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The Translocator Protein YopD in Yersinia pseudotuberculosis: A Tool and a Weapon

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The Translocator Protein YopD in Yersinia pseudotuberculosis: A Tool and a Weapon

A dissertation submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

MICROBIOLOGY AND ENVIRONMENTAL TOXICOLOGY

by

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June 2014

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Abstract

Walter Adams

The Translocator Protein YopD in Yersinia pseudotuberculosis: A Tool and a Weapon

The aim of this dissertation is to enhance our fundamental understanding of host-pathogens interactions. I investigated these interactions by studying the relationship between the innate immune response and Yersinia pseudotuberculosis. Yersinia is a genus of Gram-negative bacteria that employ a type III secretion system (T3SS), an important virulence factor for Yersinia pathogenesis. The T3SS forms a pore in the membrane of host cells and injects Yersinia effector proteins into the host cell cytoplasm to impair the host immune response. YopD is a T3SS translocator protein involved in the post-transcriptional regulation of yop synthesis and pore formation in host cell membranes, both of which are directly linked to the effective translocation of effector proteins into the host cell cytosol. YopD is also engaged in interactions with several proteins including its chaperone LcrH/SycD, its fellow translocator proteins YopB and LcrV, the regulatory protein TyeA, the effector protein YopE, and it can even self-oligomerize. As YopD is a central player in key
T3SS functions and associated protein interactions, there are many important questions that can be addressed through careful study of the YopD protein.

The first part of this dissertation utilizes YopD as a tool to understand how host cells detect T3SSs during Yersinia infection. Different models have been proposed to address how the host cell senses the presence of T3SSs. Some research indicates that the formation of a pore and subsequent K$^+$ efflux itself leads to the induction of an immune response. A competing hypothesis is that pore formation alone is insufficient and that the translocation of T3SS cargo is required for the induction of this immune response. We constructed a Y. pseudotuberculosis mutant expressing YopD devoid of its predicted transmembrane domain (YopD$_{\Delta TM}$) that formed pores in host cells but did not translocate T3SS cargo. Interestingly, this YopD$_{\Delta TM}$ mutant failed to induce significant production of pro-inflammatory cytokines, suggesting that host T3SS sensing requires either a translocation mediated event or the presence of a WT translocon.

The second part of my dissertation focuses on how YopD is required for the optimal performance of the T3SS weaponry. While the YopD amino and carboxy termini participate in pore formation and T3SS regulation, the role of the central region between amino acids 150-227 remains unknown. I assessed the contribution of the YopD central region by generating Y. pseudotuberculosis YopD$_{\Delta 150-170}$ and YopD$_{\Delta 207-227}$ mutants and analyzing their T3SS functions. These mutants formed robust pores in macrophages and displayed wildtype levels of Yop secretion in vitro.
However, both mutants exhibited defects when translocating into neutrophils and macrophages, could not prevent phagocyte ROS production, and displayed a virulence defect in disseminated *Yersinia* infection *in vivo*. These findings suggest that the YopD central region facilitates optimal T3SS effector protein delivery into target host cells to effectively disarm innate immune defenses such as ROS production. Together these findings enhance our understanding of host-pathogen interactions, both in terms of how T3SSs are detected by the host and how specific components contribute to the optimal performance of T3SS function.
Dedication and Acknowledgements

I dedicate this Ph.D. to Padre, Madre, and Cris Cross. Without them, I would have given up telling bad science jokes a looong time ago.

I would like to acknowledge all the family and friends that have helped me along the way.

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I would like to acknowledge my Thesis Committee for their support, constructive criticism, and for their support of HOTJEMs.

I would like to acknowledge all the parties and board games that helped me keep a healthy work-life balance.
I would like to acknowledge all the people who ever laughed at a science joke I told. That little pat on the back will keep this party running for a long, long time.

Chapter 2 of this dissertation is a multi-author publication. My main contributions were as follows: I performed the Ethidium Bromide Pore Formation Assays in Figure 2A-C with Laura Kwuan. I performed the translocation assays in Figure 3A-B and the TCA precipitation in Figure 3C. I performed the immune response assays in Figure 4B-D. I wrote and edited portions of the manuscript.

**Chapter 1 - Introduction**

1.1 Host-Pathogen Interactions

Microorganisms are involved in almost every facet of life and are more numerous than any other group of organisms on Earth. While the vast majority of microorganisms are beneficial or harmless to mammals, a subset of microbes are pathogenic and can cause disease or even death in their hosts. These microbial invaders employ a diverse array of strategies to promote their virulence. Accordingly, host cells are equipped with a wide array of sensors and mechanisms that detect
invading pathogens and initiate an immune response to resolve the infection. Over time, this struggle has evolved into a complex set of host-pathogen interactions that ultimately determine the progression or clearance of a disease. This thesis addresses two important questions that govern the outcome of host-pathogen interactions: 1) how do host cells detect invading pathogens and 2) what is the role of different virulence factors in bacterial pathogenesis.

1.2 Type III Secretion Systems (T3SSs) in Gram-negative Bacteria

Bacteria employ a range of mechanisms and strategies to grow, acquire nutrients, replicate, and otherwise survive in host and environmental settings. One common macromolecular nanomachine that bacteria use to grow within host organisms is the Type III Secretion System (T3SS). Extensive structural and genetic analyses of T3SSs have revealed that they can be classified into two fundamental categories, flagellar T3SSs and non-flagellar (NF) T3SSs (Blocker, Komoriya, & Aizawa, 2003; Macnab, 2004; Spreter et al., 2009). The primary function of flagellar T3SSs is to control bacteria motility. Meanwhile, NF-T3SSs serve as a transport apparatus or "injectisome", enabling bacteria to translocate bacteria effector proteins into host cells, a trait strongly correlated with virulence. Over two dozen Gram-negative bacterial species utilize NF-T3SSs as a crucial virulence factor to promote disease (Cornelis, 2006).
The T3SS is a needle like apparatus comprised of approximately 25 structural proteins that is assembled in response to specific environmental cues. The T3SSs of different bacteria are assembled in response to different environmental conditions. For example, the T3SS in *Yersinia* is induced at 37°C in the presence of millimolar levels of calcium, whereas expression of the Salmonella SPI-1 T3SS is induced in an environment at near neutral pH with high osmolarity (Altier, 2005; Susan C. Straley, Plano, Skrzypek, Haddix, & Fields, 1993). In contrast to the differing conditions that induce T3SSs, the individual components of these injectisomes are well conserved across different bacterial species. In fact, a unifying nomenclature has been employed by several groups in an attempt to bring consistency amongst the seven different T3SS families (Abby & Rocha, 2012; Abrusci, McDowell, Lea, & Johnson, 2014; Desvaux, Hébraud, Talon, & Henderson, 2009). Spanning across both the inner and outer membrane, the structure of the needle complex forms a hollow tube, through which bacterial translocator proteins are secreted. The translocator proteins form a translocon complex at the tip of the needle, which is required for the formation of pores in the membranes of host cells. Subsequently, effector proteins are thought to be translocated through the needle into the cytoplasm of host cells, where they disrupt the ensuing immune response through a range of mechanisms (Cornelis, 2006).

Despite the conservation in structure amongst T3SSs in Gram-negative bacteria, the nature and function of the effector protein expressed by each pathogen varies depending on the pathogen’s life cycle and growth niche. Extracellular pathogens such as pathogenic *Yersinia* species, *Pseudomonas aeruginosa*, and *Vibrio*
*parahaemolyticus*, employ T3SSs to inhibit uptake by professional phagocytes by employing effector proteins that disrupt the actin cytoskeleton (Cornelis, 2002b; Fällman et al., 1995; Grosdent et al., 2002; Rosqvist et al., 1990). Other extracellular bacteria, such as the plant pathogen *Pseudomonas syringae*, use the T3SS to counter host defenses and extract plant nutrients (Stavrinides, McCann, & Guttman, 2008). In contrast, *Chlamydia, Shigella*, and *Salmonella* species utilize T3SSs to enter non-phagocytic cells as well as to survive in host cells (Betts-Hampikian & Fields, 2010; Dandekar, Astrid, Jasmin, & Hensel, 2012; Schroeder & Hilbi, 2008).

It is important to note that while almost all bacteria with T3SSs are pathogenic, there have been several studies using genome sequencing data that detect the presence of type three secretion system genes in non-pathogenic organisms (M. J. Pallen, Beatson, & Bailey, 2005). These include reports of a T3SS in a non-pathogenic strain of *E. coli* and the nitrogen-fixing plant symbiont *Rhizobium* (Marie, Broughton, & Deakin, 2001; M. J. Pallen, Chaudhuri, & Henderson, 2003). Additional evidence has been discovered in the saprophytic myxobacterium *Myxococcus xanthus* and the heterotrophic nonmotile bacterium *Verrucomicrobiunm spinosum* (M. J. Pallen et al., 2005).

In a broader context, the non-virulent role function of T3SSs are more apparent when one considers the distinct categories of flagellar T3SSs and NF-T3SSs. Recent analysis by Abby and Rocha reveals several important findings regarding the relationship between these two closely related T3SSs (Abby & Rocha,
By analyzing the genomes of 699 flagellar T3SSs and 222 NF-T3SSs they found that the flagellar T3SS was primordial, indicating that the original purpose of the T3SS was to transport flagellar components through bacterial membranes and control motility (Abby & Rocha, 2012). Thus, according to this study, the NF-T3SS and its ability to inject target cells with effector proteins appears to have arisen from exaptation from the flagellar T3SS (Abby & Rocha, 2012). Taken together, while T3SSs are crucial virulence factors for a wide range of invasive microbes, these findings suggest that T3SSs have important roles outside of bacterial pathogenesis.

1.3 The Yersinia Genus

The *Yersinia* genus contains 18 different species, four of which are considered to be pathogenic to humans (Savin et al., 2014). The most notable of these pathogens is *Yersinia pestis*, the causative agent of both the bubonic plague and the pneumonic plague. Throughout history three devastating pandemics have been attributed to *Y. pestis*. The first was Justinian's plague, caused by the Antique biovar, which persisted around the Mediterranean from 541 to 750 A.D. (Kennedy, 2007). The *Y. pestis* lineage associated with Justinian’s plague is now extinct or has yet to be detected in wild rodent reservoirs as recent genetic analyses indicate this branch has no contemporary representatives (D. M. Wagner et al., 2014). Biovar Mediaevalis is responsible for the second pandemic, referred to as the *Black Death*, which began in 1345-6, lasted until 1750, and resulted in the death of approximately one third of
Europe's population (Seifert et al., 2013). Lastly, the modern plague pandemic, which originated in the Yunnan region of China in the mid-19th century, was caused by biovar Orientalis. This biovar is currently endemic to Asia, eastern and southern Africa, and the western United States (MacMillan et al., 2011; Rollins, Rollins, & Ryan, 2003; Wilschut et al., 2013). Notably, it was during this most recent pandemic that *Yersinia pestis* was first identified as the etiological cause of plague by its namesake, Alexander Yersin (Treille & Yersin, 1894).

Plague is a zoonotic disease that is maintained in animal reservoirs, most commonly associated with rodent populations. The persistence of plague in nature depends on cyclic transmission between fleas and mammals (Perry & Fetherston, 1997). Once inside the host, *Y. pestis* spreads to the regional lymph nodes, growing rapidly, and resulting in the formation of swollen lymph or bubos (Perry & Fetherston, 1997). *Y. pestis* can then disseminate into the blood stream as well as other organs including the lungs, spleen, and liver (Perry & Fetherston, 1997). An individual in whom bubonic plague has developed into secondary pneumonic plague, can infect others by transmitting infectious respiratory droplets.

Two other well-studied pathogenic *Yersinia* are the gut pathogens *Yersinia enterocolitica* and *Yersinia pseudotuberculosis*. They live in aquatic and soil environments, but are often found in contaminated meat products, especially pork, and are transmitted through the fecal-oral route (Fredriksson-Ahomaa, Hielm, & Korkeala, 1999; Laukkanen et al., 2008). Both *Y. pseudotuberculosis* and *Y.
*enterocolitica* can cause acute gastroenteritis and mesenteric lymphadenitis in healthy individuals, although these infections are usually self-limiting (Bottone, 1999; Long et al., 2010). In immunocompromised patients, such as those suffering from iron overload disease, symptoms are more severe and infection and lead to septicemia or even death (E J Bottone, 1997).

In an established mouse model of enteropathogenic *Yersinia* infection, a disseminated infection occurs over several days, the pathogenesis of which has been well characterized by several groups. Upon ingestion, enteropathogenic *Yersinia* travels through the gastrointestinal (GI) tract. During this phase the bacteria encounter an intestinal bottleneck, which significantly impacts their ability to disseminate to distal tissues such as the spleen and liver (Barnes, Bergman, Mecsas, & Isberg, 2006). Approximately 24 hours after infection, the bacteria start to colonize the ileum, cecum, and ascending colon, while also establishing a replicative niche in the intestinal lumen of the host (Barnes et al., 2006; Logsdon & Mecsas, 2003). By 48 hours post-infection, this intestinal population efficiently colonizes the mesenteric lymph nodes (MLNs), and Peyer's patches (PPs), while also seeding the spleen and liver (Logsdon & Mecsas, 2003). After 72 hours of infection, colonization of distal tissues is well established and is independent of the populations in the MLNs and PPs. The bacterial load continues to increase in the spleen and liver over the next couple of days and the mice exhibit distinct signs of morbidity including weight loss, lethargy, and scruffy fur (Logsdon & Mecsas, 2003).
Despite the many similarities between the two enteropathogens, *Y. pseudotuberculosis* is actually much more closely related to *Y. pestis* than it is to *Y. enterocolitica*. In fact, research indicates that *Y. pestis* evolved from *Y. pseudotuberculosis* as recently as 1,500-6,400 years ago, and thus, despite their different lifestyles, the two pathogens share a very high level of genetic similarity (Achtman et al., 1999; Cui et al., 2013; Harbeck et al., 2013). In contrast, *Y. enterocolitica* is estimated to have diverged from *Y. pseudotuberculosis* between 41 and 186 million years ago (Zhou & Yang, 2009).

The fourth human *Yersinia* pathogen is the recently identified species *Yersinia wautersii* (Savin et al., 2014). While different strains of *Y. wautersii* were isolated from human stool samples, data on clinical symptoms of infected individuals is not available. Despite this lack of evidence concerning the bacterial pathogenesis of this species in humans, the authors detected the presence of the *Yersinia* virulence plasmid (pYV) in one of the isolates. This is a strong indicator of the human pathogenic nature of the species, as all other three *Yersinia* human pathogens express pYV to promote their virulence (G R Cornelis et al., 1998; Gemski, Lazere, Casey, & Wohlhieter, 1980).

1.4 *Yersinia* T3SS Structure, Formation, and Regulation
The structure and ordered formation of the T3SS is discussed in section 1.4.1, whereas the underlying regulatory mechanisms responsible for this assembly is discussed in section 1.4.2.

1.4.1 Yersinia T3SS Structure and Formation

The T3SS is an important Yersinia virulence factor encoded on a 70-kb virulence plasmid (pYV). The Yersinia T3SS belongs to the Ysc (Yop secretion) family of injectisomes, one of seven injectisome families that are classified based on phylogenetic analyses of conserved T3SS proteins (Cornelis, 2006). Other members of this family include Pseudomonas aeruginosa, Photorhabdus luminescens, and Aeromonas species (Troisfontaines & Cornelis, 2005).

The entire Yersinia T3SS is a complex nanomachine with approximately 25 proteins involved in its assembly including structural components, chaperones, and other ancillary proteins (Cornelis, 2006; Diepold et al., 2010). Spanning across the inner and outer membranes, the injectisome can be divided into three distinct sections: 1) a basal body containing a cytoplasmic ATPase-C ring complex and a non-cytoplasmic portion that spans the inner membrane (IM), periplasm, and outer membrane (OM), 2) a ~55nm needle that extends outwards from the basal body into the extracellular milieu, and 3) a tripartite translocon complex that forms at the tip of the needle. Formation of the Yersinia T3SS itself begins with two independent assembly platforms. One is initiated in the OM via the protein YscC, which forms a
stable secretin ring in the membrane. YscD and YscJ are then assembled stepwise in an outside-in manner toward the IM (Diepold, Wiesand, & Cornelis, 2011). Meanwhile, another platform is constructed in the IM, starting with the assembly of YscR, YscS, and YscT. Once this complex forms, recruitment of YscV occurs, followed shortly by attachment of YscJ (Diepold et al., 2011). The closely timed recruitment of YscJ by the OM and IM platforms are thought to merge the two independent substructures, forming the membrane spanning portion of the basal body (Diepold et al., 2011).

Once this part of the basal body has assembled, formation of the cytosolic portion of the basal body can proceed (Diepold et al., 2010). The ATPase-C ring complex consists of YscK, L, N, and Q, which assemble in a single step and dock onto the IM ring, completing the export apparatus (Diepold et al., 2010). This enables secretion of YscF and subsequent needle polymerization.

The needle consists of approximately 140 7-kDa YscF subunits, with a hollow center of ~2 nm and an external diameter of 6-7 nm (Hoiczyk & Blobel, 2001). The YscF monomers are considered early substrates as they are secreted prior to middle substrates (LcrV, YopB, and YopD) or late substrates (YopHOTJEMNK and LcrQ). Upon secretion through the export apparatus, the YscF monomers are polymerized in a sequential manner to form the T3SS needle (Davis, Díaz, & Mecsas, 2010). The needle length itself is controlled by YscP, the molecular ruler protein. Recent studies propose a static model that regulates needle length with a single YscP protein (S. Wagner, Stenta, Metzger, Dal Peraro, & Cornelis, 2010). During needle assembly, YscP is partially folded and secreted, presumably N-terminus first (S. Wagner et al.,...
Needle formation continues until the YscP C-terminal domain contacts the export apparatus, after which YscU receives a signal to commence autocleavage (Frost et al., 2012; Riordan & Schneewind, 2008). Importantly, this autocleavage step results in a switch in substrate specificity between early and middle substrate secretion. Specifically, early substrates such as the needle subunits are no longer secreted, whereas middle substrates such as the translocator protein low calcium response V antigen (LcrV) are now secreted (Riordan & Schneewind, 2008; S. Wagner et al., 2010).

LcrV, along with *Yersinia* outer protein B (YopB) and YopD, are the three translocator proteins that make up the translocon complex, which forms at the tip of the T3SS needle. LcrV is secreted through the needle and forms a distinct pentameric tip complex by attaching to the distal end of the YscF polymers (Mueller et al., 2005). This establishes a hydrophilic assembly platform upon which the hydrophobic YopB and YopD can complete formation of the translocon or pore complex (Cecile Neyt & Cornelis, 1999).

In order for successful translocon formation to occur, proper LcrV-YscF interactions are crucial. Harmon *et al.* demonstrated that in a YscF mutant containing two point mutations YscF-LcrV interactions were impaired (Harmon, Murphy, Davis, & Mecsas, 2013). Further analysis revealed this mutant was unable to translocate T3SS cargo or form pores in host cell membranes (Harmon et al., 2013). Despite these deficits in T3SS function, this strain still exhibited the capacity to sense host...
cell contact as it retained the ability to secrete Yops in the presence of host cells. Together these findings indicate that *Yersinia* sensing of host cells does not depend on the formation of a WT translocon and also does not rely on normal YscF-LcrV interactions, highlighting the different requirements for specific T3SS functions.

While the entire pore complex has never been directly visualized in its native state, all three proteins are required to form pores in host cell membranes and mediate translocation of T3SS cargo (Jonas Pettersson et al., 1999; Rosqvist, Magnusson, & Wolf-Watz, 1994). Together YopB and YopD form a heteropolymeric complex that is inserted into the membrane of host cells, presumably resulting in the formation of a pore (Montagner, Arquint, & Cornelis, 2011). Once pore formation has occurred, the late substrates are translocated through the needle and into the host cell cytoplasm, presumably through the pore complex in a single step. Although this has yet to be demonstrated experimentally, two recent studies provide strong evidence for this one-step model as both T3SS cargo containing a trefoil knot or T3SS cargo fused to GFP, which folds rapidly, are unable to pass through the T3SS needle, and can be visualized within the needle complex (Dohlich, Zumsteg, Goosmann, & Kolbe, 2014; Radics, Königsmayer, & Marlovits, 2014). The late substrates themselves include 5-6 Yop effector proteins (YopHOTJEM) as well as other T3SS cargo (Guy R Cornelis, 2002b). Once inside the host cell, the different effector proteins disrupt host cell responses that result in an impaired immune response to *Yersinia* infection.
1.4.2 Regulation of the T3SS

Regulation of the T3SS in *Yersinia* is a complex, multi-factorial process that is not completely understood. The levels of regulation can be broadly grouped at the transcriptional and post-transcriptional level. A key factor for transcriptional control of the T3SS is the AraC-like transcriptional regulator LcrF. LcrF is known to bind to the promoter regions of several gene clusters crucial for T3SS function including *yop, ysc, vir, lcr* genes. (Bergman et al., 1991; China, Michiels, & Cornelis, 1990; G. Cornelis, Sluiters, de Rouvroit, & Michiels, 1989; G. Cornelis, Vanootegem, & Sluiters, 1987; Hoe, Minion, & Goguen, 1992; Lambert de Rouvroit, Sluiters, & Cornelis, 1992; Michiels et al., 1991; Skurnik & Toivanen, 1992). Control of LcrF is dictated by temperature and is mediated by two thermoregulated factors: *Yersinia* modulator A YmoA and a RNA thermosensor (Böhme et al., 2012; Hoe et al., 1992). YmoA is a small histone like protein that binds to the promoter regions of T3SS genes at 26°C (Hoe et al., 1992; Lambert de Rouvroit et al., 1992; Skurnik & Toivanen, 1992). This binding serves to preserve the intrinsic structure of DNA and effectively prevents transcription of these genes. However, when the temperature is increased to 37°C, YmoA binding affinity for DNA is reduced due to temperature-mediated changes in DNA structure (J R Rohde, Luan, Rohde, Fox, & Minnich, 1999; John R. Rohde, Fox, & Minnich, 1994). Subsequently, YmoA is quickly degraded by ClpXp and Lon proteases, further decreasing YmoA stability (Jackson, Silva-Herzog, & Plano, 2004). Together, these mechanisms significantly inhibit YmoA regulation.
Another important regulator of \textit{lcrF} is the \textit{Iron Sulfur Cluster Regulator}, IscR (Miller et al., 2014). IscR exists in two distinct forms: holo-IscR and apo-IscR, both of which are active transcription factors (Fleischhacker et al., 2012; Giel et al., 2013; Nesbit, Giel, Rose, & Kiley, 2009). Under conditions that promote sufficient iron sulfur clusters, holo-IscR is predicted to be the dominant form in the bacteria cell (Schwartz et al., 2001). It is characterized by coordination of an iron sulfur cluster, (2Fe-2S), which is loaded by the Isc Fe-S biogenesis pathway comprised of IscS, IscU, IscA, and other accessory proteins (Py & Barras, 2010). Holo-IscR is able to directly repress transcription of the \textit{iscRSUA} operon, thereby limiting expression of the Isc Fe-S biogenesis pathway when Fe-S clusters are plentiful (Giel et al., 2013; Schwartz et al., 2001). In contrast, apo-IscR lacks the [2Fe-2S] cluster and is predicted to be dominant in low iron environments (K.-C. Lee, Yeo, & Roe, 2008; Nesbit et al., 2009). Holo-IscR recognizes two distinct DNA-binding motifs, a type I and a type II, whereas apo-IscR can only recognize a type II binding motif (Fleischhacker et al., 2012; Giel et al., 2013; Nesbit et al., 2009). Modulation of the holo- and apo-IscR ratio may enable \textit{Yersinia} to respond to changing levels of bioavailable iron in different environments to control gene expression. Miller et al. recently investigated the role that IscR plays in the virulence of \textit{Yersinia pseudotuberculosis} (Miller et al., 2014). By performing bioinformatic analysis, they found a type II binding site within the \textit{lcrF} promoter region and then demonstrated that IscR can bind this site \textit{in vitro} (Miller et al., 2014). They also found that a \textit{Yersinia} mutant lacking \textit{iscR} exhibits decreased transcription of \textit{lcrF}, the \textit{virC}
operon, and many other T3SS genes (Miller et al., 2014). These data suggest that IscR regulates T3SS expression by directly promoting lcrF transcription. In the gut enteropathogenic *Yersinia* are exposed to high levels of bioavailable iron (15 mg per day) (McCance & Widdowson, 1938; Miret, Simpson, & McKie, 2003). These conditions likely support sufficient bacterial Fe-S clusters leading to repression of *iscR* through its own activity and thus little expression of the T3SS. As the *Yersinia* disseminate to distal tissues, where they require the T3SS to inactivate innate immune cells, bioavailable iron becomes severely restricted (~10^{-24} M free serum iron) (Aisen, Leibman, & Zweier, 1978; Kretchmar, Reyes, & Raymond, 1988; Martin, Savory, Brown, Bertholf, & Wills, 1987). Under these conditions, Fe-S clusters presumably become limiting, leading to derepression of *iscR*, activation of lcrF transcription, and subsequently full expression of the T3SS (Giel et al., 2013; Wu & Outten, 2009; Yeo, Lee, Lee, & Roe, 2006). In this elegant manner, *Yersinia* may be able to successfully regulate T3SS expression in response to the large changes in iron bioavailability that it experiences throughout the course of infection.

