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Inhibition Of The Ysc Type III Secretion System Of Yersinia Pestis By Compound D And Interaction Of Type III Secretion System Needle Proteins With Host Receptors

Danielle L. Jessen

INHIBITION OF THE YSC TYPE III SECRETION SYSTEM OF *YERSINIA PESTIS* BY COMPOUND D AND INTERACTION OF TYPE III SECRETION SYSTEM NEEDLE PROTEINS WITH HOST RECEPTORS

by

Danielle Louise Jessen

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This dissertation, submitted by Danielle Jessen in partial fulfillment of the requirements for the Degree of Doctor of Philosophy from the University of North Dakota, has been read by the Faculty Advisory Committee under whom the work has been done and is hereby approved.

Matthew L. Nilles, Chairperson

David S. Bradley

Catherine A. Brissette

Othman Ghribi

Katherine A. Sukalski

This dissertation is being submitted by the appointed advisory committee as having met all of the requirements of the Graduate School at the University of North Dakota and is hereby approved.

Dr. Wayne Swisher,
Dean of the Graduate School

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ABSTRACT

The type III secretion system of gram-negative bacterial pathogens is a major virulence factor and functions to modulate host immune responses. Immune modulation occurs in many ways, including direct injection of effector proteins or indirect methods such as the detection of bacterial components by host immune receptors. Knowledge of these immune modulations allows for development of treatment options in an ever-increasing antibiotic-resistance climate. The studies presented here explore both areas of immune modulation. We identify Compound D as a potent inhibitor of the type III secretion system of *Yersinia pestis*. Through evaluation of effector secretion by bacteria grown in the presence of Compound D, we establish that inhibition of secretion occurs through translocon protein YopD and is also affected by LcrQ and YopD's chaperone, LcrH. Type III Secretion inhibition by Compound D also requires a secretion active state of the type III secretion system as determined by analysis of strains that constitutively secrete effectors. The other study focuses on host recognition of bacterial proteins, specifically the needle protein of type III secretion systems. Via utilization of cells that secrete a measurable signal protein when NF-κB or AP-1 is activated, we show that needle proteins from *Yersinia pestis, Salmonella enterica* serovar Typhimurium, and *Shigella flexneri* are capable of activating cells through Toll-like receptors 2 and 4. This interaction appears to be modulated by

the N-terminus, that is reported to reside on the outside of the fully formed needle structure, exposed to host receptors. Activation of NF-κB/AP-1 correlates with production of TNF-α in response to needle proteins.

CHAPTER I

INTRODUCTION

Type III Secretion Systems

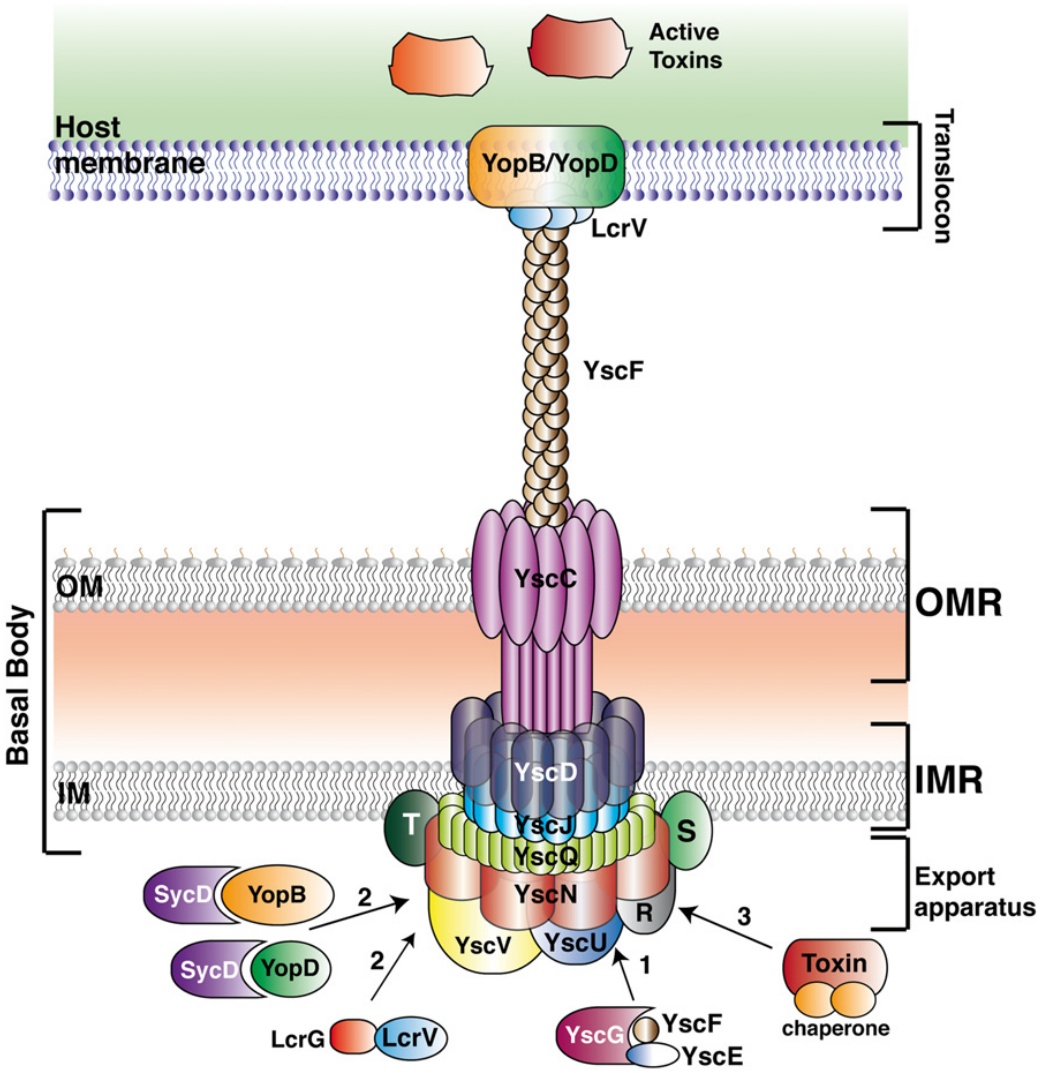
In order to manipulate the host, gram-negative bacteria utilize a number of features. One of these essential virulence factors is the type three-secretion system (T3SS). T3S systems are important in several known symbiotic relationships, demonstrating a duality of T3S functions ranging from beneficial to detrimental manipulation of eukaryotic cells (39, 87). T3SSs are found in many human pathogenic gram- negative bacteria including pathogenic strains of *Escherichia coli*, *Shigella*, *Salmonella*, *Yersinia,* and *Pseudomonas* (24, 71). T3SSs are divided into seven families based on sequence similarities. T3SSs from animal pathogens fall into three of those families: Ysc-type injectisomes, SPI-1-type injectisomes, or SPI-2-type injectisomes. Although much of the basal structures of these systems are homologous, the secreted effectors and regulation of secretion vary between each family. Ysc injectisomes are primarily found in *Yersinia* species, *P. aeruginosa*, *Vibrio*, and *Bordetella pertussis*. SPI-1 injectisomes are commonly associated with *Shigella* and *Salmonella*. SPI-2 injectisomes are associated with enterohemorraghic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC), and *Salmonella*. Many structural proteins of the

T3SS are homologous between these families; those proteins that are not homologous often still have an analogous protein with an equivalent function (24).

Structure

The T3SS is comprised of approximately 25 different proteins that make up the basal body, needle, and translocon (24). The basal body embeds in the inner and outer bacterial membrane via two ring-like structures connected by a rod structure (Figure 1) (71). The basal structural components are largely conserved between T3SSs, including bacterial flagella (24). On the cytosolic side of the basal structure an ATPase can be found that is critical for secretion of proteins (24). The internal channel of the T3SS is about 2-3 nm, only big enough for unfolded proteins to pass through (24). The number of needle complexes per bacteria varies, from 10-100 complexes, depending on the species (42).

Extending out from the basal structure is a hollow needle (24). This portion of the secretion system is made up of repeating subunits of one protein and a cap protein that sits at the tip (73). The sequence of needle proteins is largely conserved between bacterial species, except the N-terminus. X-ray crystallography and NMR have been utilized to detect structures of some needle



	Basal body		
Yersinia	YscC	YscD	YscJ
Pseudomonas	PscC	PscD	PscJ
Shigella	MxiD	MxiG	MxiJ
Salmonella	InvG	PrgH	PrgK
E. coli	EscC	EscD	EscJ

	Export apparatus						
	YscQ	YscN	YscR	YscS	YscT	YscU	YscV
	PscQ	PscN	PscR	PscS	PscT	PscU	PcrD
	Spa33	Spa47	Spa24	Spa9	Spa29	Spa40	MxiA
	SpaO	InvC	SpaP	SpaQ	SpaR	SpaS	InvA
	SepQ	EscN	EscR	EscS	EscT	EscU	EscV

	Translocon			Needle	Chaperones			
Yersinia	YopB	YopD	LcrV	YscF	SycD	LcrG	YscG	YscE
Pseudomonas	PopB	PopD	PcrV	PscF	PcrH	PcrG	PscG	PscE
Shigella	IpaB	IpaC	IpaD	MxiH	IpgC	n.i.	n.i.	n.i.
Salmonella	SipB	SipC	SipD	PrgI	SicA	n.i.	n.i.	n.i.
E. coli	EspD	EspB	EspA	EscF	CesD	n.i.	n.i.	n.i.

Figure 1. Type III secretion system structure. (Used with Permission) (50)

proteins, including MxiH from *Shigella (27),* BsaL from *Burkholderia pseudomallei* (108), and PrgI from *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) (108). The crystal structure of MxiH was used to generate a model of the T3S needle structure (7, 22, 27). The MxiH-derived model of the needle protein possesses two coiled domains with the N-terminus of the needle protein predicted to line the lumen of the T3S needle (27). The N-terminus of the needle protein in all these cases was seen to be highly mobile and disordered (8, 108) offering little data to define structures of this portion of the protein. Sun et al. reported the N-terminus in their crystal structure to be largely unorganized and not representative of the protein in its needle conformation (98). Contrary to previous models, recent work by Loquet et al. has revealed that the N-terminus of the needle protein from *Shigella* is, in fact, on the outside surface of the needle, exposing it to host elements, while the conserved carboxy end faces the lumen (63).

How needle length is determined is hypothesized by several models. Models suggest a ruler method where a specific protein dictates the length of the needle, a cup method were a specific number of needle proteins are released to create the needle, or others suggest a combination of these two models with the proteins that dictate substrate switching also involved in determining needle length (24). Length of the needle depends on the species of bacteria and studies have shown that this length is critical in the ability of the bacteria to deliver effectors to the host (24). Length of the needle is correlated with the length of major features on the outer surface of the bacteria such as adhesins (72). At the tip of the needle is a protein

that ‘caps’ the apparatus and interacts with the final portion of the structure that imbeds in the host membrane (73).

The translocon completes the T3SS. This structure is made up of two hydrophobic proteins that insert into the host membrane, thus creating a channel directly from bacteria cytosol into the host cytosol. Through this channel unfolded proteins can move from the bacteria into the targeted host cell. Some bacterial species show that these proteins make up the cap structure as well; however this has not been shown true with all T3SSs (71, 73).

Effectors

Effector molecules can mediate several functions including but not limited to bacterial uptake, alterations of the immune response, or prevention of phagocytosis (Figure 2) (39). There are hundreds of different types of effectors across all T3SS (28). These proteins mimic host cell protein function in order to irreversibly control specific functions (39). The majority of these proteins carry a N-terminal secretion signal (23) as well as a chaperone-binding domain to allow targeting to the T3SS for export (28).

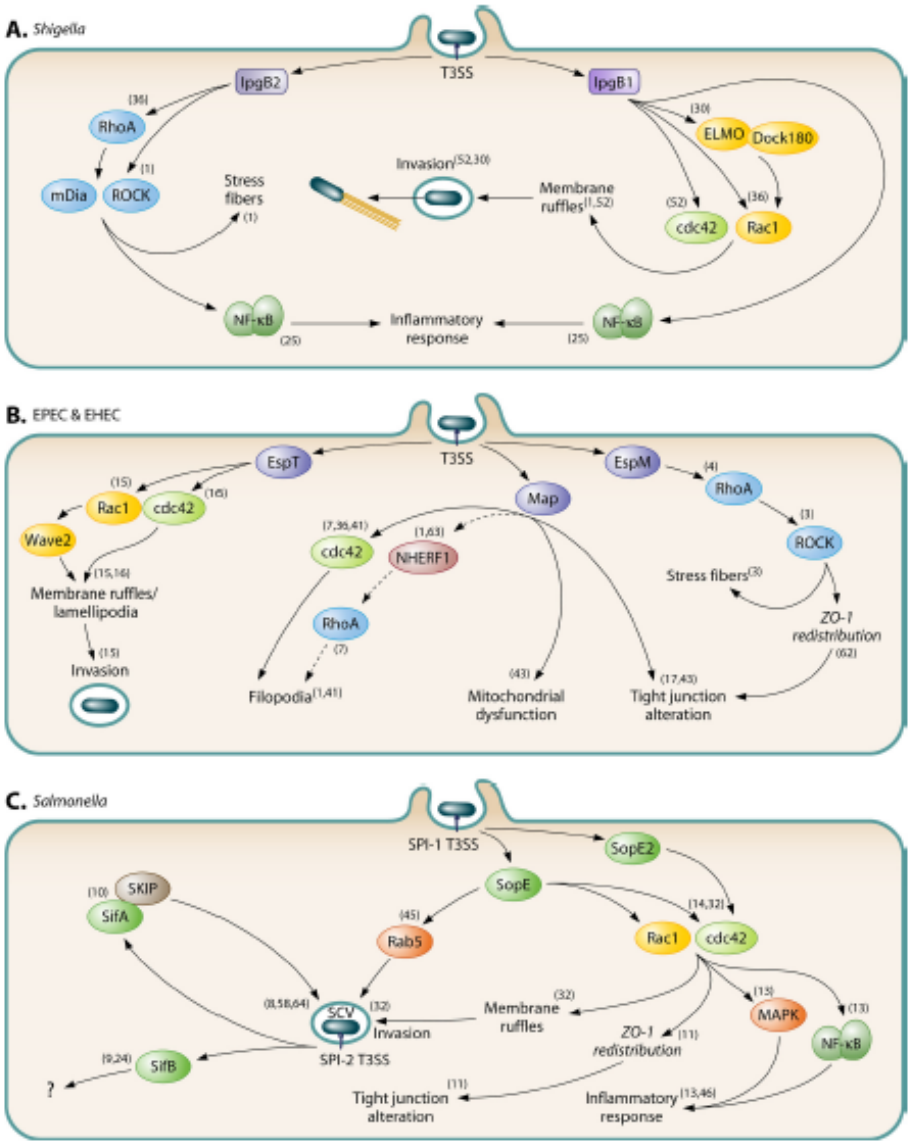


Figure 2. Cellular effects of T3SS on *Shigella,* EPEC and EHEC, and *Salmonella*. (Used with permission) (16). SopE-like and WxxxE bacterial effectors subvert host cell pathways. Using the T3SS, *Shigella* (A), EPEC/EHEC (B), and *Salmonella* (C) inject the effectors IpgB1/IpgB2, EspT/Map/EspM, and SopE/SopE2/SifA/SifB, respectively. Except for SifA and SifB, these effectors activate a cascade of signal transduction pathways, starting with activation of Rho GTPases (either Rac1, RhoA, or Cdc42), which leads to active polymerization. Membrane ruffles induced by IpgB1, EspT, SopE, and SopE2 allow bacterial engulfment and subsequent internalization into a bacterium-containing vacuole (BCV). Invasive EPEC and *Salmonella* remain in the BCV and induce the formation of intracellular actin comets and *Salmonella*-induced filaments (Sifs), respectively, while *Shigella* escapes to the cytosol, where it forms actin tails. In parallel to subversion of actin dynamics, IpgB1, IpgB2, SopE, and SopE2 induce inflammatory response, EspM, Map, SopE, and SopE2 induce tight junction alteration, and Map induces mitochondrial dysfunction. SopE and SifA, which is translocated across the SCF via the SPI-2 T3SS, play a role in maintaining the SCF. The activity of SifB remains unknown.

Regulation

Regulation of this system is crucial for delivery of effectors at the precise time needed. Most agree that host cell contact is crucial for activation; however, how this happens and through which proteins is a major debate in this field (8, 24, 50, 71, 73). Many proteins function to regulate secretion, though the particular protein and function can vary between different bacterial species. Overall, however, current theories hypothesize the importance of the needle as a regulatory element (32). In vivo, contact with the host cell membrane is required to initiate translocation of effectors (85). One hypothesis of regulation via the needle is that the signal is structurally relayed via conformational changes of the needle from the tip to the base. Another hypothesis, separate from needle protein structure, involves a protofilament, that once released, signals secretion (8). Several mutants of needle proteins have been produced that alter the regulatory control of secretion; (55, 92, 102) however, an exact mechanism has not been confirmed by analysis of these mutants.

Bacteria

Yersinia pestis

History of Plague

Yersinia pestis was separately discovered in 1894 by Alexandre Yersin and Kitasato Shibasaburō as the causative agent of plague (84). Recognized as causing the same disease long know to human cultures across the Old World as "The Plague", this bacteria became the focus of countless scientists bent on understanding its power to kill.

Previous to identification, *Y. pestis* caused three pandemics across the Old World: The Justinian Plague, The Black Death, and The Third Pandemic. The Justinian plague was predominantly bubonic and spread via trade routes from Asia to the Byzantium Empire. The Black Death ravaged the human populations from China to Europe between the 14th and 19th centuries. All three forms of the disease (bubonic, pneumonic, and septicemic) appeared during this pandemic, at times decimating the population by half in major cities. This second pandemic was so widespread and destructive that it reshaped economies, science and medicine, art, religion, and society as a whole (60). The Third Pandemic, predominantly in China, led to the identification of *Y. pestis*, the identification of the bacteria's reservoir: rodent fleas, and the method of spread

which directed development of control policies for health authorities (60, 84). This pandemic also brought plague to the United States via shipping routes from Asia (84).

Currently *Yersinia pestis* is listed as a category A biological weapon by the CDC, along with Anthrax, Smallpox, and Tularemia. Category A bacteria are identified as being easily transmitted from person to person, result in high mortality, can cause public panic, and require special action for public health preparedness (CDC). Historically *Yersinia pestis* has been used as a biological weapon, by Japan on China. Russia during the Cold war reportedly developed strains that would be effective terrorism agents; however, these strains were ordered for destruction (60).

Natural Reservoirs of *Yersinia pestis*

Yersinia pestis maintains existence in nature by transmission between susceptible rodents and their fleas, with occasional infection of incidental hosts, such as humans (84). On a whole the number of human cases worldwide result in 1,000 to 3,000 deaths per year, with most of those cases occurring in Africa (Figure 3). The last epidemic of plague was reported in India in 1994 (18). Only 52-100 people died, with mass numbers of antibiotics distributed immediately and quarantines put into place to limit the spread of disease (18).

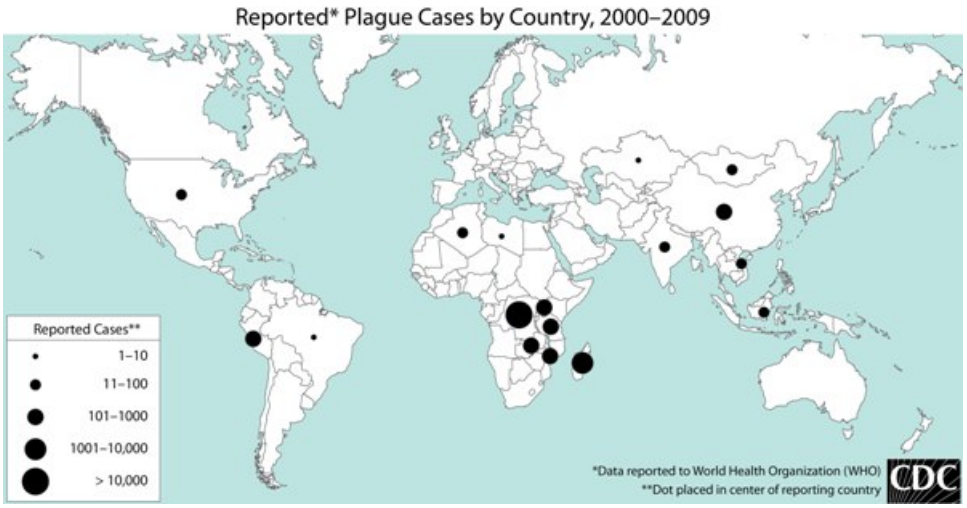


Figure 3. Reported Plague cases by country 2000-2009 (CDC).

Once brought to the United States the bacteria infected the local rodent population (84). Since then, the bacteria has spread across half of the country taking up a natural residency in the fleas of rodents such as rock squirrels, the California ground squirrel, and prairie dogs (18). Human cases are rare (1-15 per year) and only occur in the instance of a human handling or in close contact with fleas of an infected rodent or direct contact with an infected rodent or feline (38). Most of these cases are in the southwestern and western areas of the United States (Figure 4).

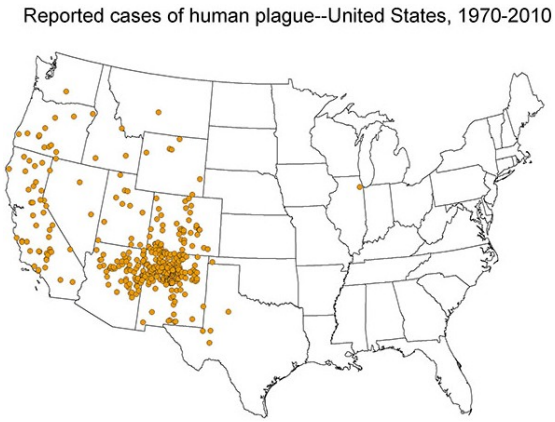


Figure 4. Reported cases of human plague – United States, 1970-2010 (CDC).

Biovars

Four known biovars of *Yersinia pestis* exist: *medievalis*, *antigua*, and *orientalis* and the non-human pathogenic biovar *microtus* (38, 62, 84, 90). These biovars are distinguished from each other by their ability to acidify glycerol and reduce nitrates (38, 84). The biovars were named in accordance to the three pandemics, however recent analysis of teeth from plague victims links the biovar *orientalis* with all three pandemics (90). *Y. pestis* strains used for the experiments of this manuscript are derived from the KIM (Kurdistan Iranian Man) strain and they are of the biovar *medievalis.*

Transmission of *Yersinia pestis*

Historically the flea *Xenopsylla cheopis,* commonly found in association with rodents, is the major vector of *Yersinia pestis (18, 38, 48, 84, 90)*. *Y. pestis* infects fleas by forming a biofilm in the flea's midgut (48). The biofilm blocks blood from getting to the flea's stomach, leaving the flea hungry and also creating a regurgitation of blood into the host (48). As the flea feeds on its host, the blood hits the biofilm block picking up bacteria and is regurgitated back into the host, thus spreading the bacteria to the host (48, 84). This host could be a rodent, in most cases, or if the opportunity presents itself, a human or other mammal (38). The still hungry flea will then move on to another site on the same host, creating multiple sites of infection with the bacteria, or move on to an additional host, continuing to spread bacteria with each bite (48). Uninfected fleas collect *Y.*

pestis by feeding on an infected individual (84). The nature of *Y. pestis* is to create a high bacteremia capable of spreading the bacteria back to uninfected fleas via a blood meal (38, 48). This high bacteremia not only returns the bacteria into the vector but also kills the host forcing the vector to move to a new warm body, thus spreading the bacteria to a new host (48, 84).

