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Assembly and Function of the *Yersinia pestis* YscKLQ Complex

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UNIVERSITY OF MIAMI

ASSEMBLY AND FUNCTION OF THE YERSINIA PESTIS YSCKLQ COMPLEX

By

Kristian L. Richards

A THESIS

Submitted to the Faculty
of the University of Miami
in partial fulfillment of the requirements for
the degree of Master of Science

Coral Gables, Florida

May 2012

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PESTIS YSCKLQ COMPLEX

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Assembly and Function of the
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Yersinia pestis, the ethological agent of plague, uses the type III secretion system (T3SS) to inject effector proteins into eukaryotic cells. Effector proteins termed *Yersinia* outer proteins (Yops) cause cytotoxicity in host cells and apoptosis in macrophages. The T3S apparatus is a complex injectisome composed of 21 essential *Yersinia* secretion (Ysc) proteins. T3S substrates are targeted for secretion by secretion signals and/or chaperone binding domains. How these substrates interact with the T3S apparatus is not known. In a recent study in *Salmonella* they found a “sorting platform” composed of the homologs to YscQ, YscK, and YscL in *Y. pestis* targets substrate/chaperone complexes to the T3S apparatus for orderly secretion. We hypothesize that a similar sorting complex exists in *Y. pestis* and is composed of a YscKLO complex and possibly the YscN ATPase and interacts with the N-terminal portion of YscD to deliver substrate/chaperone complexes to the T3S apparatus for secretion. In this study, I investigate the YscKLO complex and its interactions with the N-terminal portion of YscD and substrate/chaperone complexes. We found that the N-terminal portion of YscD interacts directly or indirectly with YscQ and YscK and that YscK interacts with the components of the sorting complex, YscQ and YscL, along with T3S substrates. Since the needle type substrate, YscF in *Y. pestis*, was not looked at in the *Salmonella* study, I decided to further investigate YscF and its two chaperones YscE and YscG. We found that the YscE/YscG chaperone is not only important for the stabilization of YscF but also for the secretion of YscF.

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Chapter One: Introduction

Plague and transmission

Yersinia spp. are gram-negative bacteria of the family *Enterobacteriaceae* (59). Three species are pathogenic to humans: *Yersinia pseudotuberculosis*, *Yersinia enterocolitica*, and *Yersinia pestis*. *Y. pseudotuberculosis* and *Y. enterocolitica* cause localized intestinal diseases. *Y. enterocolitica* causes watery or bloody diarrhea with fever. *Y. pseudotuberculosis* causes symptoms similar to infections with *Y. enterocolitica* but generally without the presence of diarrhea. These bacteria can also mimic the symptoms of acute appendicitis (34). *Y. pestis* is the etiological agent of plague (44). The plague has caused at least three massive pandemics that include the Black Death pandemic that swept Europe in the 14th century and killed a large percentage of the population of Europe. Previously some have questioned whether *Y. pestis* or possibly another pathogen was responsible for the massive numbers of deaths that occurred during this pandemic. However, a recent investigation has confirmed the presence of *Y. pestis* DNA in victims of the Black Death and a draft of the genomic DNA sequence from the bacteria isolated from these individuals has been obtained (5). Although the massive killings by the plague occurred many centuries ago, *Y. pestis*, the agent of plague, is still present in many areas of the world including areas of Africa, Asia, and the western United States (23). Plague is a flea-borne systemic disease of rodents and other mammals and there are two main forms of the disease: bubonic plague and pneumonic plague (44). Bubonic plague is transmitted by fleas and is initiated with the bite of an infected flea (24). The flea initially becomes infected upon feeding on an infected mammal, typically a small rodent. The bacteria replicate in the flea mid-gut where they

form bio-film associated aggregates (25). These aggregates bind to chitin-coated spines in the flea proventriculus and block the feeding tube of the flea. This block inhibits ingested blood from reaching the stomach of the flea. This causes the flea to bite other mammals continuously in search of a blood meal. When a blocked flea bites a mammalian host, the blood from the host will mix with the bacterial aggregates inside the flea, and is then regurgitated back into the host thus infecting it with the bacteria. Sometimes an infected flea will bite a human host and transmit the bacteria. Once inside the host, the bacteria can remain free (extracellular) or they can be engulfed by host phagocytic cells (50). These bacteria are initially transported to the regional lymph nodes. In the lymph nodes they multiply and initiate the formation of an inflamed and swollen lymph node termed a bubo (hence the name bubonic plague) (44). The bacteria initially replicate in the lymph node but eventually the bacteria, which are now primarily extracellular, enter the bloodstream where they travel to the liver and spleen. If untreated, the bacteria will replicate in the bloodstream and other organs and eventually overwhelm the host with massive bacterial growth often resulting in death. The other form of plague, pneumonic plague, is acquired through aerosolized bacteria which are inhaled into the lungs (51). The infection initiates in the lungs and spreads from there and is almost always fatal. The ability of *Y. pestis* to infect humans via aerosols, to be spread from human to human, and to cause rapid death has prompted the CDC to classify *Y. pestis* as a category A bioterrorism agent (9).

Infection and Effector Proteins

During an infection, *Y. pestis* is predominantly found extracellularly due to its ability to actively block phagocytosis by host macrophages and neutrophils (44).

Yersinia spp. use a specialized protein secretion system termed a type III secretion (T3S) system to inject virulence proteins termed effector proteins into eukaryotic cells (20).

The T3S system (T3SS) is also seen in many other gram-negative bacterial pathogens including pathogenic species of *Salmonella*, *Chlamydia*, and *Shigella*. A functional T3SS is also a component of the bacterial flagellum and is required for the secretion and assembly of the flagellar rod, hook, and filament structures (40). A complete and functional T3SS is critical for the virulence of all three human pathogenic yersiniae (36).

The injected effector proteins expressed by *Yersinia spp.* function to block bacterial phagocytosis and suppress the production of pro-inflammatory cytokines (21, 56). These effector proteins are termed *Yersinia* outer proteins (Yops). Yops can also cause cytotoxicity of many host cell types and apoptosis in macrophages. There are six identified effector Yops (7). YopH is a phosphotyrosine phosphatase (PTPase). YopH disrupts focal adhesions by dephosphorylating p130_{cas} which is a focal adhesion protein (46). YopE acts as a GTPase-activating protein (GAP) and turns off RhoA, Rac, and Cdc42 (2). YopT depolymerizes actin and modifies Rho-family proteins (28). YpkA (known as YopO in *Y. enterocolitica*) effects the cytoskeleton (15). YopJ counteracts the pro-inflammatory response and can induce apoptosis in macrophages (43). Finally YopM, a leucine-rich-repeat (LRR) protein, migrates to the nucleus of infected eukaryotic cells but its effect on the host is unknown (54).

The *Yersinia* T3SS is encoded on an approximately 70 kDa plasmid, termed pCD1 in *Y. pestis* (45). The expression of T3SS genes is tightly regulated by temperature and extracellular calcium (26). The AraC family transcriptional activator protein LcrF is required for the transcription of plasmid pCD1 T3SS genes. These genes are expressed poorly at the body temperatures of a flea (27°C), explaining why the T3SS is not required to infect the flea, but are highly expressed at mammalian body temperature (37°C). The chromosomally-encoded histone-like protein, YmoA, prevents expression of T3SS genes at 27°C. At 37°C, YmoA is rapidly degraded by the ClpXP and Lon proteases allowing for the expression of T3SS genes (30). The injection of Yops is regulated by host cell contact *in vivo* and by the level of extracellular calcium *in vitro*. The presence of millimolar levels of extracellular calcium blocks the secretion of the Yops *in vitro* (55). Importantly, the concentration of extracellular calcium present in the blood and tissues of the host is sufficient to prevent the secretion of Yops until the bacteria make contact with a eukaryotic cell. A complex composed of the secreted YopN protein, TyeA, and the SycN/YscB chaperone is required to prevent effector Yop secretion in the presence of calcium and before contact is made with a eukaryotic cell (11). These proteins are thought to block secretion at the base of the T3S apparatus (18). In addition, the extracellular needle structure composed of the YscF protein is also required to control the secretion of the Yop effector proteins (58). The YscF needle structure has been hypothesized to function as a sensor that detects extracellular calcium levels and/or host cell contact and controls the secretion of effector proteins in cooperation with the YopN/TyeA/SycN/YscB complex. Effector Yops are injected when the bacteria makes contact with a host cell.

Type III Secretion Apparatus

Yops are injected into eukaryotic cells via the T3S apparatus. The T3S apparatus in *Y. pestis* is composed of a needle-like structure and a base structure. The needle-like portion extends 40 to 60 nm from the bacterial cell surface and is topped by a needle tip (42). The needle tip is composed of LcrV which is a secreted protein. The tip is believed to help assembly of a pore-forming translocon composed of the secreted YopB and YopD proteins upon contact with a eukaryotic cell. There are 21 essential *Yersinia* secretion (Ysc) proteins that are required to assemble a functional T3S apparatus (Figure 1.1) (19).

