



The role of the *Francisella tularensis* pathogenicity island in type VI secretion, intracellular survival, and modulation of host cell signaling

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Francisella tularensis is a highly virulent gram-negative intracellular bacterium that causes the zoonotic disease tularemia. Essential for its virulence is the ability to multiply within host cells, in particular monocytic cells. The bacterium has developed intricate means to subvert host immune mechanisms and thereby facilitate its intracellular survival by preventing phagolysosomal fusion followed by escape into the cytosol, where it multiplies. Moreover, it targets and manipulates numerous host cell signaling pathways, thereby ameliorating the otherwise bactericidal capacity. Many of the underlying molecular mechanisms still remain unknown but key elements, directly or indirectly responsible for many of the aforementioned mechanisms, rely on the expression of proteins encoded by the *Francisella* pathogenicity island (FPI), suggested to constitute a type VI secretion system. We here describe the current knowledge regarding the components of the FPI and the roles that have been ascribed to them.

Keywords: *Francisella tularensis*, phagosomal escape, intracellular growth, FPI, T6SS

INTRODUCTION

Francisella tularensis is a highly infectious gram-negative intracellular bacterium that causes the disease tularemia (Oyston et al., 2004). Two main subspecies, *F. tularensis* subsp. *tularensis* and *F. tularensis* subsp. *holarctica* (also known as type A and type B, respectively), are responsible for the majority of infections and disease, which are particularly severe if caused by type A strains. Importantly, *F. tularensis* has the ability to infect a multitude of hosts, including mammals, fish, amphibians, protozoa and arthropods (reviewed in Mörner and Addison, 2001; Keim et al., 2007). Although primarily a vector-transmitted disease, *F. tularensis* infections can also occur through inhalation of contaminated materials, ingestion and skin abrasions (Dennis et al., 2001). From the site of infection, the bacteria will spread to regional lymph nodes, liver and spleen, where they rapidly grow and overwhelm the host's immune defenses (Fortier et al., 1991).

The pathogenicity of *F. tularensis* is believed to depend on its ability to replicate within host cells, particularly macrophages (Tärnvik, 1989). *F. tularensis* enters host macrophages by triggering the formation of asymmetric, spacious pseudopod loops (Clemens et al., 2005). It then modifies the endocytic pathway of the macrophage, preventing phagolysosomal fusion, upon which it escapes into the cytosol and multiplies (Golovliov et al., 2003a; Clemens et al., 2004; Santic et al., 2005a). Several factors that regulate or facilitate phagosome escape and intracellular survival of *F. tularensis* have been identified (Dean et al., 2009; Meibom and Charbit, 2009; Qin et al., 2009; Mohapatra et al., 2010), however, the underlying molecular mechanisms still remain largely unknown. A gene cluster frequently encountered in screens aimed at identifying elements important for intracellular growth and/or virulence, is the *Francisella* pathogenicity island (FPI; reviewed in Nano et al., 2004; Nano and Schmerk, 2007; Meibom and Charbit, 2009), which

has been suggested to encode a type VI secretion system (de Bruin et al., 2007; Nano and Schmerk, 2007; Bingle et al., 2008; Ludu et al., 2008; Barker et al., 2009; Bröms et al., 2009).

Note: *F. tularensis* subsp. *novicida* will be referred to as *F. novicida* and the FPI nomenclature proposed by Ludu et al. (2008) will be used throughout (Table 1; Figure 1).

THE FRANCISELLA PATHOGENICITY ISLAND DUPLICATION OF THE FPI IN STRAINS OF *F. TULARENSIS*

The 33-kb pathogenicity island, designated FPI (Figure 1), consists of 16–19 ORFs and its crucial role in the virulence of *F. tularensis* is well established (Golovliov et al., 2003b; Nano et al., 2004; Twine et al., 2005; Tempel et al., 2006; Santic et al., 2007, 2009; Weiss et al., 2007; Bönquist et al., 2008; Ludu et al., 2008; Vonkavaara et al., 2008; Bröms et al., 2009; Kraemer et al., 2009; Schmerk et al., 2009b; Åhlund et al., 2010; Bröms et al., unpublished). It was first identified in 2004 (Nano et al., 2004) by bioinformatic analysis of the partly sequenced genomes of *F. tularensis* subsp. *tularensis* strain Schu S4 (Karlsson et al., 2000; Prior et al., 2001) and *F. novicida* strain U112 (Nano et al., 2004). All subsequently sequenced *Francisella* genomes have confirmed the presence of the FPI, suggesting that it is part of the core genome (Larsson et al., 2009). However, in contrast to the rest of the genome, the FPI has lower G + C content, indicating horizontal gene transfer (Nano et al., 2004).

Interestingly, the FPI is duplicated in all the subsp. of *F. tularensis* (*F. tularensis* subsp. *holarctica*, *mediasiatica*, and *tularensis*), but is present in a single copy in *F. novicida* and *F. philomiragia* (Nano et al., 2004; Larsson et al., 2009).

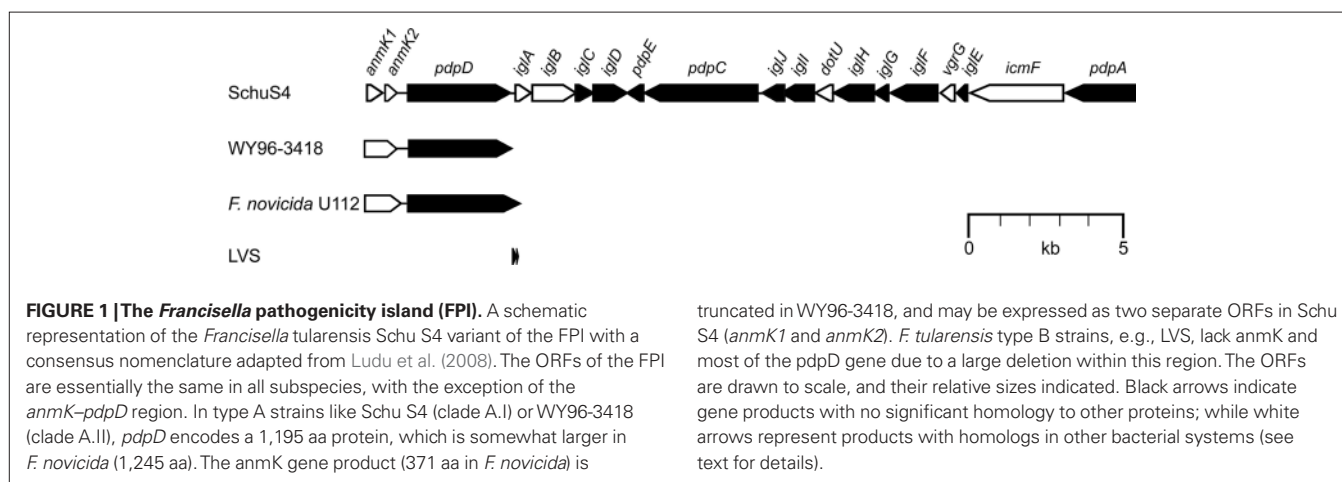
Larsson et al. (2009) recently suggested that this duplication event was facilitated by the insertion of ISFtu1 insertion sequence element adjacent to the FPI and an rRNA operon of an ancestral genome,

Table 1 | FPI gene nomenclature.

Strains and FPI gene loci ¹					Gene names and references ²			
Schu S4		LVS		U112	Nano et al. (2004)	Nano and Schmerk (2007)	Ludu et al. (2008)	Barker et al. (2009)
Locus 1	Locus 2	Locus 1	Locus 2	Locus 1				
FTT1699	FTT1344	FTL_0126	FTL_1172	FTN_1309	<i>pdpA</i>	<i>pdpA</i>	<i>pdpA</i>	<i>pdpA</i>
FTT1700	FTT1345	FTL_0125	FTL_1171	FTN_1310	<i>pdpB</i>	<i>pdpB</i>	<i>pdpB/icmF</i>	<i>pdpB/icmF</i>
FTT1701	FTT1346	FTL_0124	FTL_1170	FTN_1311	–	<i>pigA</i>	<i>iglE</i>	<i>iglE</i>
FTT1702	FTT1347	FTL_0123	FTL_1169	FTN_1312	–	<i>pigB</i>	<i>vgrG</i>	<i>vgrG</i>
FTT1703	FTT1348	FTL_0122	FTL_1168	FTN_1313	–	<i>pigC</i>	<i>iglF</i>	<i>iglF/clpV</i>
FTT1704	FTT1349	FTL_0121	FTL_1167	FTN_1314	–	<i>pigD</i>	<i>iglG</i>	<i>iglG</i>
FTT1705	FTT1350	FTL_0120	FTL_1166	FTN_1315	–	<i>pigE</i>	<i>iglH</i>	<i>iglH</i>
FTT1706	FTT1351	FTL_0119	FTL_1165	FTN_1316	–	<i>pigF</i>	<i>dotU</i>	<i>dotU</i>
FTT1707	FTT1352	FTL_0118	FTL_1164	FTN_1317	–	<i>pigG</i>	<i>iglI</i>	<i>iglI</i>
FTT1708	FTT1353	FTL_0117	FTL_1163	FTN_1318	–	<i>pigH</i>	<i>iglJ</i>	<i>iglJ</i>
FTT1709	FTT1354	FTL_0116	FTL_1162	FTN_1319	<i>pdpC</i>	<i>pdpC</i>	<i>pdpC</i>	<i>pdpC</i>
FTT1710	FTT1355	FTL_0115	FTL_1161	FTN_1320	–	<i>pigI</i>	<i>pdpE</i>	<i>pdpE/hcp</i>
FTT1711	FTT1356	FTL_0114	FTL_1160	FTN_1321	<i>iglD</i>	<i>iglD</i>	<i>iglD</i>	<i>iglD</i>
FTT1712	FTT1357	FTL_0113	FTL_1159	FTN_1322	<i>iglC</i>	<i>iglC</i>	<i>iglC</i>	<i>iglC</i>
FTT1713	FTT1358	FTL_0112	FTL_1158	FTN_1323	<i>iglB</i>	<i>iglB</i>	<i>iglB</i>	<i>iglB</i>
FTT1714	FTT1359	FTL_0111	FTL_1157	FTN_1324	<i>iglA</i>	<i>iglA</i>	<i>iglA</i>	<i>iglA</i>
FTT1715	FTT1360	FTL_0110, FTL_0109	FTL_1156, FTL_1155	FTN_1325	<i>pdpD</i>	<i>pdpD</i>	<i>pdpD</i>	<i>pdpD</i>
FTT1716	FTT1361	–	–	FTN_1326	<i>pmcA</i>	<i>pmcA</i> (<i>pmcA1</i> , <i>pmcA2</i> in Schu S4)	<i>anmK</i>	<i>anmK</i>

¹Adapted from <http://www.francisella.org/>

²*anmK* (anhydro-N-acetylmuramic acid kinase); *clpV* (virulent strain); *dot* (defect in organelle trafficking); *hcp* (hemolysin co-regulated protein); *icm* (intracellular multiplication); *igl* (intracellular growth locus); *pdp* (pathogenicity determinant protein); *pig* (pathogenicity island gene); *pmcA* (plasma membrane Ca²⁺ ATPase); *vgr* (valine-glycine repeat).



which enabled duplication of the FPI by non-reciprocal recombination. Interestingly, as deduced from the genomic locations of the units, one of them was identified as ancestral whereas the second, more recently acquired, has integrated in various locations in the different genomes. As a consequence of the duplication, FPI null mutants are easier to generate in *F. novicida*, which is also the species most extensively used to analyze the role of individual FPI proteins.

