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Running head: *YERSINIAE* VIRULENCE FACTORS

Yersiniae Virulence Factors: Type III Secretion System

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Abstract

Several Gram-negative pathogenic bacteria have evolved a complex protein secretion system termed the Type Three Secretion System (TTSS) to deliver bacterial effector proteins into host-cells that then modulate host-cellular functions. These bacterial devices are evolutionarily related to the flagellar apparatus. Although the TTSSs are substantially conserved among different species, the effector molecules they deliver are species-unique. There exist three human pathogenic *Yersiniae. Yersinia enterocolitica* and *Yersinia pseudotuberculosis* cause self-limiting gastro-enteric diseases and infect mesenteric lymph nodes, while *Yersinia pestis* is transmitted by fleas and can be aerosolized, causing the lethal disease known as plague (also known as Black Death). The TTSS is composed of over 20 proteins making up the injectisome (inserted directly into the host-cell), in addition to translocator, regulator, and modulator proteins, as well as chaperones for several effector proteins. Today, plague is still a health concern due to the ability of *Y. pestis* to be aerosolized. No effective vaccines are currently available to the public. However, research is being implemented to create a vaccine that can be widely used. The purpose of this paper is to update the state of the *Yersiniae* TTSSs by providing a review of recently published primary articles.

Yersiniae Virulence Factors: Type III Secretion System

Of three human pathogenic *Yersiniae, Y. enterocolitica* and *Y. pseudotuberculosis* enter their hosts via the oral-fecal route of infection and are self-limiting enteric pathogens, which infect the mesenteric lymph nodes (14). In contrast, *Y. pestis* is transmitted by fleabite or via the aerosol route and infect regional lymph nodes or lungs leading to the lethal disease known as plague (14).

Y. pestis is thought to have evolved from *Y. pseudotuberculosis* relatively recently (27). Molecular clock estimates, based on mutational differences, place *Y. pestis'* emergence as recently as 9,000 to 40,000 years ago. In comparing *Y. pestis* and *Y. pseudotuberculosis, Y. pestis* has two additional plasmids and has lost some chromosomal genes by deletion or inactivation. These differences enable *Y. pestis* to survive in its vector, the flea, and account for *Y. pestis'* unique route of infection (27).

Common to all three pathogenic *Yersiniae* is the presence of type III secretion system (TTSS) (14, 8). The TTSS is encoded on an approximately 70-kb virulence plasmid termed pYVE or pCD1 (8). The TTSS functions to inject bacterial effector proteins directly into the host-cell cytoplasm and has thus been referred to as an injectisome (8). This system can also be found in several Gram-negative bacteria, and although the systems are highly conserved, the effector molecules delivered into targeted host-cells are unique for each bacterial species (8).

The TTSS functions by means of a secretion apparatus composed of 20-25 different *Yersinia* secretion proteins (Yscs) (8, 18, 23). In fact, all 25 Yscs are required for a fully functioning secretion apparatus (14). It is believed that these injectisomes are environmental sensory receptors that sense the 37°C, low intracellular calcium

concentration of the host-cell, which induces the expression of the effector proteins from TTSS operons. This response is known as the low-calcium response (14). Interestingly, Ysc proteins that comprise the TTSS's injectisome are homologous to flagellar proteins and therefore are thought to have evolved divergently from the flagellar proteins (Figure 1).

Regulation and function of the *Yersiniae* TTSSs have been extensively studied. A detailed description of the TTSS and the Gram-negative bacteria who posses it was published in 1998 by Hueck (14). A more recent description of the *Yersinia* Outer Proteins (Yops) was published in 2005 by Viboud and Bliska (36). The aim of this paper is to update the state of the *Yersiniae* TTSSs.

There are 13 Yops, which are secreted by the TTSS injectisome. These proteins are grouped roughly into the following categories: (i) translocatory proteins involved in the translocation process, (ii) regulatory proteins, and (iii) effector proteins with direct anti-host functions. Interestingly, three of the effector proteins with direct anti-host functions, YopH, YpkA (YopO in *Y. enterocolitica*), and YopM, which are translocated into the host cytoplasm, are homologous to eukaryotic proteins and are thought to have been acquired from the eukaryotic host-cells. For example, YopH is a protein with tyrosine phosphatase activity and is homologous to the eukaryotic protein tyrosine phosphatase (PTPase) (11). The *Yersiniae* utilize YopH to dephosphorylate several macrophage proteins, thereby evading phagocytosis (14).

