl increased cell lysis or affected membrane integrity. This situation was even more acute for the Δ*fipB* mutant because we could detect LPS in the supernatant. There was also indication of loss of membrane integrity without added KCl in the Δ*fipB* mutant. To test this further bacteria were grown overnight in TSB/c ± KCl, incubated with different concentrations of detergents CHAPS or nOctylGlu, diluted, and then spotted on MHA/c plates. The results for the 0.25% detergent condition are shown in Figure 5. Both wild-type and Δ*fipB* strains were more sensitive to killing by detergents when grown with KCl. Schu S4 exhibited a decrease in CFU even in the absence of detergent, suggesting that KCl alone has a toxic effect on Schu S4.

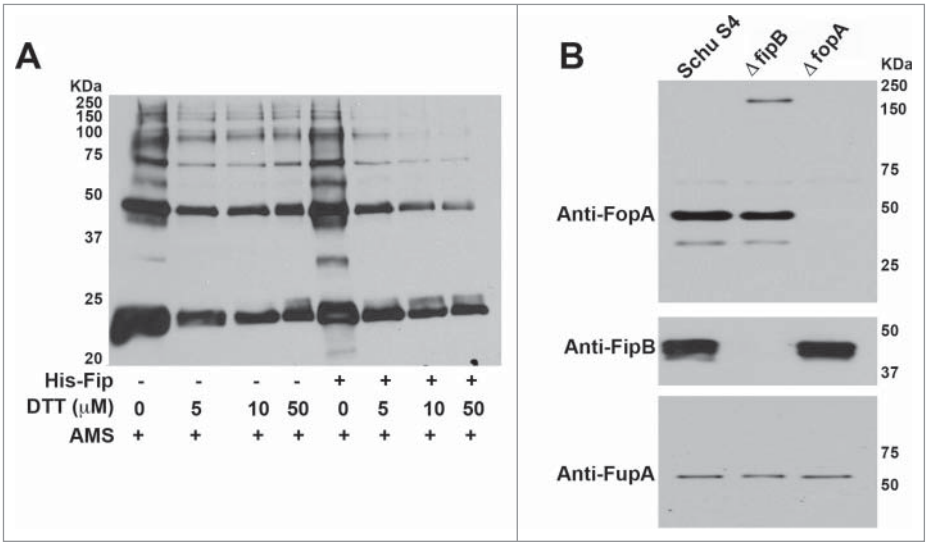

Figure 3. FipB resolved higher MW complexes of IglC and prevented higher MW complexes of FopA. Panel A) His-IglC was incubated with AMS and increasing concentrations of DTT in the presence or absence of His-FipB. Panel B) Western blot of total cell lysates of wild-type, Δ*fopA*, and Δ*fipB* strains. The same blot was incubated with anti-FopA antibody, then stripped and incubated with anti-FipB, and then stripped again and incubated with anti-FupA antibody.

There was also a decrease in CFUs in the KCl-grown Δ*fipB* mutant, though not to the same extent. This may be because the difference in the final OD_{595} in stationary phase, ± KCl was less for the Δ*fipB* mutant (Fig. S3). In sum, these results indicate that in Schu S4 strain background growth in KCl is not a viable means of assessing T6SS-mediated secretion and therefore we were unable to directly show that T6SS-mediated secretion was disrupted in a Δ*fipB* mutant.

Discussion

Among the potential FipB substrates identified in this paper were three components of the *Francisella* T6SS IglB, IglC, and PdpB. The data presented in this paper provide evidence that the T6SS of *Francisella* requires the FipB protein for proper conformation of IglB and IglC, and likely impacts the assembly of the T6SS apparatus. Both *fipB* and *iglC* mutants are highly attenuated and fail to escape the phagosome, so the avirulence of *fipB* mutants may largely be attributed to the loss of a functional T6SS.

The FipB protein has been shown to have both oxidoreductase, and isomerase activities, and is presumed to catalyze disulfide bonds in substrate proteins and also rearrange incorrectly formed disulfide bonds.[13,15,28] The FipB ortholog in the LVS strain of *F.* tularensis *holarctica* has been shown to have chaperone activity *in vitro* using the citrate synthase assay.[15] The results from the experiments presented in this paper support an isomerase or chaperone role for FipB that may or may not involve a disulfide bond in the final conformation of the protein.