Noteworthy, there is a gene cluster in *F. novicida*, encompassing ORFs FTN_0040–FTN_0052, which show homology to the FPI, albeit rudimentary, and with a different organization of the “true” FPI genes. The similarities are particularly pronounced for the core components *IglA*, *IglB*, *PdpB/IcmF*, and *DotU* where FTN_0042, FTN_0043, FTN_0040, and FTN_0051, respectively, show high (*IglA* and *IglB*) or intermediate (*PdpB* and *DotU*) protein sequence

homology (Larsson et al., 2009). Further, protein-BLAST (<http://blast.ncbi.nlm.nih.gov/>) reveals that there are genes encoding products with weak homology to PdpA, PdpC, PdpD, IglD, IglH, and IglI, interspersed with ORFs that reveal no FPI similarity. This gene cluster has not been widely discussed, nor its functionality investigated and its role in *F. novicida* virulence remains unknown.

Interestingly, combined work by several groups suggests that the second FPI locus may be functionally redundant in strains of *F. tularensis*, as transposon or deletion mutants for which only one of the two gene copies have been targeted are unaffected for virulence (Golovliov et al., 2003b; Kawula et al., 2004; Qin and Mann, 2006; Nano and Schmerk, 2007), in contrast to mutants for which both gene copies have been removed by targeted mutagenesis (Golovliov et al., 2003b; Bönquist et al., 2008; Bröms et al., 2009). In fact, Schu S4 can tolerate the loss of one of its two FPI loci (targeted deletions of regions encompassing FTT_1699–FTT_1715 or FTT_1344–FTT_1360), without substantial loss of virulence toward mice infected by the intradermal route (A. Sjöstedt and W. Conlan, unpublished). Contradicting these results is a study by Su et al. (2007), which suggests that both gene copies of *iglA*, *iglB*, or *iglC* are required for full virulence of LVS in a mouse model of respiratory tularemia and a study by Maier et al., demonstrating that *HimarFT* insertion mutants in one of the *iglB*, *iglC*, or *iglD* loci result in only partial loss of J774 monolayer integrity upon infection, in contrast to when parental LVS was used (Maier et al., 2007). Thus, it cannot be ruled out that the duplication event, and hence doubling of gene dosage, may provide some benefit to the pathogenic *F. tularensis* type A and B strains, and that this may have contributed to their differentiation from the human non-pathogenic, or rarely pathogenic, *F. novicida* and *F. philomiragia* strains, although there is little data to support this. It should be noted that FPI mutants even in highly virulent subspecies of *F. tularensis*, e.g., in the Schu S4 strain, are avirulent. Thus, despite that one CFU of the wild-type strain kills mice irrespective of route of inoculation, the lethal dose of the *iglB*, *iglC*, and *iglD* Schu S4 mutants are $\gg 10^7$ CFU. Presumably, the lack of phagosomal escape renders them avirulent (Twine et al., 2005; Kadzhaev et al., 2009). Moreover, they are non-immunogenic since mice infected with the *iglC* mutant of Schu S4 are not protected against a subsequent infection with a subspecies *tularensis* strain (Twine et al., 2005).

GENETIC DIFFERENCES WITHIN THE FPI CLUSTER

The ORFs of the FPI are essentially conserved in all subspecies, with the exception of the *anmK*–*pdpD* region (Figure 1). In *F. novicida*, *anmK* (originally denoted *pmcA*) is suggested to encode a 371 aa gene product, AnmK, which shows high identity to members of COG2377 (predicted molecular chaperone distantly related to HSP70-fold metalloproteases) (Nano and Schmerk, 2007). However, in type A strains like Schu S4 and FSC033 (clade A.I), *anmK* may be expressed as two separate ORFs due to the presence of premature stop codons at positions 190 and 328, followed by an ATG start codon at position 194. In type A strains like WY96-3418 and B38 (clade A.II), AnmK has a premature stop codon at position 328, suggesting that this group encodes a truncated form of AnmK (Nano and Schmerk, 2007). How these genetic differences affect AnmK function has not been addressed so far, but AnmK of *F. novicida* has been shown to contribute to virulence, albeit to a small degree

(Nano and Schmerk, 2007; Ludu et al., 2008). Supporting the notion that AnmK is not critical for *Francisella* pathogenicity is the fact that *F. tularensis* type B strains, virulent strains as well as LVS, lack the *anmK* gene due to a deletion that extends from 107 bp upstream of *anmK* and encompasses most of the adjacent *pdpD* gene (Nano et al., 2004; Champion et al., 2009). In *F. novicida*, *pdpD* encodes a 1,245 aa (1,195 aa in type A strains) outer membrane protein that has been shown to be important for virulence in mice (Ludu et al., 2008), while the deletion within type B strains is presumed to result in two minor ORFs (corresponding to residues 992–1124 and 1129–1195 of PdpD from type A strains) that are likely to be non-functional (Ludu et al., 2008; Champion et al., 2009). It has been speculated that the presence or absence of *pdpD* may account, in part, for the strain-to-strain specific difference in virulence between type A and type B strains (Nano et al., 2004).

FPI NOMENCLATURE – NAMING OF COMPONENTS

Over the years, the nomenclature of the FPI genes has been altered in attempts to adapt to the increasing knowledge of the function of individual proteins (Table 2). Already in 1997, the first FPI gene (later named *iglC*) was identified, and its expression shown to be prominently induced during intracellular growth (Golovliov et al., 1997). The gene was later recognized as a member of a four gene operon, which, due to its requirement for intramacrophage growth, was denoted *iglABCD* (*intracellular growth locus*) (Gray et al., 2002; Golovliov et al., 2003b, Figure 1; Tables 1 and 2). In 2004, at the time of the discovery of the FPI, a handful of the predicted ORFs were named, including *pmcA* (today *anmK*), which share homology to molecular chaperones, and the large ORFs *pdpA*, *pdpB*, *pdpC*, and *pdpD* (*pathogenicity determinant protein*) (Nano et al., 2004; Table 1). Later, *pdpB* was named IcmF, based on its homology to this crucial T6SS core component (Ludu et al., 2008). In 2007, the remaining ORFs of the island were recognized as most likely functional genes and thus denoted *pigA* to *I* (*pathogenicity island gene*) (Nano and Schmerk, 2007; Table 1). Subsequently, limited homologies have been identified between *pigB*, *pigC*, *pigF*, *pigI*, and the T6SS core components *vgrG*, *clpV*, *dotU*, and *hcp*, respectively, so these *Francisella* genes have been renamed accordingly (Ludu et al., 2008; Barker et al., 2009; Table 1). Also the remaining *pig* genes, i.e., *pigA*, *pigD*, *pigE*, *pigG*, and *pigH* have been renamed *iglE*, *iglG*, *iglH*, *iglI*, and *iglJ* respectively (Ludu et al., 2008; Barker et al., 2009; Table 1), suggesting that these genes, similar to *iglABCD*, are required for intracellular growth. However, with the exception of *iglI* of *F. novicida* (Barker et al., 2009), there is no published data to support that this is the case (Table 3). In fact, our data strongly suggest that *iglG* is dispensable for intracellular growth of *F. tularensis* strain LVS in both J774 macrophages and mouse peritoneal exudate (PEC) cells (Bröms et al., unpublished) (Table 3). Moreover, an *iglI* null mutant of LVS is able to efficiently grow within J774 cells, which is in contrast to the results observed by Barker et al. (2009) for *F. novicida* (above) (Bröms et al., unpublished; Table 3). This is an exciting finding, since it suggests that the phenotypes of FPI mutants may be species-specific, but it clearly complicates the use of a common FPI nomenclature; thus, the name IglG (Intracellular growth locus G) and IglI (Intracellular growth locus I) may perhaps be appropriate for *F. novicida*, but evidently not for LVS. Also, we do not see any obvious reason to distinguish *pdpA* from the *iglABCD*

Table 2 | Effects of FPI gene mutations on *Francisella* pathogenicity.

