Unique Substrates Secreted by the Type VI Secretion System of *Francisella tularensis* during Intramacrophage Infection

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Abstract

Gram-negative bacteria have evolved sophisticated secretion machineries specialized for the secretion of macromolecules important for their life cycles. The Type VI secretion system (T6SS) is the most widely spread bacterial secretion machinery and is encoded by large, variable gene clusters, often found to be essential for virulence. The latter is true for the atypical T6SS encoded by the *Francisella* pathogenicity island (FPI) of the highly pathogenic, intracellular bacterium *Francisella tularensis*. We here undertook a comprehensive analysis of the intramacrophage secretion of the 17 FPI proteins of the live vaccine strain, LVS, of *F. tularensis*. All were expressed as fusions to the TEM β-lactamase and cleavage of the fluorescent substrate CCF2-AM, a direct consequence of the delivery of the proteins into the macrophage cytosol, was followed over time. The FPI proteins IglE, IglC, VgrG, IglI, PdpE, PdpA, IgU and IglF were all secreted, which was dependent on the core components DotU, VgrG, and IglC, as well as IgIG. In contrast, the method was not directly applicable on *F. novicida* U112, since it showed very intense native β-lactamase secretion due to FTN_1072. Its role was proven by ectopic expression in trans in LVS. We did not observe secretion of any of the LVS substrates VgrG, IgU, IgII or IglI, when tested in a FTN_1072 deficient strain of *F. novicida*, whereas IglE, IglC, PdpA and even more so PdpE were all secreted. This suggests that there may be fundamental differences in the T6S mechanism among the *Francisella* subspecies. The findings further corroborate the unusual nature of the T6SS of *F. tularensis* since almost all of the identified substrates are unique to the species.

