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UNIVERSITY OF CALIFORNIA, SAN DIEGO

***In vitro* studies of the interaction between type III secretion system effectors and potential receptors**

A Thesis submitted in partial satisfaction of the requirements for the degree Master of Science

in

Chemistry

by

Matthew Ku

Committee in charge:

Professor Partho Ghosh, Chair
Professor Judy Kim
Professor Emily Troemel

2013

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Chair

University of California, San Diego

2013

DEDICATION

I would like to dedicate this thesis to my family.

To my parents, thank you for raising me and supporting me throughout my childhood and
young adult life

To Patrick, thank you for being my brother who I always did stuff with.

To Christina, thank you for letting me laugh at you at your expense.

To Deborah, thank you for your childhood antics that I reflect on for a laugh.

And finally, thank you to my beautiful fiancé Leti for always being supportive and seeing
the best in me despite all my shortcomings. I hope when you read this we'll actually be
married.

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ACKNOWLEDGMENTS

I would like to acknowledge Dr. Partho Ghosh for giving me the opportunity to work in his lab. I thank you for all of the suggestions you have given me throughout the years and for guiding me in the completion of this thesis. Without your patience and editing, this thesis would never have been brought to fruition.

I would also like to acknowledge my other committee members Professors Judy Kim and Emily Troemel for being willing to sit on my committee on such short notice.

I would like to acknowledge all members, past and present, of the Partho Ghosh lab. You have not only helped me discuss my research and given me suggestions for my project but have been amongst my most cherished companions in graduate school. I would like to thank Brent for his guidance in cloning and protein purification and for always laughing with me. I thank Cosmo for his energy and suggestions on pull-downs. I thank Romila for all the dancing, nicknames, and guidance on my T3SS projects. I thank Chelsea for all the food and for walking to Poole St. with me all the time. I thank Kharissa for always laughing with me, going to 7L TA meetings with me, and late night dance sessions. I thank Sumit for his knowledge and general know-how of getting results. I would also like to thank Surina, Sanghamitra, and Maher for their companionship while they were still working in the lab with me.

ABSTRACT OF THE THESIS

***In vitro* studies of the interaction between type III secretion system effectors and
potential receptors**

by

Matthew Ku

Master of Science in Chemistry

University of California, San Diego, 2013

Professor Partho Ghosh, Chair

Many Gram-negative bacterial pathogens utilize a virulence mechanism known as the type III secretion system (T3SS). The main function of the T3SS is to transport virulence factors, known as effectors, from the bacterial cytosol into the host cell. Effectors are specifically recognized by the T3SS for transport into host cells. Chaperone proteins, which bind to some effectors before transport, may aid in this specific recognition process. Although the signals that recognize effectors for transport are

known, a receptor to which these signals bind has not been identified. To identify this receptor, previously observed interactions between effectors and potential receptors in bacteria were further characterized *in vitro*. Using *in vitro* co-precipitation assays, it was found that monomers of the cytoplasmic domain of YscD, an essential inner membrane ring component of the *Yersinia* T3SS, were insufficient to bind the effector YopH, as had previously been observed for intact YscD isolated from *Yersinia*. Similarly, an unassembled form of the putative C-ring YscQ was unable to bind to chaperone-effector complexes of SycE/YopE and SycH/YopH or the free chaperone, as had been suggested by data from other organisms. These results suggest binding of an effector to a receptor with an oligomeric ring structure may only occur while the oligomeric ring is assembled. To purify oligomeric rings, a method to reconstitute the intact *Yersinia* injectisome *in vivo* using *E. coli* is described. Furthermore, a method to identify potential receptors for the effector YopE in bacteria is also described.

Chapter 1

Introduction

The type III secretion system (T3SS) is a conserved method of pathogenesis found in many Gram-negative bacteria including *Yersinia*, *Salmonella*, enteropathogenic *Escherichia coli*, and *Shigella*, amongst others. It is characterized by a long needle-like appendage that has been termed the injectisome. The main function of the T3SS is to deliver proteins (termed effectors) from the bacterial cytosol into the host cell, a process known as translocation. These effectors then function to disturb the host cell's defenses to promote infection and disease. Out of the myriad of cytosolic bacterial proteins, effectors are specifically targeted for translocation by the T3SS.

Targeting of these effectors occurs through a targeting sequence located in the ~15 N-terminal amino acids of effectors (called the signal sequence, SS) or the first 15 codons of the mRNA encoding the effectors, or both (Anderson & Schneewind 1997, Lloyd *et. al* 2001). It is hypothesized that a component or components of the injectisome act as a receptor that identifies this targeting sequence. In addition to this signal, some effectors have a region just downstream of the SS that confers binding to specific chaperones and is called the chaperone-binding (Cb) region. These chaperones are mostly dedicated to an individual effector although there are cases of multi-effector chaperones (Ghosh 2004). While not all effectors utilize a chaperone, chaperone binding appears to be important in translocation for the effectors that utilize them. It has been hypothesized that chaperone-effector binding may form a three-dimensional translocation signal identified by a receptor. In the case of the *Yersinia* effector YopE, the Cb region,

bound to its specific chaperone SycE, was shown to be important for the translocation of YopE into HeLa cells (Rodgers *et. al* 2010). Alanine substitution mutations at surface-exposed residues in the YopE Cb region caused the YopE mutants to be deficient for translocation yet still retain the ability to bind SycE and to be secreted by the T3SS. While translocation occurs naturally when the bacteria comes in contact with the host cell, secretion is a non-physiological laboratory-tractable process that results in the secretion of effectors into the extrabacterial milieu. In contrast to the Cb region, the SS region was shown to be indispensable for secretion and translocation (Rodgers *et. al* 2010), suggesting that, while the SS region targets secreted substrates to the injectisome, the Cb region aids in further identifying targets for translocation. Thus, a potential translocation receptor on the injectisome is likely to interact with the effector Cb region bound to its cognate chaperone.

Previous studies have already identified interactions between effectors and injectisome components in bacteria. The injectisome consists of an inner and outer membrane-embedded base and the extracellular-protruding needle. The inner membrane ring and its peripherally-attached components (called the export apparatus), are collectively called the basal body. In *Yersinia*, the inner membrane oligomeric ring component YscD was observed to interact with the effector YopH (Gamez *et. al* 2012). Data also suggest that a transient interaction between YscD and the specific YopH chaperone, SycH, also occurs in the secretion process. Data from other species also suggests that YscQ, the putative C-ring component of the export apparatus of the *Yersinia* TTSS, binds effectors. In *Salmonella*, a complex containing the YscQ ortholog, SpaO,

has been shown to act as a “sorting platform” to control the sequential secretion of substrates (Lara-Tejero *et. al* 2011). These interactions also appear to be mediated by chaperone proteins. Furthermore, YscQ orthologs in *Chlamydia* and *Shigella*, have also been shown to interact with secreted substrates (Morita-Ishihara *et. al* 2006, Spaeth *et. al* 2009). Although effector-injectisome interactions have previously been identified, these interactions have not been characterized *in vitro*.

This thesis focuses on reconstituting previously identified injectisome-effector interactions *in vitro* in order to provide further characterization of potential receptors for effector translocation. In this thesis, the binding of a monomeric cytoplasmic domain of YscD with YopH was tested *in vitro*. The results presented here suggest that YscD may only bind YopH while in its oligomeric form. Similarly, purified monomeric YscQ was assayed for binding with the effector-chaperone complexes SycE-YopE and SycH-YopH and the free chaperones. The results presented here indicate that either YscQ does not bind these components or may bind them only when YscQ is in its oligomeric form. Taken together, these results suggest that the binding of effectors with injectisome components may only occur while the injectisome components are in their oligomeric state

Chapter 2

Monomeric YscDc is not competent to bind YopH

2.1 – Introduction

YscD is an essential inner membrane component of the *Yersinia* injectisome. YscD consists of an N-terminal cytoplasmic portion (1-121, termed YscDc), a transmembrane region (122-142), and a large periplasmic domain (143-419). The periplasmic domain is known to interact with the outer membrane secretin YscC (Ross & Plano 2011) and the inner membrane protein YscJ. YscD likely oligomerizes into a ring-like structure that connects the inner and outer membrane rings (Spreter *et. al* 2009). Both the N- and C-terminal domains were found to be important in secretion, as truncations from either domain were found to abrogate secretion in a dominant negative manner (Ross & Plano 2011). In a dominant negative phenotype, the mutation exerts its negative effect over a wild-type background. The dominant negative phenotype caused by truncation of the YscD C-terminal 66 residues may be explained by the interaction of these residues with YscC. The mutant proteins may cause mislocalization of the protein in the periplasm, stalling the construction of the injectisome. In contrast, the specific role of YscDc in secretion is unknown.

Data suggests that YscDc may facilitate interactions with other T3SS components, including effectors. YscDc has been shown, using X-ray

crystallography, to contain a Forkhead -Associated (FHA) fold (Lountos *et. al* 2011, Gamez *et. al* 2012). FHA domains are widely known to bind proteins at a phosphothreonine although examples of non-phosphorylated protein binding to FHA domains do exist (Tong *et. al* 2010). Charge analysis of this fold in YscDc reveals that the YscDc FHA domain is unlikely to bind proteins in a phosphorylated manner (Gamez *et. al* 2012). Analysis of the crystal structure led to the identification of two loop regions in the FHA fold that are potentially significant for its function. Multiple alanine mutations in either of the two loop regions in the FHA domain (termed L3 and L4) were found to abrogate secretion without affecting the structure or fold of the protein (Gamez *et. al* 2012). This mutation also occurred in a dominant negative manner. Using affinity purification coupled to mass spectroscopy, the interactions of wild-type, L3, and L4 YscD with other T3SS components was determined. Among the interactions found were an expected one with the inner membrane protein YscJ and an unexpected interaction with the effector YopH. Surprisingly, the L3 and L4 alanine-substitution mutations caused a greater quantity of YopH to be bound. Also, a number of proteins that were not observed associated with the wild-type protein were observed bound to the mutants. These included the injectisome components YscP and YscQ and, interestingly, the specific YopH chaperone, SycH. These observations suggest that YopH binds to YscD and components in YscDc somehow facilitate the dissociation of SycH from YopH after binding.

To characterize the binding interaction between YscDc and its mutants with YopH *in vitro*, a co-precipitation assay was used. In this assay, purified monomeric

YscDc was used instead of the full-length oligomer. Previous studies have shown that the L3 and L4 mutations do not affect the fold of YscDc (Gamez *et. al* 2012). The results of this assay indicate that the monomeric form of YscDc is insufficient for binding with YopH. It is likely that YopH binding may only occur while YscD is in its multimeric ring form as opposed to the monomer.

