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Lipidation of the FPI protein IglE contributes to *Francisella tularensis* ssp. *novicida* intramacrophage replication and virulence

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Abstract

Francisella tularensis is a Gram-negative bacterium responsible for the human disease tularemia. The *Francisella* pathogenicity island (FPI) encodes a secretion system related to type VI secretion systems (T6SS) which allows *F. tularensis* to escape the phagosome and replicate within the cytosol of infected macrophages and ultimately cause disease. A lipoprotein is typically found encoded within T6SS gene clusters and is believed to anchor portions of the secretion apparatus to the outer membrane. We show that the FPI protein IglE is a lipoprotein that incorporates ^{3}H-palmitate and localizes to the outer membrane. A C22G IglE mutant failed to be lipidated and failed to localize to the outer membrane, consistent with C22 being the site of lipidation. *Francisella tularensis* ssp. *novicida* expressing IglE C22G is defective for replication in macrophages and unable to cause disease in mice. Bacterial two-hybrid analysis demonstrated that IglE interacts with the C-terminal portion of the FPI inner membrane protein PdpB, and PhoA fusion analysis indicated the PdpB C-terminus is located within the periplasm. We predict this interaction facilitates channel formation to allow secretion through this system.

Keywords

tularemia; lipoprotein; intramacrophage; virulence

Introduction

Francisella tularensis is a Gram-negative facultative intracellular bacterium that causes the disease tularemia in humans (Oyston, 2008). Tularemia is usually spread by blood-sucking vectors or is acquired through contact with an infected animal, but it can also be acquired through inhalation. *Francisella tularensis* has a low infectious dose and high morbidity/mortality associated with pulmonary infections, which has led to its designation as a

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This article describes an interesting modification of a *Francisella virulence* protein that contributes to replication within macrophages. They found that lipidation of the *Francisella* pathogenicity island protein IglE anchors it to the outer membrane where it is involved in facilitating channel formation and thus allowing secretion of other virulence factors.

Supporting Information: Additional Supporting Information may be found in the online version of this article:

category A biothreat agent. Different subspecies of *F tularensis* exhibit different levels of virulence in humans, with *F tularensis* ssp. *tularensis* causing the most serious infections and *F tularensis* ssp. *novicida* being considered avirulent in healthy humans [depending on classification, *F tularensis* ssp. *novicida* is also classified as a separate species, *F novicida*] (Sjostedt, 2007; Titball & Petrosino, 2007). *Francisella tularensis* ssp. *tularensis* has two identical copies of the *Francisella* pathogenicity island (FPI), whereas *F tularensis* ssp. *novicida* has only a single copy of the FPI (Nano *et al.*, 2004; Larsson *et al.*, 2005), but it is not clear whether this difference in FPI copy number contributes to the difference in virulence between these two subspecies.

The FPI is a cluster of 17 genes that are critical for *F. tularensis* virulence (Nano *et al.*, 2004). The FPI is required for phagosomal escape and replication within the cytosol of infected cells (Golovliov *et al.*, 2003a; Lindgren *et al.*, 2004; Santic *et al.*, 2005). Most of the FPI genes are essential for the virulence of the different subspecies of *F. tularensis* in mice by various inoculation routes (Golovliov *et al.*, 2003b; Lauriano *et al.*, 2004; Weiss *et al.*, 2007). The FPI encodes a Type VI-like Secretion System (T6SS) that facilitates *F. tularensis* phagosome escape (Barker *et al.*, 2009; de Bruin *et al.*, 2011;Broms *et al.*, 2012a). T6SS use a phage tail-like injectisome to secrete effector proteins from the bacterial cytoplasm into the cytosol of host cells or other bacteria (Mougous *et al.*, 2006; Pukatzki *et al.*, 2006; Hood *et al.*, 2010; MacIntyre *et al.*, 2010; Basler *et al.*, 2012). In the best-studied T6SS in *Vibrio cholerae*, a dynamic tubular structure attached to the membrane has been visualized that assembles, contracts, and disassembles within the bacterial cytoplasm to facilitate translocation of proteins out of the cell (Basler *et al.*, 2012).

The secretion system encoded within the FPI has a number of similarities with other T6SS, including the following: (1) IglA and IglB, two interacting proteins that are homologues of the components that constitute the contractile sheath (VipA and VipB) (Bonemann *et al.*, 2009; Broms *et al.*, 2009; Karna *et al.*, 2010); (2). PdpB (IcmF) and DotU, homologues of inner membrane proteins that are required for T6S (Barker *et al.*, 2009; de Bruin *et al.*, 2011; Zheng *et al.*, 2011); and (3). VgrG, a trimeric protein proposed to be loaded onto the tip of the puncturing device and delivered into target cells (Pukatzki *et al.*, 2007; Barker *et al.*, 2009;Broms *et al.*, 2012a). However, the FPI secretion system is also different from well-characterized T6SS in a number of ways, mainly because it appears to be missing components that are considered predictive for a functional T6SS (Boyer *et al.*, 2009). Also, the VgrG protein is significantly smaller and lacks an effector domain typically found in other VgrG proteins, and PdpB/IcmF lacks the Walker A box normally found in these proteins (Zheng & Leung, 2007; Barker *et al.*, 2009), although evidence suggests that the Walker A box is not always critical for T6S (Zheng & Leung, 2007).

Another conserved element of T6SS gene clusters is the presence of a lipoprotein that localizes to the outer membrane (Aschtgen *et al.*, 2008; Boyer *et al.*, 2009). The T6S lipoprotein from *Edwardsiella tarda* interacts with the C-ter-minus of the inner membrane protein IcmF (Zheng & Leung, 2007), and it has been proposed that this interaction between IcmF in the inner membrane and the lipoprotein in the outer membrane forms a continuous channel spanning the periplasmic space (Leiman *et al.*, 2009). In the current report, we show that the FPI protein IglE is a lipoprotein that localizes to the outer membrane and interacts

with PdpB/IcmF. IglE lipidation is required for outer membrane localization, intramacrophage replication, and virulence in mice, consistent with a conserved role of IglE in the T6-like secretion system.

Materials and methods

Strains and media

Francisella tularensis ssp. *novicida* strains are isogenic with strain U112 (ATCC 15482) and listed in Supporting Information, Table S1. *F. tularensis* ssp. *novicida* mutants KKF194 (Δ*iglE*::*ermC*) and KKF177 (Δ*dotU*::*ermC*) were created using the 'splicing by overlap extension' (SOE) method described by Liu *et al.* (Liu *et al.*, 2007). *Escherichia coli* strain DH5α was used for cloning (Hanahan, 1983), and KDZif1ΔZ was used for bacterial two-hybrid assays (Vallet-Gely *et al.*, 2005). Luria broth (LB) was used for both liquid medium and agar plates for growth of *E. coli* strains. *F. tularensis* ssp. *novicida* strains were grown on TSAP broth/agar (Liu *et al.*, 2007) or Chamberlain's defined medium (CDM) (Chamberlain, 1965) supplemented with antibiotics, as appropriate. The concentrations of antibiotics used were as follows: ampicillin 100 μg mL^{-1}; tetracycline 15 μg mL^{-1}; kanamycin 50 μg mL^{-1}; erythromycin 150 μg mL^{-1}.

