Characterization of the *Francisella tularensis* subsp. *novicida* type IV pilus

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Francisella tularensis causes the disease tularemia. Type IV pili (Tfp) genes are present in the genomes of all *F. tularensis* subspecies. We show that the wild-type *F. tularensis* subsp. *novicida* expresses pilus fibres on its surface, and mutations in the Tfp genes pilF and pilT disrupt pilus biogenesis. Mutations in other Tfp genes (pilQ and pilG) do not eliminate pilus expression. A mutation in pilE4 eliminates pilus expression, whereas mutations in the other pilin subunits pilE1–3 and pilE5 do not, suggesting that pilE4 is the major pilus structural subunit. The virulence regulator MglA is required for pilus expression, and it regulates the transcription of a putative Tfp glycosylation gene (FTN0431). However, MglA does not regulate transcription of pilF, pilT or pilE4, and a strain lacking FTN0431 still expresses pili; thus, it is unclear how MglA regulates pilus expression. Only pilF was also required for protein secretion, while pilE4 and pilT were not, indicating that there is very little overlap of the protein secretion/Tfp functions of the pil genes. The protein secretion component pilE1 was more important for *in vitro* intramacrophage growth and mouse virulence than the Tfp component pilE4. Our results provide the first genetic characterization of the novel Tfp system of *F. tularensis*.

INTRODUCTION

*Francisella tularensis* is a highly infectious bacterium that causes tularemia. *F. tularensis* is found in many different animal hosts and can be transmitted by arthropod vectors (Ellis et al., 2002). Humans can acquire the bacteria by a number of different routes, but inhalation of low doses of the organism can lead to a serious pneumonic form of disease that has a high mortality rate, and this has led to the classification of this bacterium as a category A bioterrorism agent (Dennis et al., 2001). *F. tularensis* is further divided into different subspecies (Oyston et al., 2004). *F. tularensis* subsp. *tularensis* has historically been associated with bioweapons development, and is reported to have the highest virulence for humans (McLendon et al., 2006). *F. tularensis* subsp. *novicida* is also infectious in humans but typically with a less severe outcome (Sjostedt, 2003). An attenuated 'live vaccine strain' (LVS) was derived by repeated passage of subsp. *holarctica* in the laboratory; despite the unclear nature of the attenuating mutation(s) in this strain, it is frequently used as a laboratory model for tularemia (Anthony & Kongsbavn, 1987; Eigelsbach et al., 1951). *F. tularensis* subsp. *novicida* has low virulence for humans, but maintains high virulence in mice (Kieffer et al., 2003; Lauriano et al., 2004). Whole-genome sequencing has revealed that all three subspecies are closely related, with the less virulent (for humans) subsp. *novicida* showing less genomic decay than the more virulent subsp. *holarctica* and subsp. *tularensis* (Larsson et al., 2005; Petrosino et al., 2006; Rohmer et al., 2006).

The ability of *F. tularensis* to survive and replicate within macrophages has been linked to its virulence (Anthony et al., 1991a), and inactivation of genes necessary for this ability leads to attenuation in mice (Abd et al., 2003; Baron & Nano, 1998; Golovilov et al., 2003a, b; Gray et al., 2002; Lauriano et al., 2004). The global regulator MglA controls transcription of a cluster of genes within the *Francisella* pathogenicity island (FPI) that are required for intramacrophage survival and growth (Brotcke et al., 2006; Lauriano et al., 2004; Nano et al., 2004). It is still not clear how the FPI genes contribute to intracellular...
replication. Additional factors contributing to virulence include LPS and capsule (Cowley et al., 2000; Su et al., 2007; Thomas et al., 2007), as well as a number of other genes identified through genetic screens (Maier et al., 2007; Qin & Mann, 2006; Tempel et al., 2006; Weiss et al., 2007).

The genomes of the three F. tularensis subspecies contain homologues of type IV pilus biogenesis genes, and fibres resembling type IV pili (Tfp) have been visualized on the surface of the LVS (Gil et al., 2004). The nomenclature originally proposed for F. tularensis Tfp components (Gil et al., 2004), which corresponds to that used for Neisseria spp., will be utilized here. Five potential pilin subunit genes are present (pilE1–5), as well as genes predicted to encode the outer membrane secretin PilQ, the PilF assembly ATPase, and PilG inner-membrane protein, all of which are normally required for Tfp biogenesis (Larsson et al., 2005). Also present is a gene predicted to encode PilT, an ATPase normally required for pilus retraction and twitching motility, but not pilus assembly (Burrows, 2005). F. tularensis subsp. holarctica deleted for the pilE1 (pilA) gene is severely attenuated for virulence in mice when administered by the subcutaneous route, but only slightly defective for intramacrophage replication (Forslund et al., 2006). A deletion in pilE1 is also present in the LVS, and the authors of that study suggested that the LVS pilE1 mutation may be responsible, at least in part, for the attenuation of LVS. However, the authors were unable to detect pilus fibres on the surface of either the wild-type subsp. holarctica or the pilE1 mutant strains, so the reason for the attenuated virulence was unclear at the time that these observations were reported.

