Functional analyses of pilin-like proteins from *Francisella tularensis*: complementation of type IV pilus phenotypes in *Neisseria gonorrhoeae*

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Accumulating evidence from a number of studies strongly suggests that proteins orthologous to those involved in type IV pili (Tfp) assembly and function are required for *Francisella* pathogenicity. However, the molecular mechanisms by which the components exert their influence on virulence remain poorly understood. Owing to the conservation and promiscuity of Tfp biogenesis machineries, expression of Tfp pilins in heterologous species has been used successfully to analyse organelle structure–function relationships. In this study we expressed a number of *Francisella* pilin genes in the Tfp-expressing pathogen *Neisseria gonorrhoeae* lacking its endogenous pilin subunit. Two gene products, the orthologous PilA proteins from *Francisella tularensis* subspecies *tularensis* and *novicida*, were capable of restoring the expression of Tfp-like appendages that were shown to be dependent upon the neisserial Tfp biogenesis machinery for surface localization. Expression of *Francisella* PilA pilins also partially restored competence for natural transformation in *N. gonorrhoeae*. This phenotype was not complemented by expression of the PulG and XcpT proteins, which are equivalent components of the related type II protein secretion system. Taken together, these findings provide compelling, although indirect, evidence of the potential for *Francisella* PilA proteins to express functional Tfp.

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INTRODUCTION

The Gram-negative facultative intracellular bacterium *Francisella tularensis* causes the zoonotic disease tularemia (Mörner, 1992; Tärnvik, 1989). There are at least four subspecies of *F. tularensis*, each with a unique geographical distribution (Petersen & Schriefer, 2005). *F. tularensis* subsp. *holarctica* (type B) can be found in most of Europe, Asia and North America, while *F. tularensis* subsp.

tularensis (type A) is found exclusively in North America. *F. tularensis* subsp. *mediasiatica* has been identified in Central Asia and, finally, *F. tularensis* subsp. *novicida* has been found in numerous locations in North America and in Australia (Petersen & Schriefer, 2005; Whipp *et al.*, 2003). Human tularemia infections are usually caused by subsp. *tularensis* and subsp. *holarctica*, the former being associated with more severe disease than the latter (Tärnvik & Berglund, 2003). The significantly less pathogenic subsp. *novicida* has only been reported to cause infection in immunocompromised individuals on rare occasions (Hollis *et al.*, 1989; Tärnvik, 1989). Due to its high infectivity and capacity to cause severe illness and death, *F. tularensis* is considered a Category A agent of bioterrorism (Dennis *et al.*, 2001; Khan *et al.*, 2000). Compared to other Category A bacterial pathogens, relatively little is known about the virulence factors of *F. tularensis*. However, recent comparative genomic efforts (Svensson *et al.*, 2005) and the

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Abbreviations: DATDH, 2,4-diacetamido-2,4,6-trideoxyhexose; IFM, immunofluorescence microscopy; LVS, live vaccine strain; SOEing PCR, splicing by overlapping extension PCR; T2SS, type II secretion system; TEM, transmission electron microscopy; Tfp, type IV pili.

A supplementary table of primers and four supplementary figures are available with the online version of this paper.

development of genetic tools (Golovliov *et al.*, 2003) have paved the way for progress in this research field.

The genome sequence of the virulent *F. tularensis* subsp. *tularensis* strain SCHU S4 contains several gene clusters with potential roles in virulence (Larsson *et al.*, 2005). Amongst these are those encoding proteins related to those required for assembly and secretion of type IV pili (Tfp) (Larsson *et al.*, 2005). Tfp are complex filamentous appendages defined by their shared structural, biochemical, antigenic and morphological features which are expressed by many human pathogens, including *Neisseria* species, *Pseudomonas aeruginosa* and *Vibrio cholerae* (Fullner & Mekalanos, 1999; Mattick *et al.*, 1996; Tønjum & Koomey, 1997). Tfp play central roles in the expression of many phenotypes, including multicellular behaviours, natural genetic transformation, biofilm formation, cell signalling, motility and host cell adherence (Källström *et al.*, 1998; Mattick, 2002; O'Toole & Kolter, 1998). Tfp biogenesis and the elaboration of associated phenotypes require the concerted actions of many proteins that are structurally conserved across species and genus boundaries (Strom & Lory, 1993). The pilus fibre is mainly composed of the major pilin subunit (called PilA in *P. aeruginosa*), which is expressed as a prepilin that upon cleavage by the prepilin peptidase PilD allows for proper pilus assembly and function (Strom *et al.*, 1993). Translocation of the pilus to the cell surface is then enabled by the secretin PilQ (Wolfgang *et al.*, 2000). In many instances, multiple proteins sharing structural similarities with the major pilin subunit, so-called minor pilins, are required for proper Tfp function and/or assembly (Alm *et al.*, 1996a, b; Alm & Mattick, 1996; Helaine *et al.*, 2007; Winther-Larsen *et al.*, 2001, 2005; Wolfgang *et al.*, 1998b). However, their role in Tfp biogenesis and function is still not completely understood. Furthermore, Tfp are dynamic structures that undergo retraction events mediated by members of the PilT retraction ATPase family (Nudleman & Kaiser, 2004; Skerker & Berg, 2001; Whitchurch *et al.*, 1991). Rounds of Tfp extension and retraction mediate so called twitching motility on solid and semisolid surfaces (Mattick, 2002), and susceptibility to pilus-specific phages (Bradley & Pitt, 1974; Waldor & Mekalanos, 1996).

Many components part of the type II protein secretion system (T2SS) share remarkable similarities with those involved in Tfp expression (Peabody *et al.*, 2003) and, if overexpressed, T2SS pilin-like molecules can polymerize into Tfp-like structures (Durand *et al.*, 2003; Vignon *et al.*, 2003). Despite these similarities, functional interactions between these two systems have only been reported in one instance (Lu *et al.*, 1997). Nonetheless, Tfp systems in *V. cholerae* and *Dichelobacter nodosus* have been implicated in the translocation of folded proteins from the periplasm to the extracellular milieu (Han *et al.*, 2008; Kirn *et al.*, 2003).

A number of findings suggest that *Francisella* Tfp-associated genes are involved in pathogenesis. A spontaneous mutant of *F. tularensis* subsp. *holarctica* with a

deletion within a pilin-like gene locus showed reduced virulence in mice, and a similar rearrangement at this particular locus is present in the subsp. *holarctica* live vaccine strain (LVS) (Forslund *et al.*, 2006). Interestingly, secretion of multiple proteins in *F. tularensis* subsp. *novicida* was abolished by mutations in genes predicted to encode Tfp and ancillary factors (Hager *et al.*, 2006), suggesting that Tfp in this subspecies might also be associated with a T2SS as in *D. nodosus* and *V. cholerae* (Han *et al.*, 2008; Kirn *et al.*, 2003). Surprisingly, these mutants revealed increased virulence in an animal model (Hager *et al.*, 2006). These perplexing results were reconciled by the observation that PepO, one of the secreted substrates, is a zinc protease that promotes increased vasoconstriction and potentially inhibits tissue spread and dissemination. Interestingly, PepO appears to be absent from the human-virulent *Francisella* lineages.

