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EDITORIAL

Francisella tularensis type VI secretion system comes of age

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Francisella tularensis, a Gram-negative facultative intracellular bacterium, is a causative agent of zoonotic disease tularemia. It is also listed as a category A potential bioterrorism agent.[1] As an intracellular pathogen, *F. tularensis* evolves several strategies of how to defend the host immune system which include complicated intracellular life cycle, ability to infect a variety of host cell types, high morbidity and mortality, and persistence in the environment.[2]

The pathogenicity of *F. tularensis* is associated with its ability to survive inside of the phagocytic cells and to escape into the cytoplasm.[3] Following cell entry, *F. tularensis* is located within a phagosome that begins to mature into a phagolysosome. However, *F. tularensis* is able to prevent fusion of the phagosome with the lysosome.[4] After that, the bacteria degrade the phagosomal membrane and escape into the host cell cytosol where they replicate. The molecular mechanism how the *Francisella* escapes the phagosome is not yet fully understood but it has been established that the disruption of genes located in the *Francisella* pathogenicity island (FPI) leads to the inability of bacteria to escape the phagosome.[5-10]

Pathogenicity islands (PAI) are the sources of various virulence factors that are crucial for interaction of pathogens with their respective host targets. Generally, the virulence factors repertoire encoded within PAI encompasses several functional groups involving adherence factors, siderophorins, exotoxins, invasion proteins and components of type III and IV secretion systems.[11] The FPI is a 34-kb genomic region that includes ~16–19 open reading frames (ORFs), depending on the particular *Francisella* subspecies and was first described by Nano et al.[5] This area presenting in all *Francisella* genomes suggests that it is a part of the core genome.[12] However, in contrast to the rest of the genome the FPI region is characterized by low GC content, indicating horizontal gene transfer.[5] The FPIs share more than 97% nucleotide identity across the different *Francisella* subspecies; however, while the strains highly virulent for humans exhibit the duplicated FPIs, the low virulent strains own only the single FPI copy.[5] The study of intracellular transcriptome of a highly virulent strain of *F. tularensis* during its infection cycle within murine macrophages revealed participation of the FPI gene products in both early and late induction events. This finding corroborates the FPI role in early phagocytosis as well as at the end of the cytoplasmic proliferation.[13] The first FPI identified gene was named *iglC* (intracellular growth locus C), and was found to be needed for *Francisella* intramacrophage growth.[14] Later on, further genes within the FPI, similarly to *iglC*, proved their importance for intracellular growth and accordingly were named *iglA*, *iglB*, *iglD*, *iglE*, *iglF*, *iglG*, *iglH*, *iglI* and *iglJ*. For the other group of the FPI ORFs the designation pathogenicity determinant protein (*pdp*) was adopted and the products of these ORFs were thus denoted PdpA, PdpB, PdpC and PdpD. The intensive bioinformatics search for biologic function of the FPI products revealed for some of them the homology with the components of the type VI secretion systems (T6SS) in *Vibrio cholerae* (V. *cholerae*) and *Pseudomonas aeruginosa* (*P. aeruginosa*).[15,16] T6SS is a novel tool in bacterial protein secretion and its gene cluster contains a core of 13 essential genes that are shared in about 25% of sequenced gram-negative bacterial genomes. The typical feature of all T6SSs is the presence of hemolysin co-regulated protein (Hcp) and valine-glycine repeat protein G (VgrG) in culture supernatants. Hcp and VgrG exoproteins exhibit structural similarities to a puncturing device of bacteriophage translocation machinery, therefore, instead of true secretion they are probably released upon mechanical shearing.[17] In the current T6SS model the Hcp tube is topped by VgrG trimer and the existence of this pilus-like structure is dependent on the expression