Studies of F. tularensis subsp. novicida have subsequently uncovered a role for pilE1 in protein secretion (Hager et al., 2006) and shown that secretion of a number of proteins is dependent upon some of the Tfp genes, including pilE1 (pilA), pilF, pilG and pilQ, while pilE2, pilE3 and pilT are not required for secretion. One of the secreted proteins, PepO, is a metalloprotease, and mutations in pepO or pilG (required for its secretion) result in increased bacterial spread to systemic sites following intradermal inoculation of mice. The authors propose that the Tfp gene products are required for a type II-like protein secretion system, and that secretion of PepO promotes vasoconstriction, which limits bacterial spread. Interestingly, PepO is not expressed in the human-virulent subsp. tularensis and subsp. holarctica, suggesting that the loss of this protein may have contributed to the evolution of these pathogens.

In the present study, we show that F. tularensis subsp. novicida expresses surface fibres that resemble Tfp, and that expression of these fibres requires pilF, pilT and pilE4. In contrast, mutations in pilG, pilQ, pilE1–3 and pilE5 do not prevent pilus expression. We also show that MglA regulates pilus expression, but not through regulation of pilF, pilT or pilE4 transcription. Examination of protein secretion suggested that PilF is the only component essential for both the Tfp biogenesis and type II-like protein secretion systems. Intramacrophage replication and mouse virulence were attenuated in a strain lacking protein secretion (pilE1). In contrast, a strain lacking Tfp (pilE4) had no defect in intramacrophage replication and displayed only a slight attenuation of virulence in mice. Our results illuminate the novel F. tularensis Tfp/protein secretion system, and suggest that the protein secretion system may contribute more to virulence than the Tfp biogenesis system in a mammalian host.

### METHODS

**Bacterial strains, plasmids and growth conditions.** Escherichia coli strain DH5α (Hanahan, 1983) was used for cloning manipulations. F. tularensis subsp. novicida strains used in this study are listed in Table 1. F. tularensis subsp. novicida strains were grown on TSAP medium [tryptic soybean agar powder (40 g l⁻¹) with 0.1% cysteine, 25 mg ferrous sulfate ml⁻¹, 25 mg sodium pyruvate ml⁻¹ and 25 mg sodium metasulfite ml⁻¹], or Chamberlain’s defined medium (CDM; Chamberlain, 1965). Antibiotics were used at the following concentrations: kanamycin, 50 μg ml⁻¹; tetracycline, 10 μg ml⁻¹; ampicillin, 100 μg ml⁻¹; erythromycin, 150 μg ml⁻¹. F. tularensis subsp. novicida mutants were constructed by methods described previously (Liu et al., 2007), utilizing the primers listed in Supplementary Table S1. The pilE4::T20(EZTnKan) and pilE5::T18(EZTnKan) F. tularensis subsp. novicida strains tnf1_pw06018p02q155 and tnf1_pw060510p03q137

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<th>Strain</th>
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<tr>
<td>U112</td>
<td>Wild-type</td>
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<tr>
<td>KKF34</td>
<td>U112 ΔmglA::ermC</td>
<td>Anthony et al. (1991b)</td>
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<td>KKF252</td>
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<td>KKF253</td>
<td>U112 Δ(pilE1–3)::Kan</td>
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<td>KKF255</td>
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<td>tnf1_pw060510p03q137</td>
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Plasmid construction. PCR primers are listed in Supplementary Table S1. Plasmid pKK214 (Kuoppa et al., 2001) was PCR-amplified with primers pKK214-EcoRI and pKK214-EcoNotI, followed by digestion with EcoRI and religation, which resulted in pKEK648, which lacks CmR. Then the FNT1451 promoter (Gallagher et al., 2007) was digested from pKEK886 (Liu et al., 2007) with EcoRI and NotI, and ligated into pKEK648 digested similarly, resulting in pKEK894. Finally, the pilE1 and pilE4 genes were PCR-amplified from U112 genomic DNA with primers pilE1-NcoI and pilE4-EcoRI, and pilE4-NcoI and pilE4-EcoRI, respectively. The PCR fragments were digested with NcoI and EcoRI, and ligated into pKEK894 digested similarly, to form pKEK1176 (p pilE1) and pKEK1150 (p pilE4).

Transmission electron microscopy. F. tularensis subsp. novicida strains were grown in CDM at 37 °C in 5% CO2 with shaking at 100 r.p.m. for 16 h. Bacteria were washed once with PBS, adsorbed onto polyvinyl formal-carbon-coated grids (Ernest F. Fullam) for 2 min, and fixed with 1% glutaraldehyde (Sigma-Aldrich) for 1 min. The grids were washed twice with PBS and twice with water and then negatively stained with 0.5% phosphotungstic acid (Ted Pella) for 35 s. The grids were viewed in a transmission electron microscope (FEI TECNAI 12 BioTwin G02) at 80 kV accelerating voltage, and images were taken using an AMT XR-60 CCD digital camera system.

RNA isolation and RT-PCR. Using the ChargeSwitch Total RNA Cell kit (Invitrogen), total RNA was isolated from mid-exponential phase (OD600 0.5–0.6) F. tularensis subsp. novicida cultures. The isolated RNA was treated with DNase I (Turbo DNA-free kit, Ambion) and reverse-transcribed using random primers (Invitrogen) to produce cDNA. PCR was then performed using Sybr-Green PCR-Master Mix (Applied Biosystems). Gene-specific primers (see Supplementary Table S1) were designed to amplify 150–200 bp fragments of each gene.