Central questions yet to be resolved for *Francisella* species are what components encompass the pilus machinery and which protein constitutes the major structural subunit. For the LVS, one report concluded that it expresses pilus-like appendages, and many of the genes whose products are predicted to be involved in Tfp biogenesis were shown to be transcribed (Gil *et al.*, 2004). More recently, null mutations in genes encoding a putative Tfp assembly ATPase (PilB, *P. aeruginosa* nomenclature) and a homologue for PilT were shown to prevent the formation of Tfp-like organelles in the LVS (Chakraborty *et al.*, 2008). Importantly, however, studies using the LVS are complicated by the fact that it carries mutations in genes encoding strong candidates of Tfp pilin subunits (based on structural similarities and synteny) (Forslund *et al.*, 2006). Tfp-like appendages have also been observed in *F. tularensis* subsp. *novicida*, and their expression was shown to depend on the putative assembly and retraction ATPase genes (Zogaj *et al.*, 2008). Intriguingly, mutants lacking homologues of components (PilC and PilQ, *P. aeruginosa* nomenclature) critical to surface expression of Tfp in other pathogens such as *N. gonorrhoeae* and *P. aeruginosa* were unaltered in organelle expression, but defective in protein secretion and virulence. Conversely, a mutant defective in expression of the pilin-like protein termed PilE4 retained protein secretion and virulence, but lacked Tfp-like organelles (Zogaj *et al.*, 2008). The authors concluded that Tfp expression and protein secretion can be dissociated in subsp. *novicida* and that PilE4 is probably the major subunit of Tfp. Still, conclusive evidence as to which protein constitutes the major structural subunit has yet to be presented.

In order to address some of these issues, we employed a strategy in which pilin genes from *F. tularensis* were expressed in *N. gonorrhoeae*. Based on the extensive similarity of pilins from different species and the highly conserved nature of the assembly machineries, trans-species complementation has been achieved (Elleman *et al.*, 1986; Hoyne *et al.*, 1992; Sauvonnnet *et al.*, 2000; Winther-Larsen *et al.*, 2007). Our results strongly suggest

that one particular class of pilin protein from subsp. *tularensis* and its counterpart from subsp. *novicida* is capable of forming Tfp-like appendages and complementing the Tfp-dependent phenotype of natural genetic transformation in a *N. gonorrhoeae* pilin subunit mutant.

METHODS

Bacterial strains, plasmids and growth conditions. The *F. tularensis*, *Escherichia coli* and *N. gonorrhoeae* strains used in this study are listed in Table 1. *F. tularensis* strains were grown on modified Thayer–Martin agar plates (Sandström *et al.*, 1984) and *N. gonorrhoeae* strains in GC medium or on GC agar (Freitag *et al.*, 1995) plates, both at 37 °C in a 5% CO₂ atmosphere. *E. coli* strains were grown on blood agar base (BAB; Merck) plates or in Luria–Bertani broth (LB) at 37 °C. Where appropriate, antibiotics were used at the following concentrations; kanamycin 50 µg ml⁻¹, chloramphenicol 10 µg ml⁻¹ and erythromycin 8 µg ml⁻¹. Preparation of plasmid DNA, restriction enzyme digests, ligations and transformations into *E. coli* were performed essentially as described by Sambrook *et al.* (1989).

Construction of translational fusions. The gonococcal *pilE* gene (also denoted *pilE_{GC}*) was fused in-frame to the *Francisella* pilin genes (denoted by subscript Ft for *F. tularensis* subsp. *tularensis* and Fn for *F. tularensis* subsp. *novicida*) *pilA_{Ft}*, *pilE_{Ft}*, *pilV_{Ft}*, FTT0861_{Ft}, FTT0230_{Ft}, FTT1314_{Ft} and *pilA_{Fn}* at the glycine–phenylalanine junction by overlap extension (SOEing) PCR using primers *pilE_{GC}-F*, *pilE_{GC}-R1-7*, *pilA_{Ft}/pilE_{Ft}/pilV_{Ft}/FTT0861_{Ft}/FTT0230_{Ft}/FTT1314_{Ft}/pilA_{Fn}-F2* and *pilA_{Ft}/pilE_{Ft}/pilV_{Ft}/FTT0861_{Ft}/FTT0230_{Ft}/FTT1314_{Ft}/pilA_{Fn}-R1*, where *pilE_{GC}-F* and *pilA_{Ft}/pilE_{Ft}/pilV_{Ft}/FTT0861_{Ft}/FTT0230_{Ft}/FTT1314_{Ft}/pilA_{Fn}-R1* have complementary sequences (see Supplementary Table S1, available with the online version of this paper). For *N. gonorrhoeae*, *pilE_{GC}* plasmid pPile2 was used as template and for *Francisella*, strain FSC237 (subsp. *tularensis*, SCHU S4) or FSC040 (subsp. *novicida*, U112). The fusion proteins carry the first seven residues of gonococcal *PilE_{GC}* while the remaining protein is *Francisella* derived. The amplified fragments were digested with *Bsu*361 and *Stu*I and subcloned into p2/16/1, generating plasmids pEMS25–31. The plasmids were then introduced into gonococcal strains MW24, KS101, GP111 and KS79 by transformation and selection on GC agar plates containing erythromycin as previously described (Freitag *et al.*, 1995). Direct sequencing of PCR products derived from transformants was performed using the primers p2/16/1_5' and p2/16/1_3' to ensure correct sequences (Eurofins MVG Operon).

Construction of T2SS pseudopilin transcriptional and translational fusions. The gonococcal *pilE_{GC}* gene was fused in-frame to the *Klebsiella oxytoca pulG* gene and the *P. aeruginosa* PAK *xcpT* gene at the translational start site or at the pilin peptidase cleavage site by SOEing PCR. The upstream *pilE_{GC}* region was amplified from plasmid pPile2 using primer 266 (5'-*pilE_{GC}-Sac/Bam*) together with HC2 (*pilE_{GF}pulG*), HC7 (*pilE_{ATG}pulG*), HC12 (*pilE_{GF}xcpT*) and HC21 (*pilE_{ATG}xcpT*) (Table S1). The downstream region was amplified from plasmid pCHAP231 using primers HC4 (*pilE_{GF}pulG*) and HC6 (*pilE_{ATG}pulG*) together with primer HC9. Primers HC2 and HC7 had overlapping sequences with primers HC4 and HC5, respectively. The two PCR products were joined together using the flanking primers 266 and HC10. For *xcpT*, the downstream region was amplified from plasmid pMTWT using primers HC13 (*pilE_{GF}xcpT*) and HC19 (*pilE_{ATG}xcpT*) together with HC24. Primers HC12 and HC21 had overlapping sequences with HC13 and HC19, respectively. The two PCR products were joined together using the flanking primers 266 and HC23. The amplified products were cloned into pCRII-TOPO (Invitrogen) giving rise to plasmids pHW10–13. Plasmids pHW10–13

were digested with *Sac*I, and the DNA fragments containing the *pilE::pulg* or *pilE::xcpT* fusions were cloned into plasmid p2/16/1 digested with the same enzyme. The resulting plasmids pHW14–17 were introduced into gonococcal strains by transformation and selection on GC agar plates containing erythromycin. Plasmids were sequenced at GATC Biotech (Konstanz, Germany) using custom primers. *PulG* and *XcpT* expression were verified by immunoblotting using antibodies specific for *PulG* or *XcpT*.