Requirement for:	Intracellular growth	Phagosome escape	Cytopathogenicity	Virulence		
				Chick embryos	Mice	Drosophila
<i>pdpA</i>	Y ^{1,3,20}	Y ¹	Y ¹	Y ¹	Y ^{1,18}	Y ³
<i>pdpB/icmF</i>	Y ^{3,8,9,20}	NT	Y ⁸	NT	Y ^{8,9,18}	Y ³
<i>iglE</i>	Y ^{3,6}	NT	NT	NT	Y ¹⁸	Y ³
<i>vgrG</i>	Y ^{3,5}	Y ⁵	NT	NT	Y ^{5,18}	Y ³
<i>iglF</i>	Y ^{3,7}	NT	NT	NT	Y ¹⁸	Y ³
<i>iglG</i>	N ¹⁷ , Y ^{3,7}	N ¹⁷	Y ¹⁷	NT	Y ^{17,18}	Y ³
<i>iglH</i>	Y ^{3,7}	NT	NT	NT	Y ¹⁸	Y ³
<i>dotU</i>	Y ³	NT	NT	NT	Y ¹⁸	Y ³
<i>iglI</i>	N ¹⁷ , Y ^{3,5}	Y ⁵	Y ¹⁷	NT	Y ^{5,17,18}	Y ³
<i>iglJ</i>	Y ^{3,8}	NT	Y ⁸	NT	Y ^{8,18}	Y ³
<i>pdpC</i>	N ^{3,20}	NT	NT	NT	Y ¹⁸	N ³
<i>pdpE</i>	N ^{3,17}	N ¹⁷	N ¹⁷	NT	N ^{17,18}	N ³
<i>iglD</i>	Y ^{3,15,20}	Y ¹⁵	Y ¹⁵	NT	Y ¹⁸	Y ^{3,21}
<i>iglC</i>	Y ^{3,10,11,14–16,19,20,22}	Y ^{14–16}	Y ^{10,15,22}	NT	Y ^{10,11,18}	Y ^{3,21}
<i>iglB</i>	Y ^{3,12,20}	Y ¹²	NT	NT	Y ¹⁸	Y ^{3,21}
<i>iglA</i>	Y ^{3,12,13,20}	Y ¹²	N ¹⁷	Y ¹³	Y ^{12,18}	Y ³
<i>pdpD</i>	N ^{2,3,20}	NT	NT	Y ²	Y ^{2,18}	N ³
<i>anmK</i>	N ³	NT	NT	Y ²	Y ¹⁸	N ³

“Y” indicates that the genes is required for the phenotype tested (gray boxes), “N” indicates that it is not (black boxes). NT, not tested (white boxes). For mutants, such as *iglG* and *iglI*, contradicting results exist (dark gray boxes). For strains which carry duplications of the FPI genes, the stated phenotypes and references refer to mutants that contain deletions in both copies of the FPI gene.

Supporting data from: ¹Schmerk et al. (2009a), ²Ludu et al. (2008), ³Åhlund et al. (2010), ⁴Kraemer et al. (2009), ⁵Barker et al., (2009), ⁶Rodriguez (2010), ⁷Asare and Abu Kwaik (2010), ⁸Brotcke et al. (2006), ⁹Tempel et al. (2006), ¹⁰Golovliov et al., (2003a), ¹¹Lauriano et al., (2004), ¹²Bröms et al. (2009), ¹³de Bruin et al. (2007), ¹⁴Santic et al. (2005b), ¹⁵Bönquist et al. (2008), ¹⁶Lindgren et al. (2004), ¹⁷Bröms et al. (unpublished), ¹⁸Weiss et al. (2007), ¹⁹Gray et al. (2002), ²⁰Read et al. (2008), ²¹Vonkavaara et al. (2008), ²²Lai et al. (2004).

genes required for intracellular growth, since a *pdpA* mutant of *F. novicida* also clearly exhibits very limited replication in host cells (Schmerk et al., 2009a). Moreover, the homologies reported between some of the *Francisella* proteins and T6SS core components, e.g., PigC and ClpV (Barker et al., 2009), are weak and do not provide any convincing evidence for their relatedness, nor do they provide support when it comes to assigning a function to the specific FPI protein (Bröms et al., unpublished).

For these reasons, we believe that the existing FPI nomenclature should be modified to better reflect the current knowledge of FPI protein function, but also to take into account that for many of these proteins we still have no assigned functions. In fact, since the *Francisella* T6SS is so different from all other T6SS described so far (Bingle et al., 2008) it is not unlikely that some of the FPI proteins will exhibit functions distinct from their homologous T6SS core components. For simplicity, we therefore propose that all FPI genes should be named *igl*, regardless of function, but this will be discussed more thoroughly elsewhere.

THE FPI – ITS ROLE IN PHAGOSOME BIOGENESIS, INTRACELLULAR GROWTH AND VIRULENCE

Similar to other intracellular pathogens like *Listeria monocytogenes*, *Shigella flexneri*, and *Rickettsia* spp. (Ray et al., 2009), *Francisella* rapidly escapes from the phagosome into the host cell cytosol where it

replicates (Clemens et al., 2004; Santic et al., 2005a,b; Bönquist et al., 2008). The *Francisella* containing phagosome (FCP) transiently acquires early (EEA-1 and Rab5) and late (Rab7, CD63, LAMP-1, and LAMP-2) endosomal markers, but no/little cathepsin D, suggesting limited fusion to lysosomes (Clemens et al., 2004; Santic et al., 2005a, 2008; Bönquist et al., 2008). Inhibition of the proton ATPase pump within 15–60 min post infection has been shown to result in a significant delay in phagosomal escape (Chong et al., 2008; Santic et al., 2008), indicating the importance of acidification for FCP disruption and bacterial escape. Importantly, these results have recently been challenged by Clemens et al. (2009).

A vast number of studies have identified the FPI as a key player in the ability of *F. tularensis* to modulate phagosome maturation and escape into the host cell cytosol. This is likely to be the consequence of T6SS-mediated translocation of bacterial effector protein(s) directly involved in modulation of phagosome biogenesis and lysis of the phagosomal membrane. However, none of the FPI-encoded proteins required for escape so far hold any properties of cytolysins, pore-forming toxins, or hydrolytic enzymes commonly used by intracellular pathogens to disrupt the phagosomal membrane (Ray et al., 2009). This suggests that *Francisella* may employ novel mechanisms to promote bacterial escape into the cytosol, or merely that the effector proteins are encoded outside of the FPI cluster.

Table 3 | *In silico* analysis of FPI proteins, demonstrated protein localization and protein–protein interactions.

Prediction	Bioinformatic predictions based on protein sequence						Experimental data	
	Amino acids ¹ Schu S4 (LVS/U112)	Size (kDa) ²	Secretion signal ³	TM domains ⁴	Bacterial localization ⁵	COGs ⁷	Bacterial localization	Interaction partner
<i>pdpA</i>	820	95.4	N	0	OM		Soluble ⁸	
<i>pdpB/icmF</i>	1093	127.6	N	2	OM	COG3523	IM ^{9,16}	
<i>iglE</i>	125	14.5	Y	1	Unknown (lipid attachment site ⁶)		IM, OM (latter requires a lipid attachment site) ¹⁰	
<i>vgrG</i>	164	17.5	N	0	Unknown		surface ¹¹ , secreted ¹²	
<i>iglF</i>	574, (554/576)	67.6	N	0	Unknown			
<i>iglG</i>	173	18.4	N	0	Unknown		OM ¹⁶	
<i>iglH</i>	476	55.3	N	0	Unknown			
<i>dotU</i>	207	24.6	N	1	Unknown	COG3455		
<i>iglI</i>	383	44.6	N	0	Unknown		Secreted ¹² , Soluble, IM, OM ¹⁶	
<i>iglJ</i>	257	30.9	N	0	Unknown			
<i>pdpC</i>	1328, (1328/1325)	156.2	N	0	Unknown			
<i>pdpE</i>	191, (191/188)	22.1	Y	0	Unknown		OM ¹⁶	
<i>iglD</i>	401, (398/398)	46.5	N	0	Unknown			
<i>iglC</i>	211, (209/209)	22.4	N	0	Unknown		CP ¹³ , surface ⁹	
<i>iglB</i>	514, (506/506)	58.9	N	0	Unknown	COG3517	CP ¹⁴ , surface ⁹	IglA ^{14,15}
<i>iglA</i>	196, (184/184)	21.0	N	0	CP	COG3516	CP ¹⁴ , surface ^{9,11}	IglB ^{14,15}
<i>pdpD</i>	1195, (67; 133/1245)	135.4	N	0	Unknown		OM ⁹	
<i>anmK</i>	189, 134 (NA/381)	20.4 + 15.2	N	0	OM	COG2377		

¹Number of amino acids predicted for FPI proteins from *F. tularensis* subsp. *tularensis* Schu S4. Sizes for homologous proteins from *F. tularensis* subsp. *holarctica* LVS and *F. novicida* U112 are only indicated when they deviate from the Schu S4 sequence in length.

²Predicted size in kDa of proteins from Schu S4 according to SAPS.

³SignalP 3.0 prediction of the presence (Y) or absence (N) of a secretion signal.

⁴Number of transmembrane (TM) domains predicted with TMHMM 2.0.

⁵Protein localization predicted by PSORTb.

⁶A lipid attachment site predicted (Rodriguez, 2010).

⁷Relatedness to COGs (cluster of orthologous groups; Tatusov et al., 2000).

⁸Schmerk et al. (2009a).

⁹Surface localization of IglA, IglB, and IglC occurs upon overexpression of PdpD (Ludu et al., 2008).

¹⁰Mutation of the putative site of lipidation (C22G) prevents OM localization of IglE-FLAG (Rodriguez, 2010).

¹¹Melillo et al. (2006).

¹²Barker et al. (2009).

¹³Golovliov et al. (1997).

¹⁴de Bruin et al. (2007).

¹⁵Bröms et al. (2009).

¹⁶Bröms et al. (unpublished).

NA, not applicable.

OM, outer membrane; IM, inner membrane; CP, cytoplasmic.

Since FPI mutants that fail to escape also fail to multiply, it has been difficult to determine whether the encoded gene products also contribute to multiplication, or to later stages of infection. Interestingly, FPI genes reach their maximal expression at the end of cytosolic replication, which may suggest a role for the FPI during late stages of infection, or perhaps, may provide an advantage (“ready and armed”) to bacteria that are about to infect new cells upon lysis of their current host cell (Wehrly et al., 2009).