Protein preparation. Culture supernatants of F. tularensis subsp. novicida strains were prepared according to a protocol described elsewhere (Hager et al., 2006). Protein samples corresponding to equivalent volumes of supernatant were separated by 12% SDS-PAGE and visualized by silver staining (Bio-Rad).

**RESULTS**

**F. tularensis subsp. novicida expresses Tfp**

Transmission electron microscopy was used to visualize the surface of the wild-type F. tularensis subsp. novicida U112 strain. Fibres resembling Tfp could be detected on approximately 70% of cells (Fig. 1); these fibres resembled those previously seen in subsp. holarctica LVS (Gil et al., 2004). As mentioned above, the F. tularensis subsp. novicida genome contains genes with homology to Tfp biogenesis genes (Rohmer et al., 2006). Tfp are predicted to be assembled by a complex that includes the cytoplasmic ATPase PilT, the inner-membrane protein PilG, and the outer membrane secretin PilQ (Burrows, 2005). Additionally, although not required for assembly, the ATPase PilT is typically required for pilus retraction, which contributes to twitching motility. We constructed F. tularensis subsp. novicida strains containing mutations in the Tfp genes pilF, pilG, pilQ and pilT, and visualized these strains by transmission electron microscopy.

**Intromacrophage growth assay.** F. tularensis subsp. novicida strains were used to infect the J774.1 macrophage cell line (ATCC) at an m.o.i. of ~10:1. Wells were seeded with ~10^5 J774 cells, and were infected with ~10^7 bacteria. After 1 h incubation at 37 °C and 5% CO2, gentamicin (50mg ml^{-1}) was added to the medium to eliminate extracellular organisms. The macrophage cells were lysed with 0.2% deoxycholate at 1 and 24 h post-infection, and the lysate was plated on TSAP and incubated at 37 °C, and c.f.u. were enumerated.

**Mouse virulence assays.** Groups of five female 4–6-week-old BALB/cAN(Hsd) mice (Harlan Sprague) were inoculated with a given dose of F. tularensis subsp. novicida strains delivered intranasally in 10 μl PBS, or intradermally in 100 μl. The bacterial infection doses were determined by plate counts of the inocula. Mice were monitored for 30 days post-inoculation. All experiments were approved by the institutional animal care and use committee, and performed according to federal guidelines.
The *F. tularensis* subsp. *novicida* strains containing mutations in *pilF* or *pilT* had no detectable pilus fibres on their surface (Fig. 1), indicating that PilF and PilT are required for pilus biogenesis in *F. tularensis*. In contrast, the *pilQ* and *pilG* strains expressed detectable pilus fibres. The *pilQ* mutant expressed reduced numbers of pili on its surface (~30% wild-type levels), indicating that PilQ contributes to, but is not essential for, Tfp expression. Western immunoblotting utilizing rabbit antisera against PilQ (D. Thanassi, unpublished data) confirmed that PilQ was absent from the *pilQ* mutant (data not shown). The *pilG* mutant expressed approximately wild-type levels of surface fibres, suggesting that PilG has little involvement in Tfp biogenesis.

**Evidence that PilE4 is the major pilus subunit**

The *F. tularensis* subsp. *novicida* genome contains at least five Tfp pilin subunit genes (*pilE1–5*). To determine which of these genes is the major pilin subunit, we initially constructed *F. tularensis* subsp. *novicida* strains containing mutations either in *pilE1* or in *pilE1–3* (these three genes lie adjacent to each other in the genome). The *pilE1* and *pilE1–3* mutant strains still expressed surface fibres, as determined by transmission electron microscopy (Fig. 2), demonstrating that none of PilE1, PilE2 or PilE3 is the major subunit of the pilus. The *pilE1* mutant expressed ~90% of the wild-type levels of surface fibres, while the *pilE1–3* mutant expressed only ~10% of the wild-type levels of surface fibres, suggesting that PilE2 and/or PilE3 represent minor pilus subunits that are important, but not essential, for pilus assembly. We then visualized *F. tularensis* subsp. *novicida* strains with transposon insertions in *pilE2*, *pilE3*, *pilE4* and *pilE5* (a kind gift of Professor C. Manoil, University of Washington) (Gallagher et al., 2007), and found that pili could be detected on the *pilE2*, *pilE3* and *pilE5* strains (Fig. 2; data not shown). However, no detectable fibres were found on the surface of the *pilE4* mutant (Fig. 2), suggesting that PilE4 is the major subunit of the Tfp. The *pilE4* mutant strain was complemented with a plasmid expressing *pilE4* and visualized by transmission electron microscopy. This complemented strain expressed near wild-type levels (~75%) of pili on its surface (Fig. 2). The *pilE3* and *pilE5* mutant strains expressed only ~10% of wild-type levels of surface fibres, indicating that PilE3 and PilE5 might represent minor pilin components, while the *pilE2* mutant expressed approximately wild-type levels of surface fibres.