Our data support a role for FipB in the formation of the T6SS structure. We observed an apparent increase in the amount of IglB and IglC protein in the Δ*fipB* mutant. The most likely explanation for this is that the epitopes recognized by the monoclonal antibodies used to detect these proteins are more exposed in the Δ*fipB* mutant, which is an indication that these proteins are not in the correct conformation. While there was a difference in AMS labeling of IglC in Δ*fipB* mutant, for IglB, there was no detectable difference in free sulfhydryls between the wild-type and the Δ*fipB* mutant, suggesting that for some substrates FipB may reduce incorrect disulfide bonds or prevent them from forming. The ClpV ATPase, a conserved component found in other T6SSs, but apparently absent in *Francisella* T6SS, has been shown to have a chaperone function that is required for the assembly of the IglA/IglB-equivalent protein sheath; [35] FipB could be supplying this aspect of the ClpV ATPase.

It is possible that the changes in IglB and IglC conformations are indirect, and the result the effect of FipB on another substrate, which may also have chaperone activity. However, there were no likely candidates identified by our analysis. We were able to show in an *in vitro* assay that recombinant FipB was able to reduce multimers of IglC to monomer form. While this supports the model, it should be noted that these assays have low substrate specificity.[36]

The specific mechanism of interaction between FipB and IglB and IglC needs to be further explored, but it must rely on the CXXC enzymatic active site of FipB; no substrates were purified with an AXXA mutant of FipB, and previous work has shown that the enzymatic active

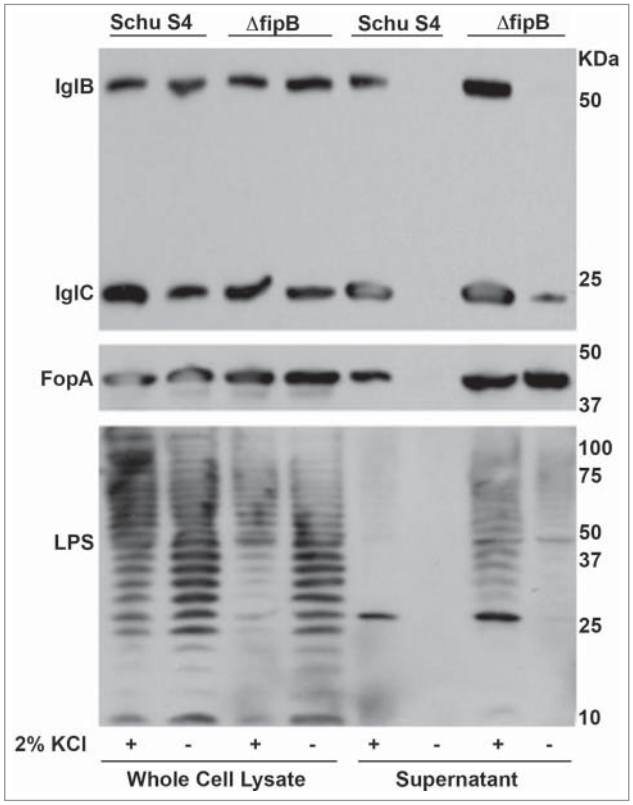

Figure 4. Detection of nonsecreted T6SS component and outer membrane constituents in the supernatants of KCl-grown bacteria. IglB, a nonsecreted T6SS component, outer membrane protein FopA, and LPS were detected in culture supernatants of KCl-grown bacteria. Bacterial cultures were grown overnight with or without 2.5 % KCl, and adjusted to the same OD_{595}. Whole cell lysates and culture supernatants were prepared as described in material and methods. The Western blot was incubated with anti-IglB and IglC antibodies, then stripped and incubated with an anti-FopA antibody. The same blot was stripped again and incubated with an anti-LPS antibody. Statistical significance was measured using an ANOVA and Dunn's multiple comparison tests (* p value <0 .05).