Gel electrophoresis and Western blot analysis. Protein samples containing SDS and β-mercaptoethanol were boiled for 5 min. All samples were separated by SDS-PAGE on 12% or 15% gels (Laemmli, 1970). Proteins were transferred to Immobilon-P Transfer Membranes (Millipore) using a Trans-Blot Semi-Dry transfer cell (Bio-Rad). Membranes were blocked in Tris-buffered saline (TBS) with 5% non-fat dry milk. Immunoblotting was performed with polyclonal rabbit antibodies against *PilA_{Ft}* (Forsslund *et al.*, 2006), *PilV_{GC}* (Winther-Larsen *et al.*, 2001), *PulG*, *XcpT* and Tfp from the gonococcal strain N400 (lot 904) (Winther-Larsen *et al.*, 2007). *P. aeruginosa* PAK *PilA*-specific serum was a gift from E. Gotschlich (Rockefeller University). *K. oxytoca* *PulG*-specific serum was a gift from A. Pugsley (Pasteur Institute, Paris) and *P. aeruginosa* *XcpT*-specific serum was a gift from A. Filloux (IBSM-CNRS, Marseille Cedex). For the alkaline-phosphatase-conjugated secondary antibody system (Roche), the visualization was accomplished by incubating the filters with 0.1% (w/v) NBT (nitro blue tetrazolium; Sigma) and 0.05% (w/v) BCIP (5-bromo-4-chloro-3-indolyl phosphate; Sigma).

Immunofluorescence, immunogold and transmission electron microscopy. Immunofluorescence microscopy was performed as described previously (Winther-Larsen *et al.*, 2005). The pili were labelled using gonococcal *PilE_{GC}* specific serum (lot 904). Samples for transmission electron microscopy were prepared as published (Winther-Larsen *et al.*, 2007) and viewed in a Hitachi (Tokyo) HU-11E-1 electron microscope. Immunogold labelling was carried out essentially as described previously (Winther-Larsen *et al.*, 2007) using rabbit antiserum specific for *PilE_{GC}* (lot 904). Images shown correspond to fields that were representative of the overall observation. For statistical analysis the percentage of piliation was calculated from at least five sets of experiments in which the number of pili was counted per 600 gonococcal cells.

Tfp purification, transformation and twitching motility assay. Transformation assays were carried out as described previously (Aas *et al.*, 2002). Pili were purified by the ammonium sulphate procedure of Wolfgang *et al.* (1998a), with modifications (Winther-Larsen *et al.*, 2007). Twitching motility assays were carried out in phenol-red-free DMEM (Gibco), supplemented with 2 mM L-glutamine, 8 mM sodium pyruvate, 5 mM ascorbic acid, 30 mM HEPES and 1 mg BSA ml⁻¹ at 37 °C on glass coverslips (Maier *et al.*, 2004).

RESULTS

The repertoire of Tfp-related pilin genes in *Francisella* genomes

The genome sequence of the *F. tularensis* subsp. *tularensis* virulent strain SCHU S4 contains multiple ORFs similar to genes encoding components involved in Tfp biogenesis and Tfp structures in other bacterial species (Larsson *et al.*, 2005). These ORFs include six genes whose predicted products have features typical for Tfp prepilins. Genome comparisons between different strains and subspecies of *Francisella* reveal several differences in the putative Tfp

Table 1. Strains and plasmids used in this study

Strain	Subspecies/alternative strain designation(s)/genotype/phenotype*	Source/reference
<i>F. tularensis</i> †		
FSC040	<i>novicida</i> , U112	Water, 1950, Utah
FSC237	<i>tularensis</i> , SCHU S4	Human ulcer, 1941, Ohio
<i>E. coli</i>		
TOP10	F ⁻ <i>mcrA</i> Δ(<i>mrr-hsdRMS-mcrBC</i>), ϕ80 <i>lacZ</i> Δ <i>M15</i> Δ <i>lacX74</i> <i>recA1</i> <i>deoR</i> <i>araD139</i> Δ(<i>ara-leu</i>)7697 <i>galU</i> <i>galK</i> <i>rpsL</i> (Sm ^r) <i>endA1</i> <i>nupG</i>	Invitrogen
DH5α	F ⁻ ϕ80 <i>dlacZ</i> Δ <i>M15</i> Δ(<i>lacZYA-argF</i>)U169 <i>deoR</i> <i>recA1</i> <i>endA1</i> <i>hsdR17</i> (r _K ⁻ m _K ⁺) <i>phoA</i> <i>supE44</i> λ ⁻ <i>thi-1</i> <i>gyrA96</i> <i>relA1</i>	Invitrogen
<i>N. gonorrhoeae</i>		
VD300		Koomey & Falkow (1987)
N400	<i>recA6</i> (<i>tetM</i>)	Tønjum <i>et al.</i> (1995)
N401	<i>recA6</i> (<i>kan</i>)	Wolfgang <i>et al.</i> (1998a)
MW24	<i>recA6</i> (<i>kan</i>) <i>pilE_{GCind}</i>	Wolfgang <i>et al.</i> (2000)
KS59	<i>recA6</i> (<i>kan</i>) <i>pilE_{GCind}</i> <i>iga::pilA_{Ft}</i>	This study
KS60	<i>recA6</i> (<i>kan</i>) <i>pilE_{GCind}</i> <i>iga::pilE_{Ft}</i>	This study
KS61	<i>recA6</i> (<i>kan</i>) <i>pilE_{GCind}</i> <i>iga::pilV_{Ft}</i>	This study
KS62	<i>recA6</i> (<i>kan</i>) <i>pilE_{GCind}</i> <i>iga::FTT0861_{Ft}</i>	This study
KS63	<i>recA6</i> (<i>kan</i>) <i>pilE_{GCind}</i> <i>iga::FTT0230_{Ft}</i>	This study
KS64	<i>recA6</i> (<i>kan</i>) <i>pilE_{GCind}</i> <i>iga::pilA_{Fn}†</i>	This study
GE2	<i>recA6</i> (<i>kan</i>) <i>pilE_{GCind}</i> <i>iga::pilE_{GC}</i>	Aas <i>et al.</i> (2002)
KS101	<i>pilE_{GCind}</i>	Aas <i>et al.</i> (2002)
KS67	<i>pilE_{GCind}</i> <i>iga::pilA_{Ft}</i>	This study
KS68	<i>pilE_{GCind}</i> <i>iga::pilE_{Ft}</i>	This study
KS69	<i>pilE_{GCind}</i> <i>iga::pilV_{Ft}</i>	This study
KS70	<i>pilE_{GCind}</i> <i>iga::FTT0861_{Ft}</i>	This study
KS71	<i>pilE_{GCind}</i> <i>iga::FTT0230_{Ft}</i>	This study
KS72	<i>pilE_{GCind}</i> <i>iga::pilA_{Fn}</i>	This study
GE101	<i>pilE_{GCind}</i> <i>iga::pilE_{GC}</i>	Wolfgang <i>et al.</i> (2000)
GP111	<i>pilE_{GCind}</i> <i>comP_{oe}</i>	Aas <i>et al.</i> (2002)
GP122	<i>pilE_{GCind}</i> <i>comP_{oe}</i> <i>iga::pilE_{GC}</i>	Aas <i>et al.</i> (2007b)
KS73	<i>pilE_{GCind}</i> <i>comP_{oe}</i> <i>iga::pilA_{Ft}</i>	This study
KS74	<i>pilE_{GCind}</i> <i>comP_{oe}</i> <i>iga::pilE_{Ft}</i>	This study
KS75	<i>pilE_{GCind}</i> <i>comP_{oe}</i> <i>iga::pilV_{Ft}</i>	This study
KS76	<i>pilE_{GCind}</i> <i>comP_{oe}</i> <i>iga::FTT0861_{Ft}</i>	This study
KS77	<i>pilE_{GCind}</i> <i>comP_{oe}</i> <i>iga::FTT0230_{Ft}</i>	This study
KS78	<i>pilE_{GCind}</i> <i>comP_{oe}</i> <i>iga::pilA_{Fn}</i>	This study
GV2	<i>pilV_{GC}::kan</i>	Winther-Larsen <i>et al.</i> (2001)
KS79	<i>pilE_{GCind}</i> <i>pilV_{GC}::kan</i>	This study
KS80	<i>pilE_{GCind}</i> <i>pilV_{GC}::kan</i> <i>iga::pilE_{GC}</i>	This study
KS81	<i>pilE_{GCind}</i> <i>pilV_{GC}::kan</i> <i>iga::pilA_{Ft}</i>	This study
KS82	<i>pilE_{GCind}</i> <i>pilV_{GC}::kan</i> <i>iga::pilE_{Ft}</i>	This study
KS83	<i>pilE_{GCind}</i> <i>pilV_{GC}::kan</i> <i>iga::pilV_{Ft}</i>	This study
KS84	<i>pilE_{GCind}</i> <i>pilV_{GC}::kan</i> <i>iga::FTT0861_{Ft}</i>	This study
KS85	<i>pilE_{GCind}</i> <i>pilV_{GC}::kan</i> <i>iga::FTT0230_{Ft}</i>	This study
KS86	<i>pilE_{GCind}</i> <i>pilV_{GC}::kan</i> <i>iga::pilA_{Fn}</i>	This study
KS45	<i>recA6</i> (<i>tetM</i>) <i>pilE_{GC}::cat</i>	Winther-Larsen <i>et al.</i> (2007)
KS199	<i>pilE_{GC}::cat</i>	This study
KS87	<i>pilE_{GC}::cat</i> <i>pilV_{GC}::kan</i>	This study
KS44	<i>pilE_{GC}::cat</i> <i>pilV_{GC}::kan</i> <i>iga::pilE_{GC}</i>	This study
KS88	<i>pilE_{GC}::cat</i> <i>pilV_{GC}::kan</i> <i>iga::pilA_{Ft}</i>	This study
KS89	<i>pilE_{GC}::cat</i> <i>pilV_{GC}::kan</i> <i>iga::pilE_{Ft}</i>	This study
KS175	<i>pilE_{GC}::cat</i> <i>pilV_{GC}::kan</i> <i>iga::FTT0861_{Ft}</i>	This study
KS90	<i>pilE_{GC}::cat</i> <i>pilV_{GC}::kan</i> <i>iga::pilA_{Fn}</i>	This study
MW4	<i>recA6</i> (<i>kan</i>) <i>pilT_{ind}</i>	Wolfgang <i>et al.</i> (1998a)
GT17	<i>recA6</i> (<i>tetM</i>) <i>pilT::mTncm17</i>	Park <i>et al.</i> (2002)
GE31	<i>pilE_{GCind}</i> <i>pilT::mTncm17</i> <i>iga::pilE_{GC}</i>	Aas <i>et al.</i> (2007b)
KS91	<i>recA6</i> (<i>kan</i>) <i>pilE_{GCind}</i> <i>pilT::mTncm17</i> <i>iga::pilA_{Ft}</i>	This study