**PilE4 varies between low virulence (novicida, LVS) and high virulence (tularensis, holarctica) strains**

Pili have previously been detected on the surface of the LVS (Gil et al., 2004), but there have been no reports of pili on the surface of wild-type subsp. *tularensis* or subsp. *holarctica* strains. An alignment of the predicted PilE4 coding sequences from subsp. *novicida*, subsp. *holarctica*, subsp. *holarctica* LVS, and subsp. *tularensis* revealed differences in the encoded subunits (Fig. 3). Specifically, the subsp. *novicida* gene encodes a large protein of 316 aa, while the subsp. *tularensis* and subsp. *holarctica* genes have identical deletions that cause a frameshift downstream of the deletion that leads to predicted truncated proteins of only 197 and 211 aa, respectively. Interestingly, the LVS gene has experienced a second deletion that places the coding sequence downstream of the first deletion back in frame, leading to a protein of 301 aa (the nucleotide alignment of *pilE4* genes is included in Supplementary Fig. S1). Notably, the predicted LVS PilE4 protein shares homology to the C terminus of *novicida* PilE4, and this homology is lacking from the *holarctica* and *tularensis* PilE4 proteins. Thus, PilE4 can be found in a longer form in low-virulence (*novicida*, LVS) strains, and a shorter truncated form in high-virulence (*tularensis*, *holarctica*) strains.

**MglA regulates Tfp expression**

MglA is a global regulator of gene expression that is required for *F. tularensis* virulence (Baron & Nano, 1998). MglA positively regulates the transcription of the FPI genes, which are necessary for intramacrophage growth, as well as that of a number of other genes (Baron & Nano, 1998; Brotcke et al., 2006; Lauriano et al., 2004; Nano et al., 2004). The *F. tularensis* subsp. *novicida* mglA strain KKF34

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**Fig. 2.** Electron micrographs of *F. tularensis* subsp. *novicida* strains. Transmission electron micrographs of *F. tularensis* subsp. *novicida* strains KKF253 (*pilE1–3*), tnfn1 _pw060510p03q137 (*pilE5*), tnfn1 _pw060418p02q155 (*pilE4*) and the *pilE4* strain transformed with plasmid pKEK1150 (*pilE4*). Bars, 500 nm.
was examined by transmission electron microscopy, which revealed that the cells expressed no detectable Tfp (Fig. 1). To determine whether any of the Tfp genes were positively regulated by MglA, RT-PCR was performed on the wild-type and mglA strains grown under identical conditions. No differences in transcript levels between the wild-type and mglA strains could be found for the pilF, pilG, pilQ, pilR, pilE1, pilE2, pilE3, pilE4 or pilE5 genes (Fig. 4; data not shown). We looked at transcript levels for two additional Tfp genes, pilO and pilP, predicted to encode proteins necessary for pilus expression, but these transcripts were also not regulated by MglA (Fig. 4).

Microarray analyses on mglA mutants of F. tularensis subsp. novicida and F. tularensis subsp. holarctica LVS have revealed a number of genes positively regulated by MglA (Brotcke et al., 2006; Charity et al., 2007). The only MglA-regulated gene identified in these studies that is annotated to potentially play a role in Tfp biogenesis corresponds to FTN_0431 (FTT0905 in Schu S4, FTL0425 in LVS). The encoded protein shares homology with the Tfp glycosylation protein PilO from Pseudomonas aeruginosa (Smedley et al., 2005). Transcript levels for FTN_0431 were lower in the mglA mutant than in the wild-type strain, confirming that MglA regulates FTN_0431 transcription (Fig. 4). RT-PCR controls included iglC, which is known to be regulated by MglA (Lauriano et al., 2003) and rpoB, which is not regulated by MglA.

Since FTN_0431 is regulated by MglA and shares homology with a Tfp glycosylation gene, we constructed an F. tularensis subsp. novicida FTN_0431 mutant, and examined it by transmission electron microscopy. The FTN_0431 mutant strain expressed pili on its surface that were similar to those of the wild-type strain (data not shown), demonstrating that this gene is not essential for Tfp expression. It remains unclear exactly how MglA regulates pilus expression.

Fig. 3. Alignment of PilE4 from different F. tularensis subspecies. PilE4 sequences from F. tularensis subsp. novicida strain U112 (accession no. YP_898046), F. tularensis subsp. holarctica strain LVS (accession no. CAJ78799), F. tularensis subsp. tularensis strain Schu S4 (accession no. YP_169863) and F. tularensis subsp. holarctica strain OSU18 (accession no. YP_762996) were aligned utilizing MULTALIN (Corpet, 1988).

Fig. 4. RT-PCR analysis of F. tularensis subsp. novicida Tfp genes. RT-PCR analysis (see Methods) was performed on total RNA prepared from F. tularensis subsp. novicida strains U112 (wild-type; WT) and KKF34 (mglA). PCR primers (see Supplementary Table S1) were specific to internal coding sequences of FTN_0415 (pilE1), FTN_0389 (pilE4), FTN_1115 (pilF), FTN_1137 (pilQ), FTN_1116 (pilG), FTN_1622 (pilT), FTN_1139 (pilO), FTN_1138 (pilP), FTN_0431, FTN_1568 (rpoB) and FTN_1322 (iglC).
**Tfp/protein secretion functional overlap**

The Tfp genes in *F. tularensis* subsp. *novicida* have been characterized elsewhere to function as a type II-like secretion system for proteins (Hager et al., 2006). The results of those authors showed that the pilE1 (*pilA*), pilF (*pilB*), pilG (*pilC*) and pilQ genes were required for secretion of several different proteins, including PepO, whereas the pilT, pilE2 and pilE3 genes were not required for protein secretion. The cell-free culture supernatants of the *F. tularensis* subsp. *novicida* Tfp gene mutants created for this report were examined for protein content in a similar manner.