Disease

Yersinia pestis causes several forms of disease depending upon the route of entry. The bubonic form of disease is most commonly recognized by the presence of buboes, i.e. swelling of the lymph nodes with a discoloring of the enlarged area (84). Other symptoms are characteristically "flu-like": high fever, low blood pressure, chills, fatigue, cough, chest pain, and dyspnea (90). Incubation time of bubonic plague is 2-6 days. The route of entry is commonly an infected fleabite with bacteria moving to a nearby lymph node via infected macrophages and then spreading via blood to the liver and spleen (2, 88). In the blood *Y. pestis* replicates to high levels causing bacteremia. High levels, $>10^7$ CFUs/ml of blood, of bacteria are critical for reinfection of the flea vector (48). These high bacterial levels however, can lead to septicemic or pneumonic plague typically resulting in sepsis and death (90). Septicemic plague has similar flu like characteristics with the addition of organ damage and shock (84).

Pneumonic plague is an infection initiated in the lung by inhaling bacteria or develops secondary to other infections that have spread to the lung (84). In

rare cases, pneumonic plague can be transmitted person to person via coughing droplets of sputum containing bacteria. Incubation time is 1-3 days with a higher incidence of death then the bubonic form. Symptoms are pneumonia-like with bloody sputum (90).

Treatment

Upon identification of the disease, the infected individual is isolated and treatment consists of commonly used broad-spectrum antibiotics (e.g. tetracycline, streptomycin, or gentamycin) (84). Historically, anyone who encountered the infected individual would be isolated and any fabric used by the infected individual would be burned to destroy infected fleas (60).

Vaccines

The CDC offers three routes to control plague: environmental management, public health education, and preventive drug therapy. Environmental management can be controlling the local rodent population. Public health education includes teaching people in plague areas how to limit rodent populations near their living areas and maintaining flea treatment of pets (84). Preventive drug therapy is given to an individual who has potentially been exposed to plague bacteria. Currently there is no approved vaccine for plague in the United States. Historically, two vaccines were developed and used. One

involved an attenuated live strain that lacked the pigmentation-locus (Pgm) and the other was a fully virulent killed strain (84). However, both of these vaccines had major problems with adverse reactions, no development of long-term immunity, and they were not able to prevent pneumonic plague (84, 91). Current vaccine research focuses on development of protein based vaccines involving the V antigen and the F1 capsule (91).

The Bacteria

Yersinia pestis is a gram negative bacillus that is part of the *Enterobacteriaceae* family. The genus *Yersinia* contains eleven species of bacteria. Three of these are deemed important for human infection. *Yersinia pestis* causes plague. *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* are both enteropathogenic species (84). Y. *enterocolitica* being the more common infectious agent of those two, but *Yersinia pseudotuberculosis* is more closely related to *Yersinia pestis* (15). These three species of bacteria share the T3SS encoding plasmid, called pCD1 *in Y. pestis*. The extra plasmids *Y. pestis* acquired (pPCP1 and pMT1), and loss of several genes created the difference between an intestinal disease and the terribly lethal plague (15).

Virulence Factors

Yersinia pestis employs many factors to cause disease; primarily, these factors are critical for evading detection or suppressing the immune system of the host. Before entering the host, the bacteria is often found in a flea where several genes, specifically acquired by *Y. pestis* as opposed to the other Yersinia species, are required. Expressed from pMT1, *Yersinia* murine toxin (Ymt), is part of the phospholipase D family of proteins and functions in blockage of the flea gut. Ymt is toxic to mice and causes circulatory failure; however, the protein is not highly expressed in a bacterial infection of the mouse and plays no known role in infection of the mammalian host (48). The *pgm* locus contains the hemin storage locus (*hms)*. These genes are functional at 28°C (temperature of the flea vector) and are critical for formation of the biofilm that creates the periventricular blockage in the midgut of the flea (48). Also encoded from the *pgm* locus is the yersiniabactin (Ybt) system that functions in iron uptake. This system is crucial for survival of the bacteria in low free iron environments (84).

As *Y. pestis* moves into the human host, the plasminogen activator protein (Pla) is utilized by the bacteria. This surface protease is expressed from pPCP1 and is responsible for dissemination of the bacteria from the site of fleabites (48, 84). Once disseminated into the host, other bacterial genes become active. These genes are activated by temperature sensitive regulation elements. Once in the host the temperature shifts from ambient temperature in the flea to the body temperature of a mammalian host (84). At this temperature *Yersinia pestis* makes a tetra-acylated LPS, instead of a hexa-acylated LPS. Tetra-acylated LPS

is non stimulatory to Toll-Like Receptor (TLR) 4 and actually antagonizes hexacylated LPS, consequently reducing innate immune cell activation, cytokine expression, and maturation of dendritic cells (2). The fraction 1 (F1) capsule, also only expressed at 37°C from pMT1, forms a gel-like layer around the bacteria and plays a major role in preventing phagocytosis (84). *Yersinia pestis* can survive and replicate in the macrophage, which appears to be critical in movement through the lymphatic system to the lymph node (2). Known factors involved in survival in the macrophage are PhoP/PhoQ and a gene located in the Pgm locus (88). Also at 37°C, the LcrF protein is produced. LcrF is responsible for the temperature-dependent activation of genes on pCD1 that encodes the type III secretion system (T3SS) (23). The LcrF transcript has a unique RNA thermosensor, which once shifted to above 30°C allows for translation to occur (10). The T3SS in *Yersinia pestis* then plays a key role in prevention of phagocytosis, manipulation of cytokine expression, and killing of immune cells (2). The details of this system are explored separately below.

The attenuated strain of *Yersinia pestis* used in these studies is the KIM8 strain. This particular strain has a spontaneous deletion of the Pgm locus that lowers the risk of working with this deadly pathogen. The pPCP1 plasmid has also been removed to efficiently detect secreted Yops. Pla effectively degrades secreted proteins (84).

T3SS

The structure of the T3SS is made up of a basal structure, the needle, and the translocon. In *Yersinia pestis* this system is encoded by the pCD1 plasmid. Also on this plasmid are effectors, chaperones, and regulatory proteins that are necessary for expression, construction, and expression of the T3S. Without the T3SS *Yersinia pestis* becomes avirulent and is easily cleared by the host immune system (23).

Structure

The base of the T3SS of *Yersinia pestis* is made up of proteins termed Ysc (Yop secretion) (Figure 1) (84). The structure is built in the outer membrane first, made up of YscC, then proceeds to building the inner ring via YscD and YscJ (30). YscQ reportedly makes up the C-ring on the cytosolic face of the basal structure (30). YscQ then interacts with the ATPase, YscN, and subsequently YscN requires YscK and YscL (51). Also essential are integral membrane proteins YscR, YscS, YscT, YscU and YscV that are thought to recognize or secrete the Ysc substrates (96).

Extending out from the base is a hollow needle structure, made up of repeating subunits of YscF. Currently, YscF has only been crystalized in complex with its chaperones YscE and YscG (99). The pore forming structure at the end of the needle is called the translocon (12, 67, 73). This structure is made up of three proteins: LcrV, YopB, and YopD (73). LcrV creates a base on the tip of the YscF proteins that make up the needle (24) and functions to help insert the

hydrophobic translocator proteins, YopB and YopD, into the host membrane (73). YopB and YopD then create a pore and allow Yops to translocate from the needle apparatus into the host cell (73). In *Yersinia* there is no evidence for the order or timing of secretion to assemble the translocon. It is presumed that due to the hydrophobic nature of YopB and YopD, these proteins are not assembled at the tip prior to cell contact (74). The translocon as a whole has yet to be isolated and visualized to confirm this assumption (73). This is contrary to the T3S system in *Shigella* where the T3S assembles its major hydrophobic translocator before cell contact (107). In secretion profiles of *Yersinia pestis,* in vitro, all three proteins are secreted into the medium.

Effectors

Effector proteins are the toxins of the T3SS. These proteins, termed Yops (Yersinia outer proteins) are translocated into the host cell and damage host responses (Figure 5) (23). Yops have a N-terminal secretion signal (39) and are translocated in an unfolded state (23).

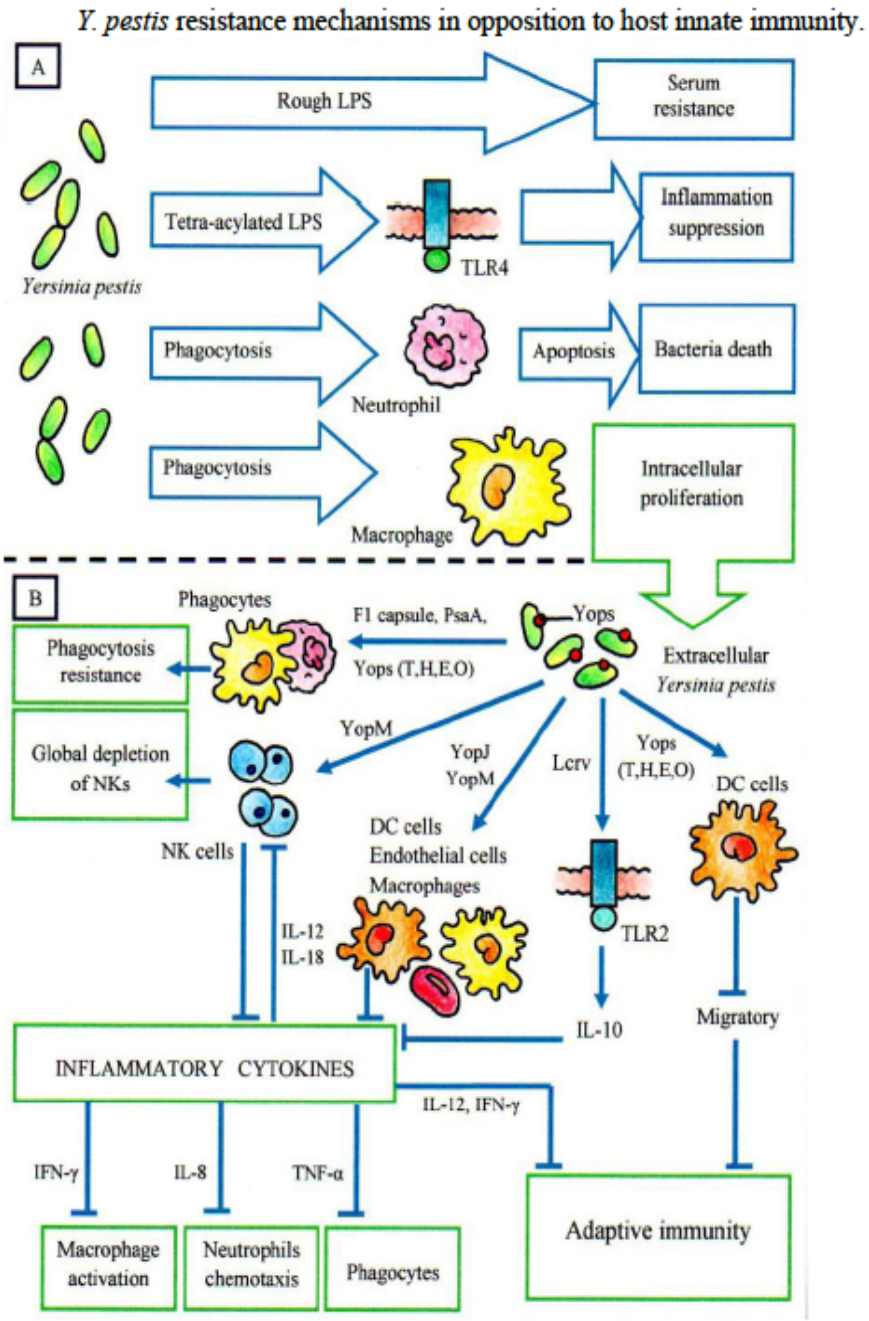


Figure 5. *Y. pestis* resistance mechanisms in opposition to host innate immunity. (Used with permission) (2). (A) Resistance mechanisms at the early stage of infection. The LPS structure varieties of *Y. pestis* during transition between flea and host temperatures make the bacteria resistant to the serum-mediated lysis and repress the proinflammatory response. In the meantime, the bacteria phagocytosed by macrophages can grow and express different virulence determinants to act on host immune response. (B) Resistance mechanisms after the release of *Y. pestis* from macrophages. The bacteria released from macrophages attain the capacity to resist phagocytosis and can inhibit the production of proinflammatory cytokines, which also attenuate the host's adaptive immunity.

YopE, YopT, and YpkA (YopO in *Y. enterocolitica*) are involved in preventing phagocytosis by interfering with Rho-GTPases. YopH is a tyrosine phosphatase that also inhibits phagocytosis. YopH is also involved in suppressing reactive oxygen species as well as disrupting adhesion proteins. YopM and YopJ inhibit proinflammatory signaling. YopM has also been implicated in a global reduction of NK cells. Neutrophil chemotaxis is inhibited by YopJ (2, 23). YopJ also induces apoptosis of macrophages (59). YopK is known to regulate the pore size from the host side of the translocon and controls the rate Yops can enter host cells (29).

Chaperones

Also critical in the regulatory function of the T3SS are chaperone proteins that are hypothesized to assist in timing and movement of proteins to the secretion apparatus and/or maintain the protein in an unfolded shape or inactive state (24). Chaperones are divided into three classes: Class III chaperones maintain the subunits of the basal structure. Class II chaperones care for the hydrophobic translocon proteins (24). Specifically in *Yersinia pestis*, LcrH/SycD is the chaperone protein for YopD and YopB (76). Along with its chaperone duties LcrH along with YopD have been implicated in post-transcriptional regulation of Yop expression (37, 109). Class I chaperones maintain the effector proteins, although some effectors appear to require no chaperone, e.g. YopM (24).

Regulation of Secretion

Regulation of the T3SS is a complex process. Under in vivo conditions cell contact is known to trigger secretion in this system (27). How that signal is relayed to the inside of the bacteria is not known, although one theory suggests a conformational change occurs in structural proteins that brings the message to appropriate regulatory cytoplasmic molecules (8). Under in vitro conditions, the *Yersinia pestis* T3S and the *Pseudomonas aeruginosa* T3S can be triggered by depleting the media of calcium (11). This response is known as the Low Calcium Response (LCR). Several proteins are involved in the regulation process of secretion from inside the bacteria. LcrG blocks secretion that can be alleviated by interaction with LcrV (44, 66, 77, 95). YopN and YopN's chaperones SycB and SycN, along with TyeA, form a complex that also regulates secretion of Yops (26, 36). YopN regulation is thought to be alleviated by secretion of YopN (44). Deletion of these regulatory proteins results in an altered ability to secrete Yops. Either secretion will not occur, such as in the case of deletion of LcrV (6), these strains are referred to as being calcium independent; or the opposite effect can occur where secretion will occur constitutively resulting in Yops secretion, for example a strain lacking LcrG (95) or YopN (36). These strains are called calcium blind strains. An additional factor that occurs in vitro when secretion is triggered is a twofold event involving a transcriptional increase in Yops expression and an overall growth restriction of the bacteria (23).

Host Response

Once *Yersinia pestis* has entered the host, via fleabite, the host's immune system, likely macrophages and neutrophils, responds to control the infection (Figure 5) (2). *Y. pestis* preferentially infects macrophages and is trafficked to the lymph node where it escapes from macrophages and begins replicating: disrupting the lymph node, the immune cell population, and possibly spreading to the blood stream. NK cells and neutrophils, however, have the capacity to kill the bacteria if they can traffic to the site of infection (2). Mast cells have also been implicated in resistance to plague although the mechanism is not known (80). At the site of the bubo, reactive nitrogen species are critical for combating the newly extracellular bacteria (93). The immune system also attempts to employ TLRs, the complement system, and the adaptive immune response. However, *Y. pestis* is very effective at evading these defenses and shutting down early innate immune system alarms (2).

Salmonella enterica

Salmonella enterica is a gram-negative pathogen that causes enteric disease in humans (13, 40). The bacteria are spread by ingestion of contaminated food, and infection causes diarrhea. There are several serovars of *enterica*: Typhi causes Typhoid fever in humans while Typhimurium causes a Typhoid like illness in mice (13). Once *Salmonella* has reached the intestine the bacteria attempts to move across the epithelium layer by invading M-cells (13). This is achieved by the

use of one of *Salmonella's* two T3SS, *Salmonella* Pathogenicity Island 1 (SPI-1) (13, 40). SPI-1 plays multiple roles in infection. Initially in infection SPI-1 effectors cause phagocytosis of the bacteria into epithelial cells and also cause an increase in inflammatory mediators and fluid movement into the intestine (Figure 2) (40). The inflammation caused by this system loosens tight junctions in the epithelial layer, which can allow more bacteria to pass into the lamina propria (13). SPI-1 is also capable of causing apoptosis of macrophages (40). However, it is also possible for *Salmonella* to survive in macrophages. This is accomplished with the other T3SS of this bacterium SPI-2. Once inside the *Salmonella* Containing Vacuole (SCV) SPI-2 effectors protect the bacteria from reactive oxygen and nitrogen species and orchestrate delivery of materials from the host cell to the SCV to facilitate bacteria growth (40).

SPI-1 and SPI-2 of *Salmonella* are found in two families of T3SS. The SPI-1 T3SS is more closely related to the T3SS found in *Shigella*, while SPI-2 resembles the *E. coli* T3SS (24). In our studies, we primarily utilized the needle proteins from both of these systems: PrgI from SPI-1 and SsaG from SPI-2. Of these two proteins only PrgI has been crystalized (25).

Shigella flexneri

Shigella is a genus of gram-negative bacteria of the *Enterobacteriacae* family. There are four species: *flexneri, sonnei, dysenteriae and boydii*. *Shigella flexneri* and *sonni* cause endemic forms of dysentery, while *Shigella dysenteriae* is associated with epidemics. These bacteria are spread by contamination of food or water and only infect humans. Symptoms associated with *Shigella* range from moderate to severe diarrhea and in more severe cases fever, abdominal cramps, and bloody mucoid stools. Death from this pathogen usually results from septic shock, severe dehydration, or acute renal failure (86).

Once inside the host *Shigella* targets the colon and moves past the epithelial layer via M-cells. After crossing the intestinal barrier the bacteria interacts with macrophages and dendritic cells. This interaction causes an increase in pro-inflammatory cytokines and chemokines. The increase in inflammation eventually leads to edema, erythema, abscess formation and musosal hemorrhages (86).

The role of the T3SS in Shigella plays out in invasion of epithelial cells and macrophages (Figure 2) (55). Effectors not only mediate uptake into the cell but also begin manipulating the immune response to favor high inflammation (86). Our studies relating to *Shigella's* T3SS focus on the needle protein, MxiH. MxiH has been crystalized and used to predict the needle structure (7, 27). Mutants of MxiH indicate that the needle protein plays a role in "sensing" host cell contact and the triggering of secretion (55).

Pseudomonas aeruginosa

Pseudomonas aeruginosa is also a gram-negative pathogen that infects humans. This pathogen is associated with several acute disease types ranging from pneumonia to infections of the urinary tract, wounds, burns, and bloodstream. Cystic fibrosis patients are keenly susceptible to *Pseudomonas* infections as well.

Like many gram-negative pathogens *Pseudomonas* also utilizes a T3SS to manipulate the host. Only four effectors of the T3SS of *Pseudomonas* exist: ExoS, ExoT, ExoU, and ExoY. These effectors are capable of preventing phagocytosis, altering cell trafficking, inhibiting cytokine release, and causing cell death (46). Ultimately *Pseudomonas'* goal is to evade innate immunity (92). The T3SS of *Pseudomonas* is closely related to the T3SS of *Yersinia* and in vitro is also activated by depletion of calcium in the environment (24). Studies by Broms et al. have revealed the ability of some *Yersinia* proteins to substitute for homologous *Pseudomonas* proteins; however, the reverse does not always work. YopD specifically can function in *Pseudomonas* however PopD, the *Pseudomonas* homolog, cannot substitute for YopD, specifically YopD's regulatory functions. This study also revealed the importance of translocon protein chaperones for proper function (14).

Small Molecule Inhibitors of T3S

The homogeneity of T3S systems between different pathogenic bacteria has led to the targeting of this system for new drug discovery. The critical role of T3S systems in disease, and the location of T3S systems on the outside of the bacteria make it a key feature to exploit to prevent disease (56). Screens for T3S inhibitors were designed to find compounds that prevented secretion of effectors but still allowed bacterial growth to decrease evolutionary pressures that can lead to resistance phenotypes (1, 45, 57, 79, 82, 100). The elimination of a major virulence factor gives the host immune system an edge over the bacteria, allowing the host to mount a successful immune response and form effective immunological memory.

Small molecule inhibitor screens identified compounds that inhibit functional activity of T3SSs, in the case of effector secretion (5, 45, 57, 79, 82, 106). These compounds must also inhibit at reasonable concentrations and have low cytotoxicity to be considered for animal use (82). Several compounds have been identified that prevent secretion and/or translocation (50); however, how the compounds accomplish this and many of the specific target proteins are unknown. Some studies were created to target specific proteins of the T3SS. YopH (33), LcrF (41), and YscN (100) have all been successful targets of such studies. The ability of these compounds to be effective in species other than *Yersinia* has yet to be established.

Our inhibitor studies utilize a small molecule inhibitor 4-4' thiobis (2-methylphenol) (Compound 2), as identified by Pan et al (83) and an isoform 2,2'-thiobis-(4-methylphenol) (Compound D). Compound 2 was found to be effective at inhibiting secretion of *Yersinia* as well as *Pseudomonas aeruginosa* although no target protein was identified (1, 83).

Host Factors and T3SS

Toll-like receptors are common innate immunity receptors on host cells (Figure 6). There are several types of receptors and each one traditionally identifies specific pathogen patterns: TLR1/TLR2 and TLR2/TLR6 work as heterodimers to recognize lipoproteins, TLR3 recognizes double stranded RNA, TLR4 works in conjunction with MD-2 and CD-14 to recognize LPS, TLR5 recognizes flagellin, TLR7 and TLR8 recognize single stranded RNA, and TLR9 interacts with unmethylated CpG (105). Once binding of the specific substrate occurs many adaptor proteins contribute to a signaling cascade that leads to activation of transcription factors, such as NF-kB (54). Activation of NF-kB leads to production of cytokines and chemokines that can prepare the host immune system to respond to the pathogen (54). There are two key adaptor proteins that TLRs utilize: MyD88 and TRIF (54). MyD88 is common to all TLRs except TLR3, which uses TRIF (105). TLR4 is unique that it can use either MyD88 or TRIF as its initial adaptor protein (54).