Plague continues to be a determent to our society. Between 1987 and 2001, 36, 876 cases of plague with 2,847 deaths were reported to the World Health Organization (WHO) (27). Closer to home, in 1992, there was an outbreak in which a plethora of

chipmunks were found dead in the four corners region of the United States: New Mexico, Arizona, Utah, and Colorado. Another reason the plague continues to be a health concern is because of *Y. pestis'* unique ability to be aerosolized (communicated via respiratory droplets) and potentially be weaponized by bioterrorists (27). While much has been accomplished in determining *Y. pestis'* mechanism of action in causing plague, more still needs to be discovered to produce an adequate vaccine.

At present, no effective plague vaccines are available. Those previously developed and used in humans confer low levels of protection, have numerous side effects, and require frequent boosting to develop immunity (27). However, LcrV is an effector protein translocated along with other Yops by the TTSS and is now being targeted as a vaccine candidate (26).

Injectisome: The TTSS needle

The TTSS employs a needle-like apparatus, termed the injectisome, to translocate effector proteins directly into the cytosol of a targeted host-cell (8). The injectisome is composed of about 25 Yscs and uses three translocator Yops, YopB, YopD and LcrV, to form a pore in the host-cell for the translocation of the effector Yops across the target cell membrane (8, 18, 23) (Refer to *Yersinia* outer proteins section below). Injectisomes consist of a basal body spanning both the inner and outer bacterial membranes and a needle (23).

Recently, many of the proteins that make up the injectisome have been shown to be homologous to flagellar proteins (14). A possible explanation for the evolution of the TTSS needle is that it divergently evolved from the basal body of the flagellar apparatus (14). After its evolution, several Gram-negative bacteria could have acquired the system

by horizontal genetic transfer. Although the secretion apparatus is highly conserved among many Gram-negative bacteria, the effector molecules that they deliver are unique to each bacterial species. Therefore, the needle complex may have divergently evolved from the flagellar proteins, whereas the effector molecules were not.

A comparison of the flagellar protein FliN and injectisome protein Ysc Q showed that FliN carries a carboxy-terminal domain of 60 amino acids that is homologous to a respectively located domain in the members of the YscQ family (14). It was also shown that FliF shares a domain with YscJ (14) (Figure 1).

Structural homologs suggest common evolutionary heritage.

(a). FliN_*Y. pestis*: 61-123
YscQ_*Y. pestis*: 236-298

FliN_*Y. pestis* IPVKLSVELGRTKMTI KELLRLSQGSVVSLDGLAG EPLDILINGYLI AQ
YscQ_*Y. pestis* LPVQVSFEVGRQIL DWHTLTS LEPGSLI DLTT PVDGEVRLLANGRLLGH

FliN_*Y pestis* GEVVVVADKYGVRI
YscQ_*Y. pestis* GRLVE IQGR LGVRI

(b). FliF *Y. pt:* 56-242
YscJ *Y. pt:* 23-213

FliF *Y. pt* LYSNLSDRDGGDIVTQ LTQLNI PY – RFADNGGA- - LL I PAEKVHETRL
YscJ *Y. pt* LYTGI SQKEGNEMLAL LRQEGLSADKEP DKDGKIKLLVEE SDVAQAI D

FliF *Y. pt* RLAQQGLP KGGAVGFE LLDQE KFGISQFSEQI - - - NYQRALEGELSRTI
YscJ *Y. pt* I LKRK GYPHE SF S TLQDVFP KDGLISS PI EELARLNYAKAQE - - ISRTL

FliF *Y. pt* GTLGPVLNVRVH LAMPKPSLFV REQK SP TASVTLALQPGRALD–DGQI
YscJ *Y. pt* S EI DGVLVARVHVVL PEEQ–NNKGKKGVAASASVFI KHAADIQF DTYI

FliF *Y. pt* NAIVYMVS SSVAGLPPGNVTVVDQTGRLLTQSDSAGRDLNASQLKFTS E
YscJ *Y. pt* P QIKQL VNNSI EGLAYDRI SV I LVPSVDVRQSSHL PRNTS I LS IQ VSEE

