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

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Francisella tularensis causes the disease tularaemia. 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*.

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INTRODUCTION

Francisella tularensis is a highly infectious bacterium that causes tularaemia. *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 biothreat 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. *holarctica* 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 tularaemia (Anthony & Kongshavn, 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; Golovliov *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

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Abbreviations: FPI, *Francisella* pathogenicity island; LVS, live vaccine strain; Tfp, type IV pili.

Two supplementary figures, showing the alignment of *pilE4* genes from different *F. tularensis* subspecies and the virulence of *F. tularensis* subsp. *novicida* *pilE1* and *pilE4* strains via intranasal inoculation in mice (repeat experiment), and a supplementary table listing the PCR primers used, are available with the online version of this paper.

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 DH5 α (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 metasilicate ml⁻¹], or Chamberlain's defined medium (CDM; Chamberlain, 1965). Antibiotics were used at the following concentrations: kanamycin, 50 μ g ml⁻¹; tetracycline, 10 μ g ml⁻¹; ampicillin, 100 μ g ml⁻¹; erythromycin, 150 μ g 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 tnfn1_pw060418p02q155 and

Table 1. *F. tularensis* subsp. *novicida* strains used in this study

Strain	Genotype	Reference or source
U112	Wild-type	Anthony <i>et al.</i> (1991b)
KKF34	U112 Δ mglA::ermC	Lauriano <i>et al.</i> (2003)
KKF73	U112 Δ pilQ::ermC	This study
KKF74	U112 Δ pilT::ermC	This study
KKF104	U112 Δ pilG::ermC	This study
KKF252	U112 Δ pilE1::Kan	This study
KKF253	U112 Δ (<i>pilE1</i> –3)::Kan	This study
KKF255	U112 Δ pilF::Kan	This study
KKF315	U112 Δ FTN_0431::Kan	This study
tnfn1_pw060418p02q155	U112 <i>pilE4</i> ::T20(EZTnKan)	Gallagher <i>et al.</i> (2007)
tnfn1_pw060510p03q137	U112 <i>pilE5</i> ::T18(EZTnKan)	Gallagher <i>et al.</i> (2007)

tnfn1_pw060510p03q137, respectively, were kindly provided by the University of Washington (Gallagher *et al.*, 2007); the transposon insertion in each strain was verified by sequencing.

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 *Cm^R*. Then the *FTN1451* 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 *pilE1-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 (*ppilE1*) and pKEK1150 (*ppilE4*).

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).

Intramacrophage 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 ~10⁵ J774 cells, and were infected with ~10⁶ bacteria. After 1 h incubation at 37 °C and 5 % CO₂, gentamicin (50 mg 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/cAnNHsd 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 delivered 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.

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 PilF, 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.

Fig. 1. Electron micrographs of *F. tularensis* subsp. *novicida* strains. Transmission electron micrographs of *F. tularensis* subsp. *novicida* strains U112 (wild-type; WT), KKF104 (*pilG*), KKF73 (*pilQ*), KKF255 (*pilF*), KKF74 (*pilT*) and KKF34 (*mglA*). Bars, 500 nm.

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 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

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 (*ppilE4*). Bars, 500 nm.