Regulation of LcrF at the post-transcriptional level also plays an important role in expression of the T3SS. *Yersinia* employs an RNA thermometer comprised of a cis-acting two-stemloop RNA structure that resides in the intergenic region of the *yscW-lcrF* transcript. At 26°C, this RNA element prevents lcrF translation by sequestering the ribosomal binding site (RBS), effectively preventing ribosomal access to this region. However, upon a shift in temperature to 37°C it is thought that the RNA stemloops undergo conformational changes, which allow ribosomal access
to the RBS and subsequent lcrF translation. This initiation of LcrF synthesis, in turn, leads to the induction a large number of LcrF-dependent virulence genes, including those involved in formation of the T3SS.

Additional regulatory checkpoints are also present during T3SS assembly. Once the T3SS basal body and YscF needle have formed, the secretory pathway is still blocked in the bacterial cytosol by LcrG (Skryzpek & Straley, 1993). LcrG positions itself at the base of the T3SS export apparatus, resulting in the accumulation of YopD, LcrH, and LcrQ. Together these proteins negatively regulate the translation of yop mRNA. However, the exact mechanism by which this occurs is unclear.

One model proposes that YopD and LcrH bind directly to the 5’ untranslated region of yop transcripts, preventing ribosomal access. This repression requires the regulatory element LcrQ to be maintained inside the cytosol of the bacteria. An alternative model postulates that YopD, LcrH, and LcrQ form a tripartite multi-regulatory complex that inhibits yop translation. The formation of this complex is yet to be demonstrated experimentally (D. M. Anderson, Ramamurthi, Tam, & Schneewind, 2002; Wulff-Strobel, Williams, & Straley, 2002).

While the specific members of the regulatory complex remain to be fully elucidated, it is clear that repression of yop translation is alleviated upon secretion of the regulatory components YopD and LcrQ. Additionally, environment cues such as the presence of host cells or low Ca$^{2+}$ conditions appear to initiate SycH binding to LcrQ (V. T. Lee, Mazmanian, & Schneewind, 2001). Presumably, this event leads to
the destabilization of the YopD-LcrH complex as well as the export of YopD and LcrH (Deborah M Anderson, Ramamurthi, Tam, & Schneewind, 2002; Cambronne, Sorg, & Schneewind, 2004). This shift to an active state is thought to be in part mediated by the autocleavage of YscU, which initiates a switch in substrate specificity from early to middle T3SS substrates (Frost et al., 2012; Riordan & Schneewind, 2008). This is thought to be followed by the induction of DNA adenine methyltransferase, which increases ClpXP protease activity against LcrG (Fälker, Schmidt, & Heusipp, 2006). LcrG degradation opens the secretory pathway, coinciding with the dissociation and secretion of YopD and LcrQ (Cambronne & Schneewind, 2002; Rimpiläinen, Forsberg, & Wolf-Watz, 1992). Presumably, this alleviates the repression of yop mRNA as now ribosomes can access the 5’-untranslated region of yop mRNA and initiate de novo synthesis of Yop effectors, which in turn are also secreted (Cambronne et al., 2004; J. Pettersson et al., 1996; Wulff-Strobel et al., 2002).

1.5. Yersinia Translocator Proteins

1.5.1 The Translocator Protein LcrV

The hydrophilic translocator protein LcrV is the first of three translocator proteins secreted by Yersinia that comprise the T3SS pore complex. Once LcrV is secreted through the T3SS needle, it polymerizes to form a pentameric complex at the tip of the needle, which is thought to serve as a scaffold upon which YopB and YopD
can assemble (Mueller et al., 2005). Structural analysis and modeling of the C and N terminal regions of LcrV indicate that the N-terminus is crucial to orchestrate the appropriate interactions with YopD and YopB for efficient pore formation (Broz et al., 2007).

1.5.2 The Translocator Protein YopB

The hydrophobic translocator protein YopB is one of three essential translocator proteins required for pore formation and translocation of T3SS cargo (Håkansson, Schesser, et al., 1996; Håkansson, Bergman, Vanooteghem, Cornelis, & Wolf-Watz, 1993). At 401 amino acids, this 46 kD protein contains two predicted transmembrane domains as well as two putative coiled-coil regions. Together, YopB in concert with YopD assemble on the LcrV scaffold to complete the pore complex. Both YopB and YopD have been shown to be inserted into liposome and host cell membranes (Håkansson, Schesser, et al., 1996; Tardy et al., 1999). This insertion event presumably results in YopBD-mediated pore formation and in injection of Yop effector proteins into the cytosol of the host cell (Håkansson, Schesser, et al., 1996; Håkansson et al., 1993; Cecile Neyt & Cornelis, 1999). Indeed, both YopB and YopD must be present to form a WT pore in host cells (Cecile Neyt & Cornelis, 1999). A closer investigation of the requirements for pore formation is discussed in Chapter 2 of this dissertation. Interestingly, researchers found that in the absence of YopD, YopB is still inserted into liposome membranes (Tardy et al., 1999). By performing
electrophysiological experiments they observed that WT *Yersinia* generated ion channels with a current of 105 pS, whereas *Yersinia* lacking YopBΔ89-217 did not induce channel formation. Interestingly, *Yersinia* lacking YopDΔ121-165 caused some membrane perturbation that was sufficient to form channel-like current fluctuations, however these clearly differed from the 105 pS WT current levels, suggesting that no stable channel structure was inserted by YopB (Tardy et al., 1999). Additional studies have revealed that a *Yersinia* strain lacking YopD induces a pro-inflammatory response by host cells, presumably due to YopB insertion (Gloria I. Viboud et al., 2003, Adams, W. Duncan, M. and Ramirez, H. unpublished data). While it is currently unclear exactly how YopB triggers a host innate immune response in the absence of YopD, it may involve specific YopB interactions with the surface of the host cell.

1.5.3 The Translocator Protein YopD

YopD is a multifunctional hydrophobic translocator protein that, together with YopB and LcrV, form the translocon or pore complex at the tip of the T3SS. At 306 amino acids, this 33.3 kD protein is slightly smaller than its YopB counterpart, but exhibits a wider range of known and potential functions (Håkansson et al., 1993; Olsson et al., 2004). While both YopB and YopD are required for pore formation and translocation of Yop effectors, YopD also serves as a negative regulator of Yop synthesis (Cambronne & Schneewind, 2002; Håkansson, Schesser, et al., 1996;
Håkansson et al., 1993; C Neyt & Cornelis, 1999). Additionally, YopB and YopD bind to each other, LcrV, YopBD chaperone protein SycD, and are known to self-oligomerize (Costa et al., 2010; Francis, Aili, Wiklund, & Wolf-Watz, 2000). YopD is also capable of directly binding with the effector proteins YopE and TyeA (Translocation of Yops into eukaryotic cells A) and interacts with YopK, although it is unknown if this interaction is direct (Cheng & Schneewind, 2000; R. Dewoody, Merritt, & Marketon, 2013; Hartland & Robins-Browne, 1998; M Iriarte et al., 1998). Also, in contrast to YopB, YopD is found in the cytosolic fraction of host cells, whereas YopB appears to only associate with the host membrane, allowing for the possibility that YopD can exhibit some effector function, although there is currently no evidence for this (Francis & Wolf-Watz, 1998).

In order to better understand how YopD integrates and performs its functions, groups have systematically mutagenized YopD and performed secondary structural analyses, thereby identifying discrete functional domains (See Fig. 1) (A. A. A. Amer, Ahlund, Forsberg, Broms, & Francis, 2011; Francis et al., 2000; Olsson et al., 2004; Tengel, Sethson, & Francis, 2002). Researchers have identified several areas thought to participate in protein-protein interactions, including a putative transmembrane domain at YopD_{128-149}, a putative coiled coil section at YopD_{256-275}, and an amphipathic region at YopD_{150-227} (Olsson et al., 2004; Raab & Swietnicki, 2008; Tengel et al., 2002). Taken together, these analyses suggest that YopD uses distinct domains to execute different functions and/or for different molecular interactions.
1.5.3.1 The N terminus of YopD: Amino acids 1-49

Limited structural information is available for the N-terminal region of YopD. While secondary structural studies have been performed on other regions of YopD, the N-terminus is largely insoluble in several buffers and thus *in vitro* biophysical analysis has not been possible (Raab & Swietnicki, 2008). Interestingly, Costa *et al.* identified a putative coiled-coil motif in the N-terminal region, albeit with a predictive probability of <70% (Costa, Amer, Fällman, Fahlgren, & Francis, 2012).
Analysis of mutants that were unable to form this putative coiled-coil domain revealed that they exhibit virulence defects in a mouse model of infection, despite displaying normal T3SS function *in vitro* (Costa et al., 2012). Another study found that deletion of small segments of the N-terminus of YopD, either amino acids 4-20 or 23-47, significantly impaired secretion of YopD, suggesting that this region contains a secretion signal (Olsson et al., 2004). While secretion was dramatically reduced, immunoblot analysis detected low levels of secreted YopD when fivefold more sample was used (Olsson et al., 2004). These same YopD mutants exhibited normal chaperone binding, *yop* regulatory control, and pore formation, but failed to translocate T3SS cargo. The fact that these YopD N-terminal mutants formed pores in host cells, despite exhibiting very low levels of secretion, should not be surprising as Edqvist *et al.* demonstrated that only minimal amounts of YopD and YopB are required for translocation and, by inference, pore formation (Edqvist, Aili, Liu, & Francis, 2007). A more in depth investigation of the amino acids 1-47 attempted to better define the nature of the YopD secretion signal (A. A. A. Amer et al., 2011). While the authors were unable to completely rule out the possibility of an mRNA-based secretion signal, their results indicate that the signal is more likely to be protein based, chaperone-independent, and may require as few as five N-terminal residues (A. A. A. Amer et al., 2011). Taken together, these findings suggest indicate that the YopD N-terminus contributes to *Yersinia* virulence and is important for classic T3SS functions including YopD secretion and translocation of T3SS cargo.
1.5.3.2 YopD Chaperone Binding Domain: Amino acids 53-149 and 278-292

The chaperone LcrH/SycD binds to two distinct regions of YopD, amino acids 53-149 and amino acids 278 to 292 (Francis et al., 2000). Thus, it is not surprising that deletion of short amino acid sections in either of these regions also prevents normal yop regulation and translocation, especially since LcrH binding is a prerequisite for normal yop control (Francis et al., 2000; Olsson et al., 2004). Despite these deficits in T3SS function, these same YopD mutants exhibit normal levels of secretion (Olsson et al., 2004). The YopD mutants lacking amino acids 53-68 or 128-149 (yopD_{Δ53-68} and yopD_{Δ128-149}, respectively) in a ΔyopK background form similar sized pores in erythrocytes compared to the parental strain.

*In silico* analysis performed by Olsson *et al.* identified a predicted putative transmembrane domain spanning amino acids 128-149 (Olsson et al., 2004). Additionally, YopE has been shown to bind to YopD amino acids 122-151 *in vitro* (Håkansson et al., 1993; Hartland & Robins-Browne, 1998). Future studies on this interaction could provide important insights on YopD structure and also help determine if this binding has any influence on *Yersinia* pathogenesis.

In contrast to the dearth of structural data for amino acids 128-149, structural information is available for the second chaperone binding domain, amino acids 278 to 292. *In silico* analysis of YopD predicted that this region contains an amphipathic α-helix domain, which was later confirmed by NMR spectroscopy (Håkansson et al., 1993; Tengel et al., 2002). Amphipathic domains have been shown to promote
association with lipid rafts, other proteins, and self-oligomerization (Madsen, Bhatia, Gether, & Stamou, 2010). More recently, it has been shown that amphipathic helices exhibit membrane curvature sensing properties. This is due to their ability to insert hydrophobic peptides into areas of defective lipid packaging, which is often associated with areas of high curvature (Hatzakis et al., 2009; Mesmin et al., 2007). Costa et al. investigated the amphipathic domain on YopD by systematically mutagenizing all of the residues present in this region and identified several residues important for YopD/YopD and YopD/LcrV interactions (Costa et al., 2010). Additionally, they demonstrated that YopD mutants lacking the amphipathic domain exhibit reduced YopD function and self-oligomerization (Costa et al., 2010). Taken together, these findings underscore the substantial contributions that individual residues and the region as a whole make to YopD functions.

1.5.3.3 Central Region of YopD: Amino Acids 150-227

Characterization of the central region of YopD and understanding its role in Yersinia pathogenesis is the main objective of the manuscript in preparation entitled "The central region of the Yersinia type III secretion system translocator protein YopD promotes Yop translocation inside phagocytes" (See Chapter 3 of this dissertation). Prior to this work, limited information existed with regard to the central region of YopD, amino acids 150-227 (YopD150-227), especially with regard to Yersinia virulence. Secondary structure analysis performed on the YopD fragment
150-287 revealed that this entire section exists in a partially unfolded state, which appears to be a requirement for successful transport of T3SS cargo (Dohlich et al., 2014; Raab & Swietnicki, 2008; Radics et al., 2014). Further investigation showed that the peptide has a well-defined secondary structure and easily converts between α-helical and random coil states at a neutral pH (Raab & Swietnicki, 2008).

Francis et al. investigated the different domains of YopD (including this central region) by generating mutants lacking small stretches of amino acids. Three of these mutants are $yopD_{150-170}$ (lacking amino acids 150-170), $yopD_{174-198}$, (lacking amino acids 174-198, and $yopD_{207-227}$ (lacking amino acids 207-227) (Francis et al., 2000). These mutants were expressed in trans under an arabinose-inducible promoter in a $yopD$ null mutant background and subjected to endogenous proteolytic digestion (Francis et al., 2000). While both the $yopD_{150-170}$ and $yopD_{174-198}$ mutants were stably expressed, matching WT YopD expression levels at 37°C, $yopD_{207-227}$ was barely detected, indicating that it was not resistant to proteolysis (Francis et al., 2000). This same trend also applied when the culture supernatants were analyzed for levels of secreted WT YopD and the YopD mutants (Francis et al., 2000). As these phenotypes were observed under an arabinose inducible promoter, an important caveat to note is that these phenotypes are not always consistent with a mutant strain that expresses the mutation on pYV. This complication was addressed in subsequent work, in which by Olsson et al. expressed these same mutants as in frame deletions on pYV (Olsson et al., 2004). Interestingly, several phenotypes exhibited by the mutants under the arabinose promoter system were not observed when the same
YopD alleles were expressed on pYV. Importantly, expression of YopD and secretion of T3SS cargo by the YopD$_{\Delta150-170}$, YopD$_{\Delta174-198}$, and YopD$_{\Delta207-227}$ were equivalent to those of WT *Yersinia* (Olsson et al., 2004). Further investigation revealed several key properties of the T3SS functions of these mutant strains. All three YopD mutants formed pores similar in size to those formed by WT YopD in erythrocytes (Olsson et al., 2004). Additionally, the YopD mutants induced comparable levels of YopE-dependent cell rounding in HeLa cells at two hours post infection (Olsson et al., 2004). The cell rounding assay suggested equivalent levels of T3SS cargo translocation amongst the mutants. The authors also performed a more sensitive translocation assay. By introducing the *Pseudomonas aeruginosa* ExoS effector protein into different YopD backgrounds, they were able to measure the translocation rate of the YopD mutant strains via detection of ExoS-mediated phosphorylation of the host cell cytosolic protein, Ras (Olsson et al., 2004). Interestingly, all three central YopD mutants translocated T3SS cargo into HeLa cells, indicating that this central region of YopD is dispensable for translocation of T3SS cargo (Olsson et al., 2004). While both the *yopD$_{\Delta174-198}$* mutant exhibited a clear hypertranslocation phenotype, both the *yopD$_{\Delta150-170}$* and *yopD$_{\Delta207-227}$* strains translocated ExoS similarly to WT levels (Olsson et al., 2004). Additional assays revealed that other T3SS functions of the *yopD$_{\Delta150-170}$* and *yopD$_{\Delta207-227}$* strains were also comparable to WT. Together with the fact that this central region of YopD is highly conserved among pathogenic *Yersinia*, these findings provided substantial impetus to investigate this region further to discern if and how this region contributes to *Yersinia* virulence.
1.5.3.4 The C-terminus of YopD: Amino acids 228-278 and 292-305

Like several other regions of YopD, the primary characterization of the YopD C-terminus is based on the generation of mutants containing in frame deletions of short segments of amino acids. Amino acids 278-292 were previously discussed in section "1.5.3.2 YopD Chaperone Binding Domain: Amino acids 53-149 and 278 to 292". YopD mutants lacking amino acids 234-254, 256-275, and 293-305 all display similar YopD functions. These YopD mutants bind the cognate chaperone LcrH, exhibit yop regulatory control, and secrete T3SS cargo. Likewise, these YopD mutants do not form pores in erythrocytes or translocate T3SS cargo into host cells.

Structural prediction software identified a putative the coiled coil domain spanning amino acids 248-277 (Håkansson et al., 1993; M. Pallen, 1997). However, this region was only predicted when low-stringency parameters were used (Olsson et al., 2004; M. Pallen, 1997). Costa et al. performed systematic mutagenesis of the C-terminus to determine how the predicted coiled-coil domain contributed to T3SS function (Costa et al., 2010). They identified a number of Yersinia mutants that exhibit normal YopD production and secretion, but that cannot form pores in red blood cells nor translocate T3SS cargo (Costa et al., 2010). Together, these findings indicate that this region plays an important role in optimal T3SS function.
1.6 *Yersinia Effector Proteins*

Following insertion of the translocator proteins YopB and YopD into the host cell membrane, 6 effector proteins are translocated into the cytoplasm of the host cell where they impair the host response to infection. Each effector executes a number of specific and sometimes overlapping functions that promote *Yersinia* pathogenesis. (Guy R Cornelis, 2002b). In addition, the protein YopK is also translocated inside host cells by the Ysc T3SS. This protein has been ascribed both effector and regulatory functions (see below).

1.6.1 YopH

YopH is a potent tyrosine phosphatase that is essential for *Yersinia* virulence (Bölin & Wolf-Watz, 1988; Guan & Dixon, 1990). The N-terminus of YopH is bound by its chaperone SycH inside the bacterium, but upon injection into the host cell, YopH targets a wide range of host proteins. These include several focal adhesion protein such as p130Cas and the focal adhesion kinase (FAK), as well as paxillin, Lck, Fyb, and SKAP-HOM. (Guy R Cornelis, 2002a). A key function of YopH is its ability to inhibit phagocytosis of *Yersinia* by host cells. This is mediated by YopH-dependent dephosphorylation of p130Cas, which leads to the disruption of actin cytoskeletal fibers (Andersson et al., 1996). YopH has also been shown to inhibit the Fc receptor-mediated respiratory burst in both neutrophils and macrophages (Bliska & Black, 1995; Ruckdeschel, Roggenkamp, Schubert, & Heesemann, 1996a).
Furthermore, several groups have reported additional roles for YopH, including the induction of apoptosis in T-cells, suppression of the production of macrophage chemoattractants, and inhibition of T-cell and B-cell activation (Alonso et al., 2004; Bruckner et al., 2005; Juris, Shao, & Dixon, 2002; Gloria I Viboud & Bliska, 2005). *In vivo* experiments found that a ΔyopH *Yersinia* mutant displays significant virulence defects in both the MLNs and Peyer’s patches at 2 days post infection relative to WT (Logsdon & Mecsas, 2003). The researchers went on to show that the ΔyopH mutant does not colonize the spleen at two and five days post infection (Logsdon & Mecsas, 2003). Additional studies found that the ΔyopH mutant is defective in colonizing both the spleen and liver at 5 and 7 days post-infection (Trülzsch, Sporleder, Igwe, Rüssmann, & Heesemann, 2004). Together, these findings highlight specific functions of YopH in inhibiting the innate and adaptive immune responses that promote *Yersinia* pathogenesis in the host.