Figure 1. The amino acid sequences for these proteins were obtained from the National Center for Biotechnology Information (NCBI) database and compared using Basic Local Alignment Search Tool (BLAST) (7, 24). Dark blue indicates identities. Aqua indicates amino acid similarities. (a)Analysis of FliN_*Y. pestis* flagellar motor switch protein and YscQ_*Y. pestis* Yop protein translocation protein Q reveals 33% identity and 53% amino acid similarity. (b) Analysis of FliF_*Y. pseudotuberculosis* (*Y. pt*) flagellar motor switch protein and YscJ_*Y. pt* Yop protein translocation protein Q reveals 23% identity and 46% amino acid similarity.

The end of the needle-like structure is made up of YscF, with YopN possibly acting as a “plug”(33). The role of LcrV, YscF, and YopN in the formation of the pore required by the injectisome to deliver the Yops in macrophages was tested by monitoring

the release of the low-molecular-weight fluorescent dye BCECF (2′, 7′-bis- (2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester, 623 Da) and of the high-molecular-weight lactate dehydrogenase (LDH, 135 kDa). Bacteria producing normal Ysc injectisomes, including the YscF needle but no translocators, did not form pores, indicating that the needle is not sufficient by itself for pore formation. YopN might form a link between the needle and the pore, guiding the effectors (18).

In a more recent study, it was found that the length of the injectisome is genetically defined (2). The length of the needle is determined by YscP, which acts as a molecular ruler (3). It is found that a minimal needle length was required for efficient functioning of the *Y. enterocolitica* injectisome and the minimal needle length correlated with the length of the major adhesin at the bacterial surface. The needle may be required for triggering type III secretion, suggesting that needle length may have evolved to match specific structures at both the bacterial and host-cell surfaces (22). YscP is also secreted and was found to be required for Yop secretion by a systematic deletion analysis where the specific region required for Yop secretion (between residues 405 and 500) was accurately localized (2).

YscU, also a key component of the TTSS injectisome, undergoes auto-cleavage of an N-terminal transmembrane domain and a long cytoplasmic C-terminal domain at a NPTH site (32). A $yscU_{N263A}$ mutant makes needles without the LcrV tip complex, essential to pore formation, and could not form translocation pores**.** The $yscU_{N263A}$ mutant was also shown to export less amounts of YscP (the molecular ruler) and consequently, made longer needles (32).

It was recently shown by electron microscopy (EM) analysis that the tip of

needles that protrude from the bacterial surface contained a distinct structure caused by LcrV bound to YscF (19). This unique localization of LcrV may explain its crucial role in the translocation process and its efficacy as the main protective antigen against plague as a vaccine candidate.

Yersinia Outer Proteins (Yops)

The Yops (toxic effector proteins) are encoded on several operons on the virulence plasmid, and their expression is induced after a temperature shift from 25ºC to 37ºC, but at 27ºC in the presence of Ca^{2+} the system remains repressed (14, 41). Normal growth of *Y. pestis* occurs at 25ºC to 27ºC (ambient temperature in flea gut). Eventually, this temperature shift and contact with the mammalian cell (low calcium), leads to the injection of the effector Yops from the bacterium into the host cell (41).

The Yops and other proteins secreted by *Yersinia* TTSS have been roughly grouped into three categories: (i) translocatory proteins involved in the translocation process and pore formation (YopB, YopD, and LcrV), (ii) proteins with direct anti-host function and are translocated directly into the eukaryotic cytosol (YopH, YopM, YopE, YopT, and YopJ) (16), and (iii) regulatory proteins, which mediate cell contact (YopN and YopK) (14, 36) (Table 1).