Table 1. cont.

Strain	Subspecies/alternative strain designation(s)/genotype/phenotype*	Source/reference
KS92	<i>recA6 (kan) pilE_{GCind} pilT::mTncm17 iga::pilE_{Ft}</i>	This study
KS93	<i>recA6 (kan) pilE_{GCind} pilT::mTncm17 iga::pilV_{Ft}</i>	This study
KS94	<i>recA6 (kan) pilE_{GCind} pilT::mTncm17 iga::FTT0861_{Ft}</i>	This study
KS95	<i>recA6 (kan) pilE_{GCind} pilT::mTncm17 iga::FTT0230_{Ft}</i>	This study
KS96	<i>recA6 (kan) pilE_{GCind} pilT::mTncm17 iga::pilA_{Fn}</i>	This study
GF101	<i>recA6 (kan) pilE_{GCind} pilF::cat iga::pilE_{GC}</i>	Aas <i>et al.</i> (2007b)
KS97	<i>recA6 (kan) pilE_{GCind} pilF::cat iga::pilA_{Ft}</i>	This study
KS98	<i>recA6 (kan) pilE_{GCind} pilF::cat iga::pilA_{Fn}</i>	This study
KS161	<i>recA6 (tetM) pilM::cat</i>	This study
KS162	<i>recA6 (kan) pilE_{GCind} pilM::cat iga::pilA_{Ft}</i>	This study
KS163	<i>recA6 (kan) pilE_{GCind} pilM::cat iga::pilA_{Fn}</i>	This study
KS164	<i>recA6 (tetM) pilN::cat</i>	This study
KS165	<i>recA6 (kan) pilE_{GCind} pilN::cat iga::pilA_{Ft}</i>	This study
KS166	<i>recA6 (kan) pilE_{GCind} pilN::cat iga::pilA_{Fn}</i>	This study
KS167	<i>pilE_{GCind} iga::pulG_{GF}</i>	This study
KS168	<i>pilE_{GCind} iga::pulG_{ATG}</i>	This study
KS169	<i>pilE_{GCind} iga::xcpT_{GF}</i>	This study
KS170	<i>pilE_{GCind} iga::xcpT_{ATG}</i>	This study
KS171	<i>pilE_{GCind} pilV_{GC}::kan iga::pulG_{GF}</i>	This study
KS172	<i>pilE_{GCind} pilV_{GC}::kan iga::pulG_{ATG}</i>	This study
KS173	<i>pilE_{GCind} pilV_{GC}::kan iga::xcpT_{GF}</i>	This study
KS174	<i>pilE_{GCind} pilV_{GC}::kan iga::xcpT_{ATG}</i>	This study
KS141	<i>pglA::kan</i>	Aas <i>et al.</i> (2007b)
KS180	<i>recA (tetM) pilE_{GC}::cat pglA::kan iga::pilA_{Ft}</i>	This study
KS181	<i>recA (tetM) pilE_{GC}::cat pglA::kan iga::pilA_{Fn}</i>	This study
GGC	<i>pglC::kan</i>	Hegge <i>et al.</i> (2004)
KS182	<i>recA (tetM) pilE_{GC}::cat pglC::kan iga::pilA_{Ft}</i>	This study
KS183	<i>recA (tetM) pilE_{GC}::cat pglC::kan iga::pilA_{Fn}</i>	This study
GGD	<i>pglD::kan</i>	Hegge <i>et al.</i> (2004)
KS184	<i>recA (tetM) pilE_{GC}::cat pglD::kan iga::pilA_{Ft}</i>	This study
KS185	<i>recA (tetM) pilE_{GC}::cat pglD::kan iga::pilA_{Fn}</i>	This study
KS142	<i>pglE_{ON}</i>	Aas <i>et al.</i> (2007b)
KS186	<i>recA (tetM) pilE_{GC}::cat pglE_{ON} iga::pilA_{Ft}</i>	This study
KS187	<i>recA (tetM) pilE_{GC}::cat pglE_{ON} iga::pilA_{Fn}</i>	This study
GGF	<i>pglF::kan</i>	Hegge <i>et al.</i> (2004)
KS188	<i>recA (tetM) pilE_{GC}::cat pglF iga::pilA_{Ft}</i>	This study
KS189	<i>recA (tetM) pilE_{GC}::cat pglF iga::pilA_{Fn}</i>	This study
KS144	<i>pglI::kan</i>	Aas <i>et al.</i> (2007b)
KS190	<i>recA (tetM) pilE_{GC}::cat pglI::kan iga::pilA_{Ft}</i>	This study
KS191	<i>recA (tetM) pilE_{GC}::cat pglI::kan iga::pilA_{Fn}</i>	This study
KS145	<i>pglO::kan</i>	Aas <i>et al.</i> (2007b)
KS192	<i>recA (tetM) pilE_{GC}::cat pglO::kan iga::pilA_{Ft}</i>	This study
KS193	<i>recA (tetM) pilE_{GC}::cat pglO::kan iga::pilA_{Fn}</i>	This study
Plasmids		
pCR2.1-TOPO	TOPO-cloning vector, Amp ^R Km ^R	Invitrogen
pCRII-TOPO	TOPO-cloning vector, Amp ^R Km ^R	Invitrogen
pCR4.0-TOPO	TOPO-cloning vector, Amp ^R Km ^R	Invitrogen
pUP6	pSSH6 plasmid with gonococcal uptake sequence	Wolfgang <i>et al.</i> (1999)
p2/16/1	pUP6 plasmid carrying the gene encoding IgA1 protease and an erythromycin-resistance cassette	Wolfgang <i>et al.</i> (2000)
pPilE2	p2/16/1 <i>iga::pilE_{GC}</i> Km ^R	Aas <i>et al.</i> (2006)
pEMS25	p2/16/1 <i>iga::pilA_{Fb}</i> Km ^R	This study
pEMS26	p2/16/1 <i>iga::pilE_{Fb}</i> Km ^R	This study
pEMS27	p2/16/1 <i>iga::pilV_{Fb}</i> Km ^R	This study
pEMS28	p2/16/1 <i>iga::FTT0861_{Fb}</i> Km ^R	This study
pEMS29	p2/16/1 <i>iga::FTT0230_{Fb}</i> Km ^R	This study