The culture supernatant of the wild-type strain closely matched that reported by Hager et al. (2006), with the major secreted proteins ChiA, PepO, CbpA and Fsp53 visible within this fraction (Fig. 5). Additionally, the mglA mutant was specifically defective for secretion of PepO, as reported previously. We confirmed that the pilF, pilG, pilQ and pilE1 mutant strains that we constructed behaved similarly to those characterized earlier, and did not secrete ChiA, PepO, CbpA and Fsp53 (Fig. 5; Hager et al., 2006). We extended the earlier findings and showed that complementation of the pilE1 mutant with pilE1 expressed from a plasmid restores secretion of ChiA, PepO, CbpA and Fsp53. Finally, secretion of ChiA, PepO, CbpA and Fsp53 in the pilE4 and FTN_0431 mutant strains was not affected, demonstrating that these genes are not involved in Tfp-related protein secretion. Our results demonstrate there is very little overlap between the Tfp components required for protein secretion and those required for the Tfp biogenesis system, with PilF representing the only factor of those tested that is apparently required for both.

**Protein secretion, but not Tfp biogenesis, contributes to intramacrophage growth and virulence**

The pilE4 subunit is required for Tfp biogenesis but not protein secretion (shown above), while the pilE1 gene is required for protein secretion (Hager et al., 2006) but plays little role in Tfp biogenesis. We therefore determined the relative roles of protein secretion and Tfp biogenesis in intramacrophage survival and growth by infecting J774 macrophages with the *F. tularensis* subsp. *novicida* pilE1 and pilE4 mutants (Fig. 6). After 24 h, the pilE1 mutant was recovered at lower numbers than the wild-type strain (~100-fold reduction, Fig. 6a) and this defect was seen in multiple experiments. In contrast, the pilE4 mutant entered and grew within macrophages in a manner similar to that of the wild-type strain (Fig. 6b). The pilE1 mutant could be complemented back to wild-type levels of macrophage entry and growth by providing pilE1 in trans on a plasmid (Fig. 6a). These results are consistent with the reported attenuation in intramacrophage growth seen with an *F. tularensis* subsp. *holarctica* pilE1 mutant (Forsslund et al., 2006). These results demonstrate that PilE1 and protein secretion, but not Tfp biogenesis, contribute to intramacrophage growth in *vivo*.

The pilE1 and pilE4 mutants were assessed for virulence by intranasal inoculation into BALB/c mice at a low dose (~10^2 c.f.u.). Inoculation with the pilE1 mutant at this dose led to lower mortality than inoculation with the pilE4 mutant, suggesting that PilE1 and protein secretion contribute to virulence in mice by this route (Fig. 7; an additional experiment is illustrated in Supplementary Fig. S2). The pilE1 and pilE4 mutants were also assessed for virulence by intradermal inoculation into BALB/c mice. At a dose of 10^6 c.f.u., both the pilE1 and pilE4 mutants appeared attenuated compared with the wild-type strain (5/5 and 3/5 survivors versus 1/5 survivors, respectively), whereas at a higher dose of 10^7 c.f.u., only the pilE1 mutant was attenuated compared with the wild-type strain (4/5 survivors versus 0/5 survivors, respectively). Thus, by both intranasal and intradermal routes, Tfp-related protein secretion plays a greater role than Tfp pilus biogenesis in *F. tularensis* subsp. *novicida* virulence.

**DISCUSSION**

**Tfp expression in *F. tularensis* subsp. *novicida***

*F. tularensis* is a potentially dangerous pathogen for humans, and yet very little is known about how it causes intramacrophage growth and intradermal virulence. Our results demonstrate that PilE1 and protein secretion, but not Tfp biogenesis, contribute to intramacrophage growth in *vivo*.

**Fig. 5.** Culture supernatant proteins of *F. tularensis* subsp. *novicida* Tfp mutants. Culture supernatant proteins of *F. tularensis* subsp. *novicida* strains U112 (wild-type; WT), KKF34 (mglA), KKF73 (pilQ), KKF74 (pilT), KKF104 (pilG), KKF255 (pilF), KKF315 (FTN_0431), KKF252 (pilE1), KKF252 transformed with pKEK1176 (pilE1+ppilE1) and tfn1_pw060418p02q155 (pilE4) were separated by 12% SDS-PAGE and visualized by silver staining. One representative from three experiments is shown. On the left are mobilities of Tfp secretion-dependent proteins ChiA, PepO, CbpA and Fsp53. Mobilities of molecular mass markers, in kDa, are given on the right.
disease. Tfp are associated with the virulence of a number of pathogens (Fullner & Mekalanos, 1999; Mattick, 2002; Taylor et al., 1987; Tonjum & Koomey, 1997), and all the sequenced genomes of \( F. \) tularensis subsp. contain Tfp genes. In the current study, we present evidence that \( F. \) tularensis subsp. \( novicida \) expresses Tfp on its surface. However, some of the Tfp genes appear to not be essential for Tfp biogenesis, but rather are required for protein secretion. Virulence assays suggest that protein secretion plays a more important role than Tfp biogenesis in \( F. \) tularensis subsp. \( novicida \) virulence. Tfp biogenesis typically requires a core set of proteins, including a cytoplasmic ATPase, PilF, an inner-membrane component, PilG, and an outer-membrane secretin, PilQ (Averhoff & Friedrich, 2003; Burrows, 2005; Mattick, 2002). We showed that while PilF is required for \( F. \) tularensis subsp. \( novicida \) Tfp biogenesis, PilG and PilQ are not essential for this process. In \( Neisseria \) meningitidis, the ATPase activity of PilF is necessary to drive polymerization of the pilin subunits within the periplasm. PilF associates with PilG in a complex at the inner membrane, and the growing polymerized pilin subunits are extruded across the outer membrane through the central cavity in PilQ (Balasingham et al., 2007; Carbonnelle et al., 2006; Collins et al., 2001, 2005; Wall et al., 1999). It is not clear how the Tfp are assembled and cross the outer membrane of \( F. \) tularensis in the absence of PilG and PilQ; no additional homologues of these proteins were found by a BLAST search. A \( pilF \) mutant of \( F. \) tularensis subsp. \( holarctica \) LVS also lacks Tfp, demonstrating the conserved nature of this factor in \( F. \) tularensis pilus biogenesis (Chakraborty et al., 2008).