IglC is the best characterized FPI protein so far, together with MglA, a positive regulator encoded outside of the FPI. Both have been shown to be essential for the ability of *F. tularensis* to modulate the biogenesis of the phagosome to avoid lysosomal fusion (Santic et al., 2005b; Bönquist et al., 2008), to escape into the host cytosol (Lindgren et al., 2004; Santic et al., 2005b), and to induce

apoptosis of J774 cells (Lai et al., 2004; **Table 3**). Consequently, *mglA* and *iglC* mutations diminish intracellular survival and growth in many different macrophage-like cell lines (Gray et al., 2002; Golovliov et al., 2003a; Lauriano et al., 2003; Lindgren et al., 2004; Santic et al., 2005b), as well as amoeba (Lauriano et al., 2004) and insect cells (Read et al., 2008; Vonkavaara et al., 2008; Santic et al., 2009; **Table 3**). To date, most of the genes located in the FPI, such as *iglA*, *iglB*, *iglC*, *iglD*, *iglI*, *iglG*, *pdpA*, *pdpB*, and *pdpD*, have been demonstrated to be required either for intramacrophage growth and/or virulence of at least one subspecies (Golovliov et al., 2003b; Lai et al., 2004; Nano et al., 2004; Tempel et al., 2006; de Bruin et al., 2007; Santic et al., 2007; Weiss et al., 2007; Bönquist et al., 2008; Chong et al., 2008; Ludu et al., 2008; Bröms et al., 2009; Schmerk et al., 2009b; Bröms et al., unpublished; **Table 3**). Interestingly,

some gene products, such as IglG, have been shown to be required for virulence but dispensable for intracellular growth, while PdpE appears to be dispensable for both processes (Bröms et al., unpublished; **Table 3**). Noteworthy, many of the earlier FPI studies lacked the tools for precise genetic analysis and proper complementation controls, and this should be taken into account when discussing the phenotypes of *Francisella* strains with lesions in FPI genes.

FPI-MEDIATED MANIPULATION OF THE HOST INFLAMMATORY RESPONSE

The pathogenesis of tularemia appears to be critically dependent on the ability of *F. tularensis* to modulate the host immune response by a number of mechanisms, including degradation of serum antibodies (Crane et al., 2009), induction of immunosuppressive cytokines and prostaglandin E2 in the lungs of infected mice (Bosio et al., 2007; Woolard et al., 2008), suppression of responses to secondary stimuli *in vivo* (Bosio and Dow, 2005), inhibition of the respiratory burst in human neutrophils (McCaffrey and Allen, 2006), impairment of phosphatidylinositol 3-kinase-mediated (Parsa et al., 2006) and IFN- γ -mediated signaling (Parsa et al., 2008) and downregulation of several TLRs in monocytes (Telepnev et al., 2003), rapid induction of apoptosis in J774 cells and mouse tissue macrophages (Lai et al., 2004; Wickstrum et al., 2009), activation of the ASC

inflammasome (Mariathasan et al., 2004; Fernandes-Alnemri et al., 2010; Jones et al., 2010; Rathinam et al., 2010), as well as rapid activation of Ras (Al-Khodor and Abu Kwaik, 2010). Several of these mechanisms are directly or indirectly linked to FPI proteins since specific FPI mutants or mutants within *mglA* show phenotypes deviant from the parent strain.

Already within 15 min after monocytic ingestion of wild-type *F. novicida*, recruitment of PKC α and PKC β 1 to the SOS2/GrB2 complex occurs, leading to activation of Ras (**Figure 2**). None of these events take place after infection with an *iglC* mutant of *F. novicida*, instead rapid activation of caspase-3 and cell death follows (Al-Khodor and Abu Kwaik, 2010; **Figure 2**). Thus, Ras activation is advantageous to the bacterium since it allows a prolonged replication in the cytosol. As it occurs so rapidly, the phagosomal localization of the *iglC* mutant may not necessarily be the reason for the aberrant Ras activation, instead, the effect may directly or indirectly relate to the function of IglC.

The proinflammatory response to *F. tularensis* in monocytic cells is TLR2-dependent (Cole et al., 2006, 2007; Katz et al., 2006; Li et al., 2006; Thakran et al., 2008; Abplanalp et al., 2009). Moreover, it has been demonstrated that *Francisella* colocalizes with TLR2 and MyD88 inside macrophages (Cole et al., 2007; **Figure 3**), signals through TLR2 from within the phagosome (Cole et al., 2008;

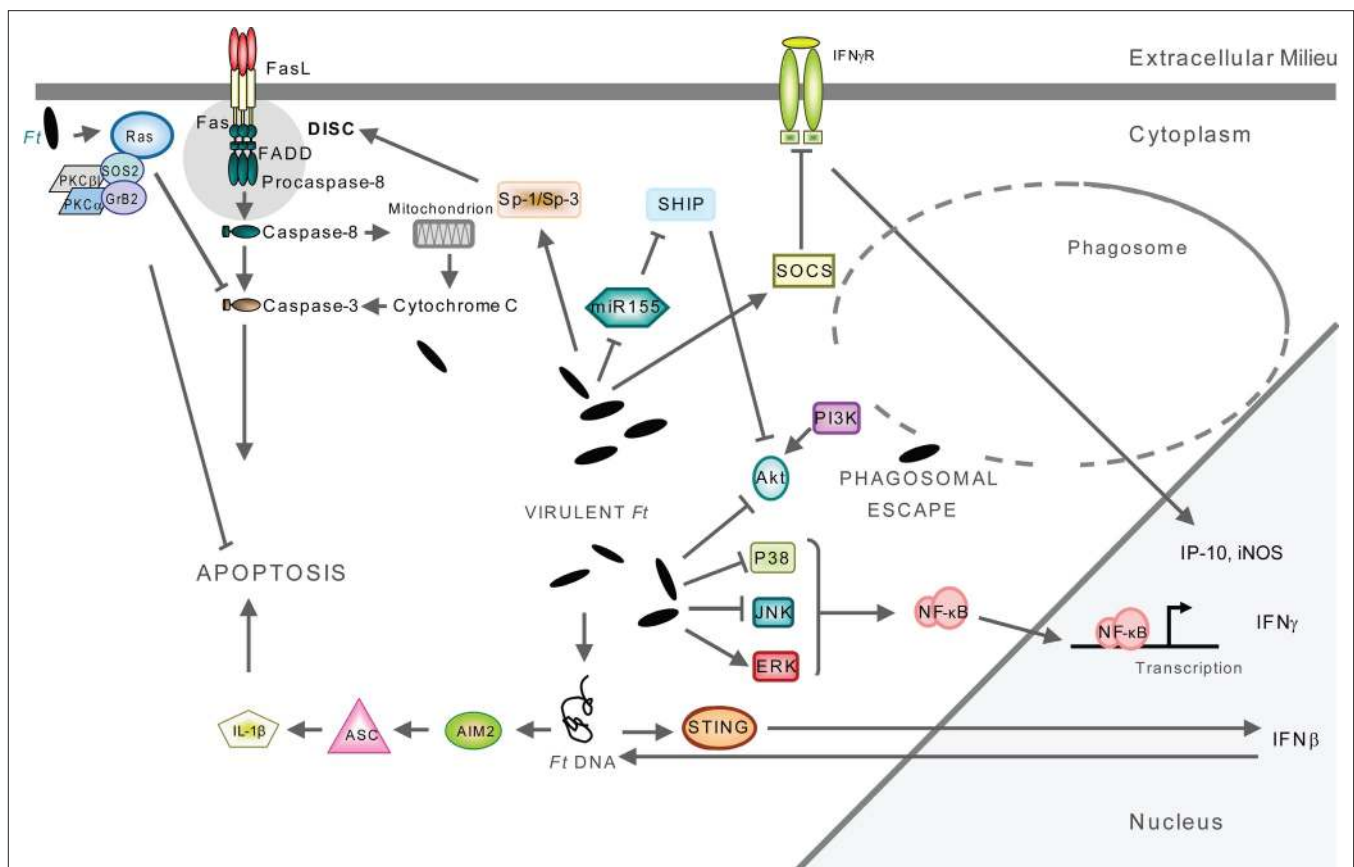


FIGURE 2 | Signaling triggered by *F. tularensis* located in the cytosol. Host cell signaling occurs after *F. tularensis* escapes from the phagosome to reside and multiply within the cytoplasm of the monocytic cells. The infection eventually

leads to apoptosis of the host cell. The engagement of a multitude of signaling pathways have been described, for details see references in the text. Only pathways that have been experimentally verified are included in the figure.

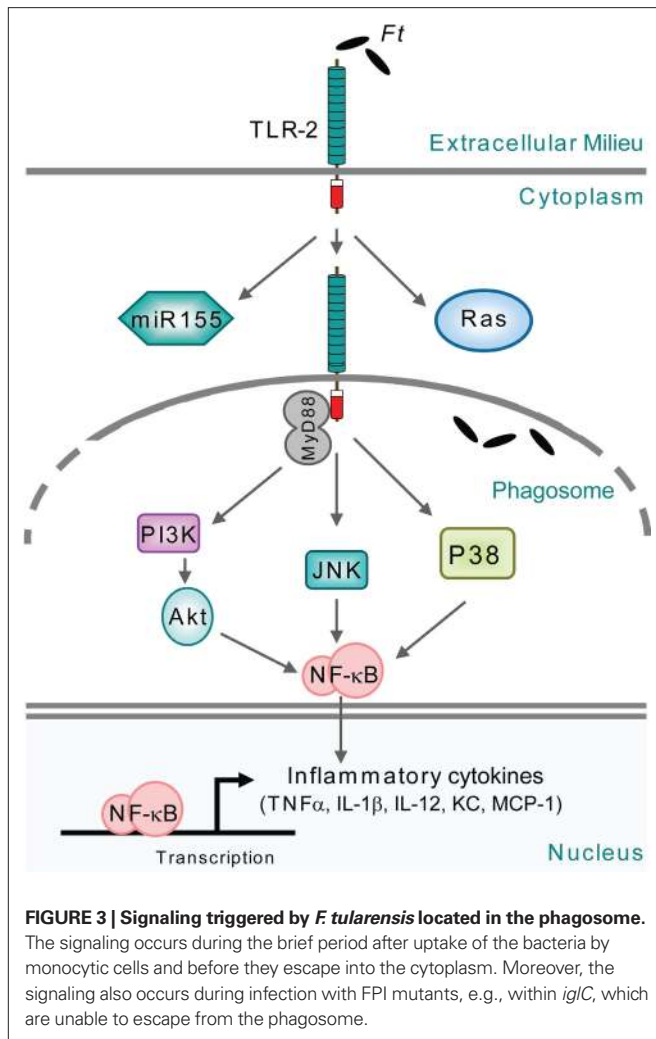


Figure 3), and that phagosome-contained *F. tularensis* demonstrate a distinct signaling pattern (Cole et al., 2008). In the latter case, macrophages infected with an *iglC* mutant showed increased expression of a subset of TLR2-dependent, proinflammatory genes, but decreased expression of other genes encoding, e.g., IFN- γ , iNOS, and IP-10 (Cole et al., 2008). The latter subset was dependent on IFN- β , indicating that this cytokine is required for full induction of the macrophage proinflammatory response (Figure 2). While the *iglC* mutant greatly increased IL-1 β mRNA expression in wild-type macrophages, IL-1 β secretion was not observed (Cole et al., 2008). Thus, TLR2-dependent expression of IFN- β , IFN- β -stimulated genes, and IL-1 β secretion requires bacterial escape from the phagosome. Noteworthy, in the absence of IFN- β , intracellular bacterial numbers were increased showing the central role of the cytokine in modulating the host response and controlling *F. tularensis* infection (Cole et al., 2008).