Plasmid construction

A list of plasmids and oligonucleotide primers used in this study can be found in Tables S1 and S2. Restriction sites used in cloning are underlined, and the FLAG tag, C22G mutation, and universal priming sites are noted in bold. *Francisella tularensis* ssp. *novicida* U112 genomic DNA was used as template in PCRs. Sequences coding for aa 23–125 of IglE and aa 590–1093 of PdpB were cloned into the bacterial two-hybrid plasmids pKEK1286 and pKEK1287 to form pKEK1542 and pKEK1613, respectively. IglE-FLAG was cloned into pKEK996 (Rodriguez *et al.*, 2008) to form pKEK1355, using primers IglE*Pci*IF and IglEFLAGEcoRIR, and then, site-directed mutagenesis (Novagen) was performed on pKEK1355 with primers IglEC22GF and IglEC22GR to create pKEK1368. The SOE reaction that amplified the Δ*iglE*::*ermC* construct in pKEK1063 and the Δ*dotU*::*ermC* construct in pKEK1003 used the FTT1346 and FTT1351 primers listed in Table S1 along with the universal primers, as described in (Liu *et al.*, 2007); these plasmids were subsequently transformed into U112 to construct KKF194 (Δ*iglE*::*ermC*) and KKF177 (Δ*dotU*::*ermC*).

The *cyaA* sequence in pKEK1069 and pKEK1072 (Barker *et al.*, 2009) was replaced with *phoA* PCR-amplified with primers PhoAFNdeI and PhoARPstI, to form pKEK1762 (*bla-phoA*) and Δ*N-bla-phoA* (pKEK1763), respectively. Sequences coding for aa 1–272 and aa 1–312 of PdpB were cloned into pKEK1762 to construct pKEK1764 and pKEK1765, respectively.

β-galactosidase and alkaline phosphatase assays

For the bacterial two-hybrid assays, plasmids expressing -Zif and -ω fusions were cotransformed into KDZif1ΔZ. Overnight cultures of these strains were diluted 1 : 100 into LB containing 0.3 mM IPTG, grown at 37 °C to OD_{600} nm 0.2–0.4, and assayed for β-

galactosidase activity (Miller, 1992). For alkaline phosphatase assays, *F tularensis* ssp. *novicida* strains were transformed with plasmids containing protein *phoA* fusions listed in Table S1, grown in TSAP supplemented with appropriate antibiotics at 37 °C, and harvested at an optical density of 600 nm of *c*. 0.3–0.4. Bacterial cells were permeabilized with chloroform and sodium dodecyl sulfate (SDS) and assayed for alkaline phosphatase activity by the method described by Michaelis (Michaelis *et al.*, 1983). Statistical significance was determined using Student's *t*-test.

Protein detection

Fractionation of *F tularensis* ssp. *novicida* whole-cell lysates into outer membrane, inner membrane, and cytoplasmic fractions was accomplished using the Sarkosyl membrane fractionation method as adapted by de Bruin *et al.* (2007). Proteins were detected by separation on a 15% SDS polyacrylamide gel, followed by Western immunoblot using anti-FLAG M2 (Sigma) monoclonal antibody, rabbit polyclonal anti-Tul4 antisera, rabbit polyclonal anti-VgrG, or rabbit polyclonal anti-PdpB [gift from F. Nano; (Ludu *et al.*, 2008)], and ECL detection reagent (Amersham-Pharmacia).

For detection of lipid incorporation into IglE, strains were grown at 37 °C in CDM with [^{3}H]palmitic acid (Moravek Biochemicals) to a final concentration of 20 μCi mL^{-1}. Overnight cultures were pelleted, washed once with PBS, and then resuspended in 1X sample buffer and boiled. Samples were separated by 15% SDS-PAGE and then fixed with 5% glacial acetic acid, 5% isopropanol, and water. The gel was then treated with Autoflour (National Diagnostics) and imaged by autoradiography. Measurement of the labeled protein at 13.2 kD normalized to the label incorporated into a constant band at *c*. 16 kD in every lane was used to quantitate the relative labeled IglE levels by densitometry of the autoradiograph.

In vivo co-immunoprecipitation

Co-immunoprecipitation experiments were performed similar to those described previously (Felisberto-Rodrigues *et al.*, 2011), with the following modifications. We utilized 250 mL *F novicida* culture at an $OD_{600\ nm}$ of 0.7 as starting material, and the solubilized supernatant was incubated with anti-FLAG antibody coupled to magnetic beads (Sigma-Aldrich) for 1 h at 4 °C.

Intramacrophage assay

Francisella tularensis ssp. *novicida* strains were used to infect the J774.1 macrophage cell line at a multiplicity of infection (MOI) of 10 : 1. The intramacrophage assay was performed as previously described (Lauriano *et al.*, 2003). Statistical significance was determined using Student's *t*-test.

Mouse virulence assays

Groups of five female 4- to 6-week-old BALB/c mice (Charles River Laboratories) were inoculated intranasally with *F tularensis* ssp. *novicida* strains in 20 μL PBS. Actual bacterial numbers delivered were determined by plate count from inocula, and an *c*. $LD_{50\ nm}$ was determined from surviving mice. Mice were monitored for 30 days after infection. The

Institutional Animal Care and Use Committee (IACUC) at UT San Antonio approved all animal procedures.

Results

IglE is a lipoprotein

Lipoproteins are synthesized in the cytosol as prolipoproteins with a characteristic leader signal sequence containing a 'lipobox' that directs it to the cytoplasmic membrane into the lipoprotein processing pathway. (Okuda & Tokuda, 2011). Processing of the prolipoprotein occurs in three steps in the cytoplasmic membrane, where a cysteine residue immediately downstream of the signal sequence is first modified with diacylglycerol, then the signal sequence is cleaved by signal peptidase II, and finally, the processed protein is N-acylated on the liberated N-terminal cysteine residue (Sankaran & Wu, 1994, 1995). Lipoproteins that subsequently localize to the outer membrane are transported across the periplasmic space by the LolABCDE complex (Okuda & Tokuda, 2011). The LipoP 1.0 Server predicts IglE is cleaved by signal peptidase II between aa 21 and 22 (Juncker *et al.*, 2003), resulting in a mature protein with C22 at the N-terminus available for lipidation, similar to T6SS lipoprotein components found in other bacteria (Fig. 1a).