We also showed that the putative ATPase PilT is required for pilus biogenesis, despite the fact that PilT is normally involved in pilus retraction and twitching motility, but not pilus biogenesis (Lauer et al., 1993; Nunn et al., 1990; Whitchurch et al., 1991). In fact, a \( P. \) aeruginosa \( pilT \) mutant strain is typically hyperpiliated, due to the inability of this strain to retract pili (Lauer et al., 1993). PilT is believed to function by disassembling the Tfp filament into pilin subunits (Burrows, 2005). The predicted PilT proteins are highly homologous between subsp. \( novicida \) and subsp. \( tularensis \) (99% identity over 342 aa), but in subsp. \( holarctica \) the \( pilT \) gene contains a point mutation at nucleotide 358 that introduces a stop codon, predicted to result in a truncated protein of 119 aa, with a possible second protein of 202 aa encoded past this stop codon. This second ORF contains the primary homology to PilT;
However, an insertion into the first ORF in *F. tularensis* subsp. *holarctica* LVS prevents Tfp expression, suggesting either that the truncated protein retains function, or that LVS has some mechanism to produce full-length PilT from the pilT pseudogene (Chakraborty et al., 2008). The requirement for PilT in both subsp. *novicida* and LVS suggests that their Tfp biogenesis systems are similar, if not identical.

Although pilG and pilQ are normally required for Tfp biogenesis, recent studies in *Neisseria* spp. (Carbonnelle et al., 2006; Winther-Larsen et al., 2005; Wolfgang et al., 2000) have re-evaluated the requirement for the various Pil components in Tfp biogenesis. These studies have shown that in the absence of PilT, some of the ‘core’ components of pilus biogenesis, including PilG and PilQ, are no longer required for Tfp synthesis. A revised model has emerged in which some of the components (e.g. PilG) actually function to antagonize the depolymerization activity of PilT, and in the absence of PilT these components are not required for Tfp biogenesis. Thus the phenotypes of the *F. tularensis* subsp. *novicida* pilG and pilQ mutants are reminiscent of *Neisseria* spp. pilT pilG and pilT pilQ mutants. This suggests that the wild-type *F. tularensis* subsp. *novicida* may be phenotypically similar to a pilT mutant *Neisseria* strain, and that the protein annotated as PilT may represent some other essential Tfp component functionally distinct from the Tfp retraction protein PilT.

**Evidence that PilE4 is the major subunit of the Tfp**

Evidence presented here suggests that the PilE4 pilin is the major subunit of the *F. tularensis* subsp. *novicida* Tfp. A pilE4 mutant completely lacked surface fibres, and could be complemented back to Tfp expression by providing pilE4 in *trans*. Definitive proof that PilE4 is a major pilin subunit will require immuno-electron microscopy with PilE4 antisera, which we currently lack. The PilE4 pilin differs from the other four putative *F. tularensis* pilins in that it is a member of the type B subfamily of Tfp, characterized by a longer leader sequence and lack of phenylalanine immediately following the predicted prepilin peptidase cleavage site (Gil et al., 2004; Hansen & Forest, 2006; Strom & Lory, 1993). PilE4 still contains the invariant glycine and glutamate residues at positions −1 and +5 with respect to the putative cleavage site found in all Tfp pilins (Gil et al., 2004). Mutations in the other four pilin subunit genes, which are members of the type A Tfp subfamily (shorter leader sequence and phenylalanine immediately following the predicted prepilin peptidase cleavage site), did not eliminate surface fibre expression, although PilE3 and PilE5 may be minor components of the pilus, since in their absence pilus expression was reduced. Thus the Tfp of *F. tularensis* subsp. *novicida* appears to be in the same subfamily as bundle-forming pili found in enteric pathogens such as *E. coli* and *Vibrio cholerae* (Burrows, 2005).