Table 1. Yops and Other Proteins Secreted by Yersinia TTSS (13, 15, 33)	
(i) Translocatory Proteins	
YopB, YopD, and LcrV	Are involved in the translocation process and pore formation
(ii) Proteins with direct anti-host function	
YopH	Is a tyrosine protein phosphatase
YopM	Binds to α-thrombin and blocks platelet activation
YopE	Directly polymerizes actin microfilaments
YopT	Is similar to YopE, YpkA/YopO are a serine/threonine protein kinases
YopJ	Interferes with mammalian signaling pathways leading to the inhibition of phagocytosis, modulation of cytokine production, and induction of apoptosis
(iii) Regulator Proteins	
YopN	Is surface exposed and involved in the low-calcium response
YopK	Seems to regulate the amount of effector Yops translocated

YopB, YopD, and LcrV: Translocators

YopB and YopD are encoded in the *lcrGVH-yopBD* operon and their transcription is induced by temperature (37°C) and millimolar concentrations of calcium (low-calcium level, mimicking the internal host-cell environment) (14). It has been shown that YopB, YopD (14), and LcrV are translocator proteins (33, 36). While these proteins are involved in translocation, they are not actually translocated into the targeted host-cells. It has also been suggested that these proteins make up the "translocation channel" in the host-cell membrane (pore-formation) mainly because of their hydrophobic domains. However, LcrV is the exception being a dimer in solution (36). The hydrophobic domains found in YopB and YopD, suggested that they are probably transmembrane proteins. Interestingly, YopB has been shown to independently disrupt artificial membranes, whereas YopD cannot. YopB may achieve this by virtue of its two hydrophobic regions (residues 166 to 188 and 228 to 250). Recently, YopB's ability to disrupt membrane was characterized by altering the hydrophobic domains of YopB and then studying the change in the virulence of the mutant strain containing the altered YopB protein (30).

In the aforementioned experiment, a helix-disrupting double consecutive proline substitution in the center of the transmembrane domain of YopB proteins was constructed. Either both hydrophobic domains were disrupted or only one hydrophobic domain was disrupted. *Y. pseudotuberculosis* expressing the mutant proteins was used to infect macrophage or epithelial cells. As it turned out, YopB with two disrupted domains resulted in a non-functional protein, which was not secreted and the YopB with only one disrupted hydrophobic domain had partially disrupted function. Ultimately, all three

functions of YopB, translocation, pore formation, and signaling, require these hydrophobic domains for insertion into the host-cell membrane (30).

The ability of YopB to insert into the host-cell membrane creating a channel along YopD explains how the injectisome can maintain contact with the host-cell without disrupting the integrity of the host-cell membrane. Furthermore, LcrV has also been found to form channels in lipid bilayers and is believed to be part of the pore-formation process making up the channel in which effector proteins are translocated (36). Also, it has been found that the interaction of YopB with the host-cell triggers a pro-inflammatory signaling response in epithelial cells infected with *y. pseudotuberculosis* (35). In fact, it was recently suggested that YopB could potentially be used to enhance the immune response in eukaryotic cells (5).

YopH, YopO (YpkA), YopE, YopT, YopM, YopJ (YopP): Effectors

The most pronounced effects by the TTSS involves the action of several effector Yops, such as YopE, YopH, YopO/YpkA, and YopT, which disrupt the target cell-signaling network and cytoskeleton rearrangement by targeting monomeric GTPases of the Rho family (8, 41). YopH, YopO (YpkA in *Y. pseudotuberculosis*), YopE, YopM, and YopJ (YopP in *Y. enterocolitica*) are directly translocated into the host-cell cytoplasm by the TTSS. Interestingly, three of these effector proteins have been found to be homologous to eukaryotic proteins (Figure 2).

YopH: A Tyrosine Protein Phosphatase

It is believed that several of these translocated effector Yops have been acquired from the host itself. The *yersiniae* are able to use these homologous host proteins, to their advantage. For example, YopH is a 468 amino acid long protein with tyrosine

phosphatase and its carboxy terminal is homologous to the eukaryotic protein tyrosine phosphatase (PTPase). In fact, YopH was shown to be the most active tyrosine phosphatase known (11). The *Yersiniae* utilize YopH to dephosphorylate several macrophage proteins contributing to their resistance to phagocytosis. One way in which YopH has been shown to evade phagocytosis has been by inhibiting the invasin interaction activated, β 1 integrin pathway. YopH has two substrate recognition sites (11). The presence of two substrate recognition sites is believed to be the evidence for why this PTPase-like protein has evolved a highly complex mechanism to locate substrates in the complex environment of the host-cell (36).