2.2 – Materials and Methods

Cloning of GST-YopH The *yopH* gene was cloned into a pGex vector containing an N-terminal Glutathione-S-Transferase (GST) tag using the Sequence and Ligase Independent Cloning (SLIC) method. The insert, composed of the *yopH* coding sequence and 24 base pairs of the target vector (pGex) sequence, was synthesized using PCR. The target plasmid (pGex) was linearized via inverse PCR. Once synthesized, both the insert and linearized vector DNA were digested with *DpnI* for one hour at 37 °C. One µg of each fragment of digested DNA was then treated with T4 DNA Polymerase (New England Biolabs) supplemented with Bovine Serum Albumin (BSA) for 30 min at 30 °C. The reaction was then quenched using 20 µM dCTP. Three µL of each digested DNA fragment was then mixed together and incubated with DNA Ligase Buffer (50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 1 mM ATP, 10 mM DTT; New England Biolabs) in total reaction volume of 10 µL for an additional 30 minutes at 37 °C before being transformed into competent *E. coli* DH5α with ampicillin resistance for selection. The correct DNA sequence was verified by DNA sequencing. (Genewiz)

Protein Expression and Purification Wild-type and mutant YscDc was expressed from a pET28b expression vector (Novagen) in *Escherichia coli* BL21 (DE3). Bacteria were grown at 37 °C in Lysogeny Broth (LB) supplemented with 50 µg/mL of kanamycin until an OD₆₀₀ of 0.6-0.8 was reached. Expression was induced using 0.5 mM Isopropyl β-D-1 thiogalactopyranoside (IPTG). The culture was grown at 20°C for 16 hours and then harvested via centrifugation (2242 x g, 20 min., 4°C) and resuspended in 1/100th of the culture's original volume in Buffer A (500 mM NaCl; 50 mM sodium

phosphate, NaPi; pH 8.0; 10 mM β -Mercaptoethanol, β ME) supplemented with EDTA-free Protease Inhibitor Cocktail (One tablet per 2 liters of culture, Roche). The cells were then lysed using an Emulsiflex-C5 (Avestin) homogenizer (2 passes at 15,000 psi). The resulting cell lysate was clarified via centrifugation (14,000 x g, 20 min, 4⁰C). The supernatant was then run over a Ni²⁺-nitriloacetic acid (NTA) column equilibrated with Buffer A containing 10 mM imidazole (wash buffer). The column was then washed with 10 column volumes (CVs) of the same wash buffer. The bound proteins were then eluted from the column using 3 column volumes of Buffer A containing 250 mM imidazole. The his-tag was cleaved by treating the protein with Precision Protease (1:50 Protease:Substrate molar ratio) supplemented with 1 mM dithiothreitol (DTT) and overnight dialysis into Buffer A. Cleaved protein was then applied to a Ni²⁺-NTA agarose column equilibrated with wash buffer and the flow-through was collected. The flow through was concentrated by ultrafiltration (3,000 molecular weight cut-off, Amicon) and then further purified using size exclusion chromatography (26/60 Superdex 200, GE Healthcare) in 50 mM NaCl, 10 mM HEPES, pH 7.5, and 10 mM β ME.

GST-YopH and free GST were expressed from a pGex expression vector in *E. coli* BL21 (DE3). Bacteria were grown at 37 ⁰C in LB supplemented with 50 μ g/mL of ampicillin until an OD₆₀₀ of 0.6-0.8 was reached. Expression was induced using 1.0 mM IPTG and the cultures grown at 25⁰C overnight. The cells were then harvested via centrifugation (2,242 x g, 20 min., 4⁰C) and resuspended in 1/100th of the culture's original volume in Phosphate Buffered Saline (PBS, 137 mM NaCl, 27 mM potassium chloride, 100 mM NaPi, pH 7.2) supplemented with Protease Inhibitor Cocktail (1 tablet

per 2 liters culture, Roche). The cells were then lysed using an Emulsiflex-C5 (Avestin) homogenizer. The resulting cell lysate was clarified via centrifugation (14,000 x g, 20 min, 4 °C). The supernatant was then run over a glutathione column equilibrated with PBS. Bound proteins were eluted using five column volumes of 10 mM Tris-HCl, 250 mM NaCl, 10 mM reduced glutathione. Purified GST-YopH was concentrated by ultrafiltration (10,000 Da molecular weight cut-off, Amicon) and further purified using size exclusion chromatography (26/60 Superdex 200, GE Healthcare) in PBS. The protein was then aliquoted, flash frozen, and stored at -80°C.

His-SycH was expressed from a pET28a expression vector in *E. coli* BL21 (DE3) made by Romila Mukerjea. Bacteria were grown at 37 °C in LB supplemented with 50 µg/mL of kanamycin until an OD₆₀₀ of 0.6-0.8 was reached. Expression was induced using 1.0 mM IPTG. The induced culture was grown at 25°C overnight and harvested via centrifugation (2,242 x g, 20 min., 4°C) and resuspended in 1/100th of the culture's original volume in Buffer A (50 mM NaPi, pH 8.0, 250 mM NaCl) supplemented with Protease Inhibitor Cocktail (1 tablet per 2 liters of culture, Roche). The cells were then lysed using an Emulsiflex-C5 (Avestin) homogenizer (2 passes at 15,000 psi). The resulting cell lysate was clarified via centrifugation (14,000 x g, 20 min, 4°C). The supernatant was then run on a Ni²⁺-nitriloacetic acid (NTA) column equilibrated with Buffer A containing 10 mM imidazole followed by washing with 10 CVs of Buffer A. The bound proteins were then eluted using buffer A containing a linear Imidazole gradient ranging from 50-300 mM. The most pure fractions were concentrated by ultrafiltration (3,000 Da molecular weight cut- off, Amicon) and further purified using

size exclusion chromatography (16/60 Superdex 200, GE Healthcare) in PBS. The protein was then aliquoted, flash frozen, and stored at -80°C .

YscDc/GST-YopH Co-precipitation Assay A 2:1 molar ratio of YscDc and YopH were incubated together for 2 hours at 4°C into a final volume of 50 μL . For a positive YopH binding control, a 2:1 molar ratio of SycH;YopH was used. The negative SycH/GST binding control was run using a 2:1 molar ratio of SycH:free GST. To the incubated proteins, 50 μL of a 50:50 slurry of GST- agarose beads and PBS was added. After an additional 2 hour incubation period at 4°C , the mixture was centrifuged ($5,000 \times g$, 25°C , 1 min) and the supernatant collected as the unbound fraction. The beads were washed four times (50 μL volume) with PBS. Bound proteins were removed from the beads by boiling for 5 minutes in SDS-PAGE loading buffer.

2.3 – Results

Preparing Proteins for the YscDc/YopH Co-precipitation Assay

One way to study the physical interaction between two proteins is through the use of an *in vitro* co-precipitation assay. This assay is often used to confirm the existence of binding interactions previously found *in vivo*. To perform this assay, one of the proteins, referred to as the “bait” protein, will contain a tag that confers binding to an immobilized affinity ligand that is specific for the tag. The other protein will remain un-tagged and serve as the “prey” protein. These bait and prey proteins are first incubated together to allow a potential binding interaction to occur. Then, the immobilized affinity ligand specific for the “bait” tag is added to the mixture and allowed to incubate binding between the tag and the ligand to occur. Following extensive washing and elution of the bound proteins, the unbound and bound fractions are separated and visualized. While the bait protein is expected to bind to the immobilized ligand, the appearance of the prey protein in the bound fraction is indicative of an interaction between the bait and prey proteins.

To prepare YscDc and YopH for the co-precipitation assay, each protein was purified separately using a glutathione or nickel agarose column and size exclusion chromatography. To prepare YopH as the “bait” protein in the co-precipitation assay, an N-terminal glutathione S-transferase (GST) tagged construct of YopH was made. GST-YopH was purified on a glutathione agarose column followed by size exclusion chromatography. The approximately 74 kDa GST-YopH band was visualized on SDS-PAGE and Coomassie Staining (Figure 2.1). Many impurity bands remained after the

purification process (Figure 2.3, lane 9), but these bands did not coincide with any of the intended “prey” proteins YscDc or SycH, and thus did not lead to concerns. Unfused GST was also purified (not shown) for use as a negative control for experiments examining binding to GST-YopH. Since SycH and YopH are known to bind one another, an N-terminal His-tagged SycH construct was also made. This construct was purified on a Ni²⁺-NTA agarose column followed by size exclusion chromatography. The approximately 17 kDa band was visualized using SDS-PAGE (Figure 2.2). An approximate 35 kDa remained after purification but this band does not coincide with any on the intended “bait” protein GST-YopH (Figure 2.3A, lane 13). Non-tagged wild-type YscDc, as well as the loop mutants L3 and L4 YscDc, were purified as described previously (Gamez *et. al* 2012).

YscDc/YopH Co-precipitation Assay

To characterize the association between YscDc and YopH, a co-precipitation assay was performed using purified GST-YopH as the “bait” and purified wild-type, L3, or L4 YscDc as the “prey” protein. Glutathione-agarose beads were used to provide the immobilized specific ligand for GST-tagged proteins. GST-YopH and YscDc were mixed in a 2:1 molar ratio and incubated for two hours. After this initial incubation period, glutathione-agarose beads were added and the mixture further incubated for two hours. The beads were then centrifuged and the supernatant was collected as the unbound fraction. Following washes with PBS, bound proteins were removed from the beads by boiling for five minutes in SDS-PAGE buffer. Visualization of the assay was done using SDS-PAGE (Figure 2.3A). To confirm the functional integrity of the GST-

YopH protein, a positive binding control using the specific-YopH chaperone SycH as the “prey” protein was also done. The presence of SycH in the bound fraction of this control confirmed the integrity of GST-YopH (Figure 2.3A, lane 8). To determine that the binding of SycH to YopH was mediated by YopH and not by the GST fusion partner, a negative binding control utilizing purified GST as the “bait” and SycH as the “prey” protein was also performed (Figure 2.3B). As evident by the lack of YscDc in the bound fractions of the assays (Figure 2.3A; lanes 2, 4, and 6), purified monomeric YscDc is not sufficient for interaction with YopH.

2.4 - Discussion

In the *Yersinia* injectisome, YscD is an inner membrane ring component that connects the outer membrane (OM) ring with the inner membrane (IM) ring. . YscD also has a cytoplasmic domain (YscDc) that contains a forkhead associated (FHA) fold (Lountos *et. al* 2011, Gamez *et. al* 2012). Alanine-substitution mutations at two loop regions in the FHA domain (termed L3 and L4) were found to abrogate secretion in a dominant negative manner (Gamez *et. al* 2012). Using affinity purification coupled to mass spectroscopy, YopH was found to associate with wild-type YscD. Interestingly, the L3 and L4 mutants associated with more YopH than wild-type and associated with the YopH chaperone SycH and the injectisome components YscQ and YscP. This suggests that YscD, specifically YscDc, may be a receptor for effectors.

In this chapter, the previously reported interaction between YscDc and the effector YopH was further characterized using an *in vitro* co-precipitation assay. Using purified monomeric, wild-type YscDc and the monomeric L3 and L4 mutants as the “prey” and GST-tagged YopH as the “bait” in a co-precipitation assay, it was found that neither wild-type YscDc nor the loop mutants L3 and L4 were sufficient for interaction with GST-YopH (Figure 2.3A, lanes 1-6). This result is unlikely to be caused by an improperly folded YopH protein, as its specific chaperone, SycH, was still able to bind to the protein (Figure 2.3A, lane 8). SycH binding to GST-YopH was not mediated through GST, as SycH was unable to bind to unfused GST alone (Figure 2.3B). This result suggests that the previously reported interaction between YscD and YopH may only occur while YscD is in its oligomeric form. Even at concentrations suitable for

crystallization, YscDc was found to exist as a monomer (Gamez *et al* 2012). This suggests that oligomerization may be initiated by its C-terminal periplasmic domain, perhaps by its interaction with the outer membrane oligomeric ring protein YscC. It is possible that interaction of YscD with YopH and other proteins may be conferred by the interface between multiple copies of YscDc. This may be tested in the future by purifying YscD in its oligomeric form and repeating the co-precipitation assay with the YscD oligomer.