To determine whether IglE is a lipoprotein, we first created an *F. tularensis* ssp. *novicida* strain lacking IglE by deleting the corresponding gene (FTN1311; Δ*iglE*::*ermC*; Materials & Methods). Western immunoblot detected expression of the immediate downstream gene product, VgrG, in the *iglE* mutant strain [Fig. S1; VgrG expression appeared slightly higher in the *iglE* mutant strain, which may be due to the promoter driving *ermC* expression (Liu *et al.*, 2007)]. This demonstrates that the Δ*iglE*::*ermC* mutation does not prevent expression of the downstream gene within this strain. The Δ*iglE F. tularensis* ssp. *novicida* strain was transformed with plasmids expressing either IglE-FLAG, or IglEC22G-FLAG, in which the putative site of lipidation was altered by site-directed mutagenesis to prevent lipidation (C22G). To determine whether IglE is a lipoprotein, the Δ*iglE* strain alone, or expressing IglE-FLAG or IglE C22G-FLAG was grown in the presence of ^{3}H palmitic acid; the wild-type U112 strain was also grown under similar conditions. Incorporation of ^{3}H palmitic acid is a frequently used technique to identify bacterial lipoproteins and has been used previously to identify *F. tularensis* Tul4 as an outer membrane lipoprotein (Sjostedt *et al.*, 1991). Whole-cell extracts were separated by SDS-PAGE, and incorporation of ^{3}H palmitate was visualized by autoradiography (Fig. 1b). Processed IglE-FLAG is predicted to have a molecular mass of 13.2 kD, which corresponds well with the band seen in lane 3, indicating that the IglE-FLAG protein incorporated ^{3}H palmitate. The *iglE* strain expressing IglE C22G-FLAG protein (lane 4) shows only background labeling, similar to the level seen in the *iglE* strain without plasmid (lane 2). A very low level of ^{3}H-labeled IglE slightly above background level (1.36-fold) could be detected in the wild-type strain (lane 1); this is likely due to the low natural levels of IglE found in the wild-type strain.

Western immunoblot of these strains with FLAG antibody (Fig. S2) indicated detectable levels of IglEC22G-FLAG expression, and this protein appeared slightly larger than the wild-type IglE-FLAG, consistent with a lack of processing. Prolipoproteins with a Cys residue substitution are localized to the cytoplasmic membrane, but fail to be modified with

diacylglycerol, and thus, the signal peptide fails to be cleaved (Sankaran & Wu, 1994); we have seen this phenomenon previously with prolipoproteins from *V. cholerae* (Morris *et al.*, 2008). This suggests that the lack of palmitate incorporation into IglEC22G is not due to the absence of IglE protein, although there are also lower levels of the unprocessed IglEC22G protein. These results are consistent with IglE being a lipoprotein that is lipidated at C22.

IglE localizes to the outer membrane

IglE is predicted to localize to the OM, because it lacks an Asp at the +2 position (Fig. 1a); an Asp at +2 usually dictates IM rather than OM localization (Okuda & Tokuda, 2011). We examined the localization of IglE by cell fractionation, using *F. tularensis* ssp. *novicida* strains expressing IglE-FLAG (Fig. 2a). We utilized both wild type and Δ*iglE* strains, as well as a strain lacking the entire FPI (ΔFPI), to determine whether other gene products within the FPI affected the localization of IglE. Inner membrane (IM), outer membrane (OM), and whole-cell (WC) samples were separated by SDS-PAGE and probed by Western immunoblot using anti-FLAG M2 antibody (Sigma), as well as antisera against the *F. tularensis* lipoprotein Tul4, which is known to localize to the OM (Sjostedt *et al.*, 1990). WC samples were matched to equivalent protein levels, and OM samples were matched to equivalent protein levels. IglE-FLAG could be detected in the OM and IM samples of the wild type and Δ*iglE* strains, similar to the localization pattern of the known lipoprotein Tul4; because the lipoprotein processing pathway proceeds through the IM, OM lipoproteins can be detected in the IM as well. These results are consistent with IglE localizing to the OM. Also, the fractionation pattern of IglE-FLAG was the same in the strain lacking the entire FPI, indicating that IglE OM localization does not require any of the other FPI proteins.

As shown above, the IglEC22G-FLAG protein is not lipidated. To determine whether lipidation of IglE is required for OM localization, we fractionated the Δ*iglE* strain expressing IglE-FLAG or IglEC22G-FLAG and performed Western immunoblot on the OM and IM fractions using anti-FLAG antibody (Fig. 2b). As expected, the IglEC22G-FLAG protein failed to localize to the OM, unlike the IglE-FLAG protein, consistent with lipidation being required for IglE OM localization. Less IglEC22G-FLAG was detected in the IM fraction, compared to the IglE-FLAG protein, and the IM-localized IglEC22G-FLAG protein appeared slightly larger than IglE-FLAG. These results can be explained by the lack of lipoprotein processing caused by the C22G mutation resulting in the pro-lipoprotein being trapped in the IM and degraded (Morris *et al.*, 2008).

Lipidation of IglE contributes to intramacrophage replication

Intramacrophage replication is a critical virulence attribute of *F. tularensis* and is dependent upon phagosomal escape. To determine the role of IglE in *F. tularensis* intramacrophage replication, the Δ*iglE* strain was examined for growth within the J774 macrophage cell line (Fig. 3). The Δ*iglE* strain was defective for intramacrophage replication, exhibiting lower levels of intracellular bacteria 24 and 48 h postinfection, in comparison with the wild-type strain U112, which replicates to high intracellular levels during this time period. Notably, the Δ*iglE* strain was not completely unable to replicate within macrophages, but rather showed a 2–3 log decrease in intracellular bacteria at 24 and 48 h postinfection. This limited intramacrophage growth phenotype is in contrast to other FPI mutants (e.g. *vgrG*, *iglC*) that

show no intracellular replication over the same time course (Barker *et al.*, 2009). The intracellular growth defect of the *iglE* strain was overcome by expression of IglE-FLAG *in trans*, indicating that FLAG-tagged IglE maintains its normal function. However, the intracellular growth defect of the *iglE* strain was not overcome by expression of IglEC22G-FLAG *in trans.* These results demonstrate that lipidation of IglE is critical for intramacrophage growth.

Lipidation of IglE is required for virulence in mice

Previously, transposon mutants with insertions in *iglE* were identified as being defective for virulence using assays screening for organ dissemination following subcutaneous injection in mice (Weiss *et al.*, 2007) or disease in fruit flies (Ahlund *et al.*, 2010). To determine the role of IglE in *F. tularensis* virulence in mice via the pulmonary route, the Δ*iglE F. tularensis* ssp. *novicida* strain and the Δ*iglE* strain expressing either IglE-FLAG or IglEC22G-FLAG were inoculated into BALB/c mice intranasally at a relatively high inoculum (*c*. 10^5 CFU) and survival monitored (Fig. 4). The wild-type U112 strain was also inoculated into BALB/c mice for comparison. A relatively low inoculum (*c*. 10^2 CFU) was utilized for the wild-type strain, due to the low $LD_{50\ nm}$ for the wild-type strain via this route. The Δ*iglE* strain was highly attenuated, causing no mortality at this inoculum. The Δ*iglE* strain expressing IglE-FLAG showed some restoration of virulence, causing 40% mortality at this inoculum. In contrast, the Δ*iglE* strain expressing IglEC22G-FLAG was highly attenuated, causing no mortality at this inoculum.

The fact that IglE-FLAG expression from the plasmid was able to completely restore intramacrophage replication (above), but not mouse virulence is likely due to the heterologous promoter driving IglE expression, and/or the high copy nature of the complementing plasmid, both of which can lead to incorrect IglE stoichiometry. Nonoptimal amounts of IglE may compromise some aspect of virulence outside of macrophages. Still, the restoration of virulence by IglE-FLAG demonstrates that the attenuated virulence of the Δ*iglE* strain is specifically due to lack of IglE. As a comparison, the wild-type strain caused the death of all animals at relatively low inoculum by 6 days postinoculation. These results demonstrate a requirement for IglE lipidation for *F. tularensis* virulence.