1.6.2 YopO/YpkA

The relatively large 82-kD effector protein YopO is comprised of at least three distinct domains: a serine/threonine kinase N-terminus, a Rho-GTPase binding domain, and an actin binding C-terminus (Barz, Abahji, Trülzsch, & Heesemann, 2000; Dukuzumuremyi et al., 2000; E.E., 1993; Håkansson, Galyov, Rosqvist, & Wolf-Watz, 1996; Juris et al., 2002). Prior to translocation, YopO binds to its dedicated chaperone, SycO in *Yersinia*. Once translocated, the serine/threonine kinase
domain requires actin binding to the last 20 amino acids of YopO to become activated (Juris et al., 2002; Navarro, Alto, & Dixon, 2005). Upon activation, the kinase domain phosphorylates Ser-47 in the GTP binding pocket of Gαq proteins, which can stimulate phospholipase-C-β and some RhoA-dependent pathways (Navarro et al., 2007). Actin binding has also been shown to be important for YopO autophosphorylation of residues Ser-90 and Ser-95, which appears to activate YopO kinase activity (Trasak et al., 2007). This kinase activity, in turn, was shown to mediate host cell rounding and inhibition of Yersinia YadA-dependent phagocytosis (Trasak et al., 2007). Studies by Prehna et al. showed that the Rho-GTPase binding domain resembles a host guanidine nucleotide inhibitor (GDI), which ultimately enables YopO to inhibit Rac1 and disrupt actin filament formation (Prehna, Ivanov, Bliska, & Stebbins, 2006). Additionally, Groves et al. also demonstrated that YopO prevents Rac activation to block Fcγ receptor-mediated phagocytosis by macrophages (Groves et al., 2010). Through these independent yet complementary mechanisms YopO disrupts the host cytoskeleton to prevent Yersinia uptake by phagocytes (Prehna et al., 2006; Trosky, Liverman, & Orth, 2008).

1.6.3 YopT

YopT is a 35.5-kD Yersinia cysteine protease encoded by some but not all species or strains of Yersinia (Schmidt, 2011; Trosky et al., 2008). The chaperone SycT is thought to bind to the N-terminal region, including residues 52 to 139, of
YopT. Once translocated into host cells, the YopT effector protein inactivates the membrane-bound Rho GTPases RhoA, Rac, and Cdc42 by cleaving their C-terminal prenylated cysteine, effectively releasing these RhoGTPases from the host cell membrane (Maite Iriarte & Cornelis, 1998; Shao et al., 2003). This cleavage event impairs the capacity of hosts cell to carry out actin assembly and remodeling, an important process for efficient phagocytosis of *Yersinia* (Aepfelbacher et al., 2003; Maite Iriarte & Cornelis, 1998; Zumbihl et al., 1999) and probably YopT mediated inhibition of pore formation (R. S. Dewoody, Merritt, & Marketon, 2013; Gloria I Viboud, Mejía, & Bliska, 2006). Additional investigations of YopT function revealed that the N-terminal residues 75 to 100 are required for YopT to bind the RhoA GTPase substrate, whereas the last 8 residues on the C-terminus are necessary for YopT maintain effector function (Isabel Sorg, Hoffmann, Dumbach, Aktories, & Schmidt, 2003).

### 1.6.4 YopJ

The effector protein YopJ is a serine/threonine acetyltransferase that downregulates the pro-inflammatory host response (Rosenzweig & Chopra, 2013). Acetylation of serine and threonine residues of MAPK and IκB activation loops, prevents phosphorylation of these same residues, an event required for induction of the respective MAPK and NF-κB signaling pathways (Mittal, Peak-Chew, & McMahon, 2006; Mukherjee et al., 2006). This disruption of host cell signaling
impairs the innate immune response to *Yersinia* infection, including dampened NF-κB activation, decreased pro-inflammatory cytokine production, and an increase in eIF2 dependent apoptosis (Monack, Mecsas, Ghori, & Falkow, 1997; Orth, 2002; Palmer, Pancetti, Greenberg, & Bliska, 1999; Schesser et al., 1998; Shrestha et al., 2012). YopJ induction of apoptosis may require disruption of TLR-2 signaling, although conflicting reports suggest that the impact of disrupting TLR-2 signaling may vary depending on the host cell type (Pandey & Sodhi, 2011; Paquette et al., 2012). YopJ has also been shown to induce cell death and intestinal barrier dysfunction through caspase-1 activation by hijacking the Nod2/RICK/TAK1 pathway (Lilo, Zheng, & Bliska, 2008; Meinzer et al., 2012).

**1.6.5 YopE**

YopE is a potent *Yersinia* effector that exhibits GTPase Activating Protein (GAP) activity. Mechanistically, YopE operates by targeting small RhoA-like G-proteins, converting them from their active GTP-bound state to an inactive GDP bound state (Bos, Rehmann, & Wittinghofer, 2007). Under *in vitro* and *in vivo* conditions, YopE can inactivate the Rho GTPases RhoA and Rac1, but seems to inhibit Cdc42 only *in vitro* (Aili, Isaksson, Hallberg, Wolf-Watz, & Rosqvist, 2006; Andor et al., 2001; Black & Bliska, 2000; Von Pawel-Rammingen et al., 2000).

YopE utilizes its GAP activity to carry out several important virulence-promoting functions, most of which involve the manipulation of actin microfilaments.
Inhibition of the Rho GTPases RhoA and Rac1 by YopE prevents host cell cytoskeletal rearrangement, which ultimately helps *Yersinia* to avoid engulfment by immune cells (Black & Bliska, 2000; Von Pawel-Rammingen et al., 2000). In fact, the rounding of host cells by 15 minutes post-infection is a reliable indirect readout for YopE effector function (Rosqvist et al., 1994; S C Straley & Cibull, 1989).

*In vivo* studies have also helped characterize the important contributions YopE makes to *Yersinia* pathogenesis. Logdson et al. demonstrated that a ∆*yopE* *Yersinia* mutant exhibits significant virulence defects in both the MLNs and PPs two days post-infection (Logsdon & Mecsas, 2003). Additional studies found that a ∆*yopE* *Yersinia* mutant fails to colonize the spleen and liver at WT levels at five and seven days post infection, respectively (Trülzsch et al., 2004).

More recent work has begun to elucidate how YopE impacts pore formation in host cells. Initial studies concluded that YopE was able to inhibit pore formation based on the observation that a *Yersinia* strain lacking *yopE* or *yopE* and *yopJ* induces greater LDH release than a wild type infection (G I Viboud & Bliska, 2001). However, LDH release is considered to occur as a subsequent event to pore formation, ultimately due to osmotic lysis of the cell. Therefore, additional pore formation assays have been utilized (Fink & Cookson, 2006; Marenne, 2003). For example, measuring release of BCECF (2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxyethyl ester), a relatively small (623 Da) fluorescent dye, (as compared to the massive 135 kDa LDH protein) may be a more direct measure of
pore formation (Marenne, 2003). Alternatively, quantification of ethidium bromide (314 Da) entry into cells also can serve as a readout of pore formation, but most likely stems from osmolysis of cells following pore formation (Clark, Virginia L., Bavoil, 2002; Fink & Cookson, 2006; Marenne, 2003; Gloria I Viboud et al., 2006). Utilizing this latter assay, Viboud et al. clearly demonstrated the strong inhibitory effect YopE has on pore formation (Gloria I Viboud et al., 2006). As cytochalasin D, a small molecule that disrupts the actin cytoskeleton, has a similar effect on pore formation, the authors proposed that the host actin cytoskeleton plays a role in limiting type III secretion once an initial injection of effector proteins has occurred (Gloria I Viboud et al., 2006).

YopE also appears to play an important role in the translocation of T3SS cargo. *Yersinia yopE*-deficient strains have been shown to translocate elevated levels of the effector YopH and the YopM-Bla fusion protein, which could be a direct result of the formation of a larger pore (Aili et al., 2008b; R. Dewoody, Merritt, Houppert, & Marketon, 2011). The GAP activity of YopE is required for this function to occur as catalytically inactive YopE<sub>R144A</sub> is unable to rescue the hypertranslocation phenotype (Aili et al., 2008b, 2006; R. Dewoody et al., 2011). While YopE is thought to exert most of its function via manipulation of the actin cytoskeleton, Hartland and colleagues demonstrated that YopE can bind YopD in vitro (R. S. Dewoody et al., 2013; Hartland & Robins-Browne, 1998). However, no direct binding of YopE to the translocon apparatus itself has been demonstrated, and thus, understanding the
molecular mechanism by which YopE regulates translocation of T3SS cargo requires further investigation.

One final function of YopE is its ability to inhibit ROS production in neutrophils and macrophages (Ruckdeschel, Roggenkamp, Schubert, & Heesemann, 1996b; Songsunthong, Higgins, Rolán, Murphy, & Mecsas, 2010). Multiple Rho GTPases are involved in ROS production, including RhoA, Rac1, and Rac2 (Bokoch, 2005). However, Rac2 is thought to play a key role, as it is required for complete assembly of the NADPH oxidase complex, a major source of ROS production (Filippi et al., 2004; Gu et al., 2003; Keith, Hynes, Sholdice, & Valvano, 2009). Songsunthong et al. demonstrated that YopE potently inhibits ROS production in neutrophil-like dHL-60 cells via inhibition of Rac2, highlighting another consequence of YopE mediated effector function (Songsunthong et al., 2010).

In summary, YopE exhibits a wide range of anti-host activities. Through its potent GTPase activity YopE is able to efficiently hamper phagocytosis, inhibit pore formation, modulate translocation, and block ROS production. Not surprisingly, a yopE-deficient strain exhibits significant colonization defects in the spleen and liver at both two and five days post-infection (Logsdon & Mecsas, 2003; Trülzsch et al., 2004). Research also has connected these in vivo phenotypes to specific defects in YopE effector function (Songsunthong et al., 2010). Together, these findings serve to highlight the substantial contributions YopE provides as an important virulence factor for Yersinia pathogenesis.
1.6.6 YopM

The effector protein YopM has long been known to be necessary for *Yersinia* virulence, but its mechanism of action eluded scientists for many years. That was until recently, when LaRock and Cookson demonstrated that YopM antagonizes caspase-1 activity and activation (Larock & Cookson, 2012). By binding directly to caspase-1, YopM is able to sequester caspase-1 in the nucleus, arresting development of the cytosolic pre-inflammasome complex (Larock & Cookson, 2012). This in turn prevents a pro-inflammatory response in macrophages, including IL-1β production, lyosomal exocytosis, and pyroptosis (Larock & Cookson, 2012). YopM may also exhibit additional roles as a scaffold protein, as it is known to directly interact with several other host proteins, including the cytoplasmic kinases, RSK1 and PRK2 (McDonald, Vacratsis, Bliska, & Dixon, 2003). In animal models of infection, a *Yersinia yopM*-deficient strain exhibits significant virulence defects in the Peyer's patches (PPs), spleen, and liver relative to wildtype infection (Trülzsch et al., 2004). Additional research showed that YopM plays important roles in disrupting PMN- and iDC-mediated clearance of *Yersinia* in the liver and spleen, respectively (Ye et al., 2011). Future studies will continue to elucidate the molecular mechanisms YopM utilizes to promote *Yersinia* virulence.

1.6.7 YopK
Identified almost three decades ago, YopK has no known enzymatic activity, or direct role in *Yersinia*-induced cytotoxicity or phagocytosis (S C Straley & Bowmer, 1986). However, recent investigations into YopK function during *Yersinia* infection have raised the possibility that YopK is the seventh *Yersinia* effector protein (R. Dewoody et al., 2011, 2013; R. S. Dewoody et al., 2013). While there has yet to be any clear ruling on its status as an effector, YopK plays an important role during *Yersinia* pathogenesis and clearly qualifies as a regulatory protein. Studies by Holmstrom and colleagues suggested that YopK plays an important role regulating T3SS cargo translocation, as a *Yersinia yopK*-deficient strain hypertranslocates YopE and YopH into host cells (Holmström et al., 1997). Accordingly, overexpression of YopK decreases YopE and YopH translocation (Holmström et al., 1997). Additional studies using Yop-β-lactamase (Bla) fusion proteins provided a more in depth analysis of the role of YopK in Yop translocation and demonstrated that a ∆yopK mutant hypertanslocates YopM-Bla into host cells and does so at a faster rate than does wildtype *Yersinia* (R. Dewoody et al., 2011).

While it is well established that YopK plays a regulatory role in T3SS translocation, the molecular basis for this is unclear. One possibility is that the role YopK plays in translocation is related to pore formation, as a *Yersinia* strain lacking multiple Yops, including YopK, was shown to induce more hemoglobin release in erythrocyte pore formation assays and to form significantly larger pores in these cells (Holmström et al., 1997). Similarly, an effectorless strain lacking YopK induces significantly more BCECF release than its parental strain (W. Adams, unpublished).
While the hemoglobin and BCECF release correlates with increased translocation, a yopK-deficient strain showed no difference in LDH release (Aili et al., 2008a; Holmström et al., 1997; W. Adams, unpublished). This highlights the importance of using multiple assays to characterize specific T3SS functions. In this instance, subtle differences in pore formation for the yopK-deficient strain seem to be unrelated to cell death (Aili et al., 2008; Holmström et al., 1997; W. Adams, unpublished). It is also interesting to speculate on the importance of pore size in determining the efficiency of T3SS cargo translocation. While it is unlikely that a change in pore size results in a change in the diameter of the T3SS needle, a larger pore could still make it easier for cargo to enter the host cell or avoid regulatory components upon entering the pore complex.

Two important observations offer some insight into the mechanism underlying YopK-mediated regulation of T3SS translocation. The first is that YopK must be present inside the host cell to regulate T3SS translocation (R. Dewoody et al., 2011). Secondly, YopK has been shown to bind YopD, a essential component of the translocation pore (R. Dewoody et al., 2013). Thus, one intriguing model that has been proposed is that YopK induces a conformational change in the T3SS itself by binding to the translocation pore, presumably via its interaction with YopD (R. S. Dewoody et al., 2013). Alternatively, YopK might physically block the translocation pore or perhaps the T3SS channel itself, effectively preventing injection of T3SS cargo (R. S. Dewoody et al., 2013).
1.7 Host Sensing of the T3SS

A fundamental question in host-pathogen interactions is how the host immune system is able to detect virulence factors of pathogenic organisms. A key strategy eukaryotic cells implement to recognize pathogens relies on their ability to identify pathogen-associated molecular patterns (PAMPs). Host cells employ an array of pattern recognition receptors (PRRs) to detect a wide range of PAMPs, which include components of the T3SS. Successful recognition of a PAMP by a PRR induces specific signaling pathways that enable the host cell to mount an immune response to the microbial intruder. Thus, when a host cell detects a specific T3SS component, an immune response is triggered in an attempt to resolve the infection. Significant research has focused on determining 1) the host PRRs are involved in sensing T3SS PAMPs and 2) the identity of these T3SS PAMPs. Distinct models incorporating different PAMPs and PPRs have been proposed to explain how T3SSs are detected. The primary objective of the publication entitled, “The Impact of Pore Formation by the Yersinia pseudotuberculosis type III secretion system on the macrophage innate immune response,” is to dissect two models of T3SS sensing in the context of a Yersinia infection (See Chapter 2 of this dissertation).

1.7.1 Pattern Recognition Receptors (PRRs)
Two important groups of PRRs that aid in the detection of PAMPs are the Toll-like receptors (TLRs) and Nod-like receptors (NLRs) (Mogensen, 2009). Several TLRs are expressed on the surface of the host cell (TLR1, TLR2, TLR4, and TLR6) and detect conserved elements of microbial membranes (O’Neill, Golenbock, & Bowie, 2013). Meanwhile, other TLRs (TLR3, TLR7, TLR8, and TLR9) localize to intracellular vesicles, where they recognize nucleic acids (O’Neill et al., 2013). Recognition of these different microbial components induces a signaling cascade through either the adaptor protein MyD88 or Trif, ultimately resulting in the production of pro-inflammatory cytokines.

NLRs are cytosolic receptors that detect a wide range of PAMPs that enter the intracellular space of host cells. All NLRs share a highly conserved central NACHT domain, but can be furthered divided into subfamilies based on the presence of other effector domains (Mogensen, 2009). For example, one important NLR subfamily consists of the NAIPs (NLR family, Apoptosis Inhibitory Proteins), which are characterized by three baculovirus inhibitor of apoptosis repeat (BIR) domains (Kofoed & Vance, 2012). While the function of the BIR domains is unknown, the NAIPs themselves have been shown to detect specific bacterial PAMPs, after which they activate a member of the NLRC subfamily, called NLRC4 (Rayamajhi, Zak, Chavarria-Smith, Vance, & Miao, 2013; Yang, Zhao, Shi, & Shao, 2013). This activation leads to the assembly of a pro-inflammatory signaling complexes referred to as the inflammasome (Kofoed & Vance, 2012). Upon inflammasome formation, secretion of the pro-inflammatory cytokines IL-1β and IL-18 occurs, as well as the
induction of pyroptosis, a pro-inflammatory form of cell death (Bergsbaken, Fink, & Cookson, 2009).

**Fig. 2.** Primary TLR-Independent Host Response Pathways that Sensing the T3SS. Host cells detect a number of different T3SS components or T3SS dependent events. A pro-inflammatory IL-8 response is induced presumably through insertion of YopB into the host cell membrane. Other membrane perturbations such as pore formation and the subsequent K⁺ efflux induce NLRP3 inflammasome activation. Meanwhile, the translocation of different T3SS cargo can be recognized by cytosolic host receptors. This recognition event induces NF-κB or NLRC4 inflammasome dependent host responses.
1.7.2 NLR Sensing of T3SSs

The majority of what is known about T3SS sensing mechanisms by host cells relies on NLR recognition events (See Fig. 2). In particular, there is a wealth of evidence supporting a role for bacterial cargo of T3SSs in activating the NLRC4 inflammasome (Rayamajhi et al., 2013). Initially researchers established that the NLRC4 inflammasome had a role in T3SS sensing by showing that cytosolic flagellin from *Salmonella enterica* Serovar Typhimurium results in NLRC4 activation and subsequent IL-1β secretion (Lightfield et al., 2008; Miao et al., 2006). Subsequent research has shown that NAIPs play a crucial role in bacterial ligand recognition upstream of NLRC4. C57BL/6 mice express four functional NAIP receptors (NAIP1, NAIP2, NAIP5, and NAIP6), while humans appear to have just one NAIP receptor (hNAIP), all upstream of the NLRC4 inflammasome (Diez et al., 2003). Bacterial flagellin was identified as the bacterial ligand for the NAIP5/6 receptor (Zhao et al., 2011). Meanwhile, Kofoed and Vance demonstrated that the host cytosolic receptor NAIP2 recognizes the *S. typhimurium* protein, PrgI, also referred to as the inner rod protein of the T3SS system (Kofoed & Vance, 2011). Further research by Suzuki *et al.* showed that the rod protein of *Shigella*, Mxil, is also recognized by NAIP2. Additional research revealed that the *S. typhimurium* needle protein PrgI as well as its homologues in *E. coli*, *Shigella flexneri*, and *Burkholderia* spp., are recognized by
NAIP1 and hNAIP in mice and humans respectively (Rayamajhi et al., 2013; Yang et al., 2013).

Several lines of evidence have suggested that NLRP3 inflammasome activation is also involved in T3SS recognition. Understanding the mechanistic basis for this proved to be quite difficult as many structurally unrelated stimuli (including nigericin, silica crystals, ATP, and pore forming toxins) induced NLRP3 activation (Muñoz-Planillo et al., 2013). Recently, Munoz-Planillo et al. demonstrated that efflux of cytosolic K$^+$ appears to be the underlying mechanism that is both induced by the various NLRP3 stimuli and also required for caspase-1 activation and the subsequent induction of the NLRP3 inflammasome (Muñoz-Planillo et al., 2013). Notably, as T3SSs form pores in the membranes of host cells, these findings suggest that at least for some pathogens, potassium efflux mediated by pore formation is sufficient to induce a pro-inflammatory immune response.

Recent studies also have proposed that K$^+$ efflux also may be a mechanism of activation for the NLRC4 /Ipaf inflammasome. However, this has not been well characterized. Senerovic et al. showed that the purified Shigella translocator protein IpaB can self-oligomerize, form pores in the membranes of host cells, destabilize lysosomal membranes, and induce intracellular K$^+$ efflux into the vacuoles of host cells (Senerovic et al., 2012). Interestingly, despite the close association between K$^+$ efflux and NALP3 inflammasome activation, this intracellular K$^+$ efflux event appears to be NALP3-independent (Senerovic et al., 2012). Instead, the authors
propose a model in which IpaB-induced K^+ efflux or vacuolar components lead to activation of the NLRC4 inflammasome.

Another study also investigated the relationship between K^+ efflux and NLRC4 activation during *P. aeruginosa* or *S. typhimurium* infection. While the report suggests that loss of intracellular K^+ is required for induction of the NLRC4 inflammasome, only a small percentage of the cells exhibited this phenotype (Arlehamn, Pétrilli, Gross, Tschopp, & Evans, 2010). The authors stated that loss of intracellular K^+ occurs after the translocation of a pathogen-associated molecule that activates the NLRC4 inflammasome (Arlehamn et al., 2010). These findings are consistent with an *in vitro* showing that K^+ efflux seemed to induce NLRC4 dependent caspase-1 activity. However, as discussed earlier in this section, a number of groups have identified specific molecules that are translocated by bacteria and detected by host cytosolic sensors that induce oligomerization of the NLRC4 inflammasome. It is important to note that these studies do not indicate that NLRC4 inflammasome oligomerization requires a K^+ efflux, and thus it remains possible that K^+ efflux is not necessary of NLRC4 activation by *Yersinia*.

While a number of studies have begun to elucidate crucial pathways for canonical inflammasome activation, a recent study has also shed light on non-canonical caspase-11 inflammasome activation. Pilla et al. discovered that during *Legionella pneumophila* infection, LPS that entered the host cytosol induces caspase-11 dependent pyroptosis in IFN-activated macrophages (Pilla et al., 2014). Additional
experiments revealed that this host response required guanylate binding proteins that appear to sense LPS and induce non-canonical inflammasome activation (Pilla et al., 2014).