In a recent study, the factors important for growth during lung infection were determined (9). Using a YopH mutant strain of *Y. pseudotuberculosis*, mice were infected intra-nasally and growth was monitored in lungs and systemic tissues. As it turned out, the YopH mutant failed to proliferate at wild-type levels four days after intranasal inoculation. This study concluded that YopH, together with YopB, was essential for development of a *Y. pseudotuberculosis* murine lung infection (9).

YpkA/YopO: A Serine/Threonine Protein Kinase

YpkA (YopO in *Y. enterocolitica*), approximately 730 amino acids, is transcribed on an operon together with YopJ/YopP (14). YpkA has a serine/threonine kinase catalytic site on the N-terminal half of the protein that is homologous to the eukaryotic serine/threonine protein kinase (36). Like YopE and YopT, YpkA interacts with RhoGTPase and thereby disrupts the actin cytoskeleton of cultured cells to prevent phagocytosis (1). Because YpkA is weakly expressed, and the effector proteins, YopE and YopH, mask its activity, a mutant with the deletion of these other two effector

proteins has to be made in order to detect the activity of YpkA (14, 36, 39). It was recently shown that a *Y. pseudotuberculosis* YpkA mutant with a deletion of its kinase domain was greatly attenuated in a murine model of infection (39).

YopE: A GTPase Activating Protein (GAP)

YopE, 219 amino acids, is highly conserved amongst all three pathogenic *Yersiniae* (14). YopE has been found to induce cytotoxic effects on host cells (4, 14, 36, 37). It was proposed that YopE exhibits GTPase-activating protein (GAP) activity towards Rac1, RhoA and Cdc42 and is essential for induction of the cytotoxic phenotype on HeLa cells (rounding, etc.) and virulence (38). Although it had been demonstrated that the cytotoxicity of YopE was due to its GAP activity toward the small GTP binding proteins, RhoA, Rac-1, and Cdc42**,** *in vitro* (1) there was no formal evidence for a direct interaction between YopE and any of these three RhoGTPases during an *in-vivo* infection. In an effort to understand the functional role of YopE *in vivo*, its GAP activity in infected eukaryotic cells was found to inactivate Rac1 as early as 5 minutes after infection. On the other hand, RhoA was downregulated approximately 30 minutes post infection and YopE had no apparent effect on the activation state of Cdc42 in infected cells. This study demonstrated that Cdc42 is not an *in vivo* target for YopE, and that YopE preferentially interacts with Rac1, and to a lesser extent with RhoA, within targeted host-cells (4). Ultimately, the major function of YopE is thought to be its contribution to the overall antiphagocytic activity of the *Yersiniae* TTSS (37).

YopT: Like YopE, also Downregulates Rho GTPase

YopT, 322 amino acids, is not expressed by sero-type strains of *Y. pseudotuberculosis* and was therefore, the last effector protein identified in 2002 (8, 14).

In 2003, a *yopE* null (complete knock-out) *Y. pseudotuberculosis* mutant was found to be much more attenuated than the *Y. enterocolitica yopE* mutant. This was most likely due to *Y. pseudotuberculosis* lacking YopT. More importantly, RhoA modification was shown to be strictly dependent on YopT without the need of any additional effector Yops (18). More recently, YopE and YopT were shown to have some level of functional redundancy by sharing overlapping virulence functions (37). Observations such as the disruption of stress fibers, cell rounding, and inhibition of phagocytosis by YopE and YopT led to this assumption (36).

In order to explore this assumption, the contributions of YopE and YopT to the pathogenesis of *Y. pseudotuberculosis* were studied in a mouse infection and tissue culture infection model. A $YopE^{+}T^{-}$ strain and a $YopE^{+}T^{+}$ strain colonized spleens of mice at similar levels after four days of oral infection. This observation suggests that YopT is not required for virulence. In sharp contrast, spleen colonization by a $YopE^{-}T^{-}$ strain was significantly reduced. However, a $YopE^{-}T^{+}$ strain colonized spleens at levels comparable to those of the $YopE^{+}T^{-}$ strain. These data suggest that YopT can promote virulence in the absence of YopE (37).

Furthermore, infection of HeLa cells with $YopE^{-}T^{-}H^{-}J^{-}$ strain expressing either YopE or YopT showed that YopE had a stronger antiphagocytic activity than YopT. This study concluded that YopE is a potent inhibitor of infection-induced signaling cascades, and YopT can only partially compensate for the loss of YopE (37), which explains why a *Y. enterocolitica yopE* mutant, still experiences attenuated virulence.