The C-terminus of PdpB is localized in the periplasm

PdpB (IcmF) has previously been shown to be an IM protein (de Bruin *et al.*, 2011), consistent with the localization of IcmF homologues in T6SS. The transmembrane prediction algorithm TMHMM (Krogh *et al.*, 2001) predicts two trans-membrane segments of PdpB, aa 10–29 and aa 273–292 (Fig. 5a). The orientation of the second transmembrane segment determines whether the PdpB C-terminus (aa 293– 1093) is located within the cytoplasm or the periplasm. To determine the orientation of the PdpB second trans-membrane segment, we utilized PhoA fusion analysis. Protein fusions of PhoA to bacterial IM proteins have been used extensively to determine membrane topology, based on the ability of PhoA to be enzymatically active only in the periplasm and not in the cytoplasm (Manoil & Beckwith, 1986). We fused two different N-terminal portions of PdpB to PhoA, either lacking (aa 1–272) or possessing (aa 1–312) the putative second transmembrane segment. As controls, we used secreted (periplasmic; Bla) and nonsecreted (cytoplasmic;

ΔN-Bla) forms of beta-lactamase [i.e. possessing or lacking the N-terminal signal sequence; (Barker *et al.*, 2009)].

Expression of aa 1–312 of PdpB fused to PhoA in *F. tularensis* ssp. *novicida* ('wild type') led to high levels of alkaline phosphatase activity (Fig. 5b), similar to levels seen with the (periplasmic) Bla-PhoA fusion. In contrast, expression of aa 1–272 of PdpB fused to PhoA only resulted in background levels of alkaline phosphatase activity, equivalent to the levels seen with the (cytoplasmic) ΔN-Bla fused to PhoA. These results are consistent with an 'inside-to-outside'-oriented transmembrane segment (aa 273–292) that localizes the PdpB C-terminus in the periplasm. To determine whether any of the other FPI proteins are important for PdpB localization, the PdpB-PhoA fusions were also expressed in a strain lacking the FPI (ΔFPI). This resulted in a loss of activity of the $PdpB^{1–312}$-PhoA fusion, suggesting that another FPI protein(s) facilitates PdpB stability and/or localization. It has previously been shown that the FPI inner membrane protein DotU is required for the stability of PdpB (de Bruin *et al.*, 2011), similar to DotU and IcmF homologues found in other T6SS (Ma *et al.*, 2009). The PdpB-PhoA fusions were expressed in a Δ*dotU* strain, which also resulted in a loss of activity of the $PdpB^{1–312}$-PhoA fusion, consistent with the stabilizing role of DotU on PdpB.

Western immunoblot analyses confirmed a lack of $PdpB^{1–312}$-PhoA protein in the strains lacking either DotU or the entire FPI (Fig. 5c). Interestingly, the $PdpB^{1–272}$-PhoA protein was unaffected by the lack of either DotU or the entire FPI, suggesting that the transmembrane region (aa 272–312) is the target of proteolytic degradation in the absence of DotU.

IglE interacts with the C-terminus of PdpB

It has been proposed that interaction between an OM lipoprotein and IcmF in the IM forms a channel spanning the periplasm that is a conserved aspect of T6SS (Leiman *et al.*, 2009; Felisberto-Rodrigues *et al.*, 2011). As determined above, the C-terminal portion of PdpB (IcmF) is located within the periplasm, which would allow for interaction with IglE. To determine whether IglE interacts with the C-terminus of PdpB, we utilized a bacterial two-hybrid system (Dove & Hochschild, 2004; Karna *et al.*, 2010), in which interactions between proteins fused to Zif and ω drive transcription of a *lacZ* reporter in the *E. coli* reporter strain KDZif1ΔZ. To prevent secretion outside the cytoplasm, IglE lacking the signal sequence (aa 23–125) was fused to ω, and the C-terminus of PdpB (aa 590–1093) was fused to Zif.

IglE-ω and PdpB-Zif interact and stimulate transcription from the engineered promoter-*lacZ* fusion present in the KDZif1ΔZ *E. coli* reporter strain (Fig. 6a). The level of beta-galactosidase activity is higher than that stimulated by the known interaction of IglA-Zif and IglB-ω (Karna *et al.*, 2010), the two FPI proteins that likely compose the contractile sheath. Either plasmid alone is insufficient to stimulate beta-galactosidase activity above background, or neither IglE-ω nor PdpB-Zif interact with another FPI protein, IglA, demonstrating the specificity of interaction.

To confirm the interaction of IglE and PdpB in *F. tularensis* ssp. *novicida in vivo*, we expressed IglE-FLAG in either the Δ*iglE* strain or a ΔFPI strain (lacking PdpB) and subjected the cell lysates to immunoprecipitation with α-FLAG. We utilized conditions similar to a previous study that demonstrated the interaction of the T6SS OM lipoprotein TssJ with the IM IcmF homologue TssM of *E. coli* (Felisberto-Rodrigues *et al.*, 2011). Immunoprecipitation of IglE-FLAG in the presence of PdpB led to co-precipitation of PdpB (Fig. 6b). Control immunoprecipitations of either IglE-FLAG in the absence of PdpB, or in the presence of PdpB and the absence of IglE-FLAG, did not result in detection of PdpB, demonstrating the specificity of this interaction. Our results are consistent with IglE interacting with the C-terminus of PdpB (IcmF) to facilitate channel formation across the periplasm.

Discussion

The ability of *F. tularensis* to escape the phagosomal compartment within macrophages is central to the virulence of this bacterium (Golovliov *et al.*, 2003a). The FPI is a cluster of 17 genes that are required for phagosomal escape, intramacrophage replication, and virulence (Nano *et al.*, 2004). The FPI genes encode a secretion system that is clearly related to Type VI secretion systems (T6SS), because of several conserved elements discussed below (Barker *et al.*, 2009). However, there are also a number of differences with other T6SS, making the FPI-encoded secretion system unique among T6SS. In fact, it has been suggested that the FPI does not encode a T6SS, based on homology searches which failed to identify several ‘signature’ proteins normally found in T6SS (Boyer *et al.*, 2009). Our results shown here suggest that a conserved element of T6SS, the periplasm-spanning IcmF-DotU-lipoprotein complex, is maintained in the FPI-encoded secretion system, providing further evidence that the FPI encodes a T6SS, albeit an unusual one.