In addition to inflammasome-dependent sensing of the T3SS, several studies have explored inflammasome independent pathways. Auerbuch et al. found that *Yersinia* devoid of effector proteins but containing a translocation competent T3SS induces a diverse TLR-independent immune response in macrophages (Auerbuch, Golenbock, & Isberg, 2009). This included induction of the NF-κB regulated genes *Mcp-1* and *c-Rel*, in addition to type-I IFN-regulated genes *Rantes, Mx-1*, and *Stat2* (Auerbuch et al., 2009). There also are examples of T3SS sensing in several other pathogens. For example, T3SS positive *Citrobacter rodentium* has been shown to induce a Nod-dependent IL-8 and Nod2-dependent TNFα response in HT29 human intestinal epithelial cells (LeBlanc et al., 2008). *Burkholderia pseudomallei*, which also expresses a T3SS, induces a Nod-independent IL-8 response in 293T cells (Hii et al., 2008). These findings highlight the importance of inflammasome-dependent responses for in the detection of T3SS by the host innate immune response.

1.7.3 NLR-Independent Sensing of T3SSs

While NLR-based pathways of T3SS sensing have been the focus of much research, host cells also seem to detect T3SS components through additional mechanisms. Recent work now has characterized the recognition of needle proteins
extracellularly through TLR2- or TLR4-dependent activation of NF-κB (Jessen et al., 2014). Other research by Viboud and Bliska demonstrated that a *Yersinia* mutant lacking YopD, but containing YopB, induces an NK-κB-dependent IL-8 pro-inflammatory signaling response (Gloria I. Viboud et al., 2003). This is presumably due to YopB insertion into the membrane of host cells, although the exact mechanism of how YopB insert into the membrane without YopD present is unclear. Nevertheless, these results highlight important mechanism of recognition of *Yersinia* T3SS recognition that have not yet been shown to be dependent on NLR signaling.
Chapter 2

Impact of host membrane pore formation by the *Yersinia pseudotuberculosis* type III secretion system on the macrophage innate immune response.

By Laura Kwuan, Walter Adams, and Victoria Auerbuch
Abstract

Type III secretion systems (T3SS) are used by Gram negative pathogens to form pores in host membranes and deliver virulence-associated effector proteins inside host cells. In pathogenic *Yersinia*, the T3SS pore-forming proteins are YopB and YopD. Mammalian cells recognize the *Yersinia* T3SS, leading to a host response that includes secretion of the inflammatory cytokine IL-1β, Toll-like receptor (TLR)-independent expression of the stress-associated transcription factor Egr1 and the inflammatory cytokine TNF-α, and host cell death. The known *Yersinia* T3SS effector proteins are dispensable for eliciting these responses, but YopB is essential. Three models describe how the *Yersinia* T3SS might trigger inflammation: (i) mammalian cells sense YopBD-mediated pore formation, (ii) innate immune stimuli gain access to the host cytoplasm through the YopBD pore, and/or (iii) the YopB-YopD translocon itself or its membrane insertion is pro-inflammatory. To test these models, we constructed a *Y. pseudotuberculosis* mutant expressing YopD devoid of its predicted transmembrane domain (YopD_{ATM}) and lacking the T3SS cargo proteins YopHEMOJTN. This mutant formed pores in macrophages, but could not mediate translocation of effector proteins inside host cells. Importantly, this mutant did not elicit rapid host cell death, IL-1β secretion, or TLR-independent Egr1 and TNF-α expression. These data suggest that YopBD-mediated translocation of unknown T3SS cargo leads to activation of host pathways influencing inflammation, cell death, and
response to stress. As the YopD<sub>ATM</sub> <i>Y. pseudotuberculosis</i> mutant formed somewhat smaller pores with delayed kinetics, an alternative model is that the wildtype YopB-YopD translocon is specifically sensed by host cells.
Introduction

Mammalian innate immune cells such as macrophages recognize and respond to numerous microbial invaders through a series of membrane-associated and cytoplasmic receptors that sense molecules unique to the microbial world (Kawai & Akira, 2011; Schroder & Tschopp, 2010; Ting, Duncan, & Lei, 2010). Based on the localization of these receptors and on the nature of their ligands, innate immune cells can distinguish between pathogenic and nonpathogenic bacteria (Vance, Isberg, & Portnoy, 2009). Given the trillions of commensal bacteria associated with the human body, the ability to induce an inflammatory response to potentially harmful pathogens while preventing chronic inflammation to beneficial commensal bacteria is of utmost importance to human health.

One clinically important, widespread, and evolutionarily ancient bacterial structure used specifically by Gram negative pathogens is the type III secretion system (T3SS)(Troisfontaines & Cornelis, 2005). The T3SSs of several bacteria, including pathogenic Yersinia and Pseudomonas aeruginosa, are recognized by macrophages (Auerbuch et al., 2009; Bergsbaken & Cookson, 2007; Miao, Ernst, Dors, Mao, & Aderem, 2008; Shin & Cornelis, 2007). This recognition leads to activation of the protease caspase-1 and secretion of the pro-inflammatory cytokine IL-1β(Bergsbaken & Cookson, 2007; Miao et al., 2008; Shin & Cornelis, 2007) as well as Toll-like
receptor (TLR)-independent induction of numerous host genes such as the stress-associated transcription factor Egr1 and the inflammatory cytokine TNF-α (Auerbuch et al., 2009; de Grado, Rosenberger, Gauthier, Vallance, & Finlay, 2001)(6, 10). T3SS-stimulated secretion of IL-1β has been suggested to be important for neutrophil recruitment and protection against P. aeruginosa in a mouse lung infection model (Lavoie, Wangdi, & Kazmierczak, 2011). This suggests that recognition of the T3SS by macrophages is an important component in defense against T3SS-expressing pathogens.

The T3SS resembles a molecular syringe composed of a basal structure that spans the bacterial inner and outer membranes and a needle-like structure that, in Yersinia, protrudes about 60 nm from the bacterial surface (Guy R Cornelis, 2006). At the tip of the Yersinia T3SS needle is LcrV, which coordinates the pore-forming proteins YopB and YopD in such a way that a pore is formed in the target host cell membrane. The LcrV-YopD-YopB translocon is thought to form a conduit that enables translocation of bacterial effector proteins called Yops directly inside the host cell cytoplasm, although this has never been visualized. The Yersinia T3SS targets cells of the innate immune system, such as macrophages (Durand, Maldonado-Arocho, Castillo, Walsh, & Mecsas, 2010; Köberle et al., 2009; Marketon, DePaolo, DeBord, Jabri, & Schneewind, 2005a). Once injected inside innate immune cells, the T3SS effector proteins interfere with uptake and killing of Yersinia and dampen some
inflammatory responses (Gloria I Viboud & Bliska, 2005). T3SS function is tightly regulated in *Yersinia* such that host cell contact is required for robust translation and secretion of Yops. YopN is a T3SS regulatory protein that is secreted through the T3SS needle and serves to prevent premature secretion of effector proteins (Hamad & Nilles, 2007). In addition, the pore-forming protein YopD participates in a complex with the regulatory protein LcrQ that inhibits translation of effector protein mRNA prior to host cell contact (D. M. Anderson et al., 2002; Cambronne & Schneewind, 2002; Williams & Straley, 1998). Once host cell contact is made, derepression of YopN secretory control allows secretion of LcrQ and translation of effector protein mRNA. Through these mechanisms and others, *Yersinia* prevents robust expression and secretion of effector proteins until they are required.

In addition to translocation of effector proteins, the T3SS of several pathogens has been shown or suggested to deliver flagellin and a T3SS structural protein called the inner rod protein inside host cells (Miao et al., 2010; Sun, Rolán, & Tsolis, 2007). Flagellin and inner rod proteins are directly recognized by cytoplasmic innate immune receptors called Naips, which together with Nlcr4/Ipaf activate caspase-1, stimulating secretion of IL-1β in addition to other responses (Kofoed & Vance, 2011; Zhao et al., 2011). However, another cytoplasmic innate immune receptor called Nlrp3/Nalp3 has been implicated in stimulating activation of caspase-1 and IL-1β secretion in response to the T3SS (Brodsky & Monack, 2009). Nlrp3 responds to a
wide variety of stimuli including bacterial pore-forming toxins, possibly through detection of potassium efflux, although the mechanism remains unclear (Brodsky & Monack, 2009; Schroder & Tschopp, 2010). The dominant pathway contributing to caspase-1 activation in response to the Yersinia T3SS has been shown to be the Nlrp3 pathway (Brodsky et al., 2010). As mice lacking caspase-1 are more susceptible to Y. pseudotuberculosis infection (Brodsky et al., 2010), elucidation of how the Yersinia T3SS triggers caspase-1 activation is critical for our overall understanding of Yersinia virulence. Furthermore, it remains unclear how the Yersinia T3SS triggers TLR-independent expression of host genes such as Egr1 (6), as these responses are thought to be independent of caspase-1.

Six known Yersinia T3SS translocated proteins, YopHEMOJT, are dispensable for triggering IL-1 secretion and TLR-independent Egr1 and TNF-α expression (Auerbuch et al., 2009). However, the pore forming protein YopB is necessary for host cell innate immune recognition of the T3SS (T3SS recognition) (Auerbuch et al., 2009; Bergsbaken & Cookson, 2007; Ryndak, Chung, London, & Bliska, 2005; Shin & Cornelis, 2007; Gloria I. Viboud et al., 2003). This requirement indicates that a YopB-mediated event, YopB membrane insertion, pore formation, and/or translocation of unknown T3SS cargo, results in T3SS recognition. Specifically, membrane disruption or potassium efflux through the T3SS pore have been suggested to underlie T3SS-triggered host cell signaling (Auerbuch et al., 2009; Brodsky et al.,
To understand the critical requirements for T3SS recognition, we sought to determine whether T3SS-mediated pore formation alone was sufficient to trigger host responses in macrophages. We identified a *Y. pseudotuberculosis* mutant that lacks YopHEMOJTN and expresses an allele of YopD devoid of its transmembrane domain (Δyop6/ΔyopN/ΔyopD_{ATM}). This mutant secreted YopB, formed 1.3-3.8 nm pores in macrophages, but was incapable of translocating T3SS cargo inside host cells. Importantly, the *Y. pseudotuberculosis* Δyop6/ΔyopN/ΔyopD_{ATM} strain did not induce IL-1β secretion, rapid host cell death, or TLR-independent expression of Egr1 and TNF-α. These data show that a translocation-incompetent YopBD pore is insufficient to stimulate a significant immune response. These findings suggest that either a wildtype YopB-YopD translocon or translocation of molecules distinct from YopHEMOJTN activate several macrophage signaling pathways that control inflammatory cytokine production, response to stress, and host cell survival.

### Materials and Methods

**Bacterial growth conditions.** *Y. pseudotuberculosis* was grown in 2xYT at 26°C/shaking overnight. The cultures were back-diluted into low calcium media.
(2xYT plus 20 mM sodium oxalate and 20 mM MgCl₂) to an OD₁₀₀ of 0.2 and grown for 1.5 hours at 26°C/shaking followed by 1.5 hours at 37°C/shaking to induce Yop synthesis as previously described (Auerbuch et al., 2009).

**Bacterial mutants.** The bacterial strains used in this study are listed in Table 1. The *Y. pseudotuberculosis* strains generated for this study were constructed by splicing by overlap extension PCR. Primers were designed using MacVector and Primer 3 software (http://fokker.wi.mit.edu/primer3/input.htm). Amplified PCR fragments, encoding ~200-400 bp of homology on either side of the intended mutation, were cloned into a SauI- and NotI-digested pSR47s suicide plasmid (pir-dependent replicon, Kan<sup>R</sup>, sacB gene conferring sucrose sensitivity)(Andrews, Vogel, & Isberg, 1998; Merriam, Mathur, Maxfield-Boumil, & Isberg, 1997). Recombinant plasmids were transformed into *E. coli* S17-1pir competent cells and later introduced into *Y. pseudotuberculosis* IP2666 via conjugation. The resulting Kan<sup>R</sup>, irgansan-resistant integrants were grown in the absence of antibiotic selection and plated on sucrose-containing media to identify clones that had lost sacB (and by inference, the linked plasmid DNA). Kan<sup>S</sup>, sucrose<sup>R</sup>, congo red-positive colonies were screened by PCR and subsequently sequenced to confirm the presence of the intended mutation. The *yopD<sub>ATM</sub>* mutation was constructed using the internal primers described in Olsson et.al 2004 (Olsson et al., 2004) along with the external primers 5’-CGGTCACTAGTGCCAGAATTGATC-3’ and 5’-
TTGGCGTCGACGTCGGTCATTCTG-3’. The full length yopD deletion mutation (leaving only the first three and last three amino acids of YopD) was constructed using the primers described in (Francis & Wolf-Watz, 1998). The ΔyopK mutation was introduced into Y. pseudotuberculosis using a pSR47s-derived suicide plasmid obtained from Dr. Molly Bergman (M. Bergman, unpublished data). Dr. Melanie Marketon kindly provided E.coli expressing YopM-Bla reporter plasmids containing a chloramphenicol (Cm)R cassette (R. Dewoody et al., 2011). YopM-Bla reporter plasmids were isolated and electroporated into Y. pseudotuberculosis. Single colonies were selected by plating on Cm and Congo Red plates.

**Primary cells and cell lines.** All animal use procedures were in strict accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the UC Santa Cruz Institutional Animal Care and Use Committee. C57Bl/6 mice were purchased from The Jackson Laboratory. Primary bone marrow-derived macrophages (BMDM) were prepared as previously described (Auerbuch et al., 2009). Immortalized C57Bl/6 and MyD88−/−/Tir−/− BMDMs were obtained from Dr. Douglas Golenbock and were grown routinely in DMEM supplemented with 10% fetal bovine serum (FBS) and 2 mM L-glutamine at 37°C/5% CO₂. Immortalized BMDMs were used for most assays to minimize animal use. However, immortalized C57Bl/6 BMDMs secreted minimal IL-1β following Y. pseudotuberculosis infection (with or
without prior TLR stimulation), so primary BMDMs were used for measuring release of IL-1β upon *Y. pseudotuberculosis* infection. However, the overall trends were similar between primary and immortalized BMDMs (W. Adams and V. Auerbuch, unpublished data).

**Hemolysis.** Red blood cell lysis induced by *Y. pseudotuberculosis* was measured as previously described, with some alterations (Davis & Mecsas, 2007a). *Y. pseudotuberculosis* low calcium media cultures (see Bacterial growth conditions, above) were spun down and resuspended at 2x10^9/ml. Sheep red blood cells (sRBC; Innovative Research) were washed three times with ice-cold phosphate-buffered saline (PBS) and resuspended in low calcium media at 2x10^9/ml. 50µl each of sRBC and bacterial suspension were added in triplicate to a 96-well round bottom plate (BD Falcon), centrifuged at 4000 rpm for 15 min at room temperature, and incubated at 37°C for 2 hrs. The supernatant was removed and the remaining pellet was resuspended in 150 µL ice-cold PBS+10% gentamicin or water. The resuspended pellets were incubated overnight at 26°C and centrifuged at 4000 rpm for 5 min at 4°C. 150 µL of the supernatant was transferred to a white-bottom, 96-well clear-bottom white plate (Corning) and absorbance was read at 545 nm on a Victor^3^ plate reader (Perkin Elmer). Data from three separate wells was averaged for each independent experiment.
**BCECF release.** BCECF release was preformed as previously described, with some alterations (Marenne, 2003). 4x10^5 immortalized C57Bl/6 BMDMs were plated in each well of a 24 well plate (BD Falcon) in 1 mL DMEM+10% FBS and incubated overnight. 20 min prior to infection, BMDMs are washed twice with 1 mL PBS and incubated with 500 µL of HBSS+10 µM BCECF-AM (Invitrogen) for 30min at 37°C/5% CO₂. Cells are washed twice in warmed phenol red-free RPMI. The BCECF-loaded BMDMs were infected at a multiplicity of infection (MOI) of 100 and the infected monolayer was centrifuged at 400xg at 4°C to initiate contact. The cells were then incubated at 37°C/5% CO₂ for 1 or 2 hours. Alternatively, 0.09% Triton x-100 was added to the cells 45 minutes prior to the completion of the experiment to achieve 100% BCECF release. The cells were centrifuged for 4 min at 250xg and 140 µL of cell culture supernatant was transferred in triplicate into a 96-well clear-bottom white plate (Corning), and BCECF fluorescence was measured using an excitation wavelength of 485 nm and an emission wavelength of 535 nm with a Victor³ plate reader (Perkin Elmer). The average of three separate BCECF fluorescence measurements was averaged for each independent experiment. Percent BCECF release was calculated as [(sample-uninfected)/(triton-uninfected)]x100.

Osmoprotectants, PEG 6000 (Spectrum), PEG 3350 (Calbiochem), sucrose (Fischer), or raffinose (Alfa Aesar), were resuspended in warm phenol red-free RPMI and added at 30 mM final concentration in a total volume of 0.5 mL (Dacheux, Goure, Chabert, Usson, & Attree, 2001).
**Ethidium Bromide Entry Assay.** 2x10^4 immortalized C57Bl/6 BMDMs were plated in each well of a 96 well clear-bottom black plate (Corning) in 100uL DMEM +10% FBS and incubated overnight. The cells were infected in triplicate at an MOI of 25 or 100 and centrifuged for 5 min at 750xg at 4°C to initiate contact. The cells were then incubated at 37°C/5% CO_2 for 2 hours. At the end of incubation period the media was aspirated and replaced with 30 µL of PBS containing 25 µg/mL ethidium bromide (EtBr) and 12.3 µg/mL Hoechst dye (Fischer). The cell monolayer was visualized using an ImageXpress \textsuperscript{MICRO} automated microscope and MetaXpress analysis software (Molecular Devices). The percent of EtBr-positive cells was calculated by dividing the number of EtBr-stained cells by the number of Hoechst-stained cells. Data from three separate wells was averaged for each independent experiment.

**Cell rounding assay.** 2x10^5 immortalized MyD88\(^{-/-}\)/Trif\(^{-/-}\) BMDMs were plated in each well of a 24 well plate in 1 mL DMEM+10% FBS and incubated overnight. MyD88\(^{-/-}\)/Trif\(^{-/-}\) BMDMs were chosen because of their flatter morphology (unpublished observations). The MyD88\(^{-/-}\)/Trif\(^{-/-}\) BMDMs were infected with *Y. pseudotuberculosis* expressing YopT at an MOI of 15. Cell rounding was monitored using a Olympus CKX41 microscope by two or three different researchers who were blinded to the identity of each sample.
**YopM translocation assay.** 2x10^4 CHO-K1 cells were plated in each well of a 96-well plate in 100 µL of F12K + 10% FBS and incubated overnight. CHO-K1 cells were infected with *Y. pseudotuberculosis* β-lactamase reporter strains for 2 hours at an MOI of 30 and incubated at 37°C/5% CO₂. 30-45 minutes prior to the end of the infection 20 µL of 6X loading solution containing CCF2-AM (Invitrogen) was added to each well and the plate was covered in foil and incubated at 30°C/5% CO₂. At the end of infection, the media was aspirated and 5 µM DRAQ5 in PBS was added to each well. The monolayers were incubated at room temperature for 5 minutes, washed once with PBS, and visualized using an ImageXpress micro automated microscope and MetaXpress analysis software (Molecular Devices). The number of YopM-Bla-positive cells was calculated by dividing the number of blue (CCF2-cleaved) cells by the number of green (total CCF2^∗^ cells). Data from two separate wells was averaged for each independent experiment.

**Type III secretion assay.** Visualization of T3SS cargo secreted in broth culture was performed as previously described (Auerbuch et al., 2009). *Y. pseudotuberculosis* low calcium media cultures were grown for 1.5 hrs at 26°C and switched to 37°C for another 2hrs. Cultures were spun down at 13,200 rpm for 10 min at room temperature. Supernatants were transferred to a new eppendorf tube. 10% final trichloroacetic acid was added and the mixture vortexed vigorously. Samples were incubated on ice for 20 mins and then spun down at 13,200 rpm for 15 min at 4°C.
The pellet was resuspended in final sample buffer (FSB) + 20% DTT. Samples were boiled for 5 min prior to running on a 12.5% SDS-PAGE gel. Sample loading was normalized for number of bacteria per sample.

**Lactate Dehydrogenase release assay.** 1.25x10^6 immortalized C57Bl/6 BMDMs were plated in each well of a 6 well plate (BD Falcon) in 2 mL DMEM+10% FBS and incubated overnight. The cells were infected at an MOI of 15. 90 µL of supernatant was transferred to an eppendorf tube every hour for four hours. After the last time point, the cells were freeze-thawed to achieve full LDH release. Supernatants were centrifuged for 1 min at 13,000 rpm and 50 µL transferred to a 96-well clear-bottom white plate (Corning). LDH release into the supernatant was measured using the CytoTox 96 Non-Radioactive Cytotoxicity Assay according to the manufacturer’s instructions (Promega). LDH release as a result of freeze-thaw was set at 100% for each sample.

**IL-1β protein detection.** 5x10^5 primary C57Bl/6 BMDMs were plated in each well of a 24 well plate in 1 mL DMEM+10% FBS and incubated overnight. The cells were infected at an MOI of 20 and 900 µl of the supernatant removed at 4 hrs, as previously described (Auerbuch et al., 2009). IL-1β protein levels in the supernatant were measured using a mouse Platinum IL-1β ELISA kit according to the manufacturer’s instructions (eBioscience).
Quantitative PCR. 1.25x10^6 immortalized MyD88^+/Trif^- BMDMs were plated in each well of a 6 well plate in 2 mL DMEM+10% FBS and incubated overnight. The cells were infected with *Y. pseudotuberculosis* at an MOI of 15, the supernatant filtered at 30 minutes through a 0.22 μm filter (Millipore) to remove non-attached and non-internalized bacteria, and total RNA harvested at two hours as previously described (Auerbuch et al., 2009). Transcript levels of TNF-α, Egr1, and 18s rRNA were measured as previously described (Auerbuch et al., 2009). Data from three separate qPCR reactions was averaged for each independent experiment.

Statistical analysis. Statistical significance of differences between groups was calculated using one-way ANOVA, followed by the Bonferroni post hoc test (95% confidence level), using KaleidaGraph (Synergy Software).