A detailed characterization of the *F. tularensis*-induced IFN- β signaling has been revealed in a series of recent publications, resulting in the identification of *F. novicida* as a prototypic agent for AIM2-mediated inflammasome activation (Fernandes-Alnemri et al., 2010; Jones et al., 2010; Rathinam et al., 2010; Ulland et al., 2010). AIM-2 activation involves recognition of cytosolic DNA,

which triggers formation of the AIM2 inflammasome by inducing AIM2 oligomerization (Fernandes-Alnemri et al., 2009; Figure 2). The AIM2-DNA complex then recruits and subsequently oligomerizes the caspase-1-activating adaptor protein ASC into the large ASC pyroptosome, which in turn activates caspase-1 leading to cleavage of IL-1 β and subsequent apoptosis (Fernandes-Alnemri et al., 2007; Figure 2). By infecting AIM2-deficient mice with *F. novicida*, it was concluded that cytosolic invasion by *F. tularensis* leads to STING-mediated production of type I interferons, such as IFN- β , which is a prerequisite for the subsequent AIM2 activation, leading to caspase-1 activation, IL-1 β and IL-18 secretion and inflammasome-mediated cell death (Gavrilin et al., 2006; Mariathasan et al., 2006; Henry et al., 2007; Cole et al., 2008; Figure 2). This chain of events critically depends on a cytosolic localization of bacteria as shown by infections with FPI mutants unable to escape the phagosome, such as *iglC*, or mutants within *mgIA*, the positive regulator encoded outside of the FPI (Gavrilin et al., 2006; Cole et al., 2008). Similarly, *F. novicida* *vgrG* and *iglI* mutants, both unable to escape from the phagosome, were recently shown to be defective for IL-1 β release (Barker et al., 2009). Thus, for *F. novicida*, there appears to be a strong correlation between the degree of phagosomal escape and inflammasome activation. In view of this, it was somewhat surprisingly that one of the studies found that the endosomal-acidification inhibitor bafilomycin completely prevented the production of type I interferons, implying the phagosome as the source of DNA for inflammasome activation (Fernandes-Alnemri et al., 2010). However, the divergent findings may be reconciled if it is assumed that the amounts of *F. novicida* DNA initially released into the cytosol upon escape *per se* are not sufficient to activate AIM2 but still can trigger production of type I interferons such as IFN- β . This will lead to monocyte activation and enhanced bacterial degradation in the phagosome, resulting in increased DNA release into the cytosol. Thereby, the accumulation of cytosolic DNA may reach a threshold required for AIM2 inflammasome activation. The role of bafilomycin in this context may be to prevent the type I interferon-enhanced killing of *F. tularensis* in the phagosome.

Francisella tularensis infection leads to interference of several signaling pathways crucial for the control of intracellular infection. One such example is the IFN- γ signaling pathway, which is essential although alone not sufficient to control *F. tularensis* infection of monocytic cells (Anthony et al., 1989; Lindgren et al., 2004; Edwards et al., 2009). The role of this pathway has been analyzed by studies of the IFN- γ -induced STAT1 activation in human and murine monocytic cells (Parsa et al., 2008; Roth et al., 2009). The interference with IFN- γ *per se* was dependent on upregulation of SOCS3, a well-known inhibitor of IFN- γ -mediated signaling, and accompanied by the suppression of IP-10 and iNOS production, resulting in increased intracellular bacterial survival (Figure 2). This signaling interference is independent of phagosomal escape and replication since mutants in *mgIA* or *iglD* were still able to efficiently suppress IFN γ -induced STAT1 (Parsa et al., 2008). The important role of IFN- γ has been corroborated by determining its role when added to *F. tularensis*-infected cell cultures. Then, it was observed that treatment of mouse PEC cells conferred a static effect on wild-type LVS bacteria, whereas the treatment had a bactericidal effect on the *iglC* mutant demonstrating that the

effect of IFN- γ is dependent on the subcellular localization of *Francisella* (Lindgren et al., 2004). There are conflicting data on the mechanisms effectuated by IFN- γ since one study reported that IFN- γ treatment did not affect the phagosomal escape and subcellular localization of Schu S4 in murine bone marrow-derived macrophages or human monocyte-derived macrophages (Edwards et al., 2009); two others that the subcellular localization of LVS was marginally affected in murine PEC (Lindgren et al., 2004), but not in murine J774A.1 cells (Bönquist et al., 2008); and a fourth that *F. novicida* was completely confined to the phagosome of human monocyte-derived macrophages (Santic et al., 2005a). Some of the discrepancies may relate to the use of different infection models and strains of *F. tularensis*, but additional studies are required to clarify this.

Mitogen-activated protein kinase (MAPK) pathways, such as p42/p44 (Erk1/2), c-Jun terminal kinase (JNK) and p38, of monocytic cells are also modulated by *F. tularensis* during infection. Hrstka et al. (2005) observed that apoptosis induced by LVS was associated with reduced p38 MAPK activity and activation of p42/p44 kinases (Figure 2). Moreover, Telepnev et al. (2005) showed that LVS is able to rapidly downregulate the initial activation of p38 and c-Jun, and hence cytokine secretion and that IglC is critical for this process. Thus, unlike the *iglC* mutant, LVS eventually downregulates the initial proinflammatory response in monocytic cells.

Moreover, phagosomal escape has been shown to be accompanied by marked downregulation of the phosphoinositol 3 kinase PI3K/Akt pathway and subsequent suppression of NF- κ B-mediated production of inflammatory cytokines (Parsa et al., 2006; Rajaram et al., 2006; Figure 2). Suppression does not occur when an *mglA* mutant is used; instead it leads to increased proinflammatory production and lower IL-10 release (Rajaram et al., 2006; Figure 3). The pathway is under negative control of the inositol phosphatase SHIP, which becomes phosphorylated upon infection by *F. tularensis* (Parsa et al., 2006; Figure 2). Interestingly, the microRNA-155 (MiR-155) that targets and negatively regulates SHIP is induced by *F. novicida* but not Schu S4 (Figure 2), thereby leading to an enhanced proinflammatory response in the former case (Cremer et al., 2009). Furthermore, the expression of the PI3K/Akt pathway members is preferentially downregulated in macrophages infected with *F. tularensis* Schu S4 compared to *F. novicida* (Butchar et al., 2008).

The PI3K/Akt pathway is also related to host-cell death. Phagosomal escape of *F. novicida* leads to activation of Sp-1/Sp-3, upregulation of Fas and recruitment of the DISC complex (Rajaram et al., 2009), which in turn activates caspases-3 and -8 leading to apoptosis (Lai and Sjöstedt, 2003; Santic et al., 2009; Figure 2). Since activation of PI3K/Akt promotes phagosome-lysosome fusion (Rajaram et al., 2009), this should limit intracellular bacterial growth and host-cell death. In corroboration of this scenario, it was found that constitutive activation of Akt, or deletion of SHIP, promotes phagolysosomal fusion and limits bacterial replication in the host cytosol, as well as preventing subsequent induction of Fas expression and cell death (Rajaram et al., 2009).

Akt is also involved in phagosome biogenesis through activation of Rab5 (Barbieri et al., 1998) and the assembly of NADPH oxidase leading to triggering of the oxidative burst (Chen et al., 2003; Hoyal et al., 2003). Thereby, modulation of the PI3K/Akt pathway

by *F. tularensis* may serve multiple purposes; downmodulation of the proinflammatory response and the oxidative burst, induction of host-cell death and limiting the biogenesis of the FCP.