YopM: Blocks Platelet Activation by Binding Thrombin

YopM, 376 amino acids, contains variable numbers of leucine rich repeat motifs

and is the only *Yersiniae* TTSS effector Yop that does not encode an enzymatic activity (36). YopM is homologous to the thrombin-binding domain of the α chain of human platelet surface glycoprotein Ib (GPIbα). This protein is also homologous to a portion of von Willebrand factor, which apart from its many functions, serves as a carrier of clotting factor VIII (14) (Figure 2). Purified YopM was shown to bind thrombin and to inhibit platelet aggregation *in vitro* (14). Although it is known that YopM plays an important role in pathogenesis, the significance of thrombin binding for *Y. pestis* pathogenesis is not clear (12). It is believed that YopM may compete with platelets for thrombin binding *in vivo* and that the resulting prevention of blood clot formation could enhance the dissemination of the bacteria throughout the body (14). Interestingly, YopM is translocated into the cytosol of the host-cell and has been shown to localize to the nucleus of the target cell (8), but it is not known how nuclear localization is related to the function of YopM (36). The most recent data indicated that YopM targets the innate immune system, as it was required for depletion of NK cells after intravenous infection of mice with *Y. pestis* (15). Studies in which gene regulation by YopM was examined, gave contradictory results as well as murine infection data (36). Figure 2 illustrates the alignment of *y. pestis'* YopM with human platelet glycoprotein Ib α (GP-Ibα).

Structural homologs suggest common evolutionary heritage.

(a). GP-Ip α: 43-132
YopM_*Y. pestis*: 273-353

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GP-Ip α       PDLPKDTT I LHLSENLLYTFSLATLMPYTRLTQ LNLDRCELTKLQV
YopM_Y. pestis PE LPQS LTFLDVSENI- - -FSGL SE L P –PNLYYLNASS NEI RSL- -

GP-Ip α       D GT LPVLGTLD LSHNQLQSLPLLGQTLPALTVLDVSFNRLT SL P
YopM_Y. pestis C D LPP SL EELNVSNNKL I ELPALP- - - PRLERL I ASFNHLAEVP
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Figure 2. The amino acid sequences for YopM and GPIbα were obtained from the NCBI database and compared using BLAST (20). Dark blue indicates identities. Aqua indicates amino acid similarities. (a)Analysis of human platelet glycoprotein Ib α (GP- Ib α) and *Y. pestis* Yop protein translocation protein M reveals 36% identity and 50% amino acid similarity.

YopJ: A Cysteine Protease

YopJ (YopP in *Y. enterocolitica*), 264 amino acids, has been shown to induce apoptosis in cultured murine macrophages (21). More recently, YopJ was found to cause apoptosis by inhibiting the mitogen-activating protein kinase (MAPK) and nuclear factor-κB (NF-κB) signaling pathways of the host-cell and blocking host proinflammatory response by suppressing cytokine production (36), and interfering with ubiquitination (tagging of proteins to be destroyed) (14). Yops counteract the MAPK and NF-κB signaling pathways of the host-cell by acting like a protease by means of binding and blocking activation of the MAPK kinases and IKK (36, 42). Because NF-κB promotes cell survival, a recent study set out to determine whether inhibition of NF-κB by YopJ was enough to cause apoptosis (42). In order to accomplish this, macrophages expressing NF-κB inhibitors were infected with *Y. pseudotuberculosis* strains either expressing YopJ or not. As it turned out, the *Y. pseudotuberculosis* strains expressing YopJ were more effective in causing apoptosis than the strains that were not expressing YopJ, suggesting that deactivation of both NF-κB and MAPK pathways are necessary for *Yersinia* to cause rapid apoptosis (42). YopJ has also been shown to be a deubiquitinating enzyme that negatively regulates signaling by removing ubiquitin moieties from critical proteins. In that regard, an *in vitro* assay demonstrated the deubiquitinating activity of purified YopJ (43).