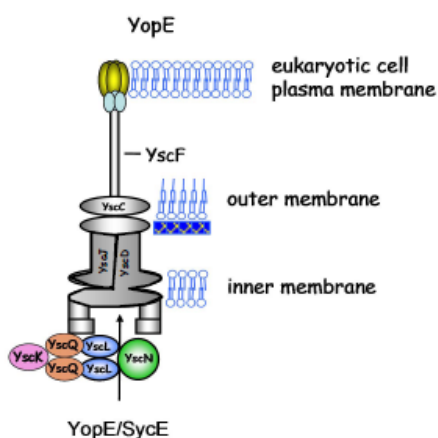


Fig. 1.1 *Y.pestis* type III secretion apparatus

The T3S apparatus is a complex structure assembled from multiple Ysc proteins. The first portion constructed is the base structure composed of YscC in the outer membrane and YscJ and YscD in the inner membrane (33). Of these components, YscD is the only one that has a significant cytosolic domain (53). YscD has been predicted to have a single transmembrane domain (residues 122 to 142), an N-terminal cytoplasmic region (residues 1 to 121), and a large periplasmic region (143 to 419). The base structure is hypothesized to surround an inner membrane secretion complex composed of YscR, YscS, YscT, YscU, and YscV (14). These inner membrane proteins are believed to form

a hole or channel through the inner membrane that serves as a passage for the secreted proteins. A complex similar to the C-ring structure in flagella is hypothesized to build onto the cytosolic domain of YscD. The cytoplasmic ring or C-ring is predicted to be composed of YscQ, which is homologous to the flagellar C-ring component FliN (31). YscQ is also predicted to interact with YscL and YscK. YscL also interacts with YscN, an ATPase required to unfold effector proteins prior to their secretion (29). YscL serves as a negative regulator of YscN ATPase activity (52). Much is still unknown about the assembly and function of these components. Once the T3S apparatus fully assembles, the apparatus becomes competent for secretion. The first class of substrates secreted is needle-type or early T3S substrates (1). The needle protein YscF is stabilized by its chaperones YscE and YscG (57). The YscE/YscG chaperone also prevents YscF from prematurely polymerizing prior to secretion. Needle assembly is triggered by the secretion of YscF. The length of the needle is regulated by the secreted protein YscP (32). YscP is a molecular ruler that determines the length of the needle and triggers a switch in substrate specificity through a direct interaction with the cytoplasmic domain of YscU, which occurs only when the needle reaches its proper length. Finally, after the switch to middle and late T3S substrates, the YopN/TyeA/SycN/YscB complex is targeted to the T3S apparatus (18). It functions to prevent effector Yop secretion until cell contact is made.

T3S substrates and chaperones

T3S substrates are targeted to the apparatus by secretion signals or chaperone binding domains and chaperones (38). Secretion signals are located at the N-terminus of the substrates within amino acids 2 to 15 (Figure 1.2).

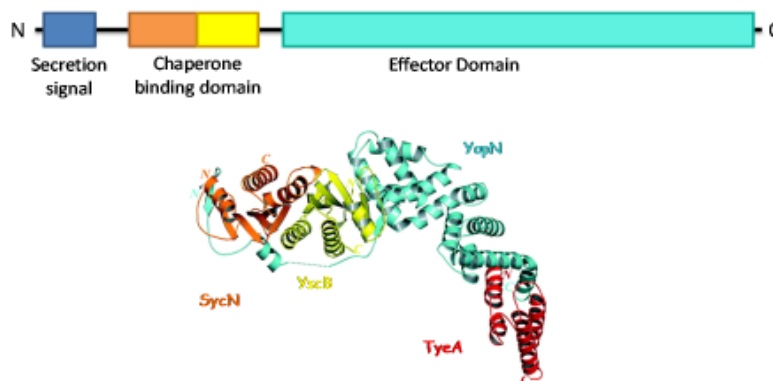


Fig. 1.2 Example that is of a T3S substrate. The T3S substrate YopN contains an N-terminal secretion signal followed by a chaperone binding domain (CBD) where its two chaperones, SycN and YscB, bind. The YopN CBD wraps around SycN/YscB chaperone keeping YopN partially unfolded and secretion competent.

These signals are not removed following export. How these signals are recognized by the T3S apparatus is still not understood but they tend to be unstructured and amphipathic in nature. T3S chaperones also play a role in targeting T3S substrates for secretion.

Chaperone binding domains are normally located following the secretion signal in the N-terminal region of the substrate (Fig. 1.2) (17). T3S chaperones have multiple roles such as preventing aggregation of substrates, preventing degradation of substrates, targeting substrates to the T3S apparatus, and preventing premature substrate interactions before secretion. Chaperones bind the chaperone binding domain of their substrates and keep them partially unfolded and secretion competent. There are three types of chaperones (17). Class I chaperones are effector-type chaperones such as SycH for YopH, SycE for YopE, and SycN and SycB for YopN. Class II chaperones are translocator-type

chaperones such as LcrH (SycD) for YopB and YopD. Class III chaperones are unique to the needle substrate, such as YscE and YscG for YscF. It is still unknown what part or component of the T3S apparatus binds or recognizes substrate/chaperone complexes. Previous studies in *Salmonella* suggested that the T3S ATPase (YscN) may recognize and unfold T3S substrates (3). Recently, Lara-Tejero *et al.* (35) published that the homologs of YscQ, YscK, and YscL in *Salmonella enterica* serovar Typhimurium (SpaO, OrgA, and OrgB respectively) interact to form a high molecular weight complex that serves as a “sorting platform” that orderly delivers T3S substrates to the T3S apparatus. This complex is formed independent of the needle complex and bacterial envelope components of the T3SS. The *Salmonella* ATPase InvC interacts with this complex but was not required for the formation of this complex. At this time no studies on the role of the YscQ, YscK, and YscL proteins in the formation of a sorting complex or the secretion of T3S substrates in *Yersinia spp.* has been investigated. We hypothesize that T3S substrate/chaperone complexes are targeted for secretion via interaction with a YscKLQ sorting complex and/or with the YscN ATPase. My studies were designed to investigate the assembly of YscK, YscL, and YscQ proteins, their interaction with other T3S components, and T3S substrate/chaperone complexes. In particular, I was interested in examining the interaction of these proteins with the early substrate YscF, the needle subunit protein, and its chaperones YscE and YscG.

Chapter Two: Results

YscD, YscQ, YscK, and YscL form a high molecular weight complex

Previous studies in our lab conducted using the yeast two-hybrid system showed that YscL interacts with YscN and YscQ, and that YscQ can interact with both YscL and YscK (29). In a yeast three-hybrid system, YscQ was able to bring together YscK and YscL while YscL was able to bring together YscN and YscQ. Thus, these components are able to interact with each other and form an ordered YscN-YscL-YscQ-YscK complex. In *Salmonella* it was recently reported that these components interacted to form a high molecular weight complex (35); however, the details of sorting complex assembly have not been investigated in any T3SS. We hypothesized that YscQ, YscK, and YscL might interact not only with each other but also with themselves to form a high molecular weight complex that might build on to the N-terminal portion of YscD in the cytoplasm (Figure 2.1).

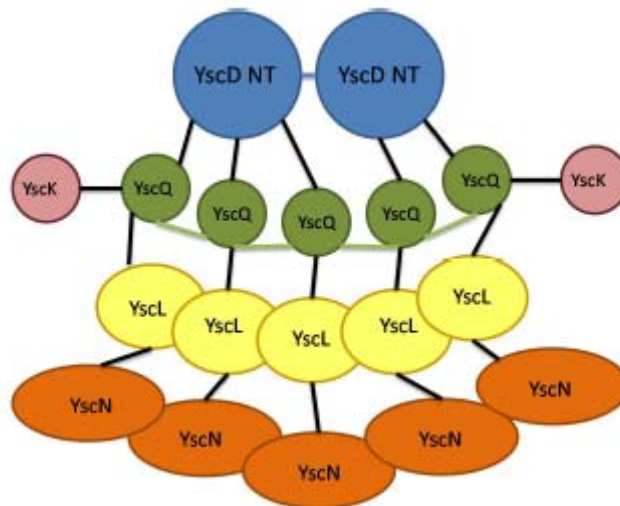


Fig. 2.1. Interaction Model of YscD-YscQ-YscK-YscL-YscN high molecular weight complex

A bacterial LexA-based one hybrid system was used to investigate the ability of YscD, YscK, YscQ, YscL, and YscN to recognize and interact with themselves (8). Previously constructed vectors encoding LexA DNA binding domain-Ysc protein hybrids (LexA-YscD_{NT}, -YscK, -YscQ, and -YscL hybrid proteins) were used to investigate the ability of these Ysc proteins to dimerize/multimerize. If the protein of interest binds to itself then the LexA DNA binding domain is brought together, can bind to DNA, and function as a repressor for a chromosomally-encoded β -galactosidase (*lacZ*) gene in *E. coli*. Thus, if the protein dimerizes or multimerizes there will be a decrease in β -galactosidase activity, measured in Miller Units (MU). Through this assay, it was confirmed that YscQ interacted with YscQ, YscL interacts with YscL, and the N-terminal portion of YscD interacts with the N-terminal portion of YscD. However, in this assay no interaction of YscK with YscK could be detected (Figure 2.2).

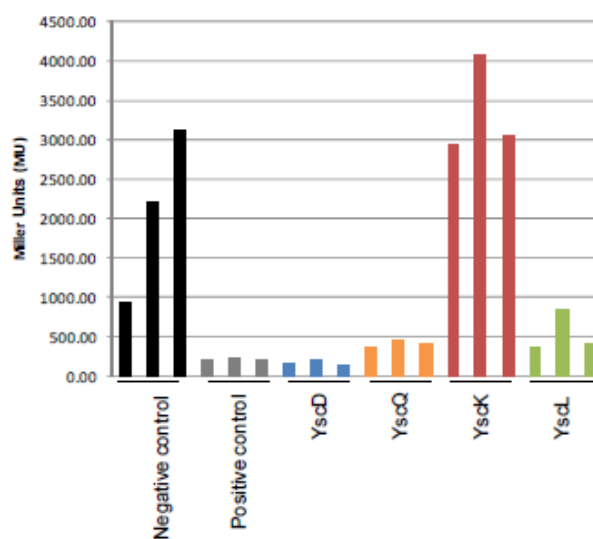


Fig. 2.2. The cytosolic portion of YscD and full-length YscQ and YscL but not YscK multimerize. *E. coli* SU101 carrying pSR658 vector alone, positive control pDD506, pSR658-YscD₂₋₁₂₁, pSR658-YscK, and pSR658-YscL were grown at 30°C. Dimerization or multimerization of LexA fusion proteins were analyzed by measuring their ability to repress β -galactosidase expression.