Results

The *Y. pseudotuberculosis* Δyop6/ΔyopN/yopD*Δ*M strain forms pores in macrophages. In order to determine the effects of pore formation on the host
response, we sought to identify *Y. pseudotuberculosis* T3SS mutants that forms pores in host cell membranes, but cannot translocate T3SS cargo. Several *Y. pseudotuberculosis* mutants were previously described to make pores in red blood cells (RBCs) but were incapable of translocating T3SS effector proteins inside nucleated cells (Olsson et al., 2004). One mutant had a small internal in-frame deletion within YopD spanning amino acids 128-149, which encompasses the predicted YopD transmembrane domain (TM) and is involved in binding to the YopBD chaperone protein LcrH/SycD (Olsson et al., 2004). We constructed an identical YopD\(_{\Delta TM}\) mutation in the *Y. pseudotuberculosis* IP2666 strain lacking the six T3SS effector proteins YopHEMOJT (\(\Delta yop6\)), as we have previously ruled out a role for YopHEMOJT in triggering IL-1\(\beta\) secretion and TLR-independent Egr1 and TNF-\(\alpha\) expression (Auerbuch et al., 2009). To verify that this mutant and several control strains can insert pores in RBCs as previously reported, we monitored hemoglobin release from sheep RBCs following infection with *Y. pseudotuberculosis* (see Materials and Methods, Fig. 1A). Consistent with Olsson et al. (Olsson et al., 2004), *Y. pseudotuberculosis* \(\Delta yop6/\Delta yopD_{\Delta TM}\) induced significant hemoglobin release, suggesting robust pore formation in RBCs.

It was critical to determine the ability of this mutant to form pores in macrophages, not just RBCs, as macrophages have been shown to recognize and respond to the *Yersinia* T3SS and are a major target of the *Yersinia* T3SS *in vivo* (Durand et al., 2004).
To test the ability of \( Y.\) \( \text{pseudotuberculosis} \) \( \Delta \text{yop6/\text{yopD}} \) \( \Delta \text{TM} \) to form pores in macrophages, we analyzed the ability of this strain to allow the release of the small fluorescent dye BCECF from immortalized, murine bone-marrow derived macrophages (BMDMs) two hours post-inoculation. Neither the \( \Delta \text{yop6/\text{yopD}} \) \( \Delta \text{TM} \) mutant nor the \( \Delta \text{yop6} \) strain (which expresses wild type YopD) could trigger release of BCECF even at a high multiplicity of infection (data not shown). These data highlight important differences in the sensitivity of pore formation assays and emphasize that requirements for pore formation in RBCs and nucleated cells may differ.

As YopN-deficient \( Y.\) \( \text{enterocolitica} \) was previously shown to induce significantly greater BCECF release than YopN-positive strains, we deleted YopN from the \( \Delta \text{yop6} \) and \( \Delta \text{yop6/\text{yopD}} \Delta \text{TM} \) mutants (Brodsky et al., 2010). Growth of the \( \Delta \text{yop6/\text{yopN}} \) and \( \Delta \text{yop6/\text{yopN/\text{yopD}}} \) \( \Delta \text{TM} \) strains was identical under the culture conditions used in the BCECF release assay (data not shown). The \( Y.\) \( \text{pseudotuberculosis} \) \( \Delta \text{yop6/\text{yopN}} \) strain induced release of 40-50% of total intracellular BCECF by one or two hours post-inoculation (Fig. 1B). \( Y.\) \( \text{pseudotuberculosis} \) \( \Delta \text{yop6/\text{yopN/\text{yopD}}} \) \( \Delta \text{TM} \) showed slower BCECF release kinetics, but did trigger release of 20-25% of total BCECF two hours post-inoculation (Fig. 1B, \( p=0.09, N=2 \); Fig. 1C, \( p<0.05, N=5 \)). These data suggest that the \( Y.\) \( \text{pseudotuberculosis} \) \( \Delta \text{yop6/\text{yopN/\text{yopD}}} \) \( \Delta \text{TM} \) mutant is capable of forming pores in macrophages.
In order to confirm this finding, we used a distinct pore formation assay that measures the entry of ethidium bromide (EtBr) inside infected BMDMs by fluorescence microscopy (Kirby, Vogel, Andrews, & Isberg, 1998). Two hours post-inoculation, the amount of EtBr entry inside BMDMs during infection with the \( Y. \) \textit{pseudotuberculosis} \( \Delta yop6/\Delta yopN/yopD_{\Delta \text{TM}} \) mutant was significantly greater than during infection with the \( yopB \)-deficient strain and ranged from 24-78% of total cells scoring positive (Fig. 2B). Taken together, these data demonstrate that the \( Y. \) \textit{pseudotuberculosis} \( \Delta yop6/\Delta yopN/yopD_{\Delta \text{TM}} \) strain forms pores in macrophages.

The \( Y. \) \textit{pseudotuberculosis} \( \Delta yop6/\Delta yopN/yopD_{\Delta \text{TM}} \) strain expresses an allele of YopD that is predicted to interact with the host membrane very differently from wildtype YopD, yet this strain can form pores in RBCs and macrophages. Likewise, other substantial modifications to the translocon complex of T3SSs have resulted in mutants that retain the capacity for membrane disruption (Matteï et al., 2011). For example, the \textit{Pseudomonas aeruginosa} YopB homologue PopB has been shown to disrupt membranes in the absence of the YopD homologue PopD (Faudry, Vernier, Neumann, Forge, & Attree, 2006). We asked whether a complete YopD deletion mutant would also form pores in mammalian cells. We constructed a \( Y. \) \textit{pseudotuberculosis} mutant carrying a full, in-frame deletion of YopD (\( \Delta yop6/\Delta yopN/\Delta yopD \)). While the average number of EtBr-stained cells during
Δyop6/ΔyopN/ΔyopD infection was greater than in the yopB-minus infection, this difference was not statistically significant by one-way ANOVA (see Materials and Methods) and was only significant at MOI 25 by the Student t-test (Fig. 2B; p=0.05). Collectively, these data suggest that a YopB-YopD_{ATM} translocon forms pores on macrophages, but that pore formation is either non-existent or just below the level of detection in the complete absence of YopD.

The *Y. pseudotuberculosis* Δyop6/ΔyopN/yopD_{Δ53-68} strain forms pores in macrophages. Olsson et al. also identified another YopD mutant, lacking amino acids 53-68, which was able to form pores in red blood cells, but unable to induce host cell rounding, much like the *yopD_{Δ128-149}* strain (Olsson et al., 2004). Investigation of the the *yopD_{Δ53-68}* mutant in a effectorless background revealed that the strain is able to form pores in erythocytes (Δyop6/ *yopD_{Δ53-68}*; Table 2)( Kwuan and Auerbuch, unpublished). Furthermore, in a ΔyopN background, both the *yopD_{Δ53-68}* (*ΔyopN/yopD_{Δ53-68}*) and *yopD_{Δ128-149}* (*ΔyopN/yopD_{Δ128-149}*) strains were able to induce BCECF release by two hours post-infection (Table 2)(Kwuan et al., 2013; Kwuan and Auerbuch, unpublished). These T3SS phenotypes were also confirmed in the ethidium bromide entry assay (Kwuan et al., 2013, Kwuan, Adams, and Auerbuch, unpublished). Together, these findings highlight subtle, but important differences in the capacity of YopD mutants to form pores in host cell membranes. Inducing hemoglobin release in erythrocytes appears to have less stringent...
requirements than causing BCECF release or ethidium bromide entry in macrophages. As such, by utilizing these pore formation assays it is possible to tease apart subtle changes in T3SS function between different YopD mutants.

**The *Y. pseudotuberculosis* Δyop6/ΔyopN/yopD_{ATM} strain forms 1.3-3.8 nm pores in macrophages.** In order to further compare the pores formed by the *Y. pseudotuberculosis* Δyop6/ΔyopN/yopD_{ATM} strain to those formed when wildtype YopD is expressed, we measured pore size using an osmoprotection assay. We infected BMDMs with *Y. pseudotuberculosis* in the presence of different size osmoprotectants and measured the amount of BCECF release two hours post-inoculation. Only osmoprotectants larger than the diameter of the T3SS pore can prevent osmotic lysis. By using different sized osmoprotectants, the approximate diameter of the pore can be determined (Gloria I. Viboud & Bliska, 2002). The size of the *Y. pseudotuberculosis* Δyop6/ΔyopN pore was 3.8-5 nm, as only PEG6000 was able to significantly block BCECF release (Fig. 1C). In contrast, the *Y. pseudotuberculosis* Δyop6/ΔyopN/yopD_{ATM} pore was 1.3-3.8 nm, as both PEG3350 and PEG6000 significantly blocked BCECF release (Fig. 1C). These data suggest that a YopB-YopD_{ATM} translocon forms smaller pores than a wildtype translocon. However, the pores formed by the Δyop6/ΔyopN/yopD_{ATM} mutant are still well within the range previously reported for T3SSs (Guy R Cornelis, 2006; Matteï et al., 2011).
The *Y. pseudotuberculosis* Δyop6/ΔyopN/yopD<sub>ΔTM</sub> strain cannot translocate ectopically-expressed T3SS effector proteins. In order to determine whether the *Y. pseudotuberculosis* Δyop6/ΔyopN/yopD<sub>ΔTM</sub> strain is capable of translocating T3SS cargo inside host cells, we measured entry of two different effector proteins inside mammalian cells: YopT and YopM. Translocation of plasmid-encoded YopT inside BMDMs was analyzed by measuring cell rounding, a downstream consequence of the RhoGTPase-disrupting activity of YopT (I Sorg, Goehring, Aktories, & Schmidt, 2001). While *Y. pseudotuberculosis* encoding wildtype YopB and YopD caused 100% of the BMDMs to round by 11 hours post-inoculation, the *Y. pseudotuberculosis* Δyop6/ΔyopN/yopD<sub>ΔTM</sub>/pYopT strain did not cause cell rounding over the background Δyop6/ΔyopN/yopB/pYopT level (40%, data not shown).

To confirm that the *Y. pseudotuberculosis* Δyop6/ΔyopN/yopD<sub>ΔTM</sub> strain cannot translocate T3SS cargo inside host cells, we measured translocation of a plasmid-encoded YopM-lactamase (YopM-Bla) reporter protein inside CHO cells using the fluorescent lactamase substrate CCF2 (R. Dewoody et al., 2011). While the *Y. pseudotuberculosis* Δyop6/ΔyopN/pYopM-Bla strain translocated YopM-Bla similarly to wildtype *Y. pseudotuberculosis*, strains lacking YopB or YopD were incapable of any YopM-Bla translocation (Fig. 3AB). Importantly, the *Y. pseudotuberculosis* Δyop6/ΔyopN/yopD<sub>ΔTM</sub>/pYopM-Bla strain was also deficient in
YopM-Bla translocation (Fig. 3AB), although it was capable of secreting YopM-Bla, YopD, and YopB into the culture supernatant upon growth under T3SS-inducing conditions (Fig. 3C). These data show that while the *Y. pseudotuberculosis* Δyop6/ΔyopN/ΔyopD_ATM strain can secrete T3SS cargo, it fails to translocate T3SS cargo inside mammalian cells.

**The *Y. pseudotuberculosis* Δyop6/ΔyopN/ΔyopD_ATM-68 strain can translocate T3SS cargo.** The ΔyopN/ΔyopD_ATM-68 strain was not the primary focus of this manuscript and thus we did not perform the same breadth of translocation assays for this mutant. In order to characterize the translocation capacity of the the Δyop6/ΔyopN/ΔyopD_ATM-68 strain, we performed the same cell rounding assay, as was done for the Δyop6/ΔyopN/ΔyopD_ATM/pYopT strain in the previous section. Surprisingly, the Δyop6/ΔyopN/ΔyopD_ATM-68/pYopT strain induced close to WT levels of cell rounding, in contrast to the Δyop6/ΔyopN/ΔyopD_ATM/pYopT strain (Table 1)(Kwuan, Adams, and Auerbuch, unpublished). This indicates that the Δyop6/ΔyopN/ΔyopD_ATM-68 strain is capable of translocating T3SS cargo. It is not clear why these results differ from those observed by Olsson et al. for their yopD_ATM-68 mutant strain, but it may be that deletion of YopN alters the translocation competency of a yopD_ATM-68 mutant (Olsson et al., 2004).
Y. pseudotuberculosis YscF mutants form pores in RBCs but fail to translocate T3SS cargo into macrophages. In addition to the YopD∆TM pore formation competent/translocation deficient Yersinia mutants, Davis and Mecsas (2007) described several YscF point mutants with the same phenotype. Specifically, the YscF point mutants yscF:D28G and yscF:K9R/I13T/K25R (Davis & Mecsas, 2007b) are both capable of inducing pore formation in RBCs as assayed by hemoglobin release. These same YscF mutants failed to induce cell rounding in macrophages, indicating that they were unable to translocate T3SS cargo (Table 1) (Davis & Mecsas, 2007b). It is important to note that these mutants were not included in the BCECF and ethidium bromide pore formation assays, so it is unclear as to what extent they can form pores in macrophages. Likewise, the size of the pore that is formed by the YscF mutants is also unknown.

The Y. pseudotuberculosis Δyop6/ΔyopN/yopD∆TM strain does not trigger rapid cell death, IL-1β secretion, or TLR-independent Egr1 and TNF-α expression in macrophages. Mammalian cells infected with Y. pseudotuberculosis ΔyopEHJ or Y. enterocolitica ΔyopHEMOPN die after several hours of infection, releasing the large cytoplasmic protein lactate dehydrogenase (Bliska, Guan, Dixon, & Falkow, 1991; R. Dewoody et al., 2011). As BCECF release precedes LDH release, LDH release has been previously ascribed to osmolysis resulting from YopBD-mediated pore formation (Bliska et al., 1991). In agreement with these previous publications, the Y.
*Yersinia pseudotuberculosis* ∆yop6/∆yopN strain induced significant LDH release from BMDMs by two hours post-inoculation (Fig. 4A). However, the *Y. pseudotuberculosis* ∆yop6/∆yopN/yopD∆TM and ∆yop6/∆yopN/yopD strains did not induce LDH release until four hours post-inoculation, and even at this late time point the amount of LDH released was lower than in the ∆yop6/∆yopN infection. These data show that *Yersinia* expressing a YopB-YopD translocon competent for pore formation but not translocation cannot induce rapid cell death (i.e.-prior to four hours post-inoculation). We propose that the rapid cell death induced by the ∆yop6/∆yopN strain may be triggered by translocation of unknown innate immune stimuli inside host cells.

The T3SSs of a number of pathogens, including *Yersinia*, have been shown to induce activation of the inflammatory protease caspase-1, leading to maturation and secretion of the inflammatory cytokine IL-1β (Brodsky & Monack, 2009). To test whether T3SS-mediated pore formation was capable of triggering IL-1β secretion, we infected BMDMs with *Yersinia* and measured the amount of IL-1β released into the supernatant four hours post-inoculation. The *Y. pseudotuberculosis* ∆yop6 strain induced a low level of IL-1β secretion (Schroder & Tschopp, 2010), as previously described (Fig. 4B). A strain lacking the T3SS cargo protein YopK induced significantly more IL-1β, which is also consistent with previous data (Brodsky et al., 2010). Importantly, while the translocation competent strain ∆yop6/∆yopN hyper-
induced IL-1β secretion to the same level as the yopK-minus strain, the Y. pseudotuberculosis Δyop6/ΔyopN/yopDΔTM strain did not induce detectable IL-1β secretion (Fig. 4B). These data indicate that T3SS-mediated pore formation in macrophages is insufficient to trigger this host signaling pathway and that potassium efflux alone is not likely to trigger IL-1β secretion during infection with Y. pseudotuberculosis.

Previous work has demonstrated that pore-forming, translocation-competent Y. pseudotuberculosis induce Egr1 and TNF-α expression in a TLR-independent manner (Auerbuch et al., 2009). To determine if pore formation alone is sufficient to induce TLR-independent Egr1 expression, we infected MyD88−/−/Trif−/− immortalized BMDMs, which are TLR-deficient, with Yersinia and measured transcription of Egr1 and TNF-α two hours post-inoculation. The Y. pseudotuberculosis Δyop6/ΔyopN strain induced a 24-fold increase in Egr1 transcription and a 7-fold increase in TNF-α transcription, while infection with the yopB-minus strain did not induce significant Egr1 or TNF-α transcription (Fig. 4CD). Importantly, the Y. pseudotuberculosis Δyop6/ΔyopN/yopDΔTM strain also did not induce significant Egr1 expression (Fig. 4C). While the Δyop6/ΔyopN/yopDΔTM mutant triggered two-fold more TNF-α expression in macrophages compared to the Δyop6/ΔyopN/ΔyopB strain, this was not statistically significant (Fig. 4D, p=1). Furthermore, the Δyop6/ΔyopN/yopDΔTM mutant did not induce more TNF-α or Egr1 expression than the yopB-minus strain.
even at a high multiplicity of infection (MOI 100; V. Auerbuch, unpublished results).

These data suggest that T3SS-mediated pore formation alone cannot trigger TLR-independent expression of the stress-associated transcription factor Egr1 or the inflammatory cytokine TNF-α.

**The *Y. pseudotuberculosis* Δyop6/ΔyopN/yopDΔ53-68 strain, but not the YscF mutants, induces TLR-independent Egr1 and TNF-α expression in macrophages.**

In order to test whether pore formation is sufficient for induction of an immune response in a wildtype YopD background, we infected MyD88−/−/Trif−/− immortalized BMDMs with either the Δyop6/ΔyopN/yopDΔ53-68 or YscF point mutant strains. We found that while the Δyop6/ΔyopN/yopDΔ53-68 strain was able to induce significant TNF-α or Egr1 expression, the YscF mutant strains were unable to induce a significant immune response (Table 2). In fact, the YscF mutant strains induced similar levels of expression as a another YscF point mutant, yscF:T70A, a negative control which cannot insert pores into RBCs. (data not shown and Table 2)(Davis & Mecsas, 2007b). These data further argue that only a translocation-competent T3SS triggers TLR-independent immune signaling in macrophages.

**Discussion**
This study reports on the identification of a *Y. pseudotuberculosis* Δyop6/ΔyopN/yopD<sub>ΔTM</sub> mutant capable of forming T3SS-mediated pores in macrophages but lacking the ability to translocate T3SS cargo inside target host cells. Pores inserted into macrophages by the Δyop6/ΔyopN/yopD<sub>ΔTM</sub> strain formed with delayed kinetics and were smaller in size than wildtype T3SS pores (1.3-3.8 nm compared to 3.8-5 nm). However, while clear pore formation in macrophages was measured by two independent assays, no macrophage response associated with innate immune recognition of the T3SS was detectable other than delayed cell death.

Experiments investigating the T3SS phenotypes of several other *Yersinia* mutants were also performed in this study. We utilized the YscF point mutants *yscF:D28G* and *yscF:K9R/I13T/K25R*, which exhibited the ability to form pores in red blood cells, but were translocation deficient, in order to confirm our results with the Δyop6/ΔyopN/yopD<sub>ΔTM</sub> strain. Indeed, the YscF point mutants were unable to induce a significant immune response in macrophages. In addition to these YscF mutants, we also characterized the T3SS functions of a Δyop6/ΔyopN/yopD<sub>Δ53-68</sub> strain. In contrast to the previous strains, the Δyop6/ΔyopN/yopD<sub>Δ53-68</sub> strain was able to form pores in host cells and translocate T3SS cargo. Interestingly, this strain was also able to induce a robust immune response in macrophages, further suggesting that translocation of T3SS cargo is a prerequisite for T3SS sensing.

As the YscF point mutant and Δyop6/ΔyopN/yopD<sub>Δ53-68</sub> strains were not the primary focus of this manuscript, their T3SS functions were not explored with the
same breadth as was the case for the Δyop6/ΔyopN/yopD<sub>STM</sub> strain. Thus, it is unclear if subtle differences exist in the T3SS functions of these mutants relative to Δyop6/ΔyopN/yopD<sub>STM</sub>, particularly with regard to pore formation. Despite this limitation, it is still possible to integrate these findings with our different models of T3SS sensing. Indeed, these results suggest that T3SS-mediated pore formation in the absence of translocation is insufficient to trigger T3SS recognition. However, because the Δyop6/ΔyopN/yopD<sub>STM</sub> strain formed pores that were measurably different than those inserted by a wildtype YopB-YopD translocon, we cannot rule out that the wildtype YopB-YopD translocon is specifically recognized by macrophages. It is possible that the YscF mutants we investigated could express a wildtype YopB-YopD translocon. However, we did not assay the pore formation capacity of these strains in macrophages or measure the size of the pores formed, and thus we cannot infer as to whether or not a wildtype YopB-YopD translocon is truly formed. Based on these data, we can revise the possible models for how the Yersinia T3SS might trigger inflammation in macrophages: (i) innate immune stimuli gain access to the host cytoplasm through the YopBD pore, and/or (ii) the wildtype YopB-YopD translocon stimulates an innate immune response. Further experiments identifying Yersinia T3SS cargo distinct from YopHEMOJTNK will refine our understanding of how the T3SS is recognized by mammalian cells.
*Y. enterocolitica* expressing wildtype YopB and an allele of YopD lacking amino acids 121-165, which encompasses the predicted transmembrane domain (TM), retained the ability to insert ion-conducting channels into planar lipid bilayers (Tardy et al., 1999). In contrast, *yopB*-minus *Y. enterocolitica* did not display channel activity. These data are consistent with our results showing that *Y. pseudotuberculosis* expressing YopD_{\Delta TM} allowed both BCECF release and EtBr entry inside macrophages while *yopB*-minus *Yersinia* did not (Fig. 1 and 2). Matteï et al. recently proposed a so-called three-tiered ring model to describe the putative structure of the LcrV-YopD-YopB translocon complex (Matteï et al., 2011). As YopD has been shown to bind both LcrV and YopB, Matteï et al. suggested that a YopD homo-oligomeric ring is superficially associated with the host membrane but links the LcrV needle tip with a YopB homo-oligomeric ring stably inserted in the host membrane (Matteï et al., 2011). Olsson et al. (Olsson et al., 2004) showed that the YopD predicted TM domain is not required for inserting pores inside RBCs, but is required to support translocation of T3SS cargo inside nucleated cells. Our study further shows that the YopD predicted TM domain is not required to insert 1.3-3.8 nm pores inside macrophages, a pore size that is slightly smaller than wildtype pores, with somewhat delayed kinetics (Fig. 1B and 1C). These data suggest that the association of the YopD oligomer with the host membrane is essential for translocation and contributes to pore formation kinetics and size. In contrast, full deletion of YopD led to no statistically significant macrophage pore formation or pore formation that was below the limit of detection (Fig. 1C and 2B). This suggests that the YopD-YopB interaction
may be important for insertion of a YopB oligomer capable of robust pore formation. Further high resolution imaging studies are needed to test this and other aspects of the three-tiered ring model.