When YopP was directly coupled to YopJ, YopP was secreted more readily than YopJ (41). Recently, a YopJ-deleted mutant strain of *Y. pestis* expressing YopP was shown to provide a better apoptotic potential. In J774A.1 cells (a BALB/c murine macrophage strain usually used for the production of monoclonal antibodies), RAW264.7

cells (a murine macrophage strain that does not demonstrate the prostaglandin (PG)-dependent autocrine regulation of tumor necrosis factor- (TNF-) secretion observed in primary resident peritoneal macrophages (RPMs)), and primary murine macrophages, better than even the most virulent strains of *Y. pestis*, EV76 and Kimberley 53 (35). It has been proposed that perhaps *Y. pestis* expressed YopJ instead of YopP because in *Y. pestis'* early stages of infection it propagates in macrophages, and, therefore, it is more advantageous for *Y. pestis* if these host-cells stay alive (41).

Still, the YopJ contribution to *Y. pestis* virulence is not clear. A recent study concluded that YopJ suppresses tumor necrosis factor α (TNF-α) induction and contributes to apoptosis of immune cells in the lymph node but is not a virulence factor in a rat model of bubonic plague (17). Following intra-dermal infection with a fully virulent *Y. pestis* strain or an isogenic *yopJ* mutant, it was determined that the deletion of *yopJ* resulted in a two-fold decrease in the number of apoptotic immune cells in the bubo, a swollen lymphnode, and a threefold increase in serum tumor necrosis factor α levels but did not result in decreased virulence, systemic spread, or colonization levels in the spleen and blood. Ultimately, the data suggest that YopJ is not essential for bubonic plague pathogenesis, but rather, the effects of YopJ appear to overlap and augment the immunomodulatory effects of other *Y. pestis* virulence factors (17).

More recently, YopJ was shown to exert its deleterious effect by catalyzing the acetylation of two serine residues in the activation loop of the MAP kinase kinase, MEK2, thereby preventing the phosphorylation of these serine residues required for activation of MEK2 and downstream signal propagation. YopJ has been shown to acetylate a threonine residue in the activation loop of both the α and β subunits of the

NF-κB pathway kinase, IKK (21). In addition, YopJ was found to not only inhibit production of the inflammatory cytokine tumor necrosis factor α (TNF-α), but also was found to inhibit the anti-inflammatory cytokine, interleukin-10 (IL-10) expression (6).

YopN and YopK: Regulator and Modulator

YopN and YopK are considered to be regulatory proteins because they mediate cell contact and also have regulatory involvement (14). YopN is surface exposed and is involved in the low-calcium response (14). TTSS secretion channels shut in the presence of millimolar concentrations of Ca^{2+} found in mammalian blood (18). YopN mutants were found to secrete large quantities of Yops even in the presence of Ca^{2+}. For this reason, YopN is thought to act as a "plug". In the presence of an appropriate secretion signal (low calcium concentration), YopN is secreted into the supernatant by the *Yersinia* TTSS followed by the other effector Yops (18).

Unlike YopN, YopK seems to regulate the amount of effector Yops that are translocated by acting as a modulator (13). YopK is encoded outside the *lcrG-lcrV-sycD-yopBD* operon, and its expression is also regulated by temperature and calcium levels. YopK is not translocated, but appears to be located in the vicinity of cell-associated bacteria during the infection process (14). YopK was shown to play a modulatory role in the translocation process when a YopK mutant expressed hypertranslocation (14).

Other Virulence Associated Factors: PNPase

Recently, it has been found that the exoribonuclease, polynucleotide phosphorylase (PNPase) is required for the optimal function of the *Yersiniae* TTSS (29). HeLa cells were used to examine the cytotoxic effect of *Y. pseudotuberculosis* and *Y. pestis pnp* mutants by comparing mutant strain cytotoxicity to that of the wild-type

strains. The kinetics of the HeLa cell cytotoxicity induced by *pnp* strains appeared delayed, thereby indicating that the *pnp* strain had compromised TTSS activity (29). More importantly, it was also shown that complementing the mutant strains with the S1 RNA-binding domain of PNPase could restore the optimal TTSS function. Furthermore, a murine infection using the above mentioned strains recapitulated attenuated virulence in the *pnp* strain (28).