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of *icmF*, *clpV*, and *dotU* genes. Additionally, VipA and VipB, other essential T6SS components, form complexes resembling bacteriophage tail sheath which contraction is crucial for Hcp and VgrG export, as well.[17] The real biologic function of Hcp is currently unknown but its structure indicates it might form a channel. As for VgrG protein in the course of *V. cholerae* infection it was found to cross-link host actin filaments and modulate the host signal transduction.[17,18] Recently it was observed that a VgrG spike can be further extended by a proline-alanine-alanine-arginine (PAAR) domain–containing protein and just the specific VgrG-PAAR combination are required for assembly of functional T6SS with different effector recruitment.[19]

The functionality of FPI encoded T6SS is still questionable. The problem is that only few FPI genes are related to conserved T6SS components while other important structures such as an AAA+ Clp-like ATPase are missing.[20] The FPI components with the greatest homology to T6SS proteins are IglA and IglB. It has been shown that these proteins mutually interact and this interaction is vital for their stability and virulence. Hence it is assumed that IglA/IglB complex is similar to *V. cholerae* VipA/VipB complex and form tubular transmembrane structure.[21] The intermediate homology exerts DotU and PdpB/Icm and, finally, *Francisella's* VgrG that is a small 17.5 kDa protein shares only limited homology with the VgrG proteins from *V. cholerae*. On the other hand *Francisella's* VgrG protein is also secreted into culture supernatants and into macrophage cytosol.[20] Cell fractionation studies of the FPI proteins localization revealed that IglC together with IglA, IglB and IglD occur in all fractions, including the location outside of bacteria. Furthermore, the IglC secretion to environmental stimuli was found to be IglA and IglG dependent.[22,23] The structural analyses then indicated overlay of IglC with Hcp3 from *P. aeruginosa*. All these pieces of information led to current view of IglC as being dominant component of the inner tube.[23] Very recent publication identified additional FPI protein IglG as putative PAAR-like protein which core of the PAAR-like domain is presumably attached to the tip of *Francisella* T6SS through interaction with VgrG protein.[24] The N-terminal extension of PAAR-like domain was shown to bind another FPI protein IglF. IglF is a secreted protein required for *in vitro* replication[25]; therefore, IglF might be new *Francisella* effector protein that the release is IglG dependent.[24]

The latest addition to the *Francisella* T6SS jigsaw puzzle is IglE protein. The IglE is a small 13.9 kDa lipoprotein encoded within the FPI. Deletion of *iglE* genes blocks intracellular proliferation of virulent *F. tularensis* strain as well as leads to *in vivo* strain attenuation in mice.[26] The defect in intracellular replication and virulence is associated with the loss of IglE lipidation that is responsible for its localization to bacterial outer membrane. Two-hybrid and *in vivo* co-immunoprecipitation analyses documented that membrane bounded IglE interacts with C-terminus of PdpB/Icm and by this way contributes to channel formation and protein secretion through the *Francisella* T6SS.[27]

In this issue of *Virulence*, Bröms et al.[28] used a mutagenesis-based approach to identify amino acids in N-terminal part of IglE that are critical for functioning of *Francisella* T6SS mediated protein secretion. In contrast to previous studies the authors exploited for the study attenuated live vaccine strain (LVS). First, they in accordance with the previous studies confirmed the outer membrane localization of IglE, its participation in *Francisella* escape from phagosome, *Francisella* failure in inflammasome activation and *in vivo* attenuated phenotype in the mouse model. They also examined the role of IglE in intracellular growth and cytopathogenicity. The previously published data on both the *F. tularensis* Schu S4 and the *F. novicida* IglE mutant described the defective bacterial replication in bone-derived macrophages and within J774 cells, respectively.[26,27] Bröms et al. study exploiting direct injection of the IglE mutant into the cytoplasm of host cells to avoid of phagosome compartment proved efficient cytosolic replication of the mutant strain. Hence, it is evident that IglE protein is important for the phagosomal escape but not for subsequent cytosolic replication. The study of Hara and Hueffer[29] examined by using immune-fluorescence microscopy the localization of FLAG-tagged FPI proteins in infected macrophage-like host cells. The FLAG-tagged IglE protein was found extracellularly in co-location with bacteria and this position was significant for time intervals 30 min and 4 h post-infection. The former interval corresponds with the escape of internalized bacteria from phagosome that supports the Bröms et al. finding. The biologic role of IglE in the latter time interval that is associated with the onset of *Francisella* proliferation has to be discovered, yet. The major point of this article was the mapping of functional domains within IglE. The authors prepared the series of IglE deletion mutants removing about 20 residues across the entire protein. Of them a region encompassing residues 44 – 125 was found to be necessary for IglE function. In the next step the authors generated a series of frameshift mutants to identify key residues within the extreme N-terminus up to 38 amino acid residue. The prepared mutants were tested for their intramacrophage growth capacity and cytopathogenic effects. The results proved that the residues 33–38 are critical for these IglE functions. To verify whether the loss of IglE function of some of generated IglE mutants reflects the defect in T6SS activity, IglC secretion was