The results reported here suggest that YscD may only bind to YopH while YscD is in its oligomeric state. This result may be indicative of a protein-binding domain that forms as a result of interactions among YscD FHA domains. Since many of the components of the injectisome occur as oligomeric structures, the function of a T3SS component may only be apparent *in vitro* by studying the protein in its oligomeric form.

2.5 – Figures

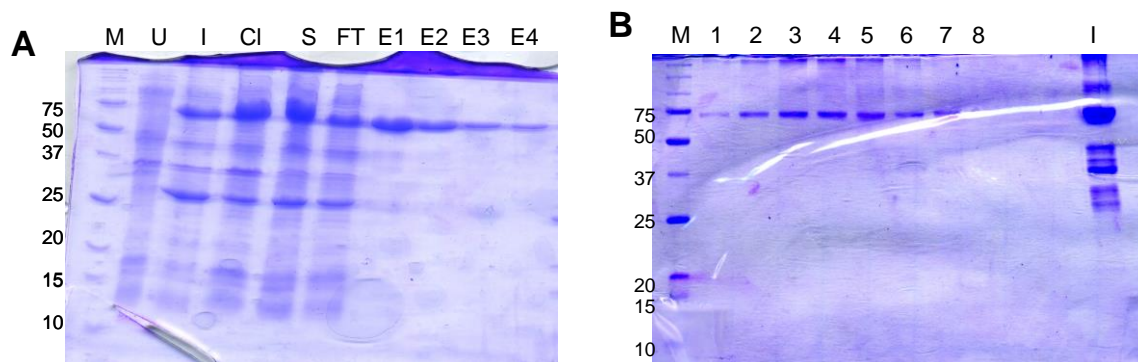


Figure 2.1 – Purification of GST-YopH

A – Glutathione Agarose Column Purification. GST-YopH was expressed and purified using a glutathione agarose column and eluted using four column volumes of 10 mM reduced glutathione. Purification was visualized using 16% SDS-PAGE and Coomassie Staining. Lanes (*from left to right*) correspond to M (Marker), U (Uninduced), I (Induced), Cl (Cell Lysate), S (Supernatant), FT (Flowthrough), E1-4 (Elutions). Eluted fractions were pooled, concentrated and further purified using size exclusion.

B – Size Exclusion Chromatography. Pooled and concentrated fractions from the glutathione agarose column were purified using size exclusion chromatography (Superdex 200, 16/60). Purification was analyzed via 16% SDS-PAGE and Coomassie Stain. Lanes 1-8 are samples taken from the single peak of the chromatograph. Lane I is the pooled, concentrated fraction from the glutathione column elution (shown in panel A).

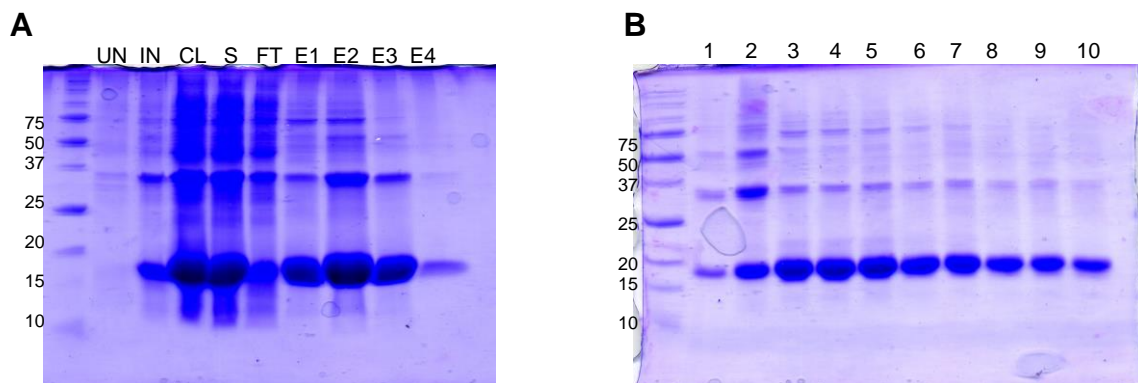


Figure 2.2 – Purification of His-SycH

A – Nickel Column Purification. Uninduced (UN), Induced (IN), Cell Lysate (CL), Supernatant (S), Flowthrough (FT), and Elution 1-4 (E1-E4) samples were shown to indicate purification of His-SycH from a pET28b expression vector in *E. coli* BL21 (DE3). Proteins were separated using 12% SDS-PAGE and visualized using Coomassie Staining.

B – Size Exclusion chromatography. Eluted fractions from nickel column purification were further purified using size exclusion chromatography (Superdex 200 16/60). Fractions corresponding to two main peaks (1-2 and 3-10) are separated using 12% SDS-PAGE and Coomassie Staining. Fractions from the second peak (8-10) were pooled and used for the assays.

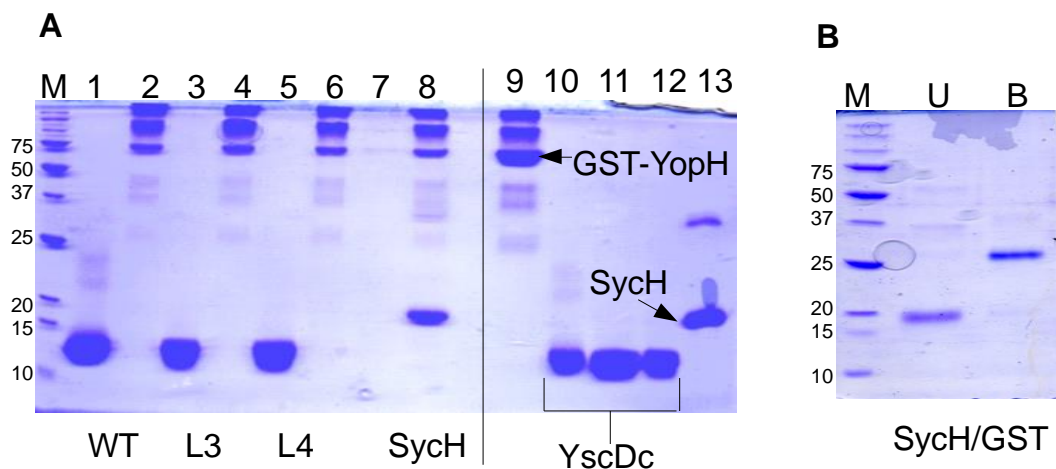


Figure 2.3 – YscDc Co-precipitation Assay with GST-YopH

A – YscDc and YopH Co-precipitation Assay. A GST coprecipitation assay (*left side*) was performed to determine binding between purified monomeric YscDc as the “prey” and GST-YopH as the “bait”. The bands were separated using 14% SDS-PAGE and visualized by Coomassie staining. The fractions were separated into unbound (*odd*) and bound (*even*) lanes. Wild-type (lanes 1-2), L3 (3-4), and L4 (5-6) YscDc and GST-YopH were assayed for binding. (as indicated on bottom of gel) A positive binding control using SycH was also done to verify that GST-YopH is still competent for binding (7-8). The lack of YscDc found in the bound fractions indicates that no detectable binding between YscDc and YopH occurred. The right side of the gel contains the inputs (from 9-13): GST-YopH, Wild-Type YscDc, L3 YscDc, L4 YscDc, and SycH.

B – Negative GST-Binding Control. A negative binding control using purified GST and His-SycH was done to show that His-SycH does not bind to the GST portion of GST-YopH. The unbound (U) and bound (B) fractions were separated by 14%SDS-PAGE and visualized by Coomassie Staining. The absence of SycH in the bound (B) lane indicates that SycH does not bind to GST.

Chapter 3

Unassembled YscQ is not sufficient to bind the Effectors YopE and YopH

3.1 - Introduction

YscQ is a component of the export apparatus of the *Yersinia* injectisome. YscQ and its orthologs are well-conserved amongst organisms utilizing TTSSs. Sequence homology to the FliM/FliN bacterial flagellum proteins suggests that it forms the C-ring on the cytoplasmic portion of the injectisome. While FliM and FliN are usually encoded by separate genes, YscQ and its homologues are usually encoded by only one gene that forms two products. It was found that the homologous FliN protein in YscQ forms as a product of an internal translation initiation site in the YscQ gene (Bzymek *et. al* 2012). This trait is also observed in the YscQ ortholog in *Salmonella*, SsaQ (Yu *et. al* 2011). The truncated form of YscQ, termed YscQ-C, was shown to form a domain-swapped homodimer that may bind to the full-length YscQ protein. Copies of this complex likely oligomerize to form the putative T3SS C-ring, which lies on the most terminal cytosolic portion of the injectisome (Morita-Ishihara *et. al* 2006).

Evidence suggests that YscQ may contribute to secretion by binding secreted substrates. Both YscQ-full length (FL) and YscQ-C were shown to be essential for secretion (Bzymek *et. al* 2012). Evidence from YscQ orthologs in other species have suggested that YscQ may bind to secreted substrates. The

YscQ ortholog in *Shigella*, Spa33, was shown to interact with effectors (Morita-Ishihara *et. al* 2006). The ortholog in *Chlamydia*, CdsQ, was shown to interact with a chaperone-effector complex (Spaeth *et. al* 2009). In *Salmonella*, a complex containing the YscQ ortholog SpaO, was shown to act as a “sorting platform” for the ordered recruitment of secreted substrates to the injectisome (Lara-Tejero *et. al* 2011). These interactions were shown to be dependent on the presence of chaperones.

To assay whether purified monomeric YscQ can bind the effectors YopE and YopH *in vitro*, a co-precipitation assay was used. Since the interaction may be mediated by their specific chaperones, SycE and SycH, respectively, the chaperones were also tested in a free form and in complex with the effectors. YscQ was purified with the heterotrimer of YscQ-FL and YscQ-C. The result of the assay show that neither the chaperones nor the chaperone-effector complexes bind to unassembled YscQ. As in the previous chapter, this result does not necessarily exclude YscQ as an effector receptor. Since YscQ is likely to form an oligomeric ring, the interactions may only occur while YscQ is in an oligomeric form or while in complex with other injectisome components. An alternative explanation for the lack of binding to YscQ may be that the interactions seen in other species may not exist in *Yersinia* species.

3.2 – Materials and Methods

Protein Expression and Purification YscQ-FL was expressed using a pET28b expression vector in *E.coli* BL21 (DE3). Bacteria were grown at 37⁰C until an OD₆₀₀ of 0.6-0.8 was reached. The cultures were induced using 0.5 mM IPTG and grown overnight at 18⁰C. The culture was then harvested via centrifugation (2,242 x g, 20 min, 4⁰C) and then resuspended in lysis buffer (100 mM NaPi, pH 8.0, 500 mM NaCl, 20 mM Imidazole). The cells were then lysed using an Emulsiflex-C5 (Avestin) homogenizer (2 passes at 15,000 psi) and the resulting cell lysate was clarified via centrifugation (14,000 x g, 20 min, 4⁰C). The supernatant was then run over a Ni²⁺-nitriloacetic acid (NTA) column equilibrated with lysis buffer followed by a wash with 10 CVs of lysis buffer. The bound proteins were then eluted using 2 column volumes of lysis buffer containing 250 mM imidazole. To cleave the his-tag, Precision Protease (1:50 mole ratio of protease to substrate) was added to the eluted protein along with 1 mM DTT and dialyzed overnight at 4⁰C into 20 mM NaPi, pH 8.0, 500 mM NaCl, and 40 mM imidazole. The cleaved protein was then added back to a Ni²⁺-NTA column equilibrated with wash buffer and the flow through was collected. This flow through was desalted using an Akta purifier into 20 mM Tris, pH 8.0, and 50 mM NaCl and then run through a Q column using a linear gradient between 50-500 mM NaCl. The most pure fractions were pooled and further purified using size exclusion chromatography (26/60 Superdex 200, GE Healthcare) into PBS. The protein was then aliquoted, flash frozen, and stored at -80⁰C.