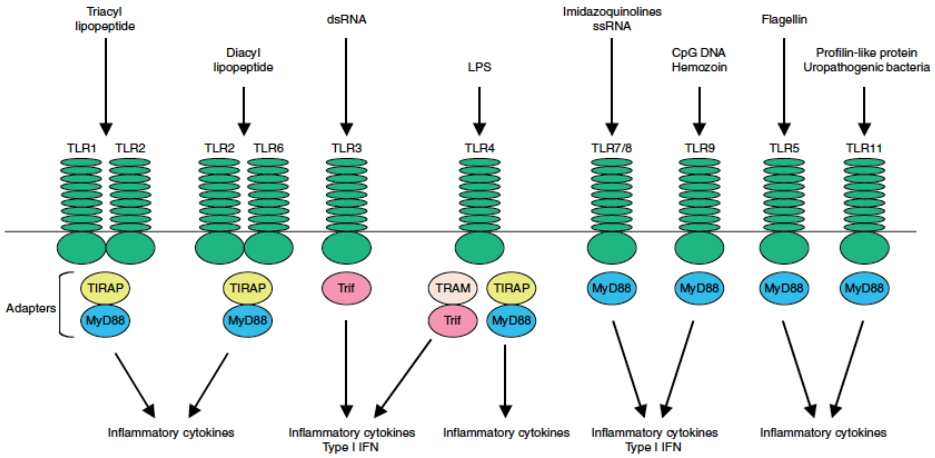


Figure 6. Toll-Like receptors (Used with permission) (54). TLR-mediated immune responses. TLR2 in concert with TLR1 or TLR6 discriminates between the molecular patterns of triacyl and diacyl lipopeptide, respectively. TLR3 recognizes dsRNA. TLR4 recognizes bacterial LPS. TLR7/8 mediates recogniation of imidazoquinolines and ssRNA. TLR9 recognizes CpG DNA of bacteria and viruses. TLR5 recognizes bacterial flagellin and mouse TLR11 recognizes components of uropathogenic bacteria and profilin-like molecule of the protozoan parasite *Toxoplasma gondii*. TLR1/2 and TLR 2/6 utilize MyD88 and TIRAP/MAL as essential adapters. TLR3 utilizes Trif. TLR4 utilizes four adapters, including MyD88, TIRAP/MAL, Trif and TRAM. TLR7/8, TLR9, TLR5, and TLR11 use only MyD88. The MyD88-dependent pathway controls inflammatory responses, while Trif mainly mediates type I IFN response. In addition, TLR7/8 and TLR9 induce type I IFN in a MyD88-dependent manner in pDCs.

Contemporary research directed towards T3S systems largely centers on the structure, regulation, and role of the translocated toxins in bacterial diseases (24). Currently few studies are being conducted on the roles of T3S structural components within the innate immune response such as TLRs. Known TLR recognized patterns within T3SS include flagellin, the major component of the bacterial flagellar shaft, known to be an important molecule that interacts with TLR5 and Nlrc4 to induce cytokine expression (70). Homologs of the needle rod protein PrgJ from *Salmonella* SPI-1 interact with Nlrc4 to induce cytokines (69). Interestingly, the equivalent protein of the SPI-2 system, SsaI, does not have the same effect (69). LcrV at the tip of the needle complex has been reported to interact with TLR2 (94); however, contradicting reports have called this response

into question (99). The needle proteins' location on the outside of the bacteria appears to be a prime location to activate host TLRs; however, no study has identified an interaction between host response elements and needle proteins.

The following research was completed to evaluate (1) if an isomer of a known T3SS small molecule inhibitor (Compound 2) could also inhibit T3SS and if so by what target and mechanism. (2) What host receptor does full length and truncated T3SS needle proteins interact with to cause an systemic increase in cytokines, as seen in previous work by our lab. We demonstrate the successful use of a novel T3SS inhibitor, Compound D, the target(s) of Compound D with a proposed mechanism, and the additional characterization of the host innate immune system response to purified needle proteins of the T3SS. The use of Compound D has proved to be useful in furthering the understanding of the T3SS. Characterization of the host innate immune system response to needle proteins will help to understand how several bacteria manipulate the immune system to cause disease. This information is useful in creating novel treatments or utilizing current approved treatments in new ways. Purified needle proteins may also become useful in artificially modulating the host immune response.

CHAPTER II

MATERIALS AND METHODS

Bacterial strains and plasmids

Bacterial strains and plasmids used in this study are listed in Table 1. All strains were stored at -80°C in 25% glycerol (v/v). Electroporation of DNA into *Y. pestis* cells was done as described previously (77). Plasmid pBAD YopD was constructed by cloning a *Nhe*I–cleaved PCR product into pBAD18. The primers used to amplify *yopD* were 5'YopD BAD *Nhe*I (5' CTC TCT CTC GTT AGC ATG ACA ATA AAT ATC AAG ACA 3') and 3'YopD BAD *Nhe*I (5' TCT CTC TCT GCA TGC TCA GAC AAC ACC AAA AGT GGC 3'). Plasmid pBAD *lcrH* was constructed by cloning a *Sac*I and *HindI*II digested PCR product into pBAD33. The primers used to amplify *lcrH* were (5' CCG AGC TCA GGA GGA AAC GAT GCA ACA AGA GAC GAC 3') and (5' CCC AAG CTT CTG GGT TAT CAA CGC ACT C 3'). $YopE_{129}$-Elk expression vector pMH141 was constructed by cloning an *Eco*R1 cleaved PCR product into a a *Sma*I *and Eco*R1 cleaved pBAD18 vector. The primers used to amplify a YopE-Elk chimera encoding DNA segment from plasmid $pYopE_{129}$-Elk {Day 2003} were AraYopEstart (5' GGA ATT CAG GAG GAA ACG ATG AAA ATA TCA TCA TTT 3') and Elk-stop (5' ACA TGC TGC TCA CTT GGC CGG GC 3'). Plasmid pMH73 for production of LcrV was

constructed by cloning a *Nde*I and *Bam*HI cleaved PCR product into pET9. The primers used to amplify *lcrV* were (5' TAC ATA TGC ATC ATC AT CAT CAT CAT GTG TTA GAG CCT ACG 3') and (5' GCG GGA TCC TCA TTT ACC AGA CGT GTC ATC TAG C 3'). Plasmid pMH166 for production of YopD were constructed by cloning a *Bam*HI and *Nde*I cleaved PCR product into pET9. The primers used to amplify *yopD* were YopDStart (5' GGA ATT CCA TAT GCA TCA TCA TCA TCA TCA TAC AAT 3') and YopDstop (5' GCG GGA TCC TCA GAC AAC ACC AAA AGC GGC 3').

Deletion of *lcrGVH* from KIM8 was accomplished using the method described by Nilles et al (78). Primers used to create deletion segment: *LcrGVs* (5' CGC GGA TCC GCT ATC TGC TCG AAC AGA 3') and *LcrG 1-5* (5' CGG GGT ACC TTA ATG GGA AGA CTT CAT AAT CTA 3') and *lcrH kpnI* (5' TGG GTA CCT AAG TGG CTT GTT CTT GGC TCA AGA GCT 3') and lcrHDS (5' CCA ACG GCG ACT TGT GTT GCC TGT GAT CCT GTA CGG 3'). Upstream segments were cut with *Kpn*I and *Bam*HI and downstream segments were cut with *Kpn*I. Segments were ligated in pLD55 after digest with *Sma*I and *Bam*HI. Resulting plasmid was used for allele exchange with pCD1 (68).

PCR primers were designed to clone a fragment of *yscF* missing the 66-bp region (encoding for the first 22 amino acids) of the N-terminus of YscF into an expression vector, pET200 (Invitrogen, Carlsbad, CA). The primers used were TrncYscFStartMT (5' CAC CCT CAA GAA GCC AGC AGA CGA TGC AAA CAA AGC GG-3') and TrnctYscFStopMT (5'- TTA TGG GAA CTT CTG TAG GAT

GCC TTG CAT TAA-3'). The resulting PCR fragment was cloned into pET200 TOPO® (Invitrogen).

Plasmids used in this study to overexpress needle proteins were constructed in pET200 TOPO® using Champion™ TOPO expression kits (Invitrogen, Carlsbad, CA). Primers for gene amplification were made Eurofins MWG Operon, Inc (Huntsville, AL). Primers used for cloning were as follows: WT PrgI forward 5'- CAC CAT GGC AAC ACC TTG GTC-3', PrgI reverse 5'- TTA ACG GAA GTT CTG AAT AAT GGC AG-3', Truncated PrgI forward 5'-CAC CTT TGA TAC GGG CGT TGA TAA TCT ACA AAC G-3', WT SsaG forward 5'-CAC CAT GGA TAT TGC ACA ATT AGT GGA TAG CTC TCC-3', SsaG reverse 5'-TCA GAT TTT AGC AAT GAT TCC ACT AAG CAT ATC C-3', Truncated SsaG forward 5'-CAC CCT CTC CCA CAT GGC GCA C-3'. Template DNA for amplification was generated using the DNeasy kit (Qiagen; Valencia, CA); the manufacturer's instructions were followed. PCR was performed using PFU Turbo® polymerase (Agilent Technologies, Santa Clara, CA). Amplified DNA was then placed in pET200 using the Champion™ TOPO expression kit, manufacturer's instructions were followed. All of the gene constructs were verified by sequencing by Eurofins MWG Operon (Brussels, Belgium). Vectors were stored in TOP10 *E. coli* by chemical transformation. WT and truncated MxiH encoding plasmids are a kind gift from Dr. William Picking, Oklahoma State University. Plasmids for protein expression were purified from TOP10 *E. coli* by Qiaprep Miniprep kit (Qiagen). Purified plasmid DNA was then transformed into the expression host, BL21(DE3) Star™ (Invitrogen, Carlsbad, CA).

Table 1: Strains and Plasmids used in this study

Strain	Relevant properties	Reference
Yersinia pestis		
KIM8	pCD1 (Lcr$^+$) pMT1 Pla$^-$	S. Straley
KIM8-3002.1	pCD1 Δ*yopB* [8-388] (Lcr$^+$) pMT1 pPCP1$^-$ Sm^r	{Skrzypek 1998}
KIM8-3002.2	pCD1 Δ*yopD* [1-305] (Lcr$^+$) pMT1 pPCP1$^-$ Sm^r	{Williams 1998}
KIM8 ΔyopK	pCD1 (Lcr$^+$) pMT1 pPCP1$^-$ Sm^r ΔyopK	Lab Stock
KIM D27.1005	pCD1 (Lcr$^+$) pMT1 Sm^r ΔlcrH	{Chen 2011}
KIM8-3002.9 2002}	pCD1 Δ*lcrQ* [Δ1−116] (Lcr$^+$) pMT1 pPCP1$^-$ Sm^r	(Wulff-Strobel
KIM8 ΔyopE	pCD1 Δ*yopE* [yopE::res, kan::res] (Lcr$^+$) pMT1 pPCP1	Lab Stock
KIM8-3002.7	pCD1 Δ*lcrG2* [Δ5-95] (Lcr$^+$) pMT1 pPCP1$^-$ Sm^r	{Nilles 1997}
KIM8-3002.8	pCD1 Δ*lcrGV2* [LcrG Δ6-95] [LcrV Δ1-268] (Lcr$^+$) pMT1 pPCP1$^-$ Sm^r	{Fields 1999}
KIM8 ΔlcrGVH	pCD1 Δ*lcrGVH* (Lcr$^+$) pMT1 pPCP1$^-$	This study
KIM8-3002.N3	pCD1 Δ*yopN* [48-197] (Lcr$^+$) pMT1 pPCP1$^-$ Sm^r	{Hamad 2006}
KIM8 ΔyscB	pCD1 Δ*yscB* [61-125] (Lcr$^+$) pMT1 pPCP1$^-$ Sm^r	G. Plano
KIM8 3002.P9	pCD1 Δ*sycN* [34-65] (Lcr$^+$) pMT1 pPCP1$^-$ Sm^r	{Day 1998}
YP814	pCD1 (Δ*sycE-yopE*::km *yscF* D46A) pPCP1$^-$ pMT1	G. Plano
E.coli		
Novablue	*recA1 endA1 hsdR17* (r_K^- m_k^+) *supE44 Thi-1 gyrA96 relA1 lac* (F' *proA*$^+$*B*$^+$) *lacI*^{q}ZΔM15::Tn10)	Novagen
BL21	F$^-$ *ompT hsdSB* (r_B^- m_B^-) *gal dcm* (DE3)	Novagen
TOP10	F-mcrA Δ(*mrr-hsdRMS-mcrBC) ψ80lacZΔM15 Δlacx74 nupG recA1 araD139 Δ(ara-leu) 7697 galE15 galK16 rpsL(Str*^{R}*) endA1λ*$^-$	Invitrogen

Table 1 cont.

Yersinia pseudotuberculosis		
YP126	Wild type, YPIII(pYV$^+$)	{Palmer 1999}
Pseudomonas aeruginosa		
PA103	Wild type	{Sato 2011}
Plasmids		
pBAD18 yopD	*araBAD*p cloning vector, Ap^r + *yopD*	This Study
pBAD33 lcrH	*araBAD*p cloning vector, Cm^r + *lcrH*	This Study
pMH139	araBAD, Ap^r *yopE$_{1-129}$-Elk*	This Study
pMH73	pET9 N-terminus His –LcrV	This Study
pMH166	pET9 N-terminus His –YopD	This Study
pET15b MxiH		Bill and Wendy Picking
pET15b *Δ1-18 MxiH*		Bill and Wendy Picking
pET15b Δ1-17 PrgI		Bill and Wendy Picking
pET200 PrgI		Drew 2012
pET200 SsaG		Drew 2012
pET200 Δ1-9 SsaG		Drew 2012
pJM119	*pET24b-YscF Km^r*	{Matson 2005}
pMNT67	*pET200-trYscF Km^r*	Toosky 2011

Media and growth conditions

Y. pestis, Y. pseudotuberculosis, P. aeruginosa were grown at 26°C in Heart Infusion Broth (HIB,Difco, Detroit MI). Overnight cultures were used to inoculate HIB to an A_{620} of 0.1 and grown at 26°C with shaking. HIB was supplemented with 1 mM $MgCl_2$, 2 mM $CaCl_2$ (referred to as cultures with Ca^{2+}) or supplemented with 2.5 μM EGTA for Ca^{2+} depletion (referred to as cultures

without Ca^{2+}). When appropriate, bacteria were grown in the presence of carbenicillin at a concentration of 50 µg/ml, chloramphenicol at a concentration of 25 µg/ml, and/or arabinose (0.2% w/v) to induce expression of genes [e.g. truncated *yopE*-Elk] from the vectors. When the A_{620} reached 0.2, Compound D or Compound 2 dissolved in DMSO was added at indicated concentrations. The cultures were then shifted to 37°C to induce T3S and incubation was continued for an additional 6 h. *Escherichia coli* strains were grown at 37°C in LB broth or on TBA plates with antibiotics added as needed. Antibiotics were used at the following concentrations: kanamycin, 50 µg/ml, and carbenicillin, 50 µg/ml.

Cell Culture

THP-1 X-Blue Cells were acquired from Invivogen, Town, CA and grown in RPMI 1640 supplemented with 10% fetal bovine serum, 100 µg/ml Normocin (Invivogen), 50 µg/ml Pen-Strep (Cellgro, Manassas, VA) at 37°C with 5% CO_2. HEK293 cells expressing human TLR2 (CD14), TLR4 (MD-2/CD14), or TLR5, were acquired from Invivogen and grown in DMEM supplemented with 4.5 g/l glucose, 10% fetal bovine serum, 50 µg/ml Pen-Strep, 100 µg/ml Normocin, 2mM L-glutamine, at 37°C with 5% CO_2. THP-1 X-Blue and HEK293 cells contain the secreted embryonic alkaline phosphatase (SEAP) reporter gene under control of NF-κB and AP-1 (THP-1 X-Blue) or NF-κB (HEK293).

His-tagged Protein Isolation

Escherichia coli BL-21(DE3) carrying plasmids for a given protein were grown overnight in non-inducing media (50xM, 1M $MgSO_4$, 40% glucose, 5% Aspartic Acid, (97)) supplemented with the correct antibiotic. Bacteria were then inoculated into auto-inducing media (50xM, 1M $MgSO_4$, 50x5052, NZ-amine S, Yeast Extract, distilled water, (97)) with antibiotic and grown to an A_{620} of 0.6-0.8. Cells were harvested by centrifugation at 4,000 x *g* for 10 min at 4°C and resuspended on ice in wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10% (w/v) glycerol). The resulting cellular suspension was then French pressed at 20,000 psi twice to lyse cells. The lysate was then clarified by centrifugation at 4,000 x *g* for 20 min. The clarified supernatant was collected and diluted with 1,000 mL of wash buffer before application to pre-equilibrated TALON metal affinity resin (Clontech, Mountain View, CA) column. The lysates were applied to the columns twice before washing with new wash buffer. Bound protein was eluted in buffer containing 50 mM sodium phosphate, 200 mM NaCl, 150 mM imidazole, and 20% glycerol (w/v). Purified protein was concentrated with Amicon Ultra Centrifugal Filters (Millipore, Billerica, MA) and dialyzed against PBS + 10% glycerol (w/v) in Slide-A-Lyzer dialysis cassettes (Thermo Fisher Scientific, Rockford, IL) before SDS/Page gel assessment of purity. Protein concentrations were determined by Bradford Protein Assay Kit (Thermo Fisher Scientific, Chicago, IL) and stored at -20°C for future use.

Secreted protein sample preparation

After 4 h of growth at 37°C in HIB, samples from *Y. pestis* cultures were taken for analysis of Yops secretion and expression. Whole cells were separated from cell-free culture supernatants by centrifugation for 5 minutes at 16400 *g* and 4°C as previously described (77). Proteins were precipitated from whole cell or culture supernatant fractions with 10% trichloroacetic acid (w/v, TCA), and subsequently dissolved at 0.1 A_{620}•ml in 2x SDS-PAGE sample buffer (77).

Protein electrophoresis and immunoblot detection

Whole cell and supernatant protein samples were used to load single lanes for SDS—polyacrylamide gel electrophoresis (SDS-PAGE) according to the method described by Laemmli (58). Samples were boiled for 10 minutes before loading on gels. Proteins separated by SDS-PAGE were then transferred to Immobilon-P membrane (Millipore Corp., Bedford, Mass) for immunoblot analysis of select proteins (LcrV, YopB, YopD, YopE, YopM, YopN, YopK, Elk) using polyclonal antibodies specific for each Yop (His-tagged LcrV (αHTV) (77), YopM (αYopM) (75), YopN (also known as LcrE (αLcrE) (77), YopE (α-YopE; gift from S.C. Straley, University of Kentucky, Lexington), and YopB (α-YopB; gift from J.B. Biliska, Stony Brook University, Stony Brook, New York), YopK (α-YopK – Lab Stock), or a monoclonal for Elk (Cell Signaling, Beverly MA). Bound primary antibodies were detected with alkaline phosphatase conjugated to secondary antibodies (goat anti-rabbit immunoglobulin G; Pierce) followed by

color development with 5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium (NBT-BCIP, Thermo Fisher Scientific, Chicago, IL).

Hemolysis Assay

Yersinia pestis deleted for *yopK* was grown overnight in BHI. The bacteria was then subcultured to an A_{620} of 0.3 and supplemented with EGTA. After growing for 1 h temperature was shifted to 37°C and grown for 3 h. Bacteria were then resuspended in 37°C PBS to a density of 50 A_{620}•ml. Sheep Blood was centrifuged at 1,000 x *g* for 10 min at room temperature, washed twice in ice-cold PBS, and resuspended to $4x10^9$ cells/ml. In 96 well plates, 50 µl of RBC's with 50 µl of bacteria were combined and some samples were treated with 60 µM of Compound D as indicated. The plate was then centrifuged at 1,000 x *g* at room temperature for 10 min. The plate was then moved to 37°C for 3.5 h. After the incubation 150 µl of PBS was added to wells (water was added to one set of cells as a control for RBC lysis) and the plate was again centrifuged at 1,000 x *g* at 4°C for 10 min. 100 µl of supernatant was transferred to a clean plate and the A_{570} read.

His-tagged Proteins interaction with Compound D

HisPur Cobalt Resin (Thermo Fisher Scientific) was mixed overnight at 4°C with PBS or His_6-LcrV or His_6-YopD. Resin/Protein mixture was eluted by

centrifugation at 700 *g* at 4°C. Compound D or Compound 2 dissolved in DMSO or DMSO alone was added to remaining resin and mixed overnight at 4°C. Compound D, Compound 2 or DMSO were eluted the next morning and added to flasks containing *Y. pestis* KIM8 to assess presence of inhibitor by the ability of the elute to effect the ability of *Y. pestis* KIM8 to secrete Yops.

Stimulation of SEAP activity in cell lines by needle proteins

THP-1 X-Blue cells were seeded at 2 x 10^6 cells/ml and HEK 293 cells at 2.5 x 10^5 cells/ml. Cells were suspended in infection medium as described by the manufacturer. Proteins were added at a final concentration of 1 µg/mL. As indicated, 20 µg/ml of antibodies (PAb (polyclonal antibody) Control, PAb hTLR2, Invivogen) or TLR4 inhibitor CL1-095 (Invivogen) were added to cell cultures, as suggested by the manufacturer, prior to addition of needle proteins. Cells were stimulated at 37°C with 5% CO_2 for 5 h or 24 h, as indicated.

Enzyme Digestion of Needle Proteins

Proteinase K (Thermo-Fisher) 40 µg/ml was used to digest needle proteins and flagellin. PBS treated with proteinase K was used as a control. Proteins were digested overnight at 37°C. Proteinase K was then inactivated with 1.6 mg/mL phenylmethanesulfonylfluoride (PMSF). Digested proteins and PBS treated were then used to stimulate THP-1 cells.

LPS Assay

ToxinSensor Chromogenic LAL Endotoxin Assay Kit (GenScript, Piscataway NJ) was used to test for endotoxin in protein samples. The Kit was used as described by the manufacturer. An equivalent amount of the endotoxin standard as found contaminating the needle protein samples was then applied to THP-1 X-Blue cells as described above, resulting in no stimulation of THP-1 X-Blue cells.

Cytokine Analysis

Cellular supernatants from THP-1 X-Blue NF-kB activation experiments were collected and stored at -80°C before analysis with Quantikine Elisa kits from R&D Systems (Minneapolis, Minnesota). The Human TNF-α kit was used as instructed by manufacturer.

SEAP Reporter Assays

Quantification of secreted embryonic alkaline phosphatase (SEAP) from the supernatant was detected using Quanti-Blue reagent (Invivogen) according to manufacturer's protocol. A microplate reader, Synergy HT (Bio-Tek, New England Ipswich, MA) quantified SEAP at 630 nm using KC4 v3.3 software (Bio-Tek).

Image acquisition and production

All immunoblots were scanned on Epson 4490 Perfection scanner at 4800 dpi using VueScan Software (v. 8.4.40; Hamrick Software, [http://www.hamrick.com]. The scanned blots were imported into Adobe Photoshop [CS5.1, Adobe Software, San Jose, CA) the images were converted to grayscale and the autolevels function was applied. Final figures were assembled in Adobe Illustrator (CS5.1) and images were downscaled to the final resolution upon export to the PNG file format.

Data Analysis and Statistics

Needle Protein data was assembled into graphs using GraphPad Prism, version 5.0d (GraphPad Software). Statistical analysis of SEAP levels was completed by using one way analysis of variance with Bonniferri or Dunnett's Multiple Comparisons posttest.