Introduction

Gram-negative bacteria have evolved various types of sophisticated machineries specialized for the secretion of macromolecules as a means to promote bacterial fitness and/or establish colonization or attachment to host cells. Out of the seven secretion machineries identified so far, the Type VI secretion system (T6SS) is the most recently identified. It occurs widely in both pathogenic and commensals and is encoded by large, variable gene clusters, often found to be essential for virulence. The latter is true for the atypical T6SS encoded by the *Francisella* pathogenicity island (FPI) of the highly pathogenic, intracellular bacterium *Francisella tularensis*. We here undertook a comprehensive analysis of the intramacrophage secretion of the 17 FPI proteins of the live vaccine strain, LVS, of *F. tularensis*. All were expressed as fusions to the TEM β-lactamase and cleavage of the fluorescent substrate CCF2-AM, a direct consequence of the delivery of the proteins into the macrophage cytosol, was followed over time. The FPI proteins IglE, IglC, VgrG, IglI, PdpE, PdpA, IgU and IglF were all secreted, which was dependent on the core components DotU, VgrG, and IglC, as well as IgIG. In contrast, the method was not directly applicable on *F. novicida* U112, since it showed very intense native β-lactamase secretion due to FTN_1072. Its role was proven by ectopic expression in trans in LVS. We did not observe secretion of any of the LVS substrates VgrG, IgU, IgII or IglI, when tested in a FTN_1072 deficient strain of *F. novicida*, whereas IglE, IglC, PdpA and even more so PdpE were all secreted. This suggests that there may be fundamental differences in the T6S mechanism among the *Francisella* subspecies. The findings further corroborate the unusual nature of the T6SS of *F. tularensis* since almost all of the identified substrates are unique to the species.
Hcp and VgrG are not only structural components of the T6SS, but may also possess effector functions. In the case of VgrGs, these functions can be attributed to C-terminal extensions that upon delivery into the host cell interfere with cellular functions. For example, the Rtx domain of F. cholerae VgrG1 cross-links actin [12], while the VIP-2 domain of A. hydrophila VgrG1 possesses actin-ADP-ribosylation activity [18]. In addition, some VgrGs carry domains that exhibit homology to bacterial cell-wall degrading enzymes, proteases as well as bacteriocins, suggesting that these proteins may have a bactericidal function [19,20,21]. In A. hydrophila, Hcp was shown to bind to the surface of macrophages and to induce IL-10 and TGF-β production, which resulted in impaired recruitment and inhibition of phagocytosis [22]. In addition, Hcp proteins with C-terminal extensions have been identified in S. enterica and E. coli, which may represent evolved Hcp proteins with effector functions [20,23,24]. Besides VgrG and Hcp, only a few T6SS-secreted proteins have been identified, most notably ExpP of E. tarda [25], the bactericidal Tse1-3 system, secreted by the HSI-1 T6SS of P. aeruginosa [26], and the Tac2 (type VI amidase effector) of Bathothrix thailandensis, which is important for growth competition against other bacteria [27]. In the latter study, 11 potential substrates secreted by the T6SS-1 system of B. thailandensis were identified, many of which may be required for mediating interbacterial interactions [27]. An aberrant variant of T6SSs is found in the highly virulent, facultative intracellular bacterium Francisella tularensis. Little is known about the molecular mechanisms of Francisella pathogenesis, but its ability to survive and replicate within macrophages appears intimately linked to its virulence [28]. Within this normally hostile cell type, the Francisella-containing phagosome appears to evade lysosome fusion and relatively quickly, the bacterium escapes into the cytoplasm and thereafter starts to proliferate [29,30,31]. The intramacrophage replication is dependent on a multitude of proteins, many of which are encoded by the Francisella pathogenicity island (FPI). This is a large, duplicated 33-kb region and a phylogenetic analysis has revealed that it constitutes the lone member of a distantly related fifth group of T6SSs [1]. Essentially all of the FPI proteins are conserved among the F. tularensis subspecies, and most of them are essential for intracellular replication as well as growth within the amoeba Acanthamoeba castellanii, a putative reservoir of F. tularensis (reviewed in [32]). The FPI encodes a truncated form of VgrG that forms multimers consistent with its suggested role as a trimeric needle complex [33]. During intracellular infection, VgrG as well as IgI, a substrate unique to Francisella, is secreted into the macrophage cytosol [34,35]. While secretion of VgrG occurred independently of the FPI, export of IgI was dependent on the FPI for F. tularensis subsp. novicida strain U112, but not for F. tularensis LVS, the live vaccine strain [34,35]. To further identify potential substrates among the FPI proteins, we undertook a comprehensive analysis of the intramacrophage secretion of FPI proteins by the LVS strain. All of the 17 FPI proteins were expressed as fusions to TEM β-lactamase, which together with the fluorescent substrate CCF2-AM, allowed us to follow their secretion into the macrophage cytosol over time. By this means, significant secretion of IgI, IgC, VgrG, IgI, PdpE, PdpA, IgJ and IgF was observed resulting in the production of blue fluorescence of infected cells. In all cases, secretion was dependent on the core components DotU, VgrG, and IgC as well as IgG. The findings further emphasize the unusual nature of the T6SS of F. tularensis and its distant relationship to other T6SSs, since all identified substrates, except for VgrG, are unique to the species. **Results** **Construction of FPI protein TEM β-lactamase fusions** In order to identify putative FPI protein substrates that are translocated by F. tularensis LVS during infection, we employed a fluorescent-based β-lactamase (TEM) translocation assay, which has previously been used to identify substrates of both Type III- and Type IV-secretion systems [36,37,38,39,40]. In this assay, each candidate gene is fused to TEM (β-lactamase) of E. coli and the bacterial strain expressing the fusion protein is used to infect host cells, which are then loaded with CCF2 substrate. Delivery of the β-lactamase fusion protein into host cell cytosol leads to cleavage of the substrate, resulting in an easily detectable change in fluorescence from green to blue emission. To generate FPI protein-TEM fusions, we first constructed vector pJEBl09, which encodes the mature β-lactamase from E. coli under the control of the constitutive groE promoter. Individual FPI genes were amplified by PCR and inserted into pJEBl09 to generate translational C-terminal fusions with the downstream β-lactamase gene. We expressed the constructs in LVS instead of its isogenic mutant background to overcome the problems with lack of complementation exhibited by many of the chimeras. In fact, out of 10 TEM-constructs that we specifically tested [IgE, VgrG, IgJF, IgJH, DotU, IgJ, IgD, IgC, IgB, IgA], only 3 (IgA, IgD, IgJ) were able to complement the corresponding mutant for growth in macrophages and/or LDH release (data not shown). Since expression of the wild-type proteins without a tag generally leads to phenotypic complementation of FPI mutants [34,35,41,42], this suggests that the 29.5 kDa TEM-tag sterically interferes with protein function. To verify that the chimeras were indeed expressed, we used TEM β-lactamase antibodies. Although the level of expression varied to some extent, a protein corresponding to the expected size of the fusion was detected in most of the samples with the exceptions of the TEM fusions of IgG and IgD, which both were somewhat smaller than their predicted size (Fig. 1). Moreover, DotU-TEM was barely detected in the bacterial pellets, while the two largest fusion proteins, PdpB-TEM and PdpC-TEM, could not be detected at all, suggesting that they may be unstable (Fig. 1 and data not shown). Since LVS encodes a truncated form of PdpD, we expressed the full-length protein from F. novicida strain U112 instead (Fig. 1). **Identification of proteins transferred by the Type VI secretion system of Francisella tularensis LVS** After verifying the expression of the fusion proteins in F. tularensis, we infected J774 macrophages with F. tularensis strains expressing the β-lactamase fusions. At indicated time points, cells were loaded with the CCF2 substrate in the presence of the drug Probenecid to prevent the substrate from being excreted by the cells. As positive and negative controls, we used LVS expressing VgrG-TEM or IgG-TEM, respectively, as we have previously shown that a CyaA fusion of VgrG, but not IgG, is secreted into macrophages during infection [34]. Translocation of the β-lactamase chimeras as reflected by the presence of cells emitting blue fluorescence signals was assessed using a live-cell microscope. At 18 h post-infection, infection with LVS alone resulted in cells emitting green fluorescence only, suggesting that the endogenous β-lactamases encoded by LVS, FTL_0879 and FTL_0957, were not secreted/and or able to cleave the β-lactam ring of CCF2-AM (data not shown), a prerequisite for the use of the assay. Similarly, no blue fluorescence was detected when cells were infected with LVS expressing IgG-TEM (Fig. 2), suggesting that it was an appropriate negative control. In contrast, infection with LVS expressing VgrG-TEM resulted in a significant, albeit small
F. tularensis
level of fusion protein expressed in an interval of 18–24 h, therefore, we focused our studies on the 18 h and 24 h, we concluded that secretion was most prominent at the 18 h.

The resulting molecules weighted 6 kDa, suggesting that the marker are indicated. The molecular weight indicated for each of the fusion protein was deduced from the primary sequence by using the SAPS server (www.ebi.ac.uk/Tools/saps/).