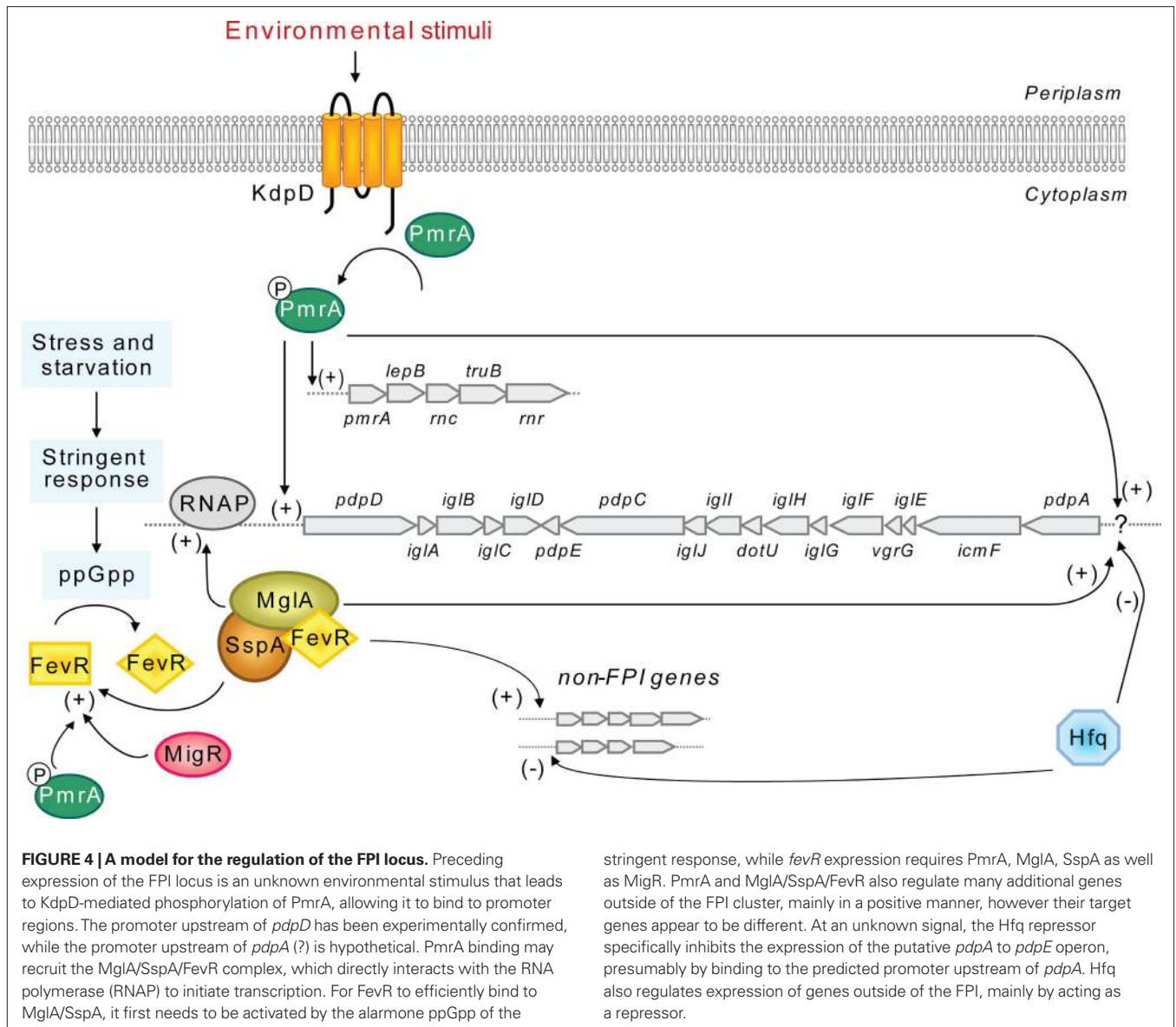
The exact roles of the FPI proteins in modulating essentially all of the aforementioned pathways described is enigmatic since it is possible that the bacterium, once in the cytosol, mediates the effects via non-FPI-dependent mechanisms, e.g., via signaling through cytosolic receptors. Thus, a possible scenario is that the essential role of the FPI proteins simply is to enable the phagosomal escape.

Regulation of the FPI – a complex story

Studies in pathogens such as *Burkholderia pseudomallei*, *Salmonella enterica*, *Vibrio cholerae*, *Pseudomonas aeruginosa*, and enteroaggregative *Escherichia coli* have led to the identification of regulatory determinants and mechanisms underlying expression of T6SS gene clusters. These complex transcriptional regulatory networks involve two-component systems (Ishikawa et al., 2009; Syed et al., 2009), quorum sensing systems (Deziel et al., 2005; Ishikawa et al., 2009; Lesic et al., 2009), alternative sigma factor σ^{54} (Pukatzki et al., 2006; Ishikawa et al., 2009; Syed et al., 2009), transcriptional activators (Deziel et al., 2005; Dudley et al., 2006; Lesic et al., 2009; Sun et al., 2010), and histone-like proteins (Lucchini et al., 2006; Castang et al., 2008). In addition, upregulation of most T6S clusters has been shown to depend on contact with, or intracellular growth within, the host cell during infection (Das et al., 2000; Parsons and Heffron, 2005; Chugani and Greenberg, 2007; Shalom et al., 2007). In *F. tularensis*, this is consistent with the demonstration that FPI genes are induced during growth within macrophages (Golovliov et al., 1997; Baron and Nano, 1998; Twine et al., 2006; de Bruin et al., 2007; Chong et al., 2008; Wehrly et al., 2009). To date, the environmental stimuli and mechanisms that govern regulation of the FPI genes and thus virulence of *Francisella* spp. are as yet unknown, although iron depletion (Deng et al., 2006; Lenco et al., 2007), and oxidative stress (Lenco et al., 2005) have been shown to induce expression of FPI genes.

Combined data from several research groups suggest the involvement of at least six regulatory proteins in controlling FPI gene expression; MglA, SspA, FevR (PigR), MigR, Hfq, and PmrA (Figure 4; Baron and Nano, 1998; Lauriano et al., 2004; Brotcke et al., 2006; Charity et al., 2007; Mohapatra et al., 2007; Buchan et al., 2009; Meibom et al., 2009). Some of these also regulate expression of genes outside the FPI, however, whereas MglA, SspA, and FevR appear to regulate the same set of genes, PmrA and Hfq control separate groups of genes (Brotcke et al., 2006; Charity et al., 2007; Guina et al., 2007; Mohapatra et al., 2007; Brotcke and Monack, 2008; Meibom et al., 2009).

Macrophage growth locus (MglA) and SspA are members of the stringent starvation protein A (SspA) family (Baron and Nano, 1998) and interact to form heterodimers that bind to the RNA polymerase, inducing the transcription of numerous genes, including *fevR* (Brotcke et al., 2006; Charity et al., 2007). FevR (*Francisella* effector of virulence regulation) has been shown to physically interact with MglA/SspA in a manner facilitated by the alarmone ppGpp, although there are conflicting data concerning this interaction (Brotcke and Monack, 2008; Charity et al., 2009). FevR positively autoregulates its own expression (Charity et al., 2009) and exhibits some homology to the helix-turn-helix region



of the MerR family of transcriptional activators, suggesting that it may bind to target DNA (Brotcke and Monack, 2008). MigR (Macrophage intracellular growth regulator) may exert its positive effect on FPI gene expression indirectly through FevR, since it has been shown to be required for *fevR* expression, in contrast to that of *mglA*, *sspA*, or *pmrA* (Buchan et al., 2009). Moreover, *fevR* expression also requires the presence of MglA, SspA as well as PmrA (Brotcke et al., 2006; Charity et al., 2007; Mohapatra et al., 2007; **Figure 4**).

Importantly, current data suggest a connection between MglA/SspA/FevR and a two-component regulatory system consisting of PmrA/KdpD (Bell et al., 2010). PmrA, which positively regulates its own transcription and that of the FPI, was originally assumed to be an orphan response regulator (Mohapatra et al., 2007; Sammons-Jackson et al., 2008). However, Bell et al. (2010) recently demonstrated that PmrA, upon phosphorylation by the histidine kinase KdpD, efficiently binds to regulated promoters of the *pmrA* operon

and FPI-encoded *pdpD*. Moreover, using coimmunoprecipitation experiments it was revealed that PmrA, MglA, and SspA are part of the same protein complex (Bell et al., 2010). Thus, although the sequence of protein binding to the regulatory complex is not clear, phosphorylated PmrA may bind to FPI gene promoters recruiting free or RNA polymerase-bound MglA, SspA and FevR, to initiate FPI gene transcription (**Figure 4**).

Finally, Hfq differs from all the other FPI regulators identified so far in that it seems to act primarily as a repressor of gene expression, both for genes located within the FPI as well as genes located outside (Meibom et al., 2009). Moreover, Meibom et al. (2009) were able to show that only a subset of FPI genes (*pdpA* to *iglJ*) are negatively regulated by Hfq (**Figure 4**), leading the authors to speculate that there is differential regulation of the two putative divergent operons in the FPI (*pdpD* to *iglD* and *pdpA* to *pdpE*). The G + C content of these two regions are significantly different from one another: 30.6% (*pdpD* to *iglD*) vs 26.6% (*pdpA* to *pdpE*),

compared to an average of 33% for the *Francisella* chromosome (Nano and Schmerk, 2007), which supports the notion of two different origins for these regions. Nevertheless, this operon organization must still be shown experimentally and putative promoter(s), in addition to that located within the intergenic region of *pdpD* and *anmK* (Bell et al., 2010), identified. Importantly, the intergenic regions between *pdpD* and *iglA* (80 nt) and *iglJ* and *pdpC* (39 nt) are also, in theory, large enough to harbor promoters, suggesting the possibility of multiple overlapping promoters within the FPI (Nano and Schmerk, 2007).

Clearly, much more work is needed in order to understand the intricate regulatory network behind *Francisella* FPI gene expression, especially how the many regulators are able to coordinate this process. The fact that these also regulate genes outside of the FPI suggests there is cross-talk between T6S and other virulence determinants, which adds further complexity to the situation. Defining how these networks are coordinated and identifying the environmental signals that induce FPI expression and T6S may also help to identify the elusive effector proteins secreted by this system. All together, this will shed light on the critical role of the FPI in *Francisella* pathogenesis.

T6S – WHAT CAN WE LEARN ABOUT THE *F. TULARENSIS* SYSTEM FROM OTHER PATHOGENS?

To date, there is limited information about the functions of the putative T6SS encoded by the *Francisella* FPI gene cluster. However, as a phenomenon highly related to pathogenesis, bacterial secretion has received great interest from the scientific society. Today several comprehensive studies on T6S in various pathogens exist, which have contributed to the understanding of these complex secretion systems. An overview is presented below.

TYPE VI SECRETION SYSTEM GENE CLUSTERS ARE UBIQUITOUS AMONG GRAM-NEGATIVE BACTERIA

When first discovered in *V. cholerae*, the T6SS gene clusters were denoted IAHP (IcmF associated homologous protein), since they include a gene with homology to *icmF* of the *Legionella pneumophila* Dot/Icm type IV secretion system (T4SS) (Das and Chaudhuri, 2003). Since then, similar clusters encompassing 15–25 ORFs have been identified by *in silico* methods in approximately 100 bacterial species (~ ¼ of all bacterial genomes sequenced to date), some encoding more than one system (Bingle et al., 2008; Shrivastava and Mande, 2008; Boyer et al., 2009). Subsequently, the importance of T6SSs for virulence has been established for pathogens like *V. cholerae*, *B. mallei*, *S. typhimurium*, *Edwardsiella tarda*, *Aeromonas hydrophila*, enteroaggregative *E. coli*, and *P. aeruginosa* (Folkesson et al., 2002; Dudley et al., 2006; Mougous et al., 2006; Pukatzki et al., 2006; Schell et al., 2007; Zheng and Leung, 2007; Suarez et al., 2008). Importantly, T6SSs are not restricted to pathogens, since they are also found in symbiotic bacteria as well as bacteria with no known association with eukaryotes, the latter suggesting that they could be used by bacteria to adapt to environmental conditions and/or acquire food nutrients (Bingle et al., 2008; Shrivastava and Mande, 2008; Boyer et al., 2009).