These studies show that in addition to the known interactions of the YscK, YscQ, YscL, and YscN proteins with each other, the N-terminal domain of YscD, YscQ, and YscL all appear to dimerize or multimerize. These studies suggest that a combination of protein-protein interactions and self-interactions is responsible for the assembly of a high molecular weight complex composed of YscK, YscQ, YscL, and YscN. This complex could interact with the T3S apparatus via interactions with the cytoplasmic portion of YscD (the only component of the base structure of the T3S apparatus that has a cytoplasmic domain) (Fig. 2.1).

The C-terminal portion of YscQ is required for dimer formation

Previous studies in *Shigella* and *Chlamydia* show that homologs of YscQ interact to form dimers (31, 41). Thus, we wanted to investigate the dimerization of YscQ in *Y. pestis* further. To locate the region of YscQ required for dimerization we conducted a deletion analysis of *yscQ* of plasmid pLexA-YscQ_{FL}, which encodes the LexA DNA-binding domain fused to full-length YscQ, and analyzed the deleted constructs using a LexA based bacterial one-hybrid system (8). Constructs removing the coding sequence for the N-terminal 25, 150, or even 200 amino acids of YscQ had little or no effect on YscQ dimerization or multimerization. However, removing as few as 57 amino acids from the C-terminal portion of YscQ abolished dimerization/multimerization (Figure 2.3).

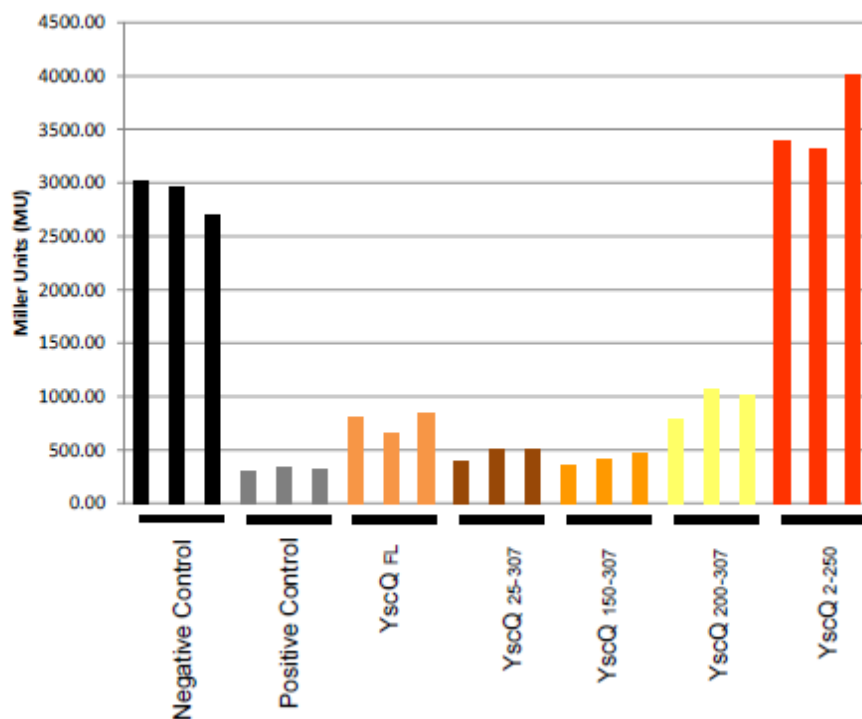


Fig. 2.3. The C-terminal region of YscQ is needed for dimerization. *E. coli* SU101 carrying pSR658 vector alone, positive control pDD506, pSR658-YscQ_{FL}, pSR658-YscQ₂₅₋₃₀₇, pSR658-YscQ₁₅₀₋₃₀₇, pSR658-YscQ₂₀₀₋₃₀₇, and pSR658-YscQ₂₋₂₅₀ were grown at 30°C. Dimerization or multimerization of LexA fusion proteins were analyzed by measuring their ability to repress β -galactosidase expression.

These results suggest that the C-terminal region of YscQ mediates the dimerization/multimerization of YscQ. Interestingly, a crystal structure is available for the C-terminal domain of the *Pseudomonas syringae* YscQ homolog HrcQ (16). This domain crystallized as a dimer, further confirming the ability of the C-terminal domain of YscQ family proteins to mediate the dimerization of these proteins.

The N-terminal cytoplasmic domain of YscD has two possible dimerization conformations

Our previous bacterial one-hybrid studies suggested that the YscD N-terminal domain also has the capacity to dimerize or multimerize (Fig. 2.2) (53). In addition, Lountos *et al.* (39) recently obtained a crystal structure for the N-terminal domain of *Y. pestis* YscD that contained two possible dimer conformations. Overall, the structure of the YscD N-terminal region was an oval structure composed of 10 β -strands and one α -helix with a prominent linker region that connects the domain to a single transmembrane domain (TM; not present in the crystal structure). One dimer conformation appears plausible as the dimerization mediated by YscD residues Arg25, Thr61, Asp62, Leu76, and Gly77 results in a dimer conformation that directs both linker regions in the same direction, presumably towards the inner membrane. The alternate dimer conformation, mediated in large part by YscD residues in the linker regions, results in a dimer conformation that has the two C-terminal linker regions pointed in opposite directions, a conformation that would make it difficult for the TM region of both monomers to cross the inner membrane. To further investigate the two predicted YscD dimerization regions, a deletion analysis of YscD using the LexA-based bacterial one-hybrid system as a dimerization reporter was used (8). Initial analysis was performed using a number of large deletions in the isolated YscD N-terminal region (Figure 2.4).

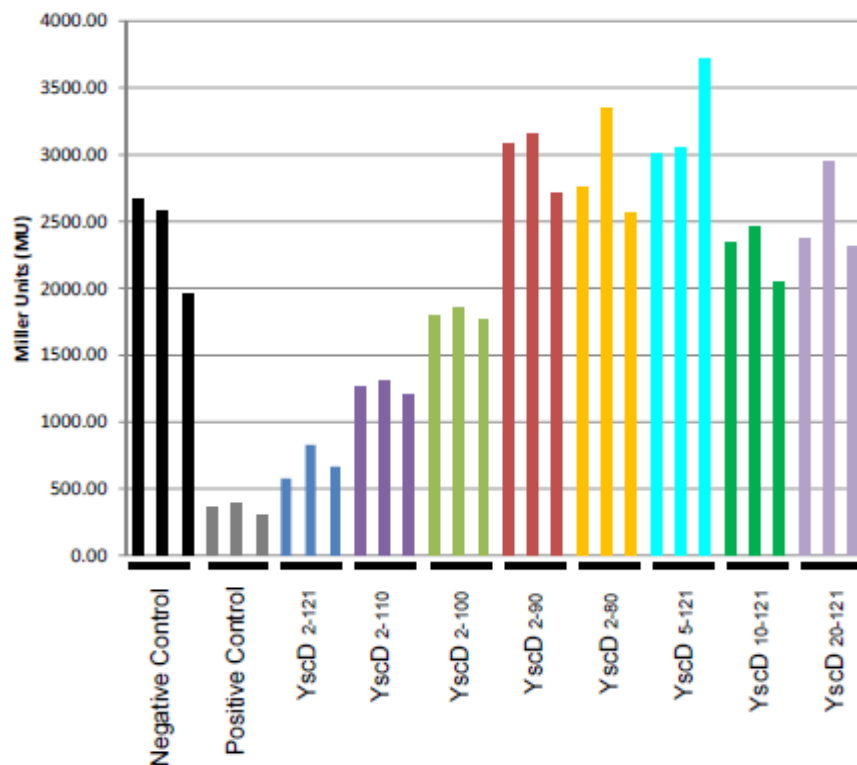


Fig. 2.4. YscD maintains dimerization at 2-100 amino acids but loses dimerization at 2-90 amino acids. *E. coli* SU101 carrying pSR658 vector alone, positive control pDD506, pSR658-YscD₂₋₁₂₁, pSR658-YscD₂₋₁₀₀, pSR658-YscD₂₋₉₀, pSR658-YscD₂₋₈₀, pSR658-YscD₅₋₁₂₁, pSR658-YscD₁₀₋₁₂₁, and pSR658-YscD₂₀₋₁₂₁ were grown at 30°C. Dimerization or multimerization of LexA fusion proteins were analyzed by measuring their ability to repress β -galactosidase expression.

The results indicate that the YscD N-terminal region dimerizes strongest with the entire YscD N-terminal region (residues 2 to 121) including the entire linker domain (residues 96 to 110) fused to the LexA DNA binding domain. Deletions of sequences encoding most of the YscD linker domain (YscD 2-110 and 2-100) reduced but did not eliminate YscD dimerization. Finally, deletions that eliminated residues 2 to 90 or more of YscD eliminated all dimerization and likely disrupt the structure of the YscD N-terminal domain (Fig. 2.4). Dimerization was also lost if short stretches of amino acids were removed from the N-terminal region of the YscD N-terminal domain with the removal of

as few as 4 amino acids resulting in the loss of dimerization (Fig. 2.4). These studies indicate that both predicted YscD dimerization conformations likely contribute to the dimerization observed in the bacterial one-hybrid studies. However, deletion of the linker residues (2-100) should enable us to specifically measure YscD dimerization mediated by the most likely physiologically relevant YscD dimer conformation that is predicted to form independent of the linker residues removed in these constructs. To confirm that the dimerization observed in the YscD 2-100 construct is mediated by the predicted YscD residues (Arg25, Thr61, Asp62, Leu76, and Gly77) key residues were mutated in both the YscD 2-100 and YscD 2-121 N-terminal LexA fusion proteins and the effects on dimerization measured using the bacterial one-hybrid system. Mutations altering single residues at the predicted dimer interface had no effect on dimerization (data not shown), thus mutants with alterations in two residues were constructed and tested. All of the mutations in the context of YscD residues 2 to 121 had no effect on YscD dimerization, presumably because the second dimerization interface was still intact in this construct (Figure 2.5).