Figure 1. Production of FPI-TEM fusion proteins in LVS. Total cell lysates of Francisella LVS harboring various FPI-TEM fusions or an empty vector control were prepared and examined by Western-blot analysis using an antibody against TEM β-lactamase (top panel) or IgIB (bottom panel).

Beta-lactamase secretion in F. tularensis subsp. novicida U112

We also wanted to verify the TEM results using another species of Francisella. For this reason, we included the F. novicida strain U112 in our study. To our surprise, infection with U112 alone using the identical experimental setup resulted in 56.1 ± 1.8% of blue fluorescent cells, suggesting that F. novicida U112, in contrast to LVS, encodes native β-lactamase(s) that is/are secreted and capable of cleaving CCF2 (Fig. 3). According to the Francisella genome database, U112 harbors two β-lactamase genes, i.e. FTN_1002 and FTN_1072, which are homologous to FTI_0957 and FTI_0879 of LVS, respectively. To determine which, if any, of these genes was responsible for the efficient cleavage of the CCF2 substrate, we included clones from a two-allele transposon mutant library [43] with insertions in either FTN_1002 or FTN_1072. The gene responsible for the blue fluorescence during infection was found to be FTN_1072, as the corresponding insertion mutant dramatically reduced the amount of blue cells to values below the cut-off of the assay (<0.5%), while insertion mutations within gene FTN_1002 had no obvious effect (Fig. 3).

From an alignment of FTN_1072 and FTI_0879, it became apparent that there are many substitutions within the LVS homologue, which may account for the inability of FTI_0879 to cleave CCF2 (Fig. 4). While altered specificity may be one explanation to these differences, another possibility is that secretion in general may be much more efficient in U112 compared to LVS. While Bina et al have shown that the encoded product of FTI_0879 is indeed secreted in LVS [44], there are no studies where the secretion efficiencies of FTN_1072 and FTI_0879 have been directly compared. To differentiate between host cells. None of the fusions caused detectable translocation in the ΔdotU, ΔvgrG or ΔiglC backgrounds, and none or very dramatically reduced translocation in the ΔiglC background (Fig. 2 and Table 1). Thus, secretion is dependent on the FPI-encoded components DotU, VgrG, IgIC and IgG. Therefore, these are likely to encode structural components of the translocation machinery and strongly suggest that secretion of FPI proteins is indeed FPI-dependent.

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these two possibilities, we therefore expressed \(FTN_{1072}\) or \(FIL_{0879}\) in trans in the \(F. novicida\) \(FTN_{1072}\) mutant. While expression of the former partially restored secretion upon infection with the \(FTN_{1072}\) mutant, resulting in 25.9±0.91% of blue fluorescent cells (~46% of U112-levels; \(P<0.001\)) (Fig. 3), expression of \(FIL_{0879}\) resulted in only 2.79±0.77% of blue fluorescent cells (~5% of U112-levels; \(P<0.001\)) (Fig. 3). These data clearly indicate that \(FIL_{0879}\) is less potent at cleaving CCF2. If altered substrate specificity is the only explanation for the observed differences in CCF2 cleavage by \(F. novicida\) and LVS, we would expect that infection with LVS expressing \(FTN_{1072}\) in trans would result in the same amount of blue fluorescent cells as for the complemented \(F. novicida\) \(FTN_{1072}\) mutant, however it turned out to be only 7.09±0.31%, i.e. 27% of \(FTN_{1072}/FTN_{1072}\)-levels (\(P<0.001\)) (Fig. 3). Thus, it appears as if also secretion of \(\beta\)-lactamases may be more efficient in \(F. novicida\) compared to LVS.

To further investigate secretion in \(F. novicida\), we introduced a selection of FPI-TEM fusions into the \(FTN_{1072}\) mutant and determined their translocation efficiencies during intramacrophage infections. The fusions included were IgI-E-TEM, IgC-TEM, VgrG-TEM, IgI-L-TEM, PdpE-TEM, PdpA-TEM, IgI-F-TEM and IgI-G-TEM, which represented substrates secreted during an LVS infection, as well as IgI-G-TEM, which was not secreted by LVS. Using the TEM assay, we demonstrated secretion of PdpE, IgI-E, IgI-G, and PdpA, but not IgI-G, at 18 h and the numbers of blue fluorescent cells obtained with these constructs at 18 h were: 16.9±1.33% (PdpE), 3.48±0.52% (IgI-E), 2.64±0.16% (IgI-G) and 0.70±0.09% (PdpA) (Fig. 2 and Table 1). Surprisingly, however, in contrast to the findings on LVS, VgrG, IgI-L, IgI-G and IgI-F were not secreted by \(F. novicida\) (Fig. 2, and Table 1). Thus, while secretion of Francisella-derived \(\beta\)-lactamases may occur more efficiently in \(F. novicida\)-infected cells than in LVS-infected cells, the same does not necessarily apply to FPI-TEM fusions. These results also suggest that PdpE, PdpA, IgI-E and IgI-G are common substrates of the T6SS of Francisella spp., but also that there are fundamental differences in the T6S mechanism of the different Francisella subspecies.

The proton motive force impacts on T6S in \(F. tularensis\)

Recently, it has been unraveled that secretion of substrates by Type III secretion systems (T3SS) as well as flagella is dependent on the proton motive force (PMF) for export of proteins across the inner membrane [45,46,47,48], while ATPases have been hypothesized to provide the initial energy required for substrate release and unfolding [47,49]. In T6SSs, the ATPases IcmF and ClpV, the latter a member of the ClpB family of AAA+ ATPases, have been suggested to energize the secretion process [50]. The two ATPases are highly conserved in T6SSs, but the Walker A box commonly present in IcmF homologues is missing from the \(F. tularensis\) IcmF/PdpB. In addition, \(F. tularensis\) also appears to lack a ClpV homologue (reviewed in [32]). Therefore it is tempting to speculate that PMF may be the main energizer of the putative T6S of \(F. tularensis\).