There is large heterogeneity among T6SS gene clusters, which are suggested to form four to five major phylogenetic groups (Bingle et al., 2008; Boyer et al., 2009). However, the phylogenetic analysis

by Bingle et al. (2008), based exclusively on *IglA* (DUF770) and *IglB* (DUF877) sequences, does not include the FPI in any of these groups, instead it is suggested to form an outgroup, indicating that it is unique from all other T6SSs. This is further accentuated by the fact that this is the only bioinformatic approach that actually identifies the FPI as a putative T6SS cluster. The current understanding of T6SSs reveals that they are related to both bacterial T4SSs and the cell-puncturing devices utilized by bacteriophages for DNA delivery (below).

CORE COMPONENTS AND SUBSTRATES OF T6SSs

T6SSs are, like T4SSs, complex multi-subunit machineries that can deliver macromolecules from the bacterial cytoplasm and into a eukaryotic host cell, traversing both bacterial and eukaryotic membranes. Such machineries include structural components of the secretin, secreted effector proteins, energizers of the secretion process as well as regulators that control the expression of these components. Most gene products of T6SS gene clusters are likely to be structural components, since when mutated, T6S is abolished. For example, in *E. tarda* 13 out of 16 genes are required for functional secretion, however their specific roles are largely unknown (Dudley et al., 2006; Schell et al., 2007; Zheng and Leung, 2007; Suarez et al., 2008; Wu et al., 2008; Pukatzki et al., 2009).

Due to the heterogeneity between the T6SS gene clusters, and the fact that empiric evidence exist from only a limited number of bacteria, it is not a trivial task to define the core T6SS components. However, most systems encode homologs to *V. cholerae* IcmF, DotU, ClpV, VipA, VipB, VgrG, and Hcp proteins (Boyer et al., 2009).

While contributing to stabilization of the *L. pneumophila* T4SS, but not being essential for its function (Sexton et al., 2004), IcmF–DotU homologs are absolutely required for functional T6S. These membrane proteins have been shown to interact and are found in all phylogenetic groups of T6SSs, suggesting that they are essential components of the secretion apparatus (Zheng and Leung, 2007; Boyer et al., 2009; Ma et al., 2009). In *Francisella*, the suggested IcmF homolog PdpB has been shown to be essential for functional T6S of *IglI* (Barker et al., 2009).

Another defining trait is a bicistronic locus encoding homologs of VipA and VipB (denoted *IglA* and *IglB* in *Francisella*). These are only found in T6SS clusters and are suggested to form a functional pair, since they have been shown to interact in a number of bacterial species including *F. tularensis* and to depend on each other for stability (de Bruin et al., 2007; Bröms et al., 2009; Aubert et al., 2010). Interestingly, VipA–VipB of *V. cholerae* and the corresponding proteins BcsL_B–BcsK_C from *B. cenocepacia* assemble into tubular structures suggested to span the bacterial membranes (Bönemann et al., 2009, 2010; Aubert et al., 2010). Whether this also occurs for *Francisella* *IglA* and *IglB* is not known, especially since it lacks a ClpV homolog, with which the *V. cholerae* and *B. cenocepacia* homologs have been shown to interact. ClpV is a ClpB-like AAA + family adenosine triphosphatase (ATPase) which is required for secretion of T6SS substrates. It forms a hexameric complex at the inner membrane, and requires an intact ATPase domain to support secretion suggesting that it is the main energizer of T6S (Schlieker et al., 2005; Mougous et al., 2006; Zheng and Leung, 2007; Bönemann et al., 2009). Intriguingly, ClpV is required for assembly of BcsL_B–BcsK_C tubules and for disassembly of VipA–VipB tubules. The latter is hypothesized to enable transport of VipA–VipB across

the inner membrane before reassembling into the tubular structure. Further, the VipA–VipB tubule structurally resembles the T4 bacteriophage tail sheath. This sheath forms an outer layer to the rigid internal tail tube that, when the sheath is contracted, penetrates the outer bacterial membrane and intermembrane peptidoglycan (Kostyuchenko et al., 2005; Leiman et al., 2009). It is conceivable that the mechanism for T6S occurs in a similar manner, since also other T6SS components bear resemblance to phage proteins (below).

The two most well studied T6SS components are VgrG (valine–glycine repeat protein G) and Hcp (hemolysin co-regulated protein). Principally, a functional T6SS is defined by its ability to export these proteins into the extracellular milieu and they represent a class of secreted substrates that also are structural components of the secretion machinery. Hcp, which is the major secreted protein of T6SSs, forms hexameric donut-shaped rings that stack on top of each other to form tubules. These have large inner diameters, forming a conduit for delivery of macromolecules, and show great resemblance to phage tail tube structures (Mougous et al., 2006; Ballister et al., 2008; Leiman et al., 2009; Pell et al., 2009). VgrG contains two domains that structurally resemble the bacteriophage T4 gp27 and gp5 proteins (Figure 5). These assemble into (gp27)₃–(gp5)₃ needle complexes where the gp5 trimer forms the needle that pierces the bacterial membrane. Presumably, a VgrG trimer forms a similar structure with the ability to puncture both bacterial and eukaryotic membranes (Dudley et al., 2006; Mougous et al., 2006; Pukatzki et al., 2006, 2007, 2009; Schell et al., 2007; Zheng and Leung, 2007; Suarez et al., 2008; Wu et al., 2008; Chow and Mazmanian, 2010).

Apart from the needle complex domains, some evolved VgrGs have C-terminal functional extensions that upon delivery into the host cell can interfere with cellular functions, e.g., the actin cross-linking domain of *V. cholerae* VgrG1 (Pukatzki et al., 2007). Besides Hcp and VgrG, there is a small number of T6SS exported substrates that are not themselves required for functional secretion and hence are more similar to the “classical” effectors of T3SSs and T4SSs, e.g., EvpP of *E. tarda* (Zheng and Leung, 2007; Pukatzki et al., 2009).

A HYPOTHETICAL MODEL FOR TYPE VI SECRETION

Based on the current knowledge of T6S, and guided by the structural resemblance to phage tail-spike organelles, models of how the system functions have been proposed (Filloux, 2009; Pukatzki et al.,

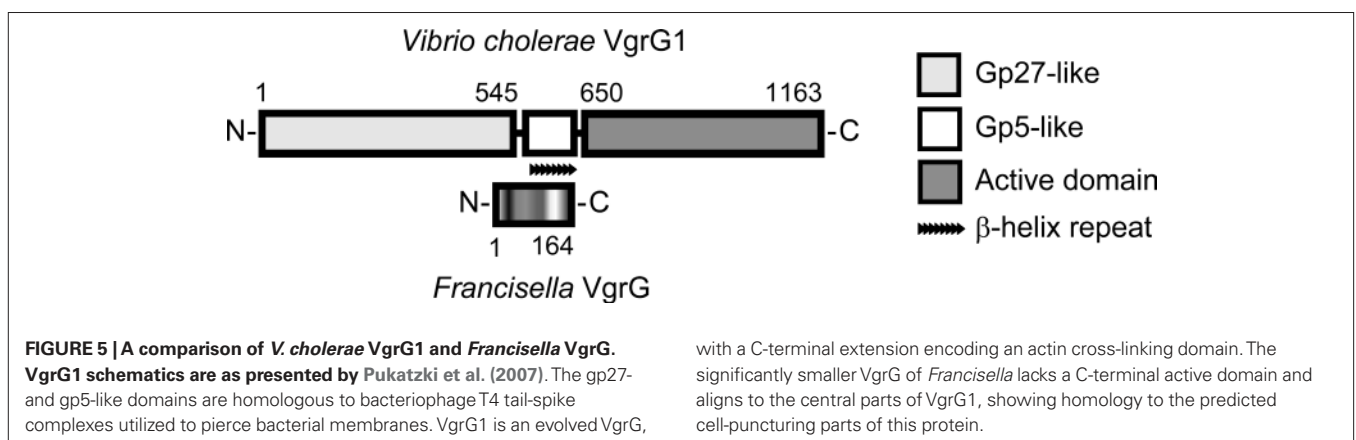
2009; Bönemann et al., 2010). A set of core components are suggested to form a pore complex in the bacterial plasma membrane, through which Hcp and VgrG are delivered to the periplasm. Here, Hcp assembles into a tubular structure, capped by the VgrG trimer and sheathed by the VipA–VipB tubule. Upon elongation of the Hcp tubule and contraction of the VipA–VipB sheath, VgrG pushes through the bacterial membrane. Depending on the localization of the bacterium, the VgrG tip may also puncture either the outer or the vesicular membrane of the host cell, allowing the delivery of the VgrG effector domain in the target cell cytosol. It is not known whether the active domain remains attached to VgrG or if it is cleaved off before it reaches its final destination. Further, the VgrG tip is too narrow to allow secretion and hence VgrG detaches from the Hcp tubule to allow Hcp and other components with effector functions to be delivered into the host cell cytosol. The hexameric ATPase ClpV has been suggested to localize to the membrane pore complex where it energizes the T6S process, controlling disassembly/assembly and export of T6S components.

EVIDENCE OF A FRANCISELLA PATHOGENICITY ISLAND ENCODED SECRETION SYSTEM

As discussed above, many of the components of the FPI are required for pathogenicity-related phenotypes such as phagosome escape, intracellular replication, cytopathogenicity and virulence in animal models (summarized in Table 2). Although information concerning the specific roles these proteins play in *Francisella* pathogenicity is scarce, they are likely to form part of a T6SS or a T6SS-like system (de Bruin et al., 2007; Bingle et al., 2008; Ludu et al., 2008; Barker et al., 2009).