CHAPTER III

RESULTS

Results inhibitor Compound D

Compound D inhibits secretion of Yops by Kim8

Several T3SS inhibitors have been identified, including an isoform of Compound D (Figure 7) (83). We intended to evaluate the ability and mechanism of Compound D to inhibit T3SS in *Yersinia pestis*. Compound D was found to inhibit secretion of YopB, YopD, YopK, YopM, and YopE at a concentration of 60 μM (Figure 8A lane 4) in *Y. pestis* KIM8. Interestingly, LcrV secretion was not inhibited, suggesting functional needles were assembled, implying that Compound D functioned after secretion of LcrV was initiated. Whole cell fractions demonstrated the presence of YopB, YopD, YopK, YopM, and YopE proteins by immunoblotting (Figure 8B) within bacteria. Expression of the analyzed proteins appeared to remain at lower levels, indicative of a non-secreting environment (Figure 8B lane 4) (85). At 20 μM Compound D low levels of Yops secretion were seen in supernatant samples and cellular levels were

more comparable to wildtype in the absence of calcium (Figure 8A-B lane 6). Compound D, like its isomer Compound 2, was found to be toxic to cultured eukaryotic cells (data not shown), resulting in an inability to examine the ability of Compound D to inhibit Yops translocation into cultured cells. An alternative method to evaluate translocation is to examine the ability of *Y. pestis yopK* strains to lyse red blood cells (47, 77). In order to test the effect of Compound D on Yops translocation into red blood cells, we tested the *yopK* strain with Compound D. Compound D was able to inhibit secretion of YopM and YopE just as seen with the wildtype (Figure 9A) with similar effects compared to wildtype on cellular protein levels (Figure 9B). Once the effect of Compound on the *yopK* strain was determined to inhibit Yops secretion like the wildtype strain, an attempt to analyze the effect of Compound D on translocation was performed using a hemolysis assay. *yopK* strains will lyse red blood cells as an indicator of active translocation (47, 77). However, Compound D lysed red blood cells (data not shown) making the effect of Compound D on translocation impossible to evaluate. Subsequently all studies analyze Yops secretion into the culture medium.

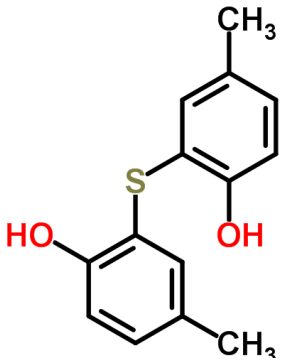


Figure 7. Compound D

2,2'-thiobis-(4-methylphenol) structure

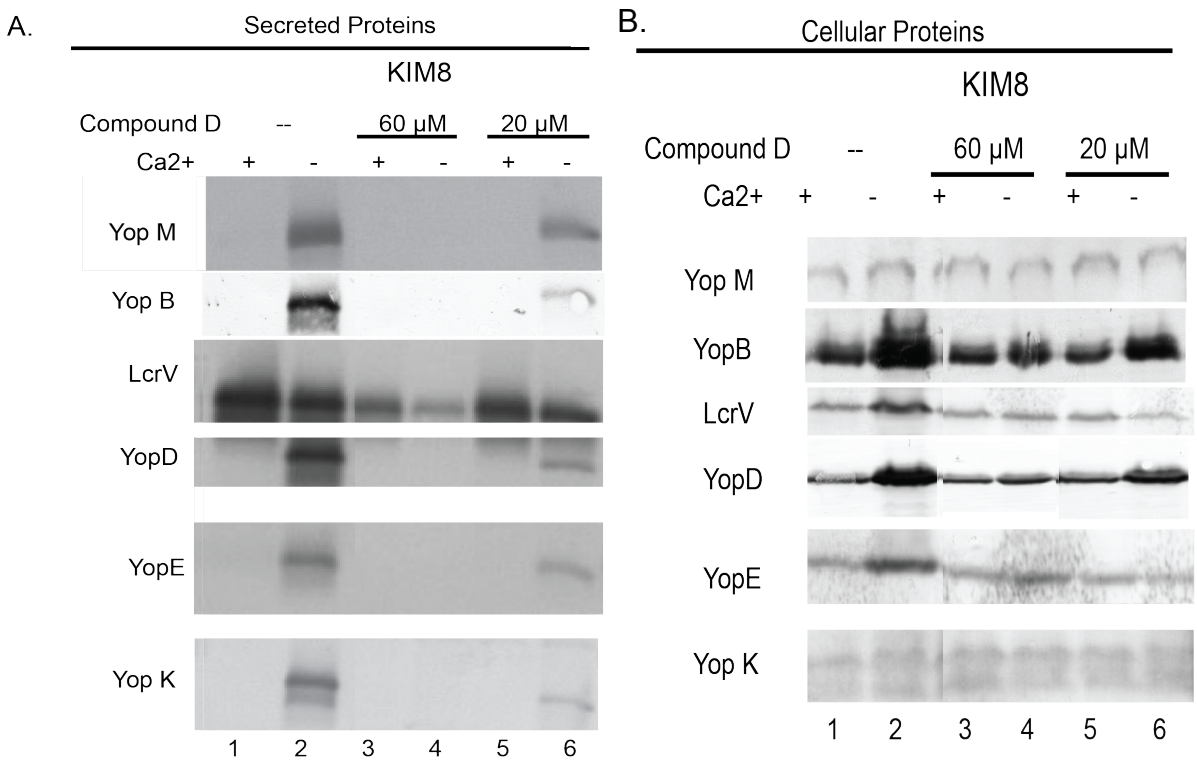


Figure 8. Compound D inhibits secretion in wildtype *Yersinia pestis* KIM8

(A) Secreted and (B) Cellular proteins from *Y. pestis* KIM8 assessed via immunoblot with antibodies to YopM, YopB, LcrV, YopD, YopE, and YopK in the absence of Compound D (lanes 1-2) and presence of Compound D at 60µM (lanes 3-4), 20µM (lanes 5-6). For panels A and B odd numbered lanes are samples in the presence of calcium and even numbered lanes are samples in the absence of calcium. All lanes are loaded with protein derived from 0.05 A_{620}•ml of bacterial culture.

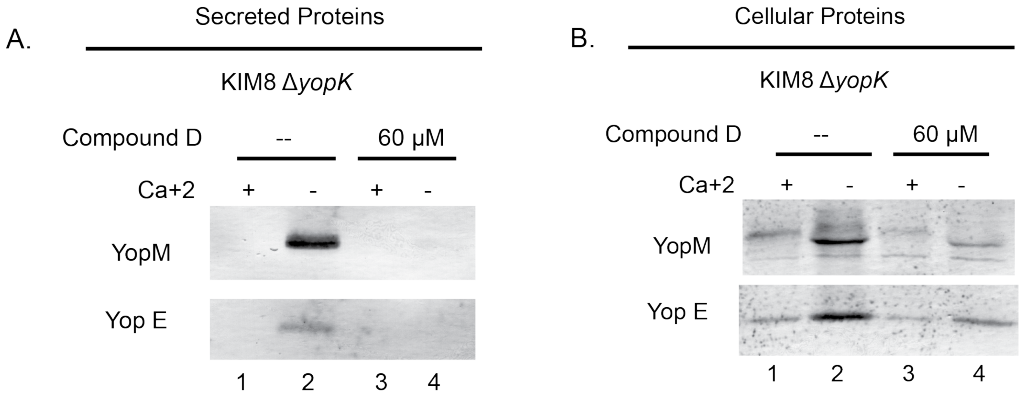


Figure 9. Compound D inhibits secretion in a *Y. pestis* Δ*yopK* strain

(A) Secreted and (B) Cellular proteins in *Y. pestis* KIM8 Δ*yopK* were assessed via immunoblots probed with antibodies to YopM and YopE. Samples lacking Compound D (lanes 1-2) and treated with 60µM Compound D (lanes 3-4). For panels A and B odd numbered lanes are samples in the presence of calcium and even numbered lanes are samples in the absence of calcium. All lanes are loaded with protein derived from 0.05 A_{620}•ml of bacterial culture.

Translocon protein YopD plays a role in Inhibition of Secretion by Compound D

Since secretion of the translocon-staging protein LcrV was unaffected by Compound D, strains lacking the other translocon proteins YopD and YopB were evaluated for the ability of Compound D to inhibit Yops secretion. The *Y. pestis* y*opB* strain behaved similar to wildtype *Y. pestis* (Figure 10A and 10B): In the presence of Compound D, Yops were present in whole cell fractions although at low levels, indicative of a non-secreting environment, and Yops were not secreted under secretion permissive circumstances. However, when the *yopD* mutant was grown under secretion inducing conditions in the presence of Compound D, Yops secretion was not inhibited as seen in the wildtype strain and the *yopB* strain (Figure 10C lanes 4 and 6), suggesting that YopD plays a role in inhibition mediated by Compound D. As expected, hyper secretion of Yops was seen in the cellular environment of the Δ*yopD* strain and the levels of cellular proteins were not affected by the inhibitor (Figure 10D). This result suggests YopD is involved in Compound D inhibition; however, YopB is not.

LcrH and LcrQ uniquely affect Compound D inhibition

Since both YopB and YopD require the chaperone LcrH (SycD) for function, the effect of Compound D on an *lcrH* deleted strain was examined. As shown by Francis et al., phenotypically distinguishing between an *lcrH* deleted and *yopD* deleted strain is difficult (37). The phenotypic effects of deleting *lcrH* could have to do with lacking YopD or YopB and not LcrH. Therefore, the lack of

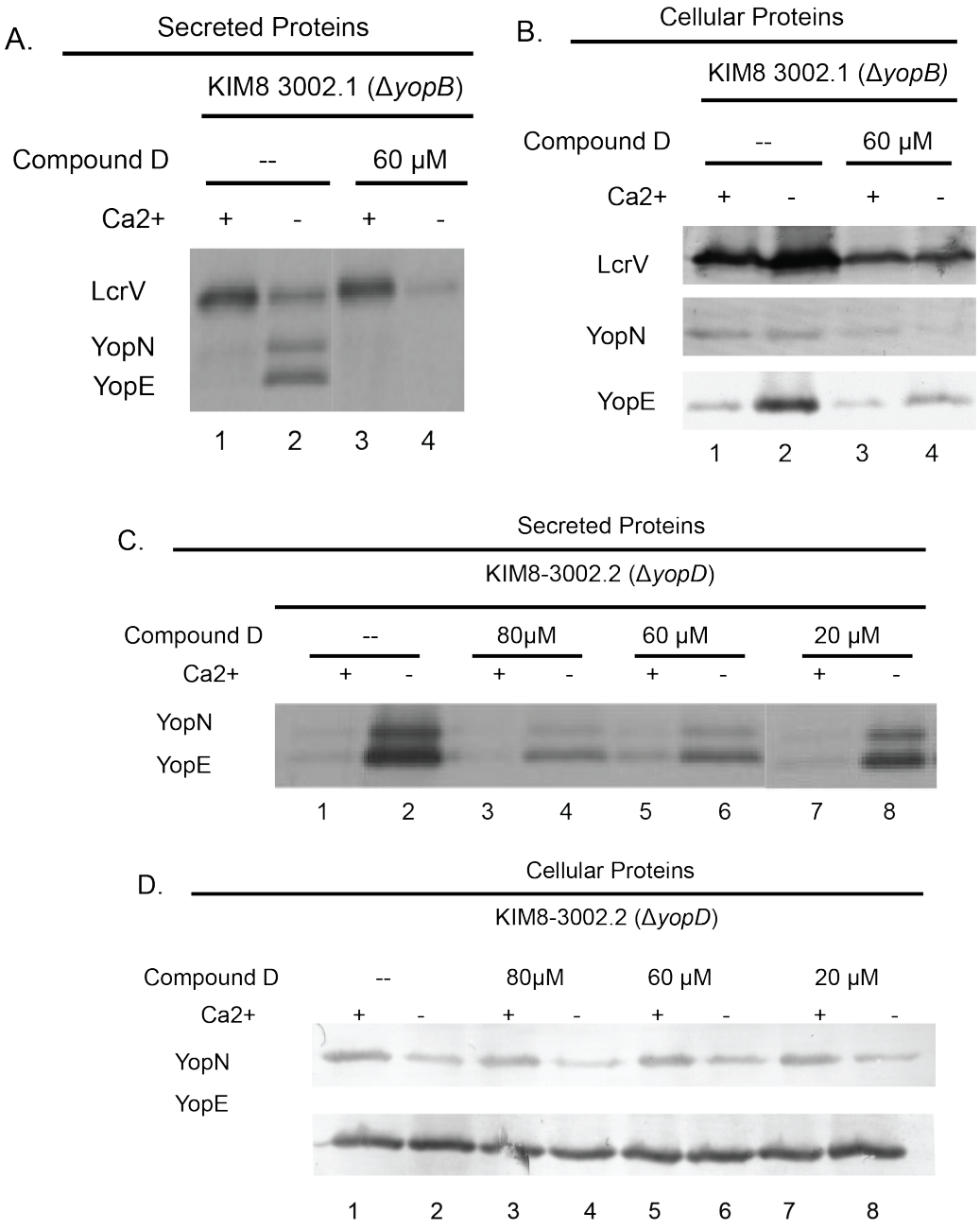


Figure 10. Compound D blocks secretion in a *Y. pestis yopB strain*, but not in a *Y. pestis yopD* strain.

(A) Secreted and (B) cellular proteins from *Y. pestis* KIM8 Δ*yopB* were assessed via immunoblots probed with antibodies to LcrV, YopN and YopE in the absence (lanes 1-2) and the presence of 60 μM Compound D (lanes 3-4). (C) Secreted and (D) cellular proteins from *Y. pestis* KIM8 Δ*yopD* were assessed via immunoblots probed with antibodies to YopN and YopE in the absence (lanes 1-2) and presence of Compound D at 80 μM (lanes 3-4), 60 μM (lanes 5-6), and 20 μM (lanes 7-8). For panels A-D odd numbered lanes are samples in the presence of calcium and even numbered lanes are samples in the absence of calcium. All lanes are loaded with protein derived from 0.05 A_{620}•ml of bacterial culture.

secretion inhibition in the *yopD* strain could be due to effects on either LcrH or YopD. Hence, an *lcrH* strain was tested with Compound D. *lcrH* strains hyper secrete Yops just as *yopD* strains (Figure 11B). When Yops secretion in the *lcrH* strain was examined in the presence of Compound D, YopE secretion was inhibited, while YopM secretion was not inhibited (Figure 11A lane 4). Interestingly, YopE has a chaperone protein, while YopM does not. This tells us that LcrH could be involved with Compound D; or a function of LcrH on YopD may be to affect the secretion of unchaperoned Yops such as YopM.

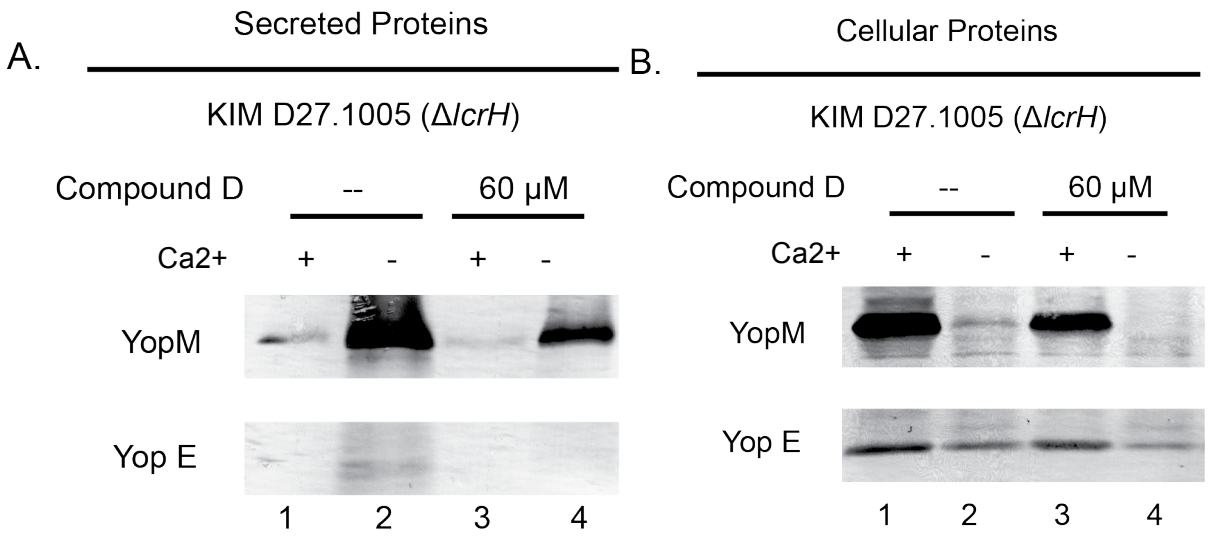


Figure 11. Compound D inhibits secretion of YopE but not YopM in a *Y. pestis lcrH* strain

(A) Secreted and (B) Cellular proteins from *Y. pestis* KIM5 Δ*lcrH* were assessed via immunoblots probed with antibodies to YopM and YopE in the absence (lanes 1-2) and the presence of 60µM Compound D (lanes 3-4). For panels A-D odd numbered lanes are samples in the presence of calcium and even numbered lanes are samples in the absence of calcium. All lanes are loaded with protein derived from 0.05 A_{620}•ml of bacterial culture.

LcrQ function relies upon YopD (109) and is thought to regulate the hierarchy of Yops secretion (110). We therefore also looked at an *lcrQ* deleted strain. Yop secretion is normal in the absence of Compound D. When the *lcrQ* strain is grown in the presence of Compound D, YopM and YopE secretion are reduced but not eliminated, and the effect appears to be greater on YopE (Figure 12A, lane 4). This result indicates that LcrQ or its function at the sorting complex could play a role in inhibition by Compound D. Expression of YopM and YopE appears to be reduced in the presence of the inhibitor, as seen by the reduction of levels evident by immunoblotting whole cell fractions (Figure 12B).

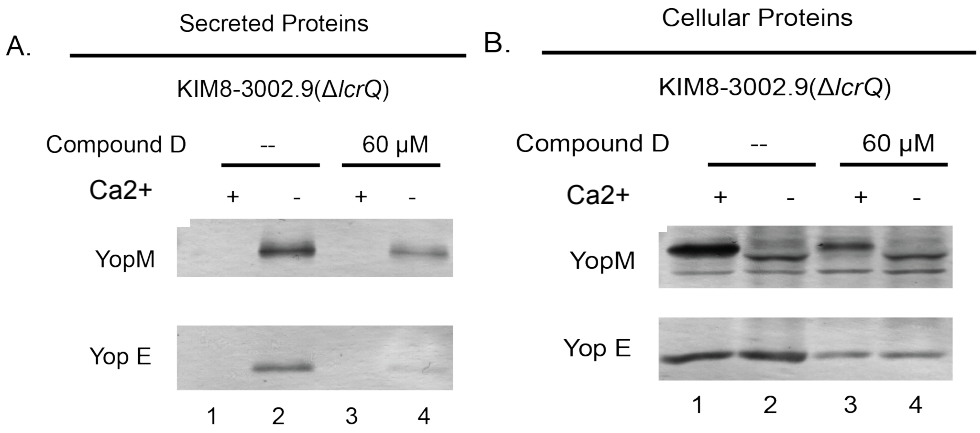


Figure 12. Compound D also inhibits secretion of YopE but not YopM in a *Y. pestis* Δ*lcrQ* strain.

(A) Secreted and (B) cellular proteins from *Y. pestis* KIM8 Δ*lcrQ* were assessed via immunoblot probed with antibodies to YopM and YopE in the absence (lanes 1-2) and the presence of 60μM Compound D (lanes 3-4). For panels A and B odd numbered lanes are samples in the presence of calcium and even numbered lanes are samples in the absence of calcium. All lanes are loaded with protein derived from 0.05 A_{620}•ml of bacterial culture.

Overexpression of YopD in KIM8 overcomes Compound D inhibition

To confirm involvement of YopD with Compound D, YopD was overexpressed in trans in *Y. pestis* strain KIM8 (Figure 13A and 13B). If YopD is the target of Compound D than overexpression of YopD might relieve the effect of Compound D. However, overexpression of YopD alone did not alleviate Compound D's ability to inhibit secretion (Figure 13A, lanes 5-8). Since overexpression of YopD alone did not alleviate the effect of Compound D in KIM8, both YopD and its chaperone LcrH were overexpressed and tested for the relief of Compound D-mediated inhibition of Yops secretion. Overexpression of YopD and LcrH overcame the inhibitory effect of Compound D (Figure 13A: lanes 13-16) in KIM8. Overexpression of LcrH alone resulted in reduced secretion and cellular expression without Compound D as previously observed, (6) and no secretion in the presence of Compound D (Figure 13A: lanes 9-12). By overexpressing YopD and LcrH, the inhibitory effect of Compound D was alleviated, indicating the extra YopD and/or LcrH sequestered the inhibitor and allowed secretion to occur.