To determine whether PMF plays a role in \(F. tularensis\) substrate export during infection, we used the membrane-permeable protonophore CCCP (carbonyl cyanide \(m\)-chlorophenylhydrazone), which is known to disrupt the PMF [51]. First, a potential toxic effect of CCCP was assessed by treating the LVS bacteria under the conditions used, CCCP does not affect growth of LVS in Chamberlain’s medium, resulting in a 6 h delay before the culture reached lag-phase (Fig. S1). A small growth restriction was also seen in the presence of as little as 1 \(\mu\)M CCCP, although the delay before reaching lag-phase was only 1 h (Fig. S1). Importantly, growth resumed at essentially the initial rate upon removal of the compound after 3 h (data not shown). Therefore, under the conditions used, CCCP does not affect \(F. tularensis\) viability. To assess the effect of CCCP on T6S-mediated export in LVS, 1 or 10 \(\mu\)M of the substance was added at 0 h, and samples were analyzed for secretion of IgI-C-TEM after 18 h. CCCP was found to have a dose-dependent effect on the secretion of IgI-C-TEM. At a concentration of 1 \(\mu\)M, the numbers of blue fluorescent cells were reduced by ~40% (\(P<0.001\)), while at the higher concentration they were ~10% of the numbers of the

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**Figure 2. Secretion of Francisella FPI proteins into J774A.1 macrophages.** Macrophages were infected either with LVS, mutants thereof or an \(F. novicida\) \(FTN_{1072}\) mutant expressing different FPI-TEM fusions. After infection, cells were washed and loaded with CCF2/AM and analyzed using live cell microscopy. TEM \(\beta\)-lactamase activity is revealed by the blue fluorescence emitted by the cleaved CCF2 product, whereas uncleaved CCF2 emits a green fluorescence.

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|          | \(F. tularensis\) | \(F. novicida\) |
|----------|------------------|----------------|
|          | LVS ΔglG ΔdotU   | \(FTN_{1072}\) |
| IgIE-TEM |                  |                |
| IgIC-TEM |                  |                |
| IgII-TEM |                  |                |
| VgrG-TEM |                  |                |
| PdpE-TEM |                  |                |
| PdpA-TEM |                  |                |
| IgIU-TEM |                  |                |
| IgIF-TEM |                  |                |
| IgIG-TEM |                  |                |
non-treated control (P<0.001) (Fig. 5). Also a decrease in secretion of PdpE-TEM, when expressed in trans from the F. novicida FTN_1072 mutant, was observed in the presence of CCCP, as FTN_1072 by 68% (P<0.001 vs the non-treated control) (Fig. 5). In contrast, CCCP had no significant impact on the secretion of the native F. novicida strain U112 regardless of concentration (Fig. 5), suggesting that it specifically targets the export of the FPI substrates. Under the conditions tested, we were unable to detect any CCCP-mediated effect on intramacrophage growth or LDH release of LVS (Fig. S2), suggesting that the PMF does not contribute to these phenotypes, but plays an important role for T6S.

**Discussion**

This study is the first comprehensive study of intracellular FPI protein secretion by *F. tularensis* and also the first *Francisella* study utilizing the TEM β-lactamase assay. There are a number of recent examples where this assay has been used for detecting intracellular translocation of bacterial proteins, even for high throughput screening of secretion, e.g., in *Legionella* and *Coxiella* [38,39]. The T6SS of *F. tularensis* is poorly understood and the limited data on secretion obtained hitherto not fully compatible. Previous data was based on the use of the CyaA reporter, but were only focused on the roles of the PdpE, IgI and VgrG proteins [34,35]. It was concluded that IgI and VgrG were both secreted in *F. novicida* and LVS, however, the data was conflicting regarding the requirement for other FPI proteins since the findings in *F. novicida* suggested that only secretion of IgI was FPI-dependent, whereas the findings on LVS concluded that IgI as well as VgrG secretion was FPI-independent [34,35]. Our present investigation using the TEM fluorescent reporter assay demonstrated that none of the eight identified substrates, IgI, IgC, VgrG, IgI, PdpE, PdpA, IgG and IgI, were exported in the absence of IgI, IgG or the T6SS core components DotU or VgrG. This indicates that the FPI indeed constitutes a secretion system and that the DotU and VgrG proteins, as their homologous core components of other T6SS, have the same essential functions for secretion in *F. tularensis*. Moreover, in contrast to Barker et al. who failed to demonstrate secretion of PdpE-CyaA [35], a PdpE-TEM fusion was secreted in our hands during LVS as well as *F. novicida* U112 infections. The discrepancy between the results obtained using the CyaA and TEM assays suggests that the different types of reporter fusions may have adverse effects on secretion, however, why the CyaA method indicated that secretion of VgrG was FPI-independent is unclear. Importantly, while the TEM-tag in several instances was found to interfere with FPI protein function in terms of its ability to support intracellular growth and/or LDH release of the corresponding LVS mutant, it did not have a general impact on the ability of the protein to be secreted as 4 out of 5 identified substrates tested in these additional assays (i.e. fusions of IgI, IgG, VgrG and IgI) failed to promote growth but were still secreted. In contrast to the previous studies that investigated secretion of FPI.