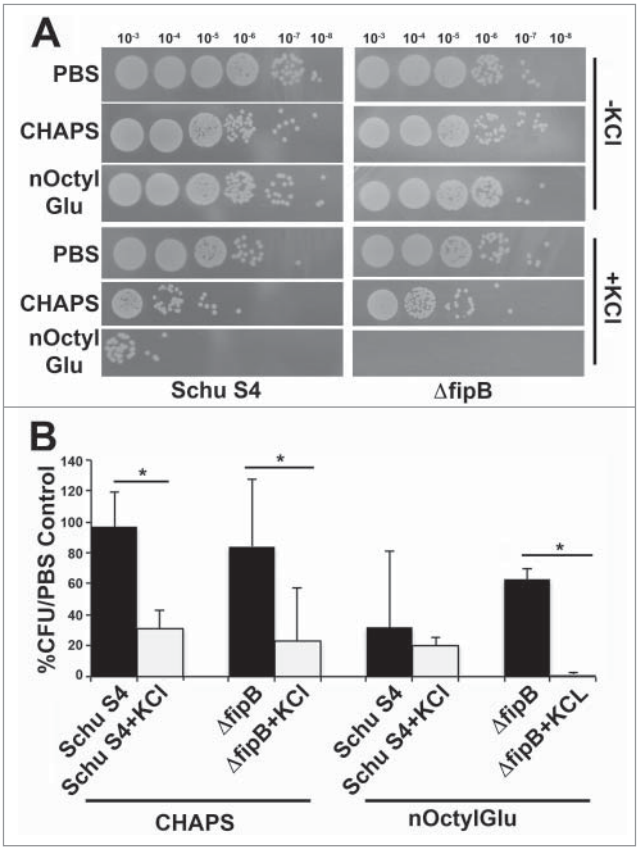

Figure 5. Growth in 2.5% KCl increases sensitivity to detergents. Schu S4 or Δ*fipB* strains were grown overnight in TSB/c with or without 2.5% of KCl. Cultures were adjusted to an OD_{595} of one, then incubated with 0.25% CHAPS or n-Octyl glucoside (nOctyl-Glu) for 90 min at RT. Serial dilutions, (by a factor of ten), were spotted on MHA/c plates and incubated at 37 °C for two d (Panel A). Quantitation of the detergent sensitivity was determined by comparing the number of recovered CFUs compared to PBS-treated controls from at least three independent experiments (Panel B). Statistical significance was measured using an ANOVA and Dunn's multiple comparison tests (* p value <0 .05).

site of FipB is essential for its role in virulence.[28] The T6SS of *Francisella* is atypical, lacking many of the conserved proteins characteristic of T6SSs.[17] The requirement of FipB may be an adaptation to the loss of some of these conserved components and has assumed the role of chaperone. Further studies of the interaction of FipB and the T6SS components will help to define its structure and assembly, which can then be contrasted and compared to other T6SSs.

We attempted to determine if T6SS was operating in the Δ*fipB* mutant by inducing secretion using growth in 5% KCl, a method that was used by Clemens *et al.* to induce T6SS effectors in *F. novicida*. However, we found that Schu S4 did not grow in 5% KCl. The bacteria tolerated growth in 2.5% KCl, but when we looked for secretion of effectors in the supernatant, we detected IglC, which was expected, but also found structural component IglB, outer membrane protein FopA, and LPS, which should not have been present (Fig. 4). Bacteria grown in the presence of 2.5% KCl were also more sensitive to detergents (Fig. 5). These results indicated that in Schu S4, and likely other *F. tularensis* subsp *tularensis* strains, growth in KCl compromises the membrane integrity and unlike *F. novicida*, this method cannot be used to assess T6SS in Schu S4. These results also suggest that regulation or control of the secretion of T6SS effectors differs between *F. novicida* and the more virulent *F. tularensis* subsp. *tularensis* strain Schu S4. In nature *F. tularensis* subsp. *tularensis* and *F. novicida* reside in different environmental niches.[37] *F. tularensis* subsp. *tularensis* is a vector borne, zoonotic bacteria, while *F. novicida* has never been isolated in wild animals or arthropods, but associated with brackish or salt water, and soil, so different environmental inducers might be expected.