SycE/YopE-His was expressed from a pET28b expression vector in *E.coli* BL21 (DE3). Bacteria were grown in LB supplemented with 50 µg/mL kanamycin at 37⁰C

until an OD₆₀₀ of 0.6-0.8 was reached. Expression was induced using 0.5 mM IPTG and the culture grown at 25 °C overnight. The culture was then harvested via centrifugation (2,242 x g, 20 min, 4°C) and resuspended in 1/100th of the culture volume in Buffer A (50 mM Sodium Phosphate, pH 8.0; 300 mM sodium chloride, NaCl; 10 mM βME) supplemented with EDTA-free Protease Inhibitor Cocktail (1 tablet per 2 liters culture, Roche). The cells were then lysed using an Emulsiflex-C5 (Avestin) homogenizer (2 passes at 15,000 psi). After lysis, the lysate was treated with DNase (20 μg/mL) for 30 minutes on ice. The resulting cell lysate was clarified via centrifugation (14,000 x g, 20 min, 4°C). The supernatant was then run over a Ni²⁺-NTA column equilibrated with Buffer A containing 25 mM imidazole. The bound proteins were eluted using 2 column volumes of Buffer A containing 500 mM imidazole. To further purify the protein and prepare the protein for co-precipitation assays, size exclusion chromatography (26/60 Superdex 200, GE Healthcare) in 50 mM NaPi, pH 8.0; 50 mM NaCl, and 10 mM βME was also done.

SycE-His was purified from a pET28b expression vector in *E. coli* BL21 (DE3). Bacteria were grown in LB supplemented with 30 mg/mL kanamycin until an OD₆₀₀ of 0.6-0.8 was reached. Expression was induced using 1.0 mM IPTG. The culture was shifted to 25°C and grown for 4 hours. Cells were harvested by centrifugation (6700 RPM, 15 min, 4°C) and resuspended in 1/100th culture volume lysis buffer (50 mM NaPi, pH 8.0, 300 mM NaCl, 10 mM βME, 0.5 mM phenylmethylsulfonyl fluoride, 20 mM Imidazole). The cells were then lysed using a Branson Sonifier 240/450, with a 90% duty cycle; output control of 9; 15 times for 30 seconds. The resulting cell lysate was

clarified via centrifugation (20,000 RPM, 20 min, 4⁰C) and then treated with DNase (20 µg/mL) for 30 min at 25⁰C. The supernatant was then run over a Ni²⁺-NTA column and eluted using a linear increasing gradient of imidazole. The protein was then further purified using size exclusion chromatography (16/60 Superdex 75, GE Healthcare) into 50 mM NaPi, pH 8.0, 500 mM NaCl, and 10 mM βME.

His-SycH and GST-YopH were purified as described in Chapter 2.2.

YscQ and SycE/YopE or SycH/YopH Co-precipitation Assay A 2:1 molar ratio of YscQ and either the lone chaperones SycE-His or His-SycH or the chaperone-effector complexes SycE/YopE-His or His-SycH/GST-YopH was incubated for 2 hours at 4⁰C in a total volume of 50 µL. A negative binding control was also done using YscQ only. After this initial incubation period, 50 µL of a 50:50 slurry of Ni²⁺-NTA agarose beads and Nickel Wash Buffer (50 mM NaPi, pH 8.0, 250 mM NaCl, 25 mM Imidazole) was added and the mixture was allowed to incubate for an additional two hours at 4⁰C on a rotating platform. The mixture was then centrifuged (5,000 x g, 25⁰C, 1 min) and the supernatant collected. The remaining beads were washed four times with wash buffer (50 mM Sodium Phosphate, pH 8.0; 250 mM NaCl; 25 mM Imidazole). Bound proteins were removed from the beads by boiling for 5 minutes in SDS-PAGE Sample Buffer.

3.3 – Results

Preparation of Proteins for Co-precipitation Assay

To prepare the proteins for the YscQ/Chaperone-effector co-precipitation assay, each of the proteins were individually purified. To prepare YscQ to function as the “prey” protein for the co-precipitation assay, a His-tagged version of YscQ was purified on a nickel agarose column. The His-tag on YscQ was then cleaved off YscQ using Precision Protease. The protein was further purified using anion exchange chromatography and size exclusion chromatography into PBS (Figure 3.1). Only one peak was observed in the size exclusion chromatograph that contained both the YscQ-FL and YscQ-C translation products were present, as observed by SDS-PAGE (Figure 3.1C). This confirmed that the purified YscQ represented a heterotrimeric complex of full-length YscQ (YscQ-FL) and YscQ-C. SycE/YopE-His (SEYE) was also purified using a nickel agarose column but the hexahistidine tag on YopE was retained to allow the SEYE complex to function as the “bait” protein. Similar to the purification of the YscQ complex, a single peak in the size exclusion chromatograph with the observation of both SycE and YopE-His proteins on SDS-PAGE confirmed the presence of the intact complex (Figure 3.2B). In lieu of purifying the SycH/YopH (SHYH) complex, a mixture of previously purified His-SycH and GST-YopH (Chapter 2.2) were used in the assay. His-SycE (SE) was previously purified (Catarina Raposo) from a nickel column and size exclusion chromatography. Some impurities remained in the proteins after purification (Figure 3.3B) but, with the exception of SEYE (Figure 3.3B, lane 3), these impurities did not affect the results of the assay. A fresh sample of SEYE was re-purified using an

identical protocol that did not contain the impurity (Figure 3.4). **YscQ Co-precipitation with SycE/YopE and SycH/YopH complexes**

As described in Chapter Two, a co-precipitation assay may be used to characterize the physical interaction between two proteins. To determine potential binding interactions between YscQ and the chaperone-effector complexes of SycE/YopE and SycH/YopH, a co-precipitation assay was performed utilizing YscQ as the “prey” protein and His-tagged versions of the chaperone-effector complexes SEYE and SHYH as the “bait”. Since potential binding of YscQ and chaperone-effectors may be mediated through the chaperone protein, free his-SycE (SE) and his-SycH (SH) were tested as well. Nickel agarose beads were used as the affinity ligand for His-tagged proteins.

YscQ was incubated along with either SE, SEYE, SH, or SHYH to allow for potential binding interactions to occur. A negative binding control utilizing YscQ with no added “bait” protein was also assayed. The mixtures were then incubated with nickel agarose beads to allow for binding of the “bait” proteins to the beads. After this incubation, the unbound fraction was collected and the beads were washed with wash buffer (50 mM NaPi, pH 8.0, 250 mM NaCl, 25 mM Imidazole). Bound proteins were removed from the beads by boiling for five minutes in SDS-PAGE buffer. The unbound and bound fractions were then visualized on SDS-PAGE. A faint band of YscQ-FL (~36 kDa band) was observed in all of the bound lanes. However, this was determined to be the non-specific binding of YscQ-FL, as the band was also observed in the negative binding control (Figure 3.3A, lane 10). As evidenced by the lack of YscQ-FL or YscQ-C in the bound fractions (Figure 3.3A, even lanes), it was determined that purified monomeric

YscQ is not sufficient for the binding of chaperone-effector complexes or the free chaperones. Although there appears to be a 36 kDa band in the bound portion of the SEYE co-precipitation assay that coincides with YscQ-FL (Figure 3.3A, lane 6), the intensity of this band does not appear to be stronger than expected given the impurity found in the input (Figure 3.3B, lane 3). To confirm that the observed band is indeed an impurity from the purification process, SEYE was re-purified without the offending impurity and the assay repeated (Figure 3.4). Although a faint band for YscQ-FL was observed in the bound lane for this assay as well, the band did not seem brighter than what was expected given the non-specific binding found in the negative control (Figure 3.3B, lane 10). Thus, it was determined to be non-specific binding of YscQ-FL to the nickel beads. The ~36 kDa impurity observed in His-SycH (Figure 3.3B, lane 4), was not observed in the assay (Figure 3.3A, lane 6). Taken together, these results show that unassembled YscQ are not competent for binding to chaperones or chaperone-effector complexes. It is also possible that the interactions observed for orthologs of YscQ with effectors may not occur in *Yersinia*.

3.4 – Discussion

YscQ is the presumed C-ring component of the *Yersinia* injectisome. The single gene of YscQ encodes two products via an internal translation initiation site (Bzymek 2012). These two products, full-length YscQ (YscQ-FL) and the truncated fragment YscQ-C, form a heterotrimeric complex. Two molecules of YscQ-C form a domain-swapped homodimer that interacts with one molecule of YscQ-FL. Twelve to fourteen copies of this complex likely oligomerize to form the putative C-ring. Both YscQ-FL and YscQ-C were shown to be essential for secretion to occur. Orthologs of YscQ in *Shigella*, and *Chlamydia* have been shown to bind secreted substrates and their chaperones. (Morita-Ishihara 2006, Spaeth 2009) SpaO, the YscQ ortholog in *Salmonella*, has even been shown to act as a “sorting platform” for the sequential secretion of substrates (Lara-Tejero 2011). This suggests that YscQ may serve as a receptor for effectors and their chaperones.

To determine whether YscQ is able to bind the chaperone-effector complexes SycE/YopE (SEYE) and SycH/YopH (SHYH), an *in vitro* co-precipitation assay utilizing untagged YscQ and His-tagged versions of the chaperone-effector complexes or the free chaperone was carried out. The His-tagged chaperone-effector complexes served as the “bait” protein and YscQ the “prey” protein. The purified YscQ was shown to be unassembled into the free heterotrimeric form (Figure 3.1C). Neither the chaperone-effector complexes SEYE and SHYH, nor the free effectors SycE (SE) and SycH (SH) were able to co-precipitate YscQ. (Figure 3.3A, even lanes) An approximately 36 kDa band that may appear to be co-precipitated with YscQ-FL (Figure 3.3A, lane 4) was

deemed to be an impurity of SEYE. This was confirmed by re-purifying SEYE without the impurity and running the assay again (Figure 3.4). The results of this assay indicate that YscQ in its unassembled form binds neither the chaperone-effector complexes nor the free chaperones.

As mentioned in the Discussion section of Chapter Two, binding of effectors to an injectisome receptor may only occur while the oligomeric form of the injectisome component is assembled. In the case of YscQ, copies of the YscQ-FL/YscQ-C heterotrimeric complex likely assemble to form the C-ring. Determination of the structure of the ring may provide further indication of where protein-binding may occur.

An alternative explanation is that the effector binding interactions seen in other T3SS-utilizing organisms may not be seen in *Yersinia spp.* While effector function varies greatly depending on the lifestyle of the species, the components comprising the injectisome appear to be well-conserved. (Cornelis 2006) As such, not all injectisome components may function in the same way in every organism. For example, an ATPase located on the export apparatus of the injectisome is well-conserved amongst T3SS-utilizing organisms. In *Salmonella*, this ATPase is known to dissociate chaperones from their effectors, presumably allowing the unfolded effector to pass through the injectisome. (Akedo 2005) It was shown that the *Salmonella* ATPase, InvC, interacts with the chaperone-effector complex SicP-SptP as well as the free chaperone SicP. Similarly, the *E. coli* ATPase EscN was able to interact with the chaperone-effector combination CesT-Tir (Gauthier & Findlay 2003). However, this interaction was not seen with the *Yersinia* ATPase YscN and the chaperone-effector complex SycE/YopE.