**Table 1.** Secretion of FPI-TEM fusions upon J774A.1 infections.

| Strain   | F. tularensis | F. novicida |
|----------|---------------|-------------|
|          | LVS | LVS | ΔigI | ΔdotU | ΔvgrG | ΔigI | FTN_1072 |
| IgI-TEM  | 1.84±0.23*** | 6.03±1.21*** | BC | BC | BC | BC | 3.48±0.32*** |
| IgC-TEM  | 0.50±0.11 | 10.6±0.52*** | 0.66±0.16* | BC | BC | BC | 2.64±0.16*** |
| VgrG-TEM | BC | BC | 2.83±0.15*** | BC | BC | BC | BC |
| IgI-TEM  | 1.30±0.17*** | 2.36±0.17*** | BC | BC | BC | BC | 16.9±1.33*** |
| PdpA-TEM | BC | BC | 1.81±0.19*** | BC | BC | BC | BC |
| IgJ-TEM  | BC | BC | 1.28±0.12*** | BC | BC | BC | BC |
| IgG-TEM  | NT | NT | 1.12±0.08*** | BC | BC | BC | BC |
| IglC-TEM | 0.52*** | 0.15*** | 1.21*** | 11.9 |

*The percentage of blue fluorescent cells was significantly different for infections with strains expressing a FPI-TEM fusion in trans compared to infections with the parental strains only (*, P<0.05; ***, P<0.001). BC = below the cut-off of the assay, i.e.<0.5%. NT = not tested.

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**Figure 3.** Secretion of *Francisella* beta-lactamases into J774A.1 macrophages. Macrophages were infected with strains of *F. tularensis* or *F. novicida* expressing beta-lactamase genes in cis or trans. After infection, cells were washed and loaded with CCF2/AM and analyzed using live cell microscopy. β-lactamase activity is revealed by the blue fluorescence emitted by the cleaved CCF2 product, whereas uncleaved CCF2 emits a green fluorescence.

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Figure 4. Alignment of β-lactamases FTL_0879 and FTN_1072 from LVS and U112 respectively. Alignments were generated using the ClustalW2 web server (http://www.ebi.ac.uk/Tools/clustalw2/index.html) and areas of amino acid identity (black boxes) or similarity (grey boxes) illustrated using the BOXSHADE 3.21 web server (http://www.ch.embnet.org/software/BOX_form.html).

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Figure 5. The impact of the PMF inhibitor CCCP on protein secretion in F. tularensis. Macrophages were infected with LVS expressing IgIC-TEM in trans, F. novicida FTN_1072 expressing PdpE-TEM in trans or U112 expressing native FTN_1072 β-lactamase in cis. After infection, cells were washed and loaded with CCF2/AM and analyzed using live cell microscopy. β-lactamase activity is revealed by the blue fluorescence emitted by the cleaved CCF2 product, whereas uncleaved CCF2 emits a green fluorescence. The experiment was repeated 6 times using duplicate samples and a representative experiment is shown. The graphs demonstrate the average proportion of blue fluorescent cells of CCCP-treated samples vs non-treated samples and the standard error of the means (SEM). The asterisks indicate that the levels of blue fluorescent cells were significantly different for CCCP-treated samples compared to non-treated samples as determined by a 2-sided t-test with equal variance (***, P≤0.001).

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The present analysis was much more comprehensive and included all of the 17 conserved FPI proteins. The data corroborated previous findings that VgrG and IglI were secreted, but also demonstrated, somewhat more unexpectedly, that secretion of IglE, IglC, PdpE, PdpA, IglJ and IglF also occurred. The findings indicate that the *F. tularensis* T6SS possesses unique substrates since all proteins except for VgrG are unique to the bacterium, although IglC was recently suggested to be a remote homologue of Hcp [42]. A majority of the identified substrates are predicted to have an unknown location within the bacterium, with the exception of PdpA, which according to PsortB may be an outer membrane (OM) protein. From empirical studies, it has been shown that PdpE as well as IglE are OM proteins, while IglC is a soluble protein, and IglI exist in both soluble and membrane fractions [32,52,53]. Only IglE has been suggested to contain a trans-membrane region, which according to TMHMM resides within its N-terminus and thus overlaps the putative signal peptide (below). While the eight proteins do not share any obvious common traits, five of them, IglE, IglC, VgrG, PdpE, and IglJ, are among the smallest of the FPI proteins. Interestingly, a very comprehensive study on the *Legionella* T4SS suggested that among 164 translocated proteins, there was a clear bias for small substrates. Thus, it is possible that it is beneficial for the bacteria to secrete smaller substrates since it most likely is more energy-efficient. Our findings based on the use of the PMF-inhibitor CCCP demonstrated that the PMF appears to play a very important role for generating the energy required for T6S in *Francisella*, which is in agreement with its critical role for other forms of protein export, e.g., that related to the T3SS and flagellar assembly [45,46,47,48]. The general belief is that substrate export via the T6SS is thought to be a Sec-independent fashion, as a one-step mechanism across both bacterial membranes [26], however, according to SignalP, both PdpE and IglE may possess N-terminal signal peptides. Since a functional T6SS was shown to be essential for their export during an LVS infection, this raises the question of whether Sec and T6SS may be connected. A precedent for this comes from a recent study on Burkholderia *cenococcum*, where the T6SS was shown to mediate the disruption of the membranes of bacteria-containing vacuoles, which then allowed the escape of proteins secreted by the Type II secretion system (T2SS) into the macrophages cytoplasm [54].