Pyroptosis is a form of caspase-1 dependent, lytic host cell death associated with secretion of inflammatory cytokines such as IL-1β (Bergsbaken et al., 2009). The ability of *Y. pseudotuberculosis* to trigger pyroptosis in macrophages is dependent on the T3SS but independent of YopHEMOJT (Bergsbaken & Cookson, 2007). Caspase-1 activation by *Yersinia* is dependent on both Nlr4 and Nlrp3 inflammasome components, although Nlrp3 seems to play the more dominant role (Brodsky et al., 2010). Recently, NLRP12 has also been implicated in stimulating IL-1β secretion after *Y. pestis* infection, but the NLPR12 ligand is unknown (Vladimer et al., 2012). Nlrc4 recognition of the *Yersinia* T3SS is independent of flagellin (Bergsbaken & Cookson, 2007). Instead, recent evidence suggests that a structural component of the *Salmonella* T3SS, called the PrgJ inner rod protein, is recognized through an Nlrc4-dependent pathway (Kofoed & Vance, 2011; Zhao et al., 2011) and the *Y. pseudotuberculosis* PrgJ homologue, YscI, activates the Nlrc4 inflammasome (E. Kofoede and R. Vance, personal communication). It is much less clear how the *Yersinia* T3SS activates Nlrp3, which is activated by a broad range of stimuli including pore forming toxins (Brodsky & Monack, 2009; Schroder & Tschopp, 2010). Efflux of potassium from host cells through pores has been postulated to be
important in Nlrp3 activation (Arlehamn et al., 2010; Brodsky & Monack, 2009; Saleh & Green, 2007; Walev, Reske, Palmer, Valeva, & Bhakdi, 1995). Shin and Cornelis (2007) previously correlated the ability of Y. enterocolitica to form pores in host cells with the ability to trigger IL-1β secretion (Shin & Cornelis, 2007).

However, the bacterial strains which were able to form pores and trigger IL-1β release in their study also had the capacity to translocate T3SS cargo inside host cells (Shin & Cornelis, 2007), raising the possibility that T3SS-mediated translocation, not pore formation itself, could trigger Nlrp3 activation. Indeed, our study indicates that a Y. pseudotuberculosis strain capable of forming 1.3-3.8 nm pores in macrophages, but not of translocating T3SS cargo, did not trigger IL-1β secretion or rapid host cell death. These pores should be capable of releasing potassium ions, although we were unable to measure potassium efflux stemming from even wildtype YopBD-mediated pore formation (data not shown). As reported previously (Brodsky et al., 2010; Zheng et al., 2011), inhibition of potassium efflux by addition of excess extracellular potassium to Y. pseudotuberculosis-infected macrophages blocked IL-1β release (Adams and Auerbuch, unpublished data). Taken together, these data suggest that, while potassium efflux plays a role in T3SS-induced IL-1β secretion, the underlying mechanism of Nlrp3 activation may be translocation of unknown T3SS cargo inside host cells. Alternatively, it is possible that a wildtype YopB-YopD complex is required to activate Nlrp3.
*Y. pseudotuberculosis* expressing YopB was previously shown to trigger NFκB and MAP kinase activation and IL-8 secretion in HeLa cells, while a YopB mutant with a disruption in one of the two YopB TM domains was defective in inducing this host signaling (Ryndak et al., 2005; Gloria I. Viboud et al., 2003). A *Y. pseudotuberculosis* mutant expressing YopD harboring an early frameshift mutation in the middle of the predicted TM domain retained the ability to trigger IL-8 secretion (Gloria I. Viboud et al., 2003). The authors of these reports suggested that YopB membrane insertion led to activation of the MAP kinase pathway. In the present study, we show that *Y. pseudotuberculosis* lacking the YopD predicted transmembrane domain, but secreting wildtype YopB, does not trigger TLR-independent Egr1 expression in macrophages (Fig. 4C). Egr1 expression has been shown to be induced by the p38 MAP kinase pathway in response to cell stress (Lim, Jain, & Cao, 1998; Rolli, Kotlyarov, Sakamoto, Gaestel, & Neininger, 1999). It is possible that YopB membrane insertion triggers MAP kinase signaling in macrophages as it does in HeLa cells, but that this leads to expression of genes distinct from Egr1. However, YopB membrane insertion may not trigger a response in macrophages. Further work analyzing the impact of the T3SS on macrophages versus intestinal epithelial cells, both of which are relevant to the enteropathogenic *Yersinia* infection cycle, will be important for understanding the impact of the innate immune response to the T3SS on gut inflammation and bacterial virulence.
Acknowledgements

This work was supported by UCSC startup funds (V.A.). W.A. was supported by the Eugene Cota-Robles Fellowship Program and by the UCSC CBSE Research Mentoring Institute.

We are grateful to Joanne Engel, Alison Davis, and Halie Miller for critical reading of the manuscript. We thank Melanie Marketon for the YopM-Bla plasmid, Molly Bergman for the ΔyopK suicide plasmid, Walter Bray for help with microscopy, Alison Davis for technical advice on the hemolysis assay, Doug Golenbock for the immortalized BMDM cell lines, and Jessica Lasky-Su for guidance on statistical analysis.
Table 1. Deletion of YopN rescues the translocation defect of a *Y. pseudotuberculosis* yopD<sub>Δ53-68</sub> mutant, but not that of a yopD<sub>Δ128-149</sub> mutant.

<table>
<thead>
<tr>
<th><em>Y. pseudotuberculosis</em> strain</th>
<th>Cell rounding&lt;sup&gt;1&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wildtype&lt;sup&gt;1&lt;/sup&gt;</td>
<td>100%</td>
</tr>
<tr>
<td>ΔyopB</td>
<td>40%</td>
</tr>
<tr>
<td>yopD&lt;sub&gt;Δ53-68&lt;/sub&gt;</td>
<td>45%</td>
</tr>
<tr>
<td>yopD&lt;sub&gt;Δ128-149&lt;/sub&gt;</td>
<td>45%</td>
</tr>
<tr>
<td>Δyop6/ΔyopN+pYopT&lt;sup&gt;2&lt;/sup&gt;</td>
<td>100%</td>
</tr>
<tr>
<td>Δyop6/ΔyopN/yopD&lt;sub&gt;Δ53-68&lt;/sub&gt;+pYopT</td>
<td>80%</td>
</tr>
<tr>
<td>Δyop6/ΔyopN/yopD&lt;sub&gt;Δ128-149&lt;/sub&gt;+pYopT</td>
<td>20%</td>
</tr>
</tbody>
</table>

<sup>1</sup> Cell rounding was observed by light microscopy and verified by independent researchers in a “blind” manner. Results are the average of 2-3 independent experiments.

<sup>2</sup> C57Bl/6 wildtype primary BMDMs were infected for 4 hours with *Y. pseudotuberculosis* expressing YopHEMOJ.

<sup>3</sup> MyD88<sup>-/-</sup>/Trif<sup>-/-</sup> immortalized BMDMs were infected for 11 hours with *Y. pseudotuberculosis* expressing only YopT. These particular BMDMs were used for detection of YopT-mediated cell rounding because of their flat morphology.
Table 2. Pore+/translocation\ Y. pseudotuberculosis do not trigger TLR-independent innate immune signaling.

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Mutant</th>
<th>Pore</th>
<th>Translocation competent</th>
<th>Immune</th>
</tr>
</thead>
<tbody>
<tr>
<td>None⁴</td>
<td>I</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>ΔyopN</td>
<td>I</td>
<td>+</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>yopDΔ150-170</td>
<td>I</td>
<td>+</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>yopDΔ53-68/ΔyopN</td>
<td>I</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>yopDΔ53-68</td>
<td>II</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>yopDΔ128-149</td>
<td>II</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>yopDΔ128-149/ΔyopN</td>
<td>II</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>yscF:D28G</td>
<td>II</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>yscF:K9R/I13T/K25R</td>
<td>II</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>yscF:T70A</td>
<td>III</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ΔyopB</td>
<td>III</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

¹ In the Y. pseudotuberculosis Δyop6 background.

² RBC lysis and/or BCECF release from macrophages.

³ Yop-mediated cell rounding assay in macrophages or [26].

⁴ TNF-α, Egr1, Axud1, and IL-10 expression in MyD88⁺⁻/Trif⁺⁻ BMDMs and IL-1β secretion in wildtype BMDMs.

⁵ Wildtype copies of yopB, yopD, and yscF.
Figure 1. Analysis of *Y. pseudotuberculosis*-induced pore formation on red blood cells and macrophages by monitoring release of hemoglobin or BCECF. (A) Red blood cells were incubated with *Y. pseudotuberculosis* and the amount of hemoglobin released as a result of pore formation was measured. The averages ± sem of the raw absorbance readings from three to nine independent experiments are shown. * p≤0.05, as determined by one-way ANOVA followed by Bonferroni post hoc test (comparing ΔyscNU, Δyop6, and Δyop6/yopD ATM).
Chapter 2 - Figure 1A

A

Lytic activity x 10^2

PBS  ΔyscNU  Δyop6  Δ6/ΔyopB  Δ6/yopDΔTM
Figure 1. Analysis of *Y. pseudotuberculosis*-induced pore formation on red blood cells and macrophages by monitoring release of hemoglobin or BCECF. (B) BCECF-loaded immortalized C57Bl/6 BMDMs were infected with *Y. pseudotuberculosis* at an MOI of 100. The amount of BCECF fluorescence released into the supernatant as a result of pore formation was measured one or two hours post-inoculation. The averages ± sem from two independent experiments are shown.
Chapter 2 - Figure 1B
Figure 1. Analysis of *Y. pseudotuberculosis*-induced pore formation on red blood cells and macrophages by monitoring release of hemoglobin or BCECF. (C) The experiment in B was repeated in the presence of 30 mM osmoprotectants of different diameters (see inset). * p≤0.05, ** p≤0.005, *** p≤0.0005, as determined by one-way ANOVA followed by Bonferroni post hoc test where each indicated group was compared to the appropriate negative and positive controls (ΔyscNU and Δyop6/ΔyopN or Δyop6/ΔyopN/ΔyopD\textsubscript{TM}).
Chapter 2 - Figure 1C
Figure 2. Analysis of *Y. pseudotuberculosis*-induced pore formation on macrophages by monitoring entry of ethidium bromide. (A-B) Entry of ethidium bromide (EtBr) inside *Yersinia*-infected immortalized C57Bl/6 BMDMs was monitored two hours post-inoculation using fluorescence microscopy. (B) The number of EtBr-positive cells out of the total Hoechst-positive cells was quantified two hours post-inoculation. The averages ± sem from three to five independent experiments are shown. * p≤0.05 and *** p≤0.0005, as determined by one-way ANOVA followed by Bonferroni post hoc test where each indicated group was compared to the appropriate negative and positive controls (Δyop6/ΔyopN/ΔyopB and Δyop6/ΔyopN).
Chapter 2 - Figure 2A
Chapter 2 - Figure 2B

Figure 2. Analysis of *Y. pseudotuberculosis*-induced pore formation on macrophages by monitoring entry of ethidium bromide. (A-B) Entry of ethidium bromide (EtBr) inside *Yersinia*-infected immortalized C57Bl/6 BMDCs was monitored two hours post-inoculation using fluorescence microscopy. (B) The number of EtBr-positive cells out of the total Hoechst-positive cells was quantified two hours post-inoculation. The averages ± sem from three to five independent experiments are shown. * p≤0.05 and *** p≤0.0005, as determined by one-way ANOVA followed by Bonferroni post hoc test where each indicated group was compared to the appropriate negative and positive controls (∆yop6/∆yopN/∆yopB and ∆yop6/∆yopN).
Figure 3. Examination of YopM-β-lactamase translocation inside CHO cells. (A-B) CHO cells were loaded with the fluorescent β-lactamase substrate CCF2 and infected with *Y. pseudotuberculosis* expressing a YopM-β-lactamase (YopM-Bla) reporter construct for two hours. Green (uncleaved CCF2) and blue (cleaved CCF2) fluorescence was measured and used to determine the percentage of CHO cells that had been injected with YopM-Bla. % YopM-Bla-positive cells = blue CCF2-cleaved cells / green total CCF2$^+$ cells x 100%. (B) Results are representative of two independent experiments. (C) *Y. pseudotuberculosis* was grown in low calcium media at 37°C to induce type III secretion in the absence of host cells. Proteins in the bacterial culture supernatant were precipitated and visualized on a polyacrilamide gel using commassie blue.
Figure 4. Analysis of LDH release, IL-1β secretion, and Egr1 and TNF-α expression in Y. pseudotuberculosis-infected macrophages. (A) Immortalized C57Bl/6 BMDMs were infected with Y. pseudotuberculosis at an MOI of 15. At various times post-inoculation, supernatants were analyzed for lactate dehydrogenase (LDH). The amount of LDH released from freeze-thaw lysis of the cell monolayer was set at 100%. Dotted line-uninfected; diamonds-Δyop6/ΔyopN/ΔyopB; circles-Δyop6/ΔyopN/ΔyopD; closed squares-Δyop6/ΔyopN; open squares-Δyop6/ΔyopN/yopDSTM. The averages ± sem of four independent experiments are shown. ** p≤0.005 and *** p≤0.0005, as determined by one-way ANOVA followed by Bonferroni post hoc test where each indicated group was compared to the appropriate negative and positive controls (Δyop6/ΔyopN/ΔyopB and Δyop6/ΔyopN).

(B) Primary C57Bl/6 BMDMs were infected with Y. pseudotuberculosis at an MOI of 20 for four hours and the amount of IL-1β in the supernatant was measured by ELISA. The averages ± sem of three independent experiments are shown. * p≤0.05 and *** p≤0.0005, as determined by one-way ANOVA followed by Bonferroni post hoc test where each indicated group was compared to the appropriate negative and positive controls (uninf and Δyop6 or Δyop6/ΔyopN).
Chapter 2 – Figure 4AB

A

% LDH release

B

pg/ml IL-1β

hours post-inoculation
Figure 4. Analysis of LDH release, IL-1β secretion, and Egr1 and TNF-α expression in *Y. pseudotuberculosis*-infected macrophages. (C-D) Immortalized MyD88<sup>+/−</sup>/Trif<sup>−/−</sup> BMDMs were infected with *Y. pseudotuberculosis* at an MOI of 10 for two hours and the amount of TNF-α and Egr1 mRNA was measured by qPCR and compared to the 18s rRNA control. The average ± sem of the ratio of mRNA:18s rRNA from four independent experiments is shown. * p≤0.05, as determined by one-way ANOVA followed by Bonferroni post hoc test (comparing ∆yop6/∆yopN/∆yopB, ∆yop6/∆yopN, and ∆yop6/∆yopN/lyopD<sub>ΔTM</sub>).
Chapter 2 – Figure 4CD
Chapter 3

The central region of the *Yersinia* type III secretion system translocator protein

*YopD promotes Yop translocation into phagocytes.*

By Walter Adams, Laura Kwuan, and Victoria Auerbuch
Summary

The *Yersinia* type III secretion system (T3SS) translocates effector proteins into host cells to manipulate immune defenses such as phagocytosis and reactive oxygen species (ROS) production. Central to the T3SS are the translocator proteins YopB and YopD, which form pores in host membranes, enabling effector protein translocation. In contrast to YopB, YopD is also important in T3SS regulation. While the YopD amino and carboxy termini participate in pore formation and T3SS regulation, the role of the central region between amino acids 150-227 remains unknown. We assessed the contribution of the YopD central region by generating *Y. pseudotuberculosis* YopDΔ150-170 and YopDΔ207-227 mutants and analyzing their T3SS functions. These mutants form robust pores in macrophages and display wildtype levels of Yop secretion *in vitro*. However, both mutants are defective in translocating YopE into neutrophils and macrophages, cannot prevent phagocyte ROS production, and display a virulence defect in disseminated *Yersinia* infection. These findings suggest that the YopD central region facilitates optimal T3SS effector protein delivery into target host cells to effectively disarm innate immune defenses such as ROS production.

Introduction

Dozens of Gram negative pathogens use a type III secretion system (T3SS) to inject bacterial effector proteins into eukaryotic host cells. This includes all three species of
*Yersinia* that are pathogenic to humans: *Yersinia pestis*, the causative agent of bubonic plague, as well as the two enteropathogens *Yersinia enterocolitica* and *Yersinia pseudotuberculosis*. While enteropathogenic *Yersinia* infections are usually self-limiting in healthy individuals, mortality rates approach 50% in immunocompromised patients (Cover & Aber, 1989). The Ysc T3SS is highly conserved in *Yersinia* and is comprised of approximately 25 structural proteins and a translocon complex made up of three translocator proteins: YopB, YopD, and LcrV (Guy R Cornelis, 2006; Diepold et al., 2010). Importantly, these three components are all essential for pore formation in host cell membranes, facilitating injection of six or seven effector proteins (YopHEMOJTK) into host cells (Guy R Cornelis, 2006; Cecile Neyt & Cornelis, 1999; J. Pettersson et al., 1996; Rosqvist et al., 1994). Once inside the host cytosol, these effector proteins disrupt a number of host processes, such as phagocytosis, expression of inflammatory cytokines, and production of reactive oxygen species (ROS), thereby promoting *Yersinia* virulence (Gloria I Viboud & Bliska, 2005).

Both neutrophils and macrophages use an NADPH oxidase complex to produce superoxide anions in response to inflammatory stimuli (Keith et al., 2009; Segal, 2005; Takeya & Sumimoto, 2003). Ultimately, this process generates a diverse set of bactericidal ROS that serve as an important component in the host response to invading pathogens. To counter this host response, *Yersinia* inhibit ROS production.
through the action of two different T3SS effector proteins. YopH is a tyrosine phosphatase that inhibits the Fc receptor-mediated respiratory burst in both neutrophils and macrophages (Bliska & Black, 1995; Ruckdeschel et al., 1996a). YopE uses its GTPase activating protein (RhoGAP) activity to inhibit the essential NADPH oxidase component Rac2, dampening ROS production in neutrophils and macrophages (Ruckdeschel et al., 1996a; Songsunthong et al., 2010). Importantly, *Y. pseudotuberculosis* YopE mutants with a specific defect in targeting Rac2 are attenuated in a mouse infection model, suggesting YopE-mediated inhibition of ROS production is important for virulence (Songsunthong et al., 2010).

YopD is a multifunctional protein known to be involved in two critical aspects of type III secretion (Olsson et al., 2004). First, YopD, together with its chaperone LcrH and the regulatory protein LcrQ, prevent translation of effector Yops prior to host cell contact, enabling Yop production upon target cell interaction (D. M. Anderson et al., 2002; Chen & Anderson, 2011). Additional studies have shown that both the amino and carboxy termini of YopD are important for this YopD regulatory function and that this control is lost when YopD is absent or mutated within these regions (Ayad A A Amer, Ahlund, Bröms, Forsberg, & Francis, 2011; Williams & Straley, 1998). Second, YopD and YopB form pores in host cell membranes, enabling translocation of effector Yops into target cells (Guy R Cornelis, 2002b; Cecile Neyt & Cornelis, 1999). A putative transmembrane domain found between amino acids 128 and 149
contributes to robust pore formation in host cell membranes. This is based off the finding that a *Yersinia* mutant lacking this region formed smaller pores in macrophages than that of WT *Yersinia*. YopD also contains a central region between amino acids 150-227, the role of which is less well understood. Deletion of YopD amino acids 171-206 was shown to be associated with enhanced effector protein translocation, while deletion of YopD amino acids 150-170 or 207-227 did not lead to any detectable phenotype *in vitro* (Olsson et al., 2004). The YopD\textsubscript{150-170} and YopD\textsubscript{207-227} regions share 100% amino acid identity with YopD from *Y. enterocolitica* and *Y. pestis*, indicating that these regions may play a specific role in *Yersinia* fitness.

In this study, we investigated the role of the YopD central region in type three secretion and *Yersinia* virulence by generating *Y. pseudotuberculosis* mutants carrying in-frame deletions of YopD amino acids 150-170 or 207-227. Our results indicate that these regions of YopD are dispensable for YopBD-mediated pore formation, yet play an important role in efficient translocation of T3SS effector proteins into phagocytes. We show that when regions YopD\textsubscript{150-170} or YopD\textsubscript{207-227} are absent, *Y. pseudotuberculosis* is defective in Yop-mediated inhibition of phagocyte ROS production and is attenuated in mice. These findings suggest that the YopD central region facilitates optimal T3SS function against cells of the innate immune system to promote *Yersinia* pathogenesis.
Results

*Y. pseudotuberculosis* yopD\textsubscript{\(150-170\)} and yopD\textsubscript{\(207-227\)} are attenuated during disseminated infection. To address the role of the YopD central region in *Y. pseudotuberculosis* type III secretion and virulence, we constructed in-frame deletions of YopD amino acids 150-170 and 207-227 in the IP2666 strain background. The yopD\textsubscript{\(150-170\)} and yopD\textsubscript{\(207-227\)} mutants secrete Yops in *vitro* to the same extent as wildtype *Y. pseudotuberculosis* (Fig. 1), consistent with previous reports (Olsson *et al.*, 2004). To determine if the YopD central region plays a role in yersiniosis, we infected C57Bl/6 mice via the intraperitoneal (I.P.) route with wildtype *Y. pseudotuberculosis* (WT) or the yopD\textsubscript{\(150-170\)} and yopD\textsubscript{\(207-227\)} mutants. Four days post-inoculation, spleens and livers were harvested and bacterial load assessed (Fig. 2). The yopD\textsubscript{\(150-170\)} and yopD\textsubscript{\(207-227\)} mutants had 50 to 250-fold fewer CFUs, respectively, in the spleen and 45 to 65-fold fewer CFUs, respectively, in the liver compared to WT. This suggests that YopD\textsubscript{\(150-170\)} and YopD\textsubscript{\(207-227\)} carry out one or more functions important for *Y. pseudotuberculosis* virulence.