The *F. tularensis* subsp. *novicida* PilE4 amino acid sequence is longer than typical pilin subunits (316 aa; Fig. 3); the homology with pilin subunits in other bacteria lies in the first ~130 aa. Interestingly, the pilE4 coding sequences of the more virulent subspecies *tularensis* and *holarctica* have experienced an internal deletion of 43 bp in relation to the *novicida* pilE4, resulting in significantly shorter PilE4 proteins (197 and 211 aa, respectively). These proteins have high homology to the N terminal 153 aa of the *novicida* PilE4, but differ in their C termini, which are encoded past the internal pilE4 deletion (Fig. 3). The pilE4 coding sequence of LVS, which was derived from *F. tularensis* subsp. *holarctica*, has experienced yet another internal deletion of 5 nt downstream of the *tularensis/holarctica* deletion, which places the coding sequence at the 3’ end of the gene back in frame. This results in a longer PilE4 (301 aa) in LVS in which the C-terminal 103 aa are identical to the C terminus of subsp. *novicida* PilE4. Tfp pili have also been visualized on the surface of LVS (Gil et al., 2004), but it is not yet clear whether these pili are also composed of PilE4. However, it is possible that the LVS-specific pilE4 deletion caused the resumption of pilus expression in LVS, which may not otherwise be expressed in the parent subsp. *holarctica* due to the truncation of PilE4.

Some of the *F. tularensis* subsp. *novicida* Tfp genes have been characterized elsewhere as contributing to the secretion of several different proteins, including the protease PepO (Hager et al., 2006). Specifically, pilF, pilG, pilQ and pilE1 have been shown to be required for protein secretion, whereas pilE2, pilE3, pilE5 and pilT have not (Forsberg & Guina, 2007; Hager et al., 2006). By analysing cell-free supernatants, we have confirmed these previous findings, and extended them to show that pilE4 and *FTN_0431* are also not required for protein secretion. Thus, the pil genes of *F. tularensis* function in both protein secretion and Tfp biogenesis, but with little overlap between these two activities: we have identified PilF as the single essential component shared between the two systems, although a mutation in pilQ reduces, but does not eliminate, Tfp expression. Type II secretion is closely related to Tfp biogenesis, and has been proposed to involve a pilus-like structure that acts like a piston to facilitate secretion of substrates across the outer membrane (Sandkvist et al., 1997). Sharing of components between protein secretion and Tfp biogenesis systems is relatively common, even in the absence of a bona fide type II secretion system, and has been documented in a number of bacteria (Hager et al., 2006; Nunn & Lory, 1991; Peabody et al., 2003).

**MglA regulation of Tfp expression in *F. tularensis***

MglA is a global regulator of virulence genes in *F. tularensis*, and it positively regulates the transcription of the FPI genes, which are necessary for intramacrophage growth (Lauriano et al., 2004). In addition, MglA regulates transcription of a large number of genes found outside the FPI (Brotske et al., 2006; Charity et al., 2007), and some of
these have also been found to be involved in intramacrophage growth. MglA also regulates the transcription of the protein secretion substrates PepO and BglX in *F. tularensis* subsp. *novicida*, and thus the secretion of these proteins is decreased in an mglA mutant (Hager et al., 2006). In this report, we identified yet another role for MglA in the expression of Tfp. An mglA mutant does not express Tfp, thus suggesting that some factor(s) required for Tfp expression is positively regulated by MglA. However, MglA does not regulate transcription of the pilF, pilT and pilE4 genes, which are required for Tfp expression.

Earlier microarray studies to identify the MglA regulon in *F. tularensis* subsp. *novicida* and *F. tularensis* subsp. holarctica LVS both identified an MglA-regulated gene annotated as a Tfp glycosylation protein (FTT0905; (Brotccke et al., 2006; Charity et al., 2007) with homology to the Tfp glycosylation protein PilO of *P. aeruginosa* (25% identity, 45% similarity; Kus et al., 2004). Although pilO is not required for *P. aeruginosa* pilus expression, evidence suggests that glycosylated pilin enhances its virulence (Smedley et al., 2005). However, PilO homologues have been shown to be important for pilus expression in Synecochyssis (Yoshihara et al., 2001) and *Pseudomonas syringae* (Roine et al., 1998). We confirmed by RT-PCR that the corresponding gene in *F. tularensis* subsp. *novicida*, FTN_0431, is positively regulated by MglA (Fig. 3), and constructed a subsp. *novicida* AFTN_0431 strain. This mutant expressed Tfp that was similar to that of the wild-type strain, and grew normally within the J774 macrophage cell line, demonstrating that the FTN_0431 gene is not required for Tfp expression in *F. tularensis* subsp. *novicida*, and is not important for intramacrophage growth. Besides the homology with Tfp glycosylases, we have no evidence that FTN_0431 is involved in Tfp expression. It is thus not clear exactly how MglA regulates Tfp expression; we consider it possible that MglA regulates the transcription or possibly translation of some novel gene(s) critical for Tfp biogenesis.