The current model of the ‘typical’ T6SS consists of two subassemblies: an inner- and outer-membrane spanning segment and a contractile bacteriophage tail-like structure (Silverman *et al.*, 2012). The membrane-associated subassembly consists of two integral cytoplasmic membrane proteins, IcmF and DotU. DotU interacts with and stabilizes IcmF, which has a long C-terminal portion that is localized within the periplasm (Ma *et al.*, 2009). The C-terminus of IcmF interacts with a lipoprotein localized to the outer membrane, and it has been proposed that this interaction forms a periplasm-spanning channel that facilitates secretion through the T6SS (Zheng & Leung, 2007; Aschtgen *et al.*, 2008; Felisberto-Rodrigues *et al.*, 2011). The results shown here, along with previous published results, suggest this architecture is conserved in the FPI-encoded secretion system (Fig. 7). The FPI proteins PdpB (IcmF) and DotU are integral inner membrane proteins, and DotU is required for the stability of PdpB (de Bruin *et al.*, 2011). We confirmed this here and further localized the PdpB region targeted for degradation in the absence of DotU to the transmembrane region (aa 272–312). We also showed that the FPI protein IglE is a lipoprotein that localizes to the outer membrane. PhoA fusion analyses demonstrated that the PdpB C-terminus is localized within the periplasm, and two-hybrid and *in vivo* co-immunoprecipitation analyses showed that the PdpB C-terminus interacts with IglE. Lack of IglE lipidation attenuates intramacrophage replication and virulence, indicating that IglE must be lipidated and anchored in the outer membrane to function. Thus, these interacting

FPI components are capable of forming a periplasm-spanning channel that is considered a core component of T6SS.

The other subassembly present in T6SS is the contractile bacteriophage tail-like structure. The contractile portion within the cytoplasm is a dynamic structure with a sheath composed of two interacting proteins (VipA and VipB in *V. cholerae*) (Basler *et al.*, 2012) The FPI proteins IglA and IglB are VipA and VipB homologues that have been demonstrated to interact with each other (de Bruin *et al.*, 2007; Broms *et al.*, 2009; Karna *et al.*, 2010). Contraction of the T6SS sheath drives the translocation of a polymer of Hcp loaded with VgrG on its tip across the outer membrane and into either host cells or other bacteria (Pukatzki *et al.*, 2007). The FPI VgrG protein shares homology with other VgrG proteins but is significantly smaller and lacks any associated effector domain normally found in VgrG proteins (Barker *et al.*, 2009). Moreover, no clear homologue of Hcp has been identified within the FPI, although it has been suggested that IglC may serve this function (de Bruin *et al.*, 2011). One of the other differences that distinguishes the FPI secretion system from other T6SS is the lack of any apparent ATPase capability in the ClpV homologue (Barker *et al.*, 2009), which is normally needed for recycling of the VipA/VipB (IglA/IglB) components of the sheath after contraction (Bonemann *et al.*, 2009). Still, evidence has accumulated that the FPI encodes a secretion system (Barker *et al.*, 2009;Broms *et al.*, 2012b) that is related to and shares many characteristics/components with T6SS (de Bruin *et al.*, 2011), and thus, the FPI represents an unusual variant of T6SS, rather than a secretion system distinct from T6SS.

The FPI T6SS is essential for *Francisella* virulence because it facilitates disruption of the phagosomal membrane and subsequent escape of the bacteria into the cytosol of infected host cells (Lindgren *et al.*, 2004). There is currently no evidence that the FPI T6SS is used for antibacterial interactions, unlike other T6SS (MacIntyre *et al.*, 2010). Perhaps, the unique nature of the FPI T6SS evolved due to its very specific function in phagosomal escape. How phagosome escape is mediated by secretion through the FPI T6SS remains unknown; several potential effectors have been identified, including VgrG, IglC, IglI, and PdpA (Barker *et al.*, 2009;Broms *et al.*, 2012b). Interestingly, IglE was also identified as a secreted protein utilizing a cytosolic β-lactamase reporter assay (Broms *et al.*, 2012b); given that IglE is a lipoprotein localized to the outer membrane, either this localization allows direct access to the reporter in the macrophage cytosol, or this lipoprotein is translocated during the secretion process out of the bacterial outer membrane.

During the preparation of this manuscript, another report was published that confirmed the lipidation and outer membrane localization of IglE in *F. tularensis* ssp. *tularensis* and *F. tularensis* ssp. *holarctica* LVS (Robertson *et al.*, 2013). These authors suggested that the C-terminus of IglE interacts with some other unidentified protein; our studies extend these observations by demonstrating that IglE interacts with the C-terminus of PdpB. This IglE (lipoprotein)–PdpB/IcmF interaction provides further evidence of the FPI encoding a T6SS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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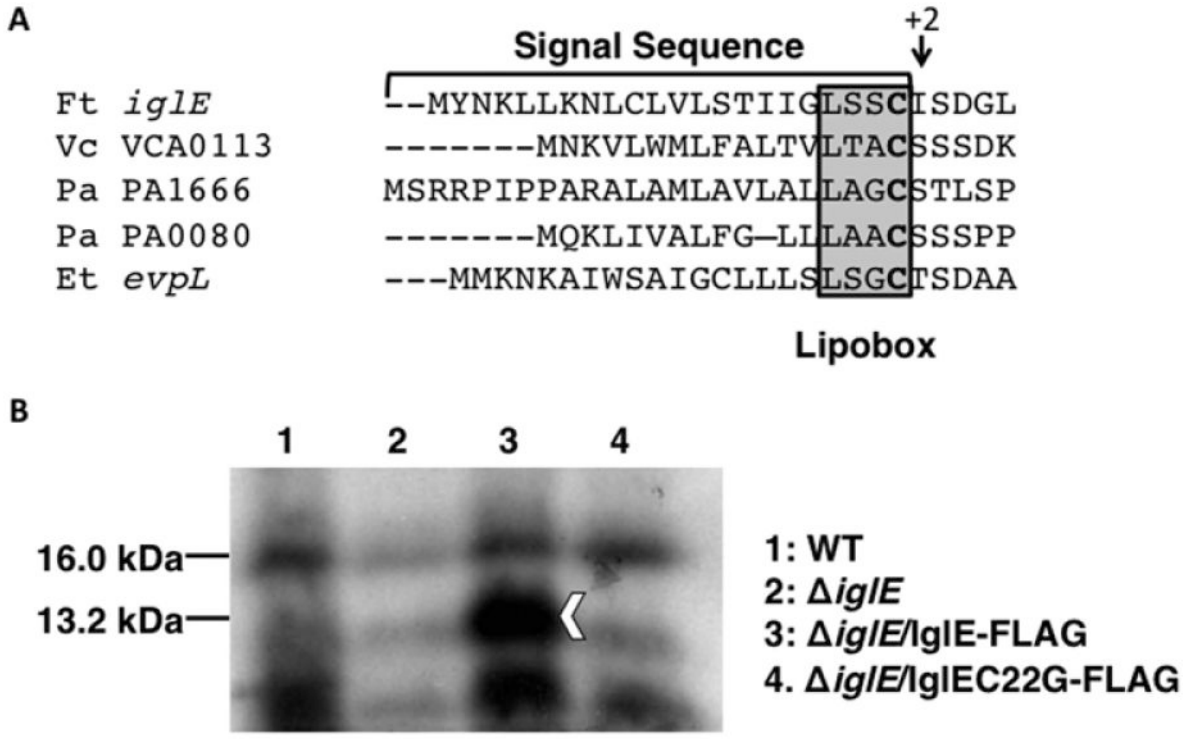

Fig. 1.