The bulk of the data was based on the 18 h time point to minimize the issue with low detection levels, since bacterial numbers are high at this time point and host cell lysis still has not occurred. Also, from a kinetic experiment, secretion was shown to be significantly lower or non-detectable at earlier time points (9 h and even more so at 3 h) for all substrates tested. Still, our data do not rule out that FPI proteins are secreted early during the phagosomal stage, however, the sensitivity of the TEM assay may not be sufficient to detect secretion at this stage. A caveat when comparing the levels of secretion of substrates is that the levels of the proteins will affect the secreted amounts. To this end, we attempted to equilibrate the expression levels by expressing all proteins under the same, strong promoter. In addition, we determined the actual levels by Western blot analysis of bacterial lysates and observed expression of most proteins, with the exception of DotU, PdpB and PdpC, which all appeared unstable when fused to TEM. Thus, this suggests that our quantification likely reflects the efficiency of the secretion machinery. Our data do not explain why the IglC and IglE proteins are more effectively secreted, but a simple explanation could be that the higher export merely reflects the need for larger quantities of these proteins to establish their essential function outside of the bacterium. For example, one could envisage that the formation of dynamic Hcp-like tubular structures on the bacterial surface may require large amounts of secreted IglC. In T3SSs, a sorting platform that promotes a secretion hierarchy among the secreted substrates has been described [55]. The sequential loading of this platform, facilitated by customized chaperones and their affinity to the platform, ensures the hierarchy in protein secretion [55]. While chaperones so far are unknown to T6SSs, the existence of mechanisms that favor the secretion of one substrate over another still cannot be ruled out.

Very few studies of T6SSs have involved a comprehensive identification of the secreted substrates to date. One notable exception is the study by Russell *et al.* where mass spectrometry was used to identify secretion of T6SS-dependent substrates by *B. thailandensis* grown in broth. In total, 11 proteins were considered to be putative T6S-substrates. In contrast, our findings are based on an intracellular pathogen that appears to very tightly regulate its T6SS-dependent secretion and may require signals unique to the intracellular environment to become activated. While there appears to be no similarities between the substrates identified in the two studies, the possibility that *F. tularensis* may encode T6S substrates outside of the FPI cannot be dismissed. Nevertheless, the substrates are likely to serve very different purposes since it was hypothesized that several of the *Burkholderia* substrates contributed to interbacterial competition, whereas all the evidence regarding the FPI proteins indicate that their critical roles are to modify the intracellular habitat to make it permissive for replication of *F. tularensis*.

Our data do not identify if the substrates also perform effector functions within the host cell. Nevertheless, even simply as part of the surface-located structure of the T6S machinery, they still could contribute to the effector function of the T6SS by directly interacting with the effectors. Precedence for this hypothesis has been provided by the demonstration of a direct interaction between the secreted effector protein EvpP and Hcp in the fish pathogen *Edwardsiella tarda* [25]. In addition, a bioinformatic analysis of *Pantoea* and *Enwinia*, which harbor up to four T6SS loci, have shown that each of them contain distinct VgrG proteins that appear to have different phylogenetic origins than the other conserved parts of the T6SS loci [21]. Since these VgrG variants in many instances contain C-terminal domains that are homologous to regions of putative T6SS effector proteins, it has been suggested that orphan VgrG proteins without C-terminal extensions may physically interact with and carry similar effector proteins through the secretion machinery, in the same way as was suggested for *Edwardsiella* Hcp. Such VgrG- and Hcp-effector combinations may perform distinct biological functions; thereby contributing to the extensive functional diversification that appears to be a hallmark of T6SSs. Analogously, the identified *F. tularensis* substrates could contribute to the diversity of effector mechanisms that have been linked to the *F. tularensis* T6SS by such an interaction, even if they are not effectors per se.

The present results indicate that the background observed with the method was minimal since less than 0.5% of the cells infected with LVS expressing any of the other TEM constructs displayed positive signals, i.e., floresced blue. They also demonstrate that bacterial lysis was not an issue at the time points tested, since this could otherwise have resulted in the unspecific delivery of TEM fusions to the host cytosol, thereby generating false positives. Moreover, the lack of any noticeable secretion in the *ΔdotU*, *ΔavgG* and *ΔiglC* backgrounds further corroborates the utility of the method. However, one drawback of using these three mutants is they lack intracellular replication, thus, the number of bacteria present will be limited and, therefore, possibly secretion may be more difficult to detect. To that end, we also investigated secretion
from the ΔiglG mutant since it replicates as well as LVS in J774 cells, however, also this mutant background led to essentially abolished secretion for most of the substrates tested. Therefore, the data clearly suggest that the FPI constitutes a secretion system and that each of the DotU, VgrG, IgGC and IgG proteins represent functional components necessary for secretion.

Another potential caveat with the experimental approach is that the quantification was performed with the LVS strain, and not a FPI mutant, since we wanted to ensure that the bacterial numbers were similar and thereby did not bias the amounts of secreted proteins. This could mean that the efficiency of the secreted fusion proteins was affected by competition with the native, non-tagged FPI proteins. This may be one explanation as to why, although the levels of cells positive for TEM secretion were higher than background levels, they still did not represent a majority of the infected cells although the infection protocol used here routinely leads to infection frequencies of >95% [56]. A recent TEM-study on the Cavasella burnetii T4SS revealed substrates with distinct secretion efficiencies that ranged from 1 to 90% when wild-type Cavasella was used to infect U937 cells [39]. Therefore, we believe that the limited secretion that we observe upon infection with Francisella may indeed be accurate, rather than stem from a technical problem with the secretion assay.