We identified 119 potential FipB substrates in the Schu S4 strain using a combination of 2D gel electrophoresis and tandem mass spectrometry. These substrates need to be validated through other methods, in part because proteins that are likely found only in the cytoplasm were identified, as well as several proteins that. are predicted to be essential. The Δ*fipB* mutant grows in culture, so substrates that are essential proteins would not be expected. Many of these essential proteins are also cytoplasmic, but it is possible that FipB may have some noncritical chaperone or disulfide bond formation role for extracytoplasmic essential proteins.

FipB substrates have also been identified in the LVS strain of *F.* tularensis *holarctica*,[14,38] (see Table S1). Straskova *et al.* used 2D gels and the sensitive protein labeling technique, ITRAQ®, to compare protein abundance in wild-type LVS and an LVS Δ*fipB* (*dsbA) strain.*[14] This was based on the assumption that if FipB were necessary for protein folding or disulfide bond formation, then its substrates would be unstable and degraded. Hiniker and Bardwell used this assumption to identify a number of *E. coli* DsbA substrates by comparing the abundance of specific proteins in wild-type and *dsbA* strains using 2D gels.[39] Straskova *et al.* were able to identify 11 proteins with altered abundance in a *dsbA* (*fipB*) LVS mutant using ITRAQ®, however, nine had increased levels, and only two were decreased. The two proteins that had decreased abundance corresponded to FipB, and FipA. FipA is a small polypeptide encoded just upstream of FipB, in the same transcriptional unit.[28] We identified four out nine of these proteins (Table S1). Ren *et al.* used a method similar to ours, co-purifying proteins with a CXXA mutant bait.[38] We identified 25 out of the 52 substrates identified by Ren *et al.* Of note, Ren *et al.* failed to identify IglC, or IglB. We used the virulent Schu S4 strain, while both other investigators used LVS, but experimental conditions may also account for the differences in the identified putative substrates.

FipB is a novel member of the DsbA family of proteins that can participate in multiple roles in disulfide bond formation and protein folding. The ability of one protein to perform these multiple functions may be an adaptation to its reduced genome. FipB's essential requirement for virulence and its ability to affect the functions of multiple proteins, including components of the T6SS, make it an attractive target for antimicrobial therapy.

Materials and methods

Bacterial strains, media and reagents

Francisella strains were grown on cysteine-supplemented Mueller-Hinton agar (MHA/c) plates, or in Trypticase Soy broth (TSB/c) and with kanamycin (Kan) (15 μg/ml) when required. Studies involving Schu S4 and derivatives of this 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) as required. Monoclonal antibodies specific for IglB, IglC, and PdpB were obtained from BEI Resources. Antibodies to FipB, FopA, and LPS were generated in house. Anti-FupA was gift from Girija Ramakrishnan.[40]

Construction of mutant strains

Mutation and plasmid constructions were performed as previously described.[9] DNA was prepared and purified using a commercial kit (Qiagen). Oligonucleotides were synthesized by Integrated DNA Technologies Inc. Restriction endonucleases and ligase were purchased from New England Biolabs. HotStart® Taq (Qiagen) was used for routine PCR. FastStartH High fidelity PCR system (Roche) was used for construction of plasmids. All cloning products were verified by DNA sequencing. Site direct mutagenesis was accomplished with a site-directed mutagenesis kit (QuikChange®). Expression of *fipB* and mutant genes was verified by Western blot with rabbit anti-FipB antibody.[9] DNA transformation was performed as previously described.[4]

Purification of his-tag FipB with substrates

Δ*fipB* complemented *in trans* with genes expressing variant His-tagged FipB (CXXA, AXXA or P286T), were grown in TSB/c with 15 μg/ml Kan for overnight at 37 °C, 200 rpm shaking. Bacteria were pelleted by centrifugation at 3,000 rpm for 30 min and then resuspended in 10 mM *N*-Ethylmaleimide (NEM) [pH 6], and incubated at room temperature (RT) for 5 min. One hundred % TCA was added to a 5% final concentration to precipitate proteins at 4 °C overnight. Precipitated proteins were harvested by centrifugation at 8,000 rpm for 20 min at 4 °C. Protein pellets were washed twice with cold acetone. Air dried pellets were alkylated by 10 mM NEM, 100 mM Tris-HCl [pH 6.0], containing 1% SDS, and protease inhibitor cocktail (Sigma) at RT for 5 min. The alkylated lysate was diluted four times with 50 mM Tris-HCl, [pH 8.0], containing 300 mM NaCl and centrifuged at 10,000 rpm for 20 min at 4 °C. Supernatants were transferred to tube containing TALON® resin pre-equilibrated with buffer A (50 mM Tris-HCl [pH 8.0], 300 mM NaCl, 0.1% of SDS) and incubated at 4 °C overnight with rocking prior to loading on a Poly-Prep Chromatography column (Bio-Rad). Columns were washed with 20X column volume of Buffer A