IglE is a lipoprotein. (a) N-terminal sequence of IglE and other T6SS lipoproteins, showing putative lipobox, N-terminal Cys (bold), and +2 residues of mature lipoproteins. (b) ^{3}H-palmitate incorporation into IglE-FLAG. *Francisella tularensis* ssp. *novicida* strains U112 (WT; lane 1), KKF194 (Δ*iglE*; lane 2), and KKF194 carrying pKEK1355 (expresses IglE-FLAG; lane 3) and pKEK1368 (expresses IglEC22G-FLAG; lane 4) were grown in the presence of ^{3}H-palmitate for 24 h. Whole-cell lysates were separated by 15% SDS-PAGE and imaged via autoradiography. Arrowhead indicates location of ^{3}H-labeled IglE-FLAG; 16 kD protein indicated was used for normalization. Quantitation and normalization indicated similar background levels of labeled 13.2-kD protein in lanes 2 and 4, 1.4-fold more labeled protein in lane 1, and 2.9-fold more labeled protein in lane 3.

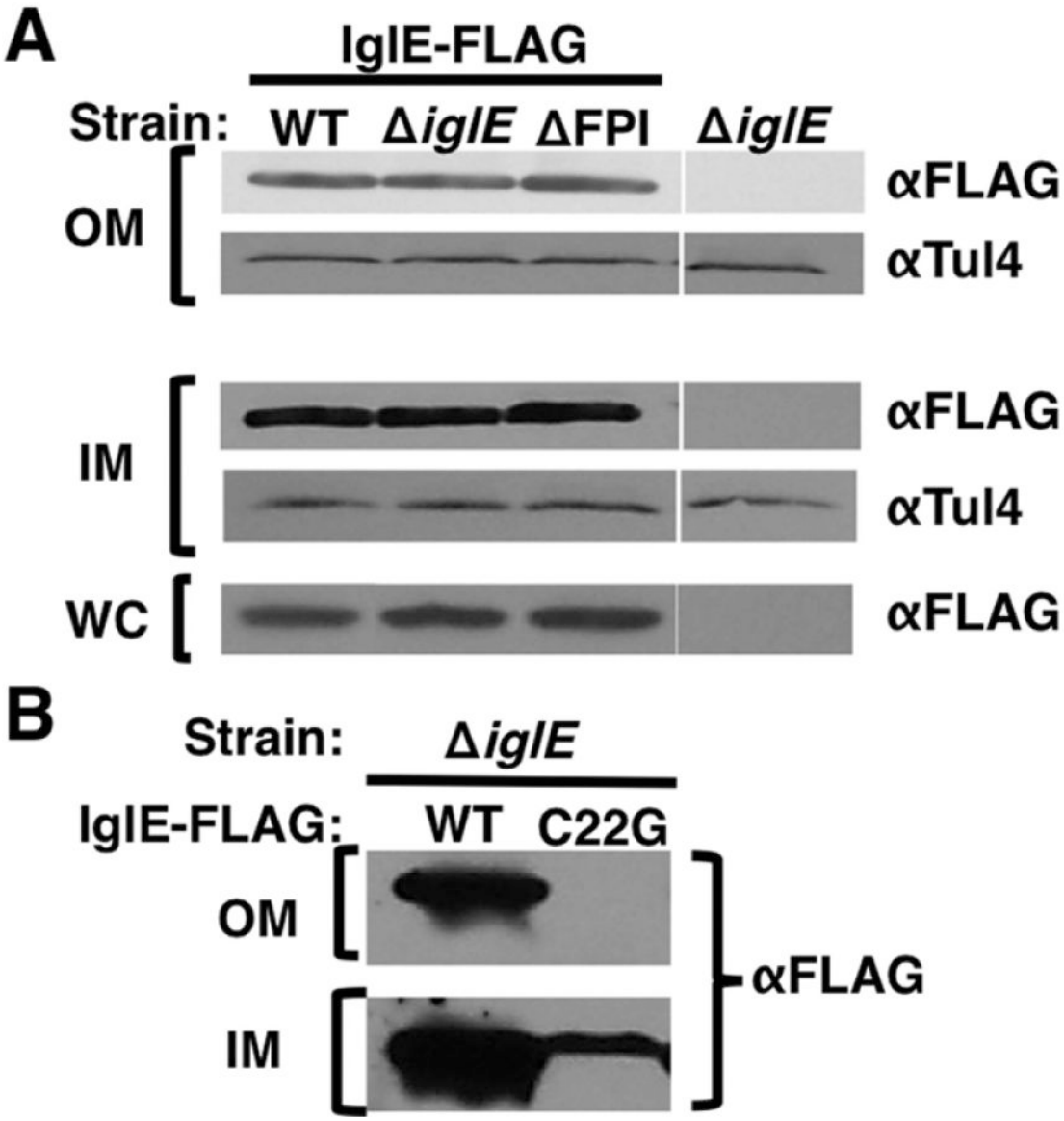

Fig. 2.

IglE localizes to the outer membrane. (a) Fractionation of IglE-FLAG. Bacterial pellets (‘WC’) and enriched inner membrane (‘IM’) and outer membrane (‘OM’) fractions were prepared from *Francisella tularensis* ssp. *novicida* strain U112 (WT), KKF194 (Δ*iglE*), and KKF219 (ΔFPI) carrying pKEK1355 (expresses IglE-FLAG), or without plasmid. Samples were separated by PAGE and subjected to Western immunoblot analysis with anti-FLAG (‘α-FLAG’) and anti-Tul4 (‘α-Tul4’) antibodies. (b) IglEC22G fails to localize to OM. Enriched inner membrane (‘IM’) and outer membrane (‘OM’) fractions were prepared from *F. tularensis* ssp. *novicida* strain KKF194 (Δ*iglE*) carrying pKEK1355 (expresses IglE-FLAG; ‘WT’) or pKEK1368 (expresses IglEC22G-FLAG; ‘C22G’). Samples were separated by PAGE and subjected to Western immunoblot analysis with anti-FLAG (‘α-FLAG’) antibodies. These are representative blots from at least three independent experiments.