*Y. pseudotuberculosis* yopD\textsubscript{\(150-170\)} and yopD\textsubscript{\(207-227\)} exhibit normal pore formation in macrophages. YopD is required to form robust pores in the membranes of host cells, facilitating Yop translocation (Neyt C and G Cornelis, 1999). To determine if the yopD\textsubscript{\(150-170\)} and yopD\textsubscript{\(207-227\)} mutants are capable of pore formation on host cells, we measured 2,7-biscarboxyethyl-5(6)-carboxyfluorescein (BCECF) release from...
macrophages one hour post-inoculation with WT or YopD mutant strains (Fig. 3). These strains lack yopHEMOJN, as BCECF release is blocked by the presence of effector proteins and YopN (Marenne, 2003). A strain unable to form a functional T3SS (Δysc NU) is unable to form pores, whereas the yopDΔ150-170 and yopDΔ207-227 mutants as well as the parental strain induce robust BCECF release. Importantly, we observed no difference in BCECF release relative to the parental strain (p>0.5), indicating that the virulence attenuation of the yopDΔ150-170 and yopDΔ207-227 mutants does not stem from a defect in T3SS-mediated pore formation.

Y. pseudotuberculosis yopDΔ150-170 and yopDΔ207-227 fail to translocate T3SS cargo at WT levels into neutrophils and macrophages. The virulence defects displayed by the yopDΔ150-170 and yopDΔ207-227 mutants suggest that T3SS function is deficient in these strains. As both YopD mutants displayed normal Yop secretion and macrophage pore formation, we hypothesized that a Yop translocation defect could explain the in vivo phenotype. Previously, a report described the translocation capacity of similar YopD mutants as being comparable to WT; however, the experiments were based on indirect measurement of effector protein translocation, such as cell rounding (Olsson et al., 2004). We took a more direct approach by measuring translocation of plasmid-encoded Yop-β-lactamase (Yop-Bla) reporter proteins into mammalian cells loaded with the fluorescent β-lactamase substrate CCF2-AM (R. Dewoody et al., 2011). Y. pseudotuberculosis pYopH-Bla and pYopE-Bla strains can secrete YopH-Bla and
YopE-Bla in vitro (data not shown). We used these Yersinia reporter strains to infect CHO cells and measure the translocation of T3SS cargo (Fig. 4A). Two hours post-inoculation, there was no significant difference between the two YopD mutants and the wildtype strain with regard to the translocation of YopH-Bla or YopE-Bla (Fig. 4B; p>0.1). Thus, under these conditions, both the yopDΔ150-170 and yopDΔ207-227 mutants are fully capable of translocating YopH-Bla and YopE-Bla at WT levels.

Yersinia preferentially target neutrophils, macrophages, and dendritic cells in vivo through their T3SS (Durand et al., 2010; Marketon et al., 2005a). To investigate the translocation capacity of the YopD mutants in physiologically relevant cell types, we performed a splenocyte translocation assay. Single cell suspensions of splenocytes (which include macrophages and neutrophils) from naïve mice were loaded with CCF2-AM, infected with different Yersinia reporter strains for one hour, and analyzed by flow cytometry. Interestingly, the yopDΔ150-170 and yopDΔ207-227 pYopE-Bla mutants translocate YopE-Bla into an average of 12 and 14% of CCF2-containing splenocytes, respectively, compared to an average of 22% for the WT strain (Fig. 4C shows one representative experiment). We stained these splenocytes with antibodies specific for macrophage and neutrophil cell surface markers and found that the yopDΔ150-170 and yopDΔ207-227 pYopE-Bla mutants translocate YopE-Bla into 30-40% fewer neutrophils and macrophages WT YopD strains (Fig. 4D). These findings
suggest that YopD_{150-170} and YopD_{207-227} are important for efficient delivery of Yops into phagocytes.

*Y. pseudotuberculosis* yopD_{Δ150-170} and yopD_{Δ207-227} are defective in inhibiting reactive oxygen species production by phagocytes. To determine whether the defect in YopE translocation displayed by the YopD central region mutants results in a decrease in YopE function, we measured the ability of *Y. pseudotuberculosis* carrying YopD mutations to inhibit phorbol myristate acetate (PMA)-induced reactive oxygen species (ROS) production by phagocytes. As previous studies have infected phagocytes with bacteria, followed by the addition of PMA, or have added PMA simultaneously with the infection, we used both approaches. When PMA is added simultaneously with the *Yersinia* to differentiated HL-60 (dHL-60) neutrophil-like cells, WT *Yersinia* inhibits 50% of ROS production while the yopD_{Δ150-170} and yopD_{Δ207-227} strains only inhibit 20-40%, with only yopD_{Δ207-227} showing statistically significance (Fig. 5A-B). Similar results were obtained using primary BMDMs (Fig. S1). Likewise, when dHL-60 cells are infected with bacteria prior to PMA addition, neither of the yopD_{Δ150-170} and yopD_{Δ207-227} strains has WT levels of ROS inhibition, although only the difference for the yopD_{Δ150-207} mutant is statistically significant (Fig. 5C-D). These data indicate that both the *Y. pseudotuberculosis* yopD_{Δ150-170} and yopD_{Δ207-227} strains are defective in their ability to inhibit ROS production in phagocytes.
We next investigated whether defective ROS inhibition in vivo plays a role in the virulence attenuation of YopD central region mutants. Mice were given either normal drinking water or drinking water containing acetovanillone, an NADPH oxidase inhibitor. We infected the mice with 1x$10^3$ WT or $yopD_{\Delta207-227}$ Y. pseudotuberculosis and found that acetovanillone treatment does not affect the bacterial load of either strain (data not shown). The identical acetovanillone dose and administration protocol was previously shown to rescue the virulence of a Salmonella strain with impaired resistance to hydrogen peroxide and nitric oxide (Song et al., 2013). However, acetovanillone only inhibits NADPH oxidase, yet neutrophils utilize both NADPH oxidase-dependent and independent pathways for ROS production (Fay, Qian, Jan, & Jan, 2006). Furthermore, the YopD central region mutants are likely to be defective in other YopE-mediated effector functions that are independent of ROS, such as inhibition of phagocytosis. We reasoned that decreasing the bacterial inoculum might increase the likelihood that the partial inhibition of ROS production afforded by acetovanillone would translate to a detectable phenotype in this context. Indeed, when a two-fold lower inoculum is used, control mice infected with WT, acetovanillone-treated mice infected with WT, and acetovanillone-treated mice infected with the $yopD_{\Delta207-227}$ mutant all have approximately one log more CFU in the spleen than control mice infected with $yopD_{\Delta207-227}$, although this difference is not statistically significant for all comparisons, as we observe more experimental variability when lower inoculums are used (Fig. 6; p=0.07, p=0.04, p=0.05, respectively). No
significant differences are observed in the liver (data not shown). Importantly, acetovanillone has no effect on wildtype *Y. pseudotuberculosis* infection of the spleen or liver (Fig. 6; spleen p=0.8), consistent with wildtype bacteria robustly inhibiting ROS production on their own without an exogenous ROS inhibitor. These findings are consistent with the hypothesis that inhibition of NADPH oxidase in phagocytes partially restore virulence to the *yopD*Δ207-227 strain. However, further experiments using NADPH oxidase deficient mice are needed to test this hypothesis.

*Y. pseudotuberculosis yopD*Δ150-170 and *yopD*Δ207-227 hyperinduce a host response in the absence of T3SS effector proteins. We previously showed that *Y. pseudotuberculosis* strains capable of translocating T3SS cargo induce a host immune response. This interaction highlights the ability of host cells to sense the presence of T3SSs and induce a pro-inflammatory immune response to resolve the infection. As both the *yopD*Δ150-170 and *yopD*Δ207-227 strains exhibit significant defects in their ability to translocate YopE into phagocytes, we investigated if these mutants are less able to inhibit a phagocytic immune response than are wildtype bacteria. We infected primary BMDMs with WT, *yopD*Δ150-170, or *yopD*Δ207-227 and quantified cytokine transcript levels. We did not see any difference in *tnfa, egr1*, or *il-10* expression between the *yopD* mutants and WT *Yersinia* (data not shown). These data are consistent with previous findings from our lab showing that both T3SS*+* and T3SS*–* *Y. pseudotuberculosis* trigger equivalent expression of these host genes when Toll-like
receptor (TLR) signaling is intact (Auerbuch et al., 2009). However, macrophages with intact TLR signaling are known to activate caspase-1-dependent pathways, such as secretion of the inflammatory cytokine IL-1β and programmed host cell death specifically in response to the *Y. pseudotuberculosis* T3SS. This activation can be inhibited by the T3SS effector protein YopM (Auerbuch et al., 2009; Brodsky et al., 2010; Larock & Cookson, 2012). However, we did not observe any significant induction of IL-1β or altered levels of host cell death by the *yopD* mutants in BMDMs (data not shown). Surprisingly, when we repeated these experiments with bacteria devoid of effector proteins (Δyop6 background), we observed strikingly different results. We infected immortalized TLR-deficient BMDMs with *Y. pseudotuberculosis* Δyop6, Δyop6/ΔyopB, Δyop6/yopDΔ150-170, or Δyop6/yopDΔ207-227 and analyzed host cell death by measuring release of LDH over time. Consistent with previous results, we observed that the T3SS+ strain lacking effector proteins (Δyop6) induce host cell cytotoxicity, while the strain deficient in targeting host cells through its T3SS (Δyop6/ΔyopB) does not (Fig. 7A). The Δyop6/yopDΔ150-170 and Δyop6/yopDΔ207-227 strains induce substantially more rapid host cell cytotoxicity (Fig. 7A), which can be decreased by reducing the inoculum (data not shown). In addition, we found that the Δyop6/yopDΔ150-170, or Δyop6/yopDΔ207-227 strains induces primary WT macrophages to secrete more IL-1β than the Δyop6 parental strain (Fig. 7B). Lastly, when TLR-deficient BMDMs are infected with the Δyop6/yopDΔ150-170, or Δyop6/yopDΔ207-227 strains, egr1 transcript levels are hyperinduced (Fig 7C). These data indicate that the absence of the YopD central region has a strong impact on host
cell signaling and survival when the T3SS effector proteins are absent, but that this effect is mitigated by at least one effector protein.

**Discussion**

The *Yersinia* translocator protein YopD is critical for T3SS regulation and pore formation on host cell membranes and facilitates delivery of T3SS cargo into target host cells. Our study investigated the contribution of the poorly-characterized central region of YopD to these fine-tuned processes by analyzing two *Y. pseudotuberculosis* mutants carrying targeted deletions within YopD\textsubscript{150-170} or YopD\textsubscript{207-227}. We found that *Y. pseudotuberculosis* mutants carrying either a yopD\textsubscript{Δ150-170} or yopD\textsubscript{Δ207-227} allele exhibit normal host membrane pore formation and secretion of Yops, suggesting that these regions of YopD are not required for these classic T3SS functions. However, the yopD\textsubscript{Δ150-170} and yopD\textsubscript{Δ207-227} mutants display significant virulence defects in a mouse model of infection, an impaired ability to translocate YopE into neutrophils and macrophages, and a diminished capacity to inhibit ROS production by these cell types. These findings suggest that the central region of YopD plays a role in facilitating translocation of Yops into phagocytes, contributing to the ability of *Y. pseudotuberculosis* to cause disseminated infection.

There are several techniques for measuring translocation of effector proteins into host cells. A previous study characterized similar *Y. pseudotuberculosis* mutants expressing the yopD\textsubscript{Δ150-170} or yopD\textsubscript{Δ207-227} alleles in a different strain background.
These researchers measured the ability of the mutants to induce YopE-mediated host cell rounding or ADP-ribosylation of the cytosolic host protein Ras by exogenously-expressed ExoS from *Pseudomonas aeruginosa* in HeLa cells (Olsson *et al.*, 2004). Results from both of these assays indicated that the YopD mutants translocate T3SS cargo at rates similar to that of *Yersinia* expressing wildtype YopD. We utilized a Yop-Bla reporter assay to measure directly the translocation of Yops into CHO cells and found that, consistent with the previous findings, the *yopD*Δ150-170 and *yopD*Δ207-227 mutants are equivalent to WT. As the YopD mutants are significantly attenuated for virulence, we reasoned that they may have a defect in their ability to inject T3SS cargo into specific cell types. Indeed, the *yopD*Δ150-170 and *yopD*Δ207-227 mutants are defective in the translocation of T3SS cargo into neutrophils and macrophages.

Previous studies have shown that *Yersinia* preferentially target neutrophils, macrophages, and dendritic cells during *in vivo* and *in vitro* infections (Durand *et al.*, 2010; Marketon, DePaolo, DeBord, Jabri, & Schneewind, 2005b). The discrepancy between the ability of *Y. pseudotuberculosis* *yopD*Δ150-170 and *yopD*Δ207-227 mutants to translocate Yops into primary phagocytes as compared to translocation into CHO cells or HeLa cells may reflect important differences in the *Yersinia* interaction with different host cell types. However, the CHO cell translocation assay was performed using 15-fold more inoculating bacteria than were used in the splenocyte translocation assay. Therefore it is possible that the *yopD*Δ150-170 and *yopD*Δ207-227 mutants have a defect in translocating YopE, and perhaps other Yops, into all target
host cells, but that this defect is masked in our CHO cell experiment. Further experiments will distinguish between these two possibilities.

As we were able to detect a substantial defect in the translocation of YopE cargo into phagocytes by the yopDΔ150-170 and yopDΔ207-227 *Y. pseudotuberculosis* mutants, we hypothesized that this should translate into impaired YopE effector function. One established function of YopE is its ability to inhibit ROS production in neutrophils and macrophages (Bliska & Black, 1995; Ruckdeschel et al., 1996b; Songsunthong et al., 2010). Indeed, both the yopDΔ150-170 and yopDΔ207-227 mutants do not inhibit ROS production to the extent that WT strains do. The defect exhibited by the yopDΔ150-170 mutant is most apparent when dHL-60s are infected with *Yersinia* for an hour, followed by PMA addition to induce ROS production (Fig.5C-D). Conversely, the yopDΔ207-227 mutant is more impaired at inhibiting ROS production when *Yersinia* and PMA are added to dHL-60s simultaneously (Fig. 5A-B), possibly reflecting subtle differences between the two YopD mutants. It is possible that the yopDΔ207-227 mutant is more defective than the yopDΔ150-170 mutant in initial injection of YopE, but that it eventually translocates higher levels of YopE into target cells than does the yopDΔ150-170 mutant. These findings establish a direct connection between the translocation defect exhibited by the mutant strains and their inability to effectively disrupt the host immune response.

We attempted to evaluate the role of ROS in an *in vivo* model. When we used an inoculum of ~500 bacteria, the yopDΔ207-227 mutant had approximately ten fold higher
CFU in the spleens of mice receiving the NADPH oxidase inhibitor acetovanillone compared to mice receiving plain water, but this difference was just shy of the significance cut-off (p=0.052). This partial rescue is not surprising given that neutrophils also utilize NADPH oxidase-independent pathways for ROS production (Fay et al., 2006). Importantly, acetovanillone had no effect on wildtype *Y. pseudotuberculosis* infection (p=0.80). This suggests that, as wildtype *Y. pseudotuberculosis* already robustly inhibits ROS production through its T3SS, inhibition of NADPH oxidase by acetovanillone has no effect. However, since the *yopD*Δ*207-227* mutant is partially defective in ROS production, acetovanillone administration boosts bacterial survival. This is consistent with a previous report showing that *Y. pseudotuberculosis* mutants specifically defective in ROS inhibition through Rac2 compete more effectively with wildtype *Y. pseudotuberculosis* in Cybb⁻/⁻ mice, which lack the gp91phox component of NADPH oxidase, than they do in wildtype mice (Songsungthong et al., 2010). Collectively, these data suggest that a defect in YopE-mediated inhibition of phagocyte ROS production partially explains the virulence attenuation of the YopD central region mutants. However, to definitively test this hypothesis, future experiments should utilize Cybb⁺/⁺ mice which completely lack NADPH oxidase activity, as opposed to using acetovanillone, which may not diffuse into all tissues to completely block NADPH-dependent ROS production.

YopE disruption of the host immune response is not limited to ROS inhibition and the YopD mutants may be unable to overcome host defenses even in the absence
of ROS production. Indeed, YopE has been shown to interfere with several host defense pathways through inactivation of RhoA and Rac1 GTPases (Andor et al., 2001; Bliska & Black, 1995). For example, an important function of YopE is its anti-phagocytic activity via Rac1 inactivation (Alrutz et al., 2001; Andor et al., 2001; Bliska & Black, 1995). It is likely that decreased YopE translocation by the \( yopD_{\Delta 150-170} \) and \( yopD_{\Delta 207-227} \) mutants leads to a defect in inhibiting both ROS production and phagocytosis, contributing to the virulence defects observed. Additionally, decreased YopE translocation may have broader effects on the injection of other T3SS cargo, as YopE is known to modulate the translocation of other Yops and \textit{Yersinia} strains lacking YopE exhibit Yop hyper-translocation (Aili et al., 2008b; Mejía, Bliska, & Viboud, 2008). Thus, it is also possible that a substantial decrease in YopE translocation results in an overall increase in the translocation of other Yop effectors. Importantly, an increase in translocation of other Yops cannot compensate for loss of YopE, as \textit{Yersinia} lacking YopE exhibits a significant virulence defect (Logsdon & Mecsas, 2003). Further experiments are necessary to determine the ability of the \( yopD_{\Delta 150-170} \) and \( yopD_{\Delta 207-227} \) mutants to translocate T3SS cargo other than YopE into primary phagocytes. The effector protein YopH also inhibits ROS production in host cells (Bliska & Black, 1995; Ruckdeschel et al., 1996a). While a \textit{Y. pseudotuberculosis} strain lacking YopE is as defective as \textit{Y. pseudotuberculosis} lacking YopB in inhibiting ROS production in neutrophils (data not shown), a defect in YopH translocation may also contribute to this process in the \( yopD_{\Delta 150-170} \) and \( yopD_{\Delta 207-227} \) mutants.
YopE has been shown to bind YopD in vitro. The hydrophobic region of YopD encompassing amino acids 122-151 is believed to be required for this interaction (Håkansson et al., 1993; Hartland & Robins-Browne, 1998). While there is no evidence to suggest that this interaction has a role in virulence, it does establish a unique relationship between translocator and effector proteins. This hydrophobic region is immediately adjacent to the missing amino acid residues in the \( yopD_{\Delta 150-170} \) Y. pseudotuberculosis mutant and slightly upstream of those missing in the \( yopD_{\Delta 207-227} \) Y. pseudotuberculosis mutant. It is possible that YopE interacts with additional regions of YopD, such as YopD\(_{150-170}\) and YopD\(_{207-227}\), and that a decreased interaction between YopE and YopD underlies the translocation defect of the \( yopD_{\Delta 150-170} \) and \( yopD_{\Delta 207-227} \) mutants. Our limited understanding of these interactions would be substantially informed by the addition of a tertiary structure for YopD. Current secondary structure analysis indicates that a YopD fragment spanning amino acids 150-278 is soluble and is partially unfolded in its native state, which may aid in YopD secretion, as T3SS cargo that do not fully unfold remain inside the T3SS needle complex (Dohlich et al., 2014; Raab & Swietnicki, 2008; Radics et al., 2014). This YopD fragment also converts between \( \alpha \)-helical and random coil states at a neutral pH upon temperature variation, which perhaps reflects a conformational change due to its interactions with LcrV and YopB during pore formation and with other T3SS cargo during translocation (Raab & Swietnicki, 2008).

YopD has been shown to interact directly or indirectly with the translocated regulatory protein YopK, which controls the rate and fidelity of T3SS cargo.
translocation (R. Dewoody et al., 2013). In the absence of YopK, *Yersinia* translocate more Yop effector protein Bla fusion constructs inside host cells. In addition, ΔyopK bacteria translocate detectable levels of a YopD-Bla reporter into host cells, whereas YopK+ bacteria do not. Interestingly, a ΔyopK mutant of *Y. pseudotuberculosis* induces elevated levels of IL-1β secretion (ref Brodsky et al., Kwuan et al.), much as we observed for the Δyop6/yopDΔ150-170 and Δyop6/yopDΔ207-227 mutants. One possibility is that the ΔyopK, Δyop6/yopDΔ150-170, and Δyop6/yopDΔ207-227 mutants hypertranslocate an immune stimulus that triggers IL-1β secretion. A group of cytosolic innate immune receptors have been shown to detect several bacterial ligands associated with the T3SS (Kofoed & Vance, 2012). These ligands include flagellin, the T3SS inner rod protein, and the T3SS needle subunit, all of which induce caspase-1 dependent activation when delivered inside mammalian cells (Miao et al., 2006; Rayamajhi et al., 2013; Yang et al., 2013). Alternatively, an as yet uncharacterized molecule also may be responsible for inducing this host response. For example, YopD is hypertranslocated into host cells in the ΔyopK mutant (R. Dewoody et al., 2013). Future studies will address whether the YopD central region mutants also hypertranslocate YopD. One intriguing possibility is that the YopD central region may serve to enhance translocation of effector Yops while limiting translocation of T3SS cargo that can serve as immune stimuli.

In conclusion, we characterized two *Yersinia* mutants, yopDΔ150-170 and yopDΔ207-227, to assess the role that the YopD central region plays in *Yersinia* pathogenesis. Despite exhibiting several wildtype in vitro phenotypes, both YopD
mutants have significant translocation defects into phagocytes. We suggest that these translocation defects prevent effective subversion of host cell defense mechanisms, such as inhibition of ROS production. Ultimately, the inability of the YopD mutants to manipulate the host immune system manifests in significant virulence attenuation in vivo. The complete conservation of YopD_{150-170} and YopD_{207-227} in all three pathogenic Yersinia species suggests that this central region may ensure optimal translocation of cargo in all three species. Future studies characterizing the capacity of YopD to influence Yop translocation will substantially enhance our fundamental understanding of type III secretion.

**Experimental Procedures**

**Bacterial growth conditions**

*Y. pseudotuberculosis* was grown in 2xYT at 26°C/shaking overnight. The cultures were back-diluted into low calcium media (2xYT with 20 mM sodium oxalate and 20 mM MgCl$_2$) to an OD$_{600}$ of 0.2 and grown for 1.5 hours at 26°C/shaking followed by 1.5 hours at 37°C/shaking to induce Yop synthesis, as previously described (Auerbuch *et al.*, 2009). Chloramphenicol was added where necessary to a final concentration of 20µg ml$^{-1}$. 