Table 1. cont.

Strain	Subspecies/alternative strain designation(s)/genotype/phenotype*	Source/reference
pEMS30	p2/16/1iga::FTT1314 _{FT} , Km ^R	This study
pEMS31	p2/16/1iga::pilA _{FT} , Km ^R	This study
pCHAP231	pBR322::(<i>pulS pulA-B pulC-O</i>), Amp ^R	d'Enfert & Pugsley (1987)
pMTWT	pMMB190::xcpT, Amp ^R	Durand <i>et al.</i> (2003)
pHW10	pCRII-TOPO::pilE _{ATG} pulG, Amp ^R Km ^R	This study
pHW11	pCRII-TOPO::pilE _{GF} pulG, Amp ^R Km ^R	This study
pHW12	pCRII-TOPO::pilE _{ATG} xcpT, Amp ^R Km ^R	This study
pHW13	pCRII-TOPO::pilE _{GF} xcpT, Amp ^R Km ^R	This study
pHW14	p2/16/1iga::pulG _{ATG} , Km ^R	This study
pHW15	p2/16/1iga::pulG _{GF} , Km ^R	This study
pHW16	p2/16/1iga::xcpT _{ATG} , Km ^R	This study
pHW17	p2/16/1iga::xcpT _{GF} , Km ^R	This study
pMCAT	pUP6 carrying the gonococcal <i>pilM</i> gene with chloramphenicol-resistance cassette inserted at amino acid A153, Km ^R Cm ^R	This study
pNCAT	pUP6 carrying the gonococcal <i>pilN</i> gene with chloramphenicol-resistance cassette inserted at <i>BclI</i> site 150 bp downstream of the start codon, Km ^R Cm ^R	This study

*Amp^R, ampicillin resistance marker; Km^R, kanamycin resistance marker; Cm^R, chloramphenicol resistance marker.

†All *Francisella* strains are from the *Francisella* Strain Collection at FOI, Umeå, and each strain has been given a strain collection number (FSC).

‡The *Francisella* subsp. *novicida* *pilA* gene (FTN_0415) has been denoted *pilA_{FT}*.

pilins between subsp. *tularensis* and subsp. *holarctica* (Fig. 1a). One locus containing three tandemly arrayed putative prepilin genes (*pilA_{FT}*, *pilE_{FT}* and *pilV_{FT}*) is rearranged in some avirulent subsp. *holarctica* strains, including the live vaccine strain (Forslund *et al.*, 2006; Svensson *et al.*, 2005). These rearrangements are associated with an in-frame deletion spanning the *pilA_{FT}* gene and the N-terminal-encoding part of *pilE_{FT}* (Svensson *et al.*, 2005).

Furthermore, the residual *pilE_{FT}* and *pilV_{FT}* genes are predicted to be pseudogenes in subsp. *holarctica* due to non-sense mutations (Fig. 1a). In addition, internal deletions and a mutation within the stop codon change the reading frame of FTT0861_{FT} (Larsson *et al.*, 2005) (Fig. 1a), thereby extending the ORF in subsp. *holarctica*. The two remaining prepilin-like ORFs, FTT0230_{FT} and FTT1314_{FT}, are identical between subsp. *tularensis* and *holarctica*.

Fig. 1. Comparison of gene clusters encoding putative Tfp pilins in *F. tularensis* subspecies *tularensis*, *holarctica* and *novicida*. (a) All *F. tularensis* subspecies have direct repeats (DRs) within the N-terminal part of the *pilA_{FT}* and *pilE_{FT}* genes (solid arrows). In some subsp. *holarctica* strains, the DRs have mediated deletion of the *pilA_{FT}* gene. *pilE_{FT}* and *pilV_{FT}* are not functional in subsp. *holarctica* due to non-sense mutations (arrowheads), and the FTT0861_{FT} pilin gene has an altered and extended reading frame due to a deletion event (Δ signs) causing mutations within the stop codon. (b) Alignment of the amino acid sequences of *F. tularensis* subsp. *tularensis* PilA_{FT} and subsp. *novicida* PilA_{FT}. The N-terminal 36 amino acids are identical while there are significant differences in the C-terminal part of these proteins. The solid arrow indicates the cleavage site of the prepilin.

Within the genome of subsp. *novicida* strain U112 (Rohmer *et al.*, 2007), most of the Tfp-related genes are found to be similar to the Tfp genes in the highly virulent subsp. *tularensis* strain SCHU S4. However, one prominent difference is in the *F. tularensis* equivalent *pilA*, *pilE* and *pilV* gene cluster. Here, the C-terminal half of the first gene, *pilA_{Ft}* (FTN_0415), is significantly different, while the N-terminal part remains homologous to SCHU S4 *pilA_{Ft}* (Fig. 1b). In addition, some sequence differences are also seen in the intergenic regions of the *pilAEV_{Ft}* cluster (Forsberg & Guina, 2007).

Expression of *Francisella* Tfp-like pilins in *N. gonorrhoeae*

Owing to the conservation and promiscuity of the biogenesis machineries, expression of Tfp pilins in heterologous species has been successfully used to analyse pilus structure–function relationships (Elleman *et al.*, 1986; Hoyne *et al.*, 1992; Sauvonnnet *et al.*, 2000; Winther-Larsen *et al.*, 2007). Here, we examined the propensities for the putative *Francisella* pilins to form Tfp in the heterologous Tfp expression system of *N. gonorrhoeae*, conditionally lacking the endogenous pilin subunit PilE (also denoted here as PilE_{GC}). To avoid potential problems related to heterologous expression levels, the *Francisella* pilin ORFs from subsp. *tularensis* strain SCHU S4 and *pilA_{Ft}* from subsp. *novicida* strain U112 were fused so as to create a glycine–phenylalanine junction to PilE_{GC}. Thus, the resulting translational fusion proteins carried the first seven residues of PilE_{GC}, but when processed by the PilD prepilin peptidase, the mature proteins are predicted to be entirely *Francisella*-derived. Despite repeated efforts, a translational fusion encoding the subsp. *tularensis* pilin FTT1314_{Ft} could not be obtained in *N. gonorrhoeae* due to accumulation of spontaneous mutations following transformation into *N. gonorrhoeae*. Immunoblot analysis verified that the remaining pilins, except for FTT0861_{Ft}, were expressed in the *N. gonorrhoeae* background (Fig. 2). Direct sequencing from genomic DNA, however, verified that the FTT0861_{Ft} clone was correct. All other pilins migrated as expected based on their calculated molecular masses, and the relative intensities of the signals varied between the antibody probes. The anti-PilE_{GC} antibodies were raised against the pilin subunits isolated from *N. gonorrhoeae* strain N400, and in a previous study it was suggested that the ability of heterologous Tfp antibodies to detect pilin subunits in immunoblotting involves exposure of a highly conserved epitope within or near the mature N-terminus following PilD cleavage (Patel *et al.*, 1991). The PilA_{Ft} antibody was raised against the C-terminal part of the PilA_{Ft} protein (Forslund *et al.*, 2006). Hence, this antibody is specific for the PilA_{Ft} pilin and would not recognize other pilins.