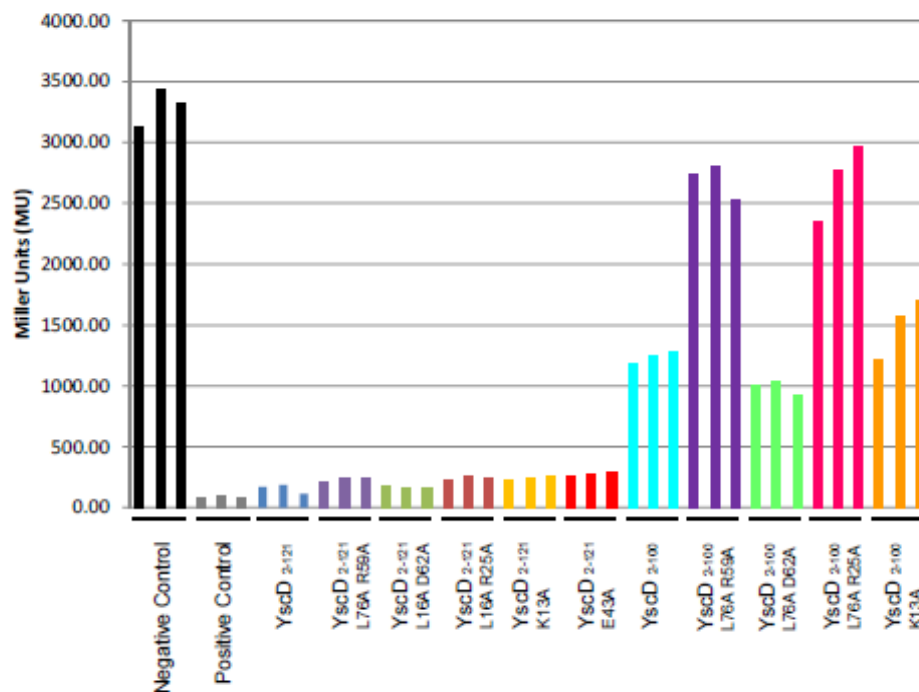


Fig. 2.5. The predicted interface between YscD_{NT} proteins that involves residues Arg25, Thr61, Asp62, Leu76, and Gly77 are important for dimerization. *E. coli* SU101 carrying pSR658 vector alone, positive control pDD506, pSR658-YscD₂₋₁₂₁, and pSR658-YscD₂₋₁₀₀ carrying residue changes as shown in the figure were grown at 30°C. Dimerization or multimerization of LexA fusion proteins were analyzed by measuring their ability to repress β-galactosidase expression.

However, when mutations that specifically altered dimerization residues L76 and R59 or L76 and R25 were introduced into the construct encoding YscD residues 2 to 100 dimerization was lost (Fig. 2.5), confirming that the residues predicted to mediate YscD dimerization via the crystal structure of YscD were required for dimerization in a LexA-based bacterial one-hybrid system. These data further suggest that YscD functions as a dimer or multimer and that the dimer conformation with both linkers pointed in the same direction represents a real dimerization interface and the likely dimerization conformation of YscD.

The N-terminal region of YscD interacts directly or indirectly with YscQ and YscK

Rhe yeast two-hybrid and bacterial one-hybrid experiments indicate that YscK, YscQ, YscL, and YscN interact with each other and with themselves to form a high molecular weight complex; however it is unknown how this complex may interact with the T3S apparatus previous studies suggest that YscQ may interact with YscD (data not shown). Therefore, we wanted to investigate the interaction of YscK and/or YscQ with the N-terminal portion of YscD in *Y. pestis*. A previously constructed plasmid (pFLAG-YscD_{FL}) encoding full-length FLAG-tagged YscD is fully functional and able to complement a $\Delta yscD$ *Y. pestis* strain (53). A plasmid encoding an N-terminal deletion of FLAG-tagged YscD (FLAG-YscD_{ΔNT}) was also available. A co-immunoprecipitation study was performed in the $\Delta yscD$ *Y. pestis* strain complemented with either a full-length FLAG-tagged YscD (YscD_{FL}) or an N-terminal deletion FLAG-tagged YscD (YscD_{ΔNT}). Bacteria were grown for 4 hours at 37°C in the absence of calcium, which allows for the assembly of the T3S apparatus. Bacterial cells were harvested and cross-linked with the cleavable amine-specific cross-linker DSP (Thermo Scientific). Cross-linked cells were lysed and membranes were solubilized with 3-14 Zwittergent detergent (EMD). After removing insoluble material by centrifugation, the lysates were rotated overnight with anti-FLAG M2 beads (Sigma-Aldrich), washed, bound proteins eluted with an excess of FLAG peptide, and samples analyzed by SDS-PAGE and immunoblot analysis. The YscQ and YscK proteins co-immunoprecipitated with the full-length FLAG-YscD protein but not with the FLAG-YscD protein deleted of its N-terminal domain (Figure 2.6).

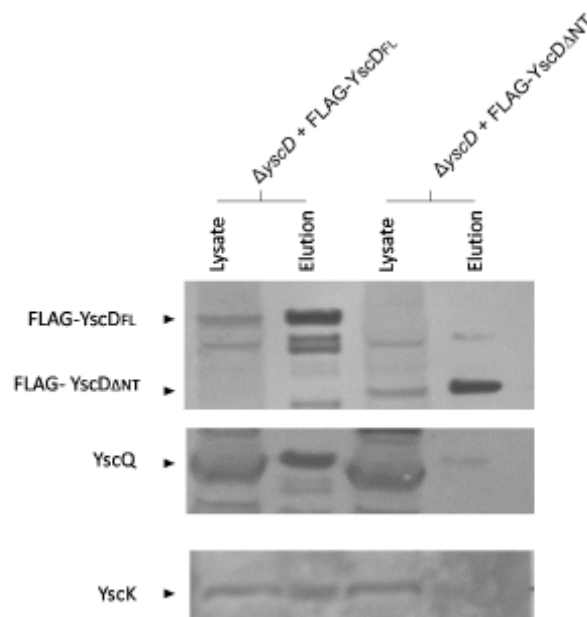


Fig. 2.6. *Y. pestis* YscQ and YscK co-immunoprecipitate with FLAG-YscD_{FL} but not FLAG-YscD_{ΔNT}. *Y. pestis* KIM5 $\Delta yscD$ strain carrying pFLAG-YscDFL and pFLAG-YscD Δ NT were grown for 4 hours at 37C in the absence of calcium. FLAG-YscD and interacting proteins were immunoprecipitated with anti-FLAG-M2 affinity gel (Sigma) and eluted with 100 μ g/ml FLAG peptide.

These studies show that the N-terminal domain of YscD is required for YscD to pull down YscQ and YscK. Although these studies do not confirm a direct interaction between YscD and YscK or YscQ, previous studies suggest that YscQ family proteins directly interact with YscD family proteins and these studies are consistent with this hypothesis (31). Thus, the YscKLQ complex likely interacts with the T3SS base structure via interactions with the cytoplasmic N-terminal portion of YscD.

YscK interacts with sorting complex components and T3S substrates

Previous studies with the yeast two-hybrid system showed that YscK interacts with YscQ and YscL and co-immunoprecipitation experiments in this study have shown that the N-terminal portion of YscD pulls down both YscQ and YscK (29). The sorting

complex is predicted to consist of YscK, YscQ, and YscL. To further confirm this and to investigate what other proteins might interact with this complex, a YscK-FLAG co-immunoprecipitation experiment was performed (Figure 2.7). FLAG-tagged YscK is fully functional and complements a $\Delta yscK$ *Y. pestis* strain (data not shown).

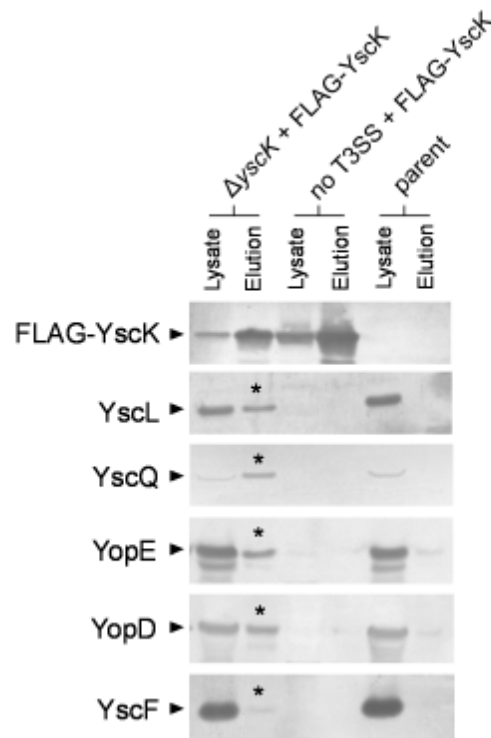


Fig. 2.7. *Y. pestis* YscL, YscQ, YopE, and YopD co-immunoprecipitate with FLAG-YscK. *Y. pestis* KIM5 (parent) and *Y. pestis* KIM5 $\Delta yscK$ and *Y. pestis* KIM6 (no pCD1 T3SS-) carrying pFLAG-YscK were grown for 4 hours at 37°C in the presence of calcium. FLAG-YscK and interacting proteins (*) were immunoprecipitated with anti-FLAG-M2 affinity gel (Sigma) and eluted with 100 μ g/ml FLAG peptide.

Y. pestis KIM5 (parent) as well as *Y. pestis* KIM5 $\Delta yscK$ and *Y. pestis* KIM6 both carrying the pFLAG-YscK plasmid were grown for 4 hours at 37°C in the presence of 2.5 mM calcium. Since the KIM6 strain does not carry plasmid pCD1 and cannot secrete Yops, calcium was added to each culture to prevent Yop secretion in all 3 cultures. Bacterial cells were harvested, cross-linked with DSP (Thermo Scientific), solubilized with 3-14 Zwittergent detergent (EMD), and centrifuged to remove insoluble material.