In contrast to the utility of the TEM assay for LVS, we could not apply it directly on the F. novicida U112 strain, since it showed very intense secretion of the native β-lactamase encoded by FTN_1072. When expressed in trans in an FTN_1072 mutant of F. novicida or in LVS, the enzyme was secreted, although more efficiently in the former strain. To determine whether secretion in general is more efficient in F. novicida, we introduced TEM-fusions of IgIE, IgGC, VgrG, IgII, PdpE, PdpA, IgJ and IgIF into FTN_1072. Surprisingly, only PdpE, IgIE, IgGC and, to a minor extent also PdpA, were secreted using the identical set-up as for LVS. This suggests that PdpA, PdpE, IgIE and IgGC are common substrates of the T6SS of Francisella spp, but that also there must be fundamental differences in T6S and/or regulation thereof in the aforementioned species. Surprisingly, among the eight proteins, PdpE is one of the least conserved substrate, with a total of 21 amino acid substitutions across the entire protein and the novicida variant is three residues shorter. IgIE and IgIF also exhibit sequence variation between the species, with a total of 23 and 24 substitutions respectively, and the F. novicida IgF homologue being 22 residues longer. In contrast, IgGC is identical between the two species, while VgrG, IgIE and IgII only contain 1, 2, and 3 substitutions respectively. Thus, sequence diversity is not a likely explanation as to why there were distinct secretion patterns in the two subspecies. Studies on other non-Francisella T6SSs have revealed that secretion appears to be tightly regulated and at times difficult to detect in the experimental systems, likely indicating that these differences also extend to the secreted substrates, as most of those identified lack apparent homologues in other bacterial systems. A reason for this may be that most of the prototypically T6SS studied so far are harbored within enterobacteria with an extracellular life style, which contrasts to the intracellular life style of F. tularensis. Instead, the FPI may represent an adaptation of a T6SS to the macrophage habitat and/or originally to alternative intracellular habitats of the bacterium such as amoeba. Future functional analysis of the substrates identified in this study, where putative effector protein(s) will be distinguished from those that constitute structural components, should provide molecular mechanisms that account for the unique intracellular life cycle of this important pathogen.

Materials and Methods

Bacterial strains, plasmids and growth conditions

Bacterial strains and plasmids used in this study are listed in Table S1. Escherichia coli strains were cultured in Luria Bertani broth (LB) or on Luria agar plates at 37°C. F. tularensis was grown on modified GC-agar base (Difco GC medium base [Becton Dickinson] complemented with hemoglobin and Iso-Vitalex) at 37°C. When necessary, tetracycline (10 μg/ml for E. coli, 5 μg/ml for F. tularensis or F. novicida), or kanamycin (50 μg/ml for E. coli, 10 μg/ml for F. tularensis or F. novicida) were used. For in vitro growth experiments of F. tularensis, LVS was grown over night in Chamberlain’s medium [62] at 37°C, 200 rpm with good aeration. Next day, bacteria were subcultured to OD600 = 0.15 and grown for an additional 24 h, during which OD600 was measured at different time points. For CCCP stress experiments, the substance was added at a final concentration of 0.1 or 10 μM to the subcultures when they had reached OD600 = 0.4. To determine whether the effects of the substance were reversible, bacteria were pelleted after 3 h, washed once in PBS and redissolved in Chamberlain’s medium lacking CCCP.

Construction of TEM expression vectors

Plasmids used in this study are listed in Table S1. Primer combinations and restriction enzymes used to generate the plasmids are listed in Table S2. All fragments were amplified by PCR amplification of the TEM coding sequence from plasmid pCKX340 [36] using primers TEM_F and TEM_R, and introducing the resulting fragment into the pKpn-koRI sites of pMOL42 [34]. Next, PCR-amplified FPI genes lacking their native stop codons were introduced as Ndel-KpnI fragments into pJE8709, to generate translational C-terminal TEM fusion proteins under the control of the constitutive gusA promoter. To express the fusion escape, as only LVS bacteria, but not ΔiglA or ΔiglC mutant bacteria, will grow upon microinjection into the cytosol of J774 cells [Meyer, Bröms and Sjöstedt, unpublished].

The T6SS of F. tularensis appears to be distinct from a bioinformatic standpoint, and in the present study we have shown that these differences also extend to the secreted substrates, as most of those identified lack apparent homologues in other bacterial systems. A reason for this may be that most of the prototypically T6SS studied so far are harbored within enterobacteria with an extracellular life style, which contrasts to the intracellular life style of F. tularensis. Instead, the FPI may represent an adaptation of a T6SS to the macrophage habitat and/or originally to alternative intracellular habitats of the bacterium such as amoeba. Future functional analysis of the substrates identified in this study, where putative effector protein(s) will be distinguished from those that constitute structural components, should provide molecular mechanisms that account for the unique intracellular life cycle of this important pathogen.
proteins in Km-resistant clones of F. novicida, the encoding gene fusions were generally excised as NdeI-EcoRI fragments from pJE809 and introduced into pKK214 [63]. Genes PTN_1072 and PTN_6079 encoding native Francisella β-lactamases were also amplified as NdeI-EcoRI fragments and introduced into this vector. Since pJEB4 possesses an intrinsic EcoRI site, we instead lifted it by NdeI-KpnI digestion from the TA cloning vector into pKK214 that originally encoded PdpE-TEM, but from which the intrinsic pfdE gene had first been excised using NdeI-KpnI digestion. All plasmids were transferred into F. tularensis or F. novicida by electroporation.