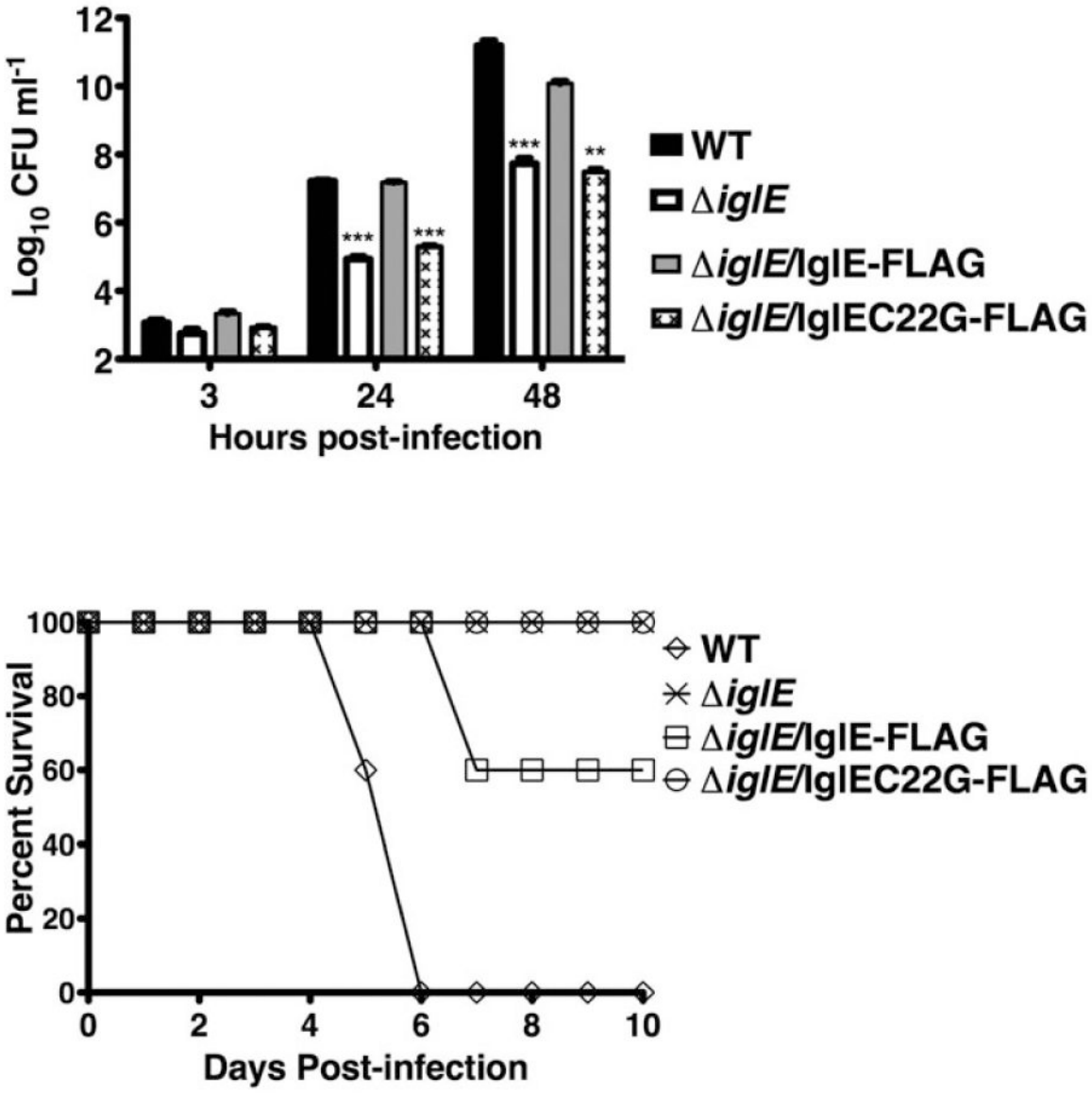

Fig. 3.

iglE Francisella tularensis ssp. *novicida* is defective for intra-macrophage replication. *Francisella tularensis* ssp. *novicida* strains U112 (WT) and KKF194 (Δ*iglE*) either without plasmid or carrying plasmids pKEK1355 (expresses IglE-FLAG) or pKEK1368 (expresses IglEC22G-FLAG) were inoculated at an MOI of *c*. 10 : 1 into J774 cells, and intracellular bacteria were enumerated at 3, 24, and 48 h. The assay was performed in triplicate. $^{**}P < 0.01$;$^{***}P < 0.001$.

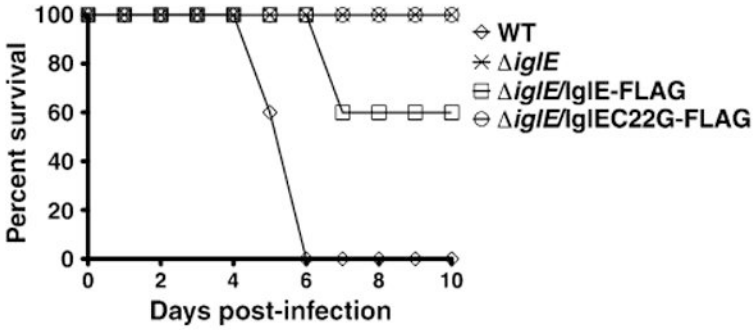

Fig. 4.

iglE Francisella tularensis ssp. *novicida* is attenuated for virulence in mice. *Francisella tularensis* ssp. *novicida* strains U112 (WT) or KKF194 (Δ*iglE*) either without plasmid or carrying plasmids pKEK1355 (expresses IglE-FLAG) or pKEK1368 (expresses IglEC22G-FLAG) were inoculated intranasally into groups of 5 female BALB/C mice and survival monitored. Inocula used were WT (120 CFU), Δ*iglE* (1.1×10^5 CFU), Δ*iglE*/IglE-FLAG ($1.2\ \Delta\ 10^5$ CFU), and Δ*iglE*/IglEC22G-FLAG (1.2×10^5 CFU).

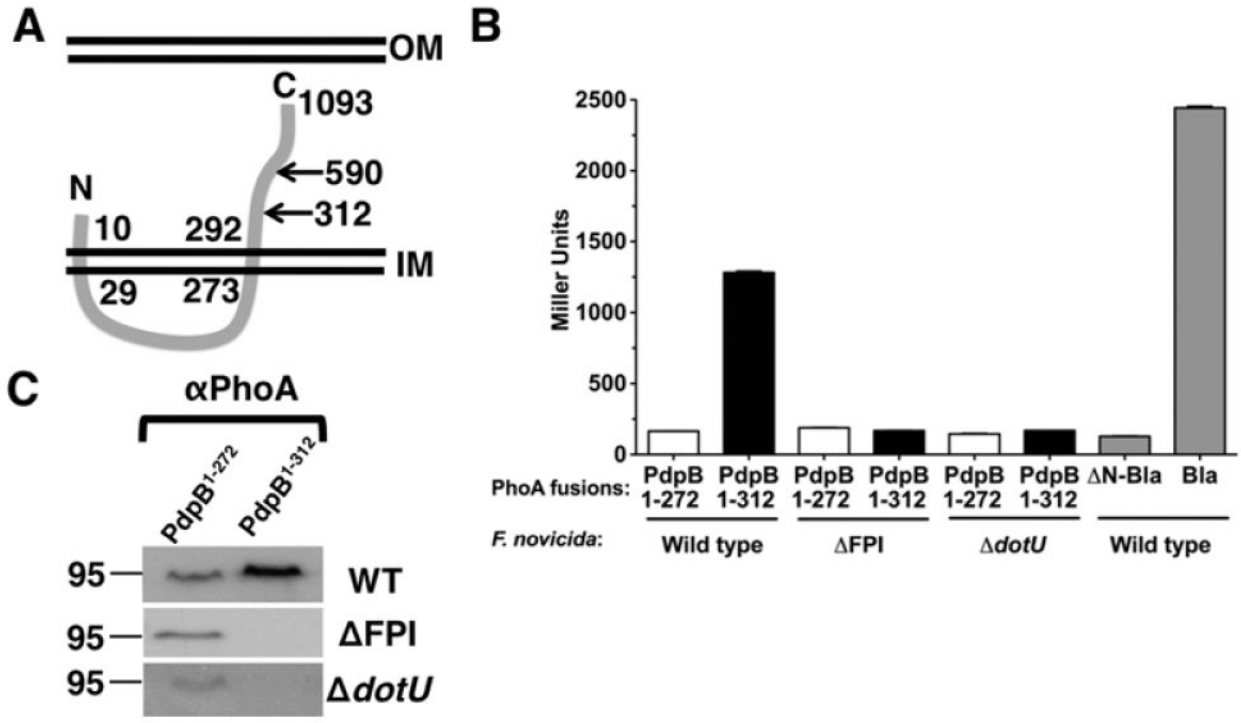

Fig. 5.