YopD and LcrH transcomplement a YopD mutant to restore inhibition by Compound D

The Δ*yopD* mutant was complemented with plasmids containing *yopD*, *lcrH*, or with *yopD* and *lcrH* (Figures 14A and 8B). Again, expression of *yopD* or *lcrH* alone in the Δ*yopD* strain did not restore the ability of Compound D to inhibit secretion (Figure 14A, lanes 5-12). Expression of *yopD* did reduce the hyper-

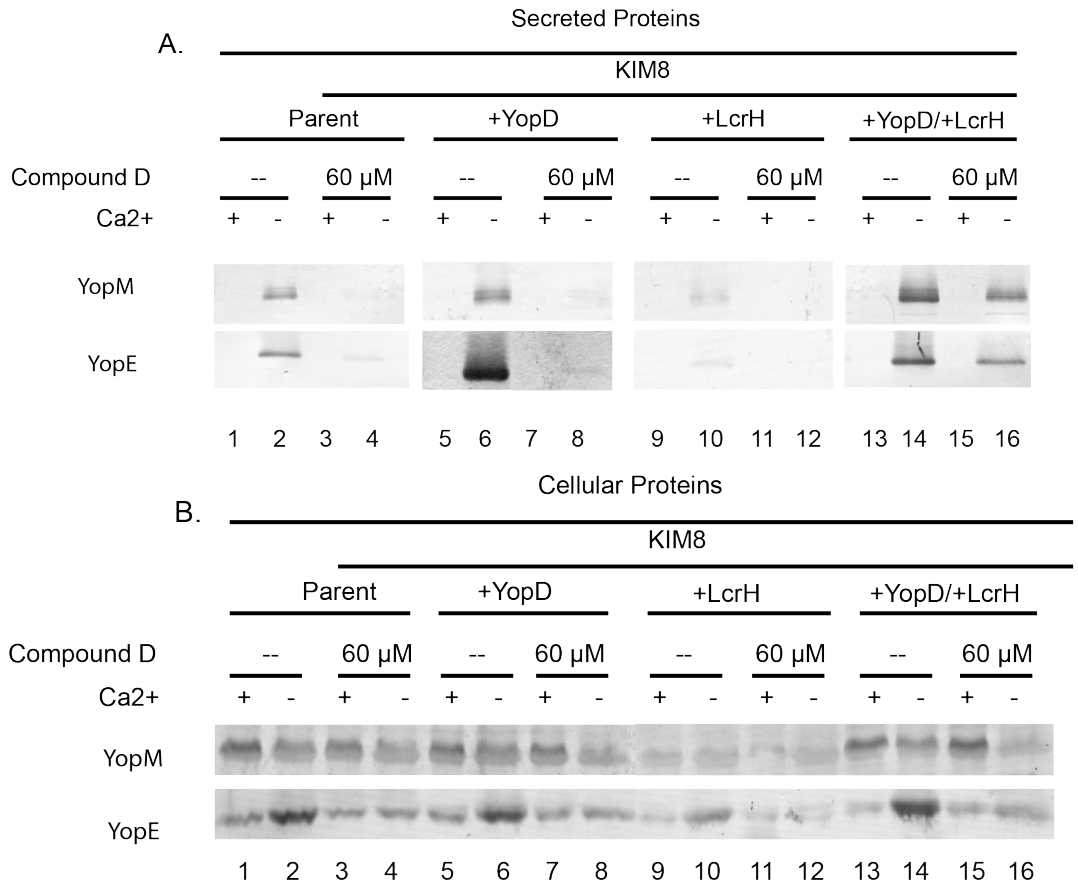


Figure 13. Overexpression of YopD and LcrH in wildtype overcomes Compound D

(A). Secreted and (B) cellular proteins from *Y. pestis* KIM8 with overexpressed *yopD*, *lcrH*, or *yopD* and *lcrH* were assessed via immunoblot with antibodies to YopM and YopE in the absence (lanes 1-2, 5-6, 9-10, and 13-14) and presence of 60 µM Compound D (lanes 3-4, 7-8, 11-12, 15-16). For panels A and B odd numbered lanes are samples in the presence of calcium and even numbered lanes are samples in the absence of calcium. All lanes are loaded with protein derived from 0.05 A_{620}•ml of bacterial culture.

expression of YopM and YopE in the Δ*yopD* strain (Figure 14B, lanes 5-8), indicating a restoration of YopD's regulatory role yet hyper secretion of Yops was still seen (Figure 14A, lanes 5-8). Interestingly, overexpression of LcrH in a *yopD* strain resulted in a dramatic reduction in cellular expression of YopM and YopE (Figure 14B, lanes 9-11), with no secretion occurring with or without the inhibitor present (Figure 14A, lanes 9-11). Co-expression of *yopD* and *lcrH* resulted in complementation to restore the ability of Compound D to inhibit secretion (Figure

14A, lanes 13-16). These results suggest that YopD or the YopD/LcrH complex are involved in inhibition by Compound D.

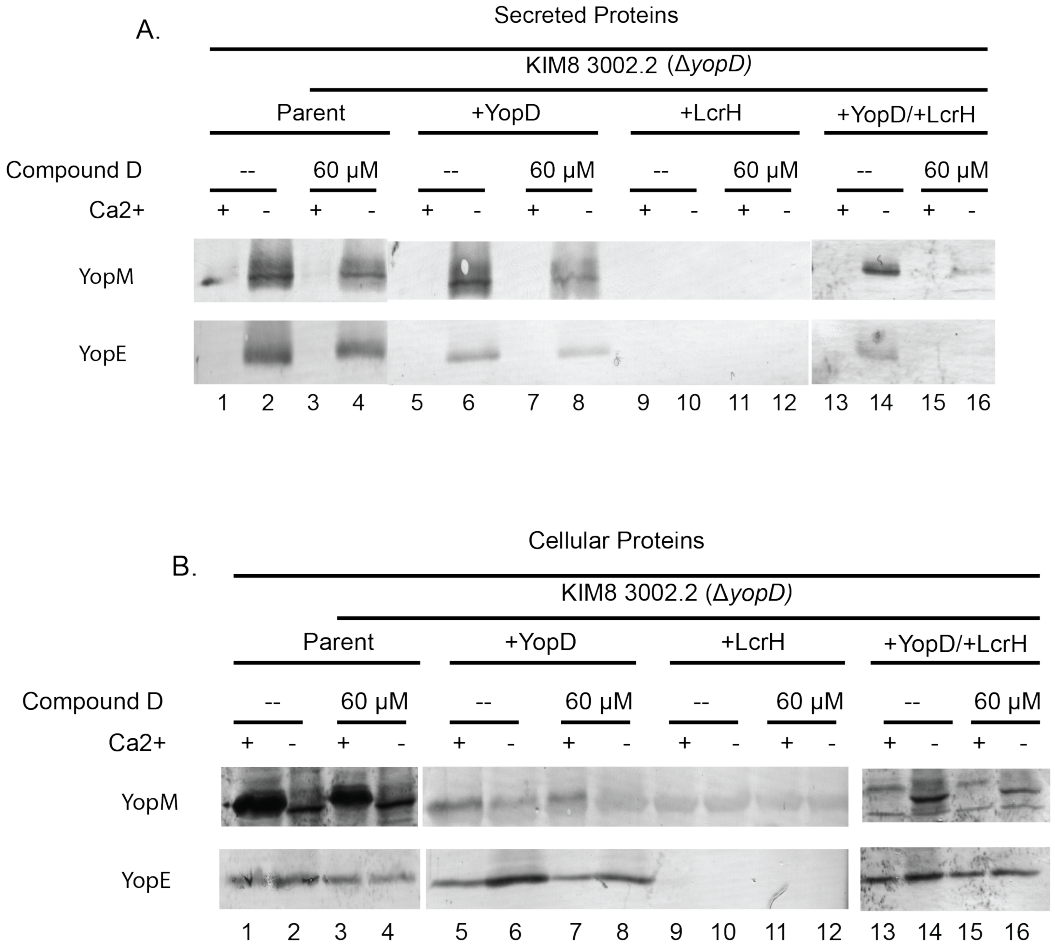


Figure 14. Transcomplementation of *Y. pestis* Δ*yopD* with YopD and LcrH restores Compound D's ability to inhibit secretion.

(A). Secreted and (B) cellular proteins from *Y. pestis* KIM8 Δ*yopD* complemented with *yopD*, *lcrH*, or *yopD* and *lcrH* were assessed via immunoblot with antibodies to YopM and YopE in the absence of Compound D (lanes 1-2, 5-6, 9-10, and 13-14) and presence of 60 μM Compound D (lanes 3-4, 7-8, 11-12, 15-16). For panels A and B odd numbered lanes are samples in the presence of calcium and even numbered lanes are samples in the absence of calcium. All lanes are loaded with protein derived from 0.05 A_{620}•ml of bacterial culture.

Interaction of YopD with Compound D

In order to evaluate if Compound D was interacting with YopD, His-tagged YopD or His-tagged LcrV in PBS or PBS alone was administered to a cobalt-immobilized metal affinity chromatography (IMAC) resin to retain poly-histidine tagged YopD on the resin columns. After washing the columns, DMSO alone, Compound D or Compound 2 dissolved in DMSO was added to the columns. The flow through was collected and added to *Y. pestis* KIM8 cultures to test if the inhibitor was present and able to inhibit secretion. *Y. pestis* KIM8 was grown and treated with Compound D that was not gel-treated as a control. Cellular proteins were all comparable (Figure 15B). The flow through from a PBS-only treated column with Compound D added was able to inhibit secretion of YopE and reduced secretion of YopM, indicating the inhibitor was able to pass through the column and not interact with the gel resin (Figure 15A, lanes 5-6). When the column was treated with his-tagged LcrV as a negative control, the inhibitor was still able to pass through and inhibit secretion as expected, showing that LcrV does not bind Compound D (Figure 15A, lanes 7-8) and that Compound D is not simply absorbed by protein. As a control a his-tagged YopD-loaded column had DMSO alone applied and the flow through was not able to inhibit secretion (Figure 15A, lanes 9-10). When Compound D and Compound 2 were administered to a his-tagged YopD-loaded column and added to KIM8 cultures, the flow-through from the Compound 2-treated column was able to inhibit secretion (Figure 15A, lanes 13-14). However, the flow-through from the Compound D-treated column was unable to inhibit secretion, suggesting that

Compound D was no longer present to inhibit secretion (Figure 15A, lanes 11-12), denoting that Compound D was retained on the his-tagged YopD-treated column. This result suggested that his-tagged YopD was able to interact with Compound D and Compound D was removed from the DMSO solution. In contrast, the related chemical Compound 2 was not retained under the same conditions suggesting the retention of Compound D is a specific interaction. These results indicated that Compound D is capable of binding to YopD.

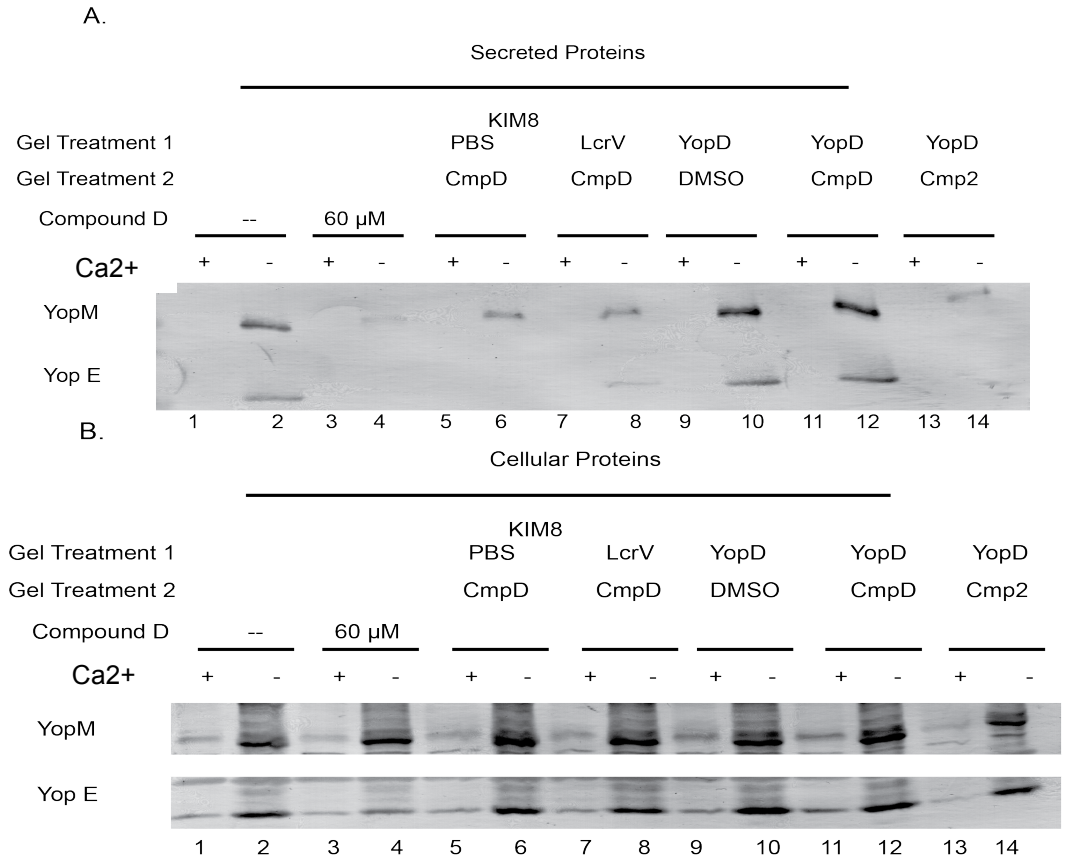


Figure 15. Compound D is pulled out of solution when exposed to YopD.

Recombinant proteins, His_6-YopD (lanes 9-14) or His_6-LcrV (lanes 7-8), or PBS (lanes 5-6) were combined with Talon affinity gel and Compound D (lanes 7-8 and 11-12) or Compound 2 (lanes 13-14) or DMSO (lanes 9-10) were exposed to gel/protein combinations. Flow through was then used to evaluate (A) secreted and (B) cellular protein of wildtype strain KIM8 to determine the presence of the inhibitors. Evaluation was done via immunoblot using antibodies to YopM and YopE. Control protein analysis was done in the absence of Compound D (lanes 1-2) and presence of 60 µM Compound D (lanes 3-4). For panels A and B odd numbered lanes are samples in the presence of calcium and even numbered lanes are samples in the absence of calcium. All lanes are loaded with protein derived from 0.05 A_{620}•ml of bacterial culture.

Secretion of YopE expressed from a non-native promoter is inhibited by Compound D

A *yopE* strain retained the ability of Compound D to inhibit Yops secretion indicating that YopE is not involved with Compound D's ability to inhibit Yops secretion. YopM and YopN secretion was still inhibited by Compound D in a *yopE* strain (Figure 16A). Since YopE was not involved in Compound D inhibition, a truncated YopE-Elk chimera expressed under a non-native promoter ($araBAD_p$) was used to analyze whether Compound D was acting at the level of secretion or expression of Yops. The YopE-Elk chimera was secreted in media lacking calcium as expected (Figure 16B: lane 2). However, in the presence of Compound D secretion of the YopE-Elk chimera was inhibited along with the natively expressed Yops (Figure 16B: lane 4), indicating inhibition of secretion is not tied to regulation of gene expression within the T3SS.

Regulatory differences in strains with unregulated Yops secretion by Compound D

Strains of *Y. pestis* with certain T3SS regulatory proteins deleted have the ability to secrete Yops despite the presence of calcium. These strains constitutively secrete Yops and are referred to as 'calcium blind' strains. Two sets of regulatory calcium blind strains representing two distinct regulatory pathways were tested in the presence of Compound D: strains with deletions of *lcrG*, or *lcrG* and *lcrV* (Figure 17A-D) and strains with deletions of *yopN, yscB, or sycN* (Figure 18A-F). For the strains lacking LcrG or LcrG and LcrV, secretion of Yops was inhibited in the secretion inducing environment of all tested strains.

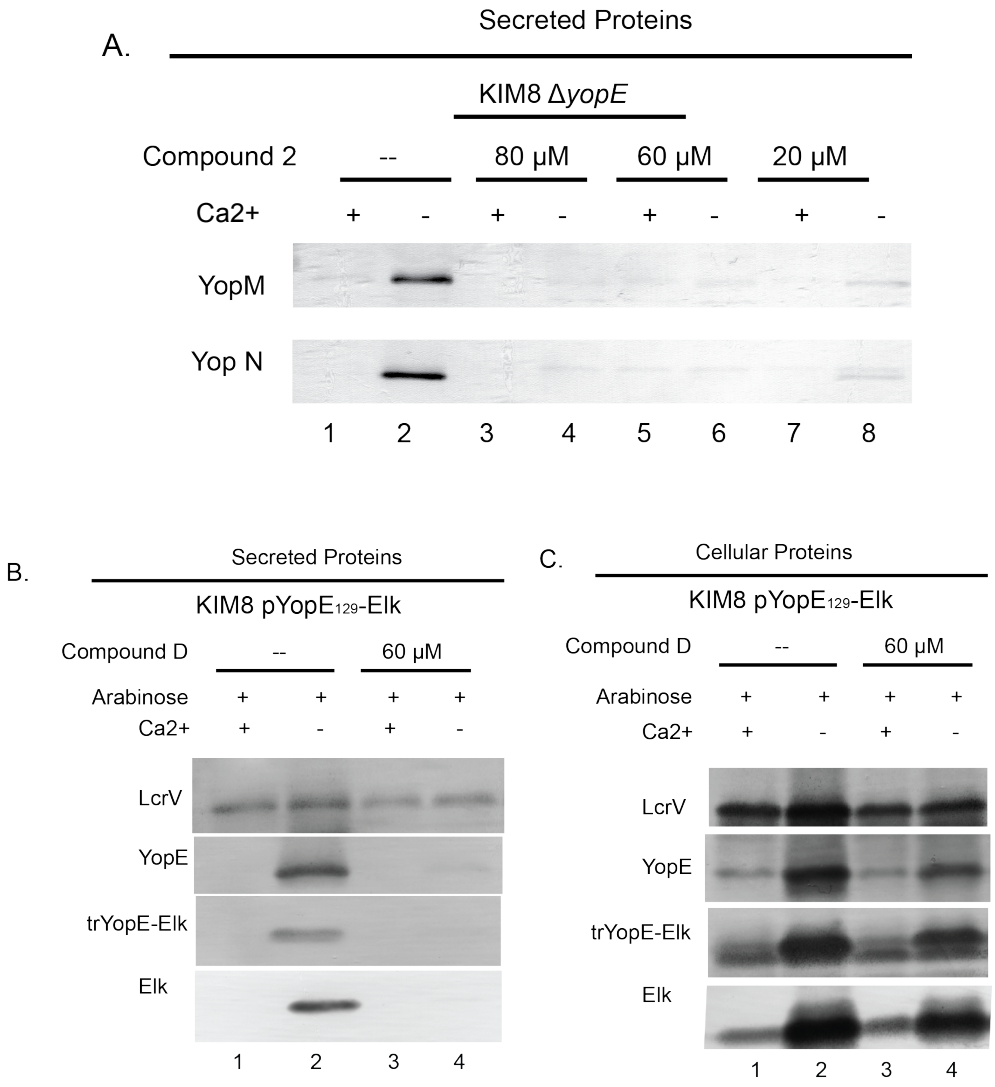


Figure 16. Compound D's inhibits secretion of a YopE-Elk Chimera

(A) Secreted proteins from *Y. pestis* KIM8 Δ*yopE* were assessed via immunoblots probed with antibodies to YopM and YopN in the absence (lanes 1-2) and presence of Compound D at 80 μM (lanes 3-4), 60 μM (lanes 5-6), and 20 μM (lanes 7-8). (B) Secretion and (C) Cellular proteins from *Y. pestis* KIM8/pYopE$_{129}$-Elk were assessed via immunoblots probed with antibodies to LcrV, YopE, and Elk in the absence (lanes 1-2) and presence of Compound D at 60 μM (lanes 3-4). For panels A-C odd numbered lanes are samples in the presence of calcium and even numbered lanes are samples in the absence of calcium. All lanes are loaded with protein derived from 0.05 A_{620}•ml of bacterial culture.

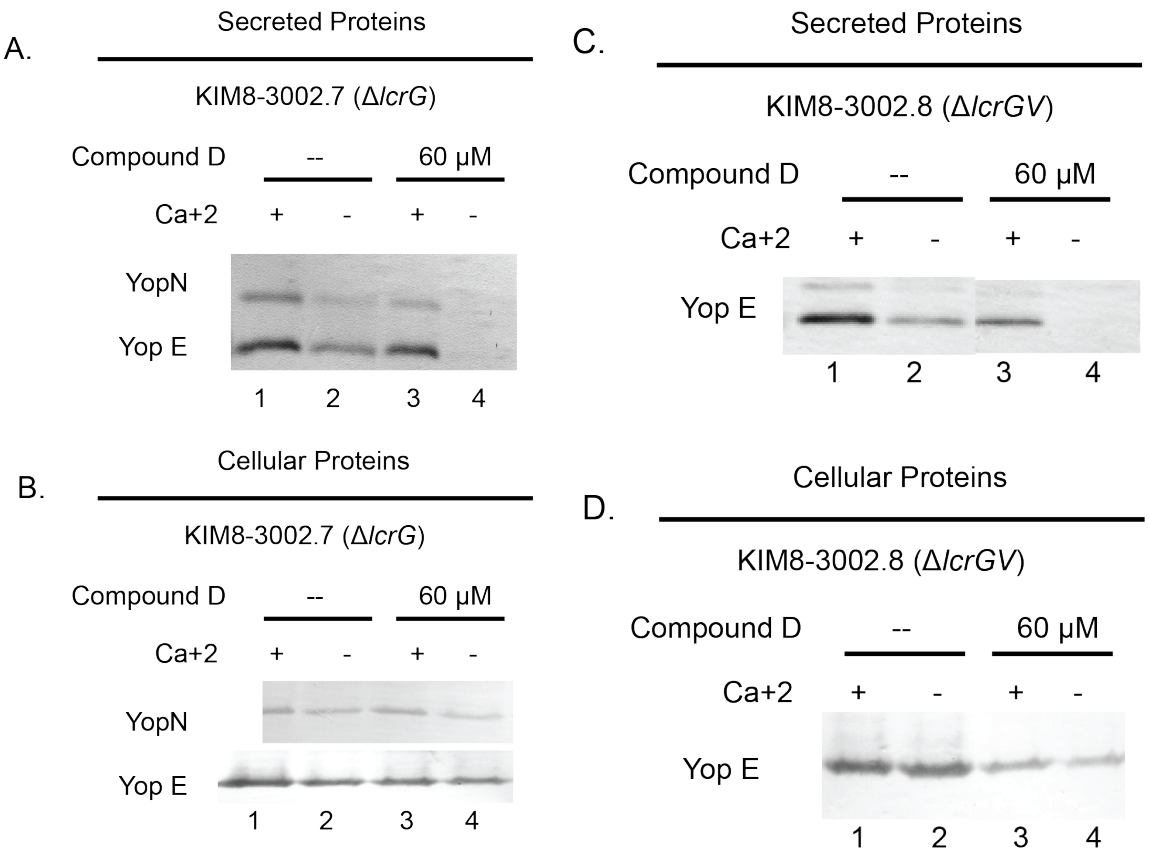


Figure 17. Compound D does not block secretion in the presence of Ca^{2+} in *Y. pestis* Δ*lcrG* or Δ*lcrGV* strain

(A and C) Secreted and (B and D) cellular proteins from regulatory mutants: Δ*lcrG* (A-B) and *ΔlcrGV* (C-D) were assessed via immunoblots probed with antibodies to YopE and/or YopN in the absence (lanes 1-2) and presence of Compound D at 60 μM (lanes 3-4). For panels A-D odd numbered lanes are samples in the presence of calcium and even numbered lanes are samples in the absence of calcium. All lanes are loaded with protein derived from 0.05 A_{620}•ml of bacterial culture.

However, secretion of Yops in the presence of calcium was not inhibited, indicating a difference in the regulation of the T3SS between the two environments. In the strains lacking YopN and its chaperones, YscB and SycN, a similar pattern was seen: secretion in the presence of calcium was not affected by Compound D. However, in the absence of calcium, YopM and YopE secretion was greatly reduced in the Δ*yopN* and Δ*yscB* strains (Figure 18A and 18C), or

completely absent in the Δ*sycN* strain (Figure 18E). Cellular levels of Yops were comparable in all strains, with a slight reduction in the non-secreting conditions. The ability of Compound D to inhibit secretion in the absence of calcium but not the presence could indicate an interaction with calcium or that a change in the T3SS structure in the absence of calcium allows the inhibitor to cause the effect of blocking secretion.

Constitutive secreting *yscF* mutant inhibited in presence and absence of calcium

Torruellas et al. identified YscF mutants that constitutively secrete Yops (102). We used a pPCP1$^-$ derivative of KIM5-3001.P61 +pYscF (D46A) (102), YP814, a strain that has YscF (D46A) expressed in cis on pCD1. YP814 was grown with Compound D to see the effect compared to the previous constitutively secreting strains. In the case of YscF D46A, Compound D was able to inhibit secretion of YopE in both the presence and absence of calcium (Figure 19, lanes 3 and 4) contrary to what we had seen in in Figures 17 and 18. The result of blocking secretion in the presence of calcium shows that Compound D activity is not affected by calcium. This specific mutant, expressing an altered needle, allowed Compound D to inhibit secretion in both the presence or absence of calcium. These data indicate that a certain needle conformation may be required for Compound D to inhibit secretion.