Western blot analysis

Bacterial lysates were prepared in Laemmli sample buffer and boiled prior to separation on 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels. Proteins were transferred onto nitrocellulose membranes using a semidyblotter (Bio-Rad laboratories, CA, USA). Membranes were probed with mouse monoclonal antibodies against TEM-β-lactamase (Abcam, Cambridge, MA) or against IgB (BEI Resources, Manassas, VA, USA), followed by a secondary goat anti-mouse antibody (Santa Cruz Biotechnology, CA, USA). For detection, the Enhanced Chemiluminescence system (ECL) (Amersham Biosciences, Uppsala, Sweden) was used.

Cultivation of macrophages and the TEM secretion assay

J774A.1 macrophages (ATCC TIB-67) were used throughout this study, cultured and maintained in DMEM (GIBCO BRL, Grand Island, NY, USA) with 10% heat-inactivated FBS (GIBCO). The day before infection, macrophages were seeded onto BD Falcon β-wells glass chambers slides (BD Biosciences, Bedford, MA, USA) in fresh culture medium at 1.5 x 10⁵ cells/well. Following incubation overnight, cells were washed, reconstituted with fresh culture medium and allowed to recover for at least 30 min. After 2 h of infection using a multiplicity of infection (MOI) of 200, the cells were washed three times and incubated in fresh medium containing 5 μg/ml gentamicin (equals time point 0 h). These conditions resulted in 1–3 bacteria/infected cell and approximately 80–100% infected cells. To study the effects of inhibitors, the medium was at this time point supplemented with 1 or 10 μM of the proton motive force inhibitor Carbonyl cyanide m-chlorophenylhydrazone (CCCP) (Sigma, MO, USA), which had been freshly dissolved in DMSO. To control for possible adverse effects, DMSO was also added to controls not treated with substances. In all cases, the final concentration of DMSO in cultures was less than 0.05%. At 3, 9, 18 h or 24 h, cells were washed twice with PBS before loading them with CCF2-AM (Promega, Madison, USA). Data are means ± standard deviations of 3 independent experiments and used to calculate the average% of blue fluorescent cells in images taken with a live-cell imaging system (ECL) (Amersham Biosciences, Uppsala, Sweden) was used.

Intracellular replication in macrophages

To determine the ability of F. tularensis to grow within macrophages, J774A.1 cells were infected for 2 h using an MOI of 200, washed three times, and incubated in the presence of 5 μg/ml gentamicin for 30 min (corresponds to time zero). When appropriate, the medium was supplemented with 1 or 10 μM CCCP onwards. At indicated time points, the macrophage monolayers were lysed in PBS with 0.1% deoxycholate, serially diluted in PBS and plated on modified GC-agar base plates for determination of viable counts. A two-sided t-test with equal variance was used to determine whether the growth of a strain differed significantly from that of LVS (mutant complementation study) or non-treated LVS (CCCP study).

LDH release assay

J774A.1 cells were infected as described in “Intracellular replication in macrophages” and supernatants were sampled at different time points and assayed for the presence of released Lactate dehydrogenase (LDH) using the CytoTox 96 Non-radioactive cytotoxicity assay (Promega, Madison, USA). Data are means ± standard deviations of 3 independent experiments and used to calculate the average% of blue fluorescent cells in images taken with a live-cell imaging system (ECL) (Amersham Biosciences, Uppsala, Sweden) was used.

Supporting Information

Figure S1 In vitro growth of F. tularensis in the presence of CCCP. LVS grown over night in Chamberlain's medium at 37°C, was subcultured to OD₆₀₀ = 0.15 and grown for an additional 24 h, during which OD₆₀₀ was measured at different time points. The PMF inhibitor CCCP was added at a final concentration of 0, 1 or 10 μM to the subcultures when they had reached OD₆₀₀ = 0.4. (TIF)

Figure S2 Intracellular growth (A) and cytopathogenicity (B) of LVS. (A) J774 cells were infected by LVS at an MOI of 200 for 2 h. Upon gentamicin treatment, cells were allowed to recover for 30 min after which they were lysed immediately (corresponds to 0 h; light gray bars) or after 9 h (dark gray bars) or 18 h (black bars) with PBS-buffered 0.1% sodium deoxycholate solution and plated to determine the number of viable bacteria (log₁₀). All infections were repeated two times and a representative experiment is shown. Each bar represents the mean values and the error bar indicates the standard deviation from triplicate data sets. The asterisk indicates that the log₁₀ number of CFU recovered from CCCP treated cells was significantly different at a given time point as determined by a 2-sided t-test with equal variance (*, P≤0.05). (B) Culture supernatants of LVS-infected or uninfected J774 cells were assayed for LDH activity at 0, 9 and 18 h post
infection and the activity was expressed as a percentage of the level of non-infected lysed cells (positive lysis control). Shown are means and standard deviations of triplicate wells from one representative experiment of two. The asterisks indicate that the cytotoxicity levels were significantly different for CCCP treated cells at a given time point as determined by a 2-sided t-test with equal variance (*, P < 0.05; **, P < 0.001).

(TIF)

Table S1 Strains and plasmids used in this study.

(DOC)

Table S2 Oligonucleotides used in this study.

(DOC)

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