U112	MIRKHSKLLQKIKGMTISELVITLTIISILAVFVTSTYSNYATRTKIANQVSLVTAHARQVYDLKKDGKDFYLN	75
LVS	MIRKHSKLLQKIKGMTISELVITLTIISILAVFVTSTYSNYATRTKIANQVSLVTAHARQVYDLKKDGKDFYLN	75
Schu S4	MIRKHSKLLQKIKGMTISELVITLTIISILAVFVTSTYSNYATRTKIANQVSLVTAHARQVYDLKKDGKDFYLN	75
FTHOSU18	MIRKHSKLLQKIKGMTISELVITLTIISILAVFVTSTYSNYATRTKIANQVSLVTAHARQVYDLKKDGKDFYLN	75
U112	DNSTVNDYGAVTKNIGESGANI IKGNAEVMIRPEAVDPDSVRWRCIVYGDGLSESSIPNHCI LGSSIFFRILKDN	150
LVS	DNSTVNDYGAVTKNIGESGANI IKGNAEVMIRPEAVDPDSVRWRCIVYGDGLSESSIPNHCI LGSSIFFRILKDN	150
Schu S4	DNSTVNDYGAVTKNIGESGANI IKGNAEVMIRPEAVDPDSVRWRCIVYGDGLSESSIPNHCI LGSSIFFRILKDN	150
FTHOSU18	DNSTVNDYGAVTKNIGESGANI IKGNAEVMIRPEAVDPDSVRWRCIVYGDGLSESSIPNHCI LGSSIFFRILKDN	150
U112	NML-----SAEENFDLNNNP	167
LVS	NMLIMIGVKFIKIIHSSVIGLSLVVMKRLNFGITL TEYLIVVI-----	193
Schu S4	NMLIMIGVKFIKIIHSSVIGLSLVVMKRLNFGITLTKYLIVVIMLQS*	197
FTHOSU18	NMLIMIGVKFIKIIHSSVIGLSLVVMKRLNFGITL TEYLIVVIMLQSYMVIEMKSLNYLMI*	211
U112	NNDWGKIHKDHPFLGDWTISGGDEEIELWNNFDRISDRDRNVAELDGDGRNEIVELSHDLASHNFKDMSLNFDYYP	242
LVS	-----AELYGDRNEIVELSHDLASHNFKDMSLNFDYYP	226
U112	RTGDDSSNFEVYLGDLIYTHNNFSQEWQKISIDLSNVENAESSKLSIREAGRDESYGALIDLNSLKITPGKIV*	316
LVS	RTGDDSSNFEVYLGDLIYTHNNFSQEWQKISIDLSNVENAESSKLSIREAGRDESYGALIDLNSLKITPGKIV*	301

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).

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*, *pilT*, *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. 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 *mgIA* 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

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 (*mgIA*), 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.

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 (Forslund *et al.*, 2006). These results demonstrate that PilE1 and protein secretion, but not Tfp biogenesis, contribute to intramacrophage growth *in vitro*.

The *pilE1* and *pilE4* mutants were assessed for virulence by intranasal inoculation into BALB/c mice at a low dose (~10² 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 contributes 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 10⁶ 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 10⁷ 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

Fig. 6. Survival and growth of *F. tularensis* subsp. *novicida* *pilE1* and *pilE4* strains within the J774 macrophage-like cell line. *F. tularensis* subsp. *novicida* strains were inoculated at an m.o.i. of $\sim 10:1$ into J774 cells, and intracellular bacteria were enumerated at 1 and 24 h. The assay was performed in triplicate; one representative from three experiments is shown. (a) Strains U112 (wild-type; WT), KKF252 (*pilE1*) and KKF252 transformed with pKEK1176 (*pilE1+ppilE1*). KKF252 showed a significant decrease in intramacrophage growth at 24 h ($*P=0.018$) compared with the wild-type strain (Student's *t* test). (b) Strains U112 (wild-type; WT) and tnfn1_pw060418p02q155 (*pilE4*).

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* subspecies 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

Fig. 7. Virulence in mice of *F. tularensis* subsp. *novicida* *pilE1* and *pilE4* strains, administered via intranasal inoculation. BALB/c mice were inoculated intranasally with $\sim 10^2$ c.f.u. *F. tularensis* subsp. *novicida* strains U112 (wild-type; WT), KKF252 (*pilE1*) and tnfn1_pw060418p02q155 (*pilE4*). The *pilE1* mutant strain showed significant attenuation ($P=0.002$, Kaplan–Meier test) compared with the wild-type strain.

(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. (Carbannelle *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 immunoelectron 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 (Brotcke *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; (Brotcke *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 pili enhance its virulence (Smedley *et al.*, 2005). However, PilO homologues have been shown to be important for pilus expression in *Synechocystis* (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* ΔFTN_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 intradermal route, similar to the results for the LVS *pilE1* (*pilA*) mutant (Forslund *et al.*, 2006), but contrary to

the previous report of enhanced virulence of the *F. tularensis* subsp. *novicida* *pilE1* mutant administered by this route (Hager *et al.*, 2006). It should be noted that an *F. tularensis* subsp. *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 C57BL6), which may also play a role.

The *pilE4* subsp. *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 *F. tularensis* subsp. *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 *F. tularensis* subsp. *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. *novicida*. Tfp may also play a more important role in the interactions of *F. tularensis* subsp. *novicida* with other hosts, including protists, or with vectors during transmission. The studies of *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 *F. tularensis*.