PilA from *F. tularensis* subsp. *tularensis* and subsp. *novicida* supports formation of Tfp-like structures in *N. gonorrhoeae*

As a first approach, transmission electron microscopy (TEM) was used to examine if the putative *Francisella*

Fig. 2. Expression of putative *Francisella* pilin genes in *N. gonorrhoeae*. Total bacterial lysates were separated by SDS-PAGE under denaturing conditions and proteins were identified by immunoblotting using anti-PilA_{Ft} (upper panel) or anti-PilE_{GC} (lower panel) antibodies. Strains used (lanes from left): MW24 (*pilE_{GC}*); GE2 (*iga::pilE_{GC}*), KS59 (*iga::pilA_{Ft}*), KS60 (*iga::pilE_{Ft}*), KS61 (*iga::pilV_{Ft}*), KS62 (*iga::FTT0861_{Ft}*), KS63 (*iga::FTT0230_{Ft}*), KS64 (*iga::pilA_{Ft}*).

pilins could form pilus-like filaments in *N. gonorrhoeae*. Structures resembling Tfp were only observed for the PilA_{Ft}-expressing strain (KS64) (Fig. 3) and at levels well below those seen for endogenous Tfp. In addition, these structures appeared as single filaments compared to the pilus bundles usually observed for wild-type gonococcal cells. Next, surface exposure of the pilin-like proteins was examined by immunofluorescence microscopy (IFM) using antibodies shown to be reactive in immunoblotting. Although no immunolabelling was detected using rabbit antibodies raised against PilA_{Ft}, antibodies raised against wild-type pili from *N. gonorrhoeae* strain N400 revealed the presence of Tfp-like appendages in strains expressing either PilA_{Ft} (KS64) or PilA_{Ft} (KS59) (Fig. 4a). No immunoreactive filaments were detected in the strains expressing PilE_{Ft} (KS60), PilV_{Ft} (KS61), FTT0861_{Ft} (KS62) or FTT0230_{Ft} (KS63) (Table 2 and IFM not shown). The immunoreactive filaments seen using the N400 Tfp antiserum in cells expressing PilA_{Ft} and PilA_{Ft} differed from those seen in the wild-type *N. gonorrhoeae* background. First, the numbers of fluorescent appendages were considerably fewer in the *Francisella* PilA-expressing backgrounds (Table 2). Second, the appendages were longer than the short pilus bundles observed in the wild-type background. To determine if the filaments seen for *Francisella* PilA by IFM were directly related to those detected by TEM, immunogold-labelling TEM using the N400 PilE_{GC} antiserum was carried out. As the appendages were specifically decorated with gold particles (Fig. 4b), we propose that the structures observed by TEM are the same as those detected by IFM.

In an attempt to confirm the composition of the structures seen for the PilA_{Ft} and PilA_{Ft} backgrounds, we carried out standard pilus purification schemes (Winther-Larsen *et al.*, 2007; Wolfgang *et al.*, 1998a). However, no pili were

Fig. 3. Piliation of wild-type and mutant gonococcal strains expressing *pilA* from *Francisella* subsp. *tularensis* and subsp. *novicida*, analysed by negative-stained TEM. Strains: GE2 (wt, *iga::pilE_{GC}*); KS59 (*iga::pilA_{Ft}*) and KS64 (*iga::pilA_{Fn}*).

recovered using these protocols. It has previously been demonstrated that levels of detectable *P. aeruginosa* PilA_{PAK}-derived pili expressed in *N. gonorrhoeae* can be dramatically increased in a mutant background lacking the PilT retraction ATPase (Winther-Larsen *et al.*, 2007). However, the use of this background in these instances had no influence on the levels of appendages detected by IFM, TEM or purification attempts (Supplementary Figs S1 and S2 and data not shown, respectively).

Evidence for *Francisella* PilA protein glycosylation in *N. gonorrhoeae*

Although epitope sharing between unrelated Tfp pilins has been documented in Western-type immunoblotting experi-

ments using heterologous anti-Tfp antisera, it seemed curious that antibodies to strain N400 Tfp would react with the PilA_{Fn}- and PilA_{Ft}-associated surface structures in *N. gonorrhoeae*. A recent study documented that immunization of rabbits with endogenous N400 Tfp results in antibodies that recognize epitopes associated with the galactose-2,4-diacetamido-2,4,6-trideoxyhexose (Gal-DATDH) disaccharide linked at serine 63 of PilE_{GC} (Vik *et al.*, 2009). There is also indirect evidence that PilA_{Ft} is post-translationally modified in its endogenous host (Forslund *et al.*, 2006). To determine if PilA_{Ft} and PilA_{Fn} might be *O*-glycosylated in *N. gonorrhoeae*, we examined the relative motilities of the proteins in defined *N. gonorrhoeae* protein glycosylation (*pgl*) mutants and variants (Aas *et al.*, 2007a). Null mutations in core genes essential for protein glycosylation (*pglC*, *pglD*, *pglF* and *pglO*)

Fig. 4. IFM of *N. gonorrhoeae* expressing various pilin genes from *Francisella tularensis*. Gonococcal cells (green) and Tfp-like structures (red) were detected using indirect IFM. Anti-PilE_{GC} specific antibodies (lot 904) were used to detect the Tfp-like structures. Strains: GE2 (*iga::pilE_{GC}*), MW24 (*pilE_{GC}*), KS59 (*iga::pilA_{Ft}*) and KS64 (*iga::pilA_{Fn}*). Note that the appearance of Tfp seen here is quite similar to that seen using the same type of technique for *E. coli* cells expressing the PulG pseudopilus as bundles (Vignon *et al.*, 2003). (b) Piliation of gonococcal strain KS64 (*iga::pilA_{Fn}*) detected by immunogold labelling and TEM using anti-PilE_{GC} (lot 904) antibodies (10 nm gold particles).

in *N. gonorrhoeae* were associated with an increase in PilA_{Fn} migration, while in a *pglA* mutant background in which only the DATDH monosaccharide is linked to proteins, the migration was intermediate between that seen in the wild-type and null mutants (Fig. 5a). Furthermore, dramatically retarded migration was observed in a *pglE_{ON}* variant background in which a Gal-Gal-DATDH trisaccharide is linked to proteins. Virtually identical results were seen for the behaviour of PilA_{Ft}, with the exception of the *pglE_{ON}* variant background, in which four distinct antigenic forms migrating more slowly than the form seen in the glycosylation null mutants were detected. Taken together, these findings suggest

that *Francisella* PilA proteins undergo glycosylation in *N. gonorrhoeae*. We then tested the potential influence of protein glycosylation on the ability of the antibodies to react with the PilA-associated appendages by IFM. For both the *Francisella* PilA_{Fn} and PilA_{Ft} proteins, the glycosylation null mutation *pglC* abrogated the labelling of the structures (Fig. 5b). However, the absence of PglC did not affect surface organelle expression, as Tfp-like structures were still visible by TEM in the PilA_{Fn}-expressing *pglC* mutant strain (Fig. 5c).