The resultant lysates were rotated overnight with anti-FLAG M2 beads (Sigma-Aldrich), washed, eluted, and analyzed by SDS-PAGE and immunoblot analysis. Both YscL and YscQ co-immunoprecipitated with FLAG-tagged YscK, confirming that YscK interacts with the other sorting complex components in *Y. pestis* (Fig. 2.7). These results indicate that a YscKLQ sorting complex is likely assembled in *Y. pestis*.

To investigate if T3S substrates interact with the sorting complex as shown previously in *Salmonella enterica*, immunoprecipitation samples were also probed for the presence of T3S substrates YopE, YopD, and YscF (Fig. 2.7) (35). YopE and YopD efficiently co-immunoprecipitated with FLAG-YscK. In addition, a small amount of YscF was also detected in the immunoprecipitates from the $\Delta yscK$ strain complemented with FLAG-YscK, suggesting that YscF may also interact with the YscKLQ complex. Thus, the YscKLQ complex interacts with T3S substrates. This has not been seen before in *Y. pestis* or any T3SS outside of the *Salmonella* SPI-1 T3SS. A *Y. pestis* strain (KIM6) lacking the virulence plasmid pCD1 and carrying pFLAG-YscK was used as a control to verify the bands detected were pCD1-specific proteins. Furthermore, the parent strain (KIM5) which is identical to the $\Delta yscK$ + pFLAG-YscK strain with the exception that YscK is not FLAG-tagged was also used to confirm that the co-immunoprecipitating proteins are being pulled down specifically due to interactions mediated via the FLAG-YscK protein (Fig. 2.7). The interaction with the needle protein YscF were not as strong as seen with other T3S substrates likely because conditions in this experiment were optimal for Yop secretion rather than needle-type secretion as samples were harvested 4 hours after a temperature shift to 37°C (7). The T3S apparatus and needle assembly is normally completed after 1 to 2 hours at 37°C (27). In the *Salmonella enterica* study they

did not look at the interaction of the sorting complex with needle substrates (35).

Furthermore, little is known about the secretion of needle-type substrates; therefore, we wanted to investigate the interactions of the YscKLQ sorting complex with YscF, and YscF secretion in general, in more detail.

YscF has an N-terminal secretion signal

Substrates are targeted to the T3S apparatus by N-terminal secretion signals or chaperone binding domains (4). We first wanted to characterize the signals that target YscF for secretion. Secretion signals are normally located within the first 2 to 15 amino acids (Fig. 1.2). Type III secretion signals tend to be amphipathic in nature and unstructured. In a study using YopE as a T3S substrate, it was found that the N-terminal secretion signal could be functionally replaced by a number of amphipathic synthetic sequences (38). However, sequences that were very polar or nonpolar generally did not function as T3S signals. We replaced the N-terminal eight residues of YscF (MSNFSGFT) with synthetic sequences (see Fig 2.8). The constructs were transformed into a $\Delta yscF$ *Y. pestis* strain and the stability and secretion of YscF and YopM were determined after 5 hours of growth at 37°C in the presence and absence of 2.5 mM calcium by SDS-PAGE and immunoblot analysis. If a very polar sequence (MSSSSSSS-YscF) or a very non-polar (MSIIIII-YscF) sequence was used then YscF was not secreted and because no functional needle was assembled YopM was also not secreted (Figure 2.8).

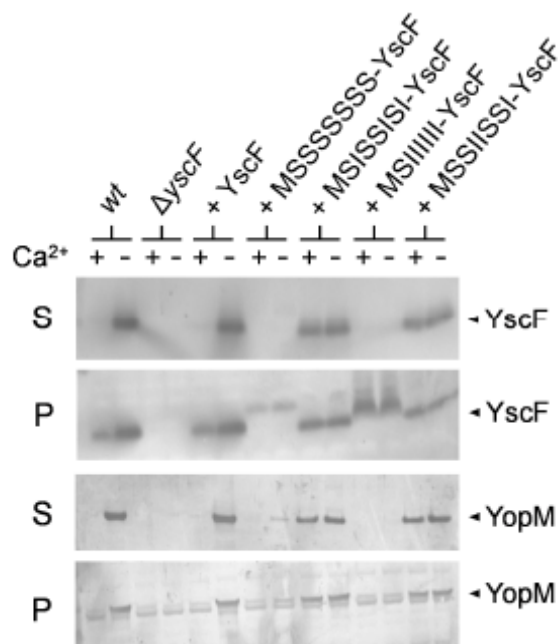


Fig. 2.8. Expression and secretion of YopM, YscF, and YscF mutants with synthetic N-terminal secretion signals. *Y. pestis* KIM5 (wt), $\Delta yscF$ strain, and $\Delta yscF$ strain carrying pBAD-YscF or pBAD-YscF MSSSSSSS, pBAD-YscF-MSiSSiSI, pBAD-YscF-MSiIIIIII, or pBAD-YscF-MSSiIISI were grown for 5 hour at 37C in the presence (+) and absence (-) of calcium. Cell pellet (P) and culture supernatant (S) fractions were analyzed by SDS-PAGE and immunoblot analysis.

However, if an amphipathic sequence was used (MSiSSiSI-YscF or MSSiIISI-YscF) YscF was secreted and the T3S apparatus was also functional for secretion of YopM (Fig. 2.8). Interestingly, the assembled T3SSs secreted YopM in both the presence and absence of calcium and are thus calcium blind. This is not unexpected as YscF has been previously shown to have a role in the regulation of the T3S process (58). Thus when the YscF secretion signal is replaced calcium regulation may also be effected. From these experiments we can conclude that YscF requires an amphipathic N-terminal secretion signal for its secretion.

YscG and YscE chaperones play a role in secretion of YscF

The YscE/YscG chaperone is needed to stabilize YscF and prevent it from prematurely polymerizing prior to its secretion (57). If YscE or YscG is not present, YscF is rapidly degraded. The YscE/YscG chaperone binds to the C-terminus of YscF (residues 51 to 87) (Figure 2.9).

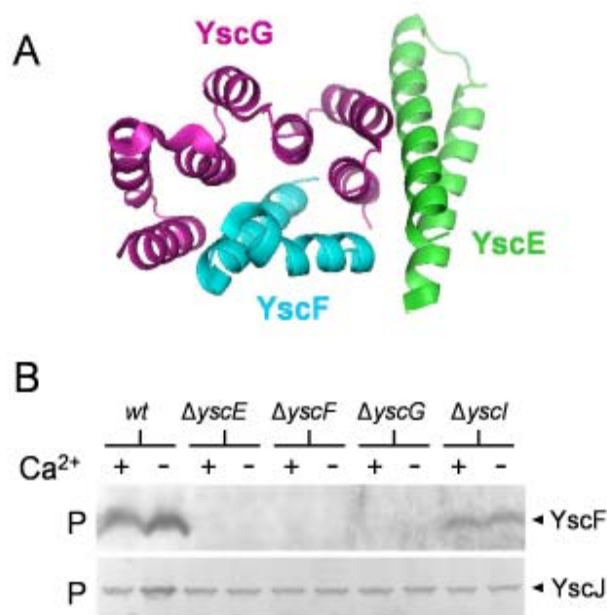


Fig. 2.9. The YscE/YscG chaperone is required for stable expression of YscF. (A) Ribbon presentation of the crystal structure of the YscEFG complex. (B) Expression of YscF and YscJ in the bacterial whole cell pellet (P) of *Y. pestis* KIM5 and $\Delta yscE$, $\Delta yscF$, $\Delta yscG$, and $\Delta yscI$ strains.

It is not known if the chaperone has a role in the secretion of YscF. This has not been investigated because if YscG or YscE are deleted YscF is degraded. We wanted to look at the possible role of YscG and YscE in the secretion of YscF. We have previously constructed and confirmed that a maltose-binding protein (MBP)-YscE and MBP-YscG are fully functional and complement $\Delta yscE$ and $\Delta yscG$ strains (12). We found that after 5 hours of growth at 37°C in the presence and absence of 2.5 mM calcium that

overexpression MBP-YscG in a $\Delta yscEG$ strain can stabilize cytosolic YscF even in the absence of YscE, however, no secretion of YscF was detected (Figure 2.10).

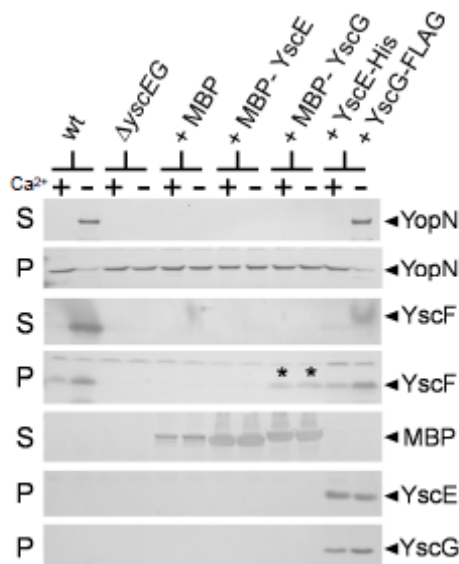


Fig. 2.10. The YscE/YscG chaperone is required for YscF secretion. Overexpression of MBP-YscG, but not MBP-YscE, stabilizes cytoplasmic YscF(*) but YscF is not secreted. Pellet (P) Culture supernatant (S).

A MBP-YscE was not able to stabilize cytosolic YscF. This shows that a complete YscE/YscG chaperone is required for YscF secretion.