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REFERENCES

- Abd, H., Johansson, T., Golovliov, I., Sandstrom, G. & Forsman, M. (2003). Survival and growth of *Francisella tularensis* in *Acanthamoeba castellanii*. *Appl Environ Microbiol* **69**, 600–606.
- Anthony, L. S. & Kongshavn, P. A. (1987). Experimental murine tularemia caused by *Francisella tularensis*, live vaccine strain: a model of acquired cellular resistance. *Microb Pathog* **2**, 3–14.
- Anthony, L. D., Burke, R. D. & Nano, F. E. (1991a). Growth of *Francisella* spp. in rodent macrophages. *Infect Immun* **59**, 3291–3296.
- Anthony, L. S., Gu, M. Z., Cowley, S. C., Leung, W. W. & Nano, F. E. (1991b). Transformation and allelic replacement in *Francisella* spp. *J Gen Microbiol* **137**, 2697–2703.
- Assalkhou, R., Balasingham, S., Collins, R. F., Frye, S. A., Davidsen, T., Benam, A. V., Bjoras, M., Derrick, J. P. & Tonjum, T. (2007). The outer membrane secretin PilQ from *Neisseria meningitidis* binds DNA. *Microbiology* **153**, 1593–1603.
- Averhoff, B. & Friedrich, A. (2003). Type IV pili-related natural transformation systems: DNA transport in mesophilic and thermophilic bacteria. *Arch Microbiol* **180**, 385–393.
- Balasingham, S. V., Collins, R. F., Assalkhou, R., Homberset, H., Frye, S. A., Derrick, J. P. & Tonjum, T. (2007). Interactions between the lipoprotein PilP and the secretin PilQ in *Neisseria meningitidis*. *J Bacteriol* **189**, 5716–5727.
- Baron, G. S. & Nano, F. E. (1998). MglA and MglB are required for the intramacrophage growth of *Francisella novicida*. *Mol Microbiol* **29**, 247–259.
- Brotcke, A., Weiss, D. S., Kim, C. C., Chain, P., Malfatti, S., Garcia, E. & Monack, D. M. (2006). Identification of MglA-regulated genes reveals novel virulence factors in *Francisella tularensis*. *Infect Immun* **74**, 6642–6655.
- Burrows, L. L. (2005). Weapons of mass retraction. *Mol Microbiol* **57**, 878–888.
- Carbonnelle, E., Helaine, S., Nassif, X. & Pelicic, V. (2006). A systematic genetic analysis in *Neisseria meningitidis* defines the Pil proteins required for assembly, functionality, stabilization and export of type IV pili. *Mol Microbiol* **61**, 1510–1522.
- Chakraborty, S., Monfett, M., Maier, T. M., Benach, J. L., Frank, D. W. & Thanassi, D. G. (2008). Type IV pili in *Francisella tularensis*: roles of *pilF* and *pilT* in fiber assembly, host cell adherence and virulence. *Infect Immun* (in press).
- Chamberlain, R. E. (1965). Evaluation of live tularemia vaccine prepared in a chemically defined medium. *Appl Microbiol* **13**, 232–235.
- Charity, J. C., Costante-Hamm, M. M., Balon, E. L., Boyd, D. H., Rubin, E. J. & Dove, S. L. (2007). Twin RNA polymerase-associated proteins control virulence gene expression in *Francisella tularensis*. *PLoS Pathog* **3**, e84.
- Collins, R. F., Davidsen, L., Derrick, J. P., Ford, R. C. & Tonjum, T. (2001). Analysis of the PilQ secretin from *Neisseria meningitidis* by transmission electron microscopy reveals a dodecameric quaternary structure. *J Bacteriol* **183**, 3825–3832.
- Collins, R. F., Frye, S. A., Balasingham, S., Ford, R. C., Tonjum, T. & Derrick, J. P. (2005). Interaction with type IV pili induces structural changes in the bacterial outer membrane secretin PilQ. *J Biol Chem* **280**, 18923–18930.
- Corpet, F. (1988). Multiple sequence alignment with hierarchical clustering. *Nucleic Acids Res* **16**, 10881–10890.
- Cowley, S. C., Gray, C. J. & Nano, F. E. (2000). Isolation and characterization of *Francisella novicida* mutants defective in lipopolysaccharide biosynthesis. *FEMS Microbiol Lett* **182**, 63–67.
- Dennis, D. T., Inglesby, T. V., Henderson, D. A., Bartlett, J. G., Ascher, M. S., Eitzen, E., Fine, A. D., Friedlander, A. M., Hauer, J. & other authors (2001). Tularemia as a biological weapon: medical and public health management. *JAMA* **285**, 2763–2773.
- Eigelsbach, H. T., Braun, W. & Herring, R. D. (1951). Studies on the variation of *Bacterium tularensis*. *J Bacteriol* **61**, 557–569.