PilA from *F. tularensis* subsp. *tularensis* and subsp. *novicida* supports competence for natural genetic transformation in *N. gonorrhoeae*

It has previously been demonstrated that the expression of *P. aeruginosa* pilin PilA_{PAK} can partially complement a defect in competence for natural transformation in a gonococcal strain lacking its endogenous pilin (Aas *et al.*, 2002). Using the same strategy, we examined whether *Francisella* pilins could function in competence for transformation in an otherwise unpiliated *N. gonorrhoeae* background. Only the strain expressing PilA_{Fn} from subsp. *novicida* (KS72) supported transformation levels just above that seen for the unpiliated control strain (Table 2). It should be noted that compared to growth on plates, strains expressing PilV_{Ft} (KS69) and pilin FTT0230_{Ft} (KS71) grew poorly in liquid culture, a property which may have a negative impact on the transformation phenotype for these strains. In a study by Aas *et al.* (2002), two pilin-like proteins, Comp_{GC} and PilV_{GC}, were shown to influence the transformation levels, but not Tfp expression, in *N. gonorrhoeae*. Whereas Comp_{GC} is essential for natural transformation, and its elevated expression leads to enhanced transformation frequencies, PilV_{GC} inhibits transformability by antagonizing CompP function. As such, *pilV_{GC}* mutants phenocopy CompP-overexpressing strains (*comp_{Poe}*). When tested in *N. gonorrhoeae comp_{Poe}* or *pilV_{GC}* backgrounds, the expression of PilA_{Fn} led to an over 500-fold increase in transformation frequency (Table 2). PilA_{Ft} expression also led to an increase in transformability, but at more modest levels (20–40-fold). None of the other four *Francisella* pilins altered transformability in either of the two backgrounds. As noted previously, the expression of both PilV_{Ft} (KS75, KS83) and FTT0230_{Ft} (KS77, KS85) was associated with poor growth in liquid culture, which could potentially also affect the results here.

The three sets of transformation experiments described above were all conducted in a genetic background where the expression of the endogenous gonococcal pilin was under the control of the *lac* promoter, although non-induced. Studies have shown that very low levels of PilE_{GC} are sufficient for high-level transformability (Aas *et al.*, 2007b; Long *et al.*, 2001). Therefore, it was possible that physiological alterations associated with high-level expression of *Francisella* pilins might perturb levels of LacI repressor, allowing residual expression of PilE_{GC}. To address this concern, the PilA_{Fn}- and PilA_{Ft}-expressing

Table 2. *Francisella* PilA pilin supports genetic transformation but not twitching motility in *N. gonorrhoeae*

Relevant genotype	Piliation (%) [*]	Transformation frequency (% of recipient cells)				Twitching motility [†]
		†	<i>compP_{oe}</i> [‡]	<i>pilV_{GC}</i> [§]	<i>pilV_{GC}</i> <i>pilE_{GC}::cat</i>	
–	0	<0.001	<0.001	<0.001	<0.001	–
<i>iga::pilE_{GC}</i>	28 ± 12	8.21 ± 4.8	25.5 ± 12	27.9 ± 9.5	26 ± 3.5	+
<i>iga::pilA_{Ft}</i>	0.5 ± 0.1	<0.001	0.04 ± 0.02	0.02 ± 0.01	0.02 ± 0.01	–
<i>iga::pilE_{Ft}</i>	0	<0.001	<0.001	<0.001	<0.001	–
<i>iga::pilV_{Ft}</i>	0	<0.001	<0.001	<0.001	ND	–
<i>iga::FTT0861_{Ft}</i>	0	<0.001	<0.001	<0.001	<0.001	–
<i>iga::FTT0230_{Ft}</i>	0	<0.001	<0.001	<0.001	ND	–
<i>iga::pilA_{Fn}</i>	9 ± 6	0.003 ± 0.001	2.24 ± 0.5	0.86 ± 0.25	1.42 ± 0.4	–
<i>iga::pulG_{GF}</i>	ND	<0.001	ND	<0.001	ND	ND
<i>iga::pulG_{ATG}</i>	ND	<0.001	ND	<0.001	ND	ND
<i>Iga::xcpT_{GF}</i>	ND	<0.001	ND	<0.001	ND	ND
<i>iga::xcpT_{ATG}</i>	ND	<0.001	ND	<0.001	ND	ND

ND, Not determined.

^{*}Piliation detected by IFM from at least five sets of experiments. Percentage calculated by counting the number of pili per 600 gonococcal cells (strains: KS101, GE101, KS67–KS72).

[†]Determined for *N. gonorrhoeae* strains with *pilE_{GC}* under the control of an inducible promoter (strains: KS101, GE101, KS67–KS72, KS167–KS170). *pilE_{GC}* was not induced during the experiments.

[‡]Same as [†], but carried out in a *CompP*-overexpressed background (strains: GP111, GP122, KS73–KS78).

[§]Same as [†], but carried out in a *pilV_{GC}* mutant background (strains: KS79–KS86, KS171–KS174).

^{||}Same as [§], but with *pilE_{GC}* mutated by insertion of a chloramphenicol-resistance cassette (strains: KS44, KS87–KS90, KS175, KS199).

pilV_{GC} strains were re-engineered so that the inducible *pilE_{GC}* allele was disrupted by a chloramphenicol-resistance gene cassette (Hegge *et al.*, 2004). The transformation frequency was essentially unaltered in this background compared to the *pilV_{GC}* inducible *pilE_{GC}* parent strains (Table 2) proving that residual *PilE_{GC}* expression could not account for the enhanced transformation frequencies.

As dynamic polymers, Tfp undergo rounds of extension and retraction events that are commonly a prerequisite for Tfp-associated genetic transformation (Wolfgang *et al.*, 1998a). In *N. gonorrhoeae*, the ability to undergo twitching motility on surfaces is directly correlated with pilus retraction activity (Merz *et al.*, 2000). However, none of the *Francisella* pilins supported twitching motility when assessed by microscopic examination for movement at the periphery of colonies or by the slide culture method (Table 2).

Expression of the T2SS pseudopilin XcpT from *P. aeruginosa* or PulG from *K. oxytoca* does not support genetic transformation in *N. gonorrhoeae*

Both the transformation and microscopy data noted above support the hypothesis that *PilA_{Ft}* and *PilA_{Fn}* are Tfp pilins. It is important to note, however, that T2SS proteins of the PulG family can, when overexpressed, result in the formation of pseudopili with an appearance similar to Tfp

(Durand *et al.*, 2003; Vignon *et al.*, 2003). Few studies, if any, have explored whether the expression of T2SS pseudopilins can support Tfp-associated functions. To examine this possibility in more detail, the T2SS proteins PulG and XcpT from *K. oxytoca* and *P. aeruginosa*, respectively, were cloned and expressed in *N. gonorrhoeae*. Like the *Francisella* pilins, both were fused at the prepilin peptidase processing site in addition to fusions generated at the putative ATG translational start codons. The expression of both PulG and XcpT in *N. gonorrhoeae* was verified using antibodies specific for each protein (Supplementary Fig. S3). The transformation assay revealed that neither PulG nor XcpT supported genetic transformation in *N. gonorrhoeae* in either a wild-type or *pilV_{GC}* mutant background (Table 2). Moreover, no pilus-like filamentous structures could be detected by TEM (data not shown).