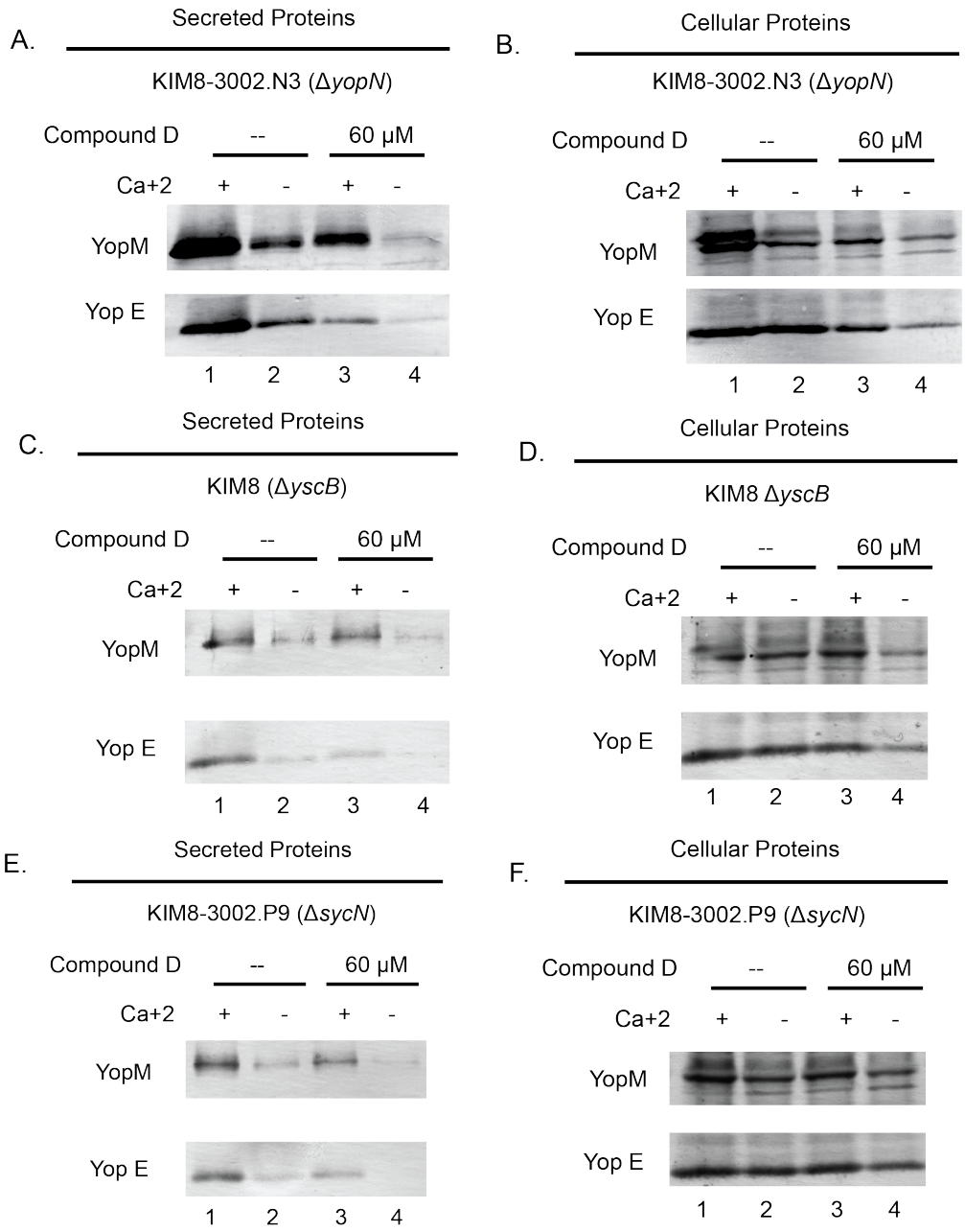


Figure 18. Compound D does not block secretion in the presence of Ca^{2+} in YopN$^-$ strains

(A,C,E) Secreted and (B,D,F) cellular proteins from regulatory mutants: *Y. pestis* Δ*yopN* (A-B), Δ *yscB* (C-D), and Δ*sycN* (E-F) were assessed via immunoblots probed with antibodies to YopE and YopM in the absence (lanes 1-2) and presence of Compound D at 60 μM (lanes 3-4). For panels A-F odd numbered lanes are samples in the presence of calcium and even numbered lanes are samples in the absence of calcium. All lanes are loaded with protein derived from 0.05 A_{620}•ml of bacterial culture.

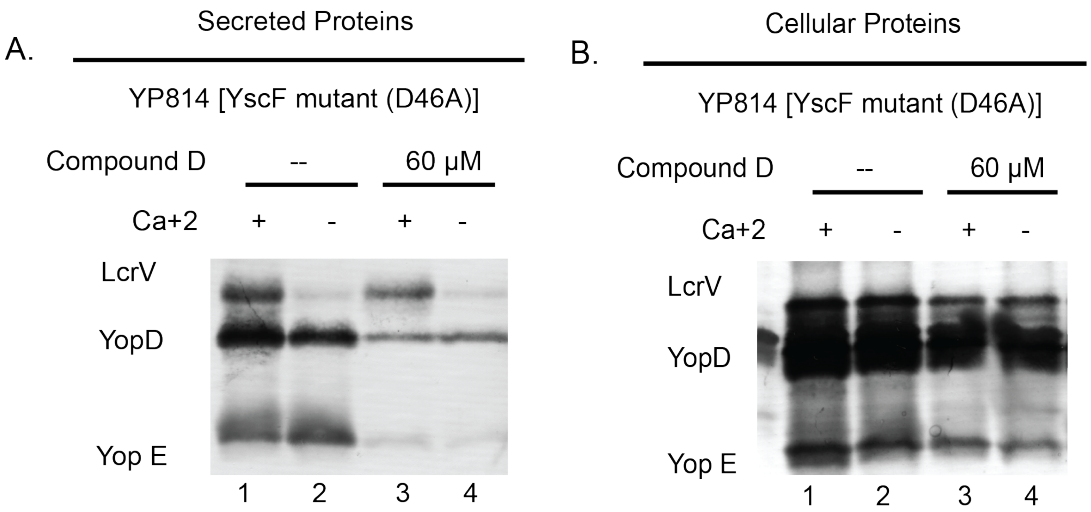


Figure 19. Yops secretion in a *Y. pestis* YscF D46A strain is inhibited by Compound D.

(A) Secreted and (B) cellular proteins from *Y. pestis* YP814, a strain expressing YscF D46A were assessed via immunoblots probed with antibodies to LcrV, YopD, and YopE in the absence (lanes 1-2) and presence of Compound D at 60 µM (lanes 3-4). For panels A and B odd numbered lanes are samples in the presence of calcium and even numbered lanes are samples in the absence of calcium. All lanes are loaded with protein derived from 0.05 A_{620}•ml of bacterial culture.

Compound 2 role in inhibiting secretion is different than Compound D

The related inhibitor, 4,4'-thiobis(3-methylphenol) (Compound 2) (Figure 20A), discovered by Pan et al. (82) was assessed in comparison to Compound D. Both compounds effectively inhibit secretion of Yops (Figure 20B). However where removing YopD can alleviate Compound D's effect, Compound 2 is unaffected (Figure 20C: lanes 1-4). YopD does not play a role in Compound 2's mechanism to inhibit secretion. YopB was also evaluated with Compound 2 and again secretion was inhibited indicating YopB is not involved (Figure20C: lanes 5-8). A constitutive Yops secreting strain lacking LcrG and LcrV was tested to see if Compound 2 was only inhibited in the absence of calcium as well. Compound 2 was able to inhibit secretion in both the presence and absence of calcium, contrary to the effect of Compound D on calcium blind strains

(Figure20D). Therefore Compound D and its isomer Compound 2, although both inhibitors of type III secretion, are accomplishing inhibition of secretion in different ways.

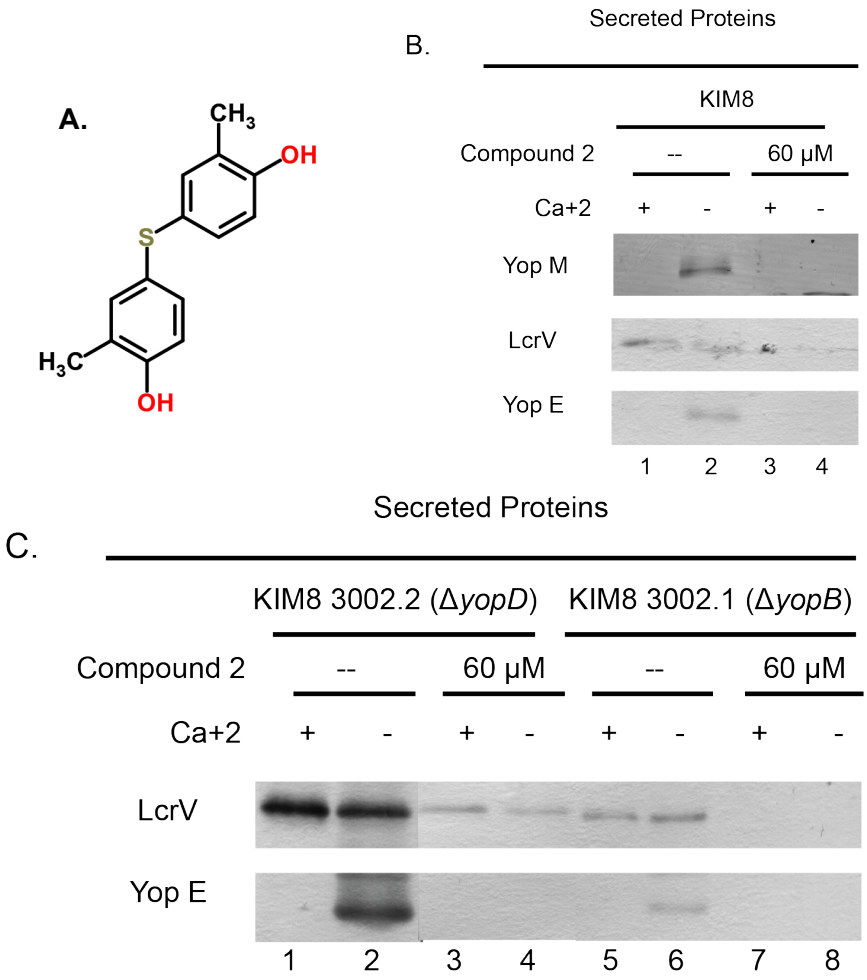


Figure 20. Compound 2 an isomer of Compound D does not target YopD.

(A).Compound 2 4-4' thiobis (2-methylphenol) structure. (B) Wildtype KIM8 was grown in the absence (lanes 1-2) and presence (3-4) of 60 µM Compound 2. Secretion was evaluated by immunoblot with antibodies to YopM, LcrV and YopE. (C) Translocon mutants YopD and YopB were grown in the absence (lanes 1-2, 5-6) and presence (3-4, 7-8) of 60 µM Compound 2 and evaluated for Yop secretion via immunoblot to antibodies of LcrV and YopE. (D) Calcium blind mutant LcrGV was also grown up in the absence (lanes 1-2) and presence (3-4) of 60 µM Compound 2 and evaluated for Yop M and YopE secretion via immunoblot. For panels B-D odd numbered lanes are samples in the presence of calcium and even numbered lanes are samples in the absence of calcium. All lanes are loaded with protein derived from 0.05 A_{620}•ml of bacterial culture.

Effects of Compound D on *Pseudomonas aeruginosa* and *Yersinia pseudotuberculosis*

Compound D's ability to affect secretion in *Pseudomonas aeruginosa* and *Yersinia pseudotuberculosis* (Figure 21A and B) was also evaluated. In both cases Compound D was able to reduce the level of secretion. Given that the two bacteria have increased growth rates compared to *Yersinia pestis*, it was not surprising that the same concentration that was proficient at completely inhibiting secretion in *Yersinia pestis* was not sufficient to inhibit secretion in *Pseudomonas aeruginosa* and *Yersinia pseudotuberculosis.* However, effector secretion levels were definitely affected by the presence of Compound D, indicating this compound is effective against other related T3SS.

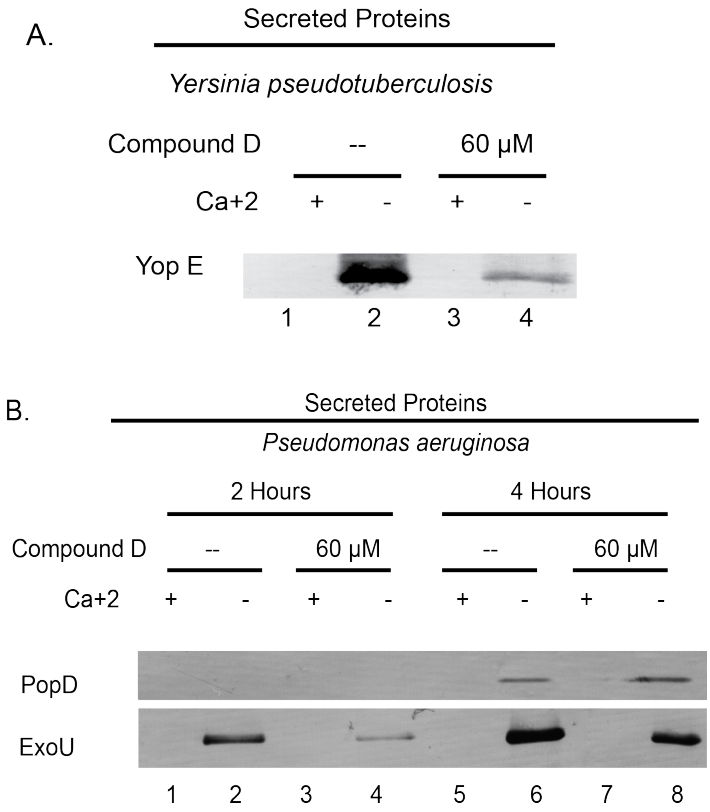


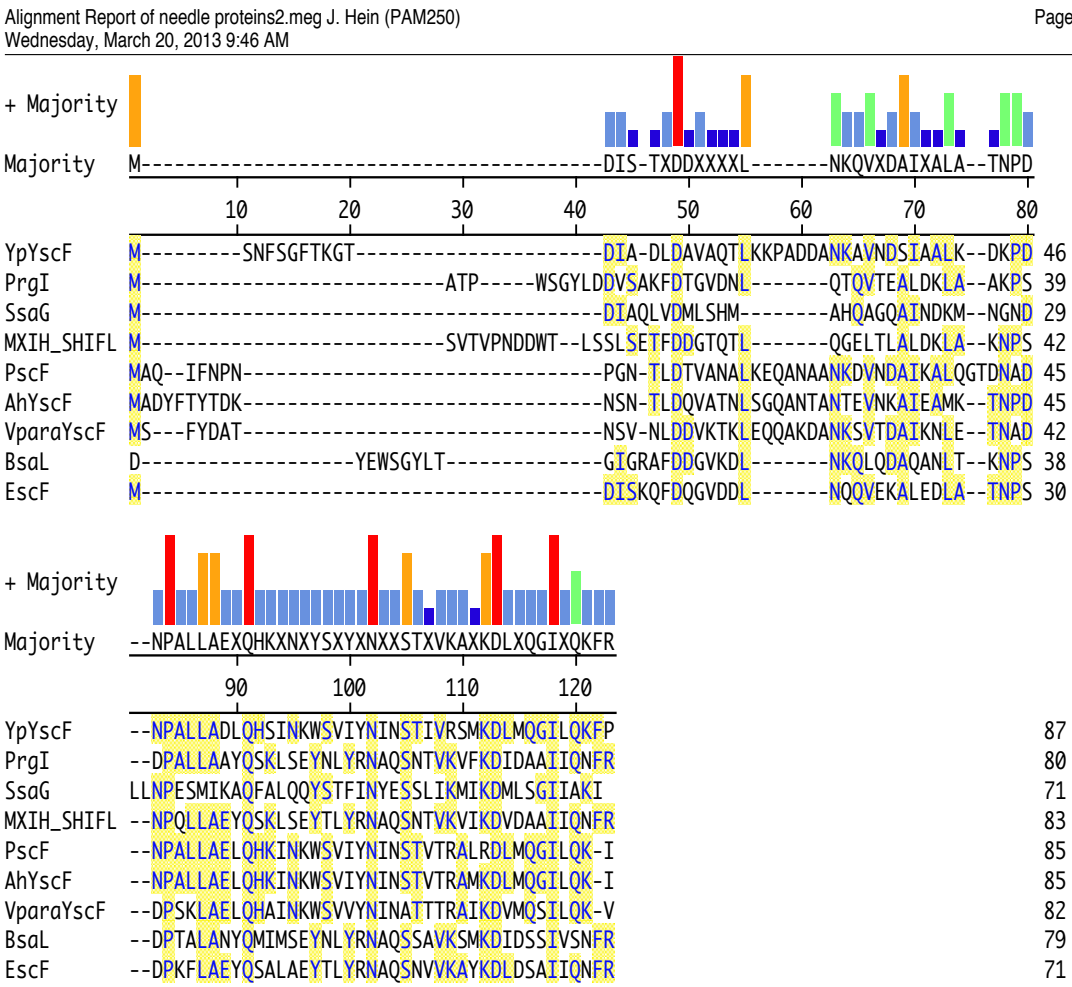
Figure 21. Compound D decreases effector secretion from *P. aeruginosa* and *Y. pseudotuberculosis*.

(A) Secreted proteins from *Yersinia pseudotuberculosis* grown in the absence (lane 1-2) and presence (lanes 3-4) of 60 uM Compound D were evaluated by immunoblot for YopE. (B) Secreted proteins from *Pseudomonas aeruginosa* at 2 hours (lanes 1-4) and 4 hours (lanes 5-8) were grown up in the absence (lanes 1-2, 5-6) and presence (lane 3-4 and 7-8) of 60 uM Compound D. Proteins were evaluated by immunoblot for ExoU and PopD. For panels A and B odd numbered lanes are samples in the presence of calcium and even numbered lanes are samples in the absence of calcium. All lanes are loaded with protein derived from 0.05 A_{620}•ml of bacterial culture.

Results of Needle Protein Activation of Host Cells

Alignment of Needle proteins

Type III secretion systems found in animal pathogens can be divided up into three main families: Ysc injectisomes (e.g. *Yersinia* spp and *Pseudomonas*), *Shigella* and *Salmonella* SPI-1 type injectisomes, and injectisomes like the systems found in *E. coli* and *Salmonella* SPI-2 (24). Our assessment of needle proteins includes at least one needle protein homolog from each T3SS family from bacteria that are human pathogens, as well as representing both *Salmonella* injectisomes: needle proteins from *Yersinia pestis* (YscF), *Salmonella enterica* serovar Typhimurium SPI-1 (PrgI), SPI-2 (SsaG), and *Shigella flexneri* (MxiH). Needle proteins of T3SS are highly conserved, except for the N-termini (Figure 22). Selected protein sequences were aligned and truncated forms of these proteins were made by deleting the N-termini corresponding to the twenty-second amino acid from the N-terminus of *Yersinia pestis* YscF. This corresponded to an 18 aa truncation of PrgI, a 15 aa truncation of MxiH, and a 9 aa truncation of SsaG, which is already naturally truncated in relation to the other needle proteins.



Decoration 'Decoration #1': Shade (with bright yellow at 50% fill) residues that match the Consensus exactly.

Decoration 'Decoration #2': Shade (with bright yellow at 50% fill) residues that match the Consensus exactly.

Figure 22. Needle proteins multiple sequence alignment demonstrates that the N-termini of T3S needle proteins are not conserved.

Needle protein sequences from several species of bacteria were aligned, using the Jotun Hein algorithm with the PAM250 matrix, with Megalign from the DNAStar Lasergene package (v. 10.1). Identical residues are shown in shaded boxes. Aligned needle proteins are from *Yersinia pestis, Yersinia pseudotuberculosis, Yersinia enterocolitica, Salmonella enterica* (both PrgI and SsaG)*, Shigella flexneri, Pseudomonas aeruginosa, Aeromonas hydrophila, Vibrio parahaemolyticus, Burkolderia pseudomallei, and E. coli.*

Response of THP-1 Cells to Needle Proteins

Evaluation of cellular responses to recombinant whole needle proteins and truncated forms of needle proteins by THP-1 X-Blue cells was accomplished by measuring SEAP production. SEAP expression is under control of NF-κB and AP-1, key transcription factors, that activate cytokine and chemokine expression critical for innate immune responses. Therefore an increase in SEAP equated to an increase in NF- κB and/or AP-1 activity. All proteins were applied at 1 μg/ml to cells. Full length MxiH significantly activated NF- κB/AP-1 as well as truncated forms of YscF and PrgI (Figure 23A). Whole YscF, PrgI, and truncated MxiH significantly activated cells; however, these proteins had lower levels of activation than their counterparts (Figure 23A). These results show that the truncated forms of YscF and PrgI increase NF-κB/AP-1 activation to higher levels in comparison to the full-length proteins. SsaG, which is only expressed within eukaryotic cells, activates cells only slightly more than the truncated form. The responses to the N-terminal truncations of YscF and PrgI suggest there may be a hidden host-activating element, whereas, the presence of the N-terminus of MxiH appears to increase interaction of MxiH and Thp-1 X-Blue cells.

When the proteins were incubated with THP-1 X-Blue cells deficient in MyD88, an adaptor protein required for TLR response, the activation of NF-κB/AP-1 was abolished (Figure 23B). The lack of response by cells lacking MyD88 indicates that the response is likely occurring through TLR recognition of the needle proteins.