- Ellis, J., Oyston, P. C., Green, M. & Titball, R. W. (2002). Tularemia. *Clin Microbiol Rev* 15, 631–646.
- Forsberg, A. & Guina, T. (2007). Type II secretion and type IV pili of *Francisella*. *Ann N Y Acad Sci* 1105, 187–201.
- Forslund, A. L., Kuoppa, K., Svensson, K., Salomonsson, E., Johansson, A., Bystrom, M., Oyston, P. C., Michell, S. L., Titball, R. W. & other authors (2006). Direct repeat-mediated deletion of a type IV pilin gene results in major virulence attenuation of *Francisella tularensis*. *Mol Microbiol* 59, 1818–1830.
- Fullner, K. J. & Mekalanos, J. J. (1999). Genetic characterization of a new type IV-A pilus gene cluster found in both classical and El Tor biotypes of *Vibrio cholerae*. *Infect Immun* 67, 1393–1404.
- Gallagher, L. A., Ramage, E., Jacobs, M. A., Kaul, R., Brittnacher, M. & Manoil, C. (2007). A comprehensive transposon mutant library of *Francisella novicida*, a bioweapon surrogate. *Proc Natl Acad Sci U S A* 104, 1009–1014.
- Gil, H., Benach, J. L. & Thanassi, D. G. (2004). Presence of pili on the surface of *Francisella tularensis*. *Infect Immun* 72, 3042–3047.
- Golovliov, I., Sjostedt, A., Mokrievich, A. & Pavlov, V. (2003a). A method for allelic replacement in *Francisella tularensis*. *FEMS Microbiol Lett* 222, 273–280.
- Golovliov, I., Baranov, V., Krocova, Z., Kovarova, H. & Sjostedt, A. (2003b). An attenuated strain of the facultative intracellular bacterium *Francisella tularensis* can escape the phagosome of monocytic cells. *Infect Immun* 71, 5940–5950.
- Gray, C. G., Cowley, S. C., Cheung, K. K. & Nano, F. E. (2002). The identification of five genetic loci of *Francisella novicida* associated with intracellular growth. *FEMS Microbiol Lett* 215, 53–56.
- Hager, A. J., Bolton, D. L., Pelletier, M. R., Brittnacher, M. J., Gallagher, L. A., Kaul, R., Skerrett, S. J., Miller, S. I. & Guina, T. (2006). Type IV pili-mediated secretion modulates *Francisella* virulence. *Mol Microbiol* 62, 227–237.
- Hanahan, D. (1983). Studies on transformation of *Escherichia coli* with plasmids. *J Mol Biol* 166, 557–580.
- Hansen, J. K. & Forest, K. T. (2006). Type IV pilin structures: insights on shared architecture, fiber assembly, receptor binding and type II secretion. *J Mol Microbiol Biotechnol* 11, 192–207.
- Kieffer, T. L., Cowley, S., Nano, F. E. & Elkins, K. L. (2003). *Francisella novicida* LPS has greater immunobiological activity in mice than *F. tularensis* LPS, and contributes to *F. novicida* murine pathogenesis. *Microbes Infect* 5, 397–403.
- Kuoppa, K., Forsberg, A. & Norqvist, A. (2001). Construction of a reporter plasmid for screening in vivo promoter activity in *Francisella tularensis*. *FEMS Microbiol Lett* 205, 77–81.
- Kus, J. V., Tullis, E., Cvitkovitch, D. G. & Burrows, L. L. (2004). Significant differences in type IV pilin allele distribution among *Pseudomonas aeruginosa* isolates from cystic fibrosis (CF) versus non-CF patients. *Microbiology* 150, 1315–1326.
- Larsson, P., Oyston, P. C., Chain, P., Chu, M. C., Duffield, M., Fuxelius, H. H., Garcia, E., Halltorp, G., Johansson, D. & other authors (2005). The complete genome sequence of *Francisella tularensis*, the causative agent of tularemia. *Nat Genet* 37, 153–159.
- Lauer, P., Albertson, N. H. & Koomey, M. (1993). Conservation of genes encoding components of a type IV pilus assembly/two-step protein export pathway in *Neisseria gonorrhoeae*. *Mol Microbiol* 8, 357–368.
- Lauriano, C. M., Barker, J. R., Nano, F. E., Arulanandam, B. P. & Klose, K. E. (2003). Allelic exchange in *Francisella tularensis* using PCR products. *FEMS Microbiol Lett* 229, 195–202.