**F. tularensis** Tfp, protein secretion, and virulence

Tfp frequently contribute to the virulence of bacterial pathogens, including *V. cholerae*, *N. meningitidis* and *E. coli* (Assalkhou et al., 2007; Mattick, 2002; Taylor et al., 1987; Tonjum & Koomey, 1997). In many cases, Tfp serve as adhesins within the host, by either binding directly to host cells or facilitating bacterial cell–cell adhesion necessary for host colonization. The closely related type II secretion machinery, which shares a number of homologous components with Tfp, is also associated with bacterial virulence. Type II secretion typically facilitates the secretion of proteins that are destructive to host tissues (e.g., toxins, proteases and lipases) from the periplasm to the extracellular milieu in Gram-negative bacteria (Sandkvist, 2001; Sandkvist et al., 1997). As demonstrated by Hager et al. (2006), some of the *F. tularensis* subsp. *novicida* proteins annotated as Tfp components participate in protein secretion of several substrates, including a protease and a glucosidase. We have demonstrated that some of the annotated Tfp components are essential for Tfp expression, but we have found little overlap between the components required for Tfp biogenesis and those required for protein secretion. This allowed us to assess the relative contributions of these systems to virulence, as measured by intramacrophage survival and virulence in mice.

The PilE1 pilin subunit is required for protein secretion, but plays little role in Tfp expression, while the PilE4 pilin subunit is essential for Tfp expression, but dispensable for protein secretion. A pilE1 mutant showed a defect in intramacrophage growth, whereas the pilE4 mutant entered and replicated in macrophages similarly to the wild-type strain. Moreover, there was an attenuation of virulence for the pilE1 mutant when administered intranasally and intradermally to mice, whereas only a slight virulence defect was observed for the pilE4 mutant administered by these routes. These results indicate a role for Tfp-related protein secretion in intramacrophage growth and virulence in mice via the pulmonary and intradermal routes of infection. These results are consistent with the earlier observations of Forslund et al. (2006), who also found a modest defect for an *F. tularensis* subsp. *holarctica* pilE1 (pilA) mutant in intramacrophage growth. However, they observed a significant virulence defect in the *holarctica* pilE1 strain when administered to mice via the subcutaneous route, which was interpreted as a role for this pilin gene in systemic spread from peripheral sites. We would further suggest that the role of PilE1 (PilA) in protein secretion is likely to be conserved among *F. tularensis* subspecies, and thus Tfp-related protein secretion is important for systemic spread from peripheral sites, but contributes less to virulence via the pulmonary route of infection.

Hager et al. (2006) reported an enhanced virulence associated with mutations in the Tfp-related protein secretion system of *F. tularensis* subsp. *novicida* following administration via the intradermal route. This was observed as increased organ burdens at earlier time points with pilG and pepO mutants, and the authors speculated that the secretion substrate protease PepO has a role in limiting recruitment of neutrophils by cleavage of pro-endothelin. However, no enhanced virulence or increased organ burden was seen when these strains were inoculated into mice via aerosol infection. In the current study, we also saw no increased virulence in mice inoculated intranasally with a pilE1 subsp. *novicida* mutant, which, like a pilG mutant, is defective in Tfp-related protein secretion. We have also constructed and evaluated a pepO mutant strain by the intranasal route, and found no evidence of enhanced virulence with this strain either (data not shown).

In our hands, the pilE1 strain is attenuated for virulence by the intranasal route. Moreover, the pilE1 mutant strain was also attenuated for virulence when mice were infected by the LVS pilE1 (pilA) mutant (Forslund et al., 2006), but contrary to
the previous report of enhanced virulence of the \textit{F. tularensis} subsp. \textit{novicida} pilE1 mutant administered by this route (Hager et al., 2006). It should be noted that an \textit{F. tularensis} subsp. \textit{novicida} pepO (‘FTT1209’) mutant constructed by another laboratory (Brotcke et al., 2006) also exhibited attenuated virulence when infected subcutaneously into mice, unlike the report of Hager et al. (2006), which showed enhanced virulence for this strain by the intradermal route of inoculation. The salient difference in virulence phenotypes of pilE1 and pepO mutants in our study and that of Brotcke et al. (2006), with those reported by Hager et al. (2006), is likely to be the nature of the pilE1 mutation. In our study the targeted mutation was constructed to have limited polar effects (Liu et al., 2007) and could be complemented for secretion and intramacrophage growth by providing pilE1 in trans, whereas in the earlier study the mutation was generated by transposon mutagenesis, and the mutation was not shown to be complemented. Another difference between these studies is the mouse strain used for intranasal infections (BALB/c versus C57BL/6), which may also play a role.

The pilE4 subsp. \textit{novicida} mutant displayed wild-type levels of growth within a macrophage cell line, and virulence similar to that of the wild-type strain when administered intranasally to mice. These results suggest that Tfp expression does not contribute to intramacrophage entry or growth, or virulence in mice via the pulmonary route, at least in \textit{F. tularensis} subsp. \textit{novicida}. Tfp expression may have a greater role via other routes of inoculation; we observed a slight attenuation when this mutant was administered intradermally into mice. Studies with \textit{F. tularensis} subsp. \textit{holarctica} LVS have found that pilT as well as pilF mutants have a significant attenuation in virulence when administered intradermally into mice (Chakraborty et al., 2008), suggesting that Tfp may contribute more to the virulence of this strain than that of subsp. \textit{novicida}. Tfp may also play a more important role in the interactions of \textit{F. tularensis} subsp. \textit{novicida} with other hosts, including protists, or with vectors during transmission. The studies of \textit{F. tularensis} virulence involving defined mutant strains have involved the mouse model of tularemia exclusively, and perhaps other animal hosts may reveal a stronger role for Tfp during pathogenesis. Further experimentation is required to elucidate the contribution of Tfp and Tfp-related protein secretion to the virulence of the other subspecies of \textit{F. tularensis}.

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