YscG mutagenesis

To further explore the role of the YscE/YscG chaperone in secretion of YscF, we performed a random mutagenesis of YscG encoded by plasmid pFLAG-YscG-YscE-6xHis (Agilent technologies). The goal of this mutagenesis was to find YscG mutants that still stabilize cytosolic YscF but do not secrete YscF. Plasmids encoding these mutants will be sequenced and analyzed using the crystal structure of the YscE/YscG/YscF complex. These mutations will likely localize to surface-exposed regions of YscG or to YscG residues involved in the contact of YscG with YscE, but will

likely not localize to YscG residues involved in binding YscF. Surface-exposed regions of YscG (or YscE) may mediate contact with other T3S components such as the YscKLQ complex. We have previously constructed a plasmid encoding a functional FLAG-YscG/YscE-6xHis chaperone that is able to complement a $\Delta yscEG$ *Y. pestis* strain (Fig. 2.10). Using the GeneMorph II EZclone mutagenesis kit (Agilent technologies) and error prone PCR we generated random mutations in *yscG* of plasmid pFLAG-YscG-YscE-6xHis. This construct was optimal because both chaperones can be detected via antibodies specific to their tags, YscG by anti-FLAG antibodies and YscE by anti-5xHIS antibodies. The unique growth restriction phenotype of *Y. pestis* was used to enrich for mutants that were defective in secretion of Yops in the absence of calcium (only mutants defective in secretion can grow under secretion inducing conditions in vitro) (47). After two rounds of selection, plasmids from randomly chosen mutants were isolated and moved into a fresh $\Delta yscEG$ *Y. pestis* mutant and tested for YscE, YscF, and YscG expression and YscF and YopN secretion. After 5 hours of growth at 37°C in the presence and absence of calcium some of the mutants were able to stabilize cytosolic YscF but did not secrete YscF or Yops (Figure 2.11).

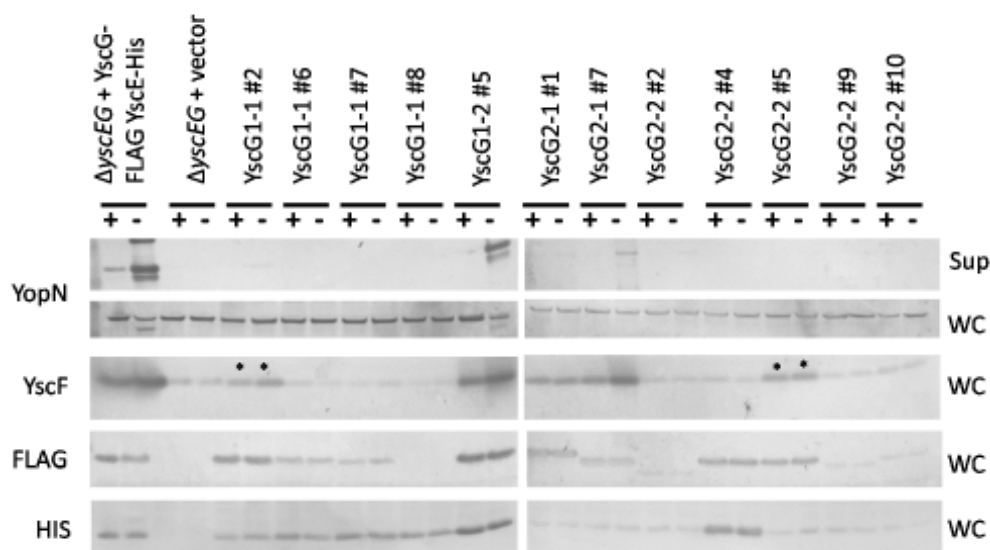


Fig. 2.11. Random mutagenesis of YscG stabilizes cytosolic YscF but does not allow secretion. *Y. pestis* KIM5 $\Delta yscEG$ strain carrying pFLAG-YscG YscE-6X HIS, pFLAG MAC (vector), or pFLAG-YscG YscE-6X HIS mutants were grown for 5 hours at 37C in the presence (+) or absence (-) of calcium. Cell pellet (P) and culture supernatant (S) fractions were analyzed by SDS-PAGE and immunoblot analysis. YscG mutants that stabilize YscF (*) but do not secrete Yops were further analyzed and sequenced.

These mutants were sequenced to find where the mutations were located (Table 2.1).

Table 2.1 Sequenced *yscG* mutations and encoded amino acid changes

Name	Amino Acid changes
YscG 1-1 #2	I28F
YscG 1-1 #6	S57C; L65I; P72H; L74I
YscG 1-1 #7	W73G; S97C
YscG 1-1 #8	G62 → STOP
YscG 1-2 #5	N51Y
YscG 2-1 #1	G16R; I28N; Q28R
YscG 2-1 #7	N6K; Q60H; L76V; R90C
YscG 2-2 #2	L34M; R101 → STOP
YscG 2-2 #4	L46P
YscG 2-2 #5	N19K; G84V; F105L
YscG 2-2 #9	N51Y; E78D; K114 → STOP
YscG 2-2 #10	M50A; A56T

Since the crystal structure of YscE/YscG chaperone is available we can also map these residues to find where they are located and if they are involved in specific protein-protein

contacts (57). Several of the YscG mutants (1-1 #8, 2-2 #2, and 2-2 #9) had mutations that resulted in stop codons within the coding sequence of *yscG* and produced a truncated non-functional YscG protein. Other YscG mutants produced a full-length YscF protein that failed to stabilize YscF. Several of these YscG mutants had mutations that changed amino acids required for the interaction of YscG with YscF (1-1 #6 and 1-1 #7). Finally, two mutants, including YscG1-1 #2 stabilized YscF but showed no YscF secretion. One of these mutants contained a single amino acid substitution I28F. I28 is one of seven hydrophobic YscF residues that mediates the contact of YscG with YscE, suggesting that YscE may be required for secretion of YscF. This is of particular interest considering our results that showed that overexpression of YscG in the absence of YscE could stabilize YscF, but YscF was not secreted. Both of these lines of evidence suggest that YscE may play a critical role in the secretion of YscF. Further studies will need to be done using additional mutants in order to further understand the role of the YscE/YscG chaperone in YscF secretion, including our goal of identifying YscE/YscG residues that mediate the interaction of the YscEFG complex with the YscKLG complex (if such mutants exist).

Discussion

T3S substrates are targeted for secretion by N-terminal secretion signals and chaperone binding domains (4). How these substrates are recognized by the T3S apparatus is still not understood. In a recent paper, a *Salmonella* SPI-1 “sorting platform” composed of the *Y. pestis* homologs of YscK, YscL, and YscQ was identified and shown to interact with T3S substrate/chaperone complexes (35). We hypothesize that there is a similar complex in *Y. pestis* that is composed of YscK, YscL, and YscQ that functions as

a sorting platform for substrate/chaperone complexes and that interacts with the cytoplasmic portion of YscD. Previous studies in our lab have shown that YscQ interacts with YscK and YscL and that YscL interacts with YscQ and the YscN ATPase (29). In this study, a bacterial LexA-based one-hybrid system was used to show that the N-terminal portion of YscD, the C-terminal portion of YscQ as well as YscL dimerize or multimerize (Fig. 2.2) (8). However, no evidence that YscK also interacts with itself was obtained (Fig. 2.2). Thus, the YscQ, YscK, YscL, and YscN T3S components not only interact with each other but also interact with themselves; thus, one can envision that these proteins could assemble into a large high molecular weight complex (see Fig 2.1). Interestingly, YscK, YscQ, YscL, and YscN are all predicted to be soluble proteins with no TM sequences (13); therefore, a complex composed of these proteins likely needs to interact with other membrane bound T3S components to complete its assembly on the T3S apparatus or to deliver T3S substrates to the apparatus. To further investigate this process *in vivo*, co-immunoprecipitation experiments were performed in *Y. pestis* strains. A FLAG-tagged full-length YscD pulled down both YscQ and YscK; however, a FLAG-YscD protein deleted for its cytoplasmic N-terminal domain did not pull down either YscQ or YscK (Fig. 2.6). These studies suggest that the predicted YscKLQ complex interacts with the membrane-bound T3S apparatus via interactions with the cytoplasmic portion of YscD.

The sorting complex identified in *Salmonella enterica* functioned to bind T3S substrate/chaperone complexes (35). The YscKLQ complex would be predicted to have a similar function in *Y. pestis*. To further investigate YscKLQ complex assembly and function in *Y. pestis*, we performed immunoprecipitation studies with a fully functional

FLAG-tagged YscK protein. As expected, both the YscQ and YscL proteins co-immunoprecipitated with FLAG-YscK, confirming the presence of an assembled YscKLQ complex in *Y. pestis*. Importantly, the T3S substrates YopD and YopE also co-immunoprecipitated with FLAG-YscK, indicating that the YscKLQ complex, like the *Salmonella* sorting platform, interacts with T3S substrate/chaperone complexes (Fig. 2.7). Unfortunately, we did not have antibodies specific for the chaperones of these two proteins (SycE for YopE and SycD for YopD) and were unable to confirm that these chaperones also co-immunoprecipitated with FLAG-YscK. Together, these studies suggest that a YscKLQ sorting complex is assembled in *Y. pestis* and that this complex interacts with T3S substrates.

Needle-type chaperones appear to be unique to the *Yersinia spp.* plasmid-encoded T3SS and other closely related T3SSs (57). These chaperones are thought to stabilize the needle protein prior to its secretion and prevent its premature polymerization. We decided to look closer at the secretion of the YscF needle protein in *Y. pestis* as this was not examined in the *Salmonella enterica* study (35). We found that a small amount of YscF co-immunoprecipitated with FLAG-YscK, even under conditions that were optimal for Yop secretion not needle secretion (Fig. 2.7). To begin to look closer at YscF secretion, we first wanted to see if YscF had an N-terminal secretion signal. We replaced the N-terminal region of YscF with synthetic secretion signals and found that YscF secretion and function is dependent upon an N-terminal amphipathic secretion signal similar to what was found with YopE in a previous study (Fig. 2.8) (38). Although, YscF with the synthetic amphipathic N-terminal sequences was secreted, initial follow-up studies suggested that needle assembly may not be complete in these strains (data not

shown). This suggests that the N-terminal region of YscF may also have a role in needle assembly. Alternatively, needle assembly may not be optimal because the synthetic T3S signal does not target YscF for secretion efficiently or may make it difficult to recognize YscF as an early substrate.