- Lauriano, C. M., Barker, J. R., Yoon, S. S., Nano, F. E., Arulanandam, B. P., Hassett, D. J. & Klose, K. E. (2004). MglA regulates transcription of virulence factors necessary for *Francisella tularensis* intraamoebae and intramacrophage survival. *Proc Natl Acad Sci U S A* 101, 4246–4249.
- Liu, J., Zogaj, X., Barker, J. & Klose, K. (2007). Construction of targeted insertion mutations in *Francisella tularensis* subsp. *novicida*. *Biotechniques* 43, 487–492.
- Maier, T. M., Casey, M. S., Becker, R. H., Dorsey, C. W., Glass, E. M., Maltsev, N., Zahrt, T. C. & Frank, D. W. (2007). Identification of *Francisella tularensis* Himarl-based transposon mutants defective for replication in macrophages. *Infect Immun* 75, 5376–5389.
- Mattick, J. S. (2002). Type IV pili and twitching motility. *Annu Rev Microbiol* 56, 289–314.
- McLendon, M. K., Apicella, M. A. & Allen, L. A. (2006). *Francisella tularensis*: taxonomy, genetics, and immunopathogenesis of a potential agent of biowarfare. *Annu Rev Microbiol* 60, 167–185.
- Nano, F. E., Zhang, N., Cowley, S. C., Klose, K. E., Cheung, K. K., Roberts, M. J., Ludu, J. S., Letendre, G. W., Meierovics, A. I. & other authors (2004). A *Francisella tularensis* pathogenicity island required for intramacrophage growth. *J Bacteriol* 186, 6430–6436.
- Nunn, D. N. & Lory, S. (1991). Product of the *Pseudomonas aeruginosa* gene *pilD* is a prepilin leader peptidase. *Proc Natl Acad Sci U S A* 88, 3281–3285.
- Nunn, D., Bergman, S. & Lory, S. (1990). Products of three accessory genes, *pilB*, *pilC*, and *pilD*, are required for biogenesis of *Pseudomonas aeruginosa* pili. *J Bacteriol* 172, 2911–2919.
- Oyston, P. C., Sjostedt, A. & Titball, R. W. (2004). Tularemia: bioterrorism defence renews interest in *Francisella tularensis*. *Nat Rev Microbiol* 2, 967–978.
- Peabody, C. R., Chung, Y. J., Yen, M. R., Vidal-Ingigliardi, D., Pugsley, A. P. & Saier, M. H., Jr (2003). Type II protein secretion and its relationship to bacterial type IV pili and archaeal flagella. *Microbiology* 149, 3051–3072.
- Petrosino, J. F., Xiang, Q., Karpach, S. E., Jiang, H., Yerrapragada, S., Liu, Y., Gioia, J., Hemphill, L., Gonzalez, A. & other authors (2006). Chromosome rearrangement and diversification of *Francisella tularensis* revealed by the type B (OSU18) genome sequence. *J Bacteriol* 188, 6977–6985.
- Qin, A. & Mann, B. J. (2006). Identification of transposon insertion mutants of *Francisella tularensis* tularensis strain Schu S4 deficient in intracellular replication in the hepatic cell line HepG2. *BMC Microbiol* 6, 69.
- Rohmer, L., Brittnacher, M., Svensson, K., Buckley, D., Haugen, E., Zhou, Y., Chang, J., Levy, R., Hayden, H. & other authors (2006). Potential source of *Francisella tularensis* live vaccine strain attenuation determined by genome comparison. *Infect Immun* 74, 6895–6906.
- Roine, E., Raineri, D. M., Romantschuk, M., Wilson, M. & Nunn, D. N. (1998). Characterization of type IV pilus genes in *Pseudomonas syringae* pv. tomato DC3000. *Mol Plant Microbe Interact* 11, 1048–1056.
- Sandkvist, M. (2001). Biology of type II secretion. *Mol Microbiol* 40, 271–283.
- Sandkvist, M., Michel, L. O., Hough, L. P., Morales, V. M., Bagdasarian, M., Koomey, M. & DiRita, V. J. (1997). General secretion pathway (*eps*) genes required for toxin secretion and outer membrane biogenesis in *Vibrio cholerae*. *J Bacteriol* 179, 6994–7003.
- Sjostedt, A. (2003). Virulence determinants and protective antigens of *Francisella tularensis*. *Curr Opin Microbiol* 6, 66–71.
- Smedley, J. G., III, Jewell, E., Roguskie, J., Horzempa, J., Syboldt, A., Stolz, D. B. & Castic, P. (2005). Influence of pilin glycosylation on *Pseudomonas aeruginosa* 1244 pilus function. *Infect Immun* 73, 7922–7931.