(Rodgers 2008) While YscQ and its orthologs are well-conserved in the T3SS, its function may differ depending on the species.

The results reported here indicate that unassembled YscQ is able to bind to neither the chaperone-effector complexes of SycE/YopE and SycH/YopH nor the free chaperones. The binding interaction may only occur while the YscQ oligomeric ring is assembled. It is also possible that these binding interactions may not occur in *Yersinia* spp.

3.5 – Figures

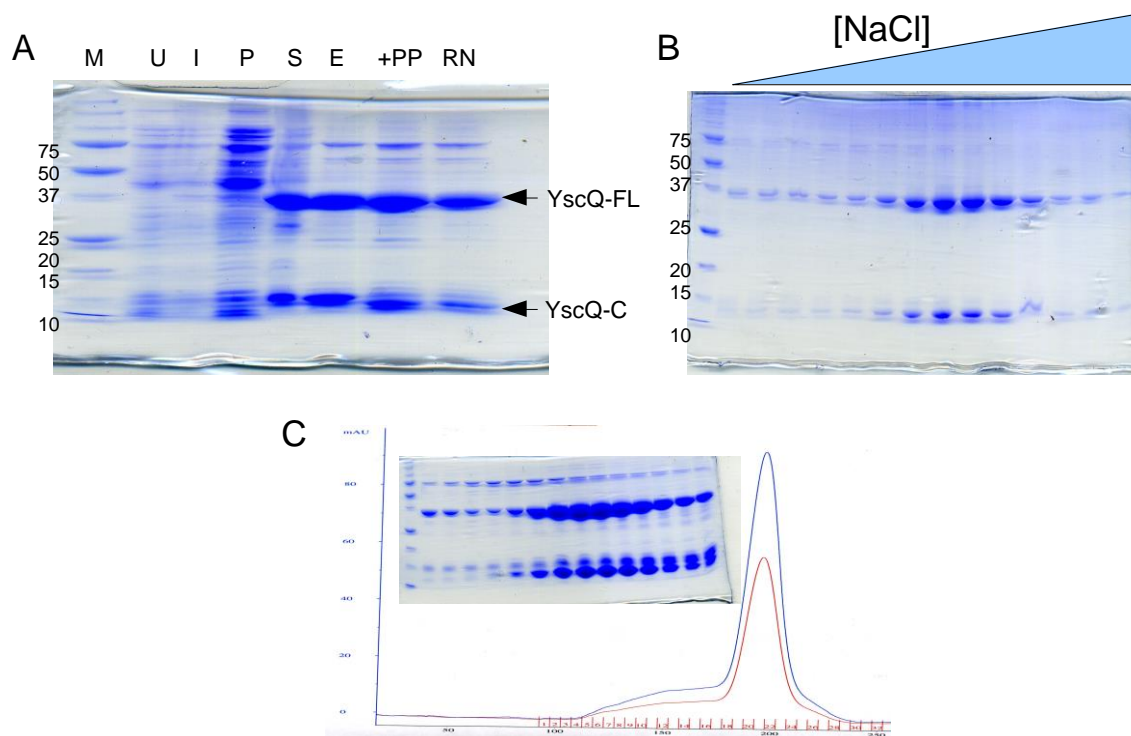


Figure 3.1 – Purification of YscQ

A – Nickel column purification and Precision protease treatment. YscQ-FL was purified using Ni-NTA agarose column purification followed by Precision protease cleavage and reverse nickel purification. Bands were separated using 12% SDS-PAGE and visualized using Coomassie staining. The lanes are as follows: M = Marker, U = Uninduced, I = Induced, P = Pellet, S = Supernatant, E = Elution, +PP = Precision Protease cleavage, RN = Reverse Nickel treatment

B – Purification using anion exchange chromatography. Further purification of cleaved YscQ was done using anion exchange chromatography (Q column) using a linear 50-500 mM NaCl gradient (as indicated by labeled gradient above lanes). Bands were separated by 12% SDS-PAGE and Coomassie staining.

C – Purification using size exclusion chromatography. YscQ was further purified using size exclusion chromatography (Superdex 200 26/60). Fractions corresponding to the single peak observed in the chromatograph were analyzed using 12% SDS-PAGE and Coomassie Staining. The Coomassie-stained gel is shown inlaid on top of the size exclusion chromatograph.

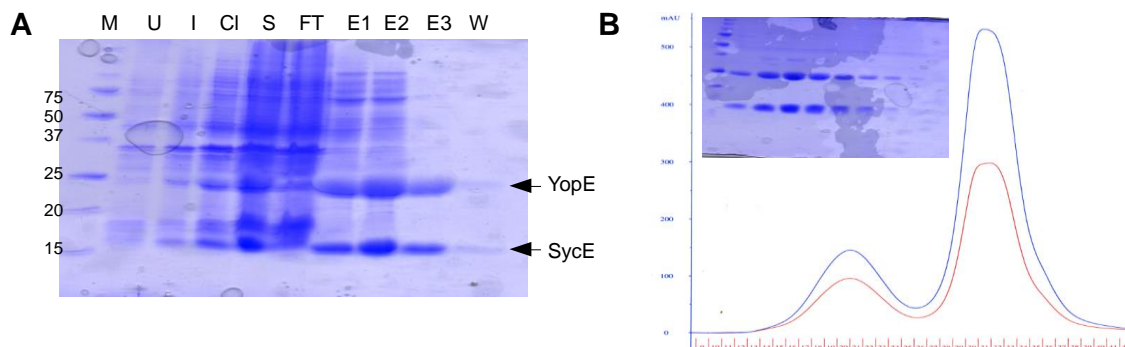


Figure 3.2 – Purification of SycE/YopE-His

A – Purification of SycE/YopE-His using Nickel Agarose Column Purification. The SycE/YopE-His protein is purified via Ni-NTA agarose column purification. The column was eluted with three column volumes of 250 mM imidazole. The proteins were separated by 16% SDS-PAGE and visualized using Coomassie Staining. The lanes are labeled as M (Marker), U (Uninduced), I (Induced), Cl (Cell Lysate), S (Supernatant), FT (Flowthrough), E (Elution), W (Wash).

B - Purification of SycE/YopE-His using size exclusion chromatography. Nickel column purified SycE/YopE-His was further purified using a size exclusion column (Superdex 200, 16/60). Proteins were separated using 16% SDS-PAGE and visualized using Coomassie Staining. The fractions visualized on the inlaid gel correspond to the larger second peak shown on the size exclusion chromatograph.

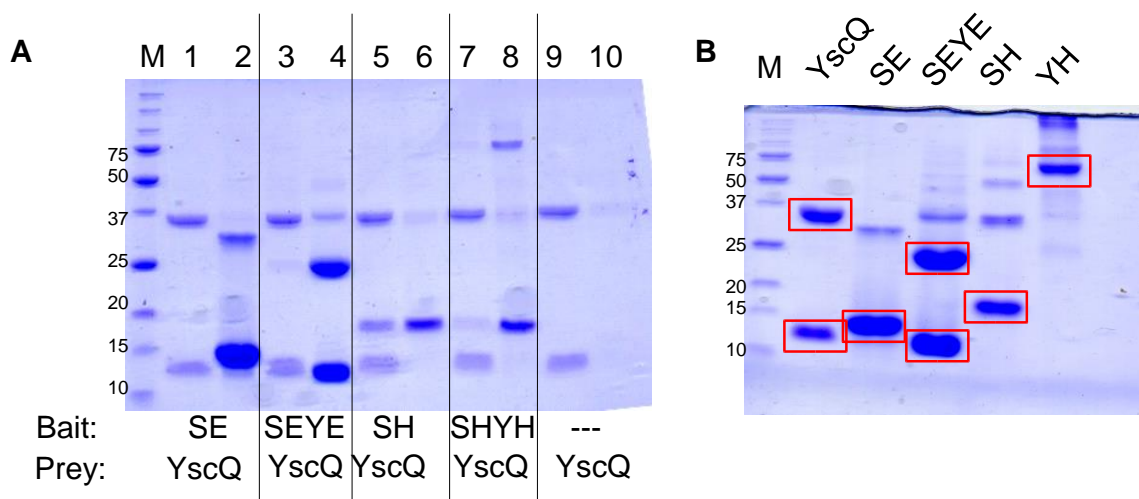


Figure 2.3 – YscQ / Chaperone-Effector Complex Co-precipitation Assay.

A – YscQ/ Chaperone-effector Co-precipitation Assay. A nickel coprecipitation assay was done to determine if the chaperone-effectors SycE/YopE and SycH/YopH bind to YscQ. The bands were separated via 14% SDS-PAGE and visualized by Coomassie Staining. Using YscQ as the untagged bait protein, the differing prey proteins consist of His-SycE (SE), SycE/YopE-His (SEYE), His-SycH (SH), and His-SycH/GST-YopH (SHYH). The lanes are organized as unbound (*odd*) and bound (*even*) fractions. The “bait” protein used with the “prey” YscQ are indicated on the bottom. A negative control with no bait protein is also run (9-10). The absence of YscQ in the bound (*even*) lanes leads to the conclusion that YscQ does not interact with any of the assayed “bait” proteins. The band located at ~35 kDa in the bound fraction of the SEYE assay lane (lane 4) was determined to be an impurity from the purification process (as shown in panel B, Lane 3).

B - Inputs of proteins for Co-precipitation Assay. The inputs for the co-precipitation assay are labeled: YscQ-FL, His-SycE, SycE/YopE-His, His-SycH, GST-YopH. The bands were separated via 14% SDS-PAGE and visualized using Coomassie Staining. Bands that are not boxed in red represent impurities from purification. With the exception of SycE/YopE-His, the impurities from the purification process do not appear to affect the determination of a positive or negative result.