In addition to its N-terminal secretion signal, YscF also has a unique heterodimeric chaperone composed of the YscE and YscG proteins (57). These chaperones prevent YscF from prematurely polymerizing prior to secretion and also stabilize YscF. If either YscE or YscG is deleted than YscF is degraded. However, it is unknown if the YscE/YscG chaperone has a role in secretion. We wanted to look closer at the role of the YscE/YscG chaperone in YscF secretion. We found that overexpression of a functional MBP-YscG protein was able to stabilize cytosolic YscF in the absence of YscE but YscF was not secreted, suggesting that YscE or a complete YscE/YscG chaperone is required for YscF secretion (Fig. 2.10). Thus, the YscE/YscG chaperone appears to have a direct role in YscF secretion similar to other T3S chaperones.

To look closer at the role of YscG in YscF secretion, we performed random mutagenesis on *yscG* of plasmid pFLAG-YscG-YscE-6xHis in a *Y. pestis yscEG* deletion strain and enriched for mutants that grew at 37°C in the absence of calcium and thus did not secrete Yops. After two rounds of selection, we randomly picked mutants, and screened them for stable YscF expression in the cytosol but no secretion of YscF or Yops. We identified a few mutants with this phenotype (Fig. 2.11). Exact mutations in *yscG* were determined through DNA sequence analysis and the available crystal structure of YscE/YscF/YscG complex was used to localize the amino acids that had been changed (57). Only one YscG mutant that stabilized YscF, but showed no YscF or Yop secretion

provided DNA sequence data suitable for further analysis (YscG 1-1 #2). This mutant contained a single amino acid change that substituted phenylalanine for YscG isoleucine 28 (I28F). YscG isoleucine 28 is one of 7 hydrophobic residues that mediate a contact between YscG and YscE. We hypothesize that this mutation disrupts the normal contacts between these proteins and produces a mutant chaperone that binds YscF but cannot deliver YscF for secretion. These results and our results with the MBP-YscG overexpression studies, suggest that YscE may play a key role in targeting the YscEFG complex to the T3S apparatus. Interestingly, co-overexpression of the *Pseudomonas aeruginosa* PscF (a homolog of YscF) and PscG proteins (a homolog of YscG) in the absence of PscE (a homolog of YscE) allowed secretion of PscF, suggesting that PscE is not required for PscF needle secretion in *Pseudomonas aeruginosa* (49). At this time it is difficult to interpret these two opposite results. It is possible that disrupting the YscE-YscG contact or overexpressing YscG in the absence of YscE alters the conformation of YscG such that YscG is no longer able to promote YscF secretion. Alternatively, YscE may be critical for targeting YscF for secretion in *Y. pestis*, but may have a slightly different role in *P. aeruginosa*. Future experiments will be aimed at identifying more YscG mutants that stabilize YscF but do not secrete YscF which should provide a clearer picture of how the YscEG chaperone targets YscF for secretion. Once such mutants are obtained, they will be used to examine the interaction of the wild type and mutant YscEFG complexes with the YscKLQ complex to determine if the mutants specifically alter this interaction. The T3S apparatus is a highly complex nanomachine and once we have a better understanding of how this machine works we can better develop treatments for infections.

Chapter 3: Experimental Methods

Bacterial strains and growth conditions

Yersinia pestis KIM and *Escherichia coli* DH5 α strains (Table 3.1) were grown in heart infusion broth (HIB) or on tryptose blood agar (TBA) plates (BD-Difco) at 27°C (*Y. pestis*) or 37°C (*E. coli*). The antibiotics ampicillin and streptomycin were used at 50 μ g/ml, chloramphenicol was used at 50 μ g/ml, and tetracycline was used at 15 μ g/ml. For growth curve and secretion assays, *Y. pestis* strains were grown in TMH medium in the presence or absence of 2.5 mM CaCl₂ for 1 hour at 27°C and then shifted to 37°C for 5 hours.

Table 3.1 Bacterial strains and plasmids

Strains or plasmid	Description	Source
<i>E. coli</i> strains		
DH5 α	F ϕ 80 lacZ Δ M15, Δ (lacZYA argF) U169, <i>endAI, recAI hsdR17 deoR supE44 thi-1 gyr96 relAI</i>	(6)
SU101	<i>lexA71::Tn5(Def)sulA211 Δ(lazIPOZYA)169/FϕlacI^qlacZΔM15:Tn9</i>	(8)
<i>Y. pestis</i> strains		
KIM5-3001	Sm ^r pCD1 pPCP1 pMT1	(37)
KIM5-3001.14	Sm ^r pCD1 (Δ <i>yscD</i>) pPCP1 pMT1	(48)
KIM5-3001.K1	Sm ^r pCD1 (Δ <i>yscK</i>) pPCP1 pMT1	This study
KIM6	Sm ^r pCD1 ⁻ pPCP1 pMT1	
KIM5-3002.P61	Sm ^r pCD1 (Δ <i>yscF</i>) pPCP1 pMT1	(58)
KIM8-3002.P40	Sm ^r pCD1 (Δ <i>yscE</i>) pPCP1 pMT1	(12)
KIM8-3002.41	Sm ^r pCD1 (Δ <i>yscG</i>) pPCP1 pMT1	(12)
KIM5-3001.K2	Sm ^r pCD1 (Δ <i>yscEG</i>) pPCP1 pMT1	This study (Day)
Plasmids		
pFLAG-CTC	Expression vector: encodes C-terminal FLAG-peptide	Sigma-Aldrich
pFLAG-MAC	Expression vector: encodes N-terminal FLAG peptide	Sigma-Aldrich

pFLAG-MAC-YscD _{FL}	HindIII-BglIII-digested PCR fragment in pFLAG-MAC vector encoding full-length N-terminal FLAG-YscD	53)
pFLAG-MAC-YscD ₁₁₉₋₄₁₉	HindIII-BglIII-digested PCR fragment in pFLAG-MAC vector encoding FLAG-YscD ₁₁₉₋₄₁₉	(53)
pFLAG-CTC-YscK	DNA sequence encoding full-length YscK in pFLAG-CTC vector	Eugenia Silva-Herzog
pFLAG-MAC-YscG YscE-6X HIS	DNA sequence encoding full-length YscG in pFLAG-MAC vector and full-length YscE tagged with 6X HIS	(10)
pFLAG-MAC-YscG1-1 #2	GeneMorph II EZClone Domain Mutagenesis Kit (Agilent Technologies) mutation of pFLAG-MAC-YscG YscE-6X HIS	This study
pFLAG-MAC-YscG1-1 #6	GeneMorph II EZClone Domain Mutagenesis Kit (Agilent Technologies) mutation of pFLAG-MAC-YscG YscE-6X HIS	This study
pFLAG-MAC-YscG1-1 #7	GeneMorph II EZClone Domain Mutagenesis Kit (Agilent Technologies) mutation of pFLAG-MAC-YscG YscE-6X HIS	This study
pFLAG-MAC-YscG1-1 #8	GeneMorph II EZClone Domain Mutagenesis Kit (Agilent Technologies) mutation of pFLAG-MAC-YscG YscE-6X HIS	This study
pFLAG-MAC-YscG1-2 #5	GeneMorph II EZClone Domain Mutagenesis Kit (Agilent Technologies) mutation of pFLAG-MAC-YscG YscE-6X HIS	This study
pFLAG-MAC-YscG2-1 #1	GeneMorph II EZClone Domain Mutagenesis Kit (Agilent Technologies) mutation of pFLAG-MAC-YscG YscE-6X HIS	This study
pFLAG-MAC-YscG2-1 #7	GeneMorph II EZClone Domain Mutagenesis Kit (Agilent Technologies) mutation of pFLAG-MAC-YscG YscE-6X HIS	This study
pFLAG-MAC-YscG2-2 #2	GeneMorph II EZClone Domain Mutagenesis Kit (Agilent Technologies) mutation of pFLAG-MAC-YscG YscE-6X HIS	This study
pFLAG-MAC-YscG2-2 #4	GeneMorph II EZClone Domain Mutagenesis Kit (Agilent Technologies) mutation of pFLAG-MAC-YscG YscE-6X HIS	This study
pFLAG-MAC-YscG2-2 #5	GeneMorph II EZClone Domain Mutagenesis Kit (Agilent Technologies) mutation of pFLAG-MAC-YscG YscE-6X HIS	This study
pFLAG-MAC-YscG2-2 #9	GeneMorph II EZClone Domain Mutagenesis Kit (Agilent Technologies) mutation of pFLAG-MAC-YscG YscE-6X HIS	This study

pFLAG-MAC-YscG2-2 #10	GeneMorph II EZClone Domain Mutagenesis Kit (Agilent Technologies) mutation of pFLAG-MAC-YscG YscE-6X HIS	This study
MBP	Expression vector encoding N-terminal Maltose Binding Protein (MBP)	Sigma-Aldrich
MBP-YscE	DNA sequence encoding full-length YscE in N-terminal Maltose Binding Protein vector	Day
MBP-YscG	DNA sequence encoding full-length YscG in N-terminal Maltose Binding Protein vector	Day
pBAD33	L-arabinose inducible expression vector	(22)
pBAD33-YscF	DNA sequence encoding full-length YscF in pBAD33 expression vector	(58)
pBAD33-MSSSSSSS-YscF	PCR fragment generated from PCR amplification around pBAD33-YscF plasmid	This study
pBAD33-MSISSISI-YscF	PCR fragment generated from PCR amplification around pBAD33-YscF plasmid	This study
pBAD33-MSIIIIII-YscF	PCR fragment generated from PCR amplification around pBAD33-YscF plasmid	This study
pBAD33-MSSIISII-YscF	PCR fragment generated from PCR amplification around pBAD33-YscF plasmid	This study
pSR658	LexA DNA-binding domain expression vector	(8)
pDD506	LexA-chloramphenicol acetyltransferase	(8)
pSR658 YscD ₂₋₁₂₁	SacI-KpnI digested PCR fragment in pSR658 encoding the LexA DNA binding domain in frame with YscD ₂₋₁₂₁	(53)
pSR658 YscD ₂₋₁₁₀	SacI-KpnI digested PCR fragment in pSR658 encoding the LexA DNA binding domain in frame with YscD ₂₋₁₁₀	This study
pSR658 YscD ₂₋₁₀₀	SacI-KpnI digested PCR fragment in pSR658 encoding the LexA DNA binding domain in frame with YscD ₂₋₁₀₀	This study
pSR658 YscD ₂₋₉₀	SacI-KpnI digested PCR fragment in pSR658 encoding the LexA DNA binding domain in frame with YscD ₂₋₉₀	This study
pSR658 YscD ₂₋₈₀	SacI-KpnI digested PCR fragment in pSR658 encoding the LexA DNA binding domain in frame with YscD ₂₋₈₀	This study
pSR658 YscD ₅₋₁₂₁	SacI-KpnI digested PCR fragment in pSR658 encoding the LexA DNA binding domain in frame with YscD ₅₋₁₂₁	This study
pSR658 YscD ₁₀₋₁₂₁	SacI-KpnI digested PCR fragment in pSR658 encoding the LexA DNA binding domain in frame with YscD ₁₀₋₁₂₁	This study
pSR658 YscD ₂₀₋₁₂₁	SacI-KpnI digested PCR fragment in pSR658 encoding the LexA DNA binding domain in frame with YscD ₂₀₋₁₂₁	This study