Expression of *Francisella* PilA-associated appendages depends on Tfp biogenesis factors

The expression of Tfp depends on several canonical Tfp assembly factors (Carbonnelle *et al.*, 2006; Martin *et al.*, 1995; Nudleman *et al.*, 2006) that are unique to Tfp systems as they are lacking in T2SSs. We expressed both *PilA_{Ft}* and *PilA_{Fn}* in a selection of mutant backgrounds lacking such components, including *PilF_{GC}*, *PilM_{GC}* or *PilN_{GC}*, to investigate whether the *Francisella*-associated

Fig. 5. Influence of the *N. gonorrhoeae* glycosylation mutants on PilA_{Ft} and PilA_{Fn}. (a) Influence on relative migration of PilA_{Ft} and PilA_{Fn}. Immunoblot of whole-cell lysates using anti-PilA_{PAK} antibodies. The strains used were as follows. Upper panel: first lane MW24 (*pilE_{GC}*), and then *iga::pilA_{Fn}* (KS64), *iga::pilE_{GC}* (GE2), *pglC* (KS183), *pglD* (KS185), *pglA* (KS181), *pglI* (KS191), *pglE_{ON}* (KS187), *pglF* (KS189), *pglO* (KS193). Lower panel: first lane MW24 (*pilE_{GC}*), and then *iga::pilA_{Ft}* (KS59), *iga::pilE_{GC}* (GE2), *pglC* (KS182), *pglD* (KS184), *pglA* (KS180), *pglI* (KS190), *pglE_{ON}* (KS186), *pglF* (KS188), *pglO* (KS192). (b) Influence on the cross-reactivity of *Francisella* PilA pilins with anti-PilE_{GC} antibodies (lot 904). Gonococcal cells (green) and Tfp-like structures (red) were detected using indirect IFM. Strains: *iga::pilA_{Ft}* (KS59), *iga::pilA_{Fn}*, *pglC* (KS182), *iga::pilA_{Fn}* (KS64), *iga::pilA_{Fn}*, *pglC* (KS183). Note that the appearance of Tfp seen here is quite similar to that seen using the same type of technique for *E. coli* cells expressing the PulG pseudopilus (Vignon *et al.*, 2003). (c) Piliation analysis by negative-stained TEM of strain *iga::pilA_{Fn}*, *pglC* (KS183). Scale bars, 200 nm.

DISCUSSION

As previously shown, homologues of Tfp structural and biogenesis components influence the pathogenesis of *F. tularensis* subsp. *holarctica* and subsp. *novicida* infections (Forslund *et al.*, 2006; Zogaj *et al.*, 2008). There are clear polymorphisms and gross differences in the content of genes encoding these components that correlate with the relative virulence of *Francisella* species (Forslund *et al.*, 2006). However, there remains a large gap in our knowledge of how these components function in shaping pathogenic potential. With the aim of beginning to resolve these issues, we conducted trans-species complementation studies and found that one particular class of pilin-like proteins in subsp. *tularensis* and *novicida* can complement Tfp-related features and functions in a strain of *N. gonorrhoeae* lacking its endogenous pilin. Despite their orthologous nature, however, PilA_{Ft} and PilA_{Fn} behaved differently in this background. While expression of both pilins individually was associated with the expression of Tfp-like appendages detectable by IFM, the levels of the appendages were lower in the PilA_{Ft}-expressing strain. Consistent with these differences, TEM detected Tfp-like organelles only in the PilA_{Fn}-expressing strain. These findings were paralleled by dramatic differences in the abilities of these two pilin-like proteins to support competence for natural genetic transformation. In backgrounds optimized to enhance DNA binding and uptake, transformability in the PilA_{Fn}-expressing strain was increased nearly 1000-fold relative to the negative control background and 50-fold relative to the isogenic PilA_{Ft}-expressing strain. Interestingly, competence for transformation has been reported for subsp. *novicida* (Gallagher *et al.*, 2008; Tyeryar & Lawton, 1970). The significance of this results in relation to our data is difficult to interpret since the components required for DNA uptake in subsp. *novicida* have yet to be characterized. It is

filament requires the endogenous *N. gonorrhoeae* Tfp assembly machinery. PilM_{GC} and PilN_{GC} are Tfp assembly factors that act in unknown capacities (Carbonnelle *et al.*, 2006), while PilF_{GC} (PilB in *P. aeruginosa*) is believed to be the subunit polymerization ATPase (O'Toole & Kolter, 1998). Pilus expression was abolished in all three biogenesis-defective mutant backgrounds (Supplementary Fig. S4).

important to note here that all available data back the notion that transformability in *N. gonorrhoeae* absolutely requires an intact Tfp extension/retraction system including a major pilin subunit (Wolfgang *et al.*, 1998a). We hypothesize accordingly that the appendages seen by IFM and TEM are in fact filamentous polymers made up of PilA_{Fn} and are structural equivalents of bona fide Tfp. This proposal is supported by the fact that antibodies raised against endogenous Tfp (composed of PilE_{GC}) that detect PilA_{Fn} protein in immunoblotting also react with the PilA_{Fn}-associated structures as seen by IFM and TEM. This proposal is further backed by the observations that these structures are not seen in cells individually lacking PilM_{GC}, PilN_{GC} and PilF_{GC}, which are associated specifically with the Tfp assembly system, as they have no counterparts in related T2SSs.

A shortcoming in determining if the structures seen in gonococci are Tfp has been the inability to purify the appendages and characterize their composition. This same situation confounds previous studies examining the relationships between Tfp-related components and the expression of extracellular appendages in both *F. tularensis* subsp. *holarctica* and subsp. *novicida*. Zogaj *et al.* (2008) identified pilus-like appendages in subsp. *novicida* and also studied the presence or absence of these structures in a variety of mutant backgrounds. Based on indirect genetic data, the authors proposed that PilE4 (orthologous to what is termed FTT0861 in this work) is the major structural subunit of Tfp. Somewhat surprisingly, the appendages were seen independently of the expression of homologues of the PilQ secretin and PilC inner-membrane protein families (*P. aeruginosa* nomenclature) that are essential for Tfp expression in most species. Similar studies of the live vaccine strain, LVS (subsp. *holarctica*), have concluded that this strain also expresses Tfp-like structures (Chakraborty *et al.*, 2008). It is difficult to assess the potential relevance/applicability of our results to the work with the LVS, as that strain carries a mutation that inactivates expression of PilA_{Fn}, the pilin-like protein we examined. In the case of subsp. *novicida*, however, our findings increase the possibility that PilA_{Fn} is the major structural component of Tfp. All studies so far have failed to purify and structurally characterize these Tfp-like structures. Therefore, it is not possible to establish which pilin is the major subunit of Tfp in the different subspecies and strains studied. However, our results provide evidence that pilin-like proteins from *Francisella* are capable of interacting with components of an established Tfp system. Additionally, comparative studies of these particular systems have a unique potential to increase our understanding of the basic genotype–phenotype relationships underlying Tfp-related biology. For example, *F. tularensis* subspecies appear to lack a homologue of the ‘canonical’ PilM Tfp assembly factor present in virtually all other type IVa pilin Tfp-expressing species (Gil *et al.*, 2004; Larsson *et al.*, 2005). Moreover, the *pilT* genes (encoding the pilus retraction ATPase in Tfp systems) in subsp. *holarctica* strains were reported to carry identical

nonsense mutations leading to a truncated protein which, based on all available data on this class of ATPases, would lead to disrupted function (Satyshur *et al.*, 2007). Similarly, the *pilE4* genes (orthologous to the gene encoding FTT0861) of subsp. *holarctica* strains carry identical frame-shift mutations leading to dramatic structural alterations in the C-terminal segment of the ORF. These latter findings are consistent with the idea that the functions served by the corresponding products are no longer associated with a fitness advantage to these strains and that the genes are undergoing decay. Thus, changes in Tfp-related genes and their products may be implicated in evolutionary events within *Francisella* species.

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