monitored. The acquired data confirmed the strong relationship between the ability of the mutant strains to fully complement the wild IglE role in *Francisella* pathogenesis and the ability to support IglC secretion. This finding further emphasizes the dependence of efficient T6SS on the presence of functional IglE protein. Additionally, the single alanine mutants were created within lipobox motif covering amino acid residues from 19–22. Again these mutants were tested by battery of cell assays to document their efficiency in infectious process. Surprisingly, despite the loss of lipidation and even low level of the IglE expression the intracellular replication was not abolished. Furthermore, in one of these mutants the C22A the blockade of IglC secretion was detected but the intracellular replication was untouched, again. Hence it can be anticipated that different parts of IglE structure can influence distinct functions.

In conclusion this study described the importance of *F. tularensis* LVS derived IglE protein for phagosomal escape, cytopathogenic effect and *in vivo* virulence. The construction of carefully designed mutants then provided information about sequence motifs critical for IglE function. The important finding was that mutated IglE proteins which were not able to complement the function of the wild type IglE variant also exerted non-functional T6SS machinery. This evidence further corroborates involvement of IglE protein in the functional T6SS assembly.

This study together with previous publications also documented that IglE was predominantly located in the outer membrane, in less amount in the inner membrane and even secreted. It is intriguing that the mutations that affected IglE lipidation and henced membrane localization did not influence microbial intracellular replication and cytopathogenicity. The authors explained this phenomenon by dual role of IglE as an outer membrane-localized form or as a non-processed secretion effector. Now the major challenge will be to find the biologic functions for both IglE roles.

Disclosure of potential conflicts of interest

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References

[1] Oyston PCF, Sjöstedt A, Titball RW. Tularaemia: bioterrorism defence renews interest in *Francisella tularensis*. Nat Rev Microbiol 2004; 2:967-78; PMID:15550942; https://doi.org/10.1038/nrmicro1045

[2] Rowe HM, Huntley JF. From the outside-in: The *Francisella tularensis* envelope and virulence. Front Cell Infect Microbiol 2015; 5:94; PMID:26779445; https://doi.org/10.3389/fcimb.2015.00094

[3] Tärnvik A. Nature of protective immunity to *Francisella tularensis*. Rev Infect Dis 1989; 11(3):440-51; PMID:2665002

[4] Checroun C, Wehrly TD, Fisher ER, Hayes SF, Celli J. Autophagy-mediated reentry of *Francisella tularensis* into the endocytic compartment after cytoplasmic replication. Proc Natl Acad Sci USA 2006; 103 (39):14578-83; PMID:16983090; https://doi.org/10.1073/pnas.0601838103

[5] Nano FE, Zhang N, Cowley SC, Klose KE, Cheung KKM, Roberts MJ, Ludu JS, Letendre GW, Meierovics AI, Stephens GJ, et al. A *Francisella tularensis* pathogenicity island required for intramacrophage growth. J Bacteriol 2004; 186:6430-6436; PMID:15375123; https://doi.org/10.1128/JB.186.19.6430-6436.2004