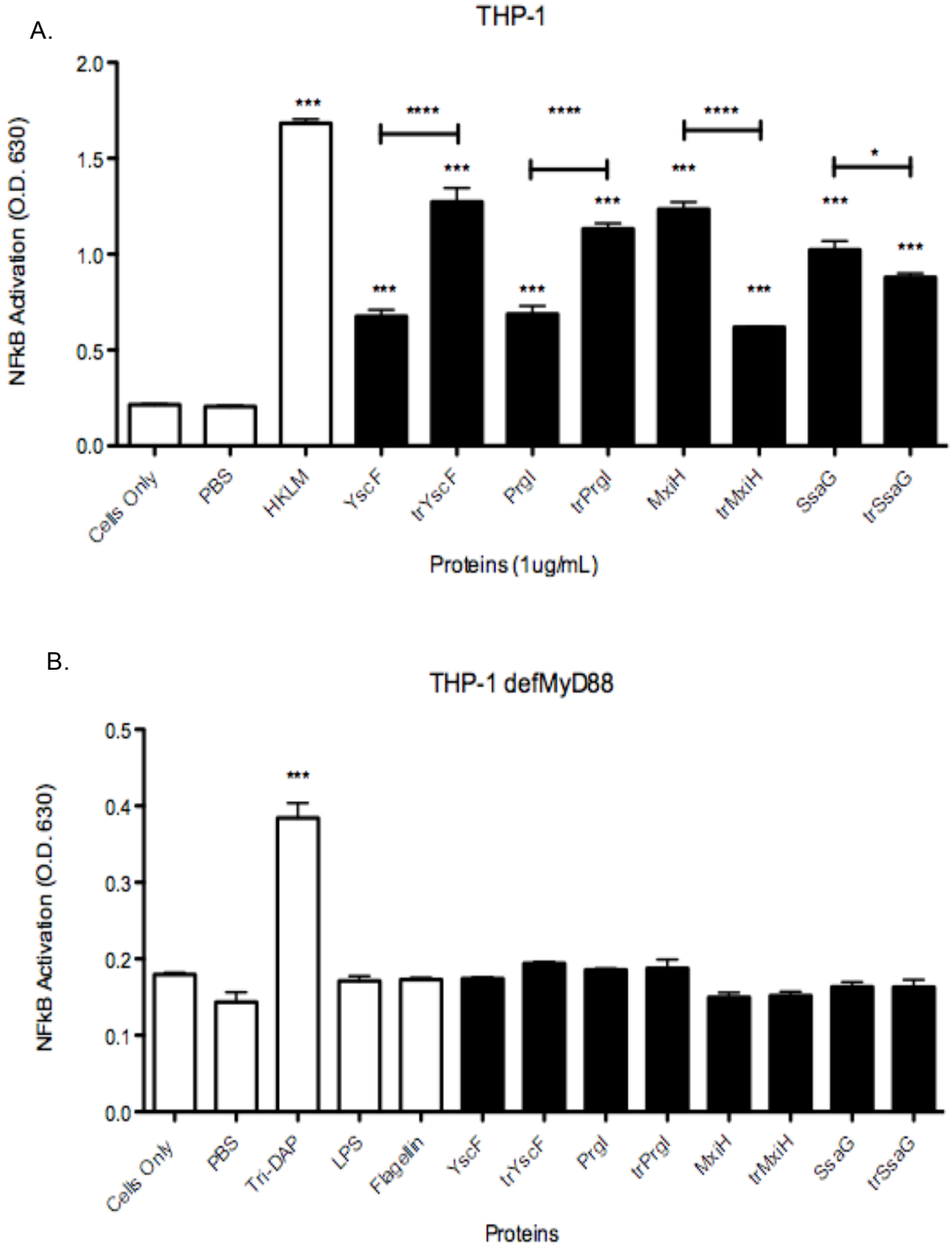


Figure 23. Activation of NF-kB in THP-1 X-Blue cells by needle proteins is MyD88 dependent.

THP-1 (A) and THP-1 defMyd88 (B) cells were seeded in wells and treated with PBS, HKLM (Heat Killed Listeria monocytogenes), LPS, Flagellin, Tri-DAP, or 1ug/ml of needle protein dissolved in PBS. SEAP levels were measured as representation of NFkB activation.

THP-1 response to digested needle proteins

Needle proteins and flagellin digested with proteinase K were added to THP-1 X-Blue cells to confirm that proteins were activating NF-κB/AP-1 (Figure 24). Proteinase K is a serine protease that cleaves after hydrophobic amino acids. Proteinase K in PBS was used as a negative control. As expected, flagellin (a TLR5 agonist; positive control) treated with proteinase K abrogated the NF-κB/AP-1 response and proteinase K alone did not activate NF-κB/AP-1. These data demonstrate that the proteinase K used did not contain a TLR agonist and that, as expected, proteinase K treatment eliminated the ability of flagellin to activate NF-kB/Ap-1 (Figure 24). The proteinase K (Figure 24) treated needle proteins also failed to illicit NF-κB/AP-1 activation; confirming that proteins are causing the cellular activation of NF-κB/AP-1.

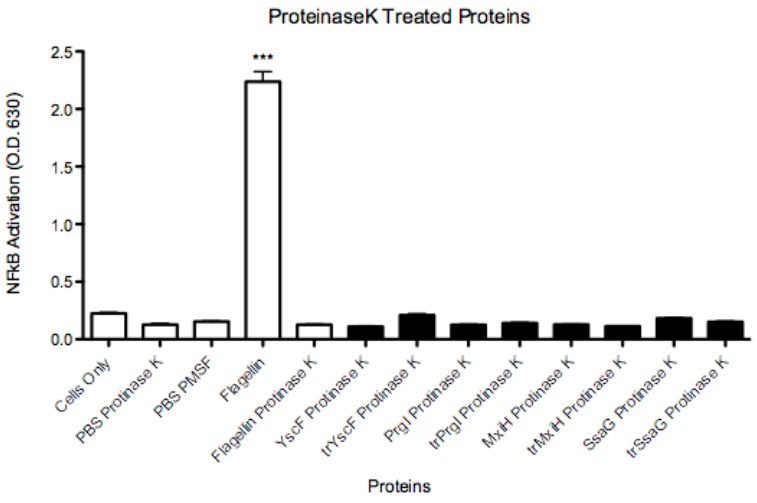


Figure 24. Activation of NF-kB in THP-1 X-Blue cells by needle proteins is abrogated by proteinase K digestion of needle proteins.

Proteins, as well as PBS and Flagellin, were digested with Proteinase K before incubation with THP-1 X-Blue cells. SEAP levels were measured as a representation of NF-kB activation.

LPS assay of needle proteins and equivalent responses by THP-1 X-Blue cells

LPS levels were analyzed to evaluate potential contamination of the needle protein samples. All samples had approximately 1 EU/mL of LPS (Figure 25A). To determine if 1 EU/mL of LPS had a significant effect on NF-κB/AP-1 activation, the standard concentrations of LPS from the kit were used to treat THP-1 X-Blue cells. The results indicated that 1 EU/mL of LPS did not activate NF-κB/AP-1 (Figure 25B). Therefore the level of LPS in the needle protein samples is not a factor in the activation levels seen in Figure 23.

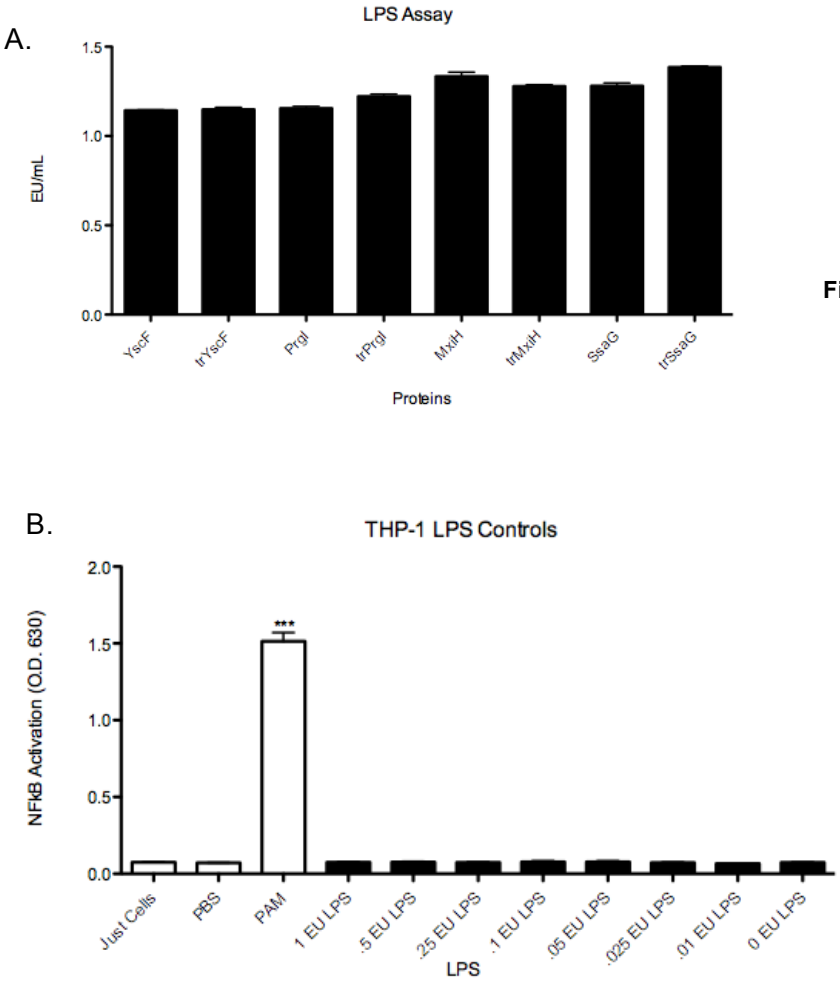


Figure 25. LPS contamination of needle proteins is minimal.

An LPS kit was used to analyze the level of LPS contamination in the needle protein preps (A). The standard LPS levels were used to treat THP-1 X-Blue cells (B). SEAP levels were measured as representation of NFkB activation.

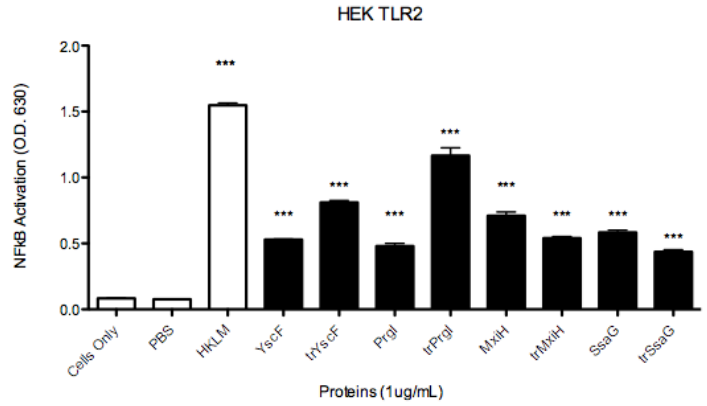
TLR Expressing HEK 293 cells response to needle proteins

To elucidate the TLRs that needle proteins could signal through, an initial screening for TLR interaction was conducted by Invivogen. HEK 293 reporter cells transfected with a selected TLR were incubated with YscF and trYscF (data not shown). These results indicated HEK 293 cells expressing TLR2 or TLR4 responded to whole and truncated forms of YscF. The NF-kB reporter in HEK 293 cells expressing TLRs 3, 5, 7, 8, or 9 did not respond to YscF or trYscF. The reporter in the HEK 293 cells responded more strongly to trYscF than full length YscF, confirming our original observations with THP-1 X-Blue cells.

To further test this result, HEK 293 cells transfected with TLR2, TLR4, or TLR5 were acquired and used to assess the needle proteins from other bacterial species as well. TLR2 expressing HEK 293 cells showed a similar pattern as seen with the THP-1 X-Blue cells where all proteins activated NF-κB/AP-1 (Figure 26A). Specifically, truncated forms of YscF and PrgI activated NF-κB/AP-1 more than full length YscF and PrgI. MxiH and SsaG behaved oppositely than the other proteins, with full length MxiH and SsaG activating slightly more than the truncated forms.

TLR4 expressing HEK 293 cells also reacted to all the tested needle proteins (Figure 26B). YscF activated slightly less than trYscF. PrgI activated less than trPrgI, similar as was observed with THP-1 X-Blue and HEK TLR2 cells. MxiH activated more than trMxiH, again similar to the other cell types. SsaG activated slightly less than trSsaG, contrary to previous cell types.

A.



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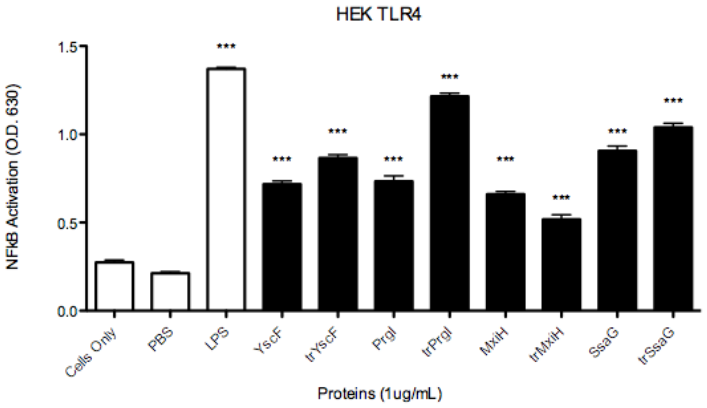

C.

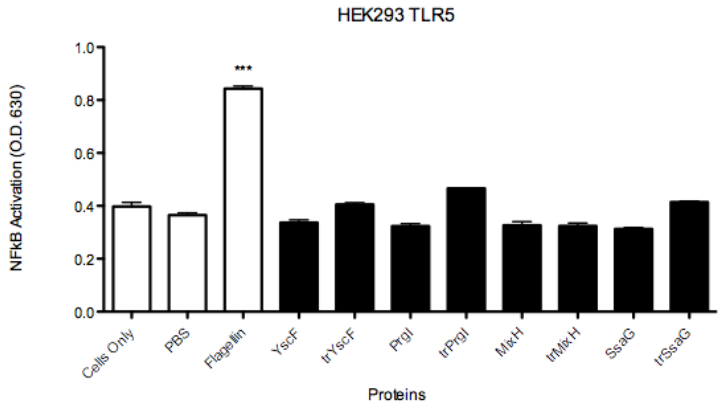

Figure 26. TLR2 and/or TLR4 are necessary for activation of NF-kB by needle proteins in HEK293 cells expressing TLR2, TLR4, or TLR5.

HEK cells that express one particular TLR were tested with 1ug/ml of needle proteins dissolved in PBS. HKLM was used as a control for TLR2 (A). LPS was the positive control for TLR4 (B). Flagellin was the positive control for TLR5 (C). SEAP levels were measured as representation of NFkB activation.

Since the HEK293/TLR2 and HEK293/TLR4 cells endogenously express TLR5, we also tested TLR5 expressing HEK 293 cells. TLR5 expressing cells showed no response to any of the needle proteins indicating that the proteins were specifically targeting TLR2 and TLR4 (Figure 26C). Taken together the data support the idea that needle proteins are recognized as a PAMP by TLR2 and potentially TLR4. Additionally, needle proteins from different bacteria trigger responses of varying magnitude and finally that the N-terminus of some needle-proteins alters the responses by TLRs.

TLR2 Antibodies block activation by needle proteins to TLR2 expressing HEK 293 cells

Antibodies to TLR2 were utilized to assess interaction of needle proteins with TLR2 in HEK293/TLR2 cells. Control antibodies and TLR2 antibodies were administered at equal concentration to HEK293 cell cultures. Administration of the isotype control antibodies followed by subsequent incubation with needle proteins (YscF, trYscF, PrgI, trPrgI, MxiH, trMxiH, SsaG, and trSsaG) resulted in no significant decrease to NF-κB/AP-1 activation by the needle proteins (Figure 27). After treatment of the cell cultures with TLR2 antibody and subsequent incubation with needle proteins (Figure 27), NF- κB/AP-1 activation was significantly decreased when compared to control antibody in the case of trYscF, PrgI, trPrgI, MxiH, trMxiH, SsaG, and trSsaG (Figure 27). YscF was not significantly decreased (Figure 27); however, YscF alone had low activation of NF-κB/AP-1 (Figure 27). These results confirm that the needle-proteins are

activating NF-κB and/or AP-1 through TLR2 as neutralization of TLR2 with antibodies abrogated the needle protein induced response in the HEK293/TLR2 cells.

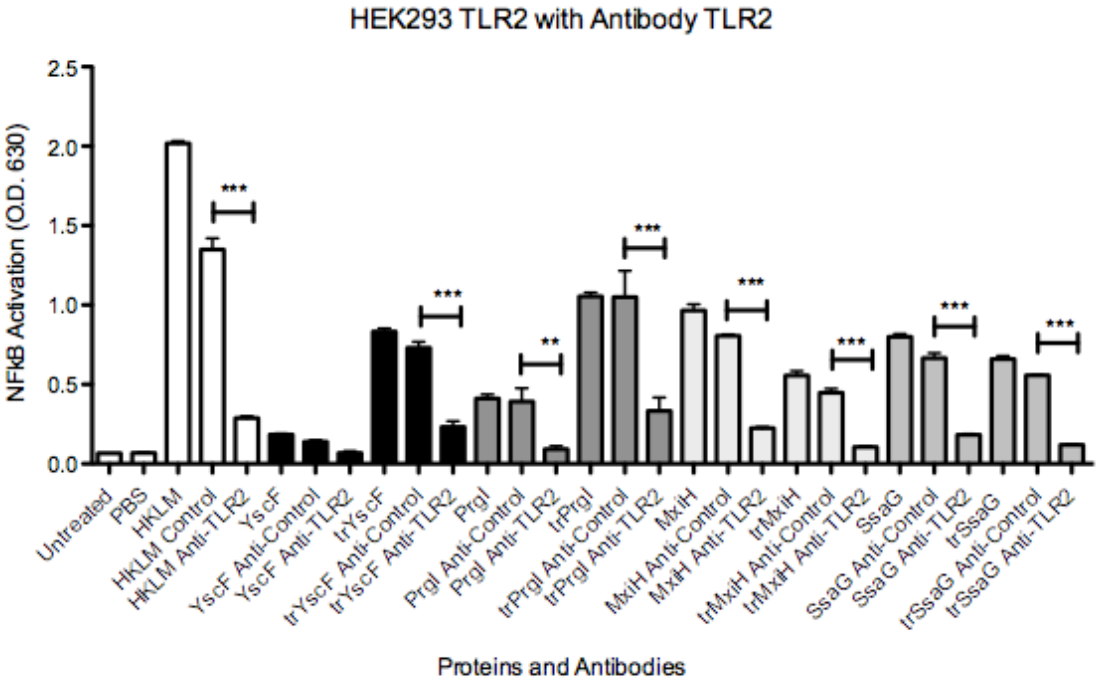

Figure 27. Antibody to TLR2 blocks NF-kB activation in response to needle proteins in HEK293 cells expressing TLR2 cells.

HEK TLR2 cells were treated with HKLM or needle proteins after no treatment, an antibody control, or antibody to TLR2. SEAP levels were measured as representation of NFkB activation.

TLR4 inhibitor CL1-095 inhibits response of THP-1 cells

TLR4 inhibitor CL1-095 was used to treat THP-1 cells prior to treatment with LPS, flagellin, or needle proteins (YscF, trYscF, PrgI, trPrgI, MxiH, trMxiH, SsaG, and trSsaG). Cells left untreated with CL1-095 reacted to LPS or proteins in the same pattern as seen in Figure 28. Those cells treated with CL1-095 did

not react to LPS. Reaction to flagellin was left intact, as expected, and reaction to needle proteins was in all cases significantly reduced, although not quite to basal levels, suggesting that activation of THP-1 cells by needle proteins can be reduced by antagonizing TLR4.

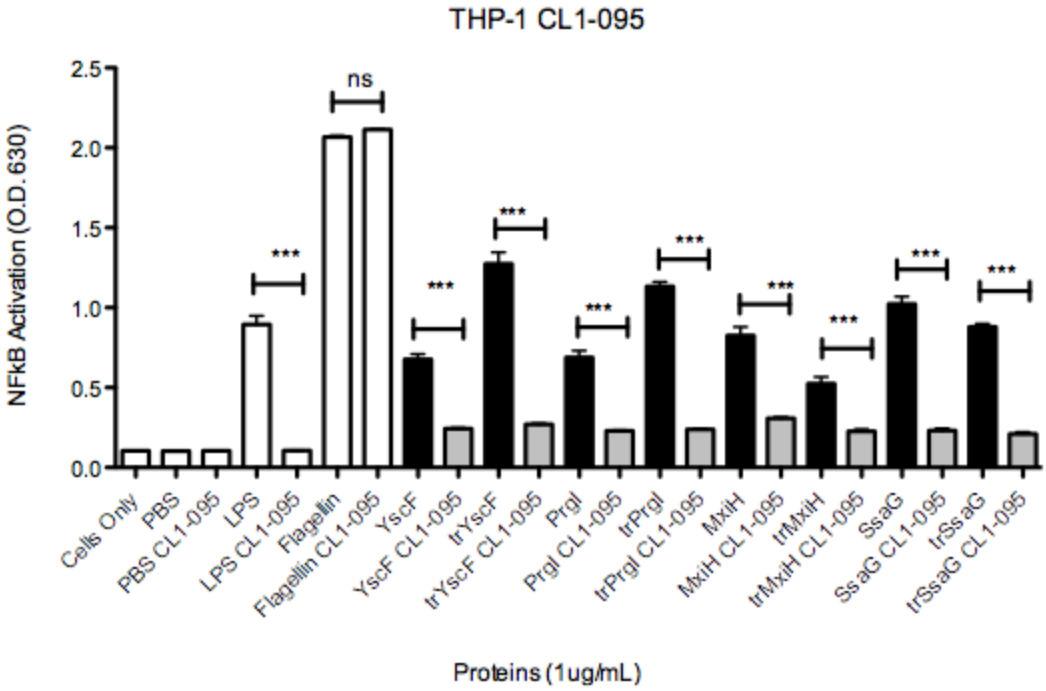

Figure 28. The TLR4 inhibitor CL1-095 blocks activation of NF-kB by needle proteins in THP-1 X-Blue cells.

THP-1 cells were treated with PBS, LPS, Flagellin, or needle proteins. Prior to treatment cells were either left untreated or treated with TLR4 inhibitor CL1-095. SEAP levels were measured as representation of NFkB activation.

TNF-α expression in response to needle proteins

THP-1 X-Blue cells were treated with PBS, LPS, full length and truncated forms of needle proteins. After 5 hours supernatants were collected and assessed via ELISA for TNF-α production. As expected, PBS treated cells produced no TNF-α and LPS treatment led to production of TNF-α (Figure 29).

Truncated forms of YscF produced significantly more TNF-α than full-length forms of YscF, consistent with the assays for NF-κB/AP-1 activation. Full length YscF did induce TNF-α but to a lower extent than trYscF. PrgI induced TNF-α however trPrgI induced slightly more (Figure 29). MxiH induced more TNF-α than trMxiH, which agrees with previous data indicating MxiH activated NF-κB/AP-1 more than trMxiH. SsaG induced more TNF-α than trSsaG, which also correlates with our NF-κB/AP-1 data. These results indicate that the increases in NF-κB activation seen consequentially leads to an increase in TNF-α levels in cell culture supernatants.

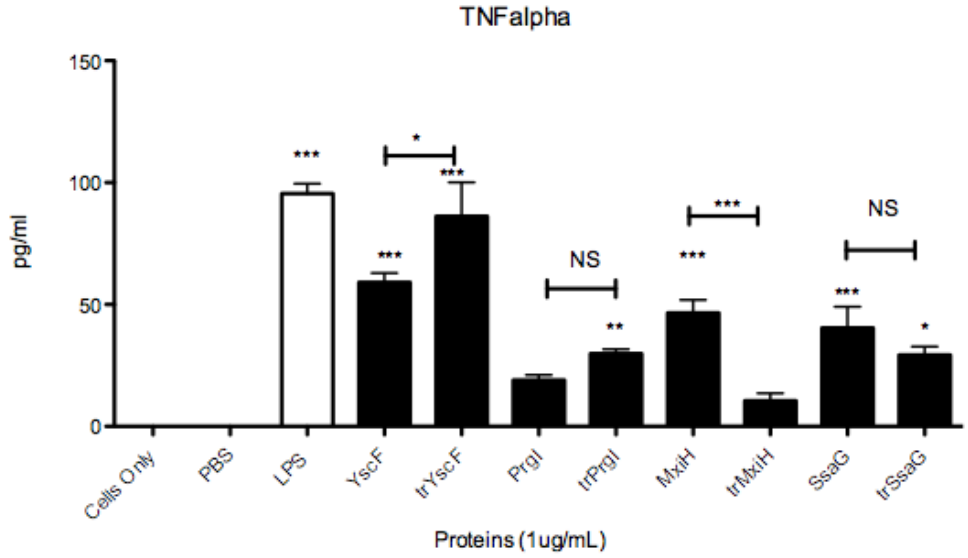

Figure 29. Activation of THP-1 X-Blue cells by needle proteins results in TNF-α secretion.