and 2 ml Buffer A containing 10 mM imidazole. Co-purified proteins were eluted with Buffer A containing 300 mM imidazole. Elutions were precipitated with TCA (10% final), then acetone washed and air-dried as described above. For samples where FipB and FipB complexes were visualized by Western Blots pellets were resuspended in 150 μl of 1X SDS-PAGE loading buffer (50 mM Tris-HCl (pH 6.8), 2% (w/v) SDS (sodium dodecyl sulfate; electrophoresis grade), 0.1% (w/v) bromophenol blue and 10% (v/v) glycerol) and with or without or 100 mM dithiothreitol (DTT), separated on a 10% SDS-PAGE and then transferred to PVDF membranes for Western immunoblotting with anti-FipB antibody followed by corresponding HRP-conjugated secondary antibodies. Blots were developed using Pierce ECL Western blotting substrate and exposed to X-ray film.

Two-dimensional (2D) gel electrophoresis

2D gel electrophoresis was performed as described by Jameson-Lee *et al.*[41] Briefly, eluted samples were boiled with SDS-PAGE loading buffer and resolved on Bio-Rad Criterion Precast gel (12 well, 4–12% acrylamide) under nonreducing conditions. The lanes were excised and treated in warm 100 mM DTT for 15 min. Free sulfhydryls were alkylated by 100 mM iodoacetamide in Laemmli loading buffer without bromophenol blue for 5 min. The gel strip was then placed in a 1-well Criterion Precast gel (6–16% acrylamide). The strip was locked in place by addition of an agarose overlay (2-D starter Kit, Bio-Rad) before gel electrophoresis. Proteins were visualized with silver stain.

Mass spectrometry sequencing

Sequencing was performed at the University of Virginia Biomolecular Research Facility. Samples were analyzed by LC-MS consisting of a Thermo Electron Obritrap Velos ETD mass spectrometer system with a Prottan nanospray ion source interfaced to a self-packed 8 cm × 75 μm id Phenomenex Jupiter 10 μm C18 reversed-phase capillary column. The data were analyzed by database searching using the Sequest search algorithm against *F. tularensis*.

4-acetoamido-4′-maleimidylstilbene 2,2′-disulfonate (AMS) labeling

The *in vivo* redox statuses of IglC and IglB were analyzed by alkylation of free thiol groups by AMS (Molecular Probes). An overnight bacterial culture grown in Chamberlain's defined media (CDM) was precipitated with 10% final concentration of TCA on ice for 1 hr. CDM was used in these experiments because this was the media used by Ren *et al.* to label FipB (DsbA) substrates in LVS.[38] However, we also performed the same experiments with bacteria grown in TSB/c and did not observe any discernable difference. Total protein was pelleted by centrifugation at 14,000 rpm for 15 min at 4 °C. The pellet was washed twice with cold acetone then resuspended in 200 μl of 100 mM Tris-HCl [pH8.0], 1% SDS, 1 mM EDTA with or without 5 mM *Tris* (2-carboxyethyl) phosphine (TCEP) and incubated at 37 °C for 1 hr. AMS was added to a final concentration of 10 mM and then incubated at RT for 2 hrs. Extra AMS was removed by TCA precipitation as described above. The pellet was resuspended in 50 μl 1% SDS, 100 mM Tris-HCl [pH8.0]. An equal volume of 2X SDS-PAGE loading buffer (100 mM Tris-HCl (pH 6.8), 4% (w/v) SDS (sodium dodecyl sulfate; electrophoresis grade), 0.2% (w/v) bromophenol blue, 20% (v/v) glycerol) was added to each sample, which was then boiled, separated on a Criterion™ Precast Gel (4–15% Tris-HCl, 1.0 mm Bio-RAD), and then transferred to a PVDF membrane for Western blot analysis with specific antibodies followed by corresponding HRP-conjugated secondary antibodies. The blot was developed using Pierce ECL Western blotting substrate and exposed to X-ray film.