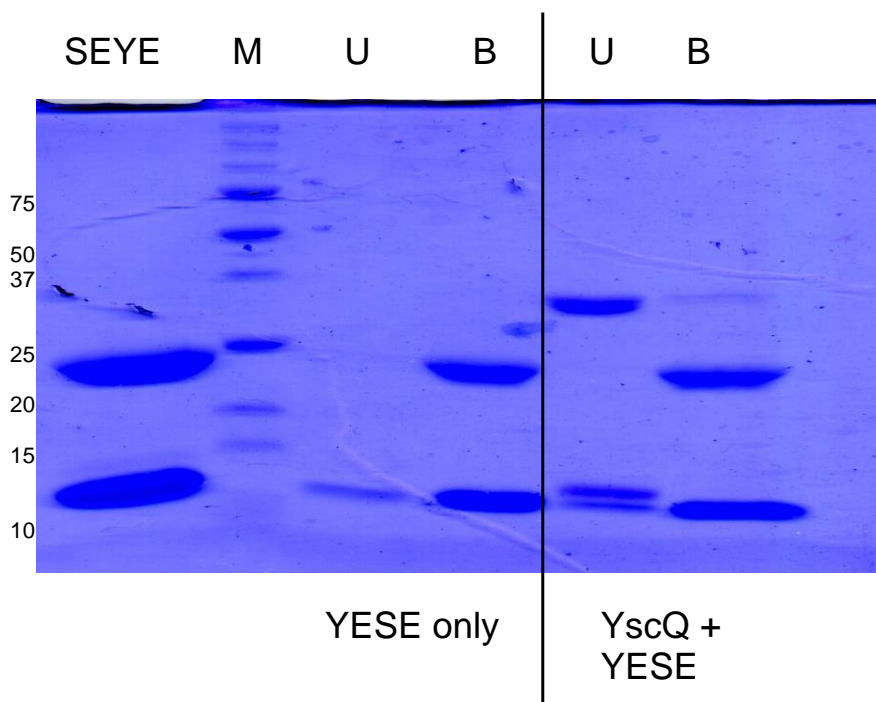


Figure 2.4 – Repurification of SycE/YopE-His and Co-precipitation assay

To confirm that the ~35 kDa band observed in the SycE/YopE-His bound fraction in the first co-precipitation assay (Figure 2.3A, lane 4) was an impurity, SycE/YopE-His was re-purified and the co-precipitation assay repeated. SEYE represents the re-purified SycE/YopE-His sample. Two co-precipitation assays were done. YESE only refers to SycE/YopE-His assayed with no YscQ “prey” protein added. The YscQ + YESE column corresponds to a repeat of the co-precipitation assay utilizing YscQ as the “prey” and YESE as the “bait.” Fractions are labeled as unbound (U) and bound (B) lanes. The proteins were separated by 14% SDS-PAGE and visualized using Coomassie Stain..

Chapter Four

A method to identify receptors for YopE targeting signals

4.1 – Introduction

Although the role of chaperones in the T3SS is not fully understood, evidence suggests that chaperone binding plays an active role in the translocation of chaperones. Although the secretion signal (the N-terminal 1-15 amino acids or mRNA codons) has been found to be competent for secretion of proteins into the extrabacterial milieu (Anderson and Schneewind 1997, Schesser *et. al* 1996, Sory *et. al* 1994) , the chaperone binding domain (Cb) was shown to be crucial for translocation of proteins into the host cell in wild-type strains of *Yersinia* (Ghosh 2004). Despite the lack of sequence similarity amongst effector chaperones, the mechanism by which they interact with their cognate effectors is similar (Ghosh 2004). Insights into the role of chaperone binding in translocation can be revealed by further studying the interaction of YopE with its specific chaperone, SycE.

YopE is one of the most highly translocated *Yersinia* effectors. Its effector function in the host cell is as a Rho-GTPase Activating Protein (Rho-GAP), which helps to disrupt the host actin cytoskeleton (Von Pawel-Rammingen *et al.* 2000). The domains of YopE consist of the N-terminal SS domain (1-15), the Cb domain (23-78), and the Rho-GAP domain (100-219). Binding of SycE occurs in a 2:1 SycE:YopE ratio (Birtalan *et. al* 2002) where the Cb region of YopE wraps around a homodimer of SycE. This binding motif is also conserved in other

chaperone-effector complexes (Luo *et. al* 2001). The interaction of SycE with YopE was shown to confer a disorder to order structure on the Cb region of YopE that was localized to the Cb region (Rodgers *et. al* 2008). Further analysis on this region has identified certain residues that remain solvent-exposed upon binding to SycE (Rodgers *et. al* 2010). Multiple alanine mutations (hereafter referred to as 3-ala and 5-ala) at these solvent-exposed residues in the YopE Cb region were found to abrogate YopE translocation but had no aberrant effect on secretion. Importantly, these YopE mutants still retained the ability to bind SycE and were present in similar quantity to that of wild-type YopE. It has been suggested that these solvent-exposed residues may be crucial for an interaction between YopE and an effector receptor located on the injectisome.

The goal of this project is to identify a potential receptor using *in vivo* studies with YopE. In contrast to the previous co-precipitation assays, no specific injectisome component is targeted. Instead, the identity of the receptor will be assayed using co-precipitation binding to the effector. A truncated form of YopE that contains the SS and Cb region will be used (*yopE*₁₋₈₀) in lieu of the full-length protein to isolate interactions with the YopE SS and Cb regions. A *yopE*-deficient strain of *Yersinia pseudotuberculosis* ($\Delta yopE$) will be transformed with a plasmid containing *yopE*₁₋₈₀ and an attached affinity tag. This tag consists of a strep-binding protein (SBP) and hexahistidine (His) tag (Figure 4.1). After expression of *yopE*₁₋₈₀-*sbp-his* in $\Delta yopE$, the binding partners of YopE will be found using affinity purification coupled to mass spectroscopy. Comparison of the proteins found to be bound to the wild-type and the mutants may lead to the identity of the effector receptor. Whichever proteins that were

found bound to the wild-type but not to the translocation-deficient mutants will be identified as a potential receptor for the translocation signal formed by chaperone binding to the Cb region.

4.2 – Materials and Methods

Cloning of truncated *yopE*₁₋₈₀ Constructs Inserts of the wild-type, 3-ala, and 5-ala *yopE* gene (1-80 amino acids) were constructed via PCR and inserted into a pBad vector containing a C-terminal Strep-Binding Protein

(SBP:ATGGATGAAAAAACCACCGGCTGGCGCGGGCGGCCATGTGGTGGGAAGGCCTGGCGGGCGAACTGGAACAGCTGCGCGCGCCTGGAACATCATCCGCAGG GCCAGCGCGAACCGGG) and hexahistidine tag using SLIC. The tags were linked together by a short linker region containing glycines and serines (GGCAGCGGCAGC). The correct DNA sequence was confirmed using DNA sequencing (Genewiz).

Expression Trial Cloned YopE-SBP-his pBad constructs were transformed into *E. coli* Top10 cells. Bacteria were grown at 37⁰C in LB supplemented with 50 µg/mL ampicillin until they reached an OD₆₀₀ of 0.8. Expression was induced using 1% arabinose at 37⁰C. Samples were taken after two and three hours. Since arabinose is metabolized by bacteria, additional arabinose was added to 1% of the total culture volume after two hours. Expression was analyzed via anti-his Western blot.

Western Blotting After running the samples on 14% SDS-PAGE, the proteins were transferred onto a polyvinylidene fluoride (PVDF) membrane using a semi-dry transfer method at 20 volts for 30 minutes. The membrane was then washed four times with 10 mL Tris-buffered saline (TBS; 50 mM Tris base, 150 mM NaCl) for five minutes per wash. The membrane was blocked for one hour at 25⁰C in blocking solution (5% weight by volume dried nonfat milk and TBS) followed by four washes with 10 mL TBST (TBS, 0.5% volume Tween). The membrane was then incubated with anti-his primary blocking

solution (1:2000 dilution of mouse anti-his monoclonal antibody with blocking solution) for one hour at 25 °C, washed four times with TBST, and then incubated with anti-mouse secondary solution (1:5000 dilution of horseradish peroxidase-conjugated goat anti-mouse antibody and blocking solution) for 45 minutes at 25 °C. The membrane was then washed four times with TBST. The blot was developed using SuperSignal West Pico Chemiluminescent Substrate and SuperSignal West Femto Maximum Sensitivity Substrate for five minutes at 25 °C then visualized using a Bio-Rad Chemidoc Imaging system.

4.3 – Results and Discussion

To study the interactions between the SS and Cb region of YopE with injectisome components, constructs containing truncated wild-type *yopE*₁₋₈₀, as well as the Cb region Alanine substitution mutants (3-Ala and 5-Ala), were constructed and tested for expression. *yopE*₁₋₈₀ contains both the SS and Cb regions. Using SLIC, inserts containing *yopE*₁₋₈₀ were cloned into a pBad vector that contained a C-terminal streptavidin binding peptide (SBP) and hexahistidine (His) tag (Figure 5.1). However, attempts to transform each of these constructs into *yopE*-deficient *Yersinia* were unsuccessful. While colonies appeared to grow on the media following the electroporation transformation procedure, they only appeared after two days of growth at 26 °C, indicating that they are likely satellite colonies. In the event that these colonies represented the correct transformed bacteria, these colonies were cultured and tested for expression of the construct. The colonies were cultured overnight at 25 °C in Brain Heart Infusion broth supplemented with 50 µg/mL kanamycin, 50 µg/mL carbenicillin, and 2.5 mM CaCl₂ (BHI-Ca). The next morning, the culture was diluted to 1/20th of the original culture volume with BHI-Ca and grown until they reached an OD₆₀₀ of 0.6. Expression was induced using 1% arabinose and the bacteria grown for three hours at 25 °C. After separating the proteins via 14% SDS-PAGE and visualization using an anti-His Western Blot, it was found that the construct was not expressed (not shown). This indicates that the construct was not expressed or the colonies tested were indeed satellite colonies.

Due to the difficulty in transformation, expression of the constructs was tested using *E. coli* Top10 cells. The constructs were chemically transformed into Top10 cells.

The bacteria were grown at 37 °C and expression was induced using 1% arabinose. After induction, the bacteria were grown for three hours at 37 °C. Although Top10 cells do not metabolize arabinose, additional arabinose was added to 1% of the total volume after two hours as a precaution. Interestingly, expression was not induced in the first two hours after induction but only after additional 1% arabinose was added (Figure 4.2, lanes 3, 6, 9). This suggests that expression may only occur at higher concentrations of arabinose.

Given that the *Yersinia* transformed earlier is indeed the correct bacteria, a number of factors may have suppressed expression. Since *Yersinia* were grown at a lower temperature (25 °C) but grown for the same amount of time, it is possible expression was not visible until the bacteria have grown to larger OD reading. Thus, growing the bacteria for a longer amount of time may yield expression. Since *Yersinia* metabolize arabinose, additional arabinose may need to be added in order to induce expression or maintain expression to a detectable level. Electroporation of *Yersinia* ($\Delta yopE$) may also have failed due to the bacteria not being electroporation-competent. Thus, additional troubleshooting regarding the protocol to make the bacteria competent may be required. These factors may be explored in the future by the investigator who will continue the work on this project.

Following the successful expression of the constructs in *Yersinia* ($\Delta yopE$), the next steps of the project will be to perform a secretion assay and perform a co-precipitation assay with streptavidin beads. To test the ability of the constructs to be secreted through the T3SS, a secretion assay will be performed. Given the conservation of the SS region, all the *yopE*₁₋₈₀ constructs (wild-type, 3-Ala, and 5-Ala) are still expected to be secreted. Following this assessment, the co-precipitation experiment will

be performed using streptavidin agarose beads. The YopE₁₋₈₀-SBP-His construct “bait” will bind to these beads (as the “bait” protein) and whichever proteins interact with YopE₁₋₈₀ (“prey” protein) will co-precipitate with this construct. The binding interactions will then be identified by mass spectroscopy. Comparison of the interactions between T3SS components and the wild-type, 3-Ala, and 5-Ala YopE constructs will help identify relevant potential translocation receptors. Interactions found with the wild-type construct but not found in the translocation-deficient 3-Ala and 5-Ala mutants may identify a receptor which specifically targets the residues that were mutated. These interactions may then be further characterized *in vitro*.