- Strom, M. S. & Lory, S. (1993).** Structure–function and biogenesis of the type IV pili. *Annu Rev Microbiol* **47**, 565–596.
- Su, J., Yang, J., Zhao, D., Kawula, T. H., Banas, J. A. & Zhang, J. R. (2007).** Genome-wide identification of *Francisella tularensis* virulence determinants. *Infect Immun* **75**, 3089–3101.
- Taylor, R. K., Miller, V. L., Furlong, D. B. & Mekalanos, J. J. (1987).** Use of *phoA* gene fusions to identify a pilus colonization factor coordinately regulated with cholera toxin. *Proc Natl Acad Sci U S A* **84**, 2833–2837.
- Tempel, R., Lai, X. H., Crosa, L., Kozlowski, B. & Heffron, F. (2006).** Attenuated *Francisella novicida* transposon mutants protect mice against wild-type challenge. *Infect Immun* **74**, 5095–5105.
- Thomas, R. M., Titball, R. W., Oyston, P. C., Griffin, K., Waters, E., Hitchen, P. G., Michell, S. L., Grice, I. D., Wilson, J. C. & Prior, J. L. (2007).** The immunologically distinct O antigens from *Francisella tularensis* subspecies *tularensis* and *Francisella novicida* are both virulence determinants and protective antigens. *Infect Immun* **75**, 371–378.
- Tonjum, T. & Koomey, M. (1997).** The pilus colonization factor of pathogenic neisserial species: organelle biogenesis and structure/function relationships – a review. *Gene* **192**, 155–163.
- Wall, D., Kolenbrander, P. E. & Kaiser, D. (1999).** The *Myxococcus xanthus pilQ* (*sglA*) gene encodes a secretin homolog required for type IV pilus biogenesis, social motility, and development. *J Bacteriol* **181**, 24–33.
- Weiss, D. S., Brotcke, A., Henry, T., Margolis, J. J., Chan, K. & Monack, D. M. (2007).** *In vivo* negative selection screen identifies genes required for *Francisella* virulence. *Proc Natl Acad Sci U S A* **104**, 6037–6042.
- Whitchurch, C. B., Hobbs, M., Livingston, S. P., Krishnapillai, V. & Mattick, J. S. (1991).** Characterisation of a *Pseudomonas aeruginosa* twitching motility gene and evidence for a specialised protein export system widespread in eubacteria. *Gene* **101**, 33–44.
- Winther-Larsen, H. C., Wolfgang, M., Dunham, S., van Putten, J. P., Dorward, D., Lovold, C., Aas, F. E. & Koomey, M. (2005).** A conserved set of pilin-like molecules controls type IV pilus dynamics and organelle-associated functions in *Neisseria gonorrhoeae*. *Mol Microbiol* **56**, 903–917.
- Wolfgang, M., van Putten, J. P., Hayes, S. F., Dorward, D. & Koomey, M. (2000).** Components and dynamics of fiber formation define a ubiquitous biogenesis pathway for bacterial pili. *EMBO J* **19**, 6408–6418.
- Yoshihara, S., Geng, X., Okamoto, S., Yura, K., Murata, T., Go, M., Ohmori, M. & Ikeuchi, M. (2001).** Mutational analysis of genes involved in pilus structure, motility and transformation competency in the unicellular motile cyanobacterium *Synechocystis* sp. PCC 6803. *Plant Cell Physiol* **42**, 63–73.

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