Oxidioreduction assay

Recombinant FipB was purified as previously described.[13] Recombinant IglC was purified from *E. coli* BL21 containing pQE60-*iglC-his* (gift from Tom Kawula) grown in Luria broth with 100 μg/ml ampicillin to 0.5 OD_{600}, then induced by 0.1 mM IPTG for additional 4 hrs. Bacteria were lysed by the addition of lysis buffer (6M urea, 0.5 M NaCl, 50 mM NaH_2PO_4, 10 U DNase and EDTA-free protease inhibitor cocktail, pH 8.0) and incubated with Talon resin (Clontech) at 4°C overnight. The resin was washed with wash buffer (6M urea, 0.5 M NaCl, 50 mM NaH_2PO_4 and protease inhibitor cocktail pH 8.0). His-tagged IglC was eluted with 300 mM imidazole, and dialyzed against 35 mM HEPES, pH 7.3. Purity was analyzed by SDS-PAGE, followed by GelCode Blue Stain (ThermoFisher). Concentration was assessed using the BCA protein kit (Pierce).

His-tagged IglC (4 μM in 50mM HEPES-NaOH, pH7.3) was incubated for 1 hr at 25°C with various concentrations of DTT (0 to 500 μM), and in the presence or absence of 4 μM His-tagged FipB. The reaction was stopped by TCA precipitation. The pellet was washed 3 times with cold acetone, air-dried, dissolved in a freshly prepared solution containing 1% SDS, 100 mM Tris-HCl, pH = 7.5, and 5 mM AMS, then incubated in the dark at 37°C for 1 hr. Samples were mixed with nonreducing 5X SDS-PAGE loading buffer and boiled for 5 min. Proteins were separated on a 12% SDS/PAGE,

transferred to a nylon membrane and incubated with anti-IglC antibody.

Preparation of KCl treated culture supernatants

Ten ml bacterial cultures were grown in TSB/c with or without 2.5 % KCl supplement for 24 hrs and then each strain was adjusted to the same OD. Fifty μl of the culture was removed and an equal volume of 2X SDS loading buffer was added, and then boiled to create the whole lysates. Bacteria were pelleted by centrifugation, and the supernatant was filtered by 0.22 μm filter, precipitated with TCA, and resuspended in 100 μl of 100 mM Tris-HCl, pH 7.5, 1% SDS. An equal volume of 2X sample buffer containing 2% 2-Mercaptoethanol was added to the tube, and then boiled. Fifty μl was applied to a 10% SDS-PAGE for protein separation, and transfer to immunoblots as described above.

Measurement of detergent sensitivity

Schu S4 or *ΔfipB* strains were grown overnight in TSB/c with or without 2.5% of KCl. Cultures were adjusted to an OD_{595} of one, then incubated with 0.25% of 3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate (CHAPS) or n-Octyl glucoside (nOctylGlu) for 30 and 90 min at RT. Factor of ten serial dilutions were spotted on MHA/c plates and incubated at 37 °C for two days. Statistical significance was measured using an ANOVA and Dunn's multiple comparison tests.

Abbreviations

AMS	4-acetoamido-4′-maleimidylstilbene 2,2′-disulfonate
CDM	Chamberlain's defined media
COG	Clusters of Orthologous Genes
CXXC	Cysteine- any amino acid- any amino acid-Cysteine
Dsb	disulfide bond
DTT	1,4-Dithiothreitol
Hcp	Hemolysin coregulated protein
IPTG	Isopropyl β-D-1-thiogalactopyranoside
LVS	Live Vaccine Strain
MAL-PEG	Methoxypolyethylene glycol maleimide
MW	molecular weight
nOctylGlu	n-Octyl glucoside
T6SS	Type six secretion system
TCEP	*Tris* (2-carboxyethyl) phosphine

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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