[6] Santic M, Molmeret M, Klose KE, Jones S, Kwaik YA. The *Francisella tularensis* pathogenicity island protein IglC and its regulator MglA are essential for modulating phagosome biogenesis and subsequent bacterial escape into the cytoplasm. Cell Microbiol 2005; 7:969-79; PMID:15953029; https://doi.org/10.1111/j.1462-5822.2005.00526.x

[7] Barker JR, Klose KE. Molecular and genetic basis of pathogenesis in *Francisella tularensis*. Ann N Y Acad Sci 2007; 1105:138-59; PMID:17395737; https://doi.org/10.1196/annals.1409.010

[8] Nano FE, Schmerk C. The *Francisella* pathogenicity island. Ann N Y Acad Sci 2007; 1105:122-37; PMID:17395722; https://doi.org/10.1196/annals.1409.000

[9] Ludu JS, de Bruin OM, Duplantis BN, Schmerk CL, Chou AY, Elkins KL, Nano FE. The *Francisella* pathogenicity island protein PdpD is required for full virulence and associates with homologues of the type VI secretion system. J Bacteriol 2008; 190(13):4584-95; PMID:18469101; https://doi.org/10.1128/JB.00198-08

[10] Buchan BW, McCaffrey RL, Lindemann SR, Allen LA, Jones BD. Identification of *migR*, a regulatory element of the *Francisella tularensis* live vaccine strain *iglABCD* virulence operon required for normal replication and trafficking in macrophages. Infect Immun 2009; 77(6):2517-29; PMID:19349423; https://doi.org/10.1128/IAI.00229-09

[11] Gal-Mor O, Finlay BB. Pathogenicity islands: a molecular toolbox for bacterial virulence. Cell Microbiol 2006; 8:1707-19; PMID:16939533; https://doi.org/10.1111/j.1462-5822.2006.00794.x

[12] Larsson P, Elfsmark D, Svensson K, Wikström P, Forsman M, Brettin T, Keim P, Johansson A. Molecular evolutionary consequences of niche restriction in *Francisella tularensis*, a facultative intracellular pathogen. PLoS Pathog 2009; 5(6):e1000472; PMID:19521508; https://doi.org/10.1371/journal.ppat.1000472

[13] Golovliov I, Ericsson M, Sandström G, Tärnvik A, Sjöstedt A. Identification of proteins of *Francisella tularensis* induced during growth in macrophages and cloning of the gene encoding a prominently induced 23-kgdalton protein. Infect Immun 1997; 65:2183-9; PMID:9169749

[14] Pukatzki S, Ma AT, Sturtevant D, Krastins B, Sarracino D, Nelson WC, Heidelberg JF, Mekalanos JJ. Identification of a conserved bacterial protein secretion system in *Vibrio cholerae* using the *Dictyostelium* host model system. Proc Natl Acad Sci U S A 2006; 103(5):1528-33; PMID:16432199; https://doi.org/10.1073/pnas.0510322103

[15] Mougous JD, Cuff ME, Raunser S, Shen A, Zhou M, Gifford CA, Goodman AL, Joachimiak G, Ordoñez CL, Lory S, Walz T, et al. A virulence locus of *Pseudomonas aeruginosa* encodes a protein secretion apparatus. Science 2006; 312:1526-3; PMID:16763151. doi:10.1126/science.1128393

[16] Bönemann G, Pietrosiuk A, Mogk A. Tubules and donuts: a type VI secretion story. Mol Microbiol 2010; 76:815-21; PMID:20444095; https://doi.org/10.1111/j.1365-2958.2010.07171

[17] Pukatzki S, Ma AT, Revel AT, Sturtevant D, Mekalanos JJ. Type VI secretion system translocates a phage tail spike-like protein into target cells where it cross-links actin. Proc Natl Acad Sci U S A 2007; 104:15508-13; PMID:17873062; https://doi.org/10.1073/pnas.0706532104