Chaperones

Yops are translocated through a narrow hollow hole in the injectisome in an unfolded state, suggesting that proteins may be secreted before acquiring their native conformations (40). Some of these translocated or secreted proteins have been shown to require small, usually acidic, accessory proteins with binding specificity towards each individual secreted protein that maintains them in an unfolded conformation required for passing through the needle (14, 40). These small chaperone proteins are required for presecretory stabilization (14). The TTSS chaperones appear to keep secreted proteins from prematurely interacting with translocatory proteins, fulfilling their role as bodyguards (their second name) (14).

Chaperones have been described for the effector proteins YopE, YopT, and YopH. SycE is the chaperone for YopE, SycT is the chaperone for YopT, and SycH is the chaperon for YopH (34). SycD is the chaperone for both YopB and YopD (10, 24, 35), while ScyN uses YscB (an injectisome protein) as a co-chaperone for secretion of YopN in *Y. pestis* (35). Recently, two crystal structures of YopN in complex with its heterodimeric secretion chaperone, SycN–YscB, and also the co-regulatory protein, TyeA, were solved. These two structures were combined to construct a theoretical model

of the YopN–SycN–YscB–TyeA complex (31). However, no chaperones had been described for YopO, YopP, YopQ (YopK in *Y. pseudotuberculosis*), and YopM. More recently, though, it was shown that these proteins did not require the assumed chaperones (35).

Infection still a problem

Bubonic plague is a lethal disease caused by *Yersinia pestis*. The Japanese weaponized *Y. pestis* from 1932 until the end of the Second World War when they used *Y. pestis*-flea bombs on civilians and prisoners (27). Instead of using the flea as a vector, some countries began to try aerosolizing *Y. pestis*. After 1969, the U.S. unilaterally ceased in the production of biological weapons. Although not proven, it is believed that the former Soviet Union created large facilities whose sole purposes were to find a way to deliver *Y. pestis* strains by aerosol (27).

The U.S. national laboratory response network detects biological terrorism agents such as *Y. pestis*. Once possible uses of biological weapons are found, they are referred to the Center for Disease Control (CDC) and the American Society for Microbiology to identify and confirm the *Y. pestis* strains (27). Streptomycin is traditionally regarded as the most affective treatment for *Y. pestis,* 1g (twice daily) for ten days and was the first antimicrobial shown to be affective against the pneumonic plague. Another drug that has been found to be affective is aminoglycosidegentamycin. Although very rare, it has been found that some strains of *Y. pestis* are antibiotic resistant, but luckily they have only been reported in Madagascar (27). Unfortunately, the plasmid responsible for the resistance has been reported to be self-transferrable to other bacteria (27).

Vaccine Production

Killed-whole-cell vaccines and live-attenuated vaccines have been created in the past, but have been found to be less than adequate. Not only do both vaccines only provide a short duration of protective immunity, but also the killed-whole-cell vaccine did not protect against primary pneumonic plague, while the live-attenuated vaccines maintained enough virulence to be unsuitable and excessively dangerous for public use (27). They offer low levels of protection, have numerous side effects, and require frequent immunization (26).

As a result, researchers are looking towards subunit vaccines as the answer. LcrV is an effecter protein translocated along with several Yops by the TTSS. This protein suppresses the host's immune system response. A subunit vaccine in development is based on LcrV (also known as the (V) antigen). In a recent study, an adenovirus-based vaccine vector expressing the V-antigen was used to immunize mice. The experimental findings were that mice immunized with a single intramuscular dose of the vaccine were protected from a lethal intranasal challenge of *Y. pestis* (26). Therefore, hope for an effective mechanism whereby infection can be halted may be on the horizon.

Conclusion

There are three human pathogenic *Yersiniae,* which share a common virulence factor termed the Type Three Secretion System present on an approximately 70-kb virulence plasmid. Many other Gram-negative bacteria possess this system, which allows them to transfer effector proteins directly into the host cell. The proteins that make up the apparatus "injected" into the host cell termed the injectisome are homologous in all the species that posses this system. However, the effector proteins are species-unique and are

believed to have been acquired from the hosts each species infect. There is still a health concern associated with *Yersiniae* especially with the plague-causing *Y. pestis* and its ability to be aerosolized, making it a potential bioterrorist weapon. Understanding the biology of the virulence factors of *Yersiniae* will allow for the development of novel preventative and therapeutic strategies to cope with *Yersiniae* infections.

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