pSR658 YscD ₂₋₁₂₁ L76A R59A	SacI-KpnI digested PCR fragment in pSR658 encoding the LexA DNA binding domain in frame with YscD ₂₋₁₂₁ L76A R59A	This study
pSR658 YscD ₂₋₁₂₁ L76A D62A	SacI-KpnI digested PCR fragment in pSR658 encoding the LexA DNA binding domain in frame with YscD ₂₋₁₂₁ L76A D62A	This study
pSR658 YscD ₂₋₁₂₁ L76A R25A	SacI-KpnI digested PCR fragment in pSR658 encoding the LexA DNA binding domain in frame with YscD ₂₋₁₂₁ L76A R25A	This study
pSR658 YscD ₂₋₁₂₁ K13A	SacI-KpnI digested PCR fragment in pSR658 encoding the LexA DNA binding domain in frame with YscD ₂₋₁₂₁ K13A	This study
pSR658 YscD ₂₋₁₂₁ E43A	SacI-KpnI digested PCR fragment in pSR658 encoding the LexA DNA binding domain in frame with YscD ₂₋₁₂₁ E43A	This study
pSR658 YscD ₂₋₁₀₀ L76A R59A	SacI-KpnI digested PCR fragment in pSR658 encoding the LexA DNA binding domain in frame with YscD ₂₋₁₀₀ L76A R59A	This study
pSR658 YscD ₂₋₁₀₀ L76A D62A	SacI-KpnI digested PCR fragment in pSR658 encoding the LexA DNA binding domain in frame with YscD ₂₋₁₀₀ L76A D62A	This study
pSR658 YscD ₂₋₁₀₀ L76A R25A	SacI-KpnI digested PCR fragment in pSR658 encoding the LexA DNA binding domain in frame This study with YscD ₂₋₁₀₀ L76A R25A	This study
pSR658 YscD ₂₋₁₀₀ K13A	SacI-KpnI digested PCR fragment in pSR658 encoding the LexA DNA binding domain in frame with YscD ₂₋₁₀₀ K13A	This study
pSR658 YscQ	SacI-PstI digested PCR fragment in pSR658 encoding the LexA DNA binding domain in frame with YscQ ₂₋₃₀₇	This study (Ross)
pSR658 YscQ ₂₅₋₃₀₇	SacI-PstI digested PCR fragment in pSR658 encoding the LexA DNA binding domain in frame with YscQ ₂₅₋₃₀₇	This study
pSR658 YscQ ₁₅₀₋₃₀₇	SacI-PstI digested PCR fragment in pSR658 encoding the LexA DNA binding domain in frame with YscQ ₁₅₀₋₃₀₇	This study
pSR658 YscQ ₂₀₀₋₃₀₇	SacI-PstI digested PCR fragment in pSR658 encoding the LexA DNA binding domain in frame with YscQ ₂₀₀₋₃₀₇	This study
pSR658 YscQ ₂₋₂₅₀	SacI-PstI digested PCR fragment in pSR658 encoding the LexA DNA binding domain in frame with YscQ ₂₋₂₅₀	This study
pSR658 YscK	SacI-KpnI digested PCR fragment in pSR658 encoding the LexA DNA binding domain in frame with YscK _{FL}	This study (Ross)

pSR658 YscL	SacI-KpnI digested PCR fragment in pSR658 encoding the LexA DNA binding domain in frame with YscL _{FL}	This study (Ross)
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β-galactosidase assays

E. coli SU101 strains harboring different pSR658 LexA fusion constructs were grown overnight in HIB and induced with 1 mM IPTG at 30°C and used to inoculate fresh cultures at an OD₆₂₀ of 0.2 the next day. Cultures were grown in HIB with 1 mM IPTG for 3 hours at 30°C and the OD₆₀₀ at harvest recorded. Bacterial cells (100 μl) were added to 500 μl β-Gal assay buffer (100 mM sodium phosphate buffer, pH 7, 10 mM KCl, 1 mM MgSO₄) and permeabilized by addition of 25 μl 0.1% SDS, BME (50 mM final concentration), and 25 μL CHCl₃. The assays were initiated by addition of 200 μl of 4 mg/ml ONPG. Reactions were stopped by addition of 500 μl of 1M Na₂CO₃ and assay times and OD₄₂₀ readings were recorded and used to calculate activity (Miller Units).

Co-immunoprecipitation assays

Y. pestis strains were grown overnight in TMH media (with or without 2.5 mM calcium) containing the proper antibiotics and used to inoculate fresh cultures at an OD₆₂₀ of 0.4 the next day. Cultures were grown in TMH for 1 hour at 27°C and then induced with 0.05 mM IPTG upon temperature shift to 37°C and grown for 4 hours (strains used for YscK-FLAG co-immunoprecipitation studies had 2.5 mM CaCl₂ present). Bacterial cells were harvested by centrifugation at 8000 rpm and resuspended in 1X PBS and cross-linked for 20 minutes with the cleavable cross-linker DSP (Thermo Scientific) at a final concentration of 2 mM. Cross-linking was stopped by the addition of 10 mM Tris-HCl

for 15 minutes. Cells were lysed by passage through a French pressure cell. Membranes were solubilized by the addition of 3-14 Zwittergent detergent (EMD) to 2% final concentration for 10 minutes. The solubilized lysates were then ultracentrifuged for 30 minutes at 60,000 rpm. Detergent soluble lysate samples were collected, diluted 1:20 with PBS and rotated overnight at 4°C with 100 µl of anti-FLAG M2 beads (Sigma-Aldrich). The next day, samples were washed 2X with 1X PBS containing 0.1% 3-14 Zwittergent detergent and eluted with 50 µg/ml FLAG peptide in 1X PBS.

YscG mutagenesis

The GeneMorph II EZClone Domain Mutagenesis Kit (Agilent Technologies) was used to randomly mutagenize *yscG* except for the ATG start and STOP codons (PCR oligonucleotides: YscG-error-F: GACGACGATGACAAAGTCAAGCTT and YscG-error-R: AGGTTTACCTCCATTGAGCCGTCA). pFLAG-YscG-YscE-6X HIS plasmid was used as template and random mutagenesis was performed with error prone PCR under medium mutation conditions. Constructs were initially transformed into the Δ *yscG* *Y. pestis* strain. Bacteria were grown overnight in TMH and used to inoculate fresh cultures at an OD₆₂₀ of 0.2 the next day. Cultures were grown in TMH for 1 hour at 27°C and temperature shifted to 37°C for 5 hours. These conditions enrich for mutants that grow and do not secrete. Cultures were then plated on TBA and calcium-chelated TBA-Magnesium Oxalate (MgOx) plates. TBA-MgOx plates were used to select for strains that grow and cannot secrete Yops. TBA and MgOx plates were grown at 37°C for two days. Two rounds of random selection were performed using this method. Random colonies from the first round of selection plated on MgOx plates were used for further

screening and analysis. Plasmids were isolated from these strains and transformed into *E. coli* DH5 α . Plasmid preps from *E. coli* DH5 α strains were used for DNA sequence analysis (Genewiz) and moved into a $\Delta yscEG$ *Y. pestis* strain. Bacteria were grown overnight in TMH and used to inoculate fresh cultures at an OD₆₂₀ of 0.2 the next day. Cultures were grown in TMH for 1 hour at 27°C and induced with 0.1 mM IPTG at temperature shifted to 37°C for 5 hours. Cultures of bacteria were harvested by centrifugation at 14,000 x g for 5 minutes at room temperature. Pellets of whole cell bacteria and TCA precipitated supernatant proteins were resuspended according to the harvest OD₆₂₀ and analyzed by SDS-PAGE and immunoblotting analysis.

SDS-PAGE and immunoblotting

Cultures of bacteria were harvested by centrifugation at 14,000 x g for 5 minutes at room temperature. Pellets of whole cell bacteria and TCA precipitated supernatant proteins were resuspended according to harvest OD₆₂₀ and analyzed by SDS-PAGE and immunoblotting as previously described. YopM and YopN were detected with rabbit polyclonal antisera (1:500) raised against full-length Yop proteins. YscF was detected with rabbit polyclonal antisera (1:500) raised against the N-terminal portion of YscF. FLAG-tagged proteins were detected with anti-FLAG M2 monoclonal antibodies (1:1,000) (Sigma-Aldrich) or rabbit anti-DDK monoclonal antibodies (1:1,000) (Origene). MBP was detected with rabbit polyclonal antibody (Sigma). 6X-HIS-tagged proteins were detected with a penta-HIS antibody (1:1,000) (Qiagen).

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