**Bacterial mutants**

The bacterial strains used in this study are listed in Table 1. *Y. pseudotuberculosis* mutants were generated by splicing using overlap extension PCR. The \( yopD_{\Delta 150-170} \) and \( yopD_{\Delta 207-222} \) mutants were constructed according to the strategy described in Olsson et al (Olsson et al., 2004). Briefly, amplified PCR fragments, encoding ~200-400 bp of homology on either side of the intended mutation, were cloned into pSR47s (Andrews et al., 1998; Merriam et al., 1997). Recombinant plasmids were introduced into *E. coli* S17-1-pir and later into *Y. pseudotuberculosis* IP2666. The resulting integrants were plated on sucrose-containing media to identify clones that had lost sacB. Kan\(^S\), sucrose\(^R\), congo red-positive colonies were screened by PCR and subsequently sequenced to confirm the presence of the intended mutation.

The \( yopD_{\Delta 150-170} \) and \( yopD_{\Delta 207-222} \) mutations was constructed using the internal primers described in (Olsson et al., 2004) along with the external primers 5’-CCAGGGAGGATCCGTTGCATTACTGAG-3’ and 5’-CACAACGTCGACTTAACTAATATT-3’. The \( \Delta yopE \) mutation was introduced into *Y. pseudotuberculosis* using a suicide plasmid generously provided by Dr. Joan Mecsas (Logsdon & Mecsas, 2003). YopE-Bla reporter plasmids, a kind gift from Dr. Melanie Marketon, were electroporated into *Y. pseudotuberculosis*. Single colonies were selected by plating on chloramphenicol and Congo Red plates.
Primary cells and cell lines

Primary bone marrow-derived macrophages (BMDM) were prepared as previously described (Auerbuch et al., 2009). Immortalized C57Bl/6 BMDMs were grown in DMEM supplemented with 10% heat-inactivated fetal bovine serum (HyClone) and 2 mM L-glutamine at 37°C/5% CO₂ (Auerbuch et al., 2009). CHO-K1 (ATCC) cells were maintained in F12K (Cellgro) supplemented with 10% heat-inactivated fetal bovine serum (HyClone) and 2 mM L-glutamine at 37°C/5% CO₂.

In vitro Yop secretion

Visualization of T3SS cargo secreted in broth culture was performed as previously described (Auerbuch et al., 2009). Y. pseudotuberculosis low calcium media cultures were grown for 1.5 hrs at 26°C followed by 37°C for 2hrs. Cultures were centrifuged at 16,000 x g for 10 min at room temperature and supernatants transferred to a new eppendorf tube. Trichloroacetic acid was added (10% final) and the mixture vortexed vigorously. Samples were incubated on ice for 20 min and centrifuged at 16,000 x g for 15 min at 4°C. The pellet was resuspended in final sample buffer (FSB) plus 20% DTT. Samples were boiled for 5 min prior to running on a 12.5% SDS-PAGE gel. Sample loading was normalized to culture density measured by OD₆₀₀.

BCECF release
BCECF release was performed as previously described (Kwuan et al., 2013). A total of 4x10^5 immortalized C57Bl/6 BMDMs were plated in each well of a 24 well plate (BD Falcon) in DMEM+10% FBS and incubated overnight. Twenty min prior to infection, BMDMs were washed twice with PBS and incubated with HBSS and 10 µM BCECF-AM (Invitrogen) for 30 min at 37°C/5% CO₂. Cells are washed twice in warmed phenol red-free RPMI. The BCECF-loaded BMDMs were infected at a multiplicity of infection (MOI) of 100 and the infected monolayer was centrifuged at 400xg at 4°C to initiate contact. The cells were then incubated at 37°C/5% CO₂ for 1 hr. Alternatively, 0.09% Triton x-100 was added to the cells 45 min prior to the completion of the experiment to achieve 100% BCECF release. The cells were centrifuged for 4 min at 250xg and 140 µL of cell culture supernatant was transferred in triplicate into a 96-well clear-bottom white plate (Corning), and BCECF fluorescence was measured using an excitation wavelength of 485 nm and an emission wavelength of 535 nm with a Victor³ plate reader (Perkin Elmer). The percentage of BCECF release was calculated as [(sample – uninfected)/(Triton X-100 – uninfected)] × 100.

Mouse infections

C57Bl/6 mice were purchased from The Jackson Laboratory. Six to eight week old C57/B6J mice were infected with ~1x10³ Y. pseudotuberculosis via intraperitoneal (I.P.), injection as previously described (Auerbuch et al., 2009). Alternatively, for
experiments involving acetovanillone (see below), mice were infected with 300-700 *Y. pseudotuberculosis*. Four days post inoculation spleens and livers were harvested, homogenized, and serial dilutions of the homogenate were plated to determine CFU per gram (CFU/g) tissue. Where indicated, 100mg ml$^{-1}$ of the NADPH oxidase inhibitor acetovanillone was added to the drinking water the night prior to infection and kept in the drinking water for the duration of the experiment (Song *et al.*, 2013).

*Yop-Bla translocation into CHO-K1 cells*

A total of $2 \times 10^4$ CHO-K1 cells were plated in each well of a 96-well plate in 100 µL of F12K + 10% FBS and incubated overnight. CHO-K1 cells were infected with the indicated *Y. pseudotuberculosis* ß-lactamase reporter strain at an MOI of 15. As an additional negative control, CHO cells were infected with a *Y. pseudotuberculosis* strain expressing a GST-Bla fusion protein (data not shown). Immediately following *Y. pseudotuberculosis* addition, the plate was spun at 110 x g for 5 min and incubated at 37°C/5% CO$_2$ for 2 hours. At 30 min post-inoculation, the supernatant was gently aspirated and replaced with fresh media. Between 30-45 min prior to the end of the infection, CCF2-AM (Invitrogen) was added to each well and the plate incubated at 30°C/5% CO$_2$. At 110 min post infection the media was aspirated and DRAQ5 added to each well. Monolayers were incubated at room temperature for 5 min, washed once with PBS, and visualized using an ImageXpress$^\text{MICRO}$ automated microscope and MetaXpress analysis software (Molecular Devices).
**Yop-Bla translocation into splenocytes**

Spleens were harvested from uninfected 6-8 week old C57Bl/6J mice. To generate single cell suspensions, spleens were placed in a six-well plate containing HBSS with Ca\(^{2+}\) and Mg\(^{2+}\) and perfused with 400 Mandl units ml\(^{-1}\) collagenase D (Roche) followed by a 30 min incubation at 37° C. Cells were passed through a 70 µm strainer and pelleted at 15,800 x g for 5 min. The pellet was resuspended in HBSS with 1mM EDTA to halt collagenase activity. Cells were treated with Red Blood Cell Lysis Buffer (Sigma) for 7 min and resuspended in 1x10\(^7\) cells ml\(^{-1}\) of RPMI + 5% FBS.

Single cell suspensions were loaded with 0.18µg ml\(^{-1}\) CCF2-AM for 2 h at 30°C, according to the manufacturer’s recommendations (Invitrogen). RPMI without phenol red was substituted for Solution C to prevent autofluorescence interference during flow cytometry. Cells were then infected with the indicated *Y. pseudotuberculosis* -lactamase reporter strains for 1 hr at an MOI of 1 and incubated at 37°C/5% CO\(_2\). A total of 2x10\(^6\) cells were resuspended in 100 µL of FACS Buffer (PBS + 5% FBS) and blocked with Mouse BD Fc Block™ (BD) for 10 min at 4°C. Cells were incubated in 100 µL of FACS buffer containing Ly-6G-APC-Cy7 and CD11b-PE-Cy5 (eBioscience) for 30 min at 4°C. Samples were washed once with FACS Buffer, centrifuged at 15,800 x g, resuspended in FACS buffer and analyzed on an LSRII flow cytometer (Becton Dickson). At least 2x10\(^5\) cells were acquired per sample and
data was analyzed using FlowJo v8.8.7 software. Cells that were not infected or labeled were used as negative controls. Fluorescence Minus One (FMO) controls were used to establish gating strategies.

*Reactive oxygen species detection*

HL-60 cells were cultured in IMDM with 20% FBS. HL-60 cells were differentiated into neutrophil-like HL-60 cells (dHL-60s) by culturing them in RPMI 1640 with 15% FBS and 1.3% DMSO for 5-6 days (Millius & Weiner, 2010). Cells were plated at 1x10^5 cells/well in HBSS in a 96-well white clear bottom tissue culture plates (Corning) and incubated overnight at 37°C/5% CO₂. Prewarmed HBSS without phenol red containing 100 μM luminol and 1 μg µl⁻¹ horseradish peroxidase was added to the dHL-60s for 30-60 min at 37°C/5% CO₂. dHL-60s were infected at MOI 15 with the indicated strain for 1 hr in HBSS without phenol red. 1μg ml⁻¹ PMA in HBSS without phenol red was added to induce ROS production. Alternatively, dHL-60s were infected at MOI 15 with the indicated strain with 1μg ml⁻¹ PMA in HBSS without phenol red simultaneously. Luminescence readings were taken immediately after infection for 30 min using a plate reader (Perkin-Elmer).

Primary bone marrow derived macrophages were primed with 50ng ml⁻¹ IFN-γ overnight and 100μM luminol was added to the media 0.5-1hr prior to infection.
Macrophages were stimulated with 1µg ml⁻¹ of zymosan to induce ROS production and infected at MOI 15. Luminescence was measured using a plate reader (Perkin-Elmer) immediately following infection every 5 min for 60 min.

**Statistical analysis**

Plotting of data and statistical analysis were performed using KaleidaGraph software. Statistical significance was determined by the unpaired Wilcoxon test for animal experiments, the student t-test for BCECF release, and one-way ANOVA with Tukey post-test for translocation and reactive oxygen species experiments.

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Table 1

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Chapter 3 - Figure 1

Figure 1. Analysis of supernatants containing T3SS cargo secreted by *Y. pseudotuberculosis* in vitro. *Y. pseudotuberculosis* strains were grown under T3SS-inducing conditions and secreted proteins were visualized by SDS-PAGE analysis and Coomassie Brilliant Blue staining. Shown is a representative gel out of three total.
Chapter 3 - Figure 2

Figure 2. Colonization of the spleen and liver by *Y. pseudotuberculosis* expressing wildtype or mutant YopD. C57Bl/6J mice were infected with 1x10^3 WT, *yopD_Δ150-170*, or *yopD_Δ207-227* *Y. pseudotuberculosis* via intraperitoneal injection. Organs were harvested four days post-inoculation and CFU per gram tissue were determined. Data from two to three independent experiments are shown. Bars indicate the geometric mean; open diamonds indicate that CFU were below the limit of detection. ** p < 0.005, *** p < 0.0001 using the Wilcoxon-Mann-Whitney non-parametric test.
Figure 3. Analysis of T3SS-mediated pore formation in macrophages.

Immortalized bone marrow derived macrophages were loaded with BCECF, infected with *Y. pseudotuberculosis*, and BCECF release was measured one hour post-inoculation. Shown is the average of five independent experiments ± standard error of the mean (SEM).
**Figure 4. Translocation of YopH-Bla and YopE-Bla reporter proteins into mammalian cells.** (A-B) CHO cells were infected with *Y. pseudotuberculosis* carrying either a YopH-Bla or YopE-Bla fusion protein at an MOI of 15 for two hours. Cells were incubated with CCF2-AM and translocation of Yop-Bla was measured by fluorescence microscopy. Blue fluorescence indicates cleaved CCF2 and green fluorescence indicates uncleaved CCF2. Representative images of YopE-Bla infections are shown with WT+YopE-Bla and ΔyopB+YopE-Bla, positive and negative controls, respectively. The average of three to five independent experiments ± SEM is shown. Scale bars indicate 100 µm.
Chapter 3 - Figure 4AB
Figure 4. Translocation of YopH-Bla and YopE-Bla reporter proteins into mammalian cells. (C-D) Single cell suspensions of splenocytes were loaded with CCF2 and were infected with *Y. pseudotuberculosis* carrying YopE-Bla at an MOI of one for one hour. Flow cytometry was used to determine the percentage of blue cells. One representative experiment is shown. (D) Splenocytes were labeled with cell surface marker-specific antibodies to identify macrophages and neutrophils. Neutrophils (Ly-6G^+CD11b^+) and macrophages (CD11b^+Ly-6G^-) were initially gated on CD11b and Ly-6G and then sub-gated on cleaved CCF2 (blue) and uncleaved CCF2 (green) to identify the % of blue cells within each cell type. Graphs show the relative % of blue^+ cells by cell type, normalizing to WT+YopE-Bla. Shown are the averages from two (uninf) or three (all other conditions) independent experiments ± SEM. * p<0.005, ** p<0.0005, and *** p<0.0001 using a one-way ANOVA, Post-hoc: Tukey HSD.
Chapter 3 - Figure 4CD
Figure 5. Inhibition of reactive oxygen species production in neutrophil-like cells by *Y. pseudotuberculosis*. Differentiated HL-60 cells (dHL-60) were incubated with luminol and horseradish peroxidase 0.5-1 hr prior to infection. PMA plus WT *Y. pseudotuberculosis* (▼), *yopD*Δ150-170 (●), *yopD*Δ207-227 (□), Δ*yopB* (○), or PMA alone (▲), or uninfected and untreated (■). *(A-B)* dHL-60 cells were treated with PMA to induce ROS production and were infected simultaneously with the indicated *Y. pseudotuberculosis* strain at MOI 15. ROS production was measured in relative light units (RLU) using a plate reader. *(A)* Shows one representative experiment and *(B)* shows the average of 8-9 independent experiments.
Chapter 3 - Figure 5AB
Figure 5. Inhibition of reactive oxygen species production in neutrophil-like cells by *Y. pseudotuberculosis*. (C-D) dHL-60s were infected with the indicated *Y. pseudotuberculosis* strain at MOI 15 for 1 h. PMA was then added to induce ROS production and ROS production was monitored using a plate reader. (C) Shows one representative experiment and (D) shows the average of three independent experiments ± SEM is shown. *p<0.05, **p<0.005, ***p<0.0005 using a one-way ANOVA, Post-hoc: Tukey HSD.
Chapter 3 - Figure 5CD
Figure 6. Impact of NADPH oxidase inhibition on colonization of the spleen and liver by Y. pseudotuberculosis strains. C57Bl/6J mice were infected with ~500 WT or yopDΔ207-227 Y. pseudotuberculosis via intraperitoneal injection. Mice were given plain drinking water or drinking water containing 100 µg ml⁻¹ acetovanillone for the remainder of the experiment. Organs were harvested four days post-inoculation and CFU per gram tissue determined. Data from three to four independent experiments are shown. Bars indicate the geometric mean; open diamonds indicate that CFU were below the limit of detection. The p values shown were determined using the Wilcoxon-Mann-Whitney non-parametric test.
Chapter 3 - Figure 6
Figure 7. Host response to *Y. pseudotuberculosis* strains in an effectorless background. (A) Immortalized C57Bl/6J BMDMs were infected with different *Y. pseudotuberculosis* strains in an effectorless background at an MOI of 15: Δ6(Δ), Δ6/ΔyopB (○), Δ6/yopDΔ207-227(●), or Δ6/yopDΔ150-170(▲). At various times post-inoculation, supernatants were analyzed for lactate dehydrogenase (LDH). One representative experiment is shown. The amount of LDH released from freeze-thaw lysis of the cell monolayer was set at 100%. (B) MyD88−/−/Trif−/− macrophages were infected with *Y. pseudotuberculosis* strains lacking the 6 known effector proteins at an MOI of 15. egr1 mRNA levels were quantified two hours post-inoculation. The average of three independent experiments ± SEM is shown. (C) MyD88−/−/Trif−/− macrophages were infected with *Y. pseudotuberculosis* strains lacking the 6 known effector proteins at an MOI of 15. IL-1β protein levels in the supernatant were quantified at four hours post-inoculation by ELISA. One representative experiment ± SEM is shown.
Chapter 3 – Figure 7
Supplementary Figure 1

Figure S1. Inhibition of reactive oxygen species production in BMDMs by Y. pseudotuberculosis. (A-B) Primary (bone marrow derived macrophages) BMDMs were incubated with luminol and horseradish peroxidase 0.5-1hr prior to infection. BMDMs were treated with zymosan to induce ROS production and were infected simultaneously with the indicated Y. pseudotuberculosis strain at MOI 15. ROS production was monitored by a plate reader for 50 minutes. PMA plus WT Y. pseudotuberculosis (▼), yopDΔ150-170 (●), or yopDΔ207-227 (○), PMA alone (▲), or uninfected and untreated (■). (A) One representative experiment is shown. (B) The average of two independent experiments at 50 minutes post-infection ± SEM is shown.
Chapter 4 – Conclusions and Future Directions

The aim of this dissertation was to enhance our fundamental understanding of host-pathogens interactions. To this end, two important questions were addressed: 1) how do host cells detect invading pathogens and 2) what is the role of specific virulence factors in bacterial pathogenesis. Before answering those questions explicitly, it was important to first provide the appropriate background information so that these questions could be put in the proper perspective.

Establishing this initial framework was the primary purpose of Chapter 1. Topics included the role of T3SSs in host-pathogen interactions, the dissection of individual components and effectors of Yersinia T3SSs, and the regulation and assembly of these injectisomes. Special emphasis was placed on the translocator protein YopD as the research from Chapter 2 and Chapter 3 both stem from different YopD mutants. Chapter 1 concluded with a brief summary of the different mechanisms that host cells use to sense T3SSs, which highlighted several important innate immune surveillance pathways.

Chapter 2 addressed the first key question posited in the introduction: How do host cells detect invading pathogens? Our findings support a translocation model of T3SS sensing, as mutants that are pore formation competent but translocation deficient do not induce a significant immune response. These findings do not rule out the possibility that the presence of a WT YopBD pore is required for T3SS sensing, as the YopD mutant we tested most extensively, \( \Delta yop6/\Delta yopN/yopD_{\text{ATM}} \), formed
slightly smaller pores and at a slower rate than do YopD-proficient strains. Other models have also been proposed to address how the host is able to sense the presence of T3SSs. One competing hypothesis to the translocation model is based on T3SS dependent pore formation. Some research indicates that host cells are able to sense the formation of a pore through subsequent $K^+$ efflux, which leads to the induction of an immune response. These contrasting observations could reflect subtle differences in host surveillance pathways that may be activated in a pathogen specific manner.

In line with our findings, several groups have recently identified different T3SS cargo that are recognized by host cell surveillance pathways and that induce a pro-inflammatory immune response. These cargo include flagellin, the T3SS inner rod protein, and the T3SS needle subunit, all of which induce caspase-1 dependent activation when delivered inside mammalian cells (Miao et al., 2006; Rayamajhi et al., 2013; Yang et al., 2013). We are actively investigating these recently characterized bacterial ligands to determine if they are responsible for the pro-inflammatory immune response we observe during infection with our $\Delta yop6/yopD_{\Delta 150-170}$, and $\Delta yop6/yopD_{\Delta 207-227}$ mutants. This research could help to better characterize established host surveillance pathways or lead to the identification of novel host mechanisms for T3SS sensing.

Chapter 3 fits loosely under the second key question posited in the introduction: What is the role of specific virulence factors in bacterial pathogenesis? While the role of the T3SS in bacterial pathogenesis is well established, this chapter
focused on a more specific question regarding the central region of the translocator protein YopD. The central region of YopD is relatively uncharacterized, and I set out to understand how this region contributes to *Yersinia* virulence by performing a detailed functional analysis.

I found that two *Yersinia* mutants lacking portions of this central region (*yopD*<sub>Δ150-170</sub> and *yopD*<sub>Δ207-227</sub>) mutants exhibited dramatic virulence defects in a mouse model of infection, indicating that this region is important for *Yersinia* pathogenesis. Furthermore, this central region is required for the optimal translocation of T3SS cargo into phagocytes. Specifically, I demonstrated that both of the *yopD*<sub>Δ150-170</sub> and *yopD*<sub>Δ207-227</sub> mutants translocated significantly less YopE-Bla into neutrophils and macrophages relative to WT. This decrease in YopE-Bla translocation by the *yopD*<sub>Δ150-170</sub> and *yopD*<sub>Δ207-227</sub> mutants correlated with their impaired ability to inhibit the production of ROS in professional phagocytes. Taken together, these findings suggest that host cells mount a more robust immune response against *Yersinia* mutants lacking this central domain, with consequent dramatic attenuation of virulence *in vivo*.

Further characterization of the *yopD*<sub>Δ150-170</sub> and *yopD*<sub>Δ207-227</sub> mutants could offer important contributions to the underlying mechanisms that mediate their virulence attenuation *in vivo*. While we provide evidence suggesting that ROS production is partially responsible for this defect, a number of other immune responses could also play a factor. It is also important to think about the pathogenesis
of a *Yersinia* infection and the subsequent host response in a tissue specific context. The $yopD_{\Delta 150-170}$ and $yopD_{\Delta 207-227}$ mutants exhibit significant virulence defects in both the spleen and liver. However the factors that mediate these defects do not necessarily have to be the same for both tissues. Ascertaining cell recruitment and cytokine production in the spleen and the liver in response to the $yopD_{\Delta 150-170}$ and $yopD_{\Delta 207-227}$ mutants may provide important insights on the specific requirements for *Yersinia* pathogenesis as well as the deficits that these *Yersinia* mutants exhibit. It is also important to remember that the mechanisms responsible for the $yopD_{\Delta 150-170}$ and $yopD_{\Delta 207-227}$ mutants themselves may be different for each mutant. This is most evident in the different *in vitro* ROS assays discussed in Chapter 3, Figure 5. These experiments reveal that the $yopD_{\Delta 150-170}$ and $yopD_{\Delta 207-227}$ mutants differ from one another in their ability to inhibit ROS production, depending on when ROS production is induced.

Future experiments will aim to understand if the central region of YopD is involved with other *Yersinia* components that modulate the translocation of T3SS cargo, including YopK and YopN. These studies will include a more in depth characterization of YopD binding interactions with other Yops and of the role of the central region of YopD in translocon assembly. These experiments will inform our understanding of the underlying mechanisms responsible for the translocation of T3SS cargo at the pore complex. Additionally, they may reveal new insights into YopD, YopK and YopN functions as individual components as well as in concert with one another.
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