4.4 – Figures

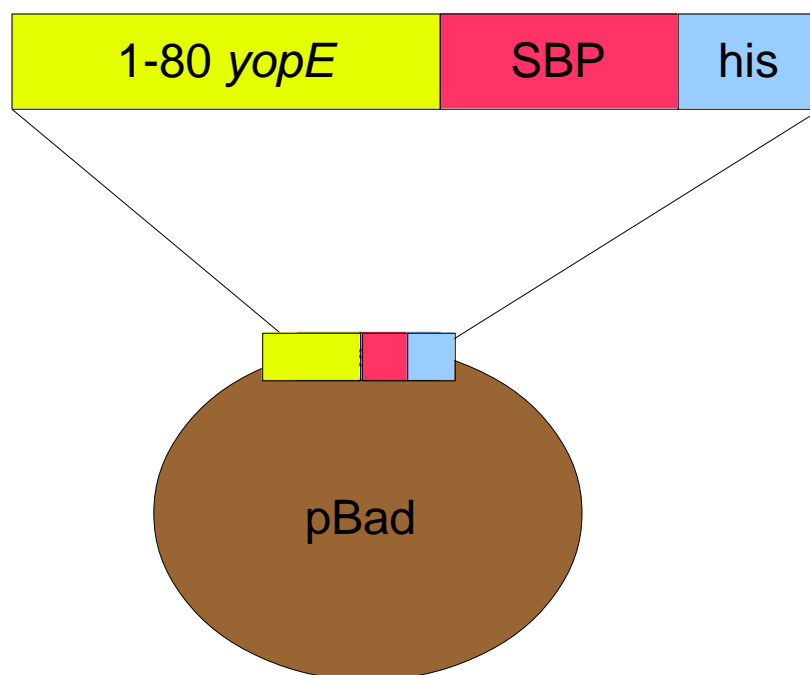


Figure 4.1 – Schematic of *yopE*₁₋₈₀ – SBP – his pBad vector

A pBad construct containing wild-type, 3-ala, 5-ala *yopE*₁₋₈₀ with a C-terminal tag containing strep-binding protein (SBP) and a hexahistidine tag (his) was made using SLIC. The final product resulted in an approximately 14 kDa protein.

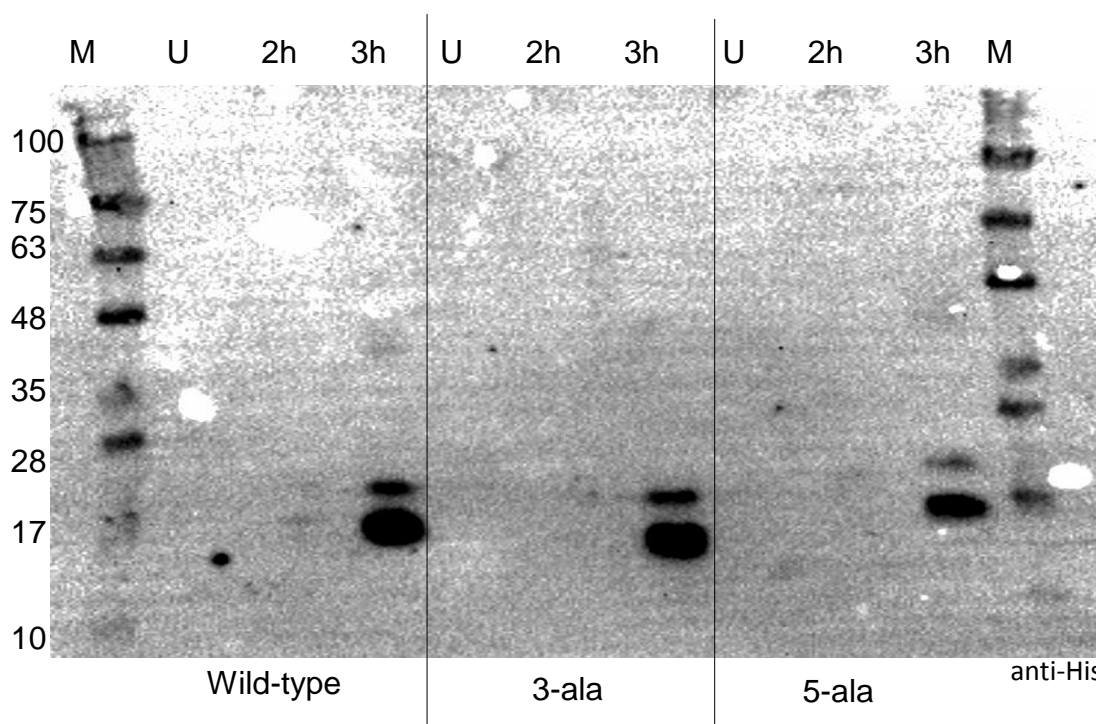


Figure 4.2 – Expression of 1-80 YopE-SBP-his in *E. Coli* Top10

The wild-type, 3-ala, and 5-ala YopE constructs were transformed and expressed in *E.coli* Top10 cells. Expression was induced using 1% arabinose at 37 degrees C. The proteins were separated by 14% SDS-PAGE and visualized using an anti-his Western Blot. After 2 hours, additional 1% arabinose was added. Samples were collected before induction (U), after 2 hours (2h), and after 3 hours (3h). The discrepancy in size observed for the 5-ala mutant is likely attributed to an uneven protein transfer or charge differences caused by the mutation.

Chapter 5

Reconstitution of the *Yersinia injectisome in vivo*

5.1 - Introduction

Since many of the components of the injectisome occur natively in an oligomeric state as part of a large complex, *in vitro* experiments involving monomeric injectisome units may be limited. Thus, the ability to reconstitute an intact injectisome *in vitro* may provide a method to produce the components in an oligomeric form. The assembly of the *Yersinia* injectisome follows a well-defined order. Assembly begins with the outer membrane ring composed of YscC and then sequentially progresses to YscD then YscJ, the components that comprise the inner membrane ring (Diepold *et. al* 2010). Only after this YscCDJ complex is formed are other components of the injectisome recruited to the structure. Data taken from the orthologous ring components in *Salmonella* suggest that a conserved structural ring-forming motif may aid in the assembly of the oligomeric rings (Spreter *et. al* 2009). Thus, the YscC oligomeric ring may aid in the assembly of the YscD oligomeric ring which subsequently may aid in the assembly of the YscJ oligomeric ring. By co-expressing YscCDJ together, the bacteria may form the oligomer structures *in vivo*. Thus, construction of the *in vivo* injectisome complex begins with YscC.

YscC is a member of the secretin family of proteins. As expected for secretin proteins, YscC forms an oligomeric ring in the outer membrane (Burghout *et. al* 2004b). Localization of YscC to the outer membrane was shown

to be dependent on the pilot protein YscW (Burghout *et. al* 2004a). Purification of YscC has previously been completed by solubilizing membranes of *Yersinia* spp. (Koster *et. al* 1997, Burghout *et. al* 2004). Soluble monomeric YscC was also previously purified heterologously, using an intein fusion tag (Goodin *et. al* 2005). However, for this project, an attempt at heterologous expression and purification of an N-terminal His-tagged YscC construct using *E. coli* was attempted. His-YscC was shown to be expressed in *E. coli* and was found to be insoluble. It is unknown whether the protein expressed is aggregated monomeric YscC or the oligomeric YscC ring.

5.2 – Materials and Methods

Cloning of his-YscC/YscW An N-terminal Precision Protease cleavable His-tag (His-PP) was added onto the YscC portion of a bicistronic pETduet vector also containing YscW. The His-PP gene sequence was cloned into the vector using the SLIC protocol (described in Chapter 1). The His-PP insert was constructed using overlapping primers that were first annealed together and then amplified using PCR. The YscC/W pETduet vector was linearized via inverse PCR.

Expression Trials The His-YscC/YscW pETduet construct was transformed into *E. coli* BL21 (DE3) and tested for expression. Bacteria were grown at 37°C in LB supplemented with 50 µg/mL carbenicillin. To test for optimal induction conditions, expression was induced using different concentrations of IPTG (0.4 and 1.0 mM), different OD₆₀₀ readings (0.4 and 1.3) before induction, and different growth conditions (2 hours at 25 °C/ 2 hours at 37 °C or 4 hours at 37 °C). The cultures were then harvested via centrifugation and lysed via sonication. The cell lysate was clarified via centrifugation. Expression was visualized after separation of proteins using 16% SDS-PAGE followed by Coomassie staining and anti-his Western blotting. 25 °C expression assays were performed as described above with the exception of induction at 0.4 mM IPTG, overnight growth at 25 °C, and varying OD₆₀₀ readings at time of induction (0.8, 1.0, 1.3).

Purification of His-YscC/W Bacteria were grown at 37°C until an OD₆₀₀ of 1.3 was reached. Expression was induced using 0.4 mM IPTG and grown for 2 hours at 25°C and 2 hours at 37°C. The culture was then harvested via centrifugation (2,242 x g, 20 min, 4

⁰C) and then resuspended in lysis buffer (100 mM NaPi, pH 8.0, 500 mM NaCl, 20 mM Imidazole, 1% Triton-X 100). The cells were then lysed using an Emulsiflex-C5 (Avestin) homogenizer (2 passes at 15,000 psi). The resulting cell lysate was clarified via centrifugation (14,000 x g, 20 min, 4 ⁰C). The supernatant was then run over a Ni²⁺-nitriloacetic acid (NTA) column equilibrated with lysis buffer containing 25 mM imidazole. To confirm the insolubility of the expressed protein, the uninduced, induced, cell pellet, and supernatant fractions were visualized via anti-his Western Blot.

5.3 – Results and Discussion

The YscCDJ complex is known to begin with construction of YscC (Diepold *et. al* 2010). Thus, the first step in assembling the complex is to express oligomeric YscC. YscC was expressed with an N-terminal his-tag along with its pilot protein YscW. YscW is known to guide YscC to the outer membrane during injectisome construction (Burghout *et. al* 2004a). It also likely mediates the oligomerization of YscC. Previous studies had also shown that, in the absence of YscW, the expression of YscC was greatly attenuated. Thus, it seems likely that YscW may be an important component for purifying oligomeric YscC. Simulating the conditions previously reported for purification of YscC in *Yersinia* (Burghout 2004b), expression of His-YscC/W appears to occur greatest at a higher OD₆₀₀ reading (Figure 5.1, Lanes 1-3) than is usually used for expression from *E. coli* BL21. Interestingly, it was also found that YscC was induced at a greater quantity when first grown at 25 °C for two hours and then moved to 37 °C for two hours, as opposed to growth at 37 °C for four hours. These conditions were used in an attempt to purify soluble His-YscC but, following lysis and centrifugation, His-YscC remained insoluble (Figure 5.2) even with the addition of TX-100, a detergent used to solubilize membranes. In an attempt to solubilize the protein, the bacteria were instead grown at 25 °C overnight after induction but His-YscC remained insoluble (Figure 5.3).

Although His-YscC was expressed, solubilizing it remains the biggest problem. This problem may occur due to the hexahistidine tag itself. Soluble YscC has previously been purified from *Yersinia* membranes (Koster *et. al* 1997, Burghout *et. al* 2004b) and was soluble when expressed in *E.coli*, using a self-cleaving intein fusion tag (Goodin *et.*

al 2005). Similar to His-tag purification using nickel affinity, the intein-fusion tag also allows the protein to be purified in a one-step affinity purification process. However, the protein purified appeared to be monomeric (Goodin *et. al* 2005). The solubility of YscC following cleavage of the intein-tag suggests that it may be the His-tag that causes YscC to aggregate.

An alternative explanation may come from the lack of expression of YscW. Expression of YscW may not only facilitate proper localization of YscC into the outer membrane but may aid in YscC ring oligomerization. In this experiment, YscW was expressed alongside YscC in a bicistronic vector. However, YscW was untagged, therefore it is difficult to determine if the protein was expressed or not. Thus, it is possible that YscC may have simply aggregated due to a lack of YscW expression.