The PdpB C-terminus is localized to the periplasm. (a) The putative transmembrane domains of PdpB as predicted by TMHMM (aa 10–29 and 273–292). The locations of PhoA fusions and the C-terminal fragment used in two-hybrid analyses are indicated. (b) *Francisella tularensis* ssp. *novicida* strains U112 (wild type), KKF219 (DFPI), and KKF177 (Δ*dotU*) were transformed with pKEK1764 (expresses $PdpB^{1–272}$-PhoA), pKEK1765 (expresses $PdpB^{1–312}$-PhoA), pKEK1763 (expresses ΔN-Bla-PhoA), and/or pKEK1762 (expresses Bla-PhoA) and assayed for alkaline phosphatase activity. The assay was performed in triplicate. C. Whole-cell lysates from strains used in (b) were separated by 10% SDS-PAGE and subjected to Western immunoblot analysis with anti-PhoA (‘α-PhoA’) antibodies.

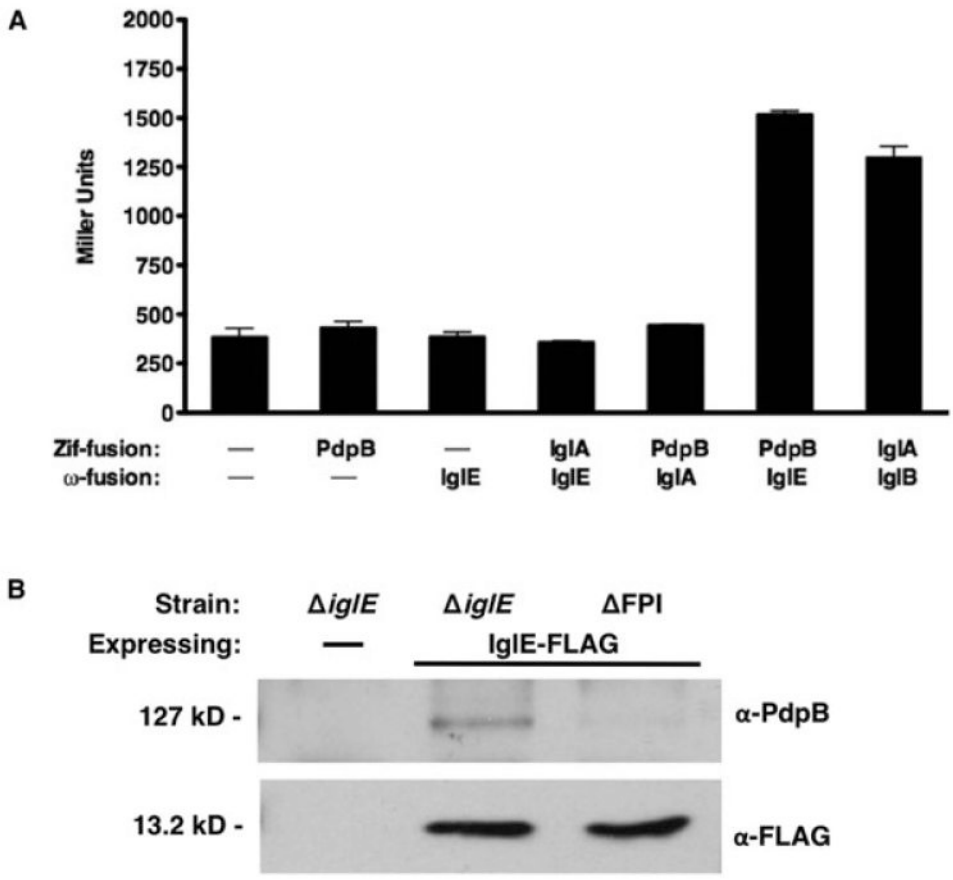

Fig. 6.

IglE interacts with the PdpB C-terminus. (a) *Escherichia coli* reporter strain KDZif1ΔZ was transformed with either empty vectors pACTR-AP-Zif and/or pBRGPω (Karna *et al.*, 2010), plasmids pKEK1613 (PdpB$^{590–1093}$-Zif), pKEK1254 (IglE$^{23–125}$-ω), pKEK1416 (IglA-Zif), and pKEK1415 (IglB-ω), and assayed for beta-galactosidase activity. All values are result of triplicate samples, $^{**}P < 0.01$. (b) Whole-cell lysates from *Francisella tularensis* ssp. *novicida* strain KKF194 (Δ*iglE*) or KKF219 (Δ*FPI*) either with no plasmid ('–') or carrying pKEK1355 (expresses IglE-FLAG) were subjected to immunoprecipitation as described in Materials & Methods with α-FLAG. Samples were separated by PAGE and subjected to Western immunoblot analysis with anti-PdpB or anti-FLAG antibodies.

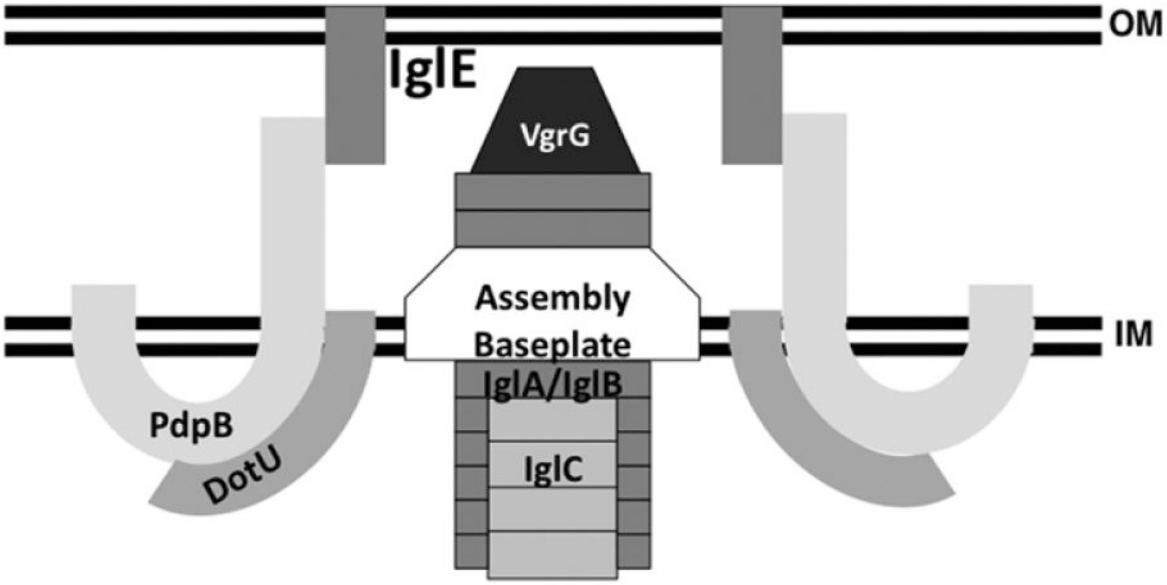

Fig. 7.
Model of the T6SS encoded by the *Francisella tularensis* FPI.