IN SILICO ANALYSIS OF THE PATHOGENICITY ISLAND GENE PRODUCTS

A bioinformatic analysis of the FPI gene products was employed to predict the presence of secretion signals and transmembrane regions, protein localization, homology to known proteins and functional domains. However, this analysis (summarized in Table 3) only gives so much away. Most FPI components reveal no homology to any known proteins, display no functional domains and the predicted localization is primarily “unknown.” Nevertheless, five of them belong to different COGs (clusters of orthologous groups) (Tatusov et al., 2000; Table 3). COG2377, represented by AnmK, includes members of the anhydro-*N*-acetylmuramic acid



with a C-terminal extension encoding an actin cross-linking domain. The significantly smaller VgrG of *Francisella* lacks a C-terminal active domain and aligns to the central parts of VgrG1, showing homology to the predicted cell-puncturing parts of this protein.

kinase family, which contribute to the fitness of pathogenic and non-pathogenic bacteria alike but is unrelated to T6S. However, the other four COGs do indeed define proteins found in T6SS clusters, i.e., the canonical functional pairs IglA–IglB (COG3516, COG3517) and PdpB/IcmF–DotU (COG3523, COG3455) (Figure 1; Table 3). Moreover, both PdpB/IcmF and DotU display predicted transmembrane domains, which is logical if they, like the homologous protein pair of the *Legionella* T4SS, localize to the inner membrane. While the predicted localization for DotU is “unknown,” PdpB is predicted to localize to the outer membrane. However, fractionation experiments have revealed that PdpB is found exclusively in the bacterial inner membrane, a localization that better agrees with its homology to IcmF (Bröms et al., unpublished; Ludu et al., 2008). Together with the requirement of PdpB/IcmF and DotU for *Francisella* virulence (Table 2), this suggests that the functions of these core components, whatever they may be, are likely to be conserved in *Francisella*.

Two FPI members, PdpE and IglE, are predicted to possess Sec secretion signals (Table 3). Both localize to the bacterial outer membrane (Bröms et al., unpublished; Rodriguez, 2010) and in the case of IglE, this localization depends on the presence of a lipid attachment site suggesting that IglE is a lipoprotein (Rodriguez, 2010).

Protein alignment studies suggest that the FPI encodes a VgrG homolog, which is significantly smaller than any known VgrG. A comparison to VgrG1 from *V. cholerae* reveals that *Francisella* VgrG aligns to the central part of this protein, corresponding to the most C-terminal part of protein gp27 and to the greater part of the membrane puncturing gp5 protein of bacteriophage T4 (Figure 5). Needless to say, the diminutive FPI VgrG has no C-terminal active domain. However, it cannot be ruled out that it still possesses an effector function, since VgrG export into the host cell cytosol was recently demonstrated (Pukatzki et al., 2007; Ludu et al., 2008; Barker et al., 2009).

It has also been suggested that PdpE encodes an Hcp-like protein (Barker et al., 2009), but this homology is faint and there has been no mechanistic evidence of PdpE executing similar functions as Hcp. In addition, Hcp-like proteins are often encoded by a monocistronic operon separated from the T6SS gene cluster. In the same study, IglF was proposed to be equivalent to ClpV, but it lacks the key ATPase domain of ClpV proteins. Overall, there is some homology between FPI members and T6SS gene clusters, but mechanistic data supporting the notion that the FPI truly encodes a T6SS is scarce.

IglA–IglB – FORMATION OF A TRANSMEMBRANE TUBULAR STRUCTURE?

The FPI components with the greatest homology to T6SS proteins are IglA and IglB, which have been shown to interact and also depend on each other for protein stability (de Bruin et al., 2007; Bröms et al., 2009). This interaction is vital for virulence and relies on a conserved α -helix within IglA (Bröms et al., 2009). Furthermore, interactions between homologous components of pathogens like *V. cholerae*, *P. aeruginosa*, *Y. pseudotuberculosis*, and uropathogenic *E. coli*, also depend on the same α -helical domain suggesting that the mechanism of interaction is conserved (Bröms et al., 2009). Hence, it is conceivable that IglA–IglB, similar to *V. cholerae* VipA–VipB and *B. cenocepacia* BcsL_B–BcsK_C, form a tubular transmembrane structure that encases the internal

components of a secretion conduit (Bönemann et al., 2009; Bröms et al., 2009; Aubert et al., 2010). There is, however, no existing evidence of IglA–IglB multimerization beyond dimers, and they primarily localize to the bacterial cytosol (Table 3), where they have been suggested to function as a chaperone complex (de Bruin et al., 2007). Nevertheless, in support of the notion that IglA and IglB may form a membrane spanning structure are reports of surface location of these components (Melillo et al., 2006; Ludu et al., 2008).

IglC – A UNIQUE EFFECTOR PROTEIN?

A comparative study of protein expression in *Francisella* grown in liquid culture or in J774 cells revealed significant induction of a 23-kDa protein, later named IglC, during intracellular growth (Golovliov et al., 1997; Gray et al., 2002). IglC has no homology to any characterized bacterial proteins (Table 3), suggesting that it may be a novel virulence factor. Furthermore, it possesses no recognizable functional motifs but adopts a unique beta-sandwich structure, with weak structural similarity to gp27, a cell-puncturing device component of bacteriophage T4 (Sun et al., 2007).

Although predominantly cytoplasmic (Golovliov et al., 1997), a small fraction of IglC localizes to the bacterial surface as determined by a biotinylation assay (Ludu et al., 2008). This led the authors to speculate that IglC could play a role in a secretion channel in the outer membrane of *Francisella*, or alternatively, in forming a pore in the host cell membrane. The biological relevance of this observation is, however, not easily assessed since IglC surface localization was demonstrated upon overexpression of PdpD (Ludu et al., 2008; Table 3). Nonetheless, IglC-export did not occur in *F. novicida* strains mutated for the T6SS core components *pdpB*, *dotU*, *iglA*, or *iglB*, suggesting that it was T6SS dependent. This was in contrast to PdpD-mediated export of IglA and IglB, which occurred also in the absence of *pdpB* or *dotU* (Ludu et al., 2008; Table 3). Still, if IglC depends on the outer membrane protein PdpD for export, and this export is an important feature of T6S, it is difficult to comprehend that a *F. novicida* *pdpD* mutant is able to grow as efficiently as the wild-type strain in all of 11 eukaryotic cell lines tested (Ludu et al., 2008).

SECRETION OF VgrG AND IglI IN MACROPHAGES

The first evidence of translocation of FPI proteins was provided in a recent study by Barker et al. (2009) who demonstrated delivery of *F. novicida* VgrG and IglI, the latter lacking homology to known bacterial proteins, into the cytosol of infected J774 macrophages. VgrG and IglI export was demonstrated by several means: translocation of C-terminal CyaA fusions was observed based on the accumulation of cAMP in the host cell cytosol, and N-terminally FLAG-tagged proteins were visualized using immunofluorescence microscopy. Moreover, the latter proteins could also be detected in the culture supernatants of bacteria grown in broth (Barker et al., 2009).

Intriguingly, in all assays, VgrG secretion occurs in an FPI-independent manner, as seen when expressed in a Δ FPI *F. novicida* strain. In contrast, export of IglI within macrophages depends on the FPI and does not occur in Δ vgrG, Δ icmF, or Δ FPI backgrounds. However, IglI-FLAG is efficiently secreted to the extracellular medium of broth-grown Δ vgrG and Δ FPI mutants. Since neither VgrG nor IglI is secreted when expressed *in trans* in *E. coli* DH5 α , this

suggests that they can exit via an FPI-independent but *Francisella*-specific export mechanism during growth in liquid culture (Barker et al., 2009). It would be interesting to investigate if the rudimentary gene cluster (FTN_0040–FTN_0052), which share homology with core components of the FPI (as described above), contributes to this export. Immunofluorescence staining of VgrG and IglI during infection shows that VgrG, when expressed in a $\Delta vgrG$ background, is released into the host cell cytosol forming a plume extending from the bacterium (Barker et al., 2009). In contrast, when secreted from an Δ FPI mutant, VgrG is localized more closely to the bacterium. Similarly, staining for IglI–FLAG in Δ iglI, Δ vgrG, or Δ FPI backgrounds also reveals a close association between the protein and the bacteria. These data could be interpreted to suggest that VgrG is truly secreted while IglI is primarily exported to the bacterial surface. Combined with the data from Ludu et al. (2008) and Melillo et al. (2006) this may imply that the FPI-encoded secretion system exports proteins to form a surface located complex including IglA, IglB, IglC, IglI, and VgrG (Barker et al., 2009).

DOES THE FPI ENCODE A TRUE T6SS?

As discussed above, there is increasing evidence that *Francisella* does encode a T6SS-like system that may export FPI proteins such as IglI but this system truly appears to be unique from all other systems described so far. First of all, it is evolutionary distinct (Bingle et al., 2008), and only 3 out of the 17 FPI proteins share significant homology to T6SS core components, i.e., IglA, IglB, and DotU (Nano and Schmerk, 2007; Boyer et al., 2009). So far, no convincing homolog of ClpV, the AAA + ATPase and putative energizer of T6SSs (Mougous et al., 2006), has been identified in

the genome, although Barker et al suggested that FTT1348 (IglF) may be a candidate (Barker et al., 2009). Moreover, while a protein with a short IcmF-related portion (PdpB) and a potential VgrG homolog have been recognized in the FPI (Nano and Schmerk, 2007; Barker et al., 2009; Boyer et al., 2009), the Walker A box commonly present in IcmF homologs is missing from *F. tularensis* PdpB (Zheng and Leung, 2007; Barker et al., 2009) and the *F. tularensis* VgrG homolog is much smaller than VgrG homologs from other species, and it also lacks the C-terminal extensions found in, e.g., the *V. cholerae* VgrG-1 and VgrG-3 proteins (Pukatzki et al., 2007; Barker et al., 2009). Altogether, these differences would suggest that the *Francisella* FPI system is clearly different compared to other T6SS. Further supporting this notion is the observation that *F. tularensis* VgrG, in contrast to all VgrG proteins reported so far, is exported into the extracellular milieu in an FPI-independent fashion (Barker et al., 2009), suggesting a novel mechanism of export. While IglI-translocation into host cells is dependent on FPI core components, it can also be secreted via an FPI-independent mechanism during *in vitro* growth.

Together, these observations raise the question whether the FPI actually encodes a true T6SS or just a system implicated in virulence that shares some components with T6SSs and T4SSs. Much more work is needed to distinguish between these possibilities.

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