After confirming the expression of a stable His-YscC oligomer, the next step of this project would include the coexpression of YscD and YscJ. The YscC oligomer may aid in the formation of oligomeric YscD and subsequently, the formation of oligomeric YscJ. Following the formation of this complex in *E. coli*, the intact His-YscCDJ complex may be purified by using a nickel column.

It was shown in Chapters two and three that monomeric injectisome components may not be suitable for binding effectors. Heterologous expression of the individual genes in *E. coli* may only lead to purification of the unassembled form of the proteins. This issue may be solved by reconstituting the base components of the injectisome *in vivo* by co-expressing the genes encoding the proteins in *E. coli*. Since assembly of the injectisome begins with YscC, the expression of YscC in *E. coli* was investigated first.

If an oligomeric form of His-YscC can be expressed, oligomeric forms of the other proteins forming the injectisome may also be attained. Performing experiments on the oligomeric forms of these components may help to characterize the binding between an effector and a receptor *in vitro*.

5.4 – Figures

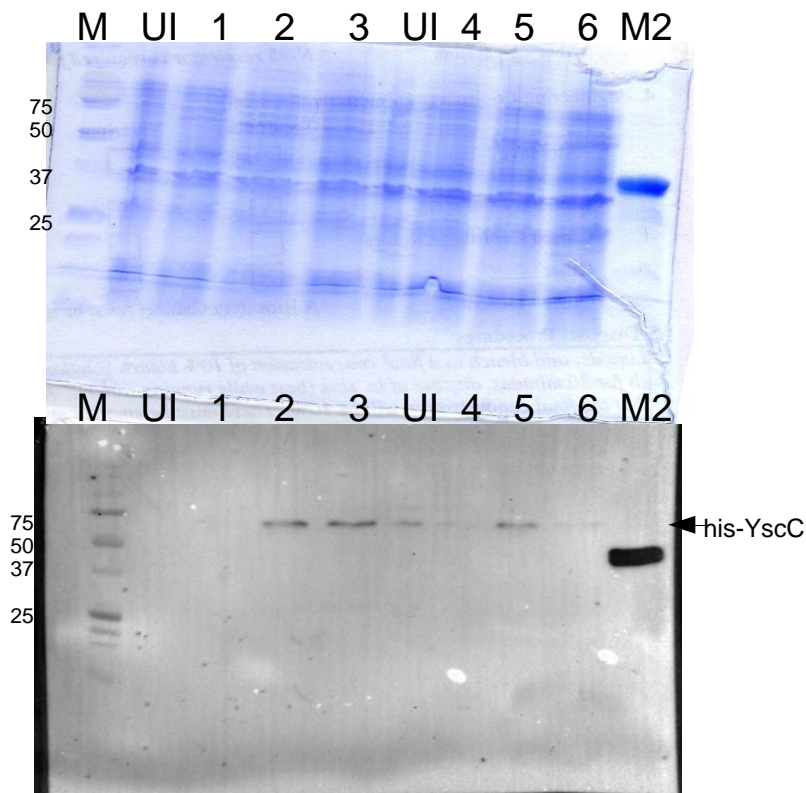


Figure 5.1 – Optimization of his-YscC/W pETduet Expression

An expression assay was used to optimize the temperature, IPTG concentration, and OD_{600} readings at time of induction. Lanes 1-3 represent samples taken from growth for two hours at 25 °C and then for two hours at 37 °C after expression is induced. Samples taken before induction are labeled UI. Lanes 4-6 represent samples taken from growth for four hours at 37 °C. Lanes 1+4 represent samples induced at $OD_{600} = 0.4$ with 0.4 mM IPTG. Lanes 2+5 represent samples induced at $OD_{600} = 1.3$ with 0.4 mM IPTG. Lanes 3+6 represent samples induced at $OD_{600} = 1.3$ with 1.0 mM IPTG. Expression was visualized using 16% SDS-PAGE stained with Coomassie Brilliant Blue (top) and by anti-his Western blot (bottom). His-YscC appears to optimally expressed with growth for two hours at 25 °C and switching to 37 °C, induction at $OD_{600} = 1.3$, and variable IPTG concentration. A purified his-tagged M-protein was added as a positive control for detection by Western Blot (kind gift from Cosmo Buffalo).

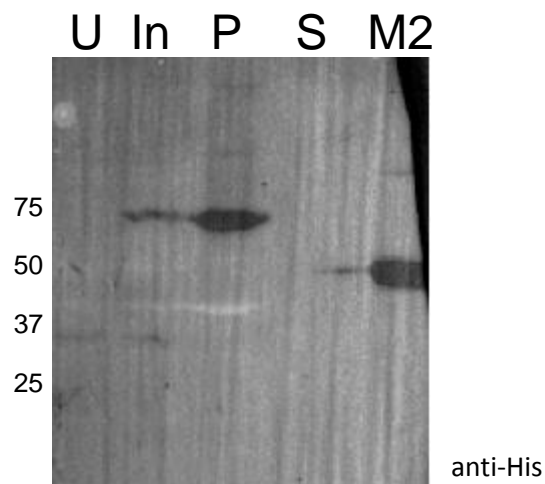


Figure 5.2 – His-YscC is insoluble

Lanes are labeled as U (Uninduced), In (Induced), P (Pellet), S (Supernatant), M2 (M2-his, Cosmo Buffalo). Using the optimized condition found previously (Figure 5.2), an attempt at purification was made and visualized using anti-his Western Blot. All of the expressed His-YscC was found in the insoluble pellet.

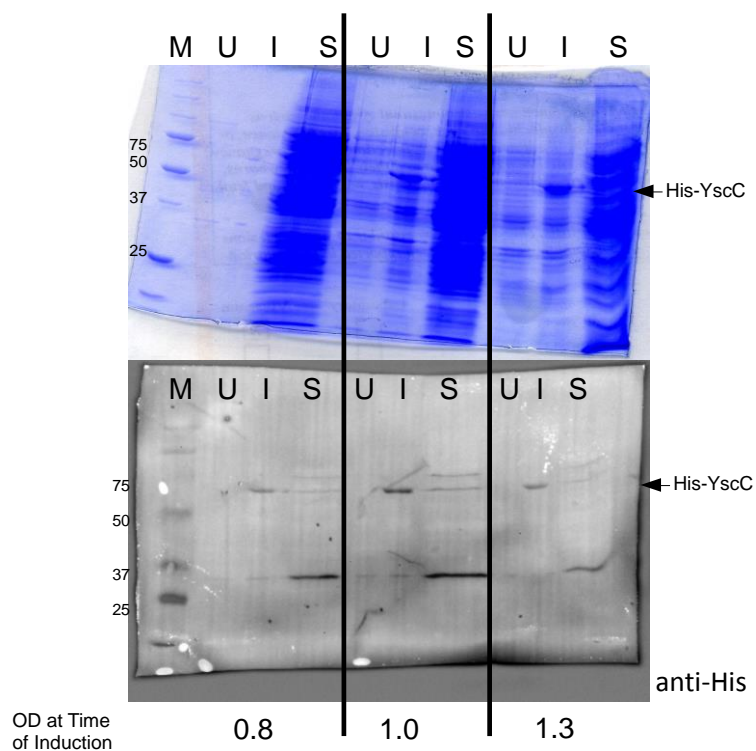


Figure 5.3 – 25 °C expression trials of His-YscC/W pETduet

In an attempt to solubilize his-YscC, an expression trial at 25 °C overnight was attempted. Bacteria were induced with 0.4 mM IPTG at different OD₆₀₀ readings: 0.8 (1-3), 1.0 (4-6), 1.3 (7-9), as indicated at the bottom of the gel. The lanes are arranged as Uninduced (U), Induced (I), and Soluble (S) fractions. The expression was visualized using 16% SDS-PAGE followed by Coomassie Brilliant Blue Staining (Top) or anti-his Western Blot (Bottom). As evidenced by the lack of His-YscC found in the supernatant lanes, growth of the bacteria at room temperature is not sufficient for solubility of His-YscC.

Chapter Six

Overall Discussion

The Type III Secretion System (T3SS) specifically identifies effectors for secretion through an N-terminal signal sequence (SS) region on the effector composed of either mRNA codons (Anderson and Schneewind 1997) or amino acid sequences (Lloyd *et. al* 2001) or both. Some effectors also utilize a chaperone protein, which bind to the effector before translocation. This chaperone binds locally to the effector at the chaperone-binding (Cb) region, located just downstream of the SS region (Rodgers *et. al* 2008). It is hypothesized that chaperone-binding to the Cb region forms a three-dimensional signal (Birtalan *et. al* 2002) that efficiently targets effectors for translocation. Although the signals that specify effectors for secretion have been identified, a receptor which binds these signals is unknown. The purpose of the experiments described in this thesis is the identification of this receptor with a focus on *in vitro* characterization of previously identified interactions between effectors and injectisome components.

In this thesis, two ring components of the *Yersinia* injectisome that were previously shown to bind effectors in bacteria were assayed for binding to effectors *in vitro*. Data found from *Yersinia* had shown that YscD binds the effector YopH (Gamez *et. al* 2012). Data from other species have also suggested that YscQ may bind to effectors (Morita-Ishihara *et. al* 2006, Spraeath *et. al* 2009) and may even function as a “sorting platform” for secreted substrates (Lara-Tejero *et. al* 2011). Using an *in vitro* co-

precipitation assay, it was found that neither the wild-type YscDc nor the L3 or L4 mutants were competent to bind to the effector YopH (Figure 2.3). Similarly, an unassembled form of YscQ was unable to bind to chaperone-effector complexes of SycH/YopH or SycE/YopE nor the free chaperones (Figure 3.3). These results suggest that effector-binding to these oligomeric ring components may only occur while the ring components are assembled.

To circumvent this issue, a method to purify oligomeric injectisome structures was pursued. The goal of this method was to reconstitute components of the *Yersinia* injectisome *in vivo* using *E. coli*. This method was designed to follow the native assembly order of the injectisome, which begins with assembly of the outer membrane ring. As described in this thesis, the first component, YscC, was over-expressed in *E. coli* but was found to be insoluble, even with the addition of detergent (Figure 5.2). Without further characterization of the expressed protein, it was unknown whether the YscC expressed was the properly folded oligomeric ring embedded into the outer membrane or an insoluble aggregate of the YscC monomer. Completion of this project may lead to the ability to purify oligomers and thus provide a means to characterize effector binding to oligomeric injectisome components.

To identify potential receptors for the YopE Cb translocation signal, a method similar to the co-precipitation assays utilized in previous chapters (Chapter 2 and 3) was described. While the co-precipitation assays described earlier focused on the characterization of two purified components, this method focuses on identifying binding interactions in bacteria. In this method, a truncated form of YopE containing only the

first 80 amino acids will be used as a “bait” protein to identify potential receptors in *Yersinia*. The wild-type *yopE*₁₋₈₀ and the 3-ala and 5-ala *yopE*₁₋₈₀ mutants were successfully cloned into a pBad vector containing a downstream SBP-His tag (Figure 4.1). However, due to a difficulty in transforming the constructs into *yopE*-deficient *Yersinia*, the expression of the constructs were assayed through expression in *E. coli* Top10 cells (Figure 4.2). Through this experiment, additional interactions between the effector targeting signals with a receptor may be identified.

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