[18] Shneider MM, Buth SA, Ho BT, Basler M, Mekalanos JJ, Leiman PG. PAAR-repeat proteins sharpen and diversify the type VI secretion system spike. Nature 2013; 500:350-3; PMID:23925114; https://doi.org/10.1038/nature12453

[19] Barker JR, Chong A, Wehrly TD, Yu JJ, Rodriguez SA, Liu J, Celli J, Arulanandam BP, Klose KE. The *Francisella tularensis* pathogenicity island encodes a secretion system that is required for phagosome escape and virulence. Mol Microbiol 2009; 74:1459-70; PMID:20054881

[20] Bröms JE, Sjöstedt A, Lavander M. The role of the *Francisella Tularensis* pathogenicity island in Type VI secretion, intracellular survival, and modulation of host cell signaling. Front Microbiol 2010; 1:136; PMID:21687753; https://doi.org/10.3389/fmicb.2010.00136

[21] Clemens DL, Ge P, Lee B-Y, Horwitz MA, Zhou ZH. Atomic structure of T6SS reveals interlaced array essential to function. Cell 2015; 160:940-951; PMID:25723168; https://doi.org/10.1016/j.cell.2015.02.005

[22] de Bruin OM, Duplantis BN, Ludu JS, Hare RF, Nix EB, Schmerk CL, Robb CS, Boraston AB, Hueffer K, Nano FE. The biochemical properties of the *Francisella* pathogenicity island (FPI)-encoded proteins IglA, IglB, IglC, PdpB and DotU suggest roles in type VI secretion. Microbiology 2011 Dec; 157(Pt 12):3483-91; PMID:21980115; https://doi.org/10.1099/mic.0.052308-0

[23] Rigard M, Bröms JE, Mosnier A, Hologne M, Martin A, Lindgren L, Punginelli C, Lays C, Walker O, Charbit A, et al. *Francisella tularensis* IglG Belongs to a Novel Family of PAAR-Like T6SS Proteins and Harbors a Unique N-terminal Extension Required for Virulence. PLoS Pathog 2016; 12:e1005821; PMID:27602570; https://doi.org/10.1371/journal.ppat.1005821

[24] Bröms JE, Meyer L, Sun K, Lavander M, Sjöstedt A. Unique substrates secreted by the type VI secretion system of *Francisella tularensis* during intramacrophage infection. PLoS One 2012; 7:e50473; PMID:23185631; https://doi.org/10.1371/journal.pone.0050473

[25] Robertson GT, Child R, Ingle C, Celli J, Norgard MV. IglE is an outer membrane-associated lipoprotein essential for intracellular survival and murine virulence of type A *Francisella tularensis*. Infect Immun 2013; 81:4026-40; PMID:23959721; https://doi.org/10.1128/IAI.00595-13

[26] Nguyen JQ, Gilley RP, Zogaj X, Rodriguez SA, Klose KE. Lipidation of the FPI protein IglE contributes to *Francisella tularensis* ssp. *novicida* intramacrophage replication and virulence. Pathog Dis 2014; 72:10-8; PMID:24616435; https://doi.org/10.1111/2049-632X.12167

[27] Bröms JE, Lavander M, Meyer L, Sjöstedt A. IglG and IglI of the *Francisella* pathogenicity island are important virulence determinants of *Francisella tularensis* LVS. Infect Immun 2011; 79:3683-3696; PMID:21690239; https://doi.org/10.1128/IAI.01344-10

[28] Bröms JE, Meyer L, Sjöstedt A. A mutagenesis-based approach identifies amino acids in the N-terminal part of *Francisella tularensis* IglE that critically control Type VI system-mediated secretion. Virulence 2017; 8(6):821-847; https://doi.org/10.1080/21505594.2016.1258507

[29] Hare RF, Hueffer K. *Francisella novicida* pathogenicity island encoded proteins were secreted during infection of macrophage-like cells. PLoS One 2014; 9:e105773; PMID:25158041; https://doi.org/10.1371/journal.pone.0105773