THP-1 cells were treated with PBS, LPS, or needle proteins. After 5 hours supernatants were collected and tested by ELISA for production of TNFα.

CHAPTER IV

DISCUSSION

To understand and combat disease, a multifaceted approach must be taken to advance our knowledge ahead of the pathogen evolution to evade our treatments. The two projects described here present a multifaceted approach to understanding *Y. pestis* virulence. Bacteria manipulate the host immune system in a targeted manner to cause disease, this manipulation is shown by the modulation of host response by the N-termini of T3SS needle proteins. These manipulations of the host immune system give an advantage to the bacterium. However, gaining understanding of how these manipulations occur can allow science to develop new treatments to contest the bacteria by giving the host immune system the advantage. Currently, new treatments such as small-molecule inhibitors to T3SS, are being developed as anti-virulence therapies in contrast to the traditional anti-metabolic and anti-biosynthetic strategies that fueled the development of modern antibiotics. Inhibiting the T3SS, a major virulence factor in many human gram-negative bacteria, leaves the pathogen unable to modulate the host immune response as effectively and allows the host to mount a successful attack on the bacteria. This method allows the immune system to effectively clear the pathogen itself without affecting the natural microbiome of the host, and also reduces the selective pressure that antibiotics

create (21). N-terminally truncated needle proteins could be developed as adjuvants or immune modulators, again shifting the advantage to the host. If host immune cells can be primed to react with N-terminally truncated needle proteins, then the host could either respond better to vaccinations or jump-start a host response to a current infection. In the current arms race we are engaged in with pathogens, advancement on many fronts will be the key to staying ahead of our adversaries.

Consequently, in researching two aspects of the T3SS, there are implications to the system that each project uniquely uncovers. Compound D, was shown to effectively inhibit the secretion of toxins in a family of T3SSs. This effect was mediated through YopD and possibly LcrQ and LcrH, and also required a secretion active conformational state of the T3SS. Compound D also revealed a functional difference between secretion by calcium blind strains in the presence and absence of calcium. Studies into the host response to needle proteins revealed that these proteins have the ability to activate host cells through TLR2 and TLR4 and that the N-terminus may modulate that interaction.

Compound D

Studies with Compound D show that Yops secretion in *Y. pestis* is inhibited by Compound D. LcrV was secreted prior to the Compound D-mediated blockage of the T3S system, indicating the secretion apparatus is functional due to its ability to secrete LcrV; however, no proteins after LcrV are secreted. This inhibition of Yops secretion requires YopD and results show that YopD was able

to pull Compound D out of solution, implying a direct interaction between the two. LcrQ and LcrH appear to also play a role in inhibition.

YopD is unique when compared to other identified targets of T3S systems, such as LcrF, ExsA, YscN and other suspected proteins of the T3S apparatus or membrane rings (34, 41, 43, 100, 106). These targeted proteins alter the T3S basal structural components or expression of the apparatus genes, as opposed to YopD, a translocon protein, presumably an early secreted protein also known to play a role in the regulation of expression of other Yops (14). YopD's regulatory role is shared with its chaperone LcrH, which is also needed for effective delivery of YopD to the secretion apparatus (37). The regulatory role of YopD and LcrH revolves around Yops translation. This protein pair may interact with mRNA of Yops and prevent their translation (20). Thus, when either of these proteins is deleted, Yop expression increases (37). However, the involvement of YopD/LcrH in Yop expression is difficult to separate from secretion induced Yop expression (32). Therefore, to elucidate whether Compound D was affecting regulation or simply physically blocking secretion becomes difficult.

In order to begin clarifying the mechanism of Compound D inhibition, several factors were investigated. Beginning at the level of transcription, a YopE-chimera, expressed under the control of a non-LCR regulated promoter, was not secreted in the presence of Compound D, implying the inhibitor is able to block secretion of a protein not under LCR control. The YopE-chimera was produced using the *araBAD*$_p$ promoter, creating a large cellular pool of the protein, which

still could not overcome the inhibition of Compound D, suggesting that a physical blockage of the apparatus is possible. This result suggests that Compound D is working at the level of secretion and not at the level of LCR regulation. The hyper production of Yops in a YopD mutant presents the idea that Compound D could be overwhelmed by YopD production to restore Yops secretion despite the presence of the inhibitor. This assertion proved false, requiring co-overexpression of YopD and its chaperone LcrH, and suggests YopD may not be the only protein required for Compound D mediated secretion inhibition.

The involvement of LcrH and LcrQ is perplexing in that both proteins are known to play multiple roles that are hard to distinguish from each other and YopD in deletion mutants. LcrH is known to be required not only for regulation but also for effective YopD and YopB delivery to the secretion apparatus. Therefore, the result of inhibition of YopE secretion, but not YopM secretion, by Compound D in the *lcrH* mutant can be interpreted multiple ways. Due to the regulatory role LcrH performs in combination with YopD in decreasing Yops translation, the lack of LcrH could affect the production of Yops. Although, this effect was only seen on YopE, since YopM was produced and secreted in this mutant. These results imply that either LcrH/YopD regulation does not equally affect all Yops or that the regulatory role is not significant for Compound D inhibition. The lack of LcrH could also affect YopD's delivery to the apparatus, although this is also confounded by the secretion of YopM but not YopE. Notably, overexpression of LcrH in figures 13 and 14 caused a dramatic decrease in cellular expression of Yops and subsequently no secretion. This was not in

conjunction with Compound D as this occurred in the absence of the inhibitor. Compound D inhibition occurs through YopD but is affected by LcrH, and in a non-LCR dependent manner.

LcrQ is also proposed to have multiple functions in the T3S system. Wulff-Strobel et al. propose that LcrQ plays a role in regulation of Yops secretion at the level of the "ysc gate" (110). This regulatory function is thought to determine substrate specificity at the gate of the apparatus, affecting the hierarchy of Yops secretion (110). If Compound D is affecting YopD and preventing secretion at the site of the "Ysc gate", it is possible that the lack of LcrQ at the gate may alter the mechanism of inhibition. However, others propose that LcrQ function is similar to YopD/LcrH function to regulate Yop production (19, 85). Similar to the *lcrH* mutant, Compound D's effect on the *lcrQ* mutant was more pronounced on YopE than YopM. It is note worthy that YopM does not require a chaperone while YopE does (104). Whether chaperones play a factor in secretion of Yops at the "ysc gate" has yet to be elucidated; although their role in determining the secretion hierarchy has been implicated by Boyd et al (9). Therefore, the results of LcrH and LcrQ affecting the production of YopE support the idea of a regulatory role being affected by Compound D to inhibit secretion. However, the lack of effect on YopM production could imply regulation by these proteins is either not equal, or the involvement of LcrH and LcrQ in inhibition by Compound D is much more complex and requires further study.

The ability of Compound D to affect effector secretion in *Pseudomonas aeruginosa* argues that the inhibition of Yops secretion by Compound D is not

directed at the regulatory effects of YopD. Broms et al. showed that PopD was unable to substitute for YopD in the *Yersinia* system due to its lack of regulatory function (14). Compound D's ability to affect secretion in *P. aeruginosa* indicates the regulatory function is not required for inhibiting secretion. These results suggest Compound D does not affect the regulatory role YopD plays in secretion but rather that Compound D works at the level of secretion through YopD.

The calcium blind strains, caused by disrupted regulation, exposed to Compound D revealed that the inhibitor could only successfully inhibit secretion with these particular strains in the environment lacking calcium. However, a calcium blind YscF D46A mutant strain indicated this was not due to a calcium effect on Compound D but likely due to a change in the secretion system. These results support the hypothesis that the removal of calcium results in a functional change in the Ysc apparatus to allow secretion and that this change involves the needle protein YscF (8). Torruellas et al. discovered mutations in YscF that allow for constitutive secretion, implying that the needle protein plays a role in calcium sensing or transmission of the calcium sensing signal to the basal structure (102). In the case of regulatory calcium blind strains (e.g. loss of LcrG or YopN function), the ability of Compound D to work in one environment and not the other implies that secretion in the presence and absence of calcium do not occur in the same manner. The removal of the regulator (LcrG or YopN) alone allows secretion by the system in the presence of calcium. While in the absence of calcium, a change in the secretion apparatus still occurs through the calcium sensing mechanism; Compound D may affect that signaling. The requirement of

an “active secretion state” for Compound D to inhibit secretion implies the mechanism of inhibition occurs at the apparatus and not at the regulatory roles of YopD, LcrH, or LcrQ.

The proposed model of inhibition by Compound D includes an interaction with YopD, which is affected by LcrH and LcrQ, and does not involve secretion regulatory proteins LcrG, LcrV, YopN, YscB, SycN. Inhibition of the T3SS can still occur for proteins not under LCR expression and regulation. The inhibition does require the sensing of calcium and the subsequent alteration in the needle apparatus. Given these results we propose the following model of inhibition by Compound D, where Compound D’s effect occurs at the site of secretion (Figure 30). The elucidation of detached control upon secretion in calcium blind strains led us to develop a separate model (Figure 31). In this model of the calcium blind strains, secretion occurs despite the absence of a signal in the presence of calcium, while in the YscF D46A the mutation signals for secretion in both calcium environments, which allows Compound D to inhibit secretion in both cases.

Interestingly the T3SS inhibitor Compound 2, an isoform of Compound D, discovered by Pan et al. (83) does not work in the same manner. Deletion of neither translocon proteins, YopD or YopB, alleviated Compound 2’s ability to inhibit secretion, nor did Compound 2 have the same effect on constitutive secretors, as it inhibited both in the presence and absence of calcium. Although

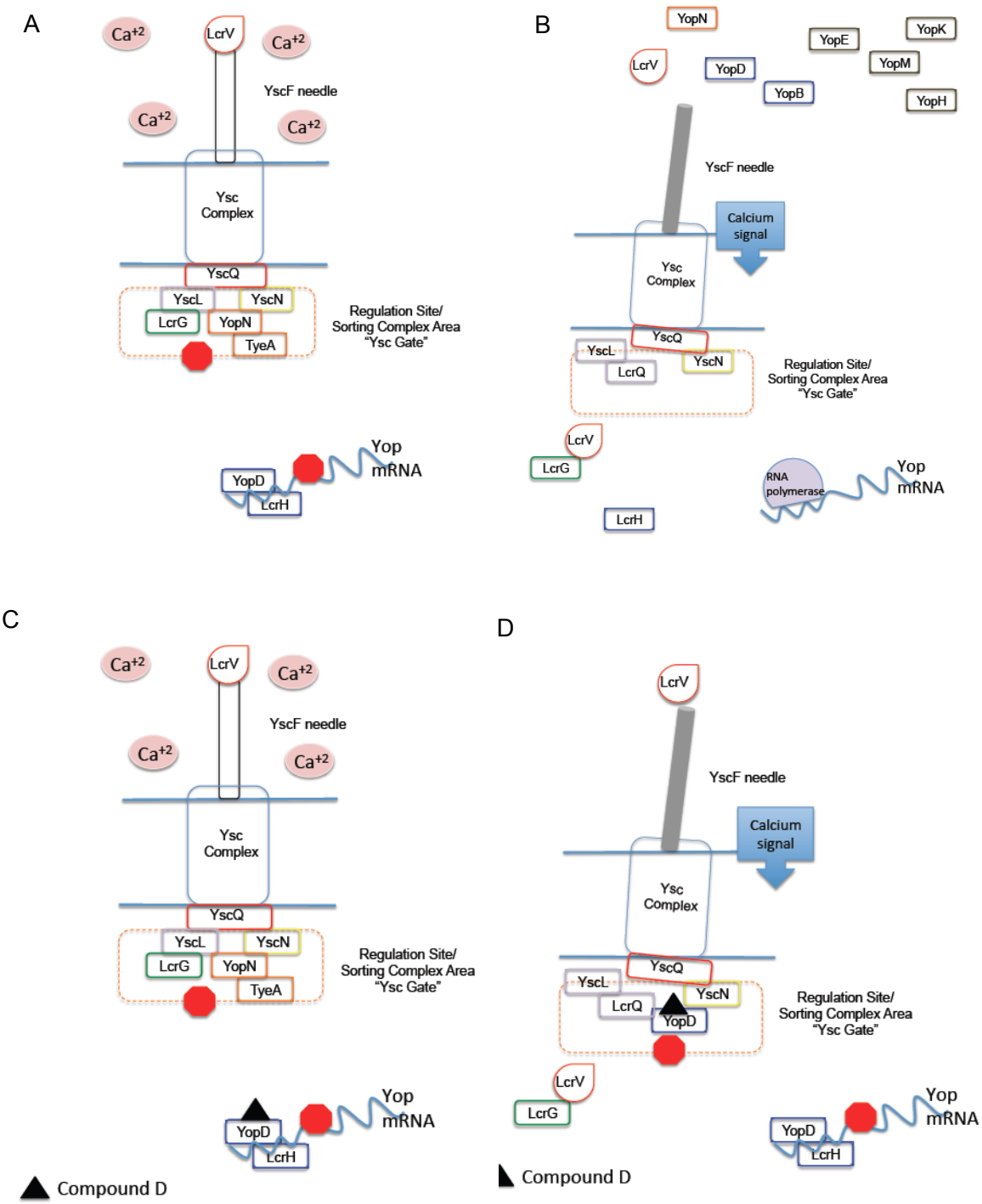

Figure 30. Mechanism for Compound D inhibition in *Yersinia pestis* T3SS

(A) Diagram of T3SS in the presence of calcium. Secretion is regulated by LcrG and YopN while Yop translation is prevented by YopD/LcrH and no secretion occurs (B) Diagram of T3SS in the absence of calcium. Calcium signal initiates secretion, LcrG is titrated away by LcrV, YopN is secreted, Yop translation is released from YopD/LcrH and Yops are secreted into medium. (C) Compound D is present however secretion is regulated by LcrG and YopN while Yop translation is prevented by YopD/LcrH and no secretion occurs. (D) In the presence of Compound D the calcium signal initiates secretion however YopD/Compound D blocks secretion at the “Ysc gate”. Translation of Yops is still inhibited because YopD levels are not lowered by secretion.

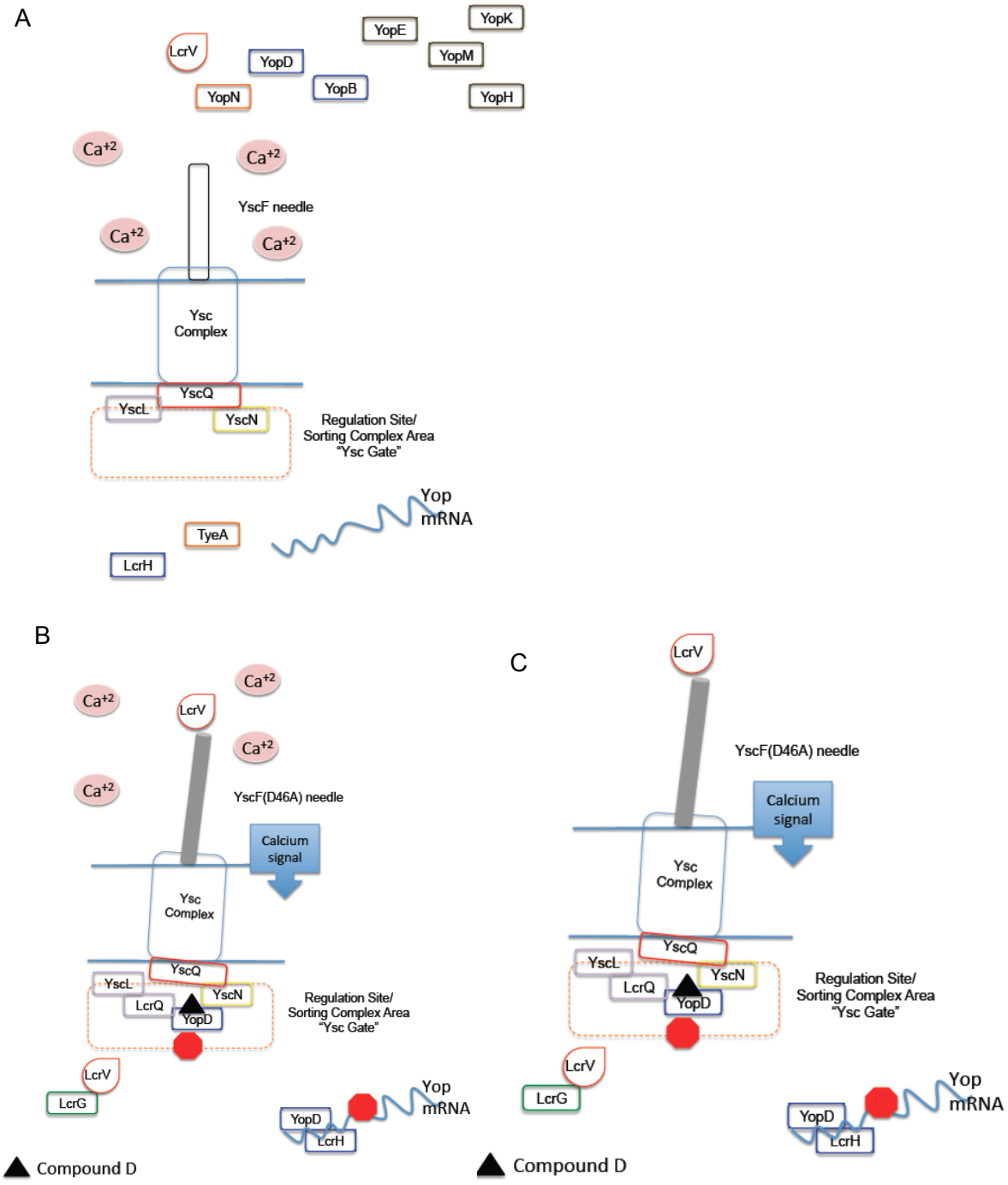

Figure 31. Mechanism for Compound D inhibition of calcium blind *Yersinia pestis* T3SS strains.

(A) Calcium blind strains lacking LcrG/YopN secrete Yops into the medium despite the presence of calcium and lack of a secretion signal. (B-C) YscF(D46A) is mutated to a locked position of sending a calcium signal, in the presence of Compound D this allows secretion to be inhibited no matter if calcium is present or not.

both compounds are able to target the T3SS in multiple strains of bacteria, they fascinatingly inhibit secretion in completely separate ways.

Unfortunately both compounds were found to be toxic: Compound 2 caused cell rounding and detachment and LDH release (83) and high serum protein binding (1), while Compound D was capable of lysing red-blood cells making translocation analysis in the presence of the inhibitor impossible. This also means these two compounds without modification would be poor candidates as potential antimicrobials. However, the unique manner in which Compound D inhibits secretion may lead us to a greater understanding of the complex regulation of this system and give insight on the deeply intertwined structural role the apparatus plays in regulation of secretion.

Host response to T3SS needle proteins

Characterization of the needle protein's (MxiH, SsaG, YscF, and PrgI) ability to induce innate immune factors revealed: 1) NF-kB/AP-1 is activated by needle proteins or portions of needle proteins. 2) Activation of NF-kB/AP-1 by needle proteins is dependent upon MyD88. 3) Activation occurred exclusively through TLR2 and TLR4. 4) Observed variation in the N-terminus of needle proteins appears to modify the interaction with TLRs.

YscF comes from the T3SS of bacteria that have anti-inflammatory infection objectives. This needle protein elicits lower levels of NF-kB/AP-1 activation and TNF-α; however, the N-terminally truncated forms have the

opposite effect and induce higher NF-kB/AP-1 activation as well as TNF-α. Interestingly, a similar phenomenon is seen with flagellin and its homologs. The N-terminus of flagellin is involved in immune evasion by some bacteria (*Helicobacter, Campylobacter* and *Bartonella)* (3).

Shigella and *Salmonella* are known to cause largely pro-inflammatory responses to the host in order to cause disease (52, 86). The *Shigella* needle-protein, MxiH, acted in accordance to this overarching goal of infection; as it induced increased NF-kB/AP-1 and TNF-α as a full-length protein. Removal of the N-terminus of MxiH actually attenuated the cellular response, indicating that the N-terminus positively adds to the pro-inflammatory environment by activating TLRs. Unexpectedly, PrgI acted similarly to YscF, less inflammatory in its natural state. It is possible other factors play a larger role in the fine-tuned pro-inflammatory response to *Salmonella.*

The *Salmonella* SPI-2 needle protein SsaG more equally activated in its full-length form and after N-terminal truncation. SsaG is already "naturally" truncated when compared to other needle proteins. Under our hypothesis that the N-termini of needle proteins modulate TLR interaction, there would be no need for an extended N-terminus because SsaG is not exposed to TLRs expressed on the outside of the host cell, since SPI-2 is only expressed once *Salmonella* is enclosed in the *Salmonella* Containing Vacuole (13) inside the host cell. Exposure to host TLRs may create pressures for the N-terminus of needle proteins to modulate host responses.

TLR4 is already known to play a unique role in *Yersinia pestis* pathogenesis because *Yersinia pestis* produces a tetra-acylated LPS (53). This change in acylation results in weaker TLR4 stimulation and subsequent deficient activation of the immune system, keeping the bacteria under the host's radar (101). TLR4's role in recognition of needle proteins was less expected than the more promiscuous TLR2. However, there are several documented cases of TLR4 interaction with pathogen associated substrates other than LPS: Respiratory Syncytial Virus (RSV) fusion protein (89), chlamydial Hsp60 (17), pneumolysin (64), *Francisella tularensis* DnaK (4), and Ebola virus glycoprotein (81), and cell wall components from *Pseudallescheria boydii* (35). Many of these pathogen associated molecular patterns (PAMPs) are well characterized as to the exact method of interaction with TLR4, and future research in our lab will hopefully elucidate how needle proteins interact with TLR4, including whether MD-2 or CD-14 is required for this interaction.

The Neisserial porin, PorB, is also a surface exposed protein. PorB is highly conserved among *Neisseria* species, except for the surface exposed loops. Much like T3S needle proteins, PorB, specifically the exposed loops, were found to interact with TLR2. The interaction with TLR2 was found to be dependent on specific amino acids and the variation between PorB of different *Neisseria* species creates unique "TLR2 binding signatures". Specific binding signatures were found to be more or less inflammatory through interaction with TLR2 (61, 65, 103). We propose that the T3SS needle proteins N-terminal variation between species acts similar to PorB, in that the unique surface

exposed N-terminus creates a unique binding signature, which modulates the host response to the bacteria.

Currently, we do not know the specifics of which amino acid sequences play a role in interactions with TLRs. We cannot at this time rule out the possibility that the binding signature is created by a change in protein structure, which could also be affected by the his-tag, as opposed to specific amino acid sequences. However, this research does describe a new class of PAMP that interacts with TLRs in an unexpected manner. This discovery has the potential to increase our understanding of host responses to T3S utilizing pathogens and to extend our knowledge of how other host processes may interact